# The Role of Interleukin-33 in Mucosal Inflammation and Fibrosis

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## Abstract

**Background:** Interleukin (IL)-33 is a newly identified member of the IL-1 cytokine family. Multiple cell types are able to produce or respond to IL-33, including non-haematopoietic structural cells, innate and adaptive immune cells. The biological activity of IL-33 was initially described as being associated with the promotion of type 2 immune responses which were characterized by the induction of $CD4^+$ T helper (Th) 2 cells. For example, exogenous administration of IL-33 in experimental models caused pathological changes in mucosal tissues such as the lung and gastrointestinal tracts; early studies reported that IL-33 can activate Th2 cells, mast cells, eosinophils or basophils to produce type 2 cytokines such as IL-4, IL-5, and IL-13. This was associated with pathological changes reminiscent of asthma, fibrosis and ulcerative colitis. Recently, a newly recognised cell population which was inducible by IL-33 and referred to as 'type 2 innate lymphoid cells' was identified and these were thought to be important for initiating type 2 immunity. However, the underlying mechanism by which IL-33 was involved in the inflammation and remodelling of diseases of the respiratory and gastrointestinal tracts remains to be fully understood.

**Hypothesis:** My hypothesis is that IL-33 is induced in the gut and lung mucosa by inflammatory signals and mediates both early inflammation and late fibrosis by amplifying the innate immune response.

**Aims:** To address this hypothesis I set out the following aims: i) to investigate the induction and effect of IL-33 via its receptor ST2 on cellular pathogenic pathways in the development of lung fibrosis (chapter 3); ii) to unravel the mechanism by which IL-33 promotes lung fibrosis (chapter 4); iii) to understand the involvement of the IL-33/ST2 pathway in ulcerative colitis (chapter 5).

**Methods:** To address these aims I used two experimental murine models. To investigate the effect of IL-33 in the fibrosis phase of airway mucosal

inflammation I used the bleomycin (BLM)-induced lung fibrosis (chapters 3 and 4). To investigate the effect of IL-33 in the acute phase of mucosal inflammation in the gut, I used dextran sulphate sodium (DSS)-induced colitis (chapter 5).

These disease models are widely accepted for laboratory investigation and I acknowledge that they do not reflect the full complexity of the human conditions. However they are extremely useful for hypothesis generation.

**Results:** My results showed

i) that IL-33 promotes the pathogenesis of bleomycin-induced lung fibrosis. This was indicated by IL-33 being constitutively expressed in lung epithelial cells but induced in macrophages by bleomycin. The specificity of this response was confirmed by using either ST2-deficient mice, or neutralising anti-IL-33 antibody treatment, which both attenuated lung fibrosis (chapter 3).

ii) that IL-33 promotes the initiation and progression of pulmonary fibrosis by recruiting and directing inflammatory cell function, and enhancing the production of pro-fibrogenic cytokines IL-13 and TGF-β in an ILC2- and M2-macrophages (chapter 4).

iii) that IL-33 signalling via ST2 induces an IL-4-dependent immune response that is pathogenic in the early stage of ulcerative colitis. I found that the clinical indices of DSS-induced experimental UC, diarrhoea and colon inflammation, were respectively impaired in ST2 knockout mice and exacerbated in WT mice by treatment with exogenous recombinant IL-33. These were associated respectively with reduced and enhanced expression of inflammatory chemokines and angiogenic cytokines *in vivo*. The exacerbation effect of treatment with recombinant IL-33 on DSS-induced acute colitis was abolished in IL-4 knockout mice (chapter 5).

**Conclusion and prospect:** Together, my results demonstrated that IL-33 expression was up-regulated in the lung and colon epithelium/endothelium in experimental BLM-induced fibrosis and DSS-induced colitis respectively. Furthermore, IL-33 exacerbated both diseases through recruiting and activating

inflammatory cells and increasing the production of type 2 cytokines. Finally, I discussed the pathological mechanisms of IL-33 in mucosal tissue based on my results and the current literature. I concluded that this insight into IL-33 biology is informative of a new potential pathogenic pathway and might be a useful biomarker of disease and that targeting IL-33 may provide a new biological therapeutic approach in these disorders (chapter 6).

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**Li D***, Guabiraba R*, Besnard AG, Komai-Koma M, Jabir MS, Zhang L, Graham G, Kurowska-Stolarska M, Liew FY, McSharry C, Xu D. IL-33 promotes ST2-dependent lung fibrosis by the induction of alternatively activated macrophages and innate lymphoid cells in mice. *Journal of Allergy and Clinical Immunology*. 2014; *doi: 10.1016/j.jaci.2014.05.011*. (*co-first authorship)

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## Author's Declaration:

The work presented in this thesis represents original work carried out by the author, with the exception of the flow cytometry result in chapter 4, which was performed in conjunction with Dr. Rodrigo Guabiraba and Dr. Anne-Gaëlle Besnard. This thesis has not been submitted in any form to any other University. Where reagents, materials or technical support has been provided by others, appropriate acknowledgement has been made in the text.

Signature: …….

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## Abbreviations:

| Abbreviation | Full Description |
|---|---|
| -/- | Knock out gene deletion |
| AAM/M2 | Alternatively activated macrophages |
| Ag | Antigen |
| AHR | Airway hyper-responsiveness |
| α-SMA | α-smooth muscle actin |
| ANOVA | Analysis of variance |
| APC | Antigen presenting cell |
| ARG1 | Arginase 1 |
| BAL | Bronchoalveolar lavage |
| BCR | B cell receptor |
| BHR | Bronchial hyper-reactivity |
| BSA | Bovine serum albumin |
| BLM | Bleomycin |
| BM | Bone marrow |
| BMM/BMDM | Bone marrow derived macrophages |
| ° C | Degree celsius |
| CCL | Chemokine ligand |
| $CCL_4$ | Carbon tetrachloride |
| CCR | Chemokine receptor |
| CD | Cluster of differentiation |
| CD | Crohn's Disease |
| cDNA | Complementary deoxyribonucleic acid |
| CG | Cathepsin G |
| CIA | Collagen-induced arthritis |
| c-kit | Stem cell growth factor receptor, SCFR (CD117) |
| CMP | Common myeloid progenitor |
| Ct | Threshold cycle |
| CTGF | Connective tissue growth factor |
| CXCL | CXC chemokine ligand |
| DAB | 3.3'-diaminobenzidine |
| DAMP | damage-associated molecular patterns |

| | |
|---|---|
| DC | Dendritic cell |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DPBS | Dulbecco’s Phosphate Buffered Saline |
| DSS | Dextran sulfate sodium |
| EAE | Experimental autoimmune encephalomyelitis |
| ECL | Enhanced chemiluminescence |
| ECM | Extracellular matrix |
| E. coli | Escherichia coli |
| EDTA | Ethylenediamine tetra-acetic acid |
| ELISA | Enzyme linked immunosorbent assay |
| EMT | Epithelial/endothelial-mesenchymal transition |
| FACS | Flow cytometry |
| FBS/FCS | Fetal bovine/calf serum |
| Fc | Fragment crystallizable (region) |
| FcεRI | High affinity IgE receptor |
| FGF | Fibroblast growth factor |
| FITC | Fluorescein isothiocyanate |
| fl | Full length |
| GATA-1 | GATA-binding factor 1 |
| G-CSF | Granulocyte colony-stimulating factor |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| HCl | Hydrogen chloride |
| H&E | Haematoxylin and eosin |
| HMGB1 | high mobility group box 1 |
| HPC | Haematopoietic progenitor cells |
| HRP | Horseradish peroxidase |
| HTH | Helix-turn-helix |
| IBD | Inflammatory bowel disease |
| ICAM-1 | Intercellular adhesion molecule-1 |
| ICOS | Inducible T cell co-stimulator |
| IFN | Interferon |

| | |
|---|---|
| Ig | Immunoglobulin |
| IGF | Insulin growth factor |
| IIP | Idiopathic interstitial pneumonia |
| IκBα | nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha |
| IL | Interleukin |
| IL-1R | IL-1 receptor |
| IL-1Ra | IL-1 receptor antagonist |
| IL-1RAcP | IL-1 receptor accessory protein |
| ILC | Innate lymphoid cell |
| i.n. | Intranasal |
| iNOS/NOS2 | Inducible nitric oxide synthase |
| i.p. | Intraperitoneal |
| IP-10 | interferon-gamma-induced protein (CXCL10) |
| IPF | Idiopathic pulmonary fibrosis |
| IRAK | IL-1 receptor-associated kinase |
| i.t. | Intratracheal |
| JAK | Janus kinase |
| JNK | c-Jun N-terminal kinase |
| KC | Keratinocyte chemoattractant (CXCL1) |
| kDa | Kilodaltons |
| KO | Knock out gene deletion |
| LCMV | Lymphocytic choriomeningitis virus |
| LPS | Lipopolysaccharide |
| LYN | Src-family kinase |
| M1 | Classically activated macrophages |
| M-CSF | Macrophage colony-stimulating factor |
| MAPK | Mitogen-activated protein kinase |
| MC | Mast cell |
| MCP-1 | Monocyte chemotactic protein-1 |
| mIL-33 | Mature IL-33 |
| m/h IL-33 | Murine/human recombinant IL-33 |
| min(s) | Minute(s) |
| MIP-1α | macrophage inflammatory protein-1 (CCL3) |

| | |
|---|---|
| MIP-2α | chemokine (C-X-C motif) ligand 2 (CXCL2) |
| MMP | matrix metalloproteinase |
| MPO | Myeloperoxidase |
| MR | Mannose receptor (CD206), M2 macrophages marker |
| mRNA | Messenger ribonucleic acid |
| MS | Multiple sclerosis |
| MyD88 | Myeloid differentiation primary response gene (88) |
| NF-HEV | Nuclear factor from high endothelial venules |
| NF-κB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NLR | nucleotide-binding oligomerization domain-like receptor |
| NLS | nuclear location sequence |
| NO | Nitric oxide |
| NOD | Nucleotide-binding oligomerization domain |
| NK | Natural killer cell |
| OVA | Ovalbumin |
| PAMP | Pathogen-associated molecular patterns |
| PB | peripheral blood |
| PBMCs | Peripheral Blood Mononuclear Cells |
| PBS | Phosphate buffered saline |
| PE | Phycoerythrin |
| PM | Pulmonary fibrosis |
| PMA | Phorbol 12-myristate 13-acetate |
| PRR | pattern recognition receptors |
| P value (P) | Probability value |
| qPCR | Quantitative polymerase chain reaction |
| RA | Rheumatoid arthritis |
| RAGE | Receptor for advanced glycation end products |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| RPE | R-phycoerythrin |
| RPMI | Roswell Park Memorial Institute |
| rpm | Rotations per minute |
| RT-PCR | Reverse transcription polymerase chain reaction |

| | |
|---|---|
| SAMP1/YitFc | Senescence accelerated mice P1/YitFc |
| s.c. | Subcutaneous |
| SCF | Stem cell factor |
| SD | Standard deviation |
| SDS | Sodium dodecyl sulphate |
| SEM | Standard error of the mean |
| sec(s) | Second(s) |
| SIGIRR | Single Ig IL-1-related molecule |
| SMC | Smooth muscle cells |
| SNP | Single-nucleotide polymorphism |
| SSc | Systemic sclerosis |
| ST2 | Tumorigenicity 2 |
| sST2 | Soluble ST2 |
| ST2(L) | Membrane bound ST2 |
| STAT | Signal transducers and activators of transcription |
| TAE | Tris-acetate-EDTA |
| TAK1 | Transforming growth factor beta activated kinase-1 |
| TBE | Tris-borate-EDTA |
| TCA | Trichloroacetic acid |
| TCA3 | T-cell activation-3 (CCL1) |
| TCR | T cell receptor |
| Tg | Transgenic |
| TGF | Transforming growth factor |
| Th | T helper (cell) |
| TIMP | Tissue inhibitors of metalloproteinases |
| TIR | Toll/interleukin 1 receptor |
| TLR | Toll-like receptor |
| TMB | 3,3',5,5'-tetramethylbenzidine |
| TNBS | trinitrobenzenesulfonic acid |
| TNF-α | Tumor necrosis factor alpha |
| TRAF | TNF receptor-associated factor |
| Treg | Regulatory T cells |
| TRIF | TIR-domain-containing adapter-inducing interferon-β |
| Tris | Tris (Hydroxymethyl)aminomethane |

| | |
|---|---|
| U | Unit |
| UC | Ulcerative colitis |
| UV | Ultraviolet |
| VCAM-1 | vascular cell adhesion molecule |
| VEGF | Vascular endothelial growth factor |
| VSMC | Vascular smooth muscle cell |
| WT | Wild type |
| w/v | Weight/volume |

# Chapter 1

# Introduction

# Chapter 1: Introduction

## 1.1 Immune system and inflammation

The human body has evolved a complementary and sophisticated immune system; including immune organs, immune cells and immune effector molecules (Murphy, 2011).

The functions of this immune system can be categorized as:

1. Immune defence: to prevent and eliminate invading pathogens, such as bacteria, viruses, fungi, parasites and other harmful elements.

2. Immune surveillance: to identify and eliminate 'altered-self material' inside the body, such as tumour cells caused by gene mutation, apoptotic and necrotic cells.

3. Immune homeostasis: to maintain immune system stability through immune tolerance and immune regulation (Abbas et al., 2011).

The processes by which the immune system recognises and eliminates antigens is called the 'immune response'. This can be divided into two types: 'innate' or 'non-specific' immunity and 'adaptive' or 'specific' immunity (Delves et al., 2011).

The immune response can protect against pathogens but can also contribute to disease if the regulatory mechanisms become dysfunctional. This abnormal immune response can cause a variety of autoimmune and hypersensitivity diseases (Murray et al., 2012).

### 1.1.1 Innate immunity

Innate immunity is also referred to as non-specific immunity. Innate immunity is the body's first line of defence formed through evolution. The innate immune response is mediated by innate immune cells including monocytes, macrophages, dendritic cells (DC), granulocytes, natural killer (NK) cells and innate lymphoid cells (ILC). These cells do not express antigen receptors but express pattern recognition receptors (PRR), thereby recognising pathogen associated molecule patterns (PAMP). For instance, macrophage and DC can recognise lipopolysaccharide (LPS) in the cell wall of Gram-negative microbes via toll like receptor 4 (TLR-4) on its membrane to initiate innate immune response (Akira et al., 2006, Abbas et al., 2011).

The innate immune response is characterised by its rapid engagement immediately after infection by innate immune cells but it lacks the antigen-specificity and memory response characteristic of the adaptive immune response. The innate immunity system includes tissue barriers, innate immune cells and innate effector immune molecules. This system exists from birth and can provide an immediate immune protection against pathogens or against damaged or mutated cells through non-specific effects (Sompayrac, 2011).

#### 1.1.1.1 Tissue barrier

Physical barriers: skin epithelium and mucosal membranes that line our digestive, respiratory and reproductive tracts form the physical barriers that restrict the entrance of pathogens (Pichery et al., 2012).

Chemical barriers: skin and mucosal tissues secrete anti-microbial molecules such as unsaturated fatty acid from sebaceous glands, lactic acid from sweat glands, lysozyme and antibiotic peptides in saliva and in gastric, respiratory and reproductive tracts (Murphy, 2011).

Haemato-encephalic (blood-brain) barrier: pia mater, choroid plexus capillary walls and astrocytes separate the circulating blood from the brain extracellular fluid (Abbas et al., 2011).

Placental barrier: syncytiotrophoblast, cytotrophoblast, villus mesenchyma and the foetal capillary walls prevent harmful molecules or microbes from entering the foetal blood (Abbas et al., 2011).

### 1.1.1.2 Innate cells

**Monocytes-macrophages**

Monocytes are white blood cells which originate in the bone marrow. Macrophages differentiate in tissues from monocyte precursors. Monocytes circulate in the peripheral blood before entering tissues to develop into tissue-specific macrophage populations such as osteoclasts, microglial cells, histiocytes, Kupffer cells and alveolar macrophages in different tissues. The primary function of macrophages is believed to involve their role as critical immune effector cells in host defence recognising microbial signals and acting as both innate effector cells and antigen-presenting cells. In addition to host defence, macrophages have also been involved in the processes of wound healing, homeostasis and immune regulation (Alber et al., 2012). Examples of these effects include phagocytosis of senescent erythrocytes, cellular debris and clearance of apoptotic cells (Aderem and Underhill, 1999).

**Activation of macrophage**

Macrophages can be further differentiated into two subset phenotypes: M1 (classically activated) or M2 (alternatively activated) under different activation conditions. In the type I immune response (T helper type 1 subset involved) such as inflammatory disease and infectious environment, macrophages undergo activation influenced by IFN-γ, IL-12 and LPS; this is termed classical activation

and macrophages are differentiated into the M1 phenotype. Classical activated M1 macrophages express inducible nitric oxide synthase (iNOS) and pro-inflammatory cytokines and are therefore potent effector cells against microorganisms and tumour cells. In the type 2 immune response (T helper type 2 subset involved) which includes allergic disorders and parasite infection, macrophages undergo an alternative activation influenced by interleukin (IL)-4 or IL-13 and differentiate into the M2 phenotype. The M2 macrophage expresses arginase I and TGF-β and is critically involved in tissue repair and fibrosis (Gordon, 2003, Mosser and Edwards, 2008, Alber et al., 2012).

**Neutrophils**

Among the white blood cells, 60-70% are neutrophils. Neutrophils are generated from bone marrow and have an average life of five days. Neutrophils are involved in the early defence against infection; they traverse blood vessel endothelium into sites of tissue infection or damage and clear microbes or cell debris by phagocytosis. Neutrophils also express pro-inflammatory cytokines and mediators and are closely associated with the inflammatory response (Godaly et al., 2001).

**Dendritic cells**

Dendritic cells (DC) are present in a variety of tissues and organs, especially where tissues are in contact with the external environment. DC is named for its branched projections like the dendrites of the nerve system. The main function of DCs is to sample antigen from tissue and transport and present this to lymphocytes in lymph nodes. There it not only provides antigen but also the co-stimulatory and cytokine signals for activation of T cells. It is the most important antigen-presenting cell (APC), thus it is considered as a crucial link between the innate and adaptive immunity. There are three major subsets of DC: conventional DCs (cDC) and monocyte-derived DCs which act as APCs, and plasmacytoid DCs (pDC) which produce type I interferons in infection (Plantinga et al., 2010, Spears et al., 2011).

### Eosinophils and basophils

The number of eosinophils and basophils is much less than the neutrophils. They are considered to be very important in the innate immunity for defending against parasite infection by releasing toxic granular and type II cytokines. Thus, they also play a critical role in the progress of allergy and asthma (Abbas et al., 2011).

### Mast cells

Mast cells are found resident in the tissues throughout the body, especially beneath mucosa and in blood vessels. They are believed to be involved in the allergic response by the release of vaso-active mediators e.g. histamine when their membrane immunoglobulin (Ig) E receptor-bound IgE are cross-linked by allergen or antigen. However, mast cells are also involved in the pro-inflammatory response and host defence against bacterial infection by producing cytokines and chemokines (Abbas et al., 2011, Menzies et al., 2011).

### Natural killer cells

Natural killer (NK) cells are derived from the same common lymphoid progenitor in the bone marrow as T cells and B cells, but do not express antigen receptors. They are considered to be important in anti-tumour and anti-virus immunity since they recognize and kill abnormal cells by inducing apoptosis. (Abbas et al., 2011)

### Innate lymphoid cells

Innate lymphoid cells (ILCs) are newly identified lymphocytes which do not express T cell or B cell antigen receptors and cell-surface markers associated with other cell lineages of the immune system (Buonocore et al., 2010, Moro et al., 2010).

On the basis of their cytokine profile associated with Th1, Th2 or Th17 cells, ILCs can be classified into three subsets (Spits et al., 2013, Walker et al., 2013):

- ILC1 include natural killer cells and interferon-γ-producing non–natural killer cells and mainly produce type I cytokine interferon-γ.
- ILC2 produce type II cytokines IL-5, IL-9 and IL-13 in response to IL-25 and IL-33.
- ILC3 include both ILCs and lymphoid tissue–inducer cells and produce Th17 cytokines IL-17A and IL-22.

**ILC2: phenotype and function**

ILC2s are closely associated with allergy and tissue fibrosis and are involved in this thesis; therefore I will focus on ILC2 cells and discuss their phenotype and functions.

ILC2 cells were identified independently by four research groups (Moro et al., 2010, Neill et al., 2010, Saenz et al., 2010, Price et al., 2010). They were derived from precursors in bone marrow which express high levels of GATA3 (Walker et al., 2013). GATA3 is required for the development of ILC2 cells since GATA3 gene-deficient mice cannot produce ILC2 cells. These cells do not express lineage (Lin) markers (CD3ε, CD4, CD8α, TCRß, TCRδ, CD5, CD19, B220, NK1.1, Ter119, Gr-1, Mac-1, CD11c and FcεRIα), however they express c-Kit, Sca-1, IL-7R, ICOS and ST2 and produce IL-5 and IL-13 in response to IL-33 or IL-25 stimulation (Salimi et al., 2013). There are no definitive markers for ILC2, therefore using these markers helps in their identification and isolation.

## 1.1.2 Adaptive immunity

The adaptive immune response is mediated by adaptive immune cells; mainly the T and B lymphocytes which express antigen receptors. It takes longer to be mobilized than the innate immune response because it is antigen-specific and requires organisational steps.

The adaptive immune response can be divided into four phases:

1. Initiation (priming) phase, T lymphocytes and B lymphocytes recognise antigens via T-cell receptor (TCR) and B-cell receptor (BCR).

2. Activation and differentiation phase, lymphocytes are started to activate, proliferate and differentiate into effector cells when received antigen, co-simulation and cytokine signals.

3. Effector-stage, antigens are eliminated by effector cells and related effector molecules.

4. Recovery phase, most effector cells are eliminated by apoptosis and only small amounts of effector cells develop into memory cells.

The adaptive immune response includes a cellular immune response; activation of lymphocytes, and a humoral immune response; production of antibody (Murphy, 2011).

### 1.1.2.1 T Cell-mediated immunity

The cellular immune response is mediated by effector T cells (mainly the $CD4^+$ and $CD8^+$ T cells) via their cell surface receptors and secreted molecules, and plays a critical role in defence particularly against intracellular microbial infection (Delves et al., 2011).

The cellular immune response is initiated by the activation of naive T cells which receive TCR signals triggered by the interaction of the T-cell receptor (TCR) and the antigen/MHC complex on antigen-presenting cells (APC). The full T cell activation also requires the signals from APC co-stimulatory molecules and cytokines. The most efficient APCs are dendritic cells (DC), but could also be macrophages or activated B cells. The activation of naive T cells then leads to their proliferation and differentiation into effector T cells. Naive $CD8^+$ T cells are differentiated into cytotoxic T cells which kill infected cells; naive $CD4^+$ T cells can be differentiated into several subsets including T helper (Th) 1, Th2, Th9, Th17 and regulatory T (Treg) cells depending on the cytokine milieu (Fig. 1-1). These cell subsets secrete specific cytokines and provide helper or regulatory roles in immunity and hypersensitivity diseases (Fig. 1-1). The tissue environment of inflammation and/or bacterial infection promotes the development of Th1 cells. Allergy and parasite infection promote Th2 cell differentiation. The cytokine environment of inflammatory and autoimmune conditions favours the development of either Th17 cells which are pro-inflammatory or Treg cells which are suppressive for T cells in immunity and disease. Most of the effector T cells are short-lived, but some could differentiate into long-lived memory T cells which are important in the protection against re-infection and in vaccine development. (Sprent and Surh, 2002, Garcia and Adams, 2005, O'Shea and Paul, 2010)

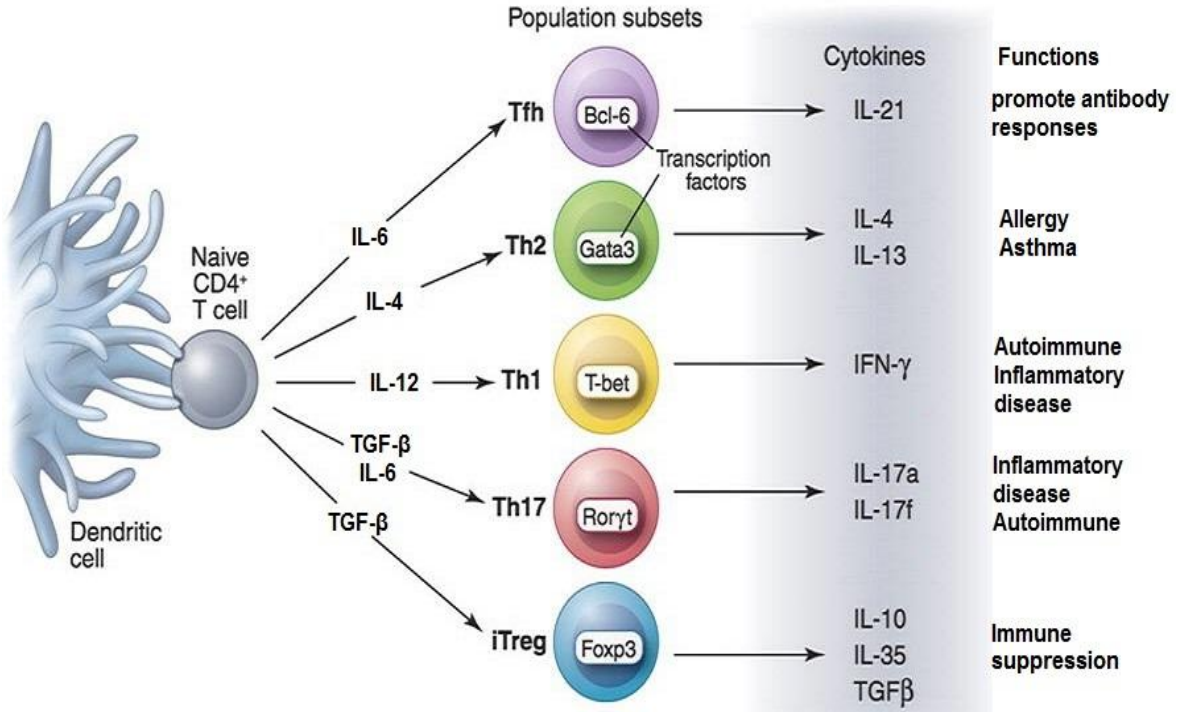



**Fig. 1-1 CD4$^+$ T cell subsets and functions.**

(Modified from O'Shea, J. J. & Paul, W. E. 2010. Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. *Science*, 327, 1098-1102.)

### 1.1.2.2 B cell-mediated humoral immunity

The humoral immune response is mediated by B cells and the antibodies they produce, and plays an important role in protection against extracellular pathogens. The activation of B cells and differentiation into antibody producing plasma cells is dependent on antigen and T cell help; T-dependent (TD) antigens require Th cells, whereas T-independent (ID) antigens do not require T cells. Peripheral B cells consist of B1 and B2 subsets, which can be distinguished by their origin, surface markers, location, and distinct antibody production profile (Martin and Kearney, 2001). The majority of B cells are conventional B2 cells which are located in the spleen or lymph nodes and produce all types of antibody in an antigen and T cell-dependent manner. In contrast, B1 cells represent about 5% of the total B cells in mice, are mainly found in the body cavities and spontaneously secrete natural IgM in a T cell-independent manner (Fagarasan and Honjo, 2000).

Antigens bound to B cell receptors (BCR) on the B cell surface trigger the first signal for B cell activation. The co-stimulatory signals generated by the interaction between CD40 ligand on T cells and CD40 on B cells and T cell secreted cytokines (IL-4, IL-5 and IL-13) are also necessary for B cell activation. Furthermore, toll-like receptor signals on the mature B cells provide an additional signal for B cell activation (O'Rourke et al., 1997).

Upon activation, conventional B cells will proliferate and differentiate into antibody-producing plasma cells. Antibodies eliminate pathogens mainly by three mechanisms:

neutralization, which prevents bacteria binding to target cell surface and forming a focus of infection, and binding bacterial toxins;

opsonisation, which enhances phagocytosis of phagocytes;

complement activation, via the classic complement activation pathway.

There are five subtypes of antibodies, namely IgM, IgA, IgG, IgD and IgE (Shapiro-Shelef and Calame, 2005, Moser et al., 2006, Ohta and Flajnik, 2006).

IgM is the low affinity high molecular weight "natural" immunoglobulin only found in blood and lymph, and clears pathogen through activating complement. IgA functions mainly as a neutralizing antibody in mucosal tissues.

IgG is the most stable antibody and induces phagocytosis as well as activates complement.

IgD is considered co-expressed with IgM.

IgE functions mainly through activating mast cells to release other chemical mediators causing reaction that can expel pathogens but may also induce hypersensitivity.

Some activated B cells do not differentiate to plasma cells post-activation; they develop into memory B cells instead. Memory B cells do not produce antibody, but are located in high numbers in secondary lymphoid tissue where they can be activated quickly when in contact with the same antigen or TLR agonists and rapidly produce a large amount of antibody (Liu et al., 1991, Hardy and Hayakawa, 2001).

### 1.1.3 Inflammatory response

The inflammatory response is primarily the host defence response to harmful stimuli, such as trauma, chemical or physical stress, or infectious agents. It is characterized by redness, swelling, heat and pain in the affected tissues. Most of these symptoms result from increased local vascular permeability to allow the access of protein-rich fluid and leukocytes.

### 1.1.3.1 Trauma

Trauma is always accompanied by a local inflammatory response. In the case of severe injury there may be systemic inflammation. The systemic inflammatory response is essential for tissue repair and has evolved in all mammals to help the healing process, but an over-reactive inflammatory response, e.g. hypersensitivity can also be pathogenic or occasionally life-threatening (Heideman and Bengtsson, 1992).

### 1.1.3.2 Infection

Infection is caused by pathogens such as viruses, prions, bacteria, fungi, parasites and other infectious agents. The immune system defends against all these pathogens through a variety of effector cells and molecules.

The first line of defence is the innate immune response which is available almost immediately to prevent or eliminate any foreign pathogens but is not specific and does not lead to immune memory. The antigen-specific adaptive immune response which is triggered by innate immunity is responsible for defending against most pathogens that cannot be dealt with by the innate immune response alone. Furthermore, adaptive immune responses include the development of immune memory post-infection to prevent future infection by the same pathogens (Murphy, 2011).

### 1.1.3.3 Sterile inflammation

Although most inflammation is triggered by invading microbes, some inflammatory responses are triggered by sterile stimuli such as dead cells and other irritant particles. The downstream reactions of inflammation are very similar whatever the initial triggers. There is often collateral damage to normal

healthy tissue during the course of inflammation especially during the innate immune response. In case of a sterile inflammatory response the disadvantages are much greater than the advantages since most sterile stimuli do no harm whereas the inflammation can be disproportionate and damaging. Prolonged acute inflammation can cause chronic remodelling and fibrosis (Rock et al., 2010).

The exact mechanisms by which sterile stimuli can trigger inflammation are still not fully understood. The best understanding so far recognises three possible interactive pathways (Fig. 1-2). These are (i) the activation of pattern recognition receptors (PRRs), as in the infectious immune response, (ii) the release pro-inflammatory cytokines and chemokines such as the IL-1 family cytokines and (iii) other receptors not common in infections such as receptors for advanced glycation end-products (Chen and Nunez, 2010).

Studies showed that the IL-1 family cytokines IL-1α and IL-1β are involved in the inflammatory response triggered by necrotic cells and irritant particles via the MyD88 pathway and blockage of the IL-1 pathway showed therapeutic potentials. However, whether other IL-1 family cytokines such as IL-18 and IL-33 are involved in the sterile inflammatory response is still largely unknown (Garlanda et al., 2013).

IL-33, a newly recognised member of the IL-1 family of cytokines, is realized when cells undergo necrosis and is considered as similar to stereotype ‘alarmin’ high-mobility group box 1 protein (HMGB1) (Moussion et al., 2008). Its role in sterile inflammation is still poorly understood despite intensive investigation of its biological effects since its discovery nine years ago (Schmitz et al., 2005). Further studies with IL-33 are needed to understand the pathogenesis of sterile inflammation and eventually help the development of new therapeutic methods.

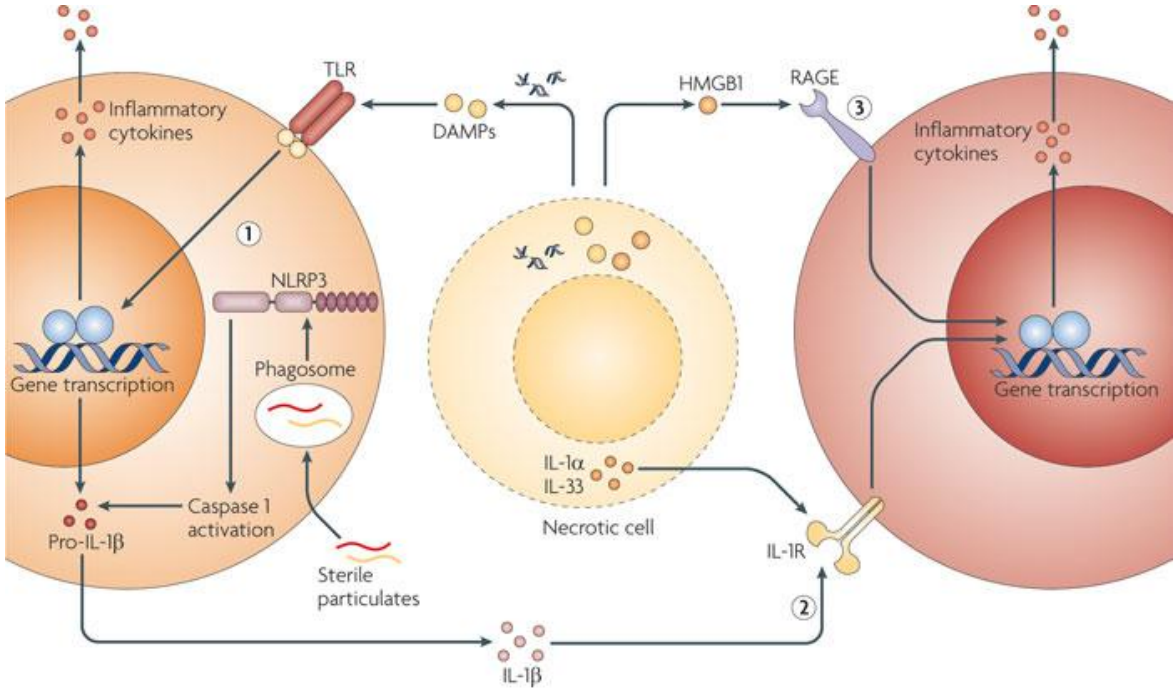



**Fig. 1-2 Necrosis induced sterile inflammation.**

When cells have been sufficiently damaged so that apoptosis cannot occur, then necrosis happens. The loss of plasma membrane integrity allows intracellular material to escape from necrotic cells. These molecules include (i) purine metabolites, cytokines like IL-1α and IL-33, (ii) IL-1 receptor agonist, and (iii) high-mobility group box 1 (HMGB1) proteins and heat shock proteins (HSP). These stimuli can trigger sterile inflammation.

(Adapted from CHEN, G. Y. & NUNEZ, G. 2010. Sterile inflammation: sensing and reacting to damage. *Nat Rev Immunol*, 10, 826-37.)

## 1.2 Interleukin-33 and its receptor system

### 1.2.1 Cytokine

Cytokines are regulatory peptides, produced by every nucleated cell (Thomson and Lotze, 2003). They are small (around 8 to 40 kDa) proteins or glycoproteins that are produced by cells in response to an activating stimulus. Up to now, more than 200 cytokine-like molecules, including 38 interleukins, have been identified. Many of the cytokines possess overlapping functions and many of the functions are still unexplored. Cytokines bind to their specific receptors and perform pleiotropic functions in different cells and in different *in vivo* contexts as shown in table 1-1. They act as a coordinated cytokine network and very likely affect every biological process, primarily in homeostasis, immunity and disease (Dinarello, 2007).

#### 1.2.1.1 Cytokine classification

Cytokines can be classified based on their source, function and structure (Thomson and Lotze, 2003, Dinarello, 2000, Dinarello, 2007).

According to their cellular sources, cytokines can be classified into:

- Lymphokines - cytokines produced by lymphocytes
- Monokines - cytokines produced by monocytes and macrophages
- Interleukins (IL) - cytokines produced by leukocytes

Based on their function, cytokines can be divided into:

- Pro-inflammatory cytokines - responsible for acute inflammation
- Anti-inflammatory cytokines - inhibiting inflammation and supporting healing
- Growth factors – required for cell growth and survival

- Chemokines – responsible for directional movement of cells

According to their structure, the cytokines can be classified into 7 cytokine families:

- The hematopoietin family (e.g. IL-2, IL-4, IL-5, IL-13, IL-15, IL-21)
- The Interferon family (IFNα, IFNß, IFNγ)
- The tumour necrosis factor (TNF) family (TNFα, LTα, LTß, RANKL, APRIL, *et al*)
- The IL-1 family (IL-1α, IL-1ß, IL-1Ra, IL-18, IL-33, IL-36α, IL-36ß, IL-36γ, IL-36Ra, IL-37, IL-38)
- The IL-17 family (IL-17, IL-25)
- The IL-10 family (IL-10, IL-22)
- The IL-12 family (IL-12, IL-23, IL-35, IL-27)
- The TGF family (TGF-α,ß)
- The chemokine family (CCL1 to 28, CXCL1 to 17, XCL1,2, CX3CL1)

## Table 1-1. Functional classification of cytokines and receptors. [a)]

| Functional class | Primary property | Other effects | Examples |
|---|---|---|---|
| Lymphocyte growth factors | clonal expansion | Th1/Th2/Th17 polarization | IL-2, IL-4, IL-7, IL-17, IL-15 |
| Th1 cytokines | ↑ Th1 responses | clonal expansion of CTL | IFN-γ, IL-2, IL-12, IL-18 |
| Th2 cytokines | ↑ Th2 responses | ↑ antibody production | IL-4, IL-5, IL-18, IL-25, IL-33 |
| Th17 cytokines | ↑ Th17 responses, IFN-γ | autoimmune responses | IL-17, IL-23, IFN-γ |
| Pro-inflammatory cytokines | ↑ inflammatory mediators | ↑ innate immune responses | IL-1α, IL-1β, TNF-α, IL-12, IL-18, IL-23, MIF, IL-32, IL-33, CD40L |
| Anti-inflammatory cytokines | ↓ inflammatory genes | ↓ cytokine-mediated lethality,↓ autoimmune disease | IL-10, IL-13, TGF-β, IL-22, IL-1Ra, IFN-α/β |
| Adipokines | pro-inflammatory | pro-atherogenic, anti-inflammatory | IL-1α, TNF-α, IL-6, leptin, adiponectin, resistin |
| gp130 signaling cytokines | growth factors | B cell activation, acute phase | IL-6, CNTF[c)], IL-11, LIF[c)], CT-1[c)] |
| Nerve growth factors | ↑ nerve/Schwann cells | B cell activation | BNDF[c)], NGF[c)] |
| Osteoclast-activating cytokines | bone resorption | immune stimulation | RANKL[c)] |
| Colony-stimulating factors | hematopoiesis | pro- and anti-inflammatory | IL-3, IL-7, G-CSF, GM-CSF, M-CSF |
| Angiogenic cytokines | neovascularization | pro-metastatic | VEGF[c)], IL-1, IL-6, IL-8 |
| Mesenchymal growth factors | fibrosis | pro-metastatic | FGF, HGF, TGF-β, BMP[c)] |
| Type II IFN | macrophage activation | ↑ MHC class II | IFN-γ |
| Type I IFN | anti-viral, ↑ MHC class I | anti-inflammatory, anti-angiogenic | IFN-α, IFN-β |
| Chemokines[b)] | ↑ cellular emigration | ↑ cell activation | IL-8, MCP-1, MIP-1α, others |

a) Does not include soluble cytokine receptors such as sTNFRp55, sTNFRp75, sIL-1R type II, IL-18 binding protein, osteoprotegerin.

b) The chemokine family includes CC and CXC chemokines with over 30 members.

c) BMP, bone morphogenic protein; BNDF, brain-derived neurotrophic factor; CNTF, ciliary neurotrophic factor; CT-1, cardiotrophin-1; LIF, leukemia inhibitory factor; NGF, nerve growth factor; RANKL, receptor activator of NF-κB ligand; VEGF, vascular endothelial growth factor.

(Adapted from DINARELLO, C. A. 2007. Historical insights into cytokines. *Eur J Immunol*, 37 Suppl 1, S34-45.)

### 1.2.1.2 The IL-1 family of cytokines

The IL-1 family consists of about 11 members including IL-1α, IL-1β, IL-1Ra, IL-18, IL-33, IL-36α, IL-36β, IL-36γ, IL-36Ra, IL-37 and IL-38 (van de Veerdonk et al., 2012). This cytokine family is characterized by containing the compiling β-trefoil structures of IL-1-like cytokine domain (Dinarello, 1994, Dinarello, 2012, Garlanda et al., 2013).

There are several important features of the IL-1 family members:

a) All have the IL-1-like domain (Garlanda et al., 2013).
b) Lack of a signal peptide that addresses the proteins to the endoplasmic reticulum and Golgi pathway. Therefore, they are released through unconventional secretory mechanisms, via secretory lysosomes, exosomes or exocytosis vesicles (Carruth et al., 1991) or when cells undergo necrosis (Hogquist et al., 1991).
c) All the members signal via a hetrodimer receptor complex consisting of a receptor and a co-receptor (Gabay et al., 2010).
d) The function of IL-1 cytokines is self-regulated either by a soluble receptor or antagonising protein (Garlanda et al., 2013).
e) Some members have dual functions as both a transcription factor in the nucleus or as a cytokine when released, for instance, IL-1α, IL-33 and IL-37 (Gabay et al., 2010, Boraschi et al., 2011, Roussel et al., 2013).

#### 1.2.1.2.1 Functions of IL-1 family members

This family plays different but important roles in immunity and disease. As shown in Table 1-2: IL-1α and IL-1β are closely associated with the inflammatory response and Th17 polarisation (Mills et al., 2013). IL-1 function can be regulated by IL-1 receptor antagonist (IL-1Ra) (Joosten et al., 1996, Palmer et al., 2003). IL-18 is mainly involved in Th1 development and response by

potentiating IL-12's effect which can be inhibited by IL-18 binding protein (IL-18BP), but IL-18 can also stimulate Th2 response in absence of IL-12 and IL-15 (Sedimbi et al., 2013, Dinarello et al., 2013). IL-36 is composed of IL-36α, IL-36ß, and IL-36γ isoforms which share the common receptor complex and play a pathogenic role in dermatitis and lung inflammation (Vigne et al., 2012, Tripodi et al., 2012). The functions of IL-36 are controlled by both IL-36 receptor antagonist (IL-36Ra) and the IL-36 binding protein IL-38 (van de Veerdonk et al., 2012). IL-37 is a recently identified immune-regulator which inhibits the inflammatory response (Nold et al., 2010). While the detailed mechanism is still poorly understood, IL-37 may do so by inducing the SMAD3, a signalling component of transforming growth factor-ß (TGF-ß) in the presence of the decoy receptor, Single Immunoglobulin IL-1 Related Receptor (SIGIRR) (Boraschi et al., 2011, Banchereau et al., 2012). Finally, IL-33 signals via ST2 and IL-1RAcP and predominantly induces a Th2 response in immunity and disease which can be regulated by soluble ST2 (sST2) receptor (Schmitz et al., 2005, Liew, 2012, Carta et al., 2013).

**Table 1-2. IL-1 family members.**

| Family name | Name | Receptor/ coreceptor | Property | Synthesized as precursor | Processing required for activity |
|---|---|---|---|---|---|
| IL-1F1 | IL-1α | IL-1RI<br>IL-1RAcP | Pro-inflammatory | Yes | No |
| IL-1F2 | IL-1β | IL-1RI<br>IL-1RAcP | Pro-inflammatory | Yes | Yes |
| IL-1F3 | IL-1Ra | IL-1RI | Antagonist for IL-1α,β | No | No |
| IL-1F4 | IL-18 | IL-18Rα<br>IL-18Rβ | Pro-inflammatory | Yes | Yes |
| IL-1F5 | IL-36Ra | IL-1Rrp2 | Antagonist for IL-36 | Yes | Yes |
| IL-1F6 | IL-36α | IL-1Rrp2<br>IL-1RAcP | Pro-inflammatory | Yes | Yes |
| IL-1F7 | IL-37 | IL-18Rα<br>IL-18BP | Anti-inflammatory | Yes | Yes |
| IL-1F8 | IL-36β | IL-1Rrp2<br>IL-1RAcP | Pro-inflammatory | Yes | Yes |
| IL1-F9 | IL-36γ | IL-1Rrp2<br>IL-1RAcP | Pro-inflammatory | Yes | Yes |
| IL-1F10 | IL-38 | IL-1Rrp2 | ? Antagonist | Yes | ? No |
| IL-1F11 | IL-33 | ST2<br>IL-1RAcP | Pro-inflammatory | Yes | No |

(Adapted from CARTA, S., LAVIERI, R. & RUBARTELLI, A. 2013. Different Members of the IL-1 Family Come Out in Different Ways: DAMPs vs. Cytokines? *Front Immunol*, 4, 123.)

## 1.2.2 IL-33

Interleukin 33 (IL-33) was identified as a new IL-1 family member by Schmitz *et al.*, in 2005.

### 1.2.2.1 IL-33 gene and protein

The gene encoding *il33* was originally identified as the *Dvs27* gene, which is upregulated in canine vasospastic cerebral arteries after experimental subarachnoid haemorrhage in 1999 (Onda et al., 1999), and as the gene for the nuclear factor from high endothelial venules (NF-HEV) described in 2003 (Baekkevold et al., 2003). The human *il33* gene is located on chromosome 9 and mouse *il33* on chromosome 19; both genes contain 7 exons (Fig. 1-3).

The human and mouse *il33* genes encode proteins of 270 and 266 amino acids with a molecular weight of IL-33 about 30 and 29.9kDa, respectively. Human and mouse IL-33 share 55% homology at the amino-acid level. Furthermore, within the IL-1 family, IL-33 most closely resembles IL-18 (Schmitz et al., 2005).

As with other members of the IL-1 family, IL-33 is produced primarily as a precursor protein, which is the full-length IL-33 (fl-IL-33). The N-terminal of fl-IL-33 contains a Helix-turn-helix (HTH) motif and a nuclear location sequence (NLS), which facilitates the transport of fl-IL-33 into the cell nucleus (Carriere et al., 2007). Fl-IL-33 also possesses several enzyme cleavage sites and can be differently processed by these proteases (Liu et al., 2013) (Fig. 1-4). It has been reported that fl-IL-33 can be processed into mature IL-33 (mIL-33) by elastase, cathepsin G and proteinase 3 which are produced by neutrophils during inflammation (Ali et al., 2007, Lefrancais et al., 2012). Cleavage creates three active forms of mIL-33: IL-$33_{95\text{-}270}$, IL-$33_{99\text{-}270}$ and IL-$33_{109\text{-}270}$; between 18 and 21 kDa in human and 20 kDa mIL-$33_{102\text{-}266}$ in mouse (Fig. 1-4). While both full-length

and mature IL-33 can bind to its receptor, it has been demonstrated that mature IL-33 has 10-fold greater bioactivity than the full-length protein (Talabot-Ayer et al., 2009). IL-33 also contains caspase 3 and 7 sites in the IL-1-like cytokine domain (Fig. 1-4) and the cleavage by these enzymes results in degradation and inactivation of IL-33 (Bae et al., 2012, Kakkar et al., 2012). Since caspase 3 and 7 are induced when cells undergo apoptosis this suggests that IL-33 may not play an important role in apoptosis.

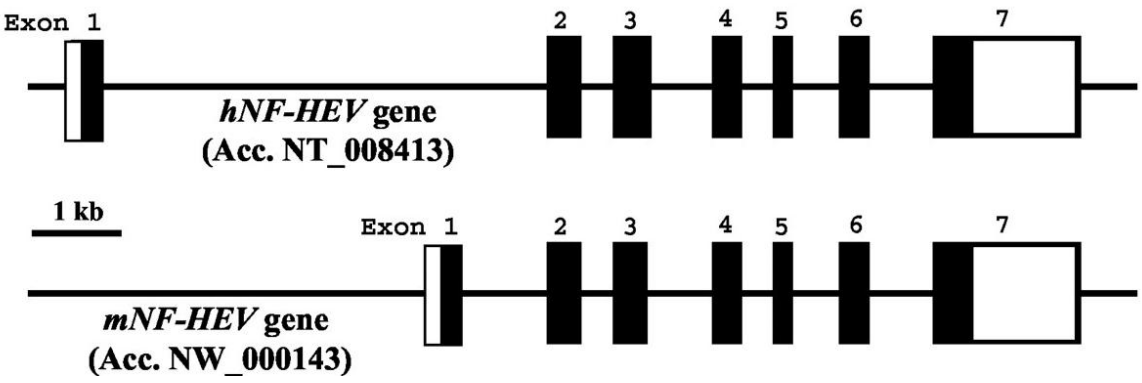



**Fig. 1-3 Genomic structure of the human and mouse NF-HEV (*il33*) genes.**

Open boxes indicate non-translated exon sequences and black boxes indicate coding exon sequence. The two genes share a similar organization with seven exons. (Adapted from BAEKKEVOLD, E. S., ROUSSIGNE, M., YAMANAKA, T., JOHANSEN, F. E., JAHNSEN, F. L., AMALRIC, F., BRANDTZAEG, P., ERARD, M., HARALDSEN, G. & GIRARD, J. P. 2003. Molecular characterization of NF-HEV, a nuclear factor preferentially expressed in human high endothelial venules. *Am J Pathol*, 163, 69-79.)

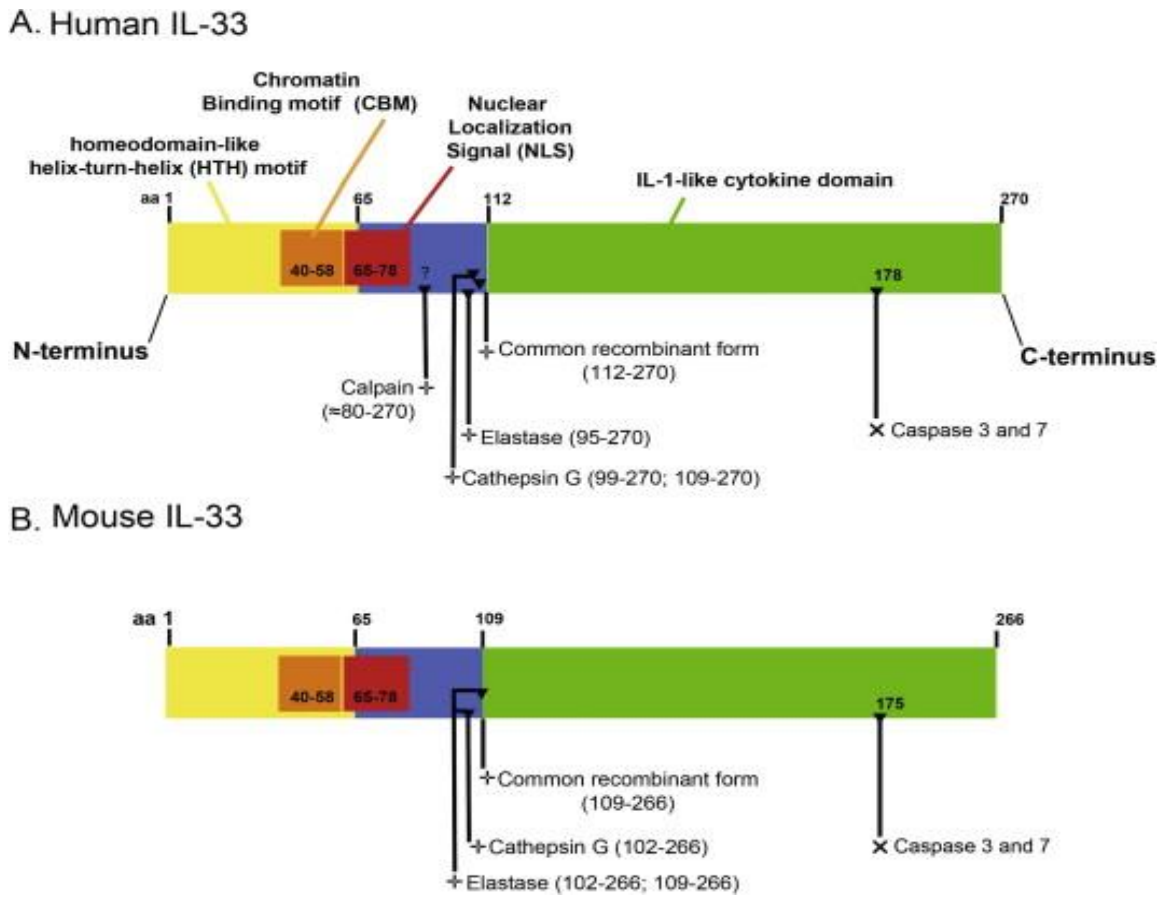



**Fig. 1-4 IL-33 protein structure and processing sites.**

Intracellular proteases (caspases or calpain) or extracellular proteases from immune cells cleave IL-33 at indicated processing sites of human (A) and mouse (B) IL-33; these cleavages either deactivate IL-33 or generate more bioactive mature IL-33.

(Adapted from Quan Liu & Hēth R. Turnquist. 2013. Implications for Interleukin-33 in solid organ transplantation. *Cytokine*, 62, 183-194.)

### 1.2.2.2 Expression, location and release of IL-33

IL-33 is widely distributed in a range of tissues, including brain, lung, heart, gut, joints, skin, spleen and lymph nodes. IL-33 is expressed in a variety of non-haematopoietic cells, mainly the innate immune cells, including fibroblasts, adipocytes, smooth muscle, endothelial and epithelial cells, macrophages and dendritic cells (Sattler et al., 2013). In contrast, adaptive immune cells (T and B lymphocytes) do not make IL-33.

IL-33 is synthesised at a very low level and is difficult to detect in serum in homeostatic conditions. However, it can be readily detected in inflamed tissue, organs and serum during trauma, and in infectious and inflammatory disorders, suggesting that IL-33 is closely associated with inflammatory conditions (Miller, 2011, Liew, 2012, Hu et al., 2013, Januzzi, 2013, Pastorelli et al., 2013).

Using an IL-33–LacZ gene trap reporter strain of mice, Melanie Pichery analyzed the expression and location of endogenous IL-33 in mice. She found that once expressed, IL-33 protein was always localized in the nucleus of producing cells (Pichery et al., 2012). This is due to fl-IL-33 protein containing the HTH and NLS domains at the N-terminal which allow the translocation of the IL-33 into the nucleus. This is consistent with the early finding that IL-33 is a nuclear factor from high endothelial venules (Baekkevold et al., 2003, Moussion et al., 2008).

As with other IL-1 family members, fl-IL-33 protein does not have a leading sequence and current evidence suggests that IL-33 can be secreted by some cells in certain culture conditions or released when cells undergo damage or necrosis (Moussion et al., 2008, Sattler et al., 2013).

It has been reported that IL-33 can be secreted without cell damage *in vitro*. Human bronchial epithelial cells cultured with extracts of the fungus Alternaria

which causes allergy can stimulate the secretion of IL-33 into the culture supernatant without affecting cell viability (Kakkar et al., 2012). While the precise mechanism is largely unknown, it has been shown that the secretion of IL-33 is dependent on the concentration of intracellular calcium and ATP in astrocytes and glial cells when stimulated with TLR ligands (Hudson et al., 2008, Zhang et al., 2011, Kouzaki et al., 2011).

How IL-33 is secreted from intact cells is incompletely understood. It is possible that IL-33 is secreted via the unconventional secretion pathway used by IL-1 family members, which uses secretory lysosomes, exosomes or exocytosis vesicles but not the classic protein secretion pathway. However, whether IL-33 can be naturally secreted by cells *in vivo* is largely unknown (Kakkar et al., 2012).

IL-33 is constitutively expressed and stored in the nucleus of cells. Current evidence suggests that IL-33 may be mainly released *in vitro* and *in vivo* when cells undergo damage or necrosis (Lefrancais et al., 2012, Lefrancais and Cayrol, 2012). The release of fl-IL-33 when cells undergo chemical or physical injury, infection or necrosis has been demonstrated in different *in vitro* conditions (Cayrol and Girard, 2009, Andronicos et al., 2012). *In vivo*, extracellular IL-33 has been detected in many inflammatory, traumatic and infectious conditions in human and mouse blood and tissue fluids, and in organs where cells have been damaged (Matsuyama et al., 2010, Le Goffic et al., 2011, Lefrancais and Cayrol, 2012, Pushparaj et al., 2013).

Once released, fl-IL-33 can be processed into mature IL-33 by the neutrophil serine proteases, cathepsin G and elastase secreted during infection or inflammation. The secreted full-length or mature IL-33 can serve as a cytokine which binds to its receptors and triggers signalling pathways and plays an important role in immunity and disease (Lefrancais et al., 2012, Luzina et al., 2012).

However, during apoptosis, IL-33 will be cleaved by caspase 3 and 7, and degraded and inactivated (Luthi et al., 2009, Ali et al., 2010). Therefore, IL-33 may mainly serve as a danger signal for trauma, inflammation and infection in the host (Lamkanfi and Dixit, 2009).

### 1.2.3 IL-33 receptors

IL-33 signals via its receptor complex consisting of ST2 and IL-1RAcp.

#### 1.2.3.1 ST2

ST2 (also designated T1 or DER4) was originally identified as an early responding protein in mouse fibroblasts (BALB/c- 3T3 cells) stimulated by serum or oncogene but not in resting cells (Tominaga, 1989, Tominaga et al., 1991). ST2 contains three immunoglobulin-like domains in the extracellular region for ligand binding, and a toll-IL-1R (TIR) domain in the cytoplasmic region for signalling, thereby belonging to the IL-1R/toll-like receptor (IL-1R/TLR) family. The ST2 gene is located close to the IL-1R genes on mouse chromosome 1 and human chromosome 2 (Tominaga et al., 1991). In the mouse, differential mRNA splicing within the ST2 gene generates two mRNAs of 2.7 and 5 kb, which translates into a shorter secreted form (soluble ST2 or sST2) and a longer, trans-membrane form (ST2L) of the protein, respectively. The sST2 is identical with the extracellular region of ST2L but with an additional nine amino acids at the C terminus (Tominaga et al., 1999). Transcription of ST2 is controlled by two distinct promoters: an upstream promoter directs transcription in haemopoietic cells such as mast cells, while a promoter 10.5 kb downstream directs expression in fibroblasts (Tominaga et al., 1991, Yanagisawa et al., 1993).

ST2L is the signal receptor for IL-33. It is expressed in a wide range of cells: innate immune cells (monocytes, macrophages, DCs, fibroblasts, eosinophils,

basophils, mast cells and ILC2) and subsets of lymphocytes (NKT, CD8, Th2 and B1 cells). ST2L was found to be expressed on Th2 but not on Th1 cells and thus may serve as a stable marker to distinguish between these two T cell lineages (Xu et al., 1998). Current evidence suggests that soluble ST2 is a decoy receptor which is antagonistic to IL-33 function *in vitro* and *in vivo*. Expression of sST2 is highly induced during trauma, inflammation and infection, which also correlates with induction and function of IL-33. Given the pro-inflammatory role of IL-33, sST2 production may have a protective effect against over-activated IL-33-mediated inflammatory responses (Liew et al., 2010, Palmer and Gabay, 2011, Salas, 2013).

The widespread gene expression of ST2 in a wide range of cell types indicates that it might serve important functions across a broad spectrum of biological systems. Indeed, increasing reports demonstrate that ST2 mediates important functions in immunity and in diseases as demonstrated using ST2 deficient mice, neutralising antibody or sST2 (Mato et al., 2009, Sedhom et al., 2013, Sattler et al., 2013).

### 1.2.3.2 IL-1RAcP

IL-1 receptor accessory protein (IL-1RAcP) was originally identified as the IL-1R co-receptor. IL-1 only binds to IL-1RI but not to IL-1RAcP (Dinarello, 1994). However, IL-1RAcP can bind to IL-1RI, thereby increasing the affinity of IL-1RI for IL-1 about 5-fold (Greenfeder et al., 1995). IL-1RAcP is also a co-receptor for IL-36 and IL-33 (Garlanda et al., 2013).

IL-33 binds to the receptor ST2 but not to IL-1RAcP. The binding and interaction of IL-33 with ST2 subsequently recruits the accessory receptor IL-1RAcP. The IL-33 binding induced receptor heterodimerization leads to the juxtaposition of the intracellular toll/IL-1 receptor (TIR) domain of both receptors which is required

for triggering downstream signalling pathways in target cells (Schmitz et al., 2005, Martin, 2013).

### 1.2.3.3 IL-33/ST2 signalling

The precise IL-33 signalling pathways are not yet fully understood. Available evidence suggests that the MyD88/IRAK/TRAF6 pathway is critically involved in IL-33 signalling (Martin, 2013) (Fig. 1-5).

The TIR-dimers of ST2 and IL-1RAcP complex initially recruit the adaptor protein MyD88 to the TIR-dimers by homotypic protein–protein interaction with its own C-terminal TIR domain. The MyD88 then recruits the IRAK-4 and IRAK-1 into the signal transduction platform which results in the activation of IRAK-4 which then phosphorylates and activates IRAK-1 (Suzuki et al., 2002, Burns et al., 2003, Loiarro et al., 2005). Once activated IRAK-1 dissociates from MyD88 and the receptor complex and interacts with Tumour Necrosis Factor receptor associated factor 6 (TRAF6) (Kollewe et al., 2004). TRAF6 then activates TAK1 which results in the activation of the classical NF-κB pathway, the activation of stress-activated protein kinase p38 and c-Jun N-terminal kinases (JNK) (Bonizzi and Karin, 2004). In parallel, extracellularly regulated kinases (ERK1/2) become activated (Schmitz et al., 2005, Funakoshi-Tago et al., 2008). Other related pathways, including the PI-3K/PKB/mTOR pathway and the JAK/STAT pathway may also be involved in IL-33 signalling in different cells (Pecaric-Petkovic et al., 2009, Salmond et al., 2012). These pathways may act synergistically to induce gene expression leading to, for example, cytokine and chemokine synthesis (Pecaric-Petkovic et al., 2009, Salmond et al., 2012, Mirchandani et al., 2012).

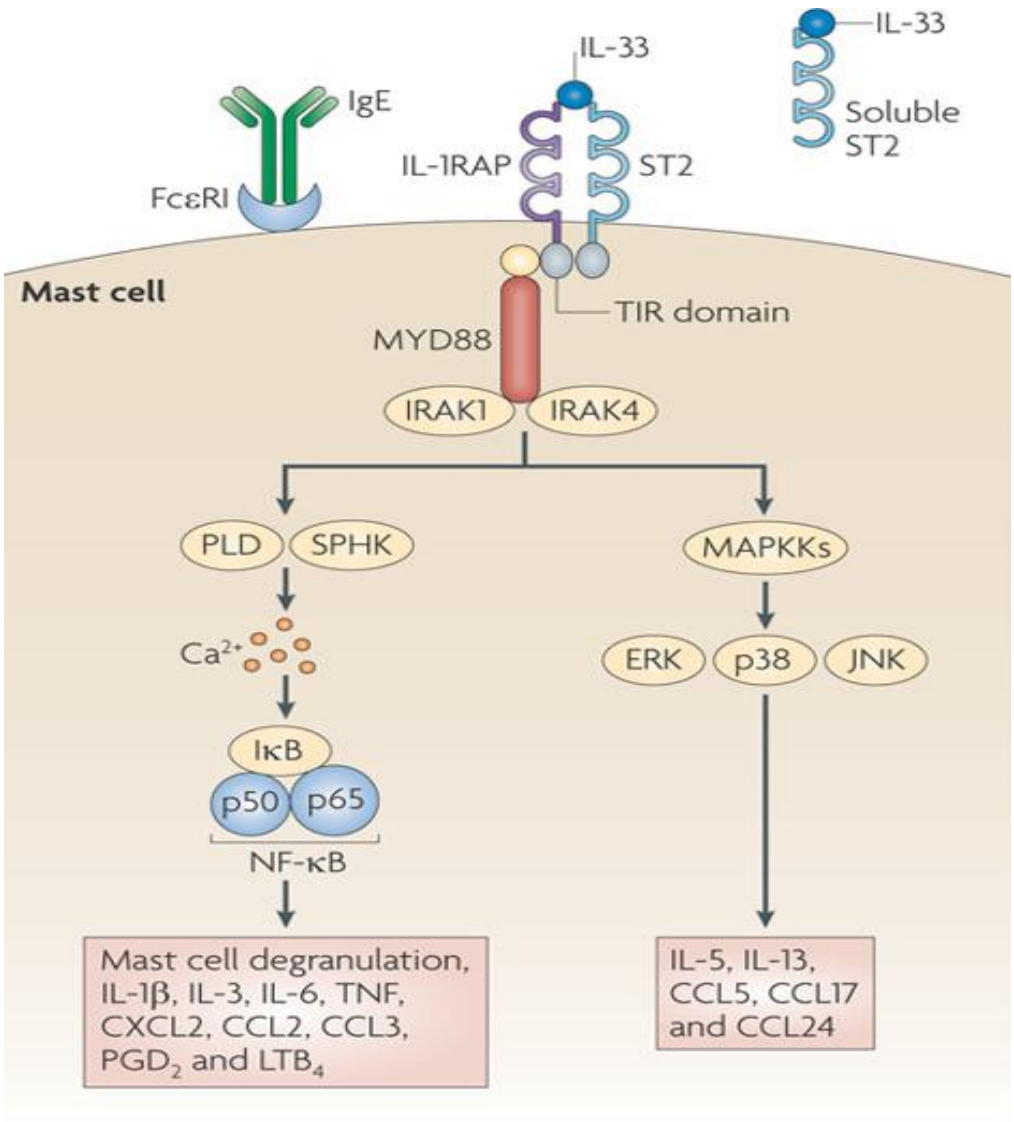



**Fig. 1-5 IL-33 signal pathways.**

IL-33 signalling via ST2 and IL-1Rap dimmers leads to the recruitment of MYD88 complex. The complex may activate two independent pathways: the pLD-SPHK pathway that leads to Ca2+ mobilization and activation of NF-κB; the MApK pathway is mediated by the activation of ERK, p38 and JNK via MApKKs. These two pathways may act synergistically to induce gene expression of other cytokines and chemokines. Soluble ST2 can act as a decoy receptor by binding to IL-33 directly.

(Adapted from LIEW, F. Y., PITMAN, N. I. & MCINNES, I. B. 2010. Disease-associated functions of IL-33: the new kid in the IL-1 family. *Nat Rev Immunol*, 10, 103-10.)

### 1.2.4 Biological functions of the IL-33 system

#### 1.2.4.1 Function of nuclear IL-33

Fl-IL-33 contains the HTH motif and is constitutively located in the nucleus of the producing cell. The function of IL-33 in the nucleus is poorly understood, however the HTH motif is necessary for fl-IL-33 to translocate into the nucleus. Full length IL-33 then can bind to heterochromatin and repress cellular gene expression in a promoter-reporter gene assay (Carriere et al., 2007, Roussel et al., 2008).

Furthermore, fl-IL-33, but not mature IL-33, interacts with the free and activated transcription factor NF-κB p65. The IL-33/NF-κB complex reduces NF-κB p65 binding to its cognate DNA response element and impairs p65-triggered transactivation. Over-expression of IL-33 reduces the IL-1–stimulated expression of NF-κB target-genes such as *IkBa, TNF-a,* and *C-REL*. Since IL-1 and NF-κB pathways play a pro-inflammatory role in many inflammatory responses by inducing other gene expressions, these results suggest that in contrast to free IL-33, the nuclear IL-33 may act as a transcriptional regulator/repressor and play an important role in gene regulation by directly binding to chromosomal DNA. While the function of nuclear IL-33 in immunity and disease is poorly understood, it has been shown that the over-expression of nuclear fl-IL-33 may contribute to the development of lung inflammation and fibrosis via an undefined mechanism (Luzina et al., 2013). Therefore, IL-33 is a dual-functional mediator, which can act as a cytokine when released and a nuclear factor when translocated to the cell nucleus (Luzina et al., 2012, Luzina et al., 2013).

I next will focus on the cytokine function of IL-33 in immunity and in disease models. The function of nuclear IL-33 will be discussed further in Chapter 6.

## 1.2.4.2 Function of cytokine IL-33

Current evidence suggests that IL-33 is a pleiotropic cytokine which signals via its receptor ST2 and can elicit different innate and adaptive immune responses depending on *in vivo* context (Sattler et al., 2013)

### 1.2.4.2.1 Effect on innate immune cells

The innate immune response is the first line of host defence against infection but is also involved in inflammatory and stress responses. Accumulating evidence demonstrates that IL-33 is predominantly involved in innate immune response in immunity and in disease. The epithelium and endothelium are the cell layers in mucosal organs primarily protective against infection and stress. IL-33 is expressed primarily in the epithelium and endothelium and can be released when cells sense inflammatory signals or undergo necrosis. The IL-33 receptor, ST2, is expressed by almost all innate cells. Therefore once released, IL-33 signalling via ST2 can induce innate immune responses by directly activating a wide range of innate immune cells including the key innate cells, eosinophils, macrophages, DCs, type 2 innate lymphoid cells (ILC2) and mast cells in the organs (Lamkanfi and Dixit, 2009, Palmer and Gabay, 2011, Mirchandani et al., 2012, Komai-Koma et al., 2012). Research findings from the author's laboratory have demonstrated several important functions of IL-33 in these key innate cells as described below.

**Eosinophils**

Eosinophils are closely associated with allergic response and contribute by secreting cytokines, chemokines and inflammatory molecules (Cherry et al., 2008). Stolarski *et al*. have shown that IL-33 is a novel factor for the differentiation of eosinophils from bone marrow progenitor cells (Stolarski et al., 2010). IL-33 can enhance ST2L expression on eosinophils, and IL-33 induced the

production of IL-13, CCL17, and TGF-beta by eosinophils *in vitro* and *in vivo*. These inflammatory mediators can then enhance the eosinophil-mediated inflammatory response in allergy and asthma *in vivo* (Salmond et al., 2012, Oh et al., 2013).

**Macrophages**

Macrophages are important innate immune cells with three key functions: phagocytosis of microbial and apoptotic cells, antigen-presentation via their MHC class I and II molecules and secretion of a wide range of cytokines, chemokines and inflammatory mediators (Martinez et al., 2008). The macrophages can differentiate into two subset phenotypes: M1 (classically activated) by IFN-γ and LPS or M2 (alternatively activated) by IL-4 and IL-13 (Gordon, 2003, Wynn and Barron, 2010, Biswas and Mantovani, 2010).

Kurowska-Stolarska *et al*. found that IL-33 potentiates IL-13-mediated polarisation of M2 and contributes to airway inflammation (Kurowska-Stolarska et al., 2009). *In vitro,* IL-33 enhanced IL-13-induced polarization of alveolar- and bone marrow-derived macrophage toward an M2 phenotype by increasing the expression of arginase I, Ym1, and chemokines CCL24 and CCL17. *In vivo*, the injection of recombinant IL-33 enhanced airway inflammation and the development of alveolar M2 cells in mice (Van Dyken and Locksley, 2013).

**Mast cells**

Mast cells are the key granular myeloid cells and are closely associated with homeostasis and disease. Mast cells are located in most organs and can be amplified during inflammatory conditions (Metcalfe et al., 1997).

Xu *et al*. found that mast cells expressed high levels of ST2 and responded directly to IL-33 to produce a spectrum of inflammatory cytokines and

chemokines *in vitro*. *In vivo*, IL-33 exacerbates inflammatory arthritis by increasing the production of pro-inflammatory cytokines and increases mast cell degranulation in the joints (Xu et al., 2008, Xu et al., 2010). Thus, the IL-33-mast cell pathway plays a pathogenic role in the exacerbation of inflammatory arthritis in mice.

Komai-Koma *et al*. found that IL-33 enhanced IgE production in naive mice depending on IL-4 mainly produced by innate cells including mast cells (Komai-Koma et al., 2012). The increased IgE then binds to IgE receptors on mast cell surface. Together the IL-33/ST2 and IgE signals triggered mast cell degranulation and systemic anaphylaxis in allergen-naive mice.

**Innate lymphoid cells**

Innate lymphoid cell 2 (ILC2s) are newly identified lymphocytes which do not express T cell or B cell antigen receptors but express ST2 and are closely associated with IL-33 function. The ILC2s produce type II cytokines IL-5, IL-9 and IL-13 in response to IL-33. ST2 signals are required for the optimal proliferation and activation of ILC2 cells (Spits et al., 2013).

Since ILC2s mainly produce IL-5 and IL-13 they play an initial and amplificatory role in innate and adaptive type 2 responses. Therefore, ILC2s are critically involved in anti-helminth infection but also in allergic disorders.

These findings suggest that IL-33 is exclusively expressed by innate immune cells and is sufficient to activate a wide range of innate immune cells via their ST2 and to initiate innate immune response.

### 1.2.4.2.2 Effect on adaptive immune cells

The adaptive immune response is induced mainly by the T (CD4 and CD8) and B lymphocytes which express antigen receptors. Among the adaptive immune cells, ST2 is selectively expressed on Th2, IL-5-producing Th cells, CD8 and B1 cells (Sattler et al., 2013).

**Th2 cells**

It has been reported before the identification of IL-33 that the orphan receptor ST2L was expressed constitutively and stably on the surface of Th2 but not Th1 cells (Xu et al., 1998). ST2L is also related to Th2 but not Th1 cell function *in vitro* and *in vivo*. Thus ST2L is a reasonable marker distinguishing Th2 from Th1 cells and is also associated with Th2 cell function. This finding has been confirmed by the report that ST2L is the IL-33 receptor and IL-33 is able to directly activate Th2 cells *in vitro* and mainly induces Th2 type immune response *in vivo* (Schmitz et al., 2005).

**IL-5$^+$ Th cells**

IL-5$^+$ Th cells are IL-5 producing CD4+ T cells: Kurowska-Stolarska *et al*. found that IL-33 directly polarizes naive CD4+ T cells into a novel T cell population which primarily produces IL-5 and IL-13 but not IL-4. This novel Th cell phenotype is different from other Th2 cells because its polarization requires ST2 and MyD88 but not IL-4/IL-4R or STAT6. Furthermore, it depends on the phosphorylation of MAPKs and NF-κB but not the induction of Th2 transcription factor GATA3. *In vivo*, adoptive transfer of the IL-33-polarized IL-5(+) IL-4(-) T cells triggered allergic airway inflammation in naive IL-4 knockout mice. Thus IL-33 selectively polarises IL-5-producing T cells which play a critical role in allergic response independent of IL-4 (Kurowska-Stolarska et al., 2008).

**CD8+ T cells**

$CD8^+$ T cells are cytotoxic T cells (CTL) which play an important role in host defence against infection and cancer. $CD8^+$ T cells do so by secreting cytotoxic granular molecules, perforin and granzymes (Green et al., 2003, Ashton-Rickardt, 2005). It has been reported that $CD8^+$ T cells express ST2 (Yang et al., 2011, Gao et al., 2013). IL-33 is necessary for $CD8^+$ T cell responses that induce protection against RNA and DNA viruses in mice (Bonilla et al., 2012). IL-33 signals through ST2 on activated CTLs, enhances clonal expansion and differentiation of CTL and is necessary for virus control (Komai-Koma et al., 2009, Yang et al., 2011).

**B1 cells**

B1 B lymphocytes produce natural IgM and play a critical role in the early defence against bacterial and viral infections. Komai-Koma *et al*. have reported that B1 but not B2 cells express the IL-33 receptor ST2. IL-33 markedly activated B1 cell proliferation and enhanced IgM, IL-5, and IL-13 production *in vitro* and *in vivo* in an ST2-dependent manner (Komai-Koma et al., 2011).

**Indirect effects on other adaptive immune cells**

It is noteworthy that IL-33 is also capable of promoting Th1 and/or Th17 type responses and B2 cell antibody production in different *in vivo* contexts in mice such as increasing IFN-γ production in presence of IL-12 and IL-17 released from effector memory T cells, by as yet undefined mechanisms (Komai-Koma et al., 2007, Yang et al., 2011, Wakahara et al., 2012). Since these cell subsets do not express ST2 it is likely that IL-33 promotes these responses indirectly by enhancing the cytokines production required for their polarisation or functions *in vivo* (Xu et al., 2008).

## 1.2.5 The role of the IL-33 system in health and disease

Growing evidence demonstrates that IL-33 system plays a critical role in health and disease. This is mainly because IL-33 is a pleiotropic cytokine and induced by

infection, stress and inflammation and ST2 is expressed on a wide range of immune cells (Sattler et al., 2013). I will mainly describe the key function of IL-33 in infectious, allergic and inflammatory disorders.

### 1.2.5.1 Role of IL-33 in infections

Current evidence suggests that IL-33 plays different roles in parasitic, bacterial and viral infections (Liew et al., 2010).

**Parasite infection**

An increasing number of reports demonstrate that the IL-33/ST2 system contributes to host defence against parasite infections (Table 1-3). It has been reported that the IL-33 system protects against *Leishmania major* (Kropf et al., 2003), *Trichuris muris* (Humphreys et al., 2008), *Toxoplasma gondii* (Jones et al., 2010), *Nippostrongylus brasiliensis* (Harvie et al., 2013) and *Strongyloides venezuelensis* (Yasuda et al., 2012). The protective effect of the IL-33 system is mainly due to its key role in the induction of Th2 responses which are required for the expulsion of the parasites (Sattler et al., 2013).

**Bacterial infection**

The IL-33 system is also critically involved in protection against some bacterial infections (Sattler et al., 2013) (Table 1-3). The IL-33 system is required for the induction of protective immunity against Gram-negative bacterial sepsis and *Pseudomonas aeruginosa* infection (Hazlett et al., 2010). These beneficial effects of IL-33 signals are due to the pleiotropic effect on a given pathogen and immune context as reported. However, the IL-33 system seems less important in protection against *Mycobacterium tuberculosis* infection, because ST2-deficient mice developed a normal host defence against this pathogen (Wieland et al., 2009). This suggests that the role of the IL-33 system in bacterial infection may

vary depending on the type of bacteria, the levels of IL-33 expressed within the infected cell type and the host immunity.

**Virus infection**

There is limited information suggesting that the IL-33 system is implicated in protection from viral infections. However, available evidence indicates that IL-33 may play different roles in different virus infections; it protects against LCMV (Bonilla et al., 2012) but promotes the infection of influenza (Le Goffic et al., 2011).

Thus, current evidence suggests that the IL-33 system may have a beneficial effect on parasite infection. However, its role in bacterial and virus infection varies depending on the types of pathogen, the levels of IL-33 expressed within the infected cell type and the host immunity. Further studies are required to gain a fuller appreciation of the role of the IL-33 system in infection.

**Table 1-3. Implication of IL-33 and its receptor ST2L in host immune defence.**

| Organism / experimental setting | Evidence for IL-33 involvement | References |
|---|---|---|
| **Parasites** | | |
| *Leishmania major* | ST2L expressing CD4 T cells localize at site of infection | (Kropf et al., 2002) |
| | ST2L signalling regulates excessive type 1 responses | (Kropf et al., 2003) |
| *Toxoplasma gondii* | Infection upregulates ST2 mRNA | (Jones et al., 2010) |
| | $ST2^{-/-}$ mice are more susceptible to infection | |
| *Trichuris muris* | Infection upregulates IL-33 expression | (Humphreys et al., 2008) |
| | IL-33 induces parasite expulsion and secretion of TSL, IL-4, IL-9, and IL-13 | |
| *Nippostrongylus brasiliensis* | ILC expand in response to IL-33 and are sufficient for worm clearance | (Neill et al., 2010, Price et al., 2010) |
| *Strongyloides venezuelensis* | Infection induces pulmonary accumulation of iLC which proliferate and produce IL-5 and IL-13 in response to IL-33 | (Yasuda et al., 2012) |
| **Bacteria** | | |
| Bacterial TLR agonists and other bacterial PAMP mimics | Upregulation of IL-33 mRNA | (Hudson et al., 2008, Nile et al., 2010, Polumuri et al., 2012, Shimosato et al., 2010, Zhang et al., 2011) |
| Lipopolysaccharides | IL-33 enhances LPS-induced inflammatory cytokine production by macrophages | (Espinassous et al., 2009) |
| *Pseudomonas aeruginosa* | IL-33 dampens inflammation and tissue damage due to M2 macrophage polarization resistance against keratitis | (Hazlett et al., 2010) |
| Experimental sepsis | Increased neutrophil recruitment and bacterial clearance | (Alves-Filho et al., 2010) |
| | Enhanced phagocytosis and killing activity | (Le et al., 2012) |
| Leptospirosis | Increased levels of sST2 are associated with bleeding and mortality in leptospirosis | (Wagenaar et al., 2009) |
| **Virus** | | |
| Viral TLR agonists and other viral PAMP mimics | Upregulation of IL-33 mRNA | (Hudson et al., 2008, Polumuri et al., 2012) |
| Influenza virus | Upregulation of IL-33 mRNA correlates with increase in pro-inflammatory cytokines | (Le Goffic et al., 2011) |

| | | |
|---|---|---|
| Dengue virus | sST2 levels are associated with disease severity | (Houghton-Trivino et al., 2010) |
| | Negative correlation between sST2 serum levels and platelet/white blood cell count | (Becerra et al., 2008) |
| LCMV | IL-33 mediates protective antiviral $CD8^+$ T cell responses | (Bonilla et al., 2012) |
| Influenza virus | Increased IL-33/ST2 expression levels | (Le Goffic et al., 2011) |
| | $ST2^{-/-}$ infected mice have decreased lung function, loss of airway epithelial integrity and impaired respiratory tissue remodelling | (Monticelli et al., 2011) |
| | Infection induces IL-33 production by alveolar macrophages | (Chang et al., 2011) |
| **Fungus/Yeast** | | |
| *Pneumocystis murina* | IL-33 induced M2 macrophages cause enhanced fungal clearance | (Nelson et al., 2011) |
| *Candida albicans* | IL-33 enhanced neutrophil recruitment and neutrophil effector functions | (Le et al., 2012) |
| *Alternaria alternata* | Infection-induced ATP release induces IL-33 secretion | (Chaturvedi et al., 2006) |

(Adapted from SATTLER, S., SMITS, H. H., XU, D. & HUANG, F. P. 2013. The evolutionary role of the IL-33/ST2 system in host immune defence. Arch Immunol Ther Exp (Warsz), 61, 107-17.)

### 1.2.5.2 Role of IL-33 in allergic diseases

**Allergy and asthma:** exogenous or transgenic expression of IL-33 in antigen-naive mice can induce a typical Th2 response and the key asthma characteristics including type II cytokine and IgE production, airway inflammation, mucus secretion, eosinophilia and airway hyper-reactivity (AHR) in antigen-dependent or independent manners (Liew et al., 2010). It is also reported that both *il33* and *st2* polymorphisms are closely associated with asthma and allergy susceptibility in human, suggesting that IL-33 system may be a new therapeutic target (Xu et al., 2013, Sattler et al., 2013).

**Anaphylaxis:** findings from the author's laboratory have demonstrated that IL-33 amplifies IgE synthesis and triggers mast cell degranulation and anaphylaxis in mice via IL-4 (Komai-Koma et al., 2012). IL-33 can induce IgE production and anaphylaxis in the presence or absence of allergen in mice. Therefore, IL-33 may play an important role in atopic or non-atopic allergy and idiopathic anaphylaxis (Miller, 2011).

**Contact hypersensitivity:** $ST2^{-/-}$ mice developed impaired oxazolone-induced contact sensitivity (CS) compared with wild-type (WT) mice. IL-33 treatment significantly exacerbated CS in mice by inducing B1 cell proliferation and function (Komai-Koma et al., 2011). Thus, IL-33 may play an important role in delayed-type hypersensitivity.

### 1.2.5.3 Role of IL-33 in chronic inflammatory disorders

**Rheumatoid arthritis**

Rheumatoid arthritis (RA): IL-33 and ST2 are highly expressed on the synovial membrane in the joints of RA patients, and their expression level correlates with

the severity of RA (Hong et al., 2011). In RA animal models, IL-33 injection exacerbated RA in WT mice but ST2 knockout mice developed impaired collagen-induced arthritis (CIA). Mast cells which express ST2 in the joints play an important role in the IL-33-promoted CIA development and exacerbation (Xu et al., 2010). Disease exacerbation was accompanied by elevated expression levels of pro-inflammatory cytokines. Results from the author's laboratory demonstrate that IL-33 is a critical pro-arthritic cytokine for inflammatory joint disease mainly via an IL-33-driven, mast-cell-dependent inflammatory pathway (Xu et al., 2008). Thus, IL-33 and its receptor system may represent a therapeutic target for RA.

**Atherosclerosis**

Atherosclerosis is a chronic inflammatory disease of the vasculature commonly leading to myocardial infarction and stroke. Miller *et al*. have shown that IL-33 and ST2 are expressed in the normal and atherosclerotic vasculature of mice and humans. Exogenous IL-33 administration impairs the development of experimental atherosclerosis in apolipoprotein (Apo) $E^{-/-}$ mice given a high-fat diet. As reported, the treatment markedly reduced the development of severe and inflamed atherosclerotic plaques in the aortic sinus and lesions. This protective effect is associated with increased concentrations of antibodies to oxidized low-density lipoprotein (ox-LDL) and reduced inflammatory cytokines. This finding suggests that IL-33 may play a protective role in the development of atherosclerosis (Miller et al., 2008).

There is evidence for the biologic relevance of both ST2 and IL-33 in other inflammatory disease, for example lung fibrosis (Mato et al., 2009) and colitis (Pastorelli et al., 2010). I will describe these examples in detail below in sections 1.3.1.2 and 1.3.2.2 and in the General Discussion.

## Table 1-4. Role of IL-33 in diseases

| | |
|---|---|
| Asthma | IL-33 levels are elevated in clinical and experimental asthma |
| | Blocking ST2 or IL-33 attenuates disease in some models |
| | Administration of IL-33 exacerbates experimental asthma and induces features of asthma in animals |
| Allergy and anaphylaxis | In the presence of IgE, IL-33 induces anaphylactic shock |
| | IL-33 causes degranulation of IgE-primed mast cells in the skin |
| | Increased expression of IL-33 by skin cells in clinical atopic dermatitis |
| Cardiovascular disease | Serum ST2 levels increased in myocardial infarction and heart failure |
| | Protective effect of IL-33 in experimental heart failure |
| | Atherosclerosis in mice is attenuated by IL-33 and exacerbated by soluble ST2 |
| Central nervous system disease | ST2 and IL-33 detected following subarachnoid haemorrhage |
| | IL-33 associated with Alzheimer's disease where expression is increased |
| Pain | IL-33 induces cutaneous and articular hypernociception |
| Arthritis | IL-33 and ST2 are increased in the synovium in rheumatoid arthritis |
| | Blocking ST2 attenuates collagen-induced arthritis |
| | IL-33 exacerbates collagen-induced arthritis |

(Adapted from LIEW, F. Y., PITMAN, N. I. & MCINNES, I. B. 2010. Disease-associated functions of IL-33: the new kid in the IL-1 family. *Nat Rev Immunol*, 10, 103-10.)

## 1.3 Pulmonary fibrosis and ulcerative colitis

### 1.3.1 Pulmonary Fibrosis

Pulmonary fibrosis is a restrictive lung disease with impaired lung function resulting from reduced alveolar inflation. Around the world, the prevalence is around 2 to 20 cases per 100 thousand of the population. One of the most common forms of lung fibrosis is idiopathic pulmonary fibrosis (IPF) (Bradley et al., 2008).

IPF is the most common form of idiopathic interstitial pneumonia (IIP), and used to be known variously as the Hamman-Rich syndrome, alveolitis fibrosis, cryptogenic fibrosing alveolitis and IIP. Epidemiologically it affects 20 per 100 thousand of the population in the USA, and 2-8 per 100 thousand of the population in Japan and Europe. The incidence of disease increases with age, and is more common in males. The average survival time after diagnosis is 2.8-3.6 years, and symptoms include difficulty in breathing, dry cough, anorexia, body weight loss, and lack of strength (Cottin, 2013, Wolters et al., 2014).

The aetiology of IPF is unknown, but may be related to chronic inflammation. Around 20% of IPF patients have a history of occupational exposure to metals and wood dust (Wilson and Wynn, 2009, Noble et al., 2012).

Fibrosis occurs when myofibroblasts produce new collagen at a rate higher than can be normally degraded, thus the total amount of collagen increases over time and finally causes permanent fibrotic scarring. The exact mechanisms of fibrosis are still largely unknown. One explanation could be that when tissue damage occurred as a result of infection, autoimmune reactions, toxins, radiation or mechanical/thermal trauma, the delicate interaction between activated immune and stromal cells to regulate normal wound healing was disrupted or over-exuberant. Fibroblasts and macrophages (M2) are believed to be key players in

the pathogenesis of fibrosis (Shepherd, 2006, Martinez et al., 2006, Meneghin and Hogaboam, 2007, Wynn, 2008, Alber et al., 2012).

Many fibrotic disorders are thought to be caused by pathogenic organisms (Meneghin and Hogaboam, 2007). The pathogen-associated molecular patterns (PAMPs) found on these organisms promote and maintain myofibroblasts activation via TLRs or NLRs (nucleotide-binding oligomerization domain-like receptors) (Otte et al., 2003, Shishido et al., 2003, Pierer et al., 2004). However some fibrotic diseases do not have a clear infectious aetiology, like idiopathic pulmonary fibrosis; paracrine cytokines derived from active leukocytes could initiate and maintain myofibroblasts activation as well (Wolters et al., 2014).

In recent years, there have been substantial studies of the mechanisms and pathogenesis of lung fibrosis, however currently lung transplantation is the only effective treatment for idiopathic pulmonary fibrosis (Wilson and Wynn, 2009, Wynn and Ramalingam, 2012, Kolb and Shargall, 2013).

#### 1.3.1.1 Immune cells in pulmonary fibrosis

Numerous cells have been reported to play a role in the development of fibrosis. Of these, the immune cells that are recruited after epithelium and/or endothelium damage may play key roles in fibrosis as follows (Wynn, 2011).

**B cell**

B cells are known to produce IL-6 upon activation. This may therefore directly promote fibrosis because IL-6 has a potential effect on tissue fibrosis as it could stimulate fibroblasts to synthesize collagen (Duncan and Berman, 1991). B cells also produce IL-10 which decreases IL-12 production thus promoting Th2 differentiation. Th2 cytokines like IL-4 and IL-13 are believed to be pro-fibrotic

mediators (Wynn, 2004, Hasegawa et al., 2005, Zhang et al., 2013). This is discussed extensively in section 1.3.1.2.

**T cell**

T cells, especially CD4+ T cells, play a prominent role in the progression of lung fibrosis. Studies on gene expression patterns of fibrotic tissues found very different profiles during Th1 and Th2 polarized conditions. Th1 cells were reported to have potential anti-fibrotic functions whereas Th2 and Th17 cells very likely play a pro-fibrotic role. The role of regulatory T cells (Treg) is complicated in the pathogenesis of fibrosis; Tregs producing IL-10 along with Th1 cytokines play an anti-fibrotic role but they could also produce TGF-ß which is a powerful pro-fibrotic cytokine (Park et al., 2005, Wynn, 2011).

**Macrophage**

Macrophages are directly involved in the process of wound healing and fibrosis. Their phagocytic role is essential for wound healing by removing microbes, cell debris and dead cells thus eliminating the causes of pro-fibrotic and pro-inflammatory responses (Duffield et al., 2013, Alber et al., 2012).

Macrophages do not produce collagen but produce matrix metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMP), especially MMP-2, 9 and 13 that could break down ECM and attenuate scarring. However MMP-9 can also up-regulate TGF-ß and IL-13 production thus promoting fibrosis. IL-13 can recruit neutrophils infiltration (Kinyanjui et al., 2013). Macrophages can increase fibroblast proliferation, survival and migration by producing TGF-ß and platelet-derived growth factor (PDGF) (Duffield et al., 2005, Gibbons et al., 2011).

Macrophages play different roles in the pathogenesis of fibrosis depending on the cytokine context. Th2 cytokines generate alternatively activated macrophages (M2) which produce arginase-1, and the arginase-1 pathway has pro-fibrotic

properties (Gibbons et al., 2011, Alber et al., 2012). The Th1 cytokines promote iNOS producing classically activated macrophages (M1) which have anti-fibrotic functions (Murray and Wynn, 2011).

**Mast cell**

Mast cell numbers are increased in the affected tissues in fibrotic diseases but the mechanism behind this relationship is unclear. There is evidence suggesting that mast cells could increase smooth muscle thickness in asthma by release of tryptase onto smooth muscle and epithelium (Amin, 2012).

Other leukocytes such as DC (Spears et al., 2011), neutrophils, basophils and eosinophils might have some effects on the development of fibrosis but the precise mechanisms are still largely unknown (Duffield et al., 2013).

### 1.3.1.2 Cytokines in pulmonary fibrosis

Cytokines play a crucial role during both the formation and inhibition of lung fibrosis (Borthwick et al., 2013). Some examples are as follows:

**TGF-β1**

TGF-β1 is the most studied and best known pro-fibrotic cytokine and it is mainly released from circulating monocytes and tissue macrophages. TGF-β1 is reported to promote fibrosis directly; it could promote fibroblasts differentiate to α-smooth muscle actin (α-SMA) positive myofibroblasts which are the key pathogenic cell type in all fibrotic diseases (Hinz et al., 2007). It also could activate mesenchymal cells to go through epithelial/endothelial-mesenchymal transition (EMT) to differentiate into collagen-synthesizing myofibroblasts (Ashcroft et al., 1999, Wynn and Ramalingam, 2012).

**Th2 cytokines**

Th2 cytokines such as IL-4 and IL-13 are considered potential fibrotic factors through both TGF-β1-independent and –dependent pathways. Besides their ability to promote fibrosis via directly activating fibroblasts to produce collagen, they also promote fibrosis indirectly via increasing the production and activation of TGF-β1 (Kaviratne et al., 2004, Wynn, 2008).

**IL-33**

The importance of IL-33 in fibrogenesis especially lung fibrosis was observed even before it was finally named 'IL-33' in 2005 (Schmitz et al., 2005) by the studies of its receptor ST2 which was first discovered in fibroblast cells (Tominaga et al., 1991). Oshikawa *et al*. reported that sST2 could modulate lung inflammation in 2002 (Oshikawa et al., 2002), Tajima *et al*. discovered that the serum level of sST2 was increased in patients with IPF and correlated with disease severity in 2003 (Tajima et al., 2003). In 2009, the pro-fibrotic role of IL-33 was observed by using sST2 over-expressing mice in BLM-induced lung fibrosis model (Mato et al., 2009). Another *in vitro* experiment also confirmed the pathological role of IL-33 (Yagami et al., 2010). The pro-fibrotic property of IL-33 was further reported in multi-walled carbon nanotube and bleomycin induced fibrosis models (Wang et al., 2011, Luzina et al., 2013).

IL-33 also plays a pathological role in other fibrotic disease in other organs for example cutaneous fibrosis (Rankin et al., 2010), hepatic fibrosis (McHedlidze et al., 2013), renal fibrosis (Akcay et al., 2011), gut fibrosis (Sponheim et al., 2010, Lopetuso et al., 2012) and pancreatic fibrosis (Masamune et al., 2010). But IL-33 seems to play a protective role in central nervous and cardiovascular systems fibrotic diseases (Miller et al., 2008, Jiang et al., 2012).

Despite extensive studies having already been done to understand the role of IL-33 in fibrotic diseases, the exact mechanism of IL-33 involvement in fibrosis is

still largely unknown; it might be due to its ability to promote other Th2 cytokines production.

**Pro-inflammatory cytokines**

The process of a progressive fibrotic response that can evolve from acute lung injury requires inflammatory factors such as TNFα, IL-1ß, IL-6, IL-8 (CXCL8), CXCL1 (KC) and CXCL2 (MIP-2) (Parmentier et al., 2000, Hirani et al., 2001). These cytokines are reported to be responsible for the initiating and prolonged progression of lung inflammation and eventual lung fibrosis. TNFα and IL-1ß are reported to promote epithelial cells differentiate to myofibroblasts in a TGF-ß1-dependent manner. IL-6 is considered to be an autocrine growth factor in fibroblasts (Wynn, 2011).

Th17 cytokines such as IL-17A and IL-21 are also reported to play a pathological role in the initiation and development of fibrosis in some animal models via recruitment of neutrophils. And there are reports about the link between IL-17A expression and TGF-ß1 (Borthwick et al., 2013, Duffield et al., 2013).

**Th1 cytokines**

Th1 cytokines, for example IFN-γ and IL-12, are reported to have anti-fibrotic properties. These cytokines could alter the polarization from type I immunity to type II immunity, thus reducing the production of Th2 cytokines (Borthwick et al., 2013).

### 1.3.1.3 Animal models

Most experimental models of lung fibrosis involve rodents and the intra-pulmonary administration of antigens or irritants (Moore and Hogaboam, 2008).

**FITC**

Intratracheal administration of fluorescein isothiocyanate (FITC) can induce pulmonary fibrosis in mice. This causes infiltration of macrophages and neutrophils into the lung interstitium. The main advantages of this model are that the lung injury site can be easily visualized, the mouse strain is independent and fibrosis formed does not self-limit; the main disadvantage is that this model is not clinically relevant (Roberts et al., 1995).

**Irradiation**

Thorax-limited exposure to a single dose of irradiation can induce lung fibrosis. The advantage of this model is that it is clinically very relevant and important, and the disadvantages are the length of time (30 weeks) necessary for the fibrotic response, and the cost (Rube et al., 2000).

**Silica**

Aerosolization or intratracheal instillation of mineral fibers can induce fibrosis in rodents. The main advantage of this model is that it is close to human diseases caused by occupational exposure to dusts and particulates; the disadvantages are that the experiment aerosolization requires specific equipment that is not widely available, and the time for fibrosis to develop is more than 60 days (Davis et al., 1998).

**Bleomycin**

The best characterized murine model of human lung fibrosis is intrapulmonary administration of bleomycin (Moore and Hogaboam, 2008, Mouratis and Aidinis, 2011). Bleomycin (BLM) is a family of compounds produced by *Streptomyces verticillis*. It has potent tumour killing properties and is used in cancer chemotherapy, but its use is limited as it causes lung fibrosis in some patients. BLM can induce lung injury by causing DNA strand breakage and inducing lipid peroxidation (Fleischman et al., 1971, Hay et al., 1991) causing alveolar cell

damage, lung inflammation and fibrosis (Izbicki et al., 2002). The advantages of this model include: i) it is the best characterized model; ii) the BLM can be delivered by a variety of methods (i.n., i.p., i.t. and i.v.); iii) this model is clinically relevant; iv) time for development of fibrosis only takes 14-28 days.

### 1.3.2 Ulcerative colitis

Inflammatory bowel disease (IBD) includes two major forms, ulcerative colitis (UC) and Crohn's Disease (CD). The epidemiology of UC in the USA shows occurrence in 10-12 per 100 thousand of the population and the incidence is higher in northern than southern locations, and greater in females. Clinical symptoms of UC include diarrhoea, abdominal pain, fever, anorexia, nausea and body weight loss. In some cases, UC can result in gastrointestinal bleeding or colorectal cancer (Podolsky, 2002, Kaser et al., 2010).

The pathogenesis of UC is not completely understood. It is believed that the chronic relapsing inflammation results from a dysregulated immune response (Maloy and Powrie, 2011, Bamias et al., 2012).

Treatment of ulcerative colitis must be personalized depending on the individual circumstances. Patients need consistent medical approach underpinned by professionals. Nutritional deficits must be corrected, plus medical therapy for progressive disease and timely surgery for refractory inflammation or complications. Treatment should be adjusted according to disease severity and clinical symptoms. The goal of treatment is mucosal healing and this probably reduces risk of complications. Cases with severe disease should be admitted to hospital for intravenous corticosteroids, fluids and electrolytes with close monitoring. The main principles of therapy for the treatment are to control active disease fast, to maintain remission, to select patients for whom surgery is appropriate, and to ensure as good a quality of life as possible (Vermeire et al., 2011, Kiesslich, 2012, Assadsangabi and Lobo, 2013).

### 1.3.2.1 Immune cells in ulcerative colitis

**Phagocyte**

Innate immune cells such as macrophages, DC and neutrophils play an important pro-inflammatory role in the development of both CD and UC (Yamamoto-Furusho and Podolsky, 2007, Kaser et al., 2010).

**B cells and T cells**

Although there is evidence of adaptive immunity involved in the development of IBD, the results from animal models using Myd88 Trif knockout mouse showed IBD to be innate immunity dependent (Brandtzaeg et al., 2006, Slack et al., 2009).

### 1.3.2.2 Cytokines in ulcerative colitis

**Pro-inflammatory cytokines**

IL-1, IL-6, IL-17 and TNF cytokines contribute to the pathogenesis of UC. The most efficient method to treat IBD is anti-TNF neutralizing antibody (Baumgart and Sandborn, 2007).

**Th1 cytokines**

IFNγ and IL-12 play an important role mainly in CD, but might have some effects in the development of UC (Baumgart and Carding, 2007).

**Th2 cytokines**

Although plenty of work has been done to investigate the role of type II cytokines including IL-4, IL-5 and IL-13 in the pathogenesis of IBD, the results are inconsistent or controversial (Stevceva et al., 2001, MacDonald et al., 2012). We still poorly understand the mechanisms of these cytokines' involvement in the IBD.

**Immune regulatory cytokines**

TGF-β and IL-10 have a protective effect on IBD as they can suppress the inflammation (Maloy and Powrie, 2011).

**IL-33**

IL-33 is expressed mainly in the epithelium and endothelium cells and it can be released from cells when in contact with inflammatory signals or when cells undergo necrosis. IL-33 signalling via ST2 can induce both varieties of immune response, both antigen-dependent and antigen–independent. IL-33 is now being considered as an amplifier of innate immune response, and there is evidence suggesting that IL-33 may play a pathogenic role in IBD (Sponheim et al., 2010, Oboki et al., 2010, Beltran et al., 2010, Seidelin et al., 2011, Grobeta et al., 2012, Sedhom et al., 2013). Thus, to investigate the role of IL-33 in the development of IBD might provide new therapeutic ideas.

## 1.3.2.3 Animal models

**SAMP1/YitFc mice**

Senescence accelerated mice (SAM) P1/YitFc (SAMP1/Fc) mice develop chronic inflammation of the ileum and therefore share many similar mechanisms with human CD. This is the best model to study human CD (Sugawara et al., 2005, Pastorelli et al., 2010).

**TNBS-induced colitis**

Intrarectal instillation of haptenating substance TNBS in ethanol can induce colitis in some strains of mice, rats and rabbits. Ethanol is used to break the mucosal barrier; TNBS is thought to haptenize colonic autologous or microbiota proteins rendering them immunogenic to the host immune system. This model is helpful to study T helper cell-dependent mucosal immune response. But the disadvantages of this model are the individual optimization of concentration if TNBS is required and the fact that it is strain-dependent (Wirtz et al., 2007).

**Oxazolone-induced colitis**

Intrarectal instillation of hapten reagent oxazolone in ethanol can induce colitis in some strains of mice and rats. Similar to TNBS-induced colitis, this model is useful to study T helper cell-dependent colitis; it is also strain-dependent and requires individual optimization (Wirtz and Neurath, 2007).

**DSS-induced colitis**

Administration of dextran sodium sulfate (DSS) in drinking water *ad libitum* can induce colitis in guinea pigs, rats, hamsters and mice, both acute and chronic depending on the dosage and time (Okayasu et al., 1990). The exact mechanism of DSS-induced colitis is still unknown, possibly the direct alteration of gut permeability, and gut bacteria may play a key role in DSS-induced colitis since under germ-free conditions mice develop very severe colitis after administration of DSS (Dieleman et al., 1994, Wirtz et al., 2007). The DSS model of colitis has many pathological similarities to human IBD especially UC. The DSS model of colitis is a cheap and easy way to mimic human IBD in animals, and is also highly reproducible and applicable to a variety of species (Mahler et al., 1998). It is a suitable model for investigating the role of permeability and epithelial destruction in the initiation of IBD (Maxwell and Viney, 2009).

## 1.4 Hypothesis and Aims

Increasing evidence suggests that the IL-33/ST2 axis plays a critical role in several chronic inflammatory disorders, including asthma, rheumatoid arthritis and atherosclerosis. However, the relevance of ST2 and IL-33 in other inflammatory and remodelling diseases, for example colitis and lung fibrosis, at the time when I started my PhD study in 2010 was largely unknown. These two diseases are common with unknown aetiology and there is therefore an important clinical unmet need to investigate them. It has been suggested that both diseases may be initiated by the dysregulation of mucosal barrier function involving the innate immune response playing a critical role in the development and perpetuation of the disorders.

**Hypotheses:**

Based on the current literature and findings described above, I hypothesised that IL-33 is induced in the gut and lung epithelium and mediates inflammation mainly by engaging the innate inflammatory response.

**Aims:**

1. To investigate the effect of IL-33 and its receptor ST2 in the development of bleomycin-induced lung fibrosis in mice (Chapter 3)
2. To elucidate the mechanism by which IL-33 signals promote bleomycin-induced lung fibrosis (Chapter 4)
3. To study the expression and function of IL-33 and ST2 in colitis (Chapter 5)
4. To understand the possible mechanism underlying pathogenic role of IL-33 in colitis (Chapter 5)
5. To improve the understanding of IL-33 in the pathogenesis of inflammatory diseases and thereby its potential therapeutic value.

# Chapter 2

# Materials and Methods

# Chapter 2: Materials and Methods

## 2.1 Mice

All mice were housed in the Biological Services facility, University of Glasgow, according to United Kingdom Home Office regulation. All experiments were undertaken in the same facility under project licenses (No. 60/3791 and 60/4405); mice were used typically starting at 6-8 weeks old. I hold a personal license (No. 60/12410). The mice strains used are summarized in Table 2-1. ST2 knockout mice were kindly supplied by Prof. F. Y. Liew (Institute of Infection, Immunity and Inflammation, University of Glasgow, Glasgow, UK) (Xu et al., 2010); IL-4 knockout and IL-4 receptor knockout mice were kindly supplied by Prof. J. Alexander (Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, UK) (Mohrs et al., 1999).

### Table 2-1. Mice strains used

| Strain | Genetic Modification | Source |
|---|---|---|
| Balb/C | Wild-type | Harlan Olac |
| Balb/C | ST2 gene knockout (ST2 KO) | Bred at University of Glasgow (Xu et al., 2010) |
| Balb/C | IL-4 gene knockout (IL-4 KO) | Bred at University of Strathclyde (Mohrs et al., 1999) |
| Balb/C | IL-4 receptor gene knockout (IL-4R KO) | Bred at University of Strathclyde (Mohrs et al., 1999) |
| C57B/6 | Wild-type | Harlan Olac |
| C57B/6 | ST2 gene knockout (ST2 KO) | Bred at University of Glasgow (Xu et al., 2010) |

## 2.2 Buffers and reagents

### 2.2.1 Cytokines

**Table 2-2. Cytokines used for *in vitro* culture**

| Cytokine | Supplier | Source | Working concentration | Use |
|---|---|---|---|---|
| M-CSF | PeproTech | Murine | 10 ng/ml | BMDM culture |
| IL-4 | PeproTech | Murine | 5—50 ng/ml | Cell stimulation |
| IL-13 | PeproTech | Murine | 5—50 ng/ml | Cell stimulation |
| IL-33 | PeproTech | Murine | 5—50 ng/ml | Cell stimulation |

### 2.2.2 Antibodies

Antibodies used for immunohistochemistry and western blot are summarized in Table 2-3; and for flow cytometry (FACS) are summarized in table 2-4.

**Table 2-3. Antibodies and isotype controls used in Immunohistochemistry and Western Blot.**

| | Produced in | Stock concentration | Dilution | Company |
|---|---|---|---|---|
| Anti-mouse IL-33 | Goat | 0.2 mg/ml | 1:40 | R&D Systems |
| Anti-mouse F4/80 | Rat | 1 mg/ml | 1:50 | AbD Serotec |
| Goat IgG | Goat | 5 mg/ml | 1:1,000 | Vector Labs Ltd. |
| Rat IgG | Rat | 1 mg/ml | 1:50 | Vector Labs Ltd. |
| Biotinylated anti-goat IgG | Rabbit | 1.5 mg/ml | 1:200 | Vector Labs Ltd. |
| Biotinylated anti-rat IgG | Rabbit | 0.5 mg/ml | 1:200 | Vector Labs Ltd. |
| Horseradish Peroxidase-linked anti-Goat IgG | Rabbit | Not specified by supplier | 1:1,000 | R&D systems |

## Table 2-4. Antibodies used in FACS

| Antigen | Label | Isotype | Stock | Volume /100 µl | Company |
|---|---|---|---|---|---|
| CD11b | PE | Hamster IgG | 0.2 mg/ml | 1 µl | eBioscience |
| CD11b | FITC | Rat IgG2b | 0.5 mg/ml | 1 µl | eBioscience |
| CD11c | PE | Rat IgG2b | 0.2 mg/ml | 1 µl | eBioscience |
| CD11c | FITC | Hamster IgG1 | 0.2 mg/ml | 2 µl | BD Bioscience |
| CD16/32 (FcBlocker) | none | Rat IgG2b | 0.5 mg/ml | 2 µl | BD Bioscience |
| CD3 | PE | Hamster IgG | 0.2 mg/ml | 1 µl | eBioscience |
| CD3 | PerCP | Rat IgG2b | 0.2 mg/ml | 1 µl | eBioscience |
| CD4 | FITC | Rat IgG2b | 0.2 mg/ml | 1 µl | eBioscience |
| CD45 | PB | Rat IgG2b | 0.5 mg/ml | 0.5 µl | BioLegend |
| CD45 | Alexa700 | Rat IgG2b | 0.2 mg/ml | 1 µl | eBioscience |
| CD45R/B220 | PE | Rat IgG2a | 0.2 mg/ml | 2 µl | eBioscience |
| CD49b/pan-NK | PE | Rat IgM | 0.2 mg/ml | 1 µl | eBioscience |
| CD206/MR | Alexa647 | Rat IgG2a | 50 µg/ml | 10 µl | AbD Serotec |
| CD278/ICOS | PerCP | Hamster IgG | 0.2 mg/ml | 1 µl | eBioscience |
| CD282/TLR2 | PE | Rat IgG2b | 0.2 mg/ml | 1 µl | eBioscience |
| ST2L/T1 | FITC | Rat IgG1 | 1 mg/ml | 2 µl | MD Bioscience |
| F4/80 | APC | Rat IgG2b | 0.2 mg/ml | 2 µl | AbD Serotec |
| F4/80 | PB | Rat IgG2a | 0.5 mg/ml | 0.5 µl | BioLegend |
| F4/80 | FITC | Rat IgG2a | 0.5 mg/ml | 0.5 µl | eBioscience |

| | | | | | |
|---|---|---|---|---|---|
| FceR1 | PE | Hamster IgG | 0.2 mg/ml | 1 µl | eBioscience |
| Gata-3 | PE | Rat IgG2b | 0.2 mg/ml | 2 µl | eBioscience |
| MHC Class II | PerCP | Rat IgG2b | 0.2 mg/ml | 0.5 µl | eBioscience |
| IL-4 | APC | Rat IgG1 | 0.2 mg/ml | 0.5 µl | eBioscience |
| IL-13 | APC | Rat IgG1 | 0.2 mg/ml | 0.5 µl | BioLegend |
| IL-33 | PE | Rat IgG2a | Not specified by supplier | 10 µl | R&D systems |

## 2.2.3 Chemicals and buffers

Chemicals and buffers used throughout the projects are listed in table 2-5 and table 2-6.

### Table 2-5. Chemicals

| Chemicals | Supplier | Usage |
|---|---|---|
| 0.01% hydrogen peroxide | Sigma-Aldrich, USA | IHC |
| 1M /1N hydrochloric acid | Sigma-Aldrich, USA | ELISA |
| 1M/2N sulphuric acid | Sigma-Aldrich, USA | ELISA |
| 3,3',5,5'-Tetramethylbenzidine (TMB) | eBioscience, USA | ELISA |
| 3,3'-diaminobenzidine tetrahydrochloride (DAB) | Vector Labs Ltd., USA | IHC |
| Agarose | Sigma-Aldrich, USA | Genotyping |
| Bleomycin (BLM) | Sigma-Aldrich, USA | *in vivo* experiments |
| Clodronate Liposomes | ClodLip BV, Netherlands | *in vivo* experiments |
| Dextran Sulphate Sodium (DSS) | ICN Biomedicals, USA | *in vivo* experiments |
| Ecoscint A Scintillation Fluid | National Diagnostics, USA | Proliferation assay |
| Ethidium Bromide | Invitrogen, UK | Genotyping |
| 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) | Sigma-Aldrich, USA | ELISA |
| Ionomycin calcium salt | Sigma-Aldrich, USA | Cell activation |

| | | |
|---|---|---|
| Isoflurane | Sigma-Aldrich, USA | *in vivo* experiments |
| Phenylmethanesulfonyl fluoride (PMSF) | Sigma-Aldrich, USA | Protein extraction |
| Phorbol 12-myristate 13-acetate (PMA) | Sigma-Aldrich, USA | Cell activation |
| Phosphatase Inhibitor Cocktail | Thermo Fisher Scientific, USA | Protein extraction |
| Protease Inhibitor Cocktail | Sigma-Aldrich, USA | Protein extraction |
| Pierce RIPA Buffer | Thermo Fisher Scientific, USA | Protein extraction |
| Sodium orthovanadate | Sigma-Aldrich, USA | Protein extraction |
| [methyl-$^{3}$H] thymidine | PerkinElmer Inc., USA | Proliferation assay |
| Tris Acetate-EDTA (TAE) | Sigma-Aldrich, USA | Genotyping |
| Trichloroacetic acid (TCA) | Sigma-Aldrich, USA | Proliferation assay |
| Tween® 20 | Sigma-Aldrich, USA | ELISA, western blot IHC |

Chemicals used throughout the projects; other chemicals not listed were obtained from Sigma-Aldrich, USA. IHC = immunohistochemistry;

## Table 2-6. Buffers

| Buffer | Constituents |
|---|---|
| Phosphate buffered saline (PBS) | 8g NaCl, 1.16g $Na_2HPO_4$, 0.2g KCl, 0.2g $KH_2PO_4$ in 1 litre distilled water, pH7.4 |
| Tris-Buffered Saline (TBS) | 2.422g Trizma base, 9g NaCl in 1 litre distilled water, pH 7.6 |
| ELISA Coating buffer | 0.1M $NaHCO_3$ pH 8.4, unless specified otherwise |
| ELISA Wash buffer | 0.05% (v/v) Tween-20 in PBS pH 7.4, unless specified otherwise |
| ELISA assay buffer | 10% FBS in PBS, unless specified otherwise |
| Radioimmunoprecipitation Assay (RIPA) buffer | 25mM Tris-HCl (pH 7.6), 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS |
| FACS buffer | 0.5% BSA and 2mM EDTA in DPBS |
| Immunohistochemistry Wash Buffer (TBST) | 0.05% (v/v) Tween-20 in PBS pH 7.6 |
| Acid Alcohol | 1 ml 37% (w/v) HCl in 100 ml 70% (v/v) Ethanol |
| Scott's tap water substitute | 3.5g Sodium hydrogen carbonate, 20g Magnesium sulphate in 1 litre distilled water |
| Chromotrope-green mixture | 0.6g Chromotrope 2R, 0.3g Fast green FCF, 0.6g phosphotungstic acid, 1 ml acetic acid in 100 ml distilled water |

Buffers used throughout the projects.

## 2.3 Tissue culture

Cells were cultured in complete medium (RPMI 1640 or DMEM, 10% heat-inactivated FBS, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 2 mM L-glutamine; Invitrogen Ltd., UK), unless otherwise specified. Cultures were incubated at 37.0°C in a humidified incubator (Heraeus Instruments, Germany) supplemented with 5% $CO_2$. Viable cells were counted with a Neubauer haemocytometer (Weber Scientific International Ltd, UK) on a Nikon Labphot microscope, staining with 0.1% (w/v) Trypan Blue (Sigma-Aldrich, USA).

### 2.3.1 Culture of mice pulmonary fibroblast cells

Primary fibroblasts of 5-6 weeks mice were obtained from freshly excised lungs using an explant technique (Peacock et al., 1992). Briefly, lungs were dissected from the thorax, laid out onto the sterile Petri dish filled with 2 ml of wash buffer (DMEM containing 100 U/ml Penicillin, 100 µg/ml Streptomycin), the tissue was then cut into ~1 $mm^3$ pieces, 25 of which were evenly distributed over the base of a 25-$cm^2$ culture flask containing 2 ml of DMEM with 20% fetal calf serum (FCS), penicillin/streptomycin. Explants were incubated in a humidified atmosphere of 5% $CO_2$ in air at 37°C. After 4 days, add 2 ml of same complete media to each flask and change the medium every 3 days afterwards; the growth of fibroblasts from tissue fragments was monitored. Once a monolayer of cells had partially covered the flask (approximately 70% confluence), tissue fragments were removed by aspiration, and cells were passaged as follows. Cells were washed twice with 2 ml of trypsin solution (0.05% trypsin / 0.02% EDTA; Invitrogen, UK), incubated at 37°C for approximately 10 minutes or until cells were rounded and began to detach from the flask, add 10 ml of culture medium (containing serum to inactivate the trypsin) and transfer to new flasks. Cells were subsequently maintained in DMEM containing 10% FCS, supplemented with antibiotics and L-glutamine as before, and used between passages 3 and 10.

### 2.3.2 Primary culture of mice bone marrow-derived macrophages

Mice were sacrificed by exposing to $CO_2$, the pelvic and femoral bones were removed from the joints, each end of the bones was cut off, and the bone marrow cells were expelled using a 26-gauge needle/5 ml syringe filled with wash buffer (PRMI 1640 containing 100 units/ml penicillin, 100 µg/ml streptomycin) to flush through both ends of the bones. The bone marrow cells were washed once with RPMI and harvested by centrifugation 1200rpm for 10 minutes, and the cells were cultured in 10 ml of RPMI 1640 supplemented with 10% FBS, 2mM L-glutamin, 100 units/ml penicillin, 100 µg/ml streptomycin, 0.05 M 2-mecaptoethanol on Petri dish (10 $x10^6$ cells per dish) in the presence of M-CSF (10ng/ml; PeproTech, USA). The cells were fed with fresh complete medium supplemented with M-CSF (10ng/ml) every 3 days. On day 7 of the culture, cells were collected with a scraper in cold PBS buffer and the purity checked using the macrophage marker (F4/80) by flow cytometry. The cell preparations should contain >95% of $F4/80^+$ macrophages.

### 2.3.3 Primary culture of lung cells

For the isolation of total lung cells, mouse lungs were extracted as described in section 2.8.2. The lungs were transferred to a Petri dish, where connective tissues and lymph nodes were removed and the remaining lung parenchyma cut into 1-2 mm cubes using curved scissors in 1 ml RPMI 1640 containing 100 units/ml penicillin. The tissue fragments were then transferred via pipette to a bijou and digested with 1 ml per lung of Digestion Buffer (RPMI 1640 completed with 100 U/ml DNAse, 1 mg/ml Collagenase D (Roche, USA)), on a mechanical shaker and incubated at 37°C for 45 minutes. The tissue was passed through an 18-gauge needle every 12 minutes to further disperse the tissue. After washing twice with RPMI 1640 then collecting the cells by centrifugation at 1200 rpm for 5 min at 4°C, the separated cells were resuspended in complete culture media and filtered through a strainer in order to get single cells. The cells ($0.5x10^6$/ml) were placed into the wells of a 24-well cell culture plate and cultured in

complete DMEM for 24 to 72 hours. The culture supernatants were harvested and stored at -20°C for cytokine analysis and the cells were placed into RNA stabilisation buffers for storage and kept at -80°C for mRNA extracted for subsequent PCR assay.

### 2.3.4 Colon cultures

Colons were removed from mice, opened longitudinally and washed in sterile PBS supplemented with antibiotics (200 units/ml penicillin, 200 µg/ml streptomycin). Three segments (1 cm in length) from the distal colon were placed in 24-well flat-bottom culture plates (Costar, USA) containing fresh RPMI 1640 supplemented with 1% penicillin/streptomycin and cultured at 37°C for 24 h. The culture supernatants were then harvested by centrifugation at 13,000 rpm for 10 minutes at 4°C and transferred to new tubes and stored at −20°C for analysis of cytokine and chemokine by Luminex or ELISA.

## 2.4 Measurement of fibroblasts proliferation

The measurement of DNA replication determined by the incorporation of tritiated [$^3$H] thymidine (PerkinElmer Inc., USA) was used to measure the cellular proliferation. Briefly mice pulmonary fibroblasts (MPFs) were grown in 10% FCS to approximately 60% confluence in 24-well or 96-well flat bottom plates and then were serum starved for 24 hours using serum-free medium to establish cell cycle synchronization (Chen et al., 2012). Cells were then cultured under different conditions for 24 or 48 hours. Cellular proliferation was determined by incorporation of [$^3$H] thymidine. Briefly, [$^3$H] thymidine (3.7 kBq/well for a 24-well plate; or 14.8 kBq/well for a 96-well plate) was added to fibroblast cultures 4 hours before the end of the experiments. After the culture, for cells grown in a 24-well plate, the medium was removed and the plate placed on ice; the cells were washed twice with 0.5 ml ice-cold PBS. The cells were further washed with 5% ice-cold trichloroacetic acid (TCA) to precipitate cellular proteins and with ice-cold 100% ethanol to extract lipid fractions. The remaining cell contents were dissolved in 0.3M NaOH. The contents of each well were transferred to a scintillation vial and 1.5 ml of Ecosint A scintillation fluid (National Diagnostics, USA) was added into each vial. The vials were vortexed thoroughly before the radioactive counts were done using a scintillation counter (PerkinElmer Inc., USA). The counts were measured in CPMs (counts per minute). For cells grown in a 96-well plate, the samples are harvested to a filtermat (PerkinElmer Inc, US) with a cell harvester (PerkinElmer Inc., US), the filtermat was air dried, then put into a plastic sample bag filled with 5 ml of Ecosint A scintillation fluid and sealed. The filtermats were then measured by a MicroBeta counter (PerkinElmer Inc., US). Counts were also measured as CPMs.

## 2.5 Recombinant IL-33 production

The His-tagged recombinant IL-33 proteins were produced in *E.coli* and affinity purified using Ni-NTA Agarose in Dr. Xu's lab as described previously (Komai-Koma et al., 2007).

### 2.5.1 Endotoxin removal

Endotoxin in the purified rIL-33 products was removed by purification with polymyxin B columns following the manufacturer's instruction: briefly, a pre-packed column (EndoTrap) was placed in a holder and remove the top cap before the bottom cap, allowing the storage solution to drain from the column (approx 8 min), making sure the EndoTrap resin does not run dry. Activate the column as follows: fill the column with regeneration buffer (approx 3 ml) and let the column drain out, repeat once; fill up the column with equilibration buffer (approx 3 ml) and drain out the column, repeat once. Then fill the column with sample (Ni-NTA agarose purified rIL-33 protein) in equilibration buffer and collect the flow through liquid immediately, the column can be repeatedly filled with up to 50 ml of samples, fill the column with 1 ml of equilibration buffer and let the column drain out to elute samples completely.

### 2.5.2 Endotoxin detection

Endotoxin levels in the rIL-33 preparations were measured using Limulus amebocyte lysate (LAL) QCL-1000 pyrogen test following the manufacturer's manual: briefly, the microplate was preheated to 37$^{o}$C in a heating block adapter (Thermo Fisher Scientific, USA). While leaving the plate in the heating block, add 50 µl of standards and samples to the wells, for blank control using 50 µl of LAL reagent water. 50 µl of LAL was then added into each well using a multi-channel pipette in the order of reagent addition. Briefly tap the plate for mixing and incubate the plate for 10 minutes followed by adding 100 µl of

preheated substrate solution into each well. 16 minutes later, stop the reaction by adding 100 µl of stop reagent in the same sequence as the previous steps. Remove the plate from the heat blocker, read the plate with an ELISA reader (Dynex Technologies, USA) using absorbance at 410 nm, record the optical density (OD) and calculate the samples' concentration based on the linear standard curve obtained by the standards on the same plate.

### 2.5.3 Protein concentration measurement

Protein concentrations of rIL-33 preparations were determined using Coomassie Protein Assay Kit (Bradford). First, perform 2-fold serial dilutions of the standard protein (Bovine serum albumin standards; Thermo Scientific, USA). Add 480 µl of Coomassie Reagent to 20 µl of the serial diluted standards, samples and PBS as blank, mix well and incubate 10 min at room temperature. Transfer 150 µl of the samples to a 96-well flat-bottom plate; the OD values were measured using an ELISA reader using 600-nm absorbance. The protein concentrations in the samples were determined by interpolating the readings to the OD values of the Standards.

### 2.5.4 Bioactivity test

To determine the bioactivity of the in-house prepared rIL-33, total splenocytes ($2x10^6$/ml) obtained from WT and ST2KO mice were stimulated with plant-bound anti-CD3 (2ug/ml) in the presence of 0, 10, 20 and 50 ng per ml of the sample rIL-33 and commercial rIL-33 (PeproTech, USA) as a standard. The cells were cultured in 24-well plate for 72 hours and supernatants were harvested. The concentrations of IL-5, the major cytokine induced by IL-33, in the culture supernatants were measured by ELISA. The IL-33 preparations which specifically induce IL-5 production in WT but not ST2KO cells were collected and used for further experiments. The relative bioactivity of home-made IL-33 was determined relative to the commercial standard.

## 2.6 Soluble collagen assay

The Sircol Collagen Assay kit (Biocolor Ltd, UK) was used to measure the soluble collagen levels in cell culture fluids or in lung tissue homogenates as recommended by the manufacturer. To measure the collagen production of fibroblast cells in vitro, the supernatants of cell culture were collected after 24h or 48h of incubation under different conditions. To measure the collagen levels in mouse lung tissue, add 1 ml of RIPA lysis buffer to frozen lung tissue samples, homogenize the tissue for 20 seconds at 19,000 rpm using an electric homogenizer (Fisher Scientific, US), centrifuge the samples 10 minutes at 12,000 rpm at 4℃, and collect supernatants for collagen assay.

The principal feature of this assay is the use of Sirius Red, which can specifically bind to collagen and be detected in alkali buffer by spectroscopy at 555 nm absorption (Streuli and Grant, 2000). Briefly, add 1.0 ml Sircol Dye Reagent to each tube containing 100 µl of samples, blanks (PBS, culture media or RIPA buffer, respectively) or standards. Cap the tubes, mix by inverting contents first and then using a mechanical shaker for 30 minutes. Transfer the tubes to a micro-centrifuge and spin at 12,000 rpm for 10 minutes to collect the precipitated collagen. Carefully invert and drain the tubes and add 750 µl ice-cold Acid-Salt Wash Reagent to the collagen-dye pellet to remove unbound dye by centrifugation at 12,000 rpm for 10 minutes and carefully remove any fluid from the lip of the tubes using cotton wool buds. Add 1000 µl Alkali Reagent to the samples, recap tubes and release the collagen-bound dye into solution using a vortex mixer. When all of the bound dye has been dissolved, transfer 200 µl of each sample to individual wells of a 96-well plate; set the micro-plate reader (Dynex Technologies, USA) to 570 nm. Measure optical density (OD) against the reagent blanks, standards and test samples, and obtain collagen concentrations by interpolating the readings into the Standard Curve.

## 2.7 Gene expression assay

## 2.7.1 Quantitative-PCR analysis

### 2.7.1.1 RNA extraction

#### 2.7.1.1.1 Isolation of total RNA from cells

A spin-column based RNA extraction kit (Qiagen, Germany) was used for this purpose, all procedures were conducted according to manufacturer's instruction manuals: briefly, add 350 µl of Buffer RLT to lyse cells (up to $5x10^6$); transfer the lysate directly into a QIAshredder spin column placed in a 2ml collection tube, centrifuge for 2 minutes at 13,000 rpm; transfer flow-through to an RNeasy spin column placed in a 2 ml collection tube, centrifuge for 15 seconds at 12,000 rpm, discard the flow-through; add 700 µl Buffer RW1 to RNeasy spin column, centrifuge for 15 seconds at 12,000 rpm, discard the flow-through; wash RNeasy spin column twice with 500 µl Buffer RPE, centrifuge at 12,000 rpm for 15 seconds the first time and 2 minutes the second time, discard the flow-through; place the RNeasy spin column in a new 1.5 ml collection tube, add 40 µl RNase-free water directly to RNeasy spin column membrane, centrifuge for 1 minute at 12,000 rpm to elute the RNA.

#### 2.7.1.1.2 Isolation of RNA from tissues

TRIzol Reagent (Invitrogen, UK) was used for the isolation of total RNA from tissues: briefly, wash the tissue preserved in RNAlater using PBS; then add 1ml TRIzol Reagent per sample (10-100 mg), homogenize the sample for 20 seconds at 19,000 rpm using an electric homogenizer (Thermo Fisher Scientific Inc., US) and incubate the homogenized sample for 5 minutes at room temperature. Purify the RNA by adding 0.2 ml of chloroform into the sample tube, shake vigorously by hand for 20 seconds, incubate 3 minutes at room temperature and centrifuge the samples at 12,000 rpm for 15 minutes at 4°C. Remove the aqueous phase of the sample by pipetting and transfer into a new tube. To precipitate the RNA, add 0.5 ml of 100% isopropanol to the aqueous phase,

incubate 10 minutes at room temperature, centrifuge the samples at 12,000 rpm for 10 minutes at 4℃; discard the supernatants, leaving only the RNA pellet. Wash the RNA with 1 ml of 75% ethanol, vortex the tubes briefly then centrifuge the tubes at 12,000 rpm for 5 minutes at 4℃. Discard the supernatants, air dry the RNA pellet for 6 minutes; re-suspend the RNA pellet in 40 µl of RNase-free water.

The purity and quantity of RNA extractions were determined by using a NanoDrop spectrophotometer (Thermo Scientific, US), and the quality was assessed by the A260/A280 ratio (1.8~2.1 was considered optimum).

### 2.7.1.2 Reverse Transcription

Reverse Transcription (RT) of RNA into cDNA was carried out using High-Capacity cDNA Reverse Transcription Kits according to manufacturer's protocol (Applied Biosystems, US). Dilute RNA samples to 1.5 µg per 10 µl using nuclease-free water. Prepare 2X reverse transcription master mix, each 10 µl mix containing 2.0 µl of 10X RT Buffer, 0.8 µl of 25X dNTP Mix, 2.0 µl of 10 RT Random Primers, 1.0 µl of MultiScribe reverse transcriptase and 4.2 µl of nuclease-free water. For a 20 µl RT reaction, mix 10 µl of 2X reverse transcription master mix with 10 µl of RNA samples in individual PCR tube, briefly centrifuge the tubes to eliminate any air bubbles; load the tubes in thermal cycler (Eppendorf, UK); perform reverse transcription reaction in the following conditions: 25℃ 10 minutes, 37℃ 120 minutes, 85℃ 5 seconds and 4℃ to stop the reaction. Keep the cDNA samples at -20℃ until further experiments.

### 2.7.1.3 Real-time PCR

Real-time polymerase chain reaction (RT-PCR) was performed using Fast SYBR Green master mix (Applied Biosystems, USA): pipette 10 µl of 2X Fast SYBR

Green master mix, 1 µl of each forward and reverse PCR primers (Tab 2.7), 2 µl of cDNA samples and 7 µl of nuclease-free water into each well of a 96-well reaction plate, briefly centrifuge the plate to eliminate any air bubbles; load the plate into an Applied Biosystems real-time quantitative PCR instrument; set the thermal cycling condition: 95℃ 20 seconds; 95℃ 3 seconds, 60℃ 30 seconds, repeat 40 cycles; calculate the results using ΔΔCT method (Livak and Schmittgen, 2001, Giulietti et al., 2001).

The primers (Integrated DNA Technologies, USA) were synthesized according to the sequences in table 2-7.

## Table 2-7. Real-time PCR primers

| Mice | forward | reverse |
|---|---|---|
| TPB | 5’-ACTATGTGGTCTTCCTGAATCC-3’ | 5’-CAAACCCAGAATTGTTCTCCTT-3’ |
| sST2 | 5’- CTTGTTCTCCCCGCAGTC-3’ | 5’- CCAATGTCCCTTGTAGTCGG-3’ |
| ST2L | 5’-TCTGTGGAGTACTTTGTTCACC-3’ | 5’-TCTGCTATTCTGGATACTGCTTTC-3’ |
| Il-13 | 5’-GAATCCAGGGCTACACAGAAC-3’ | 5’-AACATCACACAAGACCAGACTC-3’ |
| IL-33 | 5’-ACTATGAGTCTCCCTGTCCTG-3’ | 5’- ACGTCACCCCTTTGAAGC - 3’ |
| TGFß1 | 5’- CCATGAGGAGCAGGAAGG-3’ | 5’-ACAGCAAAGATAACAAACTCCAC-3’. |
| Collagen I | 5’-CATTGTGTATGCAGTGACTTC-3’ | 5’-CGCAAAGAGTCTACATGTCTAGG-3’ |
| Collagen III | 5’-TCTCTAGACTCATAGGACTGACC -3’ | 5’-TTCTTCTCACCCTTCTTCATCC-3’ |
| Collagen IV | 5’-AATCCAATGACACCTTGCAAC-3’ | 5’- TCTGGCTGTGGAAAATGTGA-3’ |

| | | |
|---|---|---|
| Arginase I | 5’-AGTGTTGATGTCAGTGTGAGC -3’ | 5’-GAATGGAAGAGTCAGTGTGGT -3’ |
| Arginase II | 5’-GTATTAATGTCCGCATGAGCATC -3’ | 5’-GTGGTTAGTAGAGCTGTGTCAG -3’ |
| iNOS | 5’- GCCTCGCTCTGGAAAGA -3’ | 5’- TCCATGCAGACAACCTT -3’ |

Real-time PCR primers used for detecting specific genes expression in mRNA.

## 2.7.2 Genotyping

### 2.7.2.1 DNA extraction

Genotyping of ST2KO mice was regularly performed by PCR. Mouse tail-tip biopsies (1cm) were taken under isoflurane anaesthetic condition. DNA from the tails of the mice was extracted using DNeasy Blood & Tissue kit according to manufacturer's instruction (Qiagen, Germany). Cut the tail tissue into small pieces and place into a 1.5 ml micro-centrifuge tube, add 180 µl Buffer ATL; then add 20 µl of proteinase K to digest the tissue. Mix thoroughly by vortexing, and incubate at 56℃ until the tissue is completely lysed (approximately 6-8 hours). After lysis, bring the samples to room temperature, vortex the tubes for 15 seconds, add 200 µl Buffer AL and mix thoroughly by vortexing; then add 200 µl of ethanol and mix well; transfer the mixture into the DNeasy Mini spin column placed in a 2 ml collection tube, centrifuge at 8000 rpm for 1 minute, discard the flow-through. Wash the DNA sample by adding 500 µl of Buffer AW1 per tube and centrifuge at 8000 rpm for 1 minute, followed by adding 500 µl of Buffer AW2 per tube and centrifuge at 14000 rpm for 3 minutes. Place the DNeasy Mini spin column in a 1.5 ml micro-centrifuge tube, add 200 µl Buffer AE directly onto the DNeasy membrane and incubate for 1 minute, centrifuge at 8000 rpm for 1 minute to elute the chromosome DNA. Determine the concentration and purity of the DNA by NanoDrop spectrophotometer.

### 2.7.2.2 PCR

Two separate PCR reactions were carried out for genotyping to identify ST2 knockout (KO; neo-cassette) mice and distinguish the wild-type (WT) mice from ST2KO mice with or without the specific size of the PCR products. Primer pairs used are shown in table 2-8.

PCR using GoTaq® Master Mixes (Promega, UK): pipette 10 µl of 2X master mix, 1 µl of forward and reverse primers, 2 µl of DNA samples and 7 µl of nuclease-free

water into PCR reaction tubes, briefly centrifuge the tubes to eliminate any air bubbles; load the tubes into a thermal cycler (Applied Biosystems, USA); perform PCR under the following reaction conditions: 94℃ 3 minutes; 94℃ 45 seconds, 60℃ 30 seconds, 72℃ 90 seconds, repeat 30 cycles; 72℃ 10 minutes and 4℃ ∞; store samples at -20℃.

The PCR products were diluted with 5X Bromophenol Blue loading buffer (Invitrogen, UK). Then 20 µl of each sample were loaded into separate wells of 2% Agarose/1X TAE gels containing 0.5 µl/ml ethidium bromide. The PCR samples were run together with a DNA ladder (Invitrogen, UK) in TAE buffer in an electrophoresis tank at 100mV for 30 min and imaged under UV light using a Gel Logic 200 Imaging System and software (Kodak, USA). Fig. 2-1 presents typical bands for WT and ST2 knock mice.

## Table 2-8. Primer pairs for genotyping

| | Forward | Reverse | PCR product |
|---|---|---|---|
| ST2 (WT) | 5'- TTGGCTTCTTTTAATAGGCCC -3' | 5'-TGTTGAAGCCAAGAGCTTACC -3' | 500 bp |
| Neo (KO) | 5'-CTATCAGGACATAGCCTTGGCTACC -3' | 5'-TGTTGAAGCCAAGAGCTTACC -3' | 200 bp |

Primer pairs used for genotyping and related sizes of PCR products of mouse tissue.

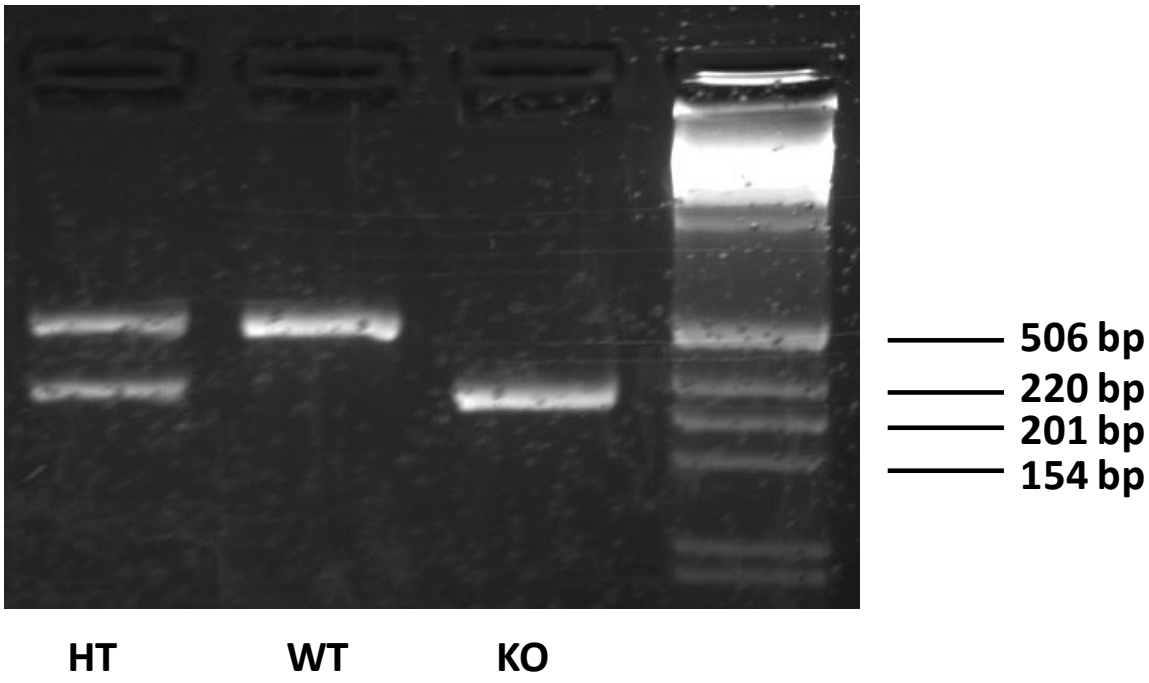



**Fig. 2-1 Genotyping**

Typical bands obtained from wild type (WT), ST2 knockout (KO) and heterozygous (HT) mice, with molecular weight ladders. The bands are approximately 500 bp for WT and 200 bp for KO.

## 2.8. Murine disease models

### 2.8.1 DSS-induced colitis

For the induction of acute colitis, female mice were fed with 3.5% (weight/volume) DSS in their drinking water from day 0 for 12 consecutive days as previously described (Wirtz et al., 2007). Some mice also received recombinant IL-33 or PBS daily by intraperitoneal injections.

The body weight, stool consistency, and rectal bleeding of the mice were monitored daily. The development of stool consistency and rectal bleeding was scored using the modified method of Cooper (Yoshihara et al., 2006) as shown in table 2-9:

**Table 2-9. Scoring system for the comparative analysis of diarrhoea**

| Score | Stool consistency and bleeding condition |
|---|---|
| **0** | *Normal; Negative Hemoccult* |
| **1** | *Soft but Still Formed; no Blood Traces in Stool* |
| **2** | *Very Soft; Blood Traces in Stool Visible* |
| **3** | *Diarrhoea; Rectal Bleeding* |

Mice were sacrificed at different time points. Tissues of the colon were removed and cleaned for histology and culture. Serum was collected for cytokine measurement.

### 2.8.2 Bleomycin (BLM)-induced lung injury

This model works optimally on C57BL/6 mice. Mice were lightly anaesthetized by exposure to 4% isoflurane gas, and then given 30 µl of a solution of bleomycin (BLM) dissolved in PBS (0.06units/25g mouse) intranasally (i.n.). The same volume of PBS was given to the control groups. The experiment lasted up to two weeks when the lung fibrosis was evident (Adamson and Bowden, 1974, Izbicki et al., 2002).

Mice were sacrificed at different time points. The bronchoalveolar lavage fluid (BALF), serum and lung tissues were collected for further analysis.

#### 2.8.2.1 Alveolar macrophage depletion

To evaluate the role of alveolar macrophages in bleomycin-induced lung fibrosis, mice were administered intranasal (i.n.) with clodronate or control liposomes (40 µl per mouse) 72 and 24 hours before bleomycin administration. All mice were sacrificed 7 days after the bleomycin injection. The success of macrophage depletion was determined by counting the macrophage numbers in lungs and BALF by FACS before and after the clodronate administration.

#### 2.8.2.2 IL-33 neutralizing

Neutralizing anti-IL-33 antibodies (Rabbit IgG) were kindly provided by Professor Mingcai Li, Medical School of Ningbo University, China. (Liu et al., 2009) To evaluate the role of IL-33 in bleomycin-induced lung fibrosis, anti-IL-33 antibody or control rabbit IgG (150 µg per mouse) were administered intraperitoneally (i.p.) 30 minutes before bleomycin administration and every five days afterwards. All mice were sacrificed 14 days after the bleomycin injection. The success of IL-

33 neutralization was determined by the reduction in IL-33 concentration in the lung tissue and BAL fluid by ELISA, before and after the antibody treatment.

## 2.9 Histological Analysis

For colon tissues: the tissues were fixed and then embedded in paraffin. Briefly, colon tissues dissected from mice were fixed for 24 hours at room temperature in 10% (v/v) formalin; then put into 70% (v/v) ethanol for 1 hour twice, 80% ethanol 1 hour twice, 90% ethanol 1 hour twice, 95% ethanol 1 hour twice, 100% ethanol 1 hour twice, Xylene 1 hour twice and then placed into pre-melted wax at 60°C for 1 hour; then the tissues were transferred into Embedding Cassettes (VWR International Limited, UK) filled up with fresh wax using Embedding Workstation (Thermo Scientific, UK). Colons were divided into right, transverse and left, and cut samples into 4 μm slices using Microtome (Bright Instrument, UK). The cut specimens were stained with haematoxylin and eosin (H&E; see details in section 2.9.1). Histological examination was performed on serial sections. Three serial sections of five to six different sites of the colon (accounting for up to 18 sections per mouse) were examined.

For lungs: The mice lung tissues were excised, fixed in 10%neutral buffered formalin for 24hr and then embedded in paraffin as described above. Sections were cut into 4 μm slices, which were stained by H&E (see details in section 2.9.1) or Gomori's Rapid One-Step Trichrome Stain (see details in section 2.9.2).

### 2.9.1 Haematoxylin and Eosin (H&E) Staining

To stain the sections using the H&E method, heat the paraffin sections first in oven for 45 minutes at 60℃. Then rehydrate sections as follows: Dewax in Xylene 3 minutes twice, 100% Ethanol 3 minutes twice, 90% Ethanol 3 minutes twice, 70% Ethanol 3 minutes twice, followed by running water 3 minutes. Stain

the sections in Harris Haematoxylin (Cell Path Ltd., UK) for 2 minutes; wash extra stain in running water 3 minutes, 1% Acid/Alcohol 3 seconds, running water 10 seconds, Scotts Tap Water substitute 30 seconds, running water 10 seconds. Counter stain in 1% eosin (Surglpath Medical Industries, Inc., UK) 2 minutes; wash off extra stain in running water. Dehydrate the sections as follows: 70% Ethanol 30 seconds, 90% Ethanol 30 seconds, 100% Ethanol 1 minute twice, Xylene 3 minutes twice. Mount coverslip over tissue section from Xylene with DPX mountant (Raymond A Lamb Ltd, UK).

The sections were then observed and images were taken using an Olympus BX41 microscope with an attached Olympus DP25 digital camera, using Cell[b] software (Olympus, Japan). The pathology changes were scored double blind.

### 2.9.2 Gomori's Rapid One-step Trichrome Stain

To stain the sections for collagen, rehydrate the sections as described above. Stain in Harris Haematoxylin 2 minutes, wash extra stain in running water for 2 minutes; stain in chromotrope-green mixture for 20 minutes, rising in 0.2% acetic acid 2 dips; wash extra stain in running water for 3 minutes. Dehydrate sections and mount coverslip as described above.

Lung sections from mice were processed and the pathology conditions were determined using 1~4 scoring systems (Daniels et al., 2004) as shown in table 2-10:

**Table 2-10. Scoring system for comparative analysis of fibrosis**

| Score | |
|---|---|
| 1 | *no fibrosis* |
| 2 | *occasional small subpleural foci* |
| 3 | *moderate interalveolar septal thickening and subpleural foci* |
| 4 | *continuous interalveolar and subpleural fibrosis* |

## 2.9.3 Immunohistochemistry

***The staining of IL-33 and F4/80 in lung tissue:***

As above, heat the paraffin sections in an oven at 60℃ for 45 minutes. Rehydrate the sections: Dewax in Xylene 3 minutes twice, 100% Ethanol 3 minutes twice, 90% Ethanol 3 minutes twice, 70% Ethanol 3 minutes twice, wash buffer (TBST; 1X TBS, 0.05% Tween-20) 3 minutes. Blocking endogenous peroxidase activity: incubate the sections in 0.5% hydrogen peroxide/methanol at room temperature for 30 minutes; wash in wash buffer for 5 minutes twice. Block Fc receptors: use wax pen (Vector Labs, Ltd., USA) to draw a ring around sections, add 50 µl of Fc-blocker (anti-mouse CD16/32; Innovex Biosciences, Inc., USA) per section, incubate 30 minutes at room temperature; quick wash in TBST. To prevent non-specific bindings: add 100 µl per section of 2.5% rabbit serum (Vector Labs, Ltd., USA) in wash buffer containing 200 µl/ml of Avidin D block (Vector Labs, Ltd., USA) and incubate for 30 minutes at room temperature; briefly wash in wash buffer; add a block solution of 2.5% rabbit serum in wash buffer containing 200 µl/ml of Biotin Block (Vector Labs, Ltd., USA) diluents 100 µl per section and incubate for 30 minutes at room temperature; wash once after the incubation. Addition of primary antibody/isotype control: add 100 µl diluted 1°Ab or control antibody (Table 2-3) to the appropriate tissue section;

incubate the sections at 4℃ overnight. Bring the sections to room temperature for 1 hour. Wash the sections in wash buffer for 5 minutes twice. Addition of Biotinylated secondary antibody: add 100µl diluted species specific Biotinylated 2°Ab (1:200 dilution) to the tissue section; incubate for 30 minutes at room temperature and wash in wash buffer for 5 minutes twice. Addition of Avidin/Biotin Complex: add the Avidin/Biotin complex (Vector Labs, Ltd., USA) in wash buffer (50 µl per ml of Avidin and Biotin prepared 30 minutes before use) to the sections and incubate at room temperature for 30 minutes and wash in wash buffer for 5 minutes twice. Visualize using DAB: add 100 µl DAB (Vector Labs Ltd., USA) to the section and allow the stain to develop; stop the reaction by washing the section in wash buffer for 5 minutes; wash in running water for 5 minutes. Counter stain: stain the sections in Haematoxylin for 5 seconds, wash in running water. Dehydrate sections: 70% Ethanol 30 seconds, 90% Ethanol 30 seconds, 100% Ethanol 1 minute twice, Xylene 3 minutes twice. Mount coverslip over tissue section from Xylene with DPX mountant. The antibodies and negative controls used throughout the projects are listed in table 2-3; all antibodies were diluted with 2.5% rabbit serum (Vector Labs, Ltd., USA) and 2.5% mice serum (Thermo Scientific, USA) in wash buffer.

## 2.10 Tissue homogenising

To measure the protein levels in the tissue samples, less than 1 g of frozen samples was put into 1 ml ice cold RIPA lysis buffer completed with protease inhibitors. Homogenize the tissue for 20 seconds at 19,000 rpm using an electric homogenizer (Thermo Fisher Scientific Inc., US); incubate the homogenized sample for 5 minutes at 4℃; centrifuge the samples at 12,000 rpm for 10 minutes at 4℃; transfer the supernatants to new tubes and measure the protein concentration using Coomassie Protein Assay Kit (Bradford); store the samples at -70℃ for further collagen or cytokine measurements.

## 2.11 Lung digestion for flow cytometry

For the isolation of total lung cells, mouse lungs were extracted as described in section 2.8.2. The lungs were transferred to a Petri dish, cut into small pieces using scalpels in 1 ml RPMI 1640 containing 100 units/ml penicillin. The tissue fragments were then transferred via pipette to 24-well-plate and digested with 2 ml per lung of digest media (RPMI 1640 completed with 100 units/ml penicillin, 0.5 g/ml DNAse, 0.125 mg/ml Liberase TL (Roche, USA)), on a mechanical shaker and incubated at 37°C for 60 minutes. The digested material was then passed through a 100 μM Cell Strainer (Corning, USA) and fresh cold medium added to stop the digestion reaction. The cells were washed once by centrifugation at 1200 rpm for 10 minutes and then resuspended in 1.5 ml of red blood cell lysis buffer for 1 minute. Fresh medium was added to the cells and they were washed again. A single cell suspension was prepared in 10 ml of complete media for further experiments.

## 2.12 Broncho Alveolar Lavage Fluid (BALF) analysis

The post mortem mouse lungs were lavaged twice *in situ* with 0.8 ml of PBS via a catheter and syringe. After lavage, the total number of cells was counted using a

haemocytometer and the cell type was identified by differential cell count. For the determination of cell types, BALF was centrifuged at 1400rpm for 5 minutes, the supernatant was collected and stored at −20°C for further analysis of soluble collagen and cytokines; the remaining cells were resuspended and counted, and $5x10^4$ cells were used to make a slide smear using a cyto-centrifuge (Fisher Scientific Ltd., UK) at 450 rpm for 6 minutes. The slide preparations were stained with Rapid Romanowsky Stain (TCS Biosciences Ltd. UK) and a differential cell count was made based on the morphology of at least 400 cells under a Motic B1 microscope (Motic, Germany).

## 2.13 Cytokine measurements

### 2.13.1 Enzyme-linked immunosorbent assay

Murine cytokines IL-1β, IL-4, IL-5, IL-10, IL-12, IL-13 and IFN-γ were analyzed by ELISA using Ready-SET-Go! ELISA kits (eBioscience, USA) and TGFβ1, IL-33, ST2, CCL2, CXCL1 and CXCL9 using R&D DuoSet ELISA Development kits (R&D systems, USA). All ELISAs were conducted according to the manufacturers' protocol.

For IL-1β, IL-4, IL-5, IL-10, IL-12, IL-13 and IFN-γ: Coat an ELISA plate (Costar 9018, Corning, USA) with 100 µl/well of capture antibody in Coating Buffer (0.1M $NaHCO_3$) and incubate at 4°C overnight. Wash the wells 5 times with >300µl/well Wash Buffer (1X PBS, 0.05% Tween-20), blot plate on absorbent paper to remove any residual buffer. Block the wells with 200 µl/well Assay Diluent (1X PBS, 10%FBS), incubate at room temperature for 1 hour. Wash as step 2. Add 100 µl per well of 2-fold serial dilutions of the top standards to make the standard curve and blanks (PBS, RIPA buffer or media, respectively) as control. Incubate the plate at room temperature for 2 hours followed by wash as previous described. Add 100 µl/well of Biotin-conjugated detection antibody diluted in Assay Diluent and incubate at room temperature for 1 hour. Wash as previously steps. Add 100 µl per well of Avidin-HRP diluted in Assay Diluent and incubate at room temperature for 30 minutes. Wash the plate for a total of 7 times. Add 100 µl of Substrate Solution (Tetramethylbenzidine Substrate Solution) to each well, incubate at room temperature for 15 minutes. Stop the reaction by adding 100 µl per well of Stop Solution (1M HCl). Read the plates using an ELISA reader (Tecan, Switzerland) at 450 nm and subtract the value read at 570 nm. Measure the OD against the reagent blanks, standards and test samples, and obtain the relative cytokine concentrations by interpolating the readings to the Standard Curve.

For TGFβ1, IL-33, ST2, CCL2, CXCL1 and CXCL9: Coat the ELISA plate (Costar 9018, USA) with 100 µl per well of capture antibody in Coating Buffer (1X PBS)

and incubate overnight at room temperature. Wash the plate 5 times with >300µl/well Wash Buffer (1X PBS, 0.05% Tween-20). Block the wells with 300µl/well of Block Buffer (1X PBS, 5% Tween-20) at room temperature for 1 hour. Wash as step 2. To detect TGFß1 levels in cell culture or BAL fluid, add 0.1 ml 1N HCl to 0.5 ml sample and incubate at room temperature for 10 minutes, then neutralize the samples by adding 0.1ml 1.2N NaOH/0.5M HEPES. For serum samples, add 0.1 ml 2.5N Acetic Acid/10 M Urea to 0.1ml serum sample and incubate at room temperature for 10 minutes, then neutralize the samples by adding 0.1ml 2.7N NaOH/1M HEPES. Add 100 µl per well of 2-fold serial dilutions of the standards, blanks and samples to the appropriate wells and incubate at room temperature for 2 hours. Wash as step 2. Add 100 µl per well of detection antibody diluted in Reagent Diluent and incubate at room temperature for 2 hours. Wash as step 2. Add 100µl/well of Streptavidin-HRP diluted in Reagent Diluent and incubate at room temperature for 20 minutes. Wash the plates 5 times and then add 100 µl of Substrate Solution (Tetramethylbenzidine Substrate Solution) to each well and incubate at room temperature for 15 minutes. Stop the reaction by adding 50 µl per well of Stop Solution (2N $H_2SO_4$). Read the plate and obtain the concentrations as above.

## 2.13.2 Luminex

The concentrations of multi cytokine and chemokines were also detected by a Magnetic beads-based (20-plex) cytokine fluorescence assay method (Invitrogen, UK) according to the manufacturer's instructions, using a Luminex platform (Bio-Rad Laboratories Inc., UK). The 20-plex cytokine assay can detect 20 mediators, including: cytokines: IL-1α, IL-1ß, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, TNF-α, INFγ; chemokines: MIG, MIP-1α, KC, IP-10, MCP-1; inflammatory mediators: FGF-Basic, VEGF, and GM-CSF.

First of all, the protein standard was reconstituted with 0.5 ml of Assay Diluent and then the serially diluted reconstituted standard to make a standard curve. Pre-wet the assay wells by adding 200 µl of Working Wash Solution into

designated wells; incubate plate 20 seconds at room temperature; aspirate the Working Wash Solution from the wells using the vacuum manifold (do not exceed 5 mm Hg). Vortex the antibody beads for 30 seconds, then sonicate using Ultrasonic water bath (Dawe Instruments, UK) for 40 seconds immediately prior to use in the assay; pipette 25 µl of the antibody bead solution into each well, keep the plate from light. Wash the beads twice by adding 200 µl Working Wash Solution to the well and dry the filter plate using vacuum manifold. Pipette 50 µl Incubation Buffer into each well, add 100 µl of appropriate standard dilution and blank to wells designated for the standard curve, add 50 µl Assay Diluent followed by 50 µl sample to each well designated for the samples; cover the filter plate with an aluminium foil and incubate the plate for 2 hours at room temperature on an orbital shaker (500-600 rpm); wash the filter plate twice. Add 100 µl of 1X Biotinylated Detector Antibody to each well and incubate the plate for 1 hour at room temperature on an orbital shaker. Wash the filter plate twice. Add 100 µl of Streptavidin-RPE to each well and incubate the plate for 30 minutes at room temperature with shaker. Wash the filter plate three times. Add 100 µl of Working Wash Solution to each well, shake the plate (500-600 rpm) for 3 minutes to re-suspend the beads. Uncover the plate and insert the plate into the XY platform of Luminex instrument, and analyze the samples. Determine the concentration of the samples from the Bio-Plex standard curve fitting software (Bio-Rad Laboratories, UK).

### 2.13.3 Western blotting

Fibroblast cells cultured in 24-well plate were washed twice using 500 µl ice-cold PBS followed by 50 µl of ice-cold RIPA buffer supplemented with protease inhibitor cocktail, 1 mM PMSF, and 1 mM sodium orthovanadate. Cells lysis was then collected using a cell scraper and the protein concentrations were measured using Coomassie Blue method. The protein samples were denatured by mixing with NuPAGE LDS sample buffer containing NuPAGE reducing agent and incubated at 70°C for 10 min. An equal amount (~25 µg) of protein samples was loaded onto 10% NuPAGE Bis-Tris gel placed in an XCell Surelock Mini-Cell Electrophoresis System (Invitrogen, UK). Fill the inner chamber of the

electrophoresis system with 200 ml of running buffer supplemented with 500 µl of NuPAGE antioxidant and the outer chamber with running buffer. The proteins were electrophoretically separated for 1 h (150 V). The separated proteins were transferred onto a nitrocellulose membrane for 1 h (30 V) in an XCell II blot module (Invitrogen, UK) containing transfer buffer. The membrane was then washed in PBS and blocked with 10% (w/v) non-fat milk powder in PBS for 1 h at room temperature. After 3 × 5 minutes washes in PBS, the membrane was probed with primary antibody diluted in 5% (w/v) non-fat milk powder in PBS-Tween 2 hours at room temperature. The antibodies used were affinity purified polyclonal rabbit anti-mouse IL-33 Ab (1:1000, R&D System). The membrane was washed in 3 × 5 minutes washes in PBS-Tween and probed with horseradish peroxidase-conjugated secondary antibody (1:4000) in PBS-Tween for 1 hour at room temperature. After the 3 × 5 minutes washes in PBS, the membrane was incubated in chemiluminescent substrate (Amersham ECL, Amersham Bioscience) for 30 seconds. The membrane was then placed between two layers of acetate film and exposed to X-ray film (Kodak BioMax Light Film, Sigma-Aldrich). The film was then developed in a Kodak X-OMAT developer.

## 2.14 Flow Cytometry

For surface staining, the cells from each sample were washed with Fluorescence Assisted Cell Sorting (FACS) buffer (0.5% BSA and 2mM EDTA in DPBS), resuspended in 100 µl of FACS buffer and incubated for 30 minutes at 4°C with FcBlocker to prevent non-specific antibody binding to Fc receptors. Wash the samples once with 1 ml FACS staining buffer per tube, and stain with relevant fluorochrome-conjugated antibody (Table 2-3) or isotype controls for 30 minutes at 4°C in the dark (always avoiding light from this step). Wash the samples twice with 2 ml of FACS buffer, and resuspended in 400 µl of FACS buffer. The cells were then collected and analyzed with a FACSCalibur flow cytometer (BD Bioscience, USA).

For intracellular staining, cells were stimulated with PMA (50 ng/mL) and Ionomycin (1 µg/mL) for 4 hours before staining. Stain the cells with suitable cell surface antigens as described above. Wash once, then fix the cells by adding 100 µl of IC Fixation Buffer (eBioscience, USA) and incubate for 20 min at 4°C in the dark, wash twice by adding 2 ml of Permeabilization Buffer (eBioscience, USA). Resuspend the cells with 100 µl of Permeabilization Buffer, add the appropriate amount of fluorochrome-labeled antibodies for the detection of intracellular antigen and incubate for 20 min at room temperature, wash once by adding 2 ml of Permeabilization Buffer. Then wash by adding 2 ml of FACS staining buffer, resuspending the cells with 400 µl of staining buffer for the acquiring. To exclude dead cells, 2 µl of Via-probe (BD Bioscience, USA) was added to each tube immediately before acquiring and analysis on Beckman Coulter CyAn Flow Cytometer. The data was analysed by using FlowJo software (Tree Star Inc., USA). The anti-mouse antibodies used throughout the projects are listed in Table 2-4.

## 2.15 Statistical analysis

Data were analyzed by using one-way Analysis of Variance (ANOVA) followed by Tukey's or Newman-Keuls post-hoc analysis. One way ANOVA was used to examine mean differences between two or more groups, in order to compare every mean to every other mean. All results showed throughout were displayed as mean + standard error mean (SEM) from 5 to 7 mice unless stated otherwise. Data are representative of at least 2 separated experiments. A p value of less than 0.05 was considered to indicate statistical significance.

The experimental group size was determined by disease incidence and from the expected difference among experimental groups from preliminary experiments or from literature search wherever possible, also taking into account resources available and ethical animal use. A power of 0.8 was used to calculate the experiment group sizes (Festing and Altman, 2002, Charan and Kantharia, 2013).

# Chapter 3

# The pathogenic role of IL-33 in bleomycin-induced lung fibrosis in mice

# Chapter 3: The pathogenic role of IL-33 in bleomycin-induced lung fibrosis in mice

## 3.1 Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic interstitial pneumonia of unknown cause and poor prognosis (Martinez et al., 2006). While the etiology of IPF is still not fully understood, it has histological similarities to other forms of lung fibrosis which result from chronic inflammatory reactions induced by variety of stimuli including persistent infections, autoimmune reactions, allergic response, chemical toxins, radiation and tissue injury (Scotton and Chambers, 2007, Wynn and Ramalingam, 2012). No drugs have been approved so far for the treatment of IPF, although several new drugs are currently undergoing clinical trials (Noble et al., 2012). Patients with IPF live for an average of 2-3 years after the diagnosis (Ley et al., 2011, Cottin, 2013). There is an imperative to understand the underlying pathogenesis in order to direct novel therapies for IPF and other fibrotic diseases.

Bleomycin (BLM) is an anti-neoplastic antibiotic isolated from *Streptomyces verticillus* and is a cancer chemotherapy drug (Umezawa et al., 1967). Its major side effect is pulmonary toxicity (Muggia et al., 1983, Hay et al., 1991, Sugiyama and Kumagai, 2002). Bleomycin is reported to cause lung damage by direct breakage of DNA strands and by the induction of reactive oxygen species (ROS) therefore inducing oxidative stress (Moeller et al., 2008). While this limits its clinical application, using BLM to induce lung fibrosis in animals has provided a useful model to study human lung fibrosis. This animal model was established on dogs first, and then extended to rodents (Fleischman et al., 1971, Adamson and Bowden, 1974, Frost et al., 1983).

The lung fibrosis is characterized by excessive accumulation of fibroblasts and extracellular matrix (ECM) components, including collagens. Many inflammatory chemokines and cytokines are involved in the mechanism of fibrogenesis,

especially Th2 type cytokines (IL-4 and IL-13) which promote fibroblast activation and collagen production (Wynn, 2003, Barron and Wynn, 2011, Wynn and Ramalingam, 2012, Wynn, 2011, Borthwick et al., 2013).

IL-33, a member of the IL-1 cytokine family, is a dual functional factor. Full-length IL-33 contains a nuclear target domain and may act as a transcription factor (Lefrancais et al., 2012, Lefrancais and Cayrol, 2012). However, the pathophysiological role of nuclear IL-33 is still largely unknown (Lefrancais and Cayrol, 2012). Mature cytokine IL-33 may serve as an 'alarmin' which is released when cells sense inflammatory signals or undergo necrosis (Moussion et al., 2008, Lamkanfi and Dixit, 2009, Oboki et al., 2010, Liew, 2012) The cytokine IL-33 then binds to its receptors and mainly induces type 2 cytokines (IL-5 and IL-13) and promotes a type 2 response in inflammatory and allergic diseases (Komai-Koma et al., 2007, Kurowska-Stolarska et al., 2008). It is reported that the IL-33/ST2 axis may also play a profibrotic role in different organs, including the lung (Yanaba et al., 2011, Lopetuso et al., 2012, Santulli et al., 2012, Cevikbas and Steinhoff, 2012, Zhu and Carver, 2012, Roussel et al., 2013). It has been shown that over-expressing soluble ST2 could attenuate BLM-induced lung fibrosis in transgenic mice (Mato et al., 2009). However, the role of IL-33, as a cytokine, in the pathogenesis of lung fibrosis has not been defined although as a nuclear factor, it was recently reported to be a pro-fibrotic factor (Luzina et al., 2013).

The aim of the work presented in this chapter was to determine the effect of IL-33 as a cytokine in BLM-induced lung fibrosis and any associated mechanism. I used two experimental approaches:

1) using BLM to induce fibrosis in ST2 deficient mice,

2) treating mice with neutralising anti-IL-33 antibody before inducing fibrosis,

Our results demonstrated that the IL-33/ST2 axis is essential for the development and exacerbation of BLM-induced pulmonary fibrosis.

## 3.2 Bleomycin up-regulates IL-33 expression in lung tissue

As IL-33 may serve as an alarm cytokine released to indicate tissue damage, I sought first to find out if bleomycin could induce the expression of ST2 and IL-33 in lung cells *in vitro* and in lung tissue *in vivo*.

I first studied the expression of IL-33 and ST2 *in vitro*. The lung tissues from normal C57B/6 mice were collected and digested into single cells and cultured with BLM for 24 hours. The culture supernatants were collected to measure the concentration of IL-33 and cells for the mRNA expression levels of IL-33 and its receptor ST2L mRNA by real-time PCR. BLM induced the expression of both IL-33 and its receptor ST2L in total lung cells in a dose-dependent manner (Fig. 3-1).

To study the induction of IL-33 and ST2 expression *in vivo*, groups of WT C57B/6 mice were given BLM or control PBS on day 0, and sacrificed at different times up to 14 days (Fig. 3-2). The BAL fluid (BALF) and lung tissues were collected and the protein and mRNA levels of IL-33 were determined by IHC, ELISA and real-time PCR. The tissue samples were processed and stained for IL-33 as described in Chapter 2. The IHC results demonstrated that airway epithelial cells were constitutively positive for IL-33 staining (Fig. 3-3a). However, BLM also induced the production of IL-33 protein in cells located in the alveoli of airway compared to PBS controls (Fig. 3-3b). The location and morphological appearance of these cells suggest that the IL-33+ cells in the alveoli of airway were likely to be alveolar macrophages.

The dynamics of IL-33 expression were explored by a kinetic study demonstrated that BLM induced the expression of IL-33 starting from day 1 and lasting for at least 14 days (Fig. 3-4a). The BALF of mice treated with BLM contained significantly higher concentration of IL-33 compared to control group (Fig. 3-4b).

Together these results demonstrated that IL-33 as well as its receptor ST2 is up-regulated by the BLM challenge in lung tissue, indicating that the IL-33/ST2 axis may play an important role in BLM-induced fibrosis.

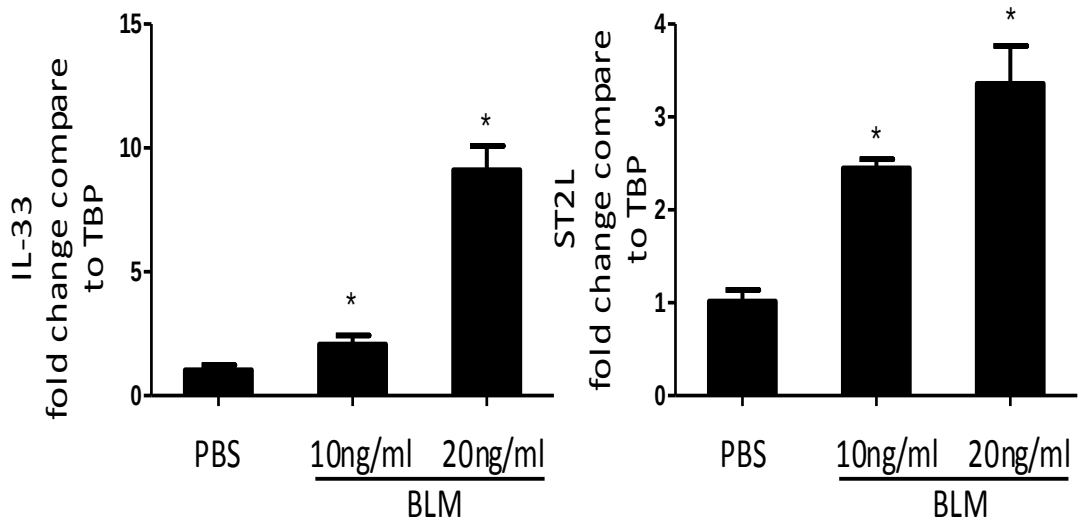



**Fig. 3-1 BLM increases IL-33 and ST2 expression in total lung cells.**

Total lung cells from normal C57B/6 mice were digested and cultured with different doses of BLM for 24 hours. The cells were then harvested and digested for PCR assay as described in Chapter 2. IL-33 and ST2L expression were measured by real-time PCR. Data are representative of three experiments, mean ± SEM, n=3 per group, *P < 0.05 compared to PBS group.

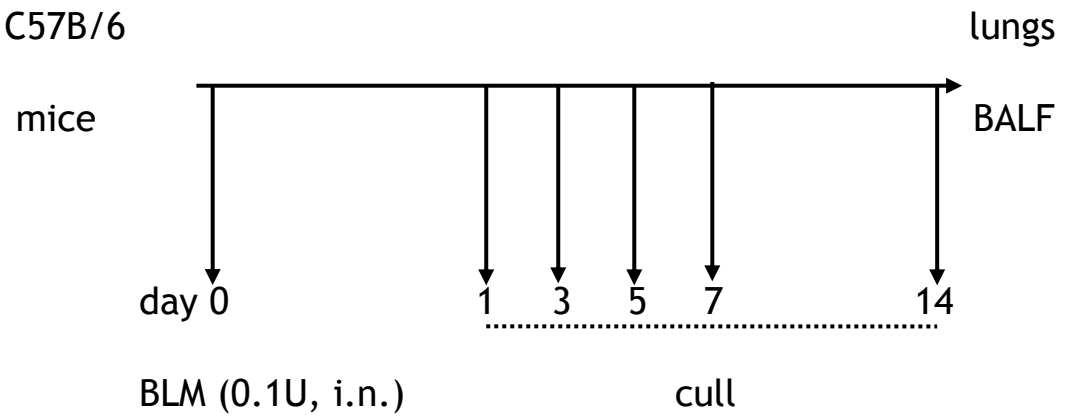



**Fig. 3-2 Experimental plan used to induce fibrosis in WT mice.**

Male WT C57B/6 mice were administered with 0.1U BLM or control PBS i.n. on day 0. The mice were monitored daily. BAL fluid (BALF) and lungs were collected at cull times; Cytokine concentrations in BALF were measured by ELISA; lung tissue was used for histology analysis, digested in RIPA buffer for measurement of collagen and cytokines or digested in Trizol for PCR analysis as described in Chapter 2.

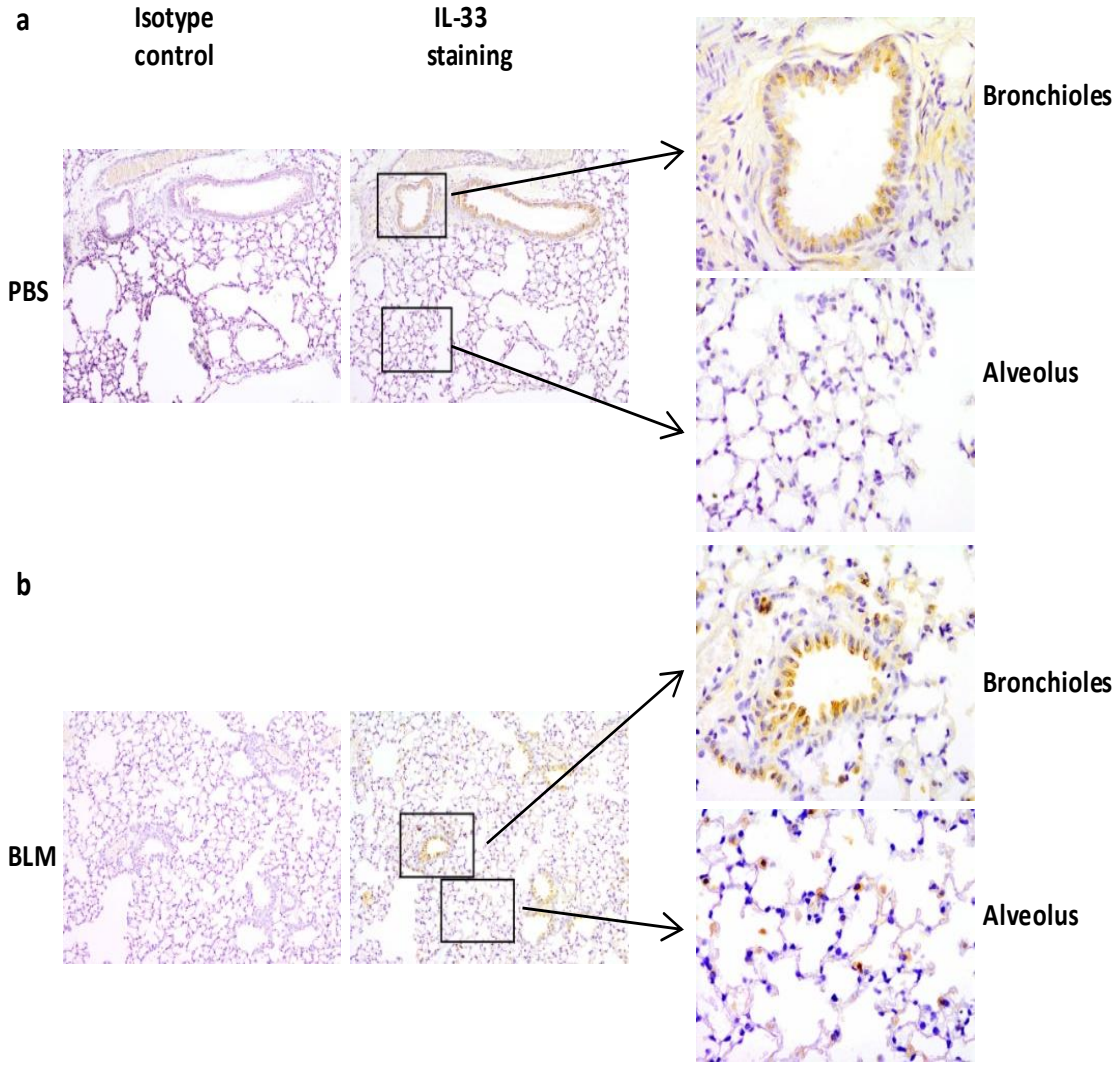



**Fig. 3-3 BLM increases IL-33 expression in lung tissue.**

Lung tissue samples from groups of male mice treated with BLM or PBS as control were harvested 7 days after BLM/PBS administration. The tissue samples were processed and stained for IL-33 as described in Chapter 2. Staining showed that IL-33 positive cells are consistently existed among bronchioles but increased in the alveolus area. (original magnification x100, enlarged to x400)

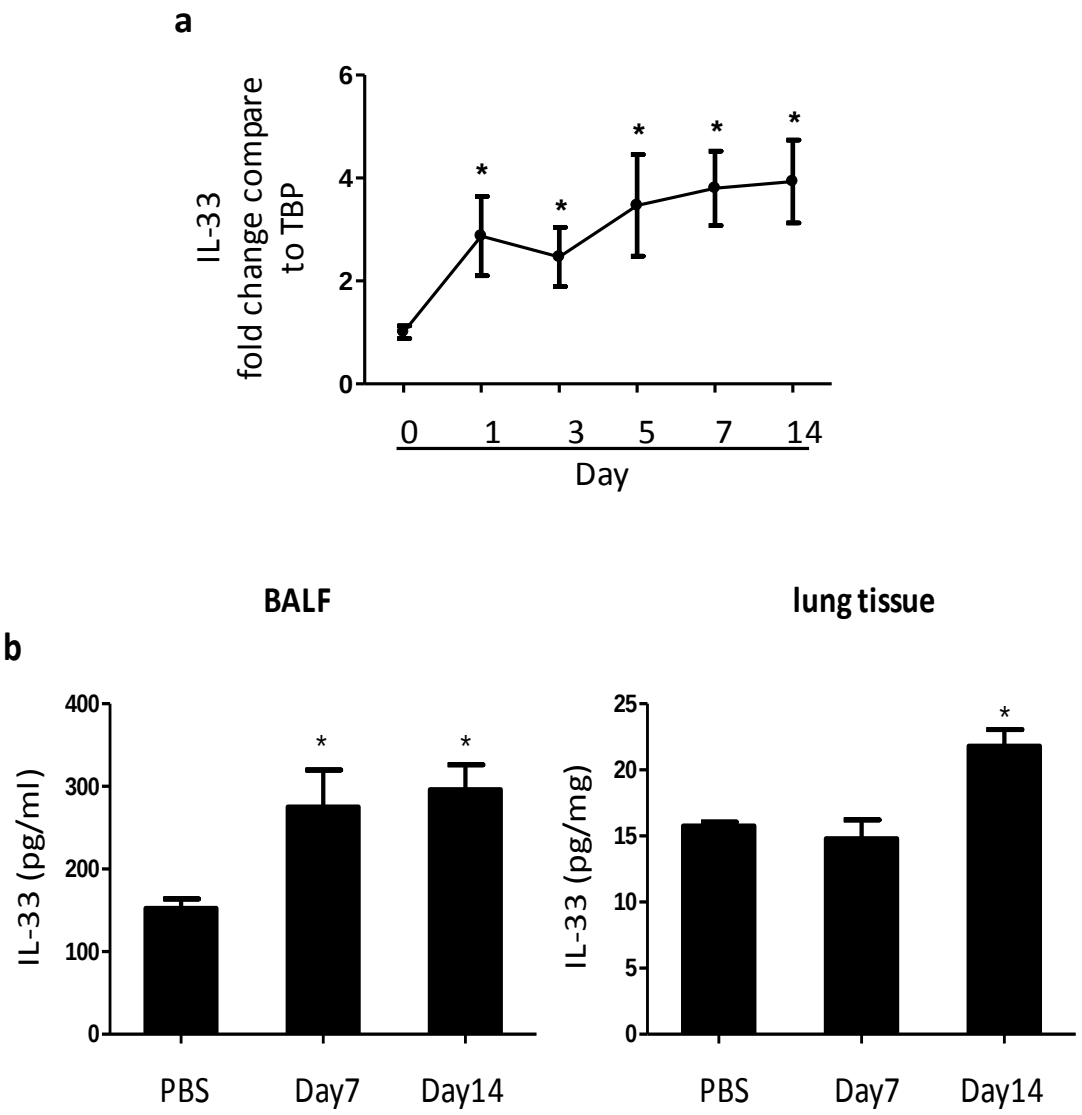



**Fig. 3-4 BLM increases IL-33 mRNA expression in lung tissue.**

Lung tissue and BALF fluid samples from groups of C57B/6 mice given BLM or PBS control were harvested from day 1 to day 14 after BLM administration. The tissue samples were directly digested using RIPA buffer and TRIzol. The level of IL-33 gene expression was measured using real-time PCR (a). The IL-33 concentrations in BALF (left) and lung tissue (right) were measured using ELISA (b). Data are representative of three experiments, mean ± SEM, n= 6 mice per group, *P < 0.05 compared to PBS group.

## 3.3 ST2 deficient mice show impaired BLM-induced lung fibrosis

We next investigate the importance of IL-33 specificity and signalling by examining its receptor ST2 in the pathogenesis of fibrosis. We did this by using ST2 gene-deleted mouse strain on a C57B/6 background in BLM-induced fibrosis model. Groups of WT and ST2-/- mice were given BLM or PBS as control on day 0. The mice were sacrificed on day 7 and day 14 (Fig. 3-5). Lung tissues and BALF were collected for analysis.

WT mice that received BLM developed lung inflammation (Fig. 3-6a) and fibrosis (Fig. 3-7a) from day 7 and further enhanced on day 14 compared to PBS control groups. This was evidenced by significantly increased inflammatory leukocyte infiltration and collagen deposition in the lung which quantified by the histological inflammatory and fibrosis scores, (Fig. 3-6b and 3-7b). The soluble collagen content and collagen III gene expression in the lung tissue of WT mice were also enhanced statistics (Fig. 3-8). Importantly, all the BLM-induced lung inflammation, fibrosis and collagen production in the same-treated ST2-/- mice were significantly reduced compared to the WT controls (Fig. 3-7 and 3-8). Furthermore, ST2-/- mice that received BLM also had significantly lower leukocyte counts in the BAL fluid, and lower proportions of macrophages, neutrophils and lymphocytes on day 14 compared to control groups (Fig. 3.9). Moreover, the reduced lung inflammation and leukocyte infiltrations in the lung of ST2-/- mice were also accompanied by significantly reduced levels of IL-33, IL-1β and inflammatory chemokines (CXCL1, CXLC2 and CCL2) compared to WT control (Fig. 3-10).

Taken together, these results suggest that ST2 signals play a pathological role in the development of BLM-induced fibrosis in mice and in the enhanced production of IL-33 and other inflammatory cytokines and chemokines in lung tissue.

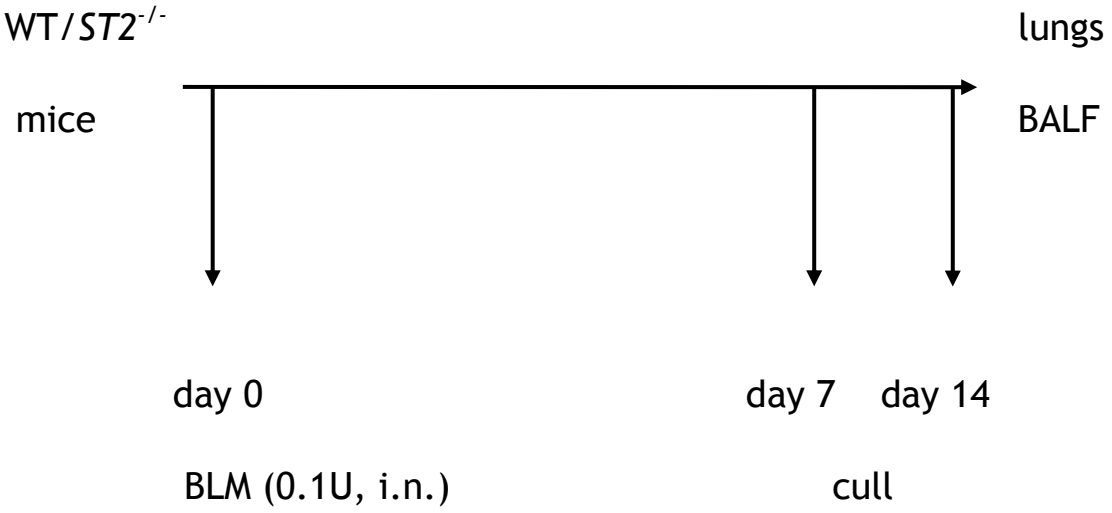



**Fig. 3-5 Experimental plan used to induce fibrosis in WT and ST2$^{-/-}$ mice.**

Male WT and ST2$^{-/-}$ C57B/6 mice were administered with 0.1U BLM or PBS control i.n. on day 0. The mice were monitored daily. BAL fluid and lung tissue were collected on day 7 and day 14. Cytokine concentrations in BALF were measured by ELISA; lung tissue was used for histology analysis, digested in RIPA buffer for measurement of collagen and cytokines or digested in Trizol for PCR analysis as described in Chapter 2.

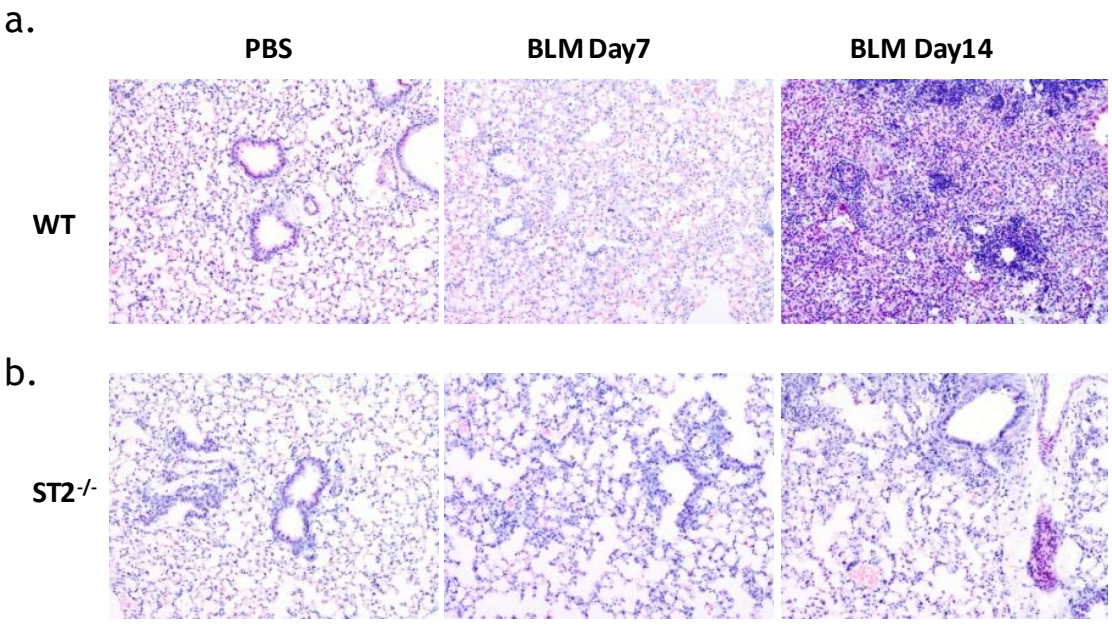
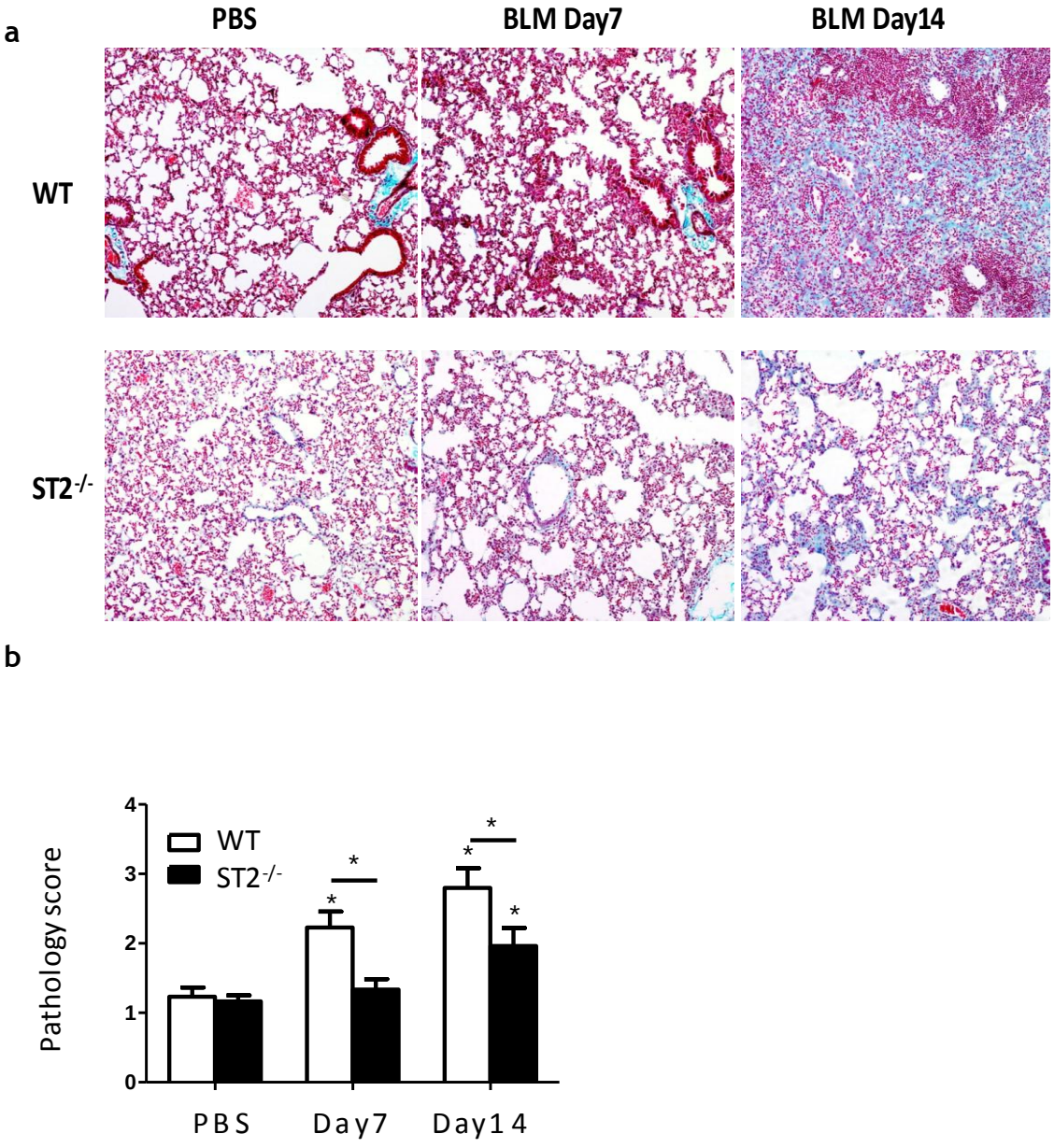



**Fig. 3-6 ST2$^{-/-}$ mice have impaired bleomycin-induced lung inflammation**

Lung tissue from WT and ST2$^{-/-}$ C57B/6 mice was harvested 7 days and 14 days after BLM administration. The lung tissue histological sections from WT mice (a) or ST2$^{-/-}$ mice (b) were stained with H&E as described in Chapter 2. Representative pictures of mean histological change were shown from each group (original magnification x100).




## Fig. 3-7 ST2$^{-/-}$ mice have impaired BLM-induced lung fibrosis

Lung tissue from male WT and ST2$^{-/-}$ C57B/6 mice was harvested on days 7 and 14 after BLM administration. The tissue histology sections were stained with Trichrome as described in chapter 2 (a). Representative pictures of mean histological change were shown from each group (original magnification x100). Pathological scores were determined as described in Chapter 2 (b). Data are representative of three experiments, mean ± SEM, n= 6 mice per group, *P < 0.05 compared to PBS groups.

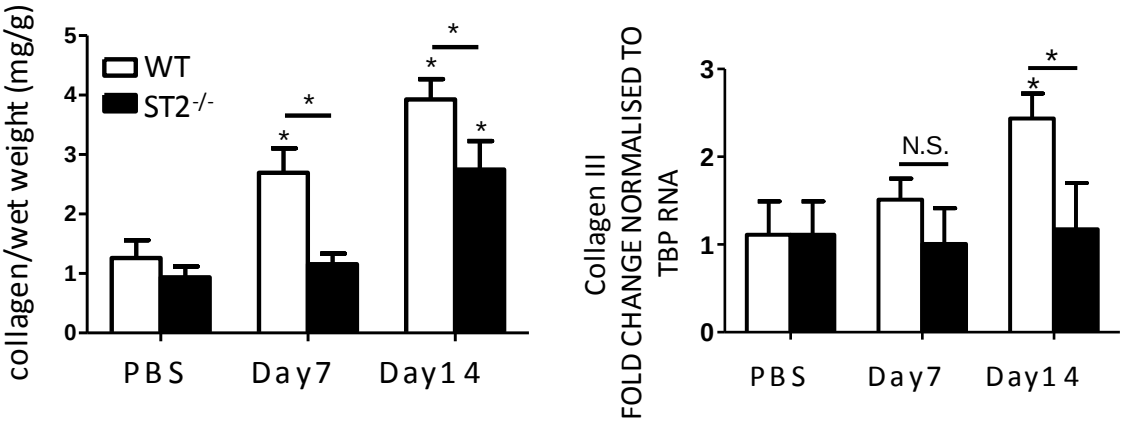



**Fig. 3-8 ST2$^{-/-}$ mice showed reduced collagen content of lungs after BLM administration.**

Lung tissue from male WT and ST2-/- C57B/6 mice was harvested on days 7 and 14 after BLM administration. The collagen content in the lung tissue was measured using a Sircol soluble collagen assay; collagen III mRNA expression was measured using real-time qPCR as described in chapter 2. Data are representative of three experiments, mean ± SEM, n= 6 mice per group, *P < 0.05 compared to PBS groups.

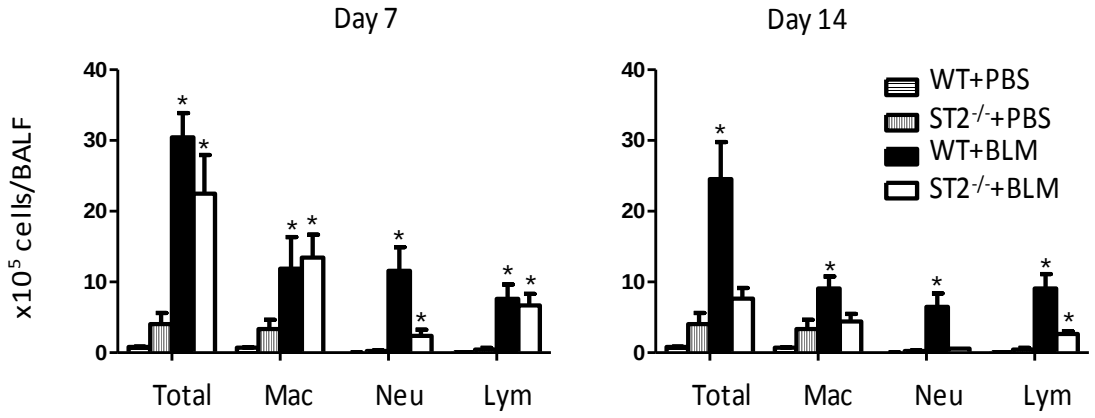



**Fig. 3-9 ST2$^{-/-}$ mice have reduced inflammatory lung lavage cytology following administration of bleomycin**

BAL of WT and ST2-/- C57B/6 mice was harvested 7 days and 14 days after BLM administration. Cells in the BAL fluid were stained and counted as described in chapter 2. Data are representative of three experiments, mean ± SEM, n= 6 mice per group, *P < 0.05 compared to PBS groups.

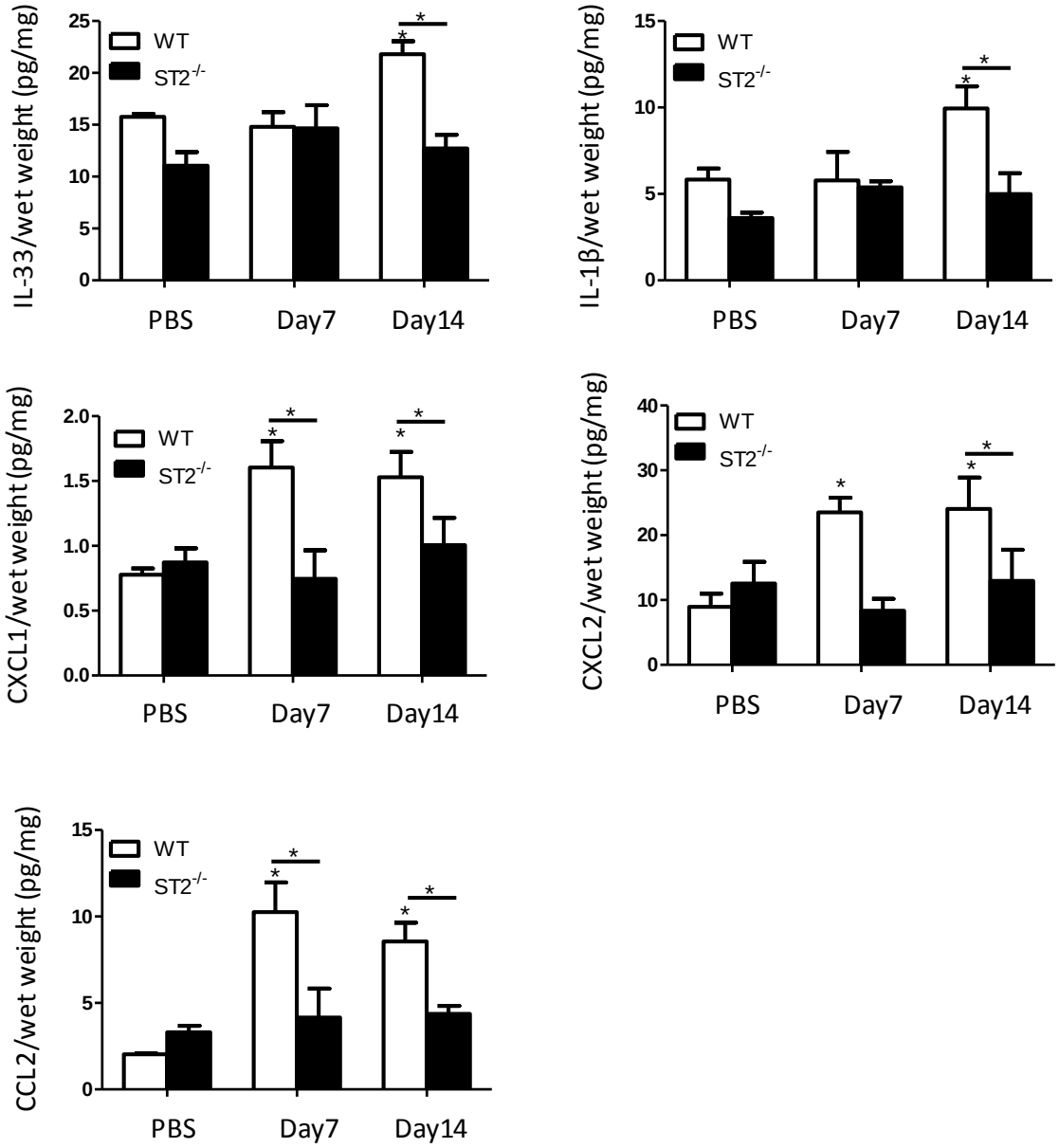



**Fig. 3-10 ST2$^{-/-}$ mice have reduced lung inflammatory cytokine and chemokine profiles in BLM-induced fibrosis.**

Lung tissues were collected on days 7 and 14 after administration of bleomycin. The tissues were lysed and the extract was measured for IL-1ß, IL-33, CXCL1, CXCL2 and CCL2 concentrations. Data are representative of three experiments, mean ± SEM, n= 6 mice per group, *P < 0.05 compared to PBS groups.

## 3.4 IL-33 neutralizing antibody suppresses BLM-induced lung fibrosis

We further assessed the importance of endogenous IL-33 in the development of BLM-induced fibrosis by treating the mice with anti-IL-33 antibody as described previously (Qiu et al., 2013). Groups of WT C57B/6 mice were administered intranasally with BLM or control PBS on day 0, some mice were also treated with anti-IL-33 antibody or control rabbit IgG intraperitoneally on day 0, 5 and 10. Mice were sacrificed on day 14 and lung and BALF samples were collected and analysed as described in chapter 2 (Fig. 3-11).

In WT mice given bleomycin, those that were treated with anti-IL-33 antibody developed significantly less lung inflammation (Fig. 3-12) and fibrosis (Fig 3-13) compared to those treated with non-specific IgG control. This was demonstrated by significantly decreased histological inflammatory and fibrosis scores, soluble collagen content and collagen III gene expression (Fig. 3-14). In the BAL fluid, leukocyte numbers including macrophages and neutrophils were significantly reduced on day 14 by the anti-IL-33 antibody treatment (Fig. 3-15). The anti-IL-33 antibody treatment significantly reduced IL-33 protein levels in lung tissue (Fig. 3-16), and also concentration of IL-1 and inflammatory chemokine production in the lung tissue, consistent with the reduced IL-1 and inflammatory chemokine production in ST2-/- mice (Fig. 3-16 and 3-10).

These results therefore demonstrated that endogenous cytokine IL-33 is critically involved in the pathological progress of BLM-induced fibrosis in mice, especially during the inflammatory phase preceding fibrosis.

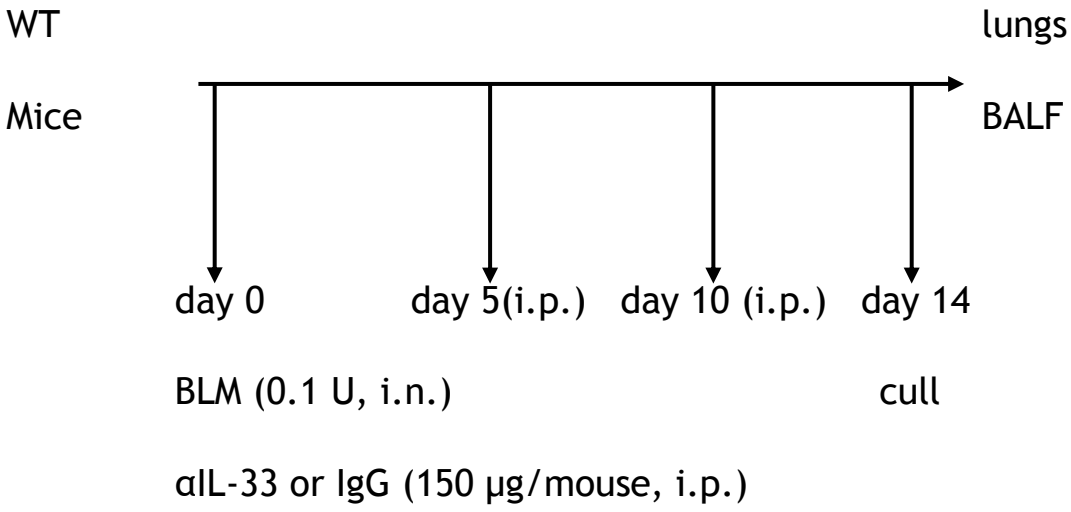



**Fig. 3-11 Experimental plan for the treatment of BLM-induced fibrosis in mice using anti-IL-33 antibody**

WT C57B/6 mice were administered with or without 0.1U BLM i.n. on day 0. The antibody or control rabbit IgG (150 μg/mouse) was given intraperitoneally 30 minutes before BLM on day 0 and also on day 5 and day 10. The mice were monitored daily and culled on day 14. Cytokine concentrations in BALF were measured by ELISA; lung tissue was used for histology analysis, digested in RIPA buffer for measurement of collagen and cytokines or digested in Trizol for PCR analysis as described in Chapter 2.

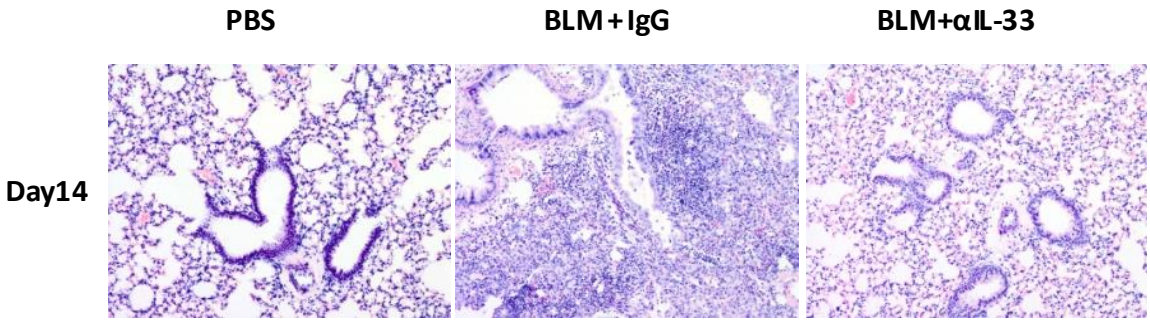



**Fig. 3-12 anti-IL-33 antibody treated mice have reduced development of BLM-induced airway inflammation.**

Lung tissue from male WT mice was harvested 14 days after BLM and antibody administration. The tissue histological sections were stained with H&E as described in chapter 2. Representative pictures of mean histological change were shown from each group (original magnification x100).

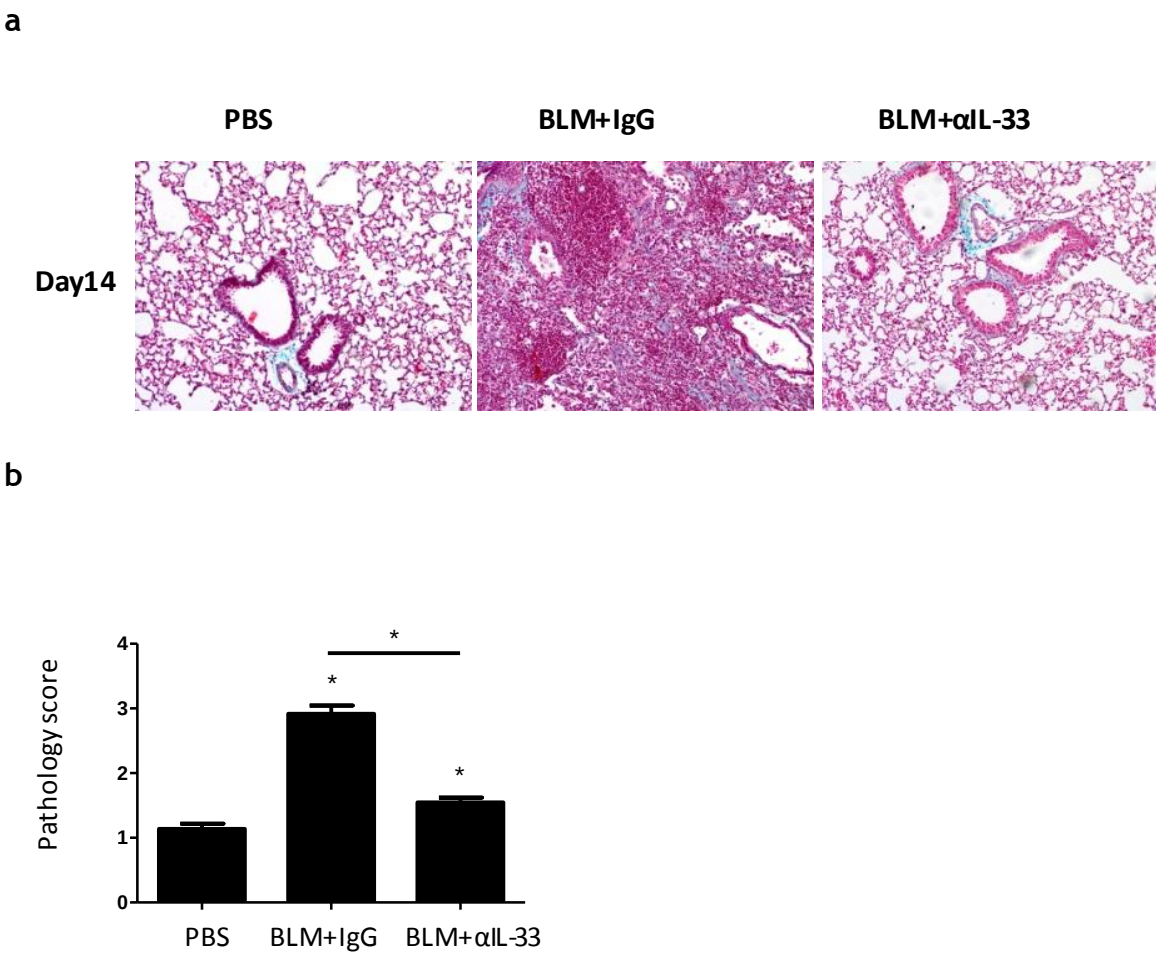



**Fig. 3-13 αIL-33 antibody treated mice have ameliorated BLM-induced lung fibrosis.**

Lung tissue male WT mice were harvested 14 days after BLM and antibody administration. a. The tissue samples sections were stained with Trichrome as described in chapter 2. b. Pathological scores were determined as described in chapter 2; representative pictures of mean histological change were shown from each group (original magnification x100). Data are representative of three experiments, mean ± SEM, n=3 per group, *P < 0.05.

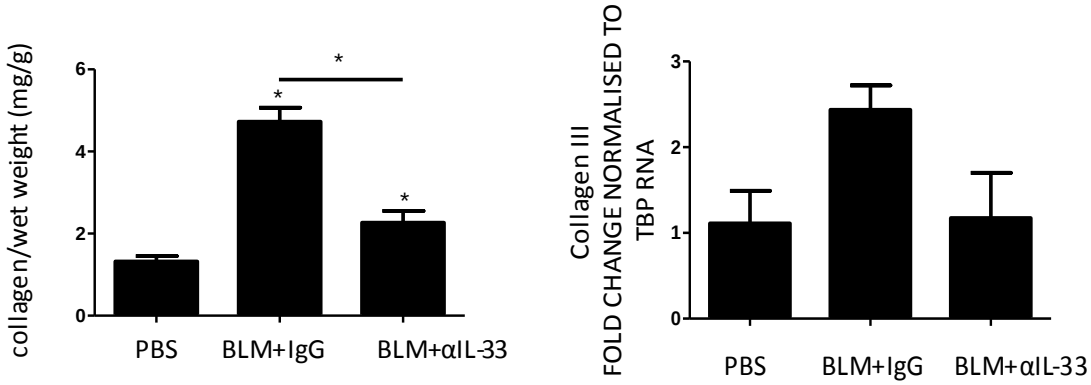



**Fig. 3-14 αIL-33 treatment reduces collagen content of lungs in BLM-induced fibrosis mice.**

The mice were treated as in Fig. 3-11. Lung tissues were collected on day 14, collagen content in the lung tissues was measured using Sircol soluble collagen assay; collagen III expression was measured using real-time PCR. Data are representative of three experiments, mean ± SEM, n= 6 mice per group, *P < 0.05.

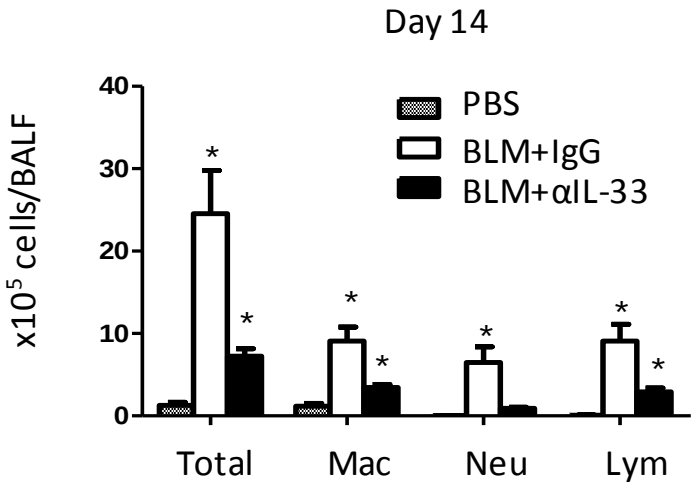



**Fig. 3-15 αIL-33 treatment reduces BLM-induced leukocyte infiltrations in the lung.**

Leucocytes in the BAL fluid were stained and counted as described in chapter 2. Data are representative of three experiments, mean ± SEM, n= 6 mice per group, *P < 0.05 compared to PBS group.

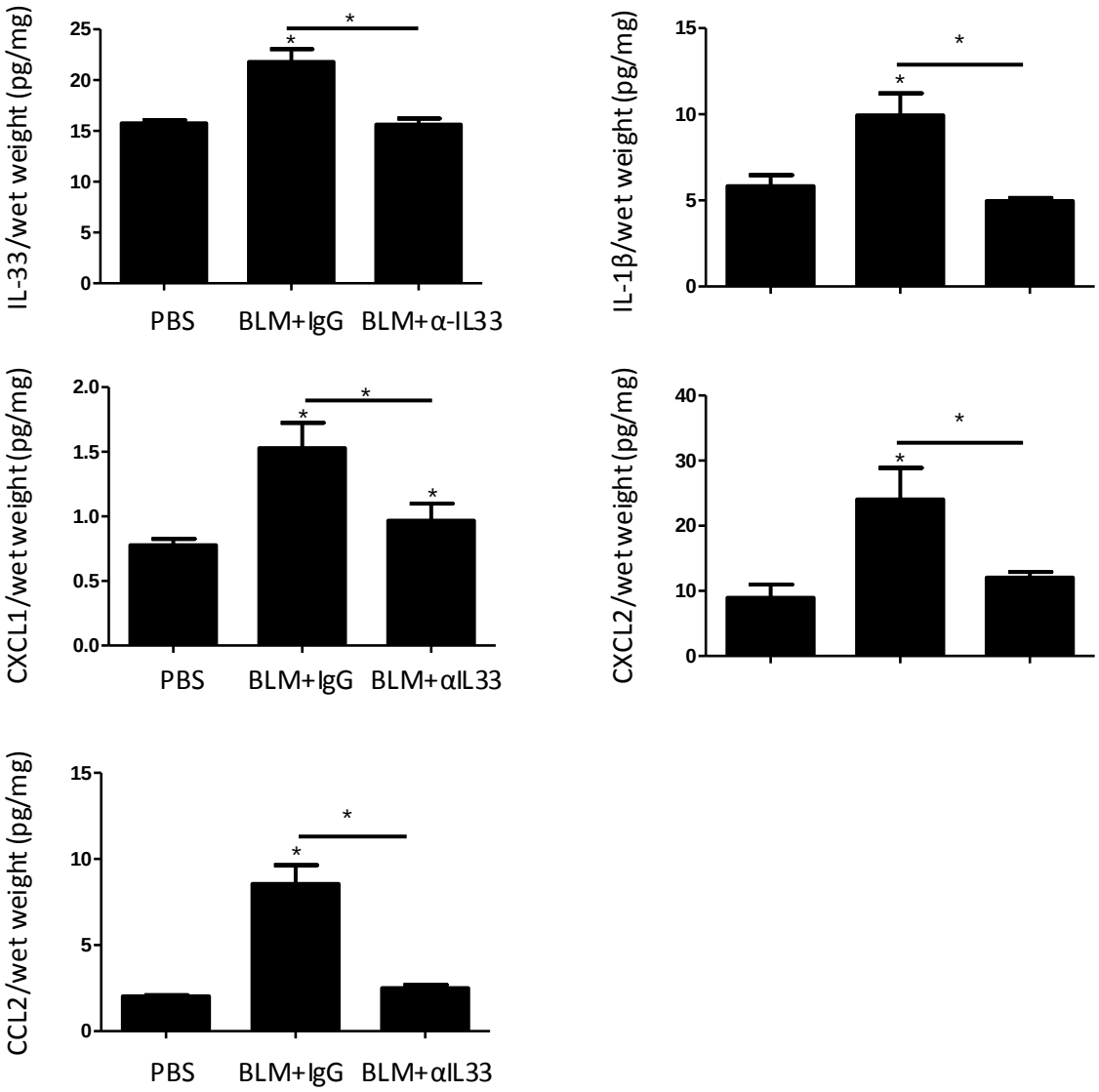



**Fig. 3-16 αIL-33 treatment reduces inflammatory cytokines and chemokines in BLM-induced lung fibrosis mice.**

Lung tissues were collected on day 14 and lysed using RIPA buffer, the IL-1β, IL-33, CXCL1, CXCL2 and CCL2 concentrations were measured using ELISA. Data are representative of three experiments, mean ± SEM, n= 6 mice per group, *P < 0.05.

## 3.5 Chapter Discussion

By using three different experimental approaches, we demonstrated that IL-33 is a key pro-inflammatory factor in lung fibrosis possibly through its ability to promote inflammation. The main findings from this work described in this chapter are summarised:

i) Administration of BLM stimulates IL-33 mRNA expression in the lung.
ii) Treatment with IL-33 neutralizing antibody prevents IL-33 expression and BLM-induced lung fibrosis.
iii) ST2 deficient mice develop impaired lung fibrosis and IL-33 expression after BLM administration.

These results together suggest that IL-33 expression induced by bleomycin is necessary for the development and severity of BLM-induced fibrosis.

We observed that IL-33 was constitutively expressed in normal mouse lung tissue, predominantly in airway epithelium. Administration of bleomycin induced an increased expression of lung tissue IL-33 within one day and this lasted for at least 14 days. Thus, the constitutive low levels of IL-33 could be increased and prolonged *in vivo*. However, the cellular source of this increased IL-33 expression is not fully understood. The histological results suggest that lung epithelial cells constitutively express IL-33 independently of BLM as reported before (Kurowska-Stolarska et al., 2009). In contrast, location and morphological appearance suggests that the increased expression of IL-33 in lung tissue cells inducible by BLM appeared related to the alveolar macrophages as seen by IHC of lung tissue histology using anti-IL-33 antibody. This antibody has been demonstrated in several papers to specifically detect IL-33 in the tissues (Pastorelli et al., 2010, Lefrancais et al., 2012). The specific staining of the antibody was also confirmed by using the appropriate antibody isotype control in the IHC. Additional validation experiments that would further confirm the specificity of the antibody would include, for example, using the same antibody

and method to stain lung tissue from IL-33 knockout or IL-33 over-expressing mice, or by blocking this anti-IL-33 antibody with increasing amounts of recombinant IL-33 before staining which would demonstrate elimination of specific staining.

Furthermore, in order to identify the cellular sources of IL-33, an effort to double stain the IL-33-positive cells in lung tissue sections with a combination of antibodies that recognises both the macrophage marker F4/80 as well as IL-33 did not show double staining. It is possible that there were technical problems which I didn't understand. I therefore couldn't confirm that the IL-33-positive cells are macrophages, because mouse type II pneumocytes were also reported to be IL-33-positive, which do not express F4/80 (Kim et al., 2012). In contrast to this report, we did not observe IL-33 positive type II pneumocytes in healthy control mouse lung tissue. However, analysing cells dispersed from mouse lung tissue using flow cytometry, we demonstrated that bleomycin significantly increased the frequency and number of cells expressing IL-33 that also stained positive for cytokeratin suggesting that they were epithelial cells, as well as cells expressing IL-33 that also stained with F4/80 suggesting they were macrophages and this was increase did not occur in St2-/- mice (Li et al., 2014, Fig. E7 and Fig. 4). These data indicate that both lung epithelial cells and macrophages are the likely source of endogenous IL-33 in the bleomycin-induced fibrosis.

It is still unclear how the IL-33/ST2 pathway is involved in lung fibrosis. It was recently reported that increased IL-33 expression is associated with increased leukocyte infiltration of lung tissue, including macrophages and neutrophils (Salmond et al., 2012). It is known that these cells are associated with the pathogenesis of lung fibrosis and migrate to lung tissue by chemotaxis in response to chemokines including CXCL1 and CXCL2 (Rot and von Andrian, 2004). Interestingly, we found that IL-33 could induce the mRNA expression and synthesis of these chemokines early in the development of bleomycin-induced lung fibrosis. Thus, IL-33 may increase inflammatory cell infiltration to the lung at least in part by enhancing the expression of related chemokines.

A potential role for IL-33 in lung fibrosis has been described in a recent report (Luzina et al., 2013). This group demonstrated that adenovirus-delivered full-length (fl) IL-33 which was located in the nucleus promoted lung fibrosis via an undefined, but ST2-independent, mechanism. This observation is complementary to the work of this chapter and suggests that while the nuclear IL-33 and cytokine IL-33 may have distinct biological effect (Luzina et al., 2012, Lefrancais and Cayrol, 2012), both the full length and mature-IL-33 are pro-fibrogenic but may induce fibrosis via distinct mechanisms. Additional studies will be necessary to resolve this dual role and likely help clarify the overall mechanism of how IL-33 contributes to fibrosis. In contrast to our result, the study of Luzina et al showed that ST2-deficiency had no effect on the development of BLM-induced fibrosis (Luzina et al., 2013, Li et al., 2014). The discrepancies may be in part due to the strain of mice used. The mouse strain, including the ST2 gene-deleted mice, used in our study was the commonly used, fibrosis-sensitive C57B/6 strain, whereas the ST2 knockout mice used in the Luzina et al. study were of a BALB/c background which are resistant to the fibrotic stage of BLM (Gur et al., 2000). Furthermore, the flIL-33 was expressed and delivered by a non-replicating adenoviral transfection agent and the role of this vehicle itself was not fully characterized in the production of IL-33 or in BLM-induced fibrosis (Luzina et al., 2013). More work is needed to clarify these issues.

To understand the possible mechanisms of idiopathic pulmonary fibrosis, several animal models have been developed. These display some of the fibrotic changes and the putative early inflammatory changes but they do not recapitulate the pathogenic feature of human IPF. While valuable in the understanding of clinical IPF, these models have shown the limitation in the modelling of human IPF. This is mainly because of the pulmonary anatomical and physiological differences between human and animal but also because the aetiology of IPF is unknown. The bleomycin-induced lung fibrosis model is the most commonly employed and referenced model of lung fibrosis (Mouratis and Aidinis, 2011). This model is proposed to mimic some of the histological hallmarks of human interstitial lung fibrosis that might be informative of IPF, and to help to study and understand

the pathogenic roles of immune system, including the contribution of activities of cytokines, signalling pathways and immune cells to pulmonary fibrosis (Moeller et al., 2008, Gibbons et al., 2011). However, there are limitations of this model when used to mimic IPF. It is acknowledged that this model failed to recapitulate some important aspect of human IPF, for example, the experimental animal models are often associated with a dense inflammatory infiltrate whereas this is not typical in human IPF which can be less inflammatory and more fibrotic perhaps due to chronicity. (Umezawa et al., 1967, Moore and Hogaboam, 2008, Degryse and Lawson, 2011, Mouratis and Aidinis, 2011, Smith, 2013). The lack of fibroblastic foci and hyperplastic epithelium; and the neutrophil rich inflammatory process in post-bleomycin injury that is not common in human IPF (Moore and Hogaboam, 2008, Degryse and Lawson, 2011). Furthermore, the animal model is unable to reproduce the chronic aspect of IPF due to the spontaneous remission of the disease in the model when a single dose of bleomycin is administered.

The clinical significance of IL-33 in fibrosis is unknown. IL-33 can be detected in the lungs in several chronic fibrotic diseases, including interstitial lung disease (ILD), scleroderma lung disease, cystic fibrosis and systemic sclerosis (Yanaba et al., 2011, Luzina et al., 2012, Luzina et al., 2013). Our findings extend these observations and provide compelling evidence for a mechanistic role for IL-33 in the development of fibrosis and may be a new therapeutic target. For example, our results suggest that blocking the IL-33/ST2 pathway by using either neutralising anti-IL-33 antibody or soluble ST2 receptor to antagonist IL-33 function may have a beneficial effect on fibrosis.

However, the precise mechanism by which the IL-33/ST2 pathway induces the development and exacerbation of lung fibrosis is largely unknown and some of the possible mechanisms are addressed in the next chapter.

# Chapter 4

# Mechanisms by which IL-33 promotes bleomycin-induced lung fibrosis

# Chapter 4: Mechanisms by which IL-33 promotes bleomycin-induced lung fibrosis

## 4.1 Introduction

In Chapter 3, I found that IL-33 plays a fibrogenic role in BLM-induced lung fibrosis in mice. However, the mechanism by which IL-33 promotes fibrosis was unclear and will be investigated in this Chapter.

In Chapter 3, I found that BLM induced the expression of IL-33 protein in cells located in the alveoli of airway compared to PBS controls (Fig. 3-3b). The location and morphological appearance of these cells suggested that they were likely to be alveolar macrophages. Furthermore, in BLM-induced fibrosis WT mice treated with recombinant IL-33 had enhanced BLM-induced lung fibrosis, and $ST2^{-/-}$ mice had reduced lung fibrosis and reduced numbers of macrophages (Fig. 3-7, 3-9, 3-19 and 3-21). In the present chapter I will explore the roles of macrophage phenotype and also type 2 innate lymphoid cells in the process of fibrosis.

Macrophages can be polarized into two phenotype subsets by classical activation (M1) and alternative activation (M2), dependent on different environmental conditions. In the presence of type 1 cytokines (IFN-γ) and bacterial LPS, they can be polarized into M1; whereas in the presence of type 2 cytokines (IL-4, IL-13), they can be polarized into M2. The M1 macrophages mainly express inducible nitric oxide synthase (iNOS) and produce pro-inflammatory cytokines which together play an important role against infection. On the other hand, M2 macrophages express arginase I (ARGI) and produce pro-fibrotic cytokine and are closely involved in tissue repair and remodelling (Gordon, 2003, Biswas and Mantovani, 2010, Alber et al., 2012). Our group found that macrophages express ST2, and that IL-33 is able to potentiate IL-13-polarised M2 development (Kurowska-Stolarska et al., 2009). The potential role of macrophages and in

particular the M2 macrophages in the IL-33-mediated fibrosis will be investigated in this Chapter.

Group 2 innate lymphoid cells (ILC2s) or nuocytes are a newly discovered category of innate immune cells that express high levels of ST2 and IL-25 receptor by which they can be activated and recruited by IL-33 and IL-25 respectively (Neill et al., 2010, Moro et al., 2010, Price et al., 2010). ILC2s are mainly found in lymphoid tissues associated with barrier surfaces such as skin, gut and lung, and can produce type 2 cytokines (Chang et al., 2011), such as IL-13, which are reported to be involved in the polarizing of M2 macrophages and development of fibrosis (Doherty et al., 1993, Bogdan et al., 1997, Wynn and Ramalingam, 2012). The involvement of ILC2 in IL-33-mediated fibrosis will also be investigated in this chapter.

It is known that both IL-33 and ST2 are expressed in fibroblasts (Xu et al., 2008). However, the role of IL-33/ST2 in the function of fibroblasts is less well understood. Therefore, the roles of IL-33/ST2 in fibroblast function *in vitro* and in fibrosis *in vivo* were also investigated.

Profibrotic cytokines, in particular IL-13 and TGF-β, play a critical role in organ fibrosis, including IPF and BLM-induced lung fibrosis (Borthwick et al., 2013). These cytokines may cause fibrosis by promoting fibroblast proliferation and collagen production (Wynn, 2011). It is known that IL-33 is able to induce IL-13 (Liew, 2012, McHedlidze et al., 2013), however, whether and how IL-33 induces TGF-β is less well understood. More importantly, whether and how these cytokines contribute to IL-33-promoted lung fibrosis is currently unknown.

Thus, the aim of this project is to understand the mechanism by which IL-33 affects BLM-induced fibrosis. I will focus mainly on its role in the key inflammatory cells associated with fibrosis, including the alveolar macrophage

and ILC2s. Our results demonstrated that IL-33 mediated lung fibrosis mainly by promoting the function of ILC2s and macrophage.

## 4.2 IL-33 promotes bleomycin-induced lung fibrosis via alveolar macrophages

Using immune-histochemical staining of mouse lung tissue after BLM instillation, we found that cells in the alveoli were induced to express IL-33, and that these IL-33 positive cells had alveolar macrophage morphology (Fig. 3-2). In order to confirm if they were macrophages, adjacent lung tissue sections (Fig. 3-2) were stained for the macrophage marker F4/80 using specific antibody (Fig. 4-1). The F4/80 positive cells were also located in the alveoli and had the same morphology as the IL-33-expressing cells in figure 3-2 (Fig. 4-1). An effort to stain the tissue sections simultaneously with both anti-IL-33 and anti-F4/80 failed due to technical problems (data not shown), therefore we solved this problem by using flow cytometry to analyze dispersed lung cells double stained with anti-IL-33 and F4/80 antibodies. To do this, WT and $ST2^{-/-}$ mice were given BLM or PBS for control and the lung tissues were harvested. The tissues were digested to generate single cell suspensions and then stained with anti-IL-33 and -F4/80 antibodies. BLM significantly increased the $IL\text{-}33^{+}/F4/80^{+}$ macrophage numbers in lung tissue of WT mice compared to the control mice (Fig. 4-2, Li et al., 2014). However, the induction of $IL\text{-}33^{+}$ macrophages in lung tissues by BLM was abrogated in the $ST2^{-/-}$ mice compared to WT mice (Fig 4-2). These results suggest that IL-33/ST2 signals are required for the expression of IL-33 and recruitment of macrophages in BLM-induced fibrosis.

To define the importance of macrophages in the BLM-induced and IL-33 exacerbated lung fibrosis, we depleted alveolar macrophages firstly by treating the mice with clodronate liposomes before the administration of BLM and IL-33 in mice (Van Rooijen and Sanders, 1994). Clodronate is the drug bisphosphonate and in liposome is cytotoxic to macrophages when phagocytosed. This method has been shown to effectively deplete only alveolar macrophages when given i.n. to mice (Kurowska-Stolarska et al., 2009). The experiment was performed as planned (Fig. 4-3). The administration of clodronate significantly decreased lung macrophages, lung inflammation (Fig. 4-4) and fibrosis (Fig. 4-5) in both BLM and IL-33 plus BLM groups compared to the PBS liposome control group. The

increased collagen content and collagen III mRNA expression induced by BLM and IL-33 plus BLM were also attenuated by the clodronate depletion of alveolar macrophage compared to PBS liposome controls (Fig. 4-6). Furthermore, clodronate depleted ~80% of alveolar macrophages compared with the control group (Fig. 4-7). Intriguingly, alveolar macrophages depletion also resulted in the reduction of the number of BLM-induced and IL-33-enhanced neutrophils and lymphocytes in the BAL fluid (Fig. 4-7), suggesting that alveolar macrophages play a pivotal role in the recruitment of these inflammatory leukocytes by BLM and IL-33 in fibrosis (Fig. 4-7).

These findings suggest that although IL-33 plays a role in the pathogenesis of BLM-induced fibrosis, it cannot replace the function of alveolar macrophages in the fibrogenic progress, and IL-33 is unlikely to exacerbate BLM-induced lung fibrosis through any cell types except alveolar macrophages.

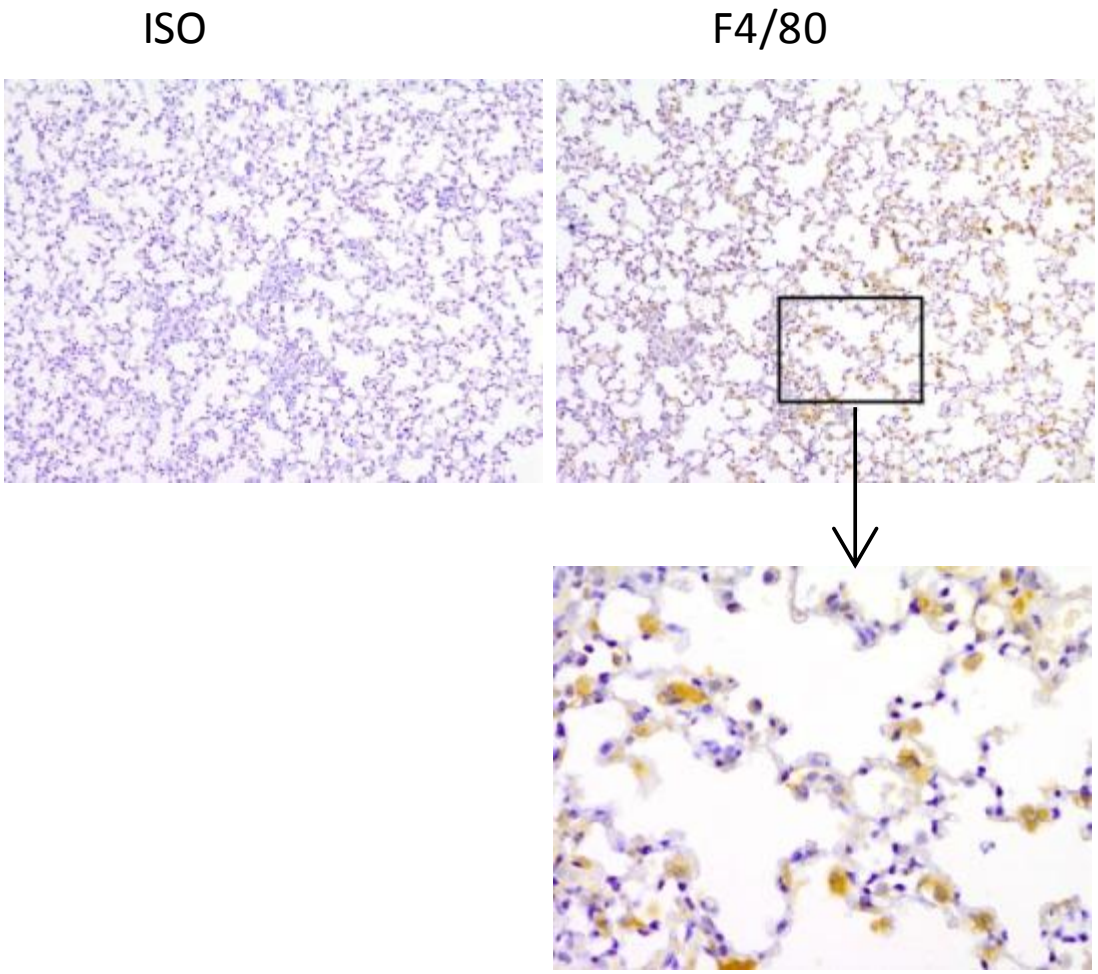



**Fig. 4-1 BLM administration enhances F4/80 positive macrophages in lung tissue**

Lung tissue samples from groups of mice were harvested 7 days after BLM administration. The tissue samples were processed and stained for F4/80 using specific antibody as described in chapter 2, section 2.13. (original magnification x100, enlarged to x400)

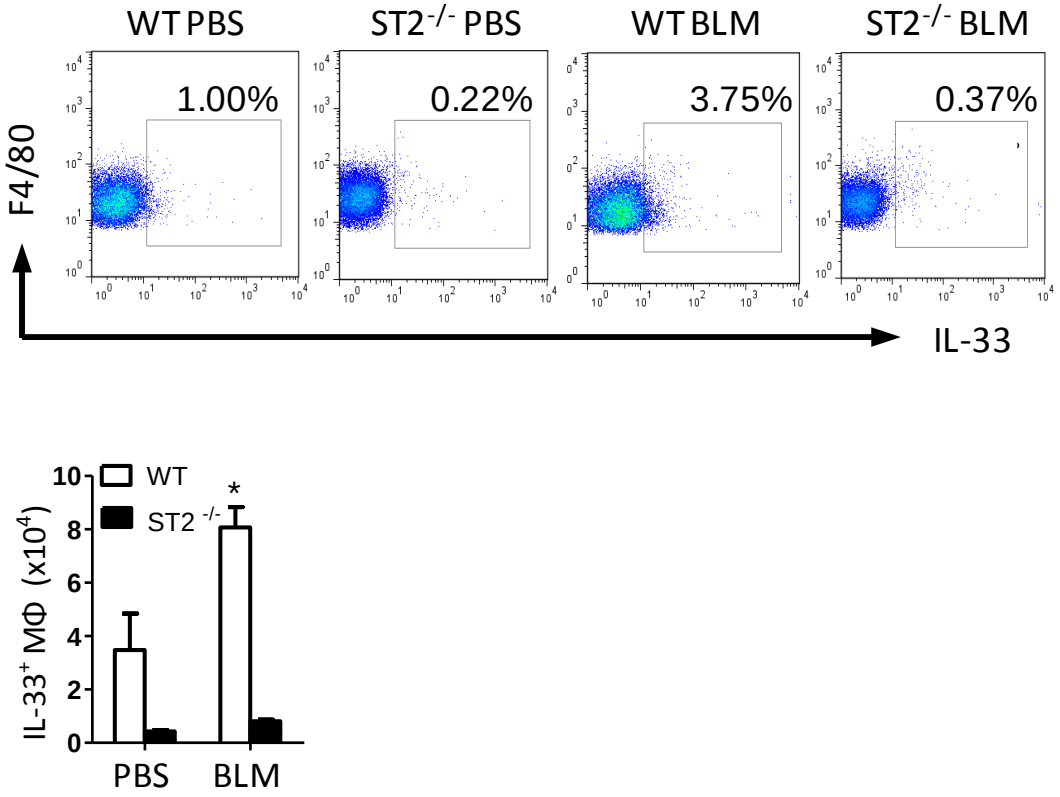



**Fig. 4-2 ST2$^{-/-}$ mice with reduced number of IL-33 positive macrophages in lung tissue.**

WT and ST2$^{-/-}$ mice were given BLM on day 0 and culled on day 7. The lung tissues were dispersed and stained with both IL-33 and F4/80 using appropriate antibodies, and analysed using FACS as described in chapter 2, section 2.13. *p<0.05 compared to PBS groups.

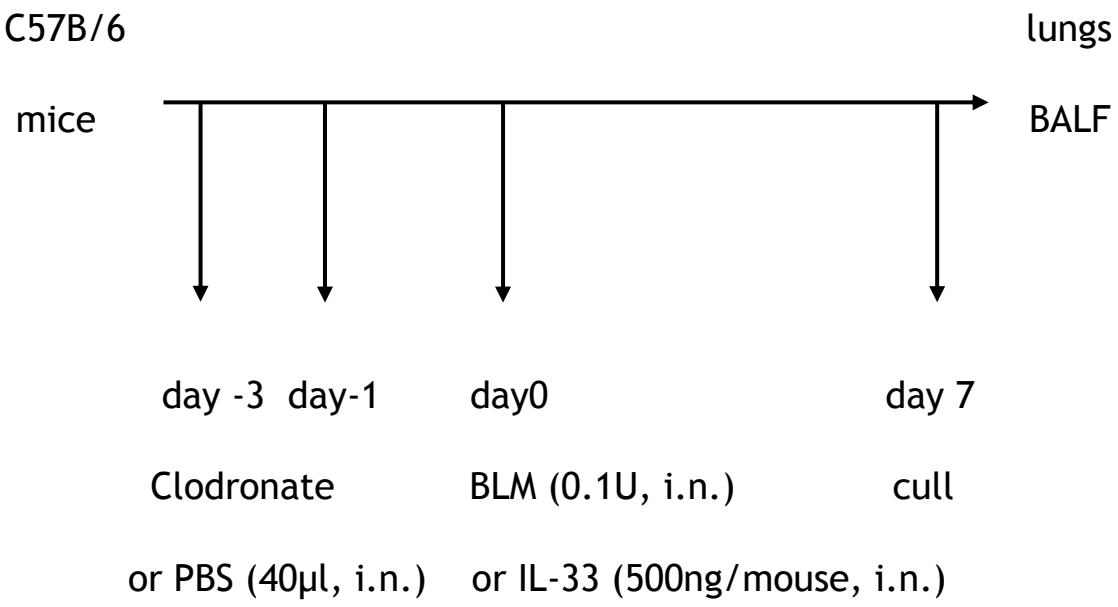



**Fig. 4-3 Experimental plan to deplete macrophages using clodronate liposomes in BLM-induced fibrosis in mice.**

The depleting effect of clodronate liposomes typically lasts up to 7 days *in vivo*. Two weeks prior to the first clodronate injection and during the experiment, mice were kept in filtered cages and given sterile food and water. C57B/6 mice were administered with clodronate liposome or control liposome (40µl) i.n. 3-day and 1-day before 0.1U bleomycin (BLM) i.n./PBS on day 0. Some mice were also given IL-33 with BLM at the same time. The mice were monitored daily, culled on day 7. BAL fluid and lung tissue were collected and analyzed as described in chapter 2.

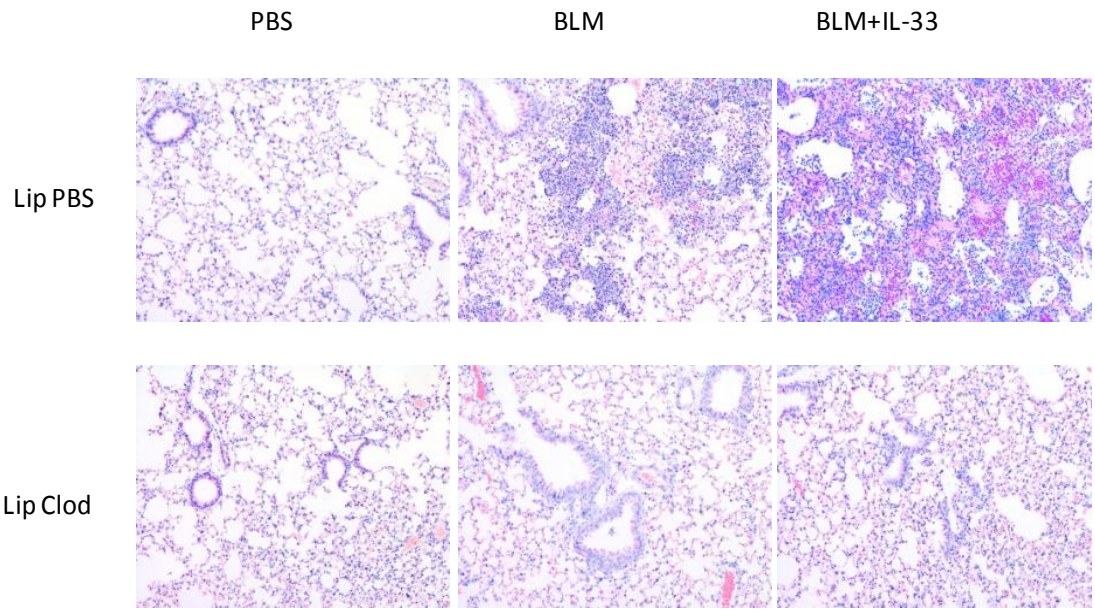



**Fig. 4-4 Clodronate depletion of macrophages reduces lung inflammation and fibrosis in mice given BLM and IL-33.**

Mice were treated with clodronate or control liposomes (40µl) 3 days and 1 day before the BLM and IL-33 administration as shown in Fig 4-3. The lung tissues were harvested 7 days after BLM and IL-33 administration. The lung tissue sections were stained with H&E as described in chapter 2, section 2.9.1. Representative pictures of mean histological change are shown from each group. (original magnification x100)

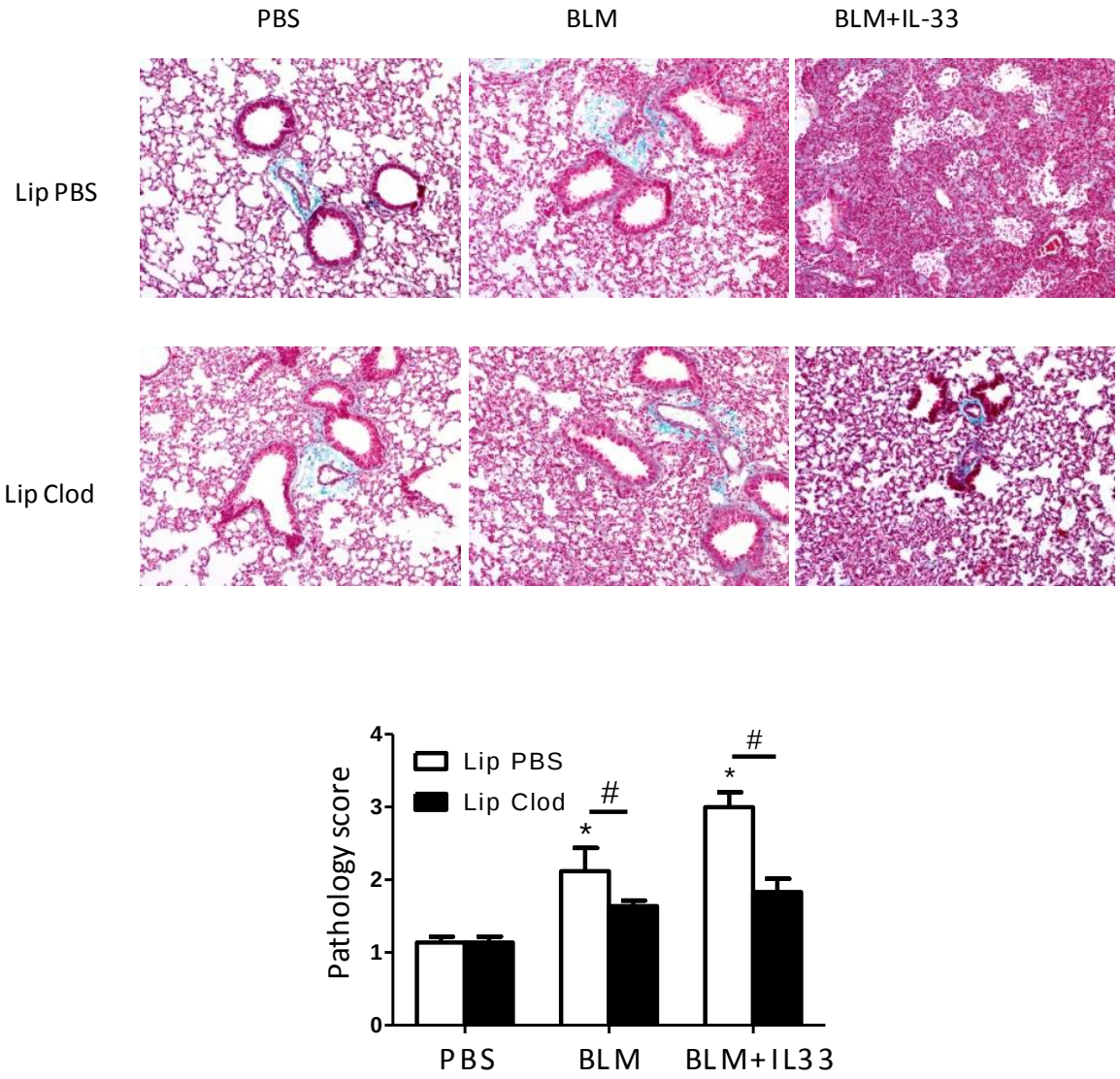



**Fig. 4-5 The depletion of macrophages reduces BLM and IL-33 enhanced lung fibrosis in mice.**

Groups of mice were treated as described in Fig. 4-3. The lung tissues were harvested 7 days after PBS, BLM or BLM plus IL-33 administration. The tissue sample sections were stained with trichrome and the pathological score was determined as described in chapter 2, section 2.9. Representative pictures of mean histological change are shown from each group (original magnification x100). Data are representative of two experiments, mean ± SEM, n= 6 mice per group, * $p<0.05$ compared to PBS group; # $p<0.05$ compared to BLM alone group.

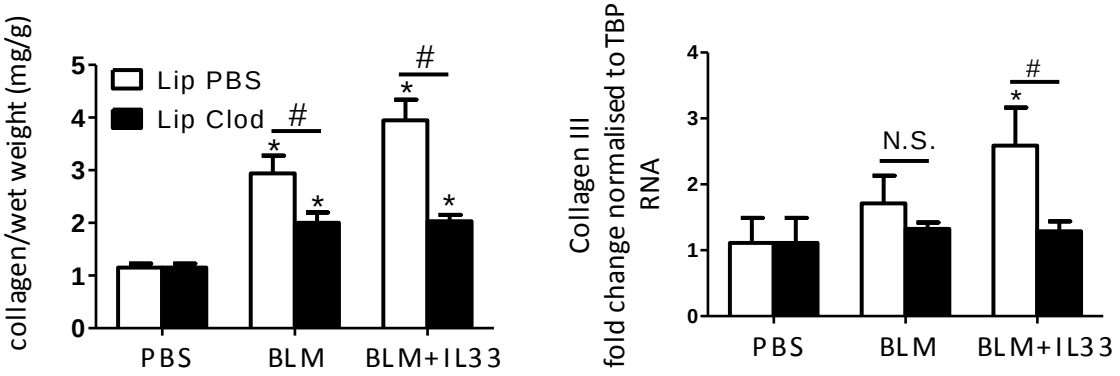



**Fig. 4-6 Clodronate depletion of macrophages reduces lung tissue collagen content and mRNA expression after administration of BLM and IL-33.**

Groups of mice were treated as shown in Fig. 4-3. Lung tissues were collected on day 7 after BLM, and collagen content was measured using the Sircol soluble collagen assay. Collagen III expression was measured using real-time qPCR. Data are representative of two experiments, mean ± SEM, n= 6 mice per group, *p<0.05 compared to PBS group; # p<0.05 compared to BLM alone group.

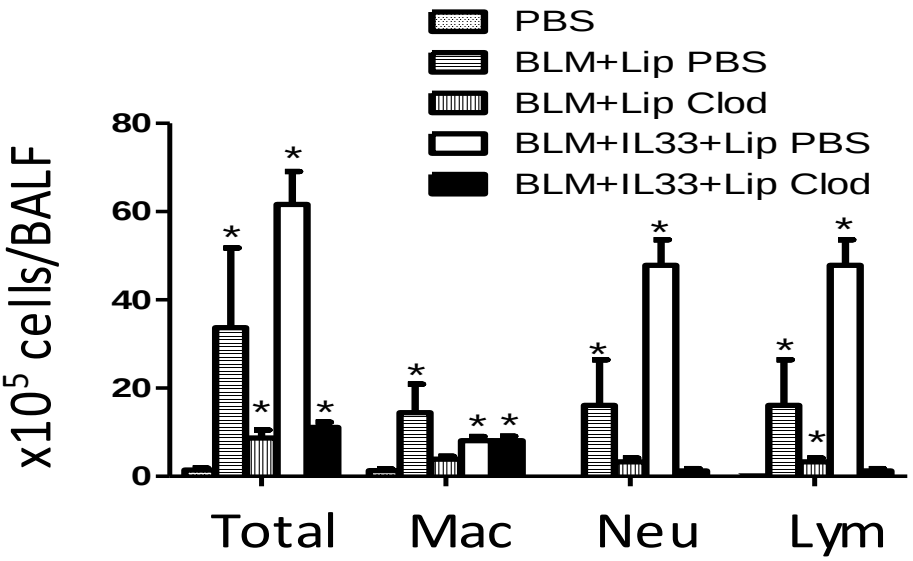



**Fig. 4-7 The depletion of macrophages reduces BLM and IL-33 enhanced leukocyte infiltration in mice.**

Groups of mice were treated as shown in Fig. 4-3. Briefly, liposome clodronate or control PBS liposomes were instilled i.n. to mice 3 days and 1 day before BLM. After mice were culled 7 days after BLM, cells from BAL fluid (BALF) were stained and counted as described in chapter 2, section 2.12. Data are representative of two experiments, mean ± SEM, n= 6 mice per group, *P < 0.05 compared to PBS group.

## 4.3 IL-33 amplifies the polarization of M2 macrophage in BLM-induced lung fibrosis.

After establishing the essential role of macrophages in BLM-induced and IL-33-exacerbated lung fibrosis, we further investigate the involvement of subsets of macrophages in the IL-33-mediated effect on BLM-induced fibrosis *in vitro* and *in vivo*.

We initially determined whether and how IL-33 and BLM can polarize M2 *in vitro* using bone marrow-derived macrophages (BMDM). BMDMs were polarized with IL-13, IL-33 or BLM alone or together for 48 hours. The levels of M2 cytokines, IL-4, IL-13 and TGF-β1, and the surrogate cell markers inducible nitric oxide synthase (iNOS) and arginase I (ARGI) for M1 and M2 respectively were measured by ELISA or qPCR.

IL-33 enhanced IL-13 production in BMDM (Fig. 4-8a). IL-13 markedly enhanced IL-4 but only slightly enhanced TGF-β1 production in BMDM (Fig. 4-8b). The IL-33 alone elevated TGF-β1 but not IL-4. However, it is noteworthy that IL-13 plus IL-33 synergistically enhanced TGF-β1 production in BMDM (4.8b). BLM alone could induce TGF-β1 production but failed to induce/enhance IL-13 and IL-4 production in BMDM.

IL-13, which polarizes M2 (Van Dyken and Locksley, 2013), enhanced the expression of the M2 marker ARG1 but reduced the expression of the M1 marker iNOS, compared to controls (Fig. 4-9a and b). IL-33 or BLM alone failed to induce either M1 or M2 markers. However, IL-13 plus IL-33 markedly enhanced ARG1 but not iNOS expression compared to IL-13 alone (Fig. 4-9a and b). These results suggest that IL-33 may induce IL-13 production by macrophages and subsequently enhance IL-13-mediated M2 polarization *in vitro*.

In order to demonstrate that M2 macrophages are involved in the development of fibrogenesis *in vivo*, we then determined the M1 and M2 proportions using the surrogate markers iNOS and arginase I (ARGI), for M1 and M2, respectively, in lung tissue. The lung tissues were collected from the experiments described in Chapter 3 using $ST2^{-/-}$ mice (Fig. 3-5) and anti-IL-33 antibody treated mice (Fig. 3-11). The expression of ARGI was markedly reduced in lung tissue of ST2 deficient mice on day 14 (Fig. 4-10a) and anti-IL-33 antibody treated mice on day 7 (Fig. 4-10b) compared to controls. In contrast, the iNOS expression was enhanced in the lung of $ST2^{-/-}$ mice on day 14 (Fig. 4-10a) and anti-IL-33 antibody treated mice on day 7 compared to control groups (Fig. 4-10b). We next determined the frequencies of macrophages and M2 macrophages and IL-33-expressing M2 in fibrotic lung tissue of WT and $ST2^{-/-}$ mice by flow cytometry. The $CD45^{+}$ leucocytes in the digested lung tissues were gated first as in Fig. 4-11a and then the $F4/80^{+}$ macrophages and $CD206^{+}$ M2 and $IL\text{-}33^{+}$ M2 levels were determined by FACS (Fig. 4-11 b and c). The FACS analysis showed that while the percentage of $F4/80^{+}$ macrophages was slightly but significantly reduced in $ST2^{-/-}$ compared to WT mice (Fig. 4-12a), the percentage of M2 macrophages in $ST2^{-/-}$ mice was markedly reduced 7 days after being given BLM (Fig. 4-12b). More interestingly, most of the M2 macrophages were expressing IL-33 in WT mice, which was nearly completely abolished in the $ST2^{-/-}$ mice (Fig. 4-12c). These results suggested that IL-33/ST2 signals are required for the induction of M2 macrophages and M2 IL-33 expression but not the M1 macrophages in lung tissue in mice.

Together with the result from alveolar macrophage depletion experiments, we conclude that IL-33 signals may promote BLM-induced fibrosis by polarizing M2 in mice.

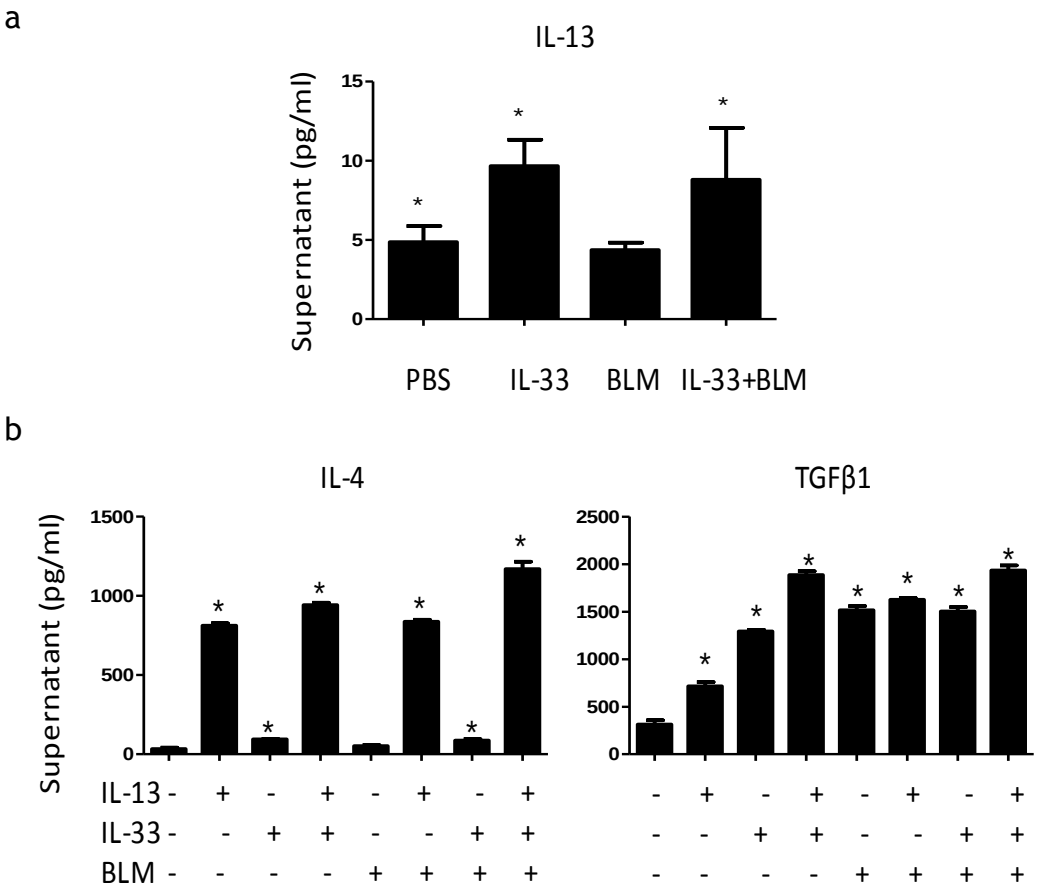



**Fig. 4-8 The role of IL-33 in the induction of profibrogenic cytokine productions in BMDM *in vitro*.**

BMM were stimulated with IL-13, IL-33 or BLM for 48 hours, the supernatants were harvested and IL-4, IL-13 and TGF-β1 concentrations were measured by ELISA as described in section 2.12.1. Data are representative of two experiments, mean ± SEM, n= 6 mice per group, *P < 0.05 compared to media group.

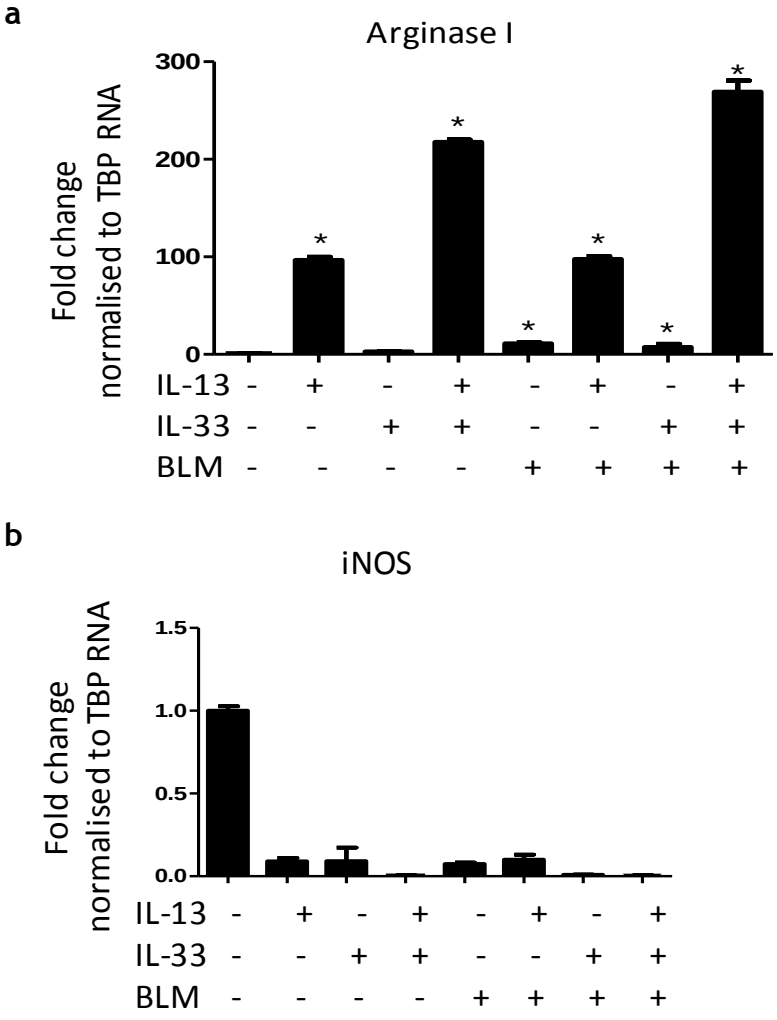



**Fig. 4-9 IL-33 potentiates IL-13 induced M2 but not M1 polarisation in BMDM *in vitro*.**

BMMs were stimulated by IL-13, IL-33 or BLM alone or together for 24 hours and then ARGI and iNOS expression measured by qPCR as described in chapter 2, section 2.7.1. Data are representative of two experiments, mean ± SEM, n= 6 mice per group, *P < 0.05 compared to media group.

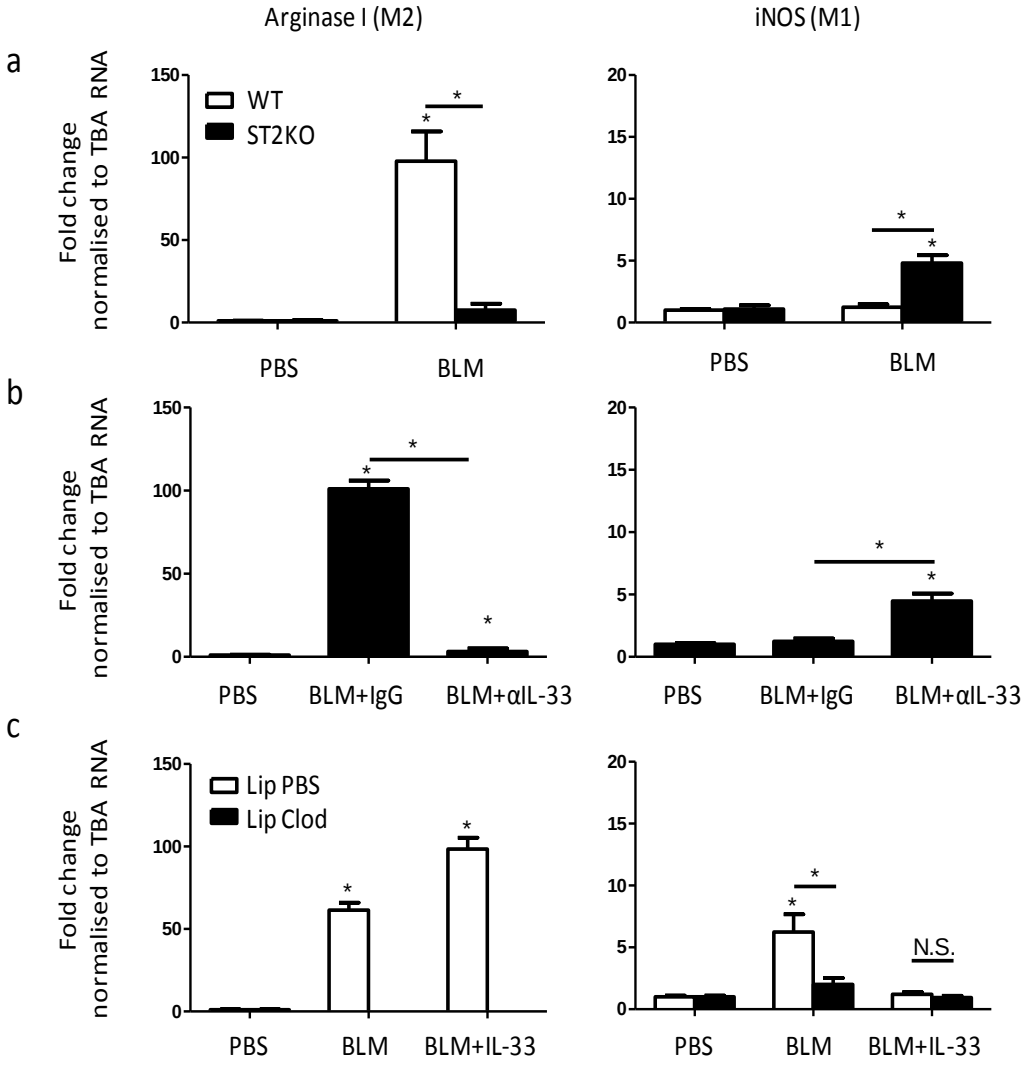



**Fig. 4-10 ST2 deficiency or anti-IL-33 treatment reduces M2 marker ARGI and enhances M1 marker iNOS expression in fibrotic lungs.**

Group of mice were treated as in Fig. 3-5, 3-11 and 4-3. Lungs were collected and expression of ARGI and iNOS were analyzed by qPCR as described in chapter 2. Data are representative of two experiments, mean ± SEM, n= 6 mice per group, *P < 0.05 compared to PBS group.

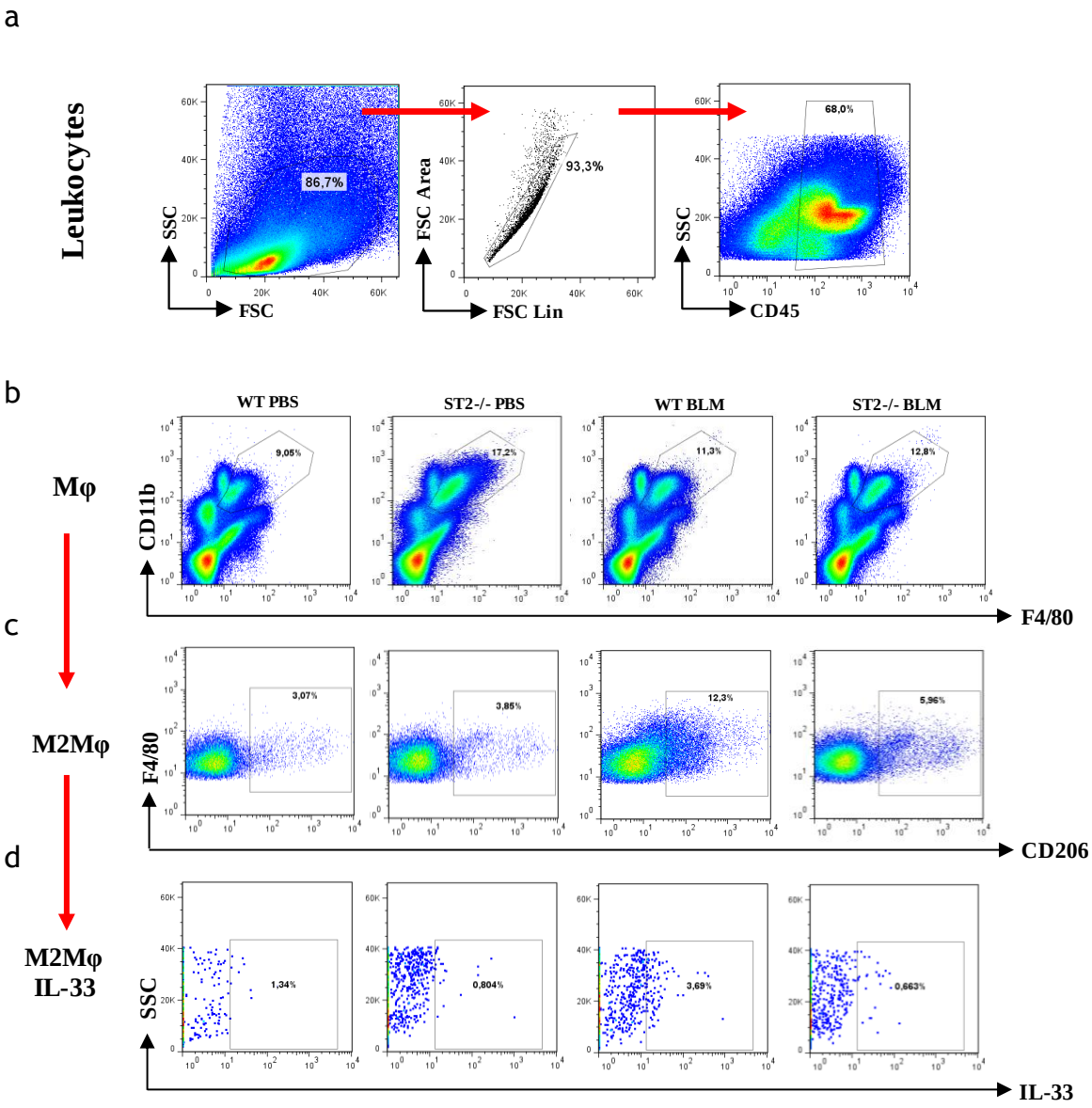
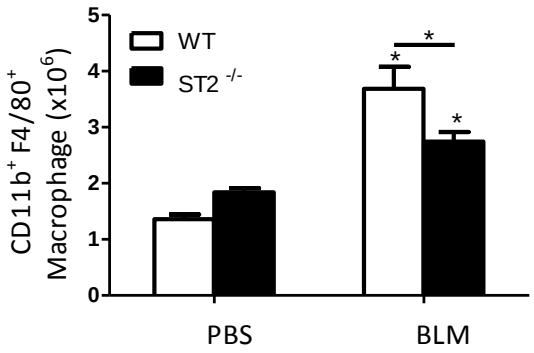


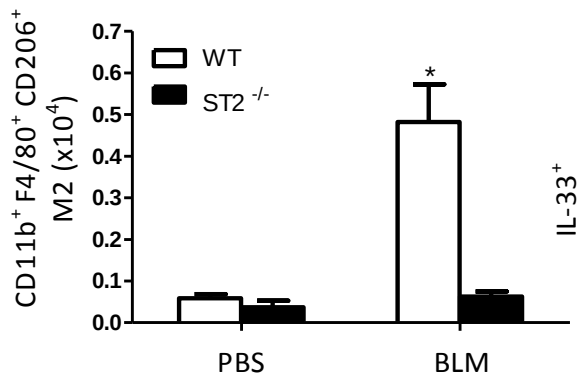

**Fig. 4-11 FACS gating strategy for subsets of macrophages in the lung digests.**

Lungs from WT and ST2$^{-/-}$ mice were collected and digested, and stained with appropriate antibodies for FACS analysis as described in chapter 2.

a




b




c

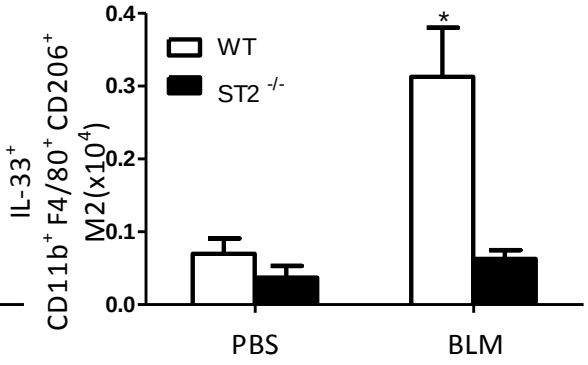



**Fig. 4-12 ST2$^{-/-}$ mice reduce cell numbers of macrophages, M2 macrophages and IL-33 producing macrophages in the lung digests.**

Lungs from WT and ST2$^{-/-}$ mice were collected and digested, and stained with appropriate antibodies for FACS analysis as described in chapter 2. Data are representative of two experiments, mean ± SEM, n= 6 mice per group, *P < 0.05 compared to PBS group.

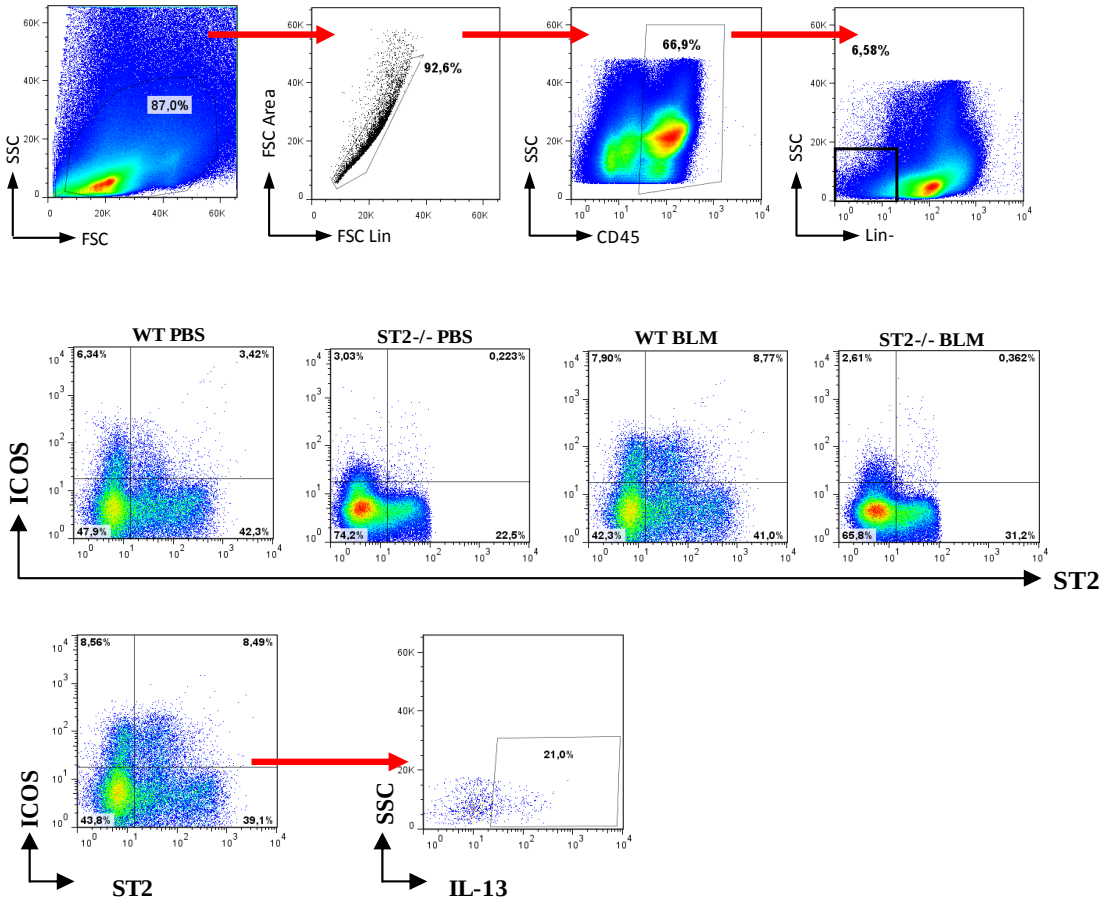
## 4.4 IL-33 signals increase IL-13-expressing Type 2 innate lymphoid cells in BLM-induced lung fibrosis

ILC2 is a newly discovered innate cell subset which produces type 2 cytokines, including IL-13, and plays an initiating role in Th2 response and fibrosis (Neill et al., 2010, Spits and Di Santo, 2011). We found that macrophages, perhaps the M2, are required for IL-33 to exacerbate lung fibrosis and M2 can be polarised by IL-13 and IL-33. We next investigated the role of IL-33 in the induction of IL-13 expressing ILC2 in BLM-induced fibrosis.

Lung fibrosis was induced in WT and $ST2^{-/-}$ mice by BLM as above. The lungs were harvested and digested as described in section 2.13, and the dispersed cells were labelled and gated for $CD45^{+}ICOS^{+}ST2^{+}$ and $IL\text{-}13^{+}$ ILCs as indicated in Fig. 4-13a. Our results showed that giving BLM markedly enhanced lung ILC2 numbers in WT mice as early as day 3 with further enhancement by day 7 (Fig. 4-13b). However, the enhancement of lung ILC2 was completely abolished in $ST2^{-/-}$ mice (Fig. 4-13b). Furthermore, the IL-13 producing ILCs were also increased in WT but not in $ST2^{-/-}$ mice 3 days after receiving BLM (Fig. 4-13b).

These results demonstrated the ILC2 cell numbers are enhanced in the BLM-induced lung fibrosis and IL-33 signals play an important role in ILC2 function. These ILC2 may provide an additional source of IL-13 in M2 polarisation and lung fibrosis.

a

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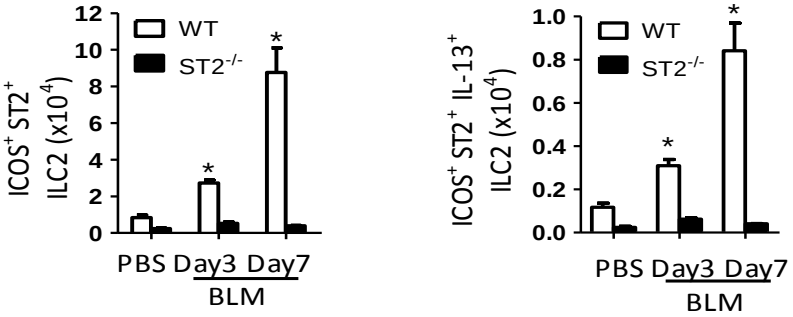



**Fig. 4-13 ST2 is required for the induction of ILC2 in the fibrotic lung in mice.**

Lungs from BLM-treated WT and ST2-/- mice were collected and digested, stained with appropriate antibodies for FACS analysis as described in chapter 2. a) FACS gating strategy for ILC2s and numbers of ILC2s in the lung digests. b) The numbers of IL-13+ILC2. Data are representative of two experiments, mean ± SEM, n= 6 mice per group, *P < 0.05 compared to PBS group.

## 4.5 IL-33 induced pro-fibrogenic cytokine production in BLM-induced fibrosis

Both IL-13 and TGF-ß1 are well-known cytokines with pro-fibrotic functions (Borthwick et al., 2013). However, it is less known a) how and when these cytokines are induced in the context of fibrosis; b) the relationship between IL-13 and TGF-ß1 in this context; c) whether IL-33 is able to induce these cytokines in fibrosis. We sought to answer these questions by the following experiments.

In section 4.3, we initially determined that IL-33 and BLM could induce pro-fibrotic cytokines *in vitro* in bone marrow-derived macrophages (BMDM). BMDM were stimulated with IL-13, IL-33 or BLM alone or together for 48 hours. The levels of M2 cytokines, IL-4, IL-13 and TGF-ß1 were measured by ELISA or qPCR.

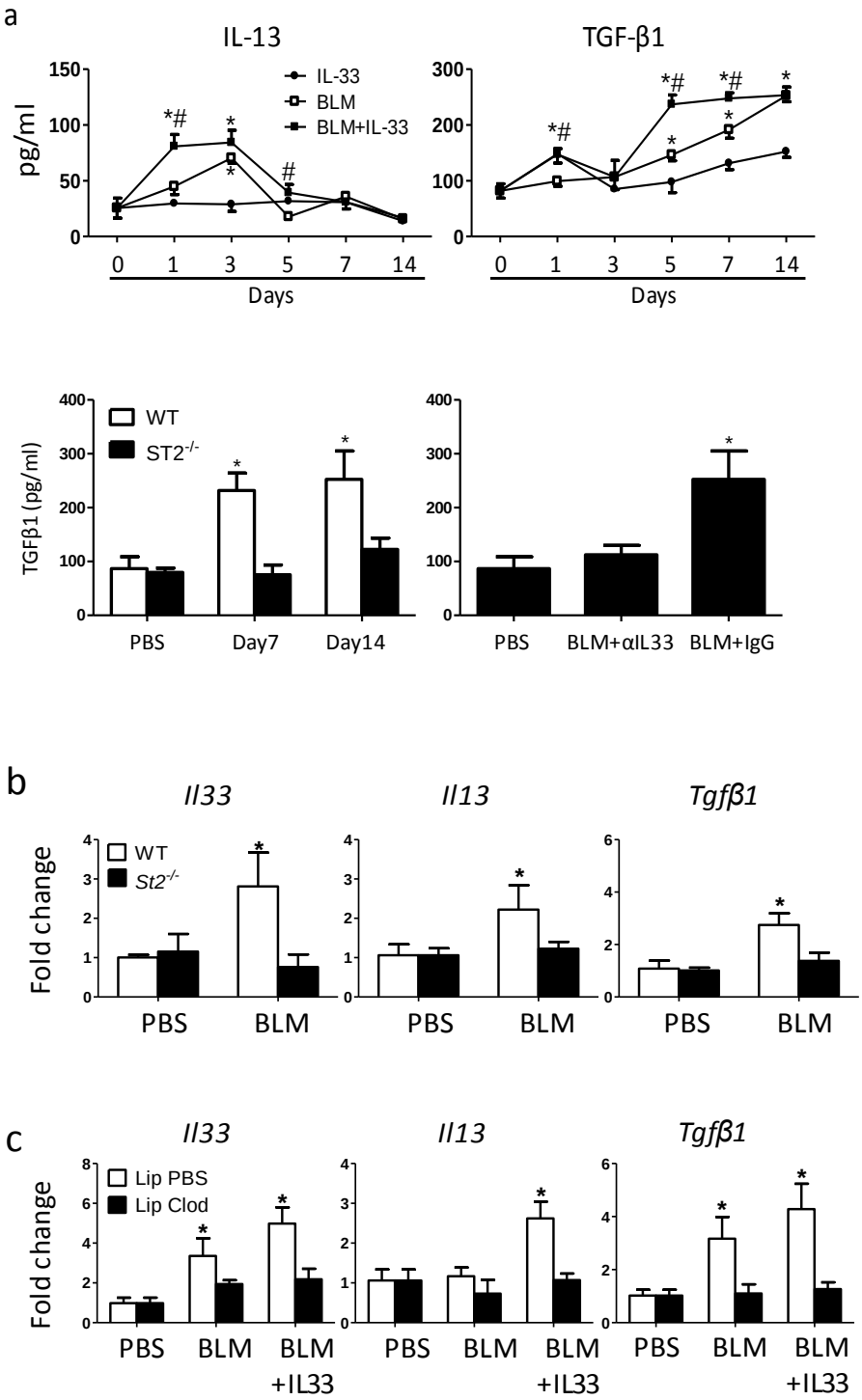
We found that IL-33 alone enhanced IL-13 production in BMDM compared to controls (Fig. 4-8a). IL-13 alone significantly enhanced IL-4 but slightly enhanced TGF-ß1 production in BMDM (Fig. 4-8b). The IL-33 elevated TGF-ß1 but not IL-4 production was further enhanced by IL-13 in BMDM (Fig. 4-8b). BLM alone could induce TGF-ß1 production but failed to induce/enhance IL-13 and IL-4 production alone or together with IL-13 and IL-33 in BMDM. Therefore, it was IL-13 and IL-33 that mainly induce pro-fibrotic cytokines in macrophages.

We found in Chapter 3 that IL-33 could exacerbate BLM-induced fibrosis. We then determined the kinetics of production of IL-13 and TGF-ß1 in the BAL fluid from mice given either BLM or IL-33 alone or together in mice. BLM alone induced IL-13 production which appeared from day 1 and declined on day 5 (Fig. 4-14a). BLM-induced TGF-ß1 appeared later on day 5 and lasted at least 14 days. The BLM-induced IL-13 and TGF-ß1 production was further enhanced in their combination with IL-33 (Fig. 4-14a). However, IL-33 alone had no effect on the production of these cytokines at this stage (first two weeks post BLM) (Fig. 4-

14a). These results suggest that IL-33 may induce IL-13 first and this IL-13 subsequently induces TGF-β1 in the context of lung fibrosis.

As described in section 3.3 and 4.2, ST2 deficiency and macrophage depletion ameliorated BLM-induced fibrosis. We further determined the IL-13 and TGF-β1 mRNA expression in lung tissue from these experiments. In alveolar macrophage depleted mice, the expressions of IL-33, IL-13 and TGF-β1 induced by BLM and BLM plus IL-33 were nearly completely abolished compared to control mice (Fig. 4-14b). Furthermore, the expression levels of IL-13, IL-33 and TGF-β1 induced by BLM were also abolished in $ST2^{-/-}$ group (Fig. 4-12c).

These results suggested that BLM induces pro-fibrotic cytokines mainly via macrophages and is dependent on ST2 signals.




## Fig. 4-14 BLM induces ST2-dependent production of profibrotic cytokines by macrophages

BALF was collected as shown in Fig. 3-2, 3-5 and 3-11 and the content of IL-13 and TGF-ß1 were measured by ELISA (a). The mRNA expressions of i*l33, il13* and *tgfß1* were measured by qPCR in lung tissue collected as shown in Fig. 3-2 and 4-3(b). Data are representative of two experiments, mean ± SEM, n= 6 mice per group, *P < 0.05 compared to PBS groups.

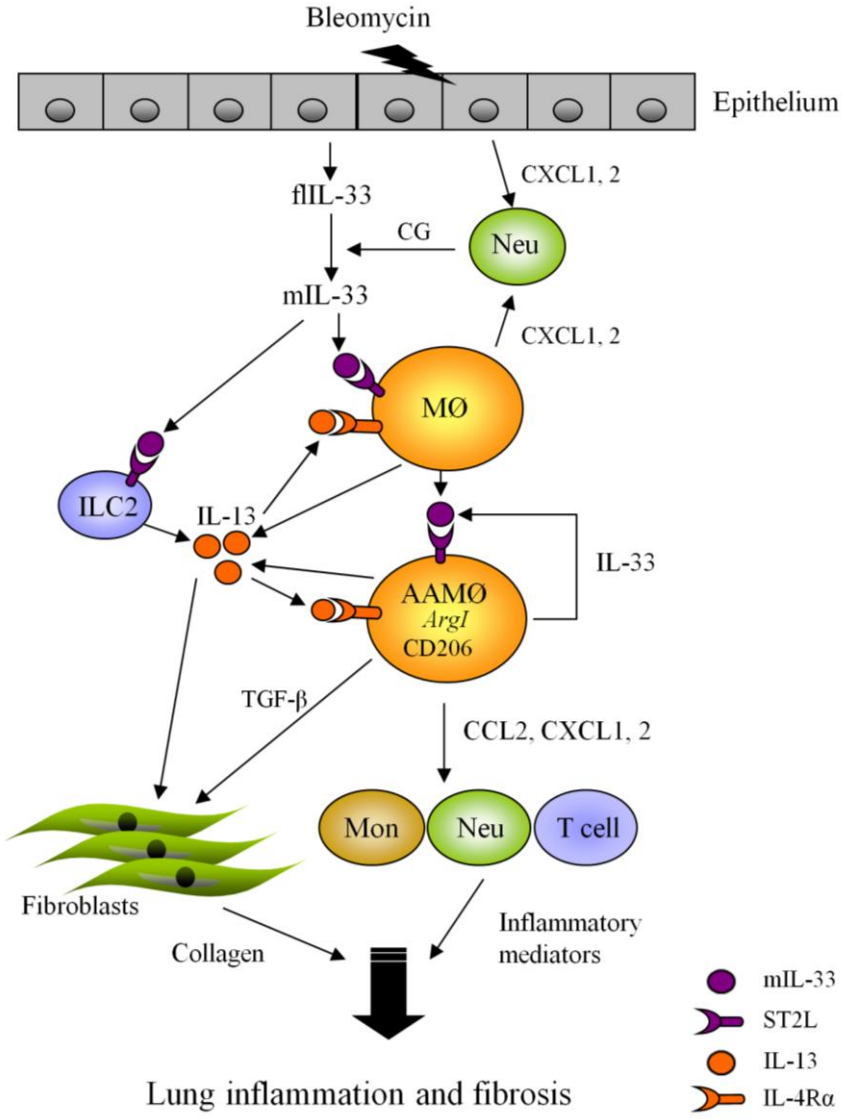



**Fig. 4-15 Schematic representation of a potential mechanism of BLM-induced IL-33 synthesis and lung fibrosis**

BLM triggers the release of flIL-33 from damaged airway epithelial cells and the recruitment of neutrophils. Neutrophil cathepsin G (CG) then processes flIL-33 to mIL-33. The mIL-33 stimulates early ST2-dependent ILC2 cell expansion and IL-13 production. IL-33 and IL-13 then synergistically polarize alveolar macrophages into the M2 phenotype that produces TGF-ß1, IL-13 and more IL-33, which in turn activates fibroblasts to proliferate and overproduce collagen. IL-33 also enhances macrophage production of chemokines which induce the infiltration of neutrophils and lymphocytes into the lung, which together may exacerbate lung inflammation and the development of fibrosis.

## 4.6 Chapter Discussion

The main findings from the work described in this chapter are:

i) Exacerbation effect of IL-33 on BLM-induced lung fibrosis is abolished by alveolar macrophage depletion.
ii) IL-33 could polarize M2 macrophages which promote BLM-induced lung fibrosis.
iii) IL-33 signals increased the number of lung interstitial ILC2 in BLM-induced lung fibrosis and ILC2 may provide a source of early IL-13 in fibrosis.
iv) IL-33 induces fibrogenic cytokines in BLM-induced lung fibrosis.

Alveolar macrophages, in particular M2 macrophages, are closely associated with the pathogenesis of fibrosis by producing fibrogenic cytokine and chemokines (Wynn and Barron, 2010, Alber et al., 2012, Gibbons et al., 2011). Our previous results in Chapter 3 demonstrated that IL-33 signals play a critical role in BLM-induced lung fibrosis and suggested that IL-33 may be expressed in alveolar macrophages. In this chapter we demonstrated that macrophages are one of the main sources of IL-33, because lung macrophages express IL-33 and macrophage depletion reduced IL-33 production in the lung. Furthermore, ST2 signals are required for the optimal induction of IL-33 in tissue, suggesting that IL-33 is produced by macrophages in an autocrine manner. However, at the moment, we do not have direct evidence to show the importance of macrophage-derived IL-33 in BLM-induced lung fibrosis. Future work will be required to help resolve this question, for example, by adoptive transfer of macrophages from WT or IL-33 transgenic mice, using macrophages from IL-33-/- mice as controls, into the lung of IL-33-/- recipients to see if the macrophage-derived IL-33 is sufficient to drive the fibrotic process in the BLM model.

Finally, since epithelial cells are the first cell layer that comes into contact with BLM in the airway and they constitutively express IL-33, epithelial cells may also contribute to the cytokine pool of IL-33 in this context.

Macrophages can be polarized to different subsets, one of the subsets, M2 macrophages, producing IL-13 and TGF-β1. Our results are consistent with previous reports (Kurowska-Stolarska et al., 2009) that IL-33 plus IL-13 could induce M2 polarization. These could explain how IL-33 is critical in the induction of fibrotic cytokines and chemokines by M2 macrophages and thereby promoting the pathogenesis of lung fibrosis.

Our work also showed that the IL-33/ST2 axis induces the production of key chemokines CXCL1, CXCL2 and CCL2 in BLM-induced fibrosis. It is known that CXCL1 and CXCL2 are chemoattractants for the migration of neutrophils, and CCL2 for macrophages and lymphocytes in fibrotic lung tissue caused by BLM (Car et al., 1994, Rot and von Andrian, 2004, Strieter et al., 2007). And IL-33 could induce the production of CXC chemokines alone within the mucosal surface (Guabiraba et al., 2014). These results might explain how IL-33 recruited pro-inflammatory leukocytes to the lung which contribute to the early inflammation and subsequent fibrosis. In addition, we found that alveolar macrophage depletion also abolished the infiltration of lymphocytes and neutrophils, suggesting that alveolar macrophages play a central role in the recruitment of inflammatory cells into lung tissue.

We found that IL-33 could also increase the level of pro-fibrogenic cytokines IL-13 and TGF-β1 in lung tissue in an ST2 and macrophage-dependent manner (Fig. 4-14). Furthermore, our kinetic cytokine expression experiment showed that BLM and IL-33 first induce the expression of IL-13 and then production of TGF-β1 in BAL fluid, suggesting that the TGF-β1 may be induced in macrophage by IL-33-derived IL-13. This agrees with the previous reports showing that IL-13 can induce TGF-β1 expression *in vivo* (Fichtner-Feigl et al., 2006). As shown in section 2.13.1, the TGF-β1 we measured was total TGF-β1 not active TGF-β1

which needed to be measured using receptor-based assay (Albro et al., 2013). As the concentration of active TGF-β1 is a more accurate description of the biologically functionality of TGF-β1 and these two measures are not necessarily interchangeable (Gibbons et al., 2011, Mackinnon et al., 2012), more work is required to determine the nature of the TGF-β1 induced by IL-33.

Our results showed that the IL-33/ST2 pathway is required for the recruitment of ILC2s and for the ILC2s to produce IL-13. This is consistent with published reports showing that IL-33 signals promote early ILC2 development and proliferation in different contexts *in vivo* (Moro et al., 2010, Spits and Di Santo, 2011, Salmond et al., 2012, McHedlidze et al., 2013, Pastorelli et al., 2013, Molofsky et al., 2013). Since IL-13 is needed for M2 polarization and for fibroblast function, our results suggest that IL-33 may initially induce IL-13 production by ILC2, and IL-13 then subsequently amplify M2 and fibroblast function in lung fibrosis (Fig. 4-15). To further understand the role of ILC2 in IL-33- and BLM-induced pulmonary fibrosis, we amplified the ILC2 cell numbers *in vivo* by injection of recombinant IL-33, daily for 5 days, which is a well-established method for the enrichment of ILC2 for adaptive transfer experiments. The ILC2 were purified by cell sorting and were adoptively transferred into normal recipient mice 1 day after BLM (Li et al 2014). The ILC2 transfer resulted in more severe bleomycin-induced lung inflammation and fibrosis in the recipient mice (Li et al 2014). The pathogenic changes were accompanied by increased inflammatory cell infiltration and collagen production, and the mRNA expression of collagen 3, Il13 and Tgfβ1 in the lung tissue compared to controls. Together, these data demonstrate that ILC2 cells may contribute to the BLM- and IL-33-mediated pulmonary fibrosis, perhaps by providing an additional source of IL-13 in this context (Fig 4-15).

Fibroblasts are the key cell in the development of fibrosis mainly by producing collagen and cellular matrix molecules (Wynn, 2008, Wynn, 2011, Wynn and Ramalingam, 2012, Shepherd, 2006). It is known that fibroblasts express both IL-33 and ST2 (Xu et al., 2008). However, the role of IL-33 in fibroblast function is largely unknown. I therefore generated primary fibroblasts from mouse lungs as

described in Chapter 2 to study the effect of IL-33 on fibroblast function *in vitro*. I found that stimulation by recombinant IL-33 seemed to have no significant effect on the proliferation, migration and collagen production of cultured fibroblasts in normal conditions, suggesting that IL-33 alone may be unable to affect fibroblast functions directly and those fibroblasts may mainly produce IL-33 for other cells (data not shown). Therefore, based on the current evidence, the promoting role of IL-33 system in lung fibrosis may be mainly attributed to its effect on the induction of lung inflammation and indirect activation of fibroblast function via enhance IL-13 and TGFβ-1 production in this context (Fig 4-15). However, we cannot eliminate the possibility that IL-33 may synergise with other factors/cytokines to promote fibroblast function, for instance, that IL-33 needs IL-13 to polarize M2; and that stimulation of hepatic stellate cells with IL-13 could increase their proliferation and expression of pro-fibrotic genes (McHedlidze et al., 2013). More work is needed to clarify the role of IL-33 in lung fibroblast function.

In summary, our experiments demonstrated that IL-33 amplified BLM-induced pulmonary fibrosis via inducing inflammation through recruiting a number of inflamed immune cells, in particular the ILC2s and alternatively activating macrophages. IL-33 recruits ILC2s which produce IL-13, and IL-13 plus IL-33 induce M2 macrophages, and M2 macrophages produce IL-13 and TGFβ1 which promote the collagen production and deposition which accelerate the lung fibrosis (Fig 4-15).

# Chapter 5

# The pathogenic role of IL-33 in DSS-induced colitis in mice

# Chapter 5: The pathogenic role of IL-33 in DSS-induced colitis in mice

## 5.1 Introduction

Ulcerative colitis (UC) is one of the most common idiopathic disorders in inflammatory bowel disease (IBD) (Hanauer, 2006). The etiology of UC is still largely unknown. Current evidence suggests that UC is caused by environmental, genetic and immunological factors (Hanauer, 2006, Podolsky, 2002, Baumgart and Carding, 2007). The initiation of UC may be due to barrier dysfunction and unusual angiogenesis in gut epithelium caused by environmental or infectious factors (Podolsky, 2002, Papadakis and Targan, 2000, Sanchez-Munoz et al., 2008, Bamias et al., 2012). Despite considerable research, few therapeutic options have emerged, and there remains considerable unmet clinical need for the treatment of the disorder (Frei et al., 2012).

Dextran sodium sulphate (DSS)-induced colitis is the best characterised animal model of human UC (Okayasu et al., 1990, Wirtz et al., 2007, Maxwell and Viney, 2009). The pathological process in mice given DSS is similar to that in patients with UC, including diarrhoea, chronic inflammation and ulcerations (Wirtz et al., 2007). The mechanism of DSS-induced colitis is not fully understood; DSS has a direct toxic effect on gut epithelial cells and this might cause gut damage and inflammation (Okayasu et al., 1990, Dieleman et al., 1994). Acute DSS-induced colitis is T and B lymphocyte independent, so it is an ideal model to investigate the role of gut epithelial cells and the innate immune response in the development of inflammatory colitis (Dieleman et al., 1994, Ghosh, 2004).

Current studies show that type II cytokines such as IL-4, IL-5 and IL-13 play an important role in the development of UC (Maloy and Powrie, 2011, Targan and Karp, 2005, Papadakis and Targan, 2000, Mizoguchi et al., 1999, Heller et al., 2005, Fuss et al., 1996). IL-4 deficient mice developed much less severe colitis using the DSS-induced colitis, suggesting that IL-4 has direct effects on the development and severity of colitis potentially by modulating innate cell

function in colon tissue (Mizoguchi et al., 1999, Stevceva et al., 2001). Type I cytokines including IFN-γ and pro-inflammatory cytokines (IL-1, IL-6, IL-17 and TNFα) might also contribute to the pathogenesis of colitis, but mainly in the chronic stage (Maloy and Powrie, 2011, Obermeier et al., 1999, Bauer et al., 2010, Sanchez-Munoz et al., 2008, Papadakis and Targan, 2000). However, the mechanism by which the type 1 and 2 cytokines are induced by DSS and the cell source of these cytokines are unresolved. In this regard, IL-33, a newly described type II cytokine inducer, might play a critical role in colitis (Schmitz et al., 2005, Beltran et al., 2010, Pastorelli et al., 2010, Sedhom et al., 2013).

IL-33 is predominantly expressed and released by the epithelial and endothelial cells after sensing of inflammatory signals e.g. undergoing necrosis (Schmitz et al., 2005). Its receptor ST2 is expressed in all innate leukocytes recognised to date, but only in selective adaptive leukocytes (Schmitz et al., 2005, Liew et al., 2010, Liew, 2012, Kurowska-Stolarska et al., 2008, Xu et al., 1998, Stolarski et al., 2010, Neill et al., 2010, Komai-Koma et al., 2012). Thus the IL-33/ST2 pathway can directly activate eosinophils, macrophages, innate lymphoid cells (ILCs), mast cells and thereby trigger innate immune responses (Schmitz et al., 2005, Liew et al., 2010, Liew, 2012, Kurowska-Stolarska et al., 2008, Xu et al., 1998, Stolarski et al., 2010, Neill et al., 2010, Komai-Koma et al., 2012). IL-33 can also induce Th1- and Th17-mediated immune responses in pro-inflammatory disorders through unknown mechanisms (Xu et al., 2008, Verri et al., 2008). Recent reports show that the mRNA expression of IL-33 is up-regulated in human biopsy specimens from UC patients in which the increase is specifically in intestinal epithelial cells (Pastorelli et al., 2010, Beltran et al., 2010, Kobori et al., 2010, Seidelin et al., 2011). Furthermore, IL-33-deficient mice showed impaired development of experimental colitis after DSS administration (Oboki et al., 2010). There is also evidence suggesting that the IL-33/ST2 pathway plays an important role in IBD in general, but the underlying mechanism is unknown (Seidelin et al., 2011, Oboki et al., 2010, Palmer and Gabay, 2011, Vermeire et al., 2011, Pastorelli et al., 2010, MacDonald et al., 2012, Pastorelli et al., 2013).

The aim of this part of the overall PhD project is to investigate whether IL-33 has a role in the development of UC using an acute model of DSS-induced colitis in mice, and to investigate any associated mechanisms. In summary; our experimental data suggested that IL-33 exacerbated colitis by the induction of pro-inflammatory and angiogenic cytokines and chemokines in an ST2 and IL-4 dependent manner.

## 5.2 DSS enhances IL-33 and other inflammatory cytokine and chemokine production in colonic tissue

Pro-inflammatory cytokines and inflammatory chemokines are closely associated with the pathogenesis of colitis (Bamias et al., 2011). Group of WT BALB/c mice were fed with or without DSS for 7 continuous days, and then the colon tissues were removed and placed into organ culture to measure spontaneous cytokine release (Fig. 5-1). After 24 hours of culture, the supernatants were collected and the levels of cytokines and chemokines were measured by ELISA. Some specimens of colon were also digested using RIPA buffer to measure the intracellular IL-33 concentration in the tissue. The group treated with DSS had significantly higher concentration of IL-33 in both colon tissue culture supernatants and lysed tissue cell cultures compared to similar cultures from sham-treated control group (Fig. 5-2). Similar phenomena were observed for type II cytokines (IL-4 and IL-13) and pro-inflammatory factors (TNFα, KC and MIP-2); the type I cytokine IFN-γ had the same trend as the type II cytokines (Fig. 5-3).

Together these results demonstrated that IL-33 as well as other Th2 type cytokines, pro-inflammatory cytokines and inflammatory chemokines are induced and released in DSS stimulated colonic tissue of mice. They suggest that IL-33 may be involved in the initiation of pathogenesis of colitis.

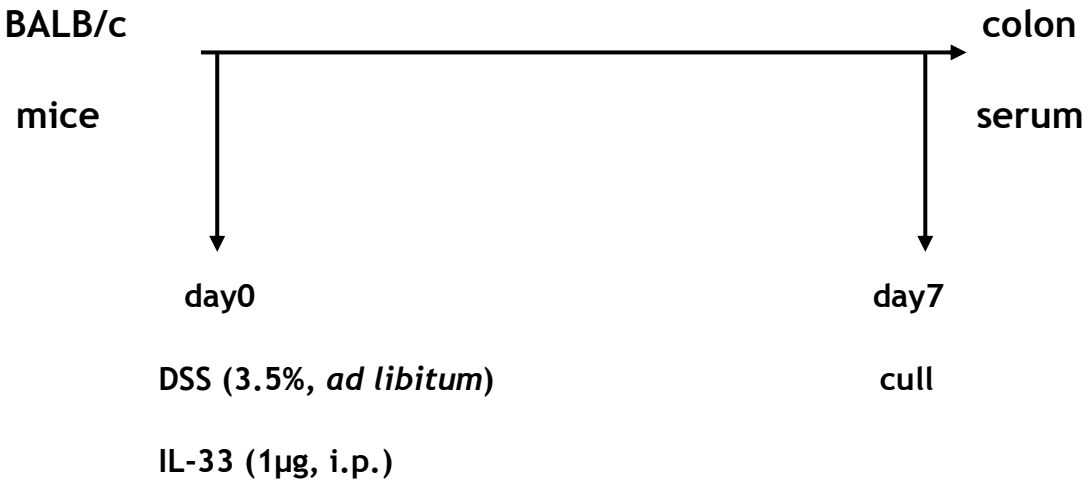



**Fig. 5-1 Experimental plan used to induce acute colitis in WT mice.**

Female WT BALB/c mice were fed with or without 3.5% (weight/volume) DSS in their drinking water from day 0 for 7 continuous days. Some mice were injected i.p. with 1μg IL-33 or PBS daily from day 0 to day 7. The body weight, stool consistency and bleeding condition were monitored daily. Serum and colon were collected and analysed as described in chapter 2.

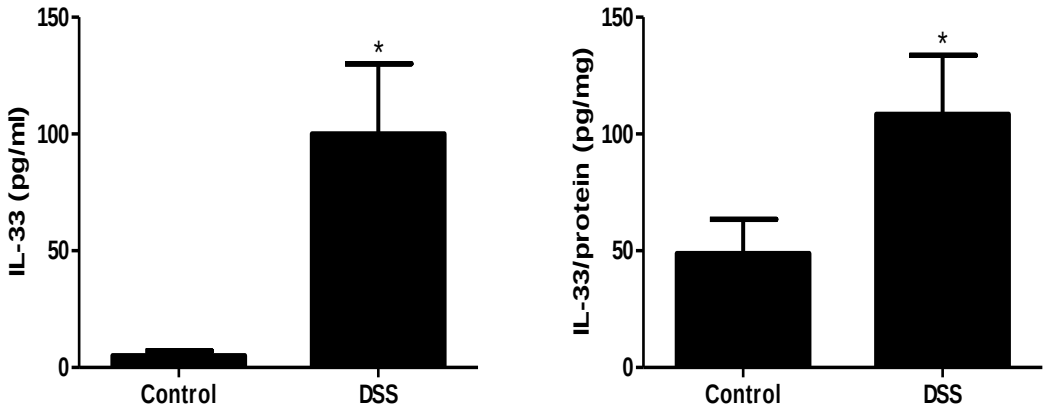



**Fig. 5-2 DSS induces IL-33 production *ex vivo* in mice.**

Colon tissue samples from groups of female BALB/c mice were harvested 7 days after DSS administration. The tissue samples were directly digested using RIPA buffer or cultured for 24 hours before the supernatant was collected. The IL-33 concentrations in supernatant (left) and cell lysis (right) were measured using ELISA as described in chapter 2. Data are representative of two experiments, mean ± SEM, n= 5 mice per group, *P < 0.05 compared to PBS group.

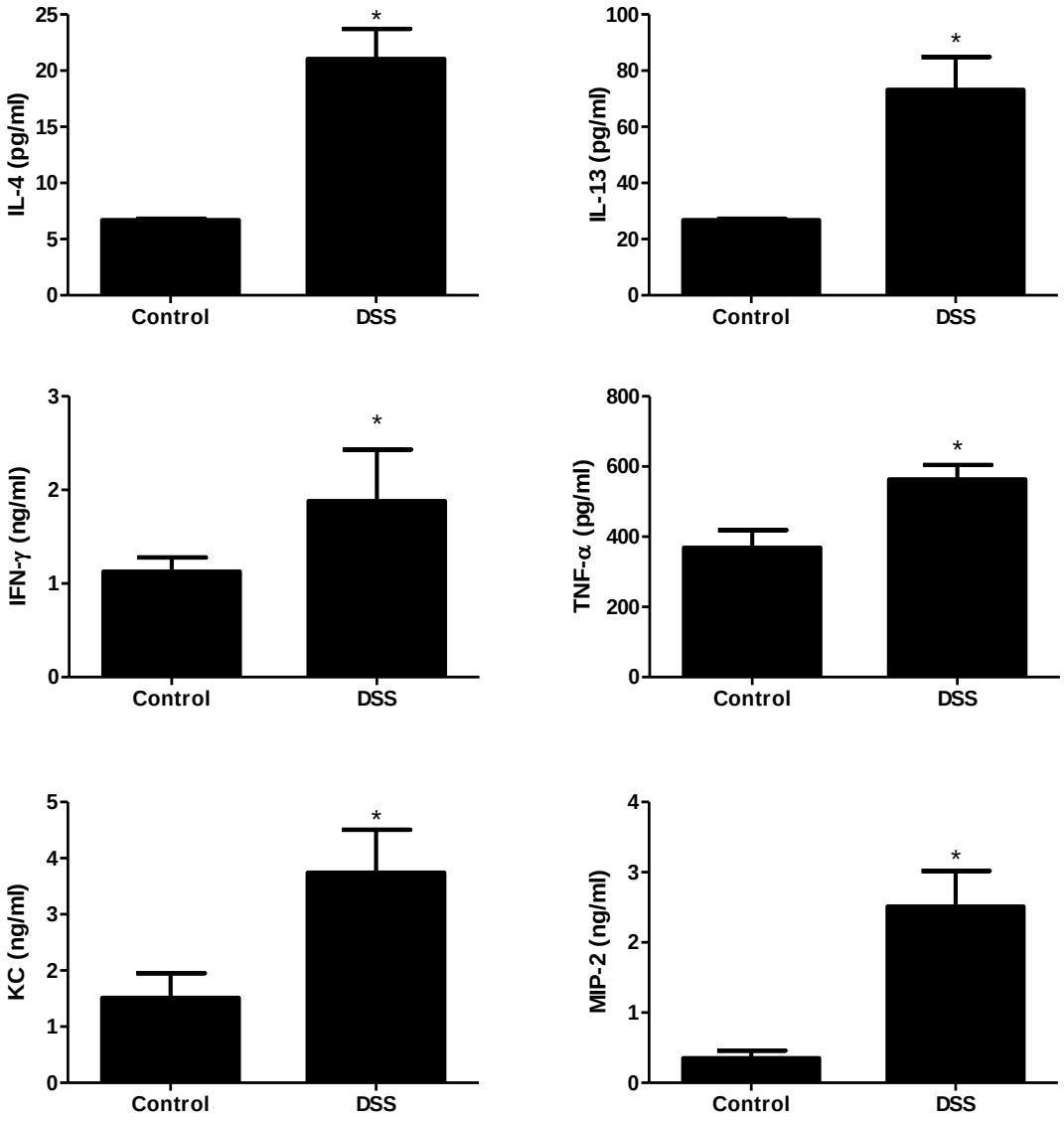



**Fig. 5-3 DSS induces cytokine and chemokine production in colon tissue of mice.**

Colon tissue samples from groups of female WT BALB/c mice fed with or without DSS were harvested on day 7. The tissue samples were cultured for 24 hours before the supernatant was collected. The cytokine/chemokine concentrations in the supernatant were measured using ELISA as described in chapter 2. Data are representative of two experiments, n= 5 mice per group, mean ± SEM, *P < 0.05 compared to PBS group.

## 5.3 ST2 gene-deficient mice show impaired DSS-induced colitis

### 5.3.1 ST2 deficiency mice develop less severe colitis

We next investigate the importance of IL-33 signalling in the pathogenesis of colitis by using ST2, the IL-33 receptor, deficient mice in an acute DSS-induced colitis model. Groups of WT and $ST2^{-/-}$ mice were given DSS or not in their drinking water and the development of clinical parameters of colitis were monitored daily for up to 20 days (Fig. 5-4).

As shown in Fig. 5-5, WT mice but not ST2-/- mice developed colitis-related diarrhoea 10 days after DSS administration. However, diarrhoea became evident at 20 days, which was significantly delayed by 10 days in $ST2^{-/-}$ mice. Colon shortening is a pathology sign of gut inflammation (Kojouharoff et al., 1997, Axelsson et al., 1998). Compared to the mice without DSS, both DSS-treated WT and $ST2^{-/-}$ mice had significantly shortened colon length loss. However the colon length was longer in $ST2^{-/-}$ group than that in the WT mice (Fig. 5-6). The histological analysis of colon tissue showed clearly inflammation in both groups (Fig. 5-7). Consistent with the report (Oboki et al., 2010), the body weight changes between WT and $ST2^{-/-}$ mice were not significantly different (Fig. 5-8) at this time point.

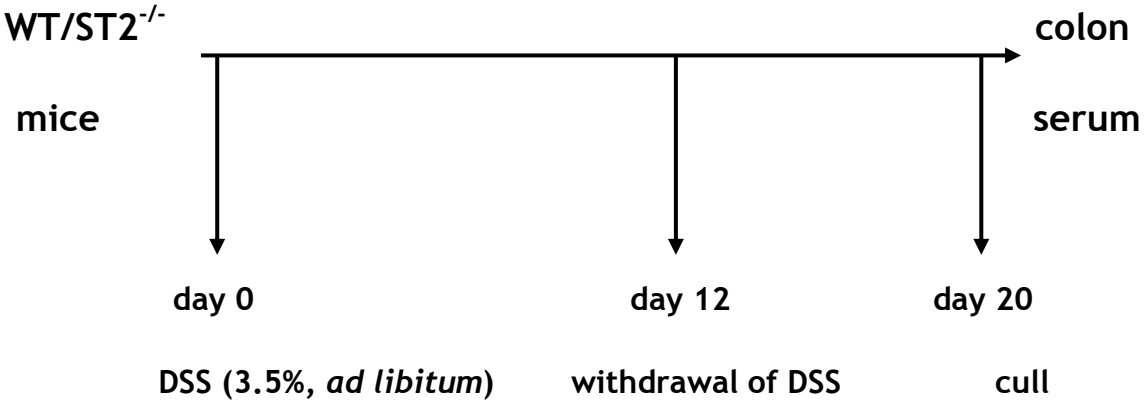



**Fig. 5-4 Experimental strategy used to induce colitis in WT and $ST2^{-/-}$ mice.**

Female WT and $ST2^{-/-}$ BALB/c mice were given or not 3.5% (weight/volume) DSS in their drinking water from day 0 for 12 continuous days. The body weight loss was calculated as percentage of the baseline body weight as on day 0. The body weight, stool consistency and bleeding condition were monitored daily. Serum and colon were collected and analysed as described in chapter 2.

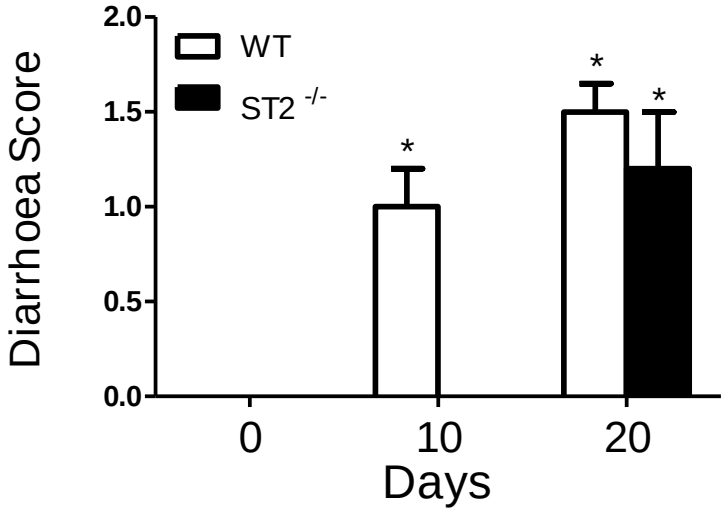



**Fig. 5-5 Diarrhoea score of DSS-induced colitis in WT and ST2$^{-/-}$ mice.**

Female WT and ST2$^{-/-}$ BALB/c mice were given 3.5% (weight/volume) DSS in their drinking water from day 0 for 12 continuous days and culled on day 20. The stool consistency and bleeding condition was scored as follows: 0 (normal, negative hemoccult); 1 (soft but still formed, no blood traces in stool); 2 (very soft, blood traces in stool visible); 3 (diarrhea, rectal bleeding). Data are representative of three experiments, mean ± SEM, n= 5 mice per group, *P < 0.05 compared to control group.

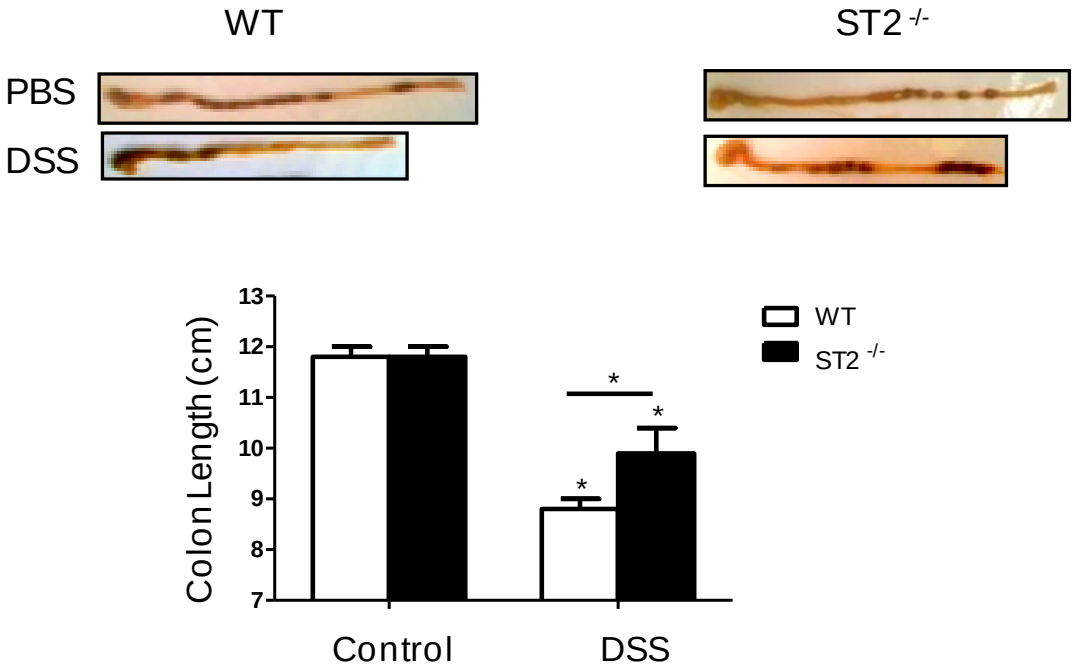



**Fig. 5-6 Comparison of the colon length of different treated WT and ST2$^{-/-}$ mice.**

The colon tissues were collected on day 20 and the length measured as in chapter 5. Data are representative of three experiments, mean ± SEM, n= 5 mice per group, *P < 0.05 compared to PBS group.

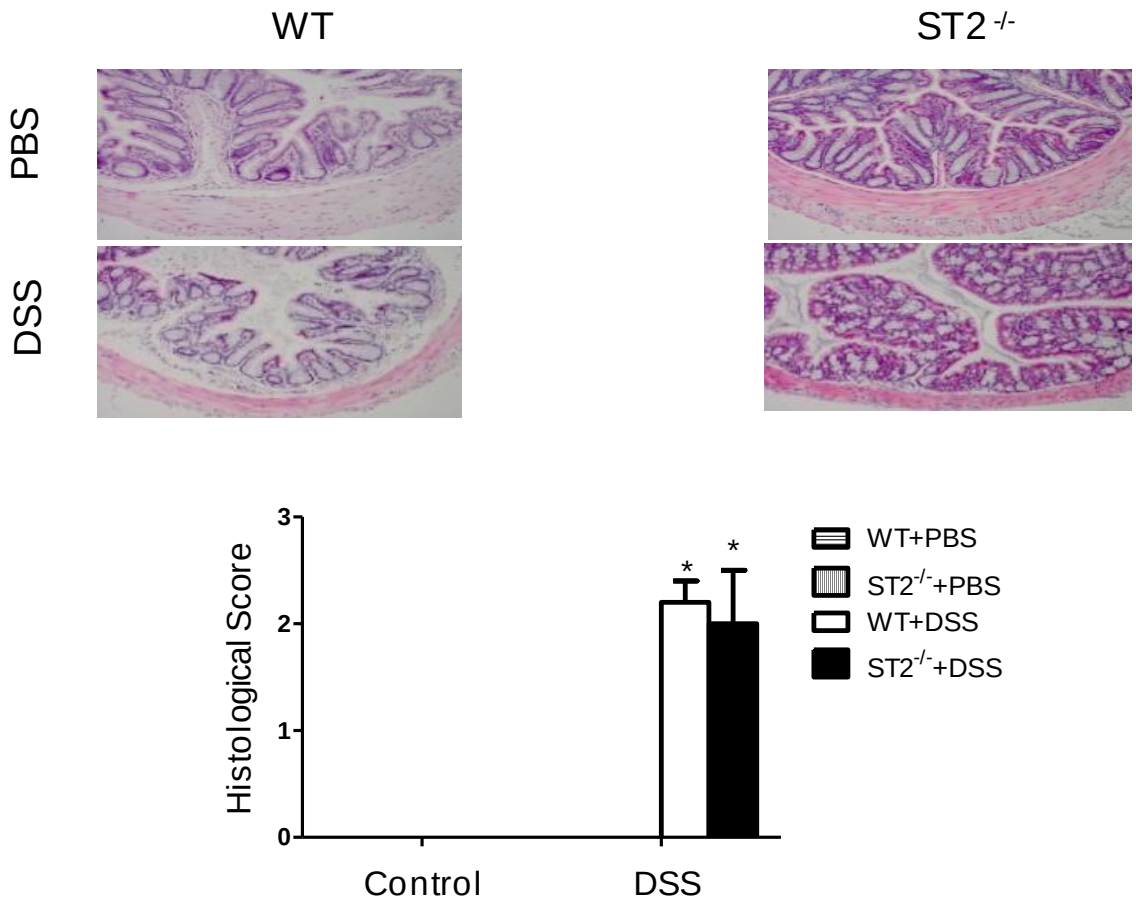



**Fig. 5-7 Histological analysis of DSS-induced colitis in WT and ST2$^{-/-}$ mice.**

The colon tissues were collected on day 20. The colon sections were stained with H&E and scored as described in chapter 2. Data are representative of three experiments, mean ± SEM, n= 5 mice per group, *P < 0.05 compared to control group.

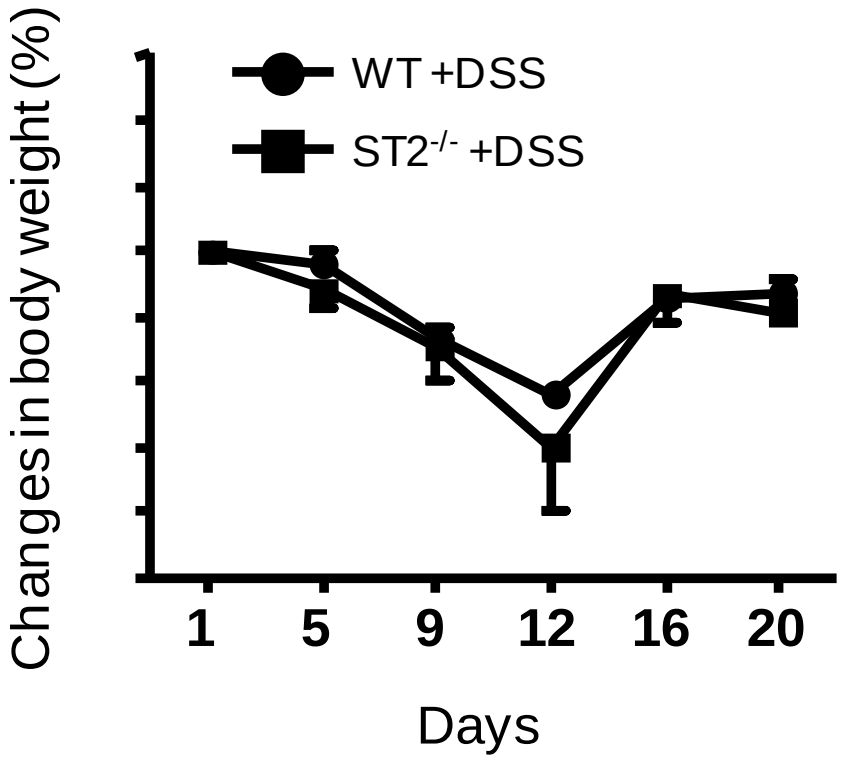



**Fig. 5-8 Body weight changes of DSS-induced colitis in WT and ST2$^{-/-}$ mice.**

Female WT and ST2$^{-/-}$ BALB/c mice were given 3.5% (weight/volume) DSS in their drinking water from day 0 for 12 continuous days and culled on day 20. The body weight changes were calculated as the percentage of the baseline body weight as on day 0. Data are representative of three experiments, mean ± SEM, n= 5 mice per group, *P < 0.05 compared to control group.

### 5.3.2 ST2 deficiency mice show reduced inflammatory cytokines and chemokines in DSS-induced colitis

Local and systemic inflammatory cytokines and chemokines play a critical role in colitis (Hanauer, 2006). We further assessed the serum cytokine and chemokine profile in WT and $ST2^{-/-}$ DSS colitis mice by 20-plex Luminex. Compared to control, WT mice treated with DSS had significantly increased concentration of IL-6, IL-13, IL-17 and VEGF (Fig. 5-9). There were no significantly increased concentrations of these in $ST2^{-/-}$ mice (Fig. 5-9).

Also compared to control, DSS-administered WT mice had significantly increased, but $ST2^{-/-}$ mice had significantly decreased serum IL-4 concentration (Fig. 5-10). However, DSS treated WT and $ST2^{-/-}$ mice had similarly enhanced serum IL-12 levels compared to control (Fig. 5-10). The levels of IFN-γ, chemokines CXCL9 and CXCL10, and the immune suppressive cytokine IL-10 were unchanged compared to WT control (Fig. 5-11).

These results demonstrated that ST2 is necessary for the development of DSS-induced colitis suggests that IL-33/ST2 pathway plays a pathogenic role in acute colitis.

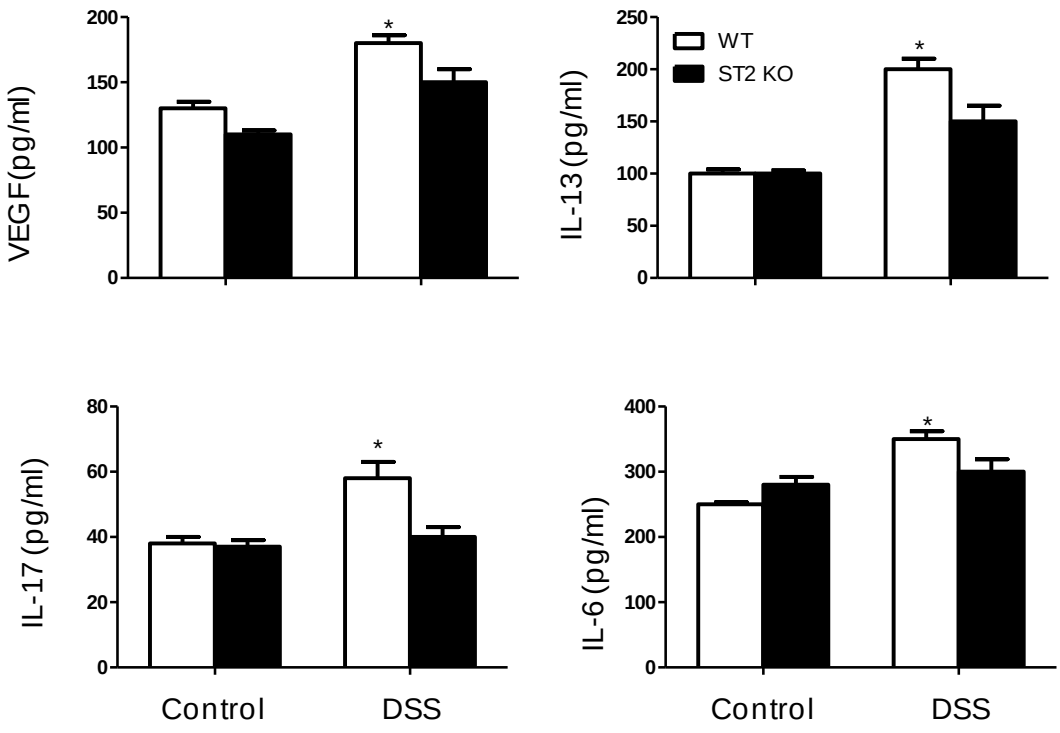



**Fig. 5-9 IL-17 and IL-6 levels in WT and ST2$^{-/-}$ mice.**

Serum samples from groups of female WT and ST2$^{-/-}$ BALB/c mice fed with or without DSS were harvested on day 20. The IL-6 and IL-17 concentrations in serum samples were measured using Luminex as described in chapter 5. Data are representative of three experiments, mean ± SEM, n= 5 mice per group, *P < 0.05 compared to control group.

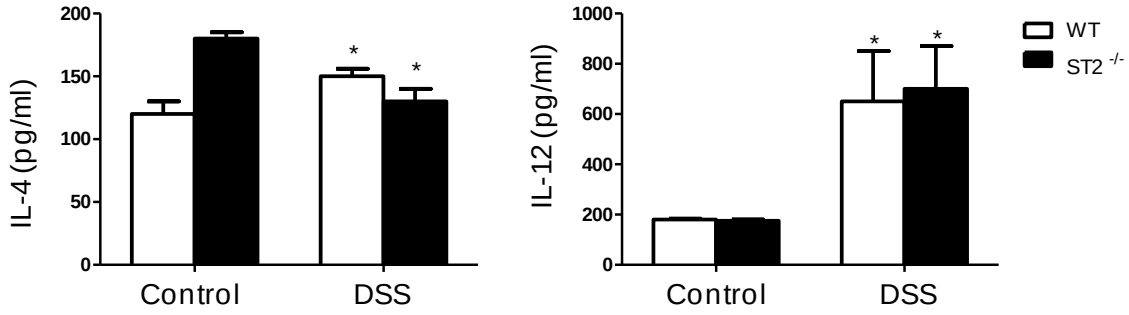



**Fig. 5-10 Serum IL-4 and IL-12 levels in WT and ST2$^{-/-}$ mice.**

Serum samples from groups of female WT and ST2$^{-/-}$ BALB/c mice fed with or without DSS were harvested on day 20. The IL-4 and IL-12 concentrations in serum samples were measured using Luminex as described in chapter 5. Data are representative of three experiments, mean ± SEM, n= 5 mice per group, *P < 0.05 compared to PBS group.

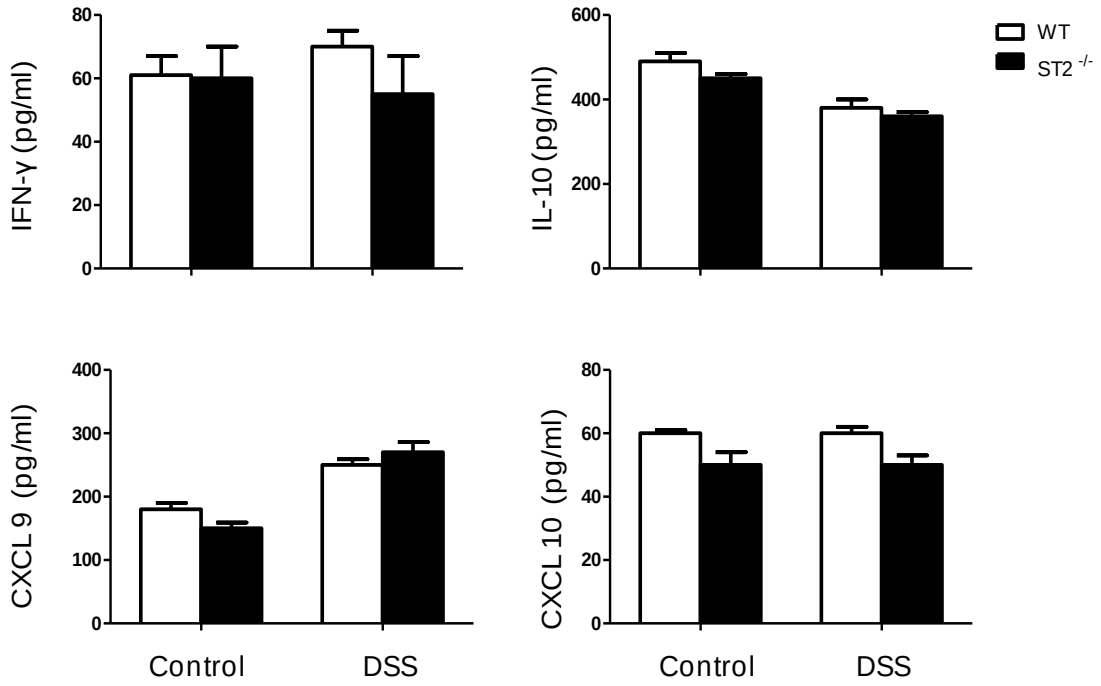



**Fig. 5-11 IL-10, IFN-γ, CXCL9 and CXCL10 levels in WT and ST2$^{-/-}$ mice.**

Serum samples from groups of female WT and ST2$^{-/-}$ BALB/c mice fed with or without DSS were harvested on day 20. The IL-10, IFN-γ, CXCL9 and CXCL10 concentrations were measured using Luminex as described in chapter 5. Data are representative of three experiments, mean ± SEM, n= 5 mice per group, *P < 0.05 compared to control group.

## 5.4 Exogenous IL-33 exacerbates DSS-induced colitis in mice

### 5.4.1 Exogenous IL-33 enhances severity of colitis in mice

We next investigate the importance of IL-33 in the pathogenesis of colitis by administration of exogenous IL-33 by directly injecting IL-33 recombinant protein (rIL-33) into the mice intraperitoneally (i.p.). Groups of mice were given either PBS, IL-33, DSS alone or DSS plus IL-33. The developments of clinical parameters of colitis were monitored daily up to 20 days (Fig. 5-12). As shown in Fig. 5-13, the administration of PBS or IL-33 did not caused diarrhoea in mice from day 10 compared to controls. As before, administration of DSS caused diarrhoea which was significantly enhanced by exogenous IL-33 on day 20 (Fig. 5-13). Exogenous IL-33 and DSS reduced the colon length; however DSS plus IL-33 further decreased the colon length as compared to the control groups (Fig. 5-14). The histological analysis of colon tissue showed that IL-33 and DSS alone significantly and IL-33 plus DSS group markedly enhanced gut inflammation in mice (Fig. 5-15). As reported before (Oboki et al., 2010), there was no significant difference in body weight changes between DSS alone and DSS plus IL-33 groups (Fig. 5-16). Taken together, our results demonstrated that the severity of DSS-induced colitis was significantly increased in the presence of IL-33.

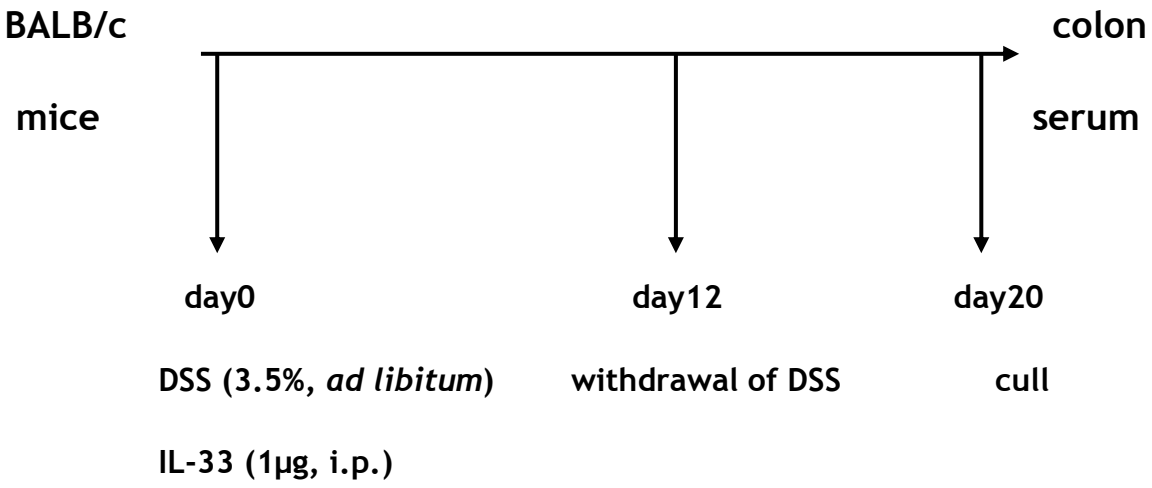



**Fig. 5-12 Experimental strategy used to induce colitis in BALB/c mice.**

Female BALB/c mice were given or not 3.5% (weight/volume) DSS in their drinking water from day 0 for 12 continuous days. Some mice were injected i.p. with 1μg IL-33 or PBS daily from day 0 to day 20. The body weight, stool consistency and bleeding condition were monitored daily. Serum and colon were collected and analysed as described in chapter 2.

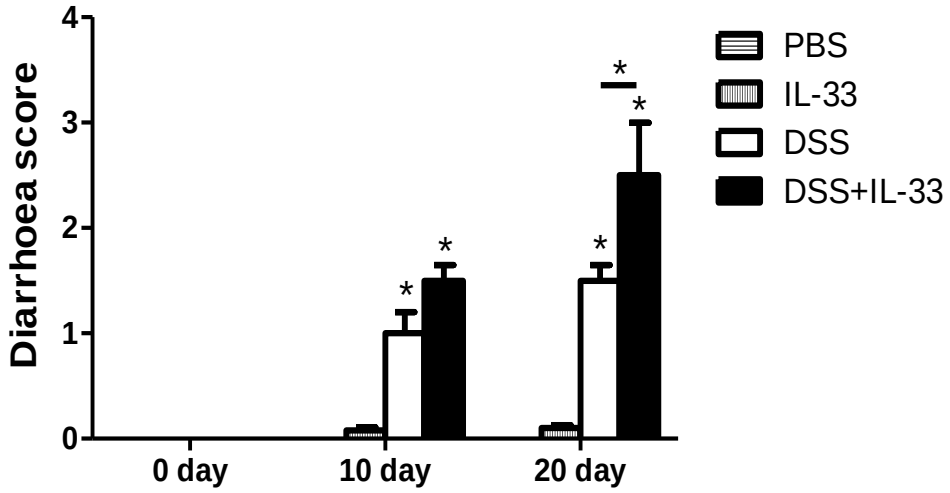



**Fig. 5-13 Diarrhoea score in mice with or without DSS/IL-33**

Groups of female mice were given DSS, IL-33 alone or DSS plus IL-33. The stool consistency and bleeding condition were scored as follows: 0 (normal, negative hemoccult); 1 (soft but still formed, no blood traces in stool); 2 (very soft, blood traces in stool visible); 3 (diarrhoea, rectal bleeding) as described in chapter 2. Data are representative of three experiments, mean ± SEM, n= 5 mice per group, *P < 0.05 compared to PBS alone group.

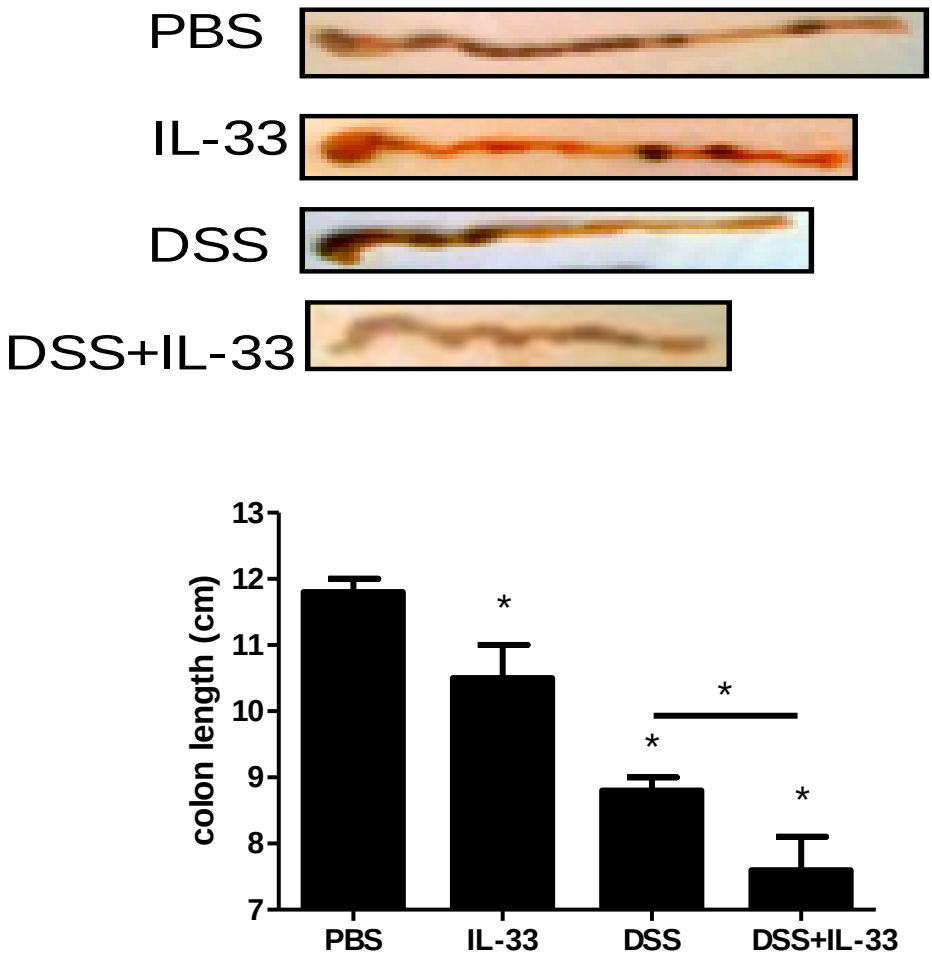



**Fig. 5-14 Comparison of the colon length of mice administered PBS, DSS, IL-33 alone or DSS plus IL-33.**

The colon tissues were collected on day 20 and the length measured as in chapter 2. Data are representative of three experiments, mean ± SEM, n= 5 mice per group, *P < 0.05.

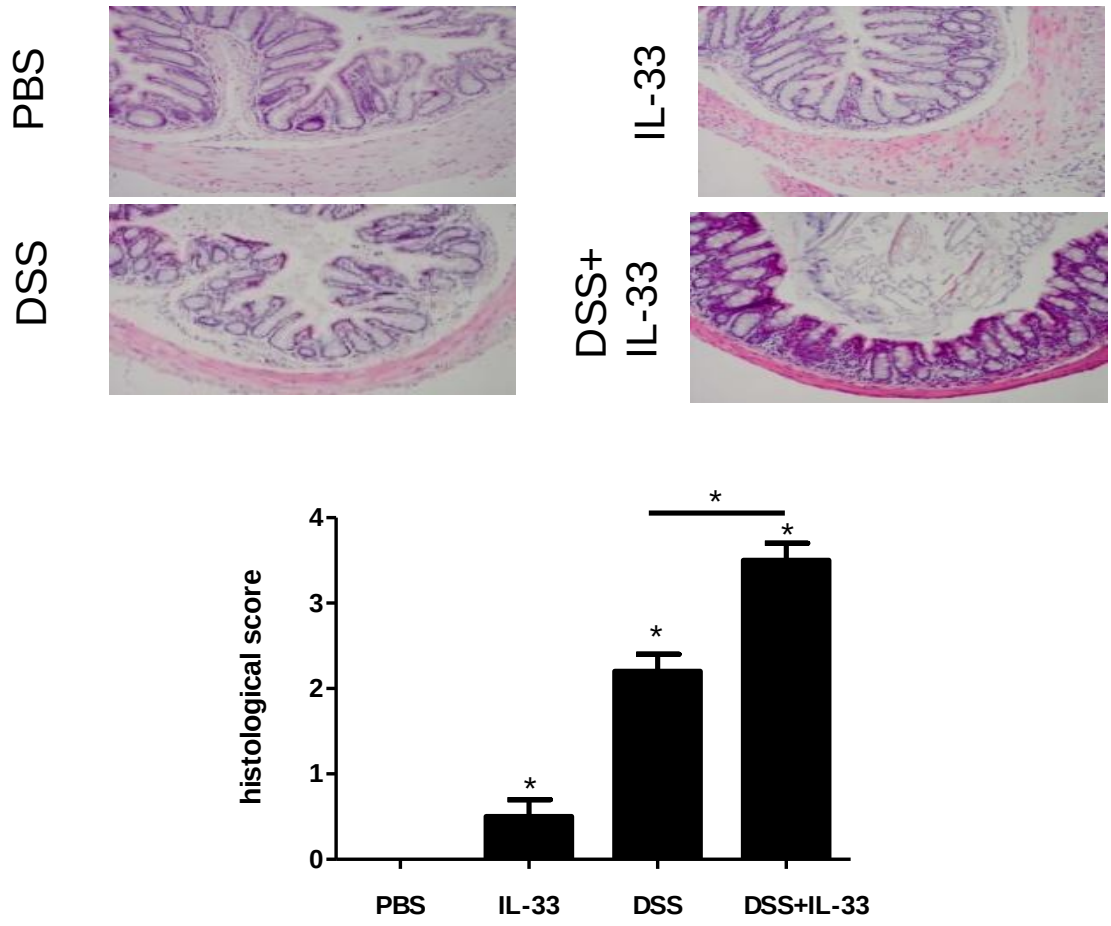



**Fig. 5-15 Histological score of DSS, IL-33 and DSS plus IL-33 induced colitis in mice.**

The colon tissues were collected on day 20. The colon sections were stained with H&E and scored as described in chapter 2. Data are representative of three experiments, mean ± SEM, n= 5 mice per group, *P < 0.05.

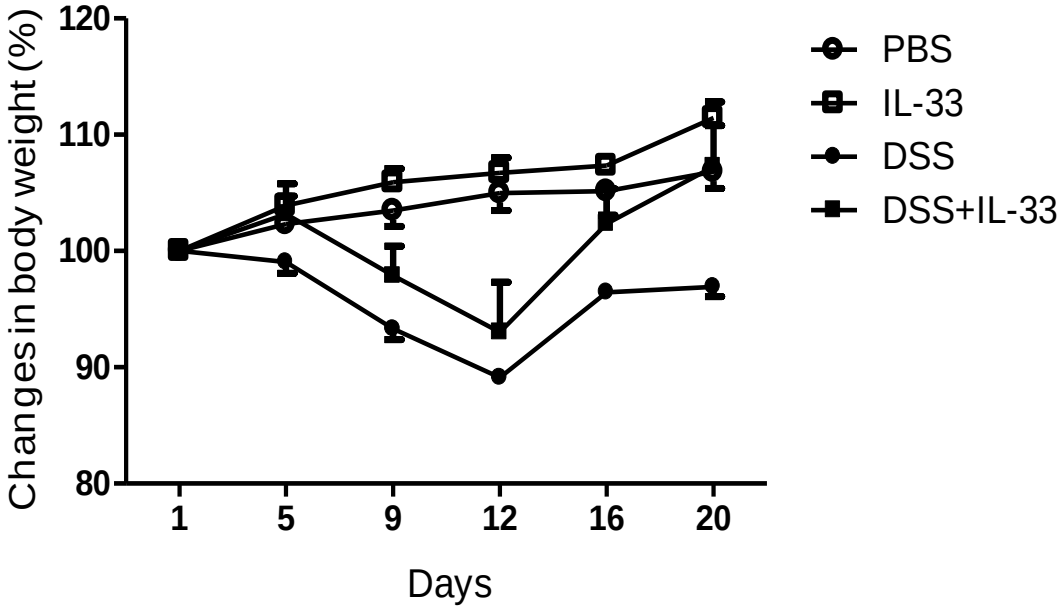



**Fig. 5-16 Body weight changes in mice given DSS, IL-33 and IL-33 induced colitis.**

Female BALB/c mice were given 3.5% (weight/volume) DSS in their drinking water from day 0 for 12 continuous days and culled on day 20; IL-33 (1μg/day/mouse) was injected intraperitoneally. The body weight changes were calculated as the percentage of the baseline body weight as on day 0. Data are representative of three experiments, mean ± SEM, n= 5 mice per group.

### 5.4.2 Exogenous IL-33 enhances inflammatory cytokine and chemokine production in mice

We compared the serum cytokine and chemokine concentrations in mice given PBS, IL-33, DSS alone and DSS plus IL-33 by 20-plex Luminex. IL-33 alone induced IL-13 (Fig. 5-17), reduced IL-10 and IFN-γ (Fig. 5-18) production, but had no significant effect on other 17 cytokines tested. DSS administration induced IL-4, IL-13 and other inflammatory cytokines in colon tissue (Fig. 5-3), there was no equivalent increase in the concentration in the serum except IL-12 (Fig. 5-17 and 5-18), suggesting that DSS mainly induces these cytokines locally in the colon. However, DSS plus IL-33 induced higher concentrations of Th2 type cytokine IL-4 and IL-13 (Fig. 5-17), inflammatory cytokines (IL-6 and IL-17, Fig. 5-17) but lower levels of IL-10 and IFN-γ (Fig. 5-18) than the control groups (Fig. 5-17 and 5-18). IL-33 alone also increased the levels of angiogenic cytokine (VEGF), chemokines CXCL9 but not CXCL10 in the serum (Fig. 5-19) compared to PBS controls. Whereas DSS alone had no significant effect on VEGF and chemokine production, DSS plus IL-33 group induced higher levels of VEGF and chemokines (CXCL9, CXCL10) in the serum (Fig. 5-19).

Together, these results demonstrated that IL-33 is an important inducer of the key pathogenic Th2 type cytokines (IL-4, IL-13), Th17 type cytokines (IL-6, IL-17) and angiogenic cytokines (VEGF) and chemokines (CXCL9, CXCL10) in colitis *in vivo*. The results suggest that IL-33 may promote colitis by inducing pro-inflammatory and angiogenic cytokines and chemokines and reducing Th1 type cytokine (IFN-γ) and immune suppressive cytokine (IL-10) expression in DSS-induced colitis.

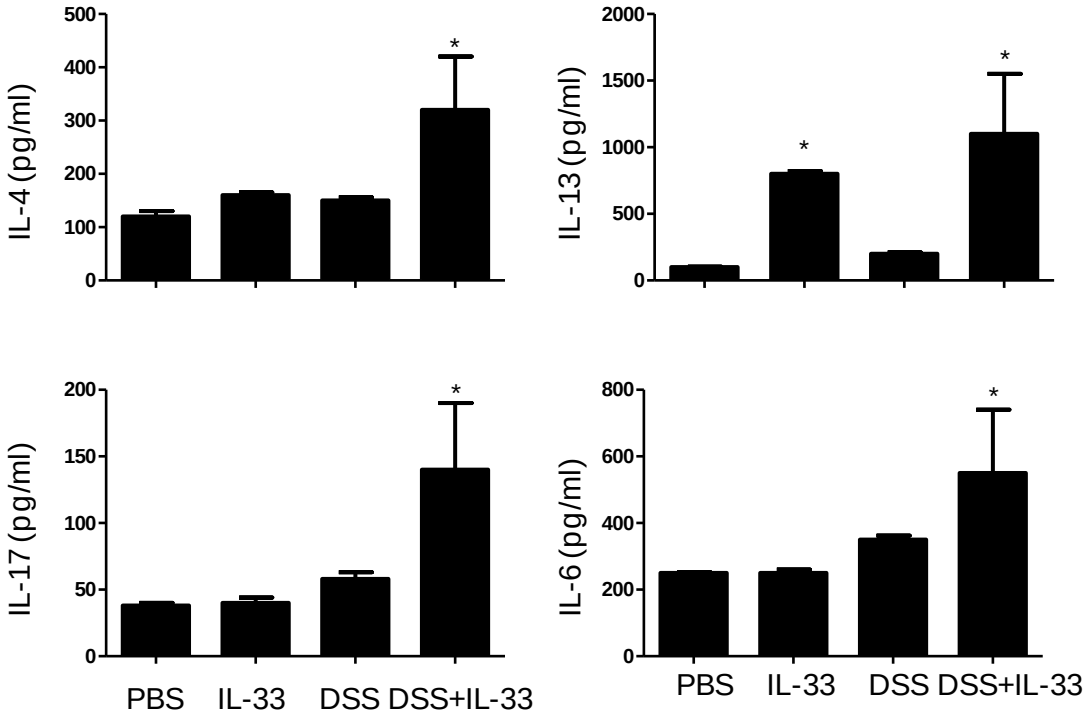



**Fig. 5-17 Serum cytokine IL-4, IL-13, IL-17 and IL-6 levels in mice *in vivo*.**

Serum samples from groups of female BALB/c mice receiving PBS, IL-33 alone, DSS alone or DSS plus IL-33 were harvested on day 20. The IL-4, IL-13, IL-17 and IL-6 concentrations in serum samples were measured using Luminex as described in chapter 5. Data are representative of three experiments, mean ± SEM, n= 5 mice per group, *P < 0.05 compared to PBS alone group.

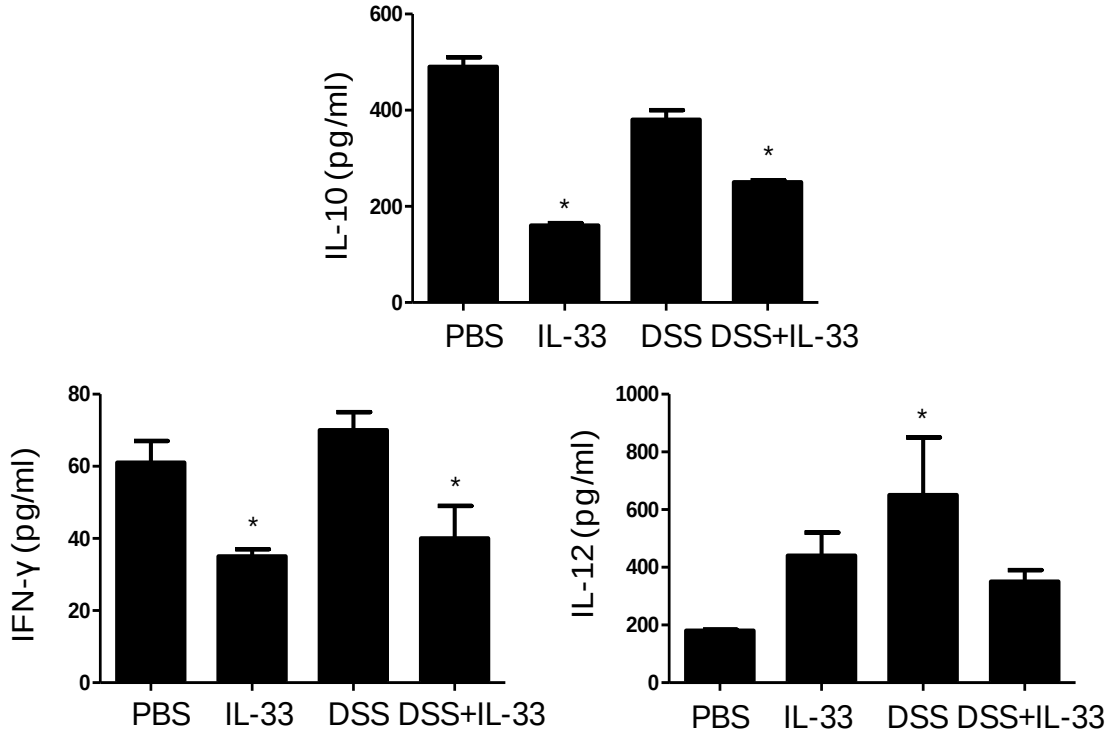



**Fig. 5-18 Serum IL-10, IFN-γ and IL-12 levels in mice.**

Serum samples from groups of BALB/c mice given PBS, IL-33 alone, DSS alone or DSS plus IL-33 were harvested on day 20. The IL-10, IFN-γ and IL-12 concentrations in serum samples were measured using Luminex as described in chapter 5. Data are representative of three experiments, mean ± SEM, n= 5 mice per group, *P < 0.05 compared to PBS alone group.

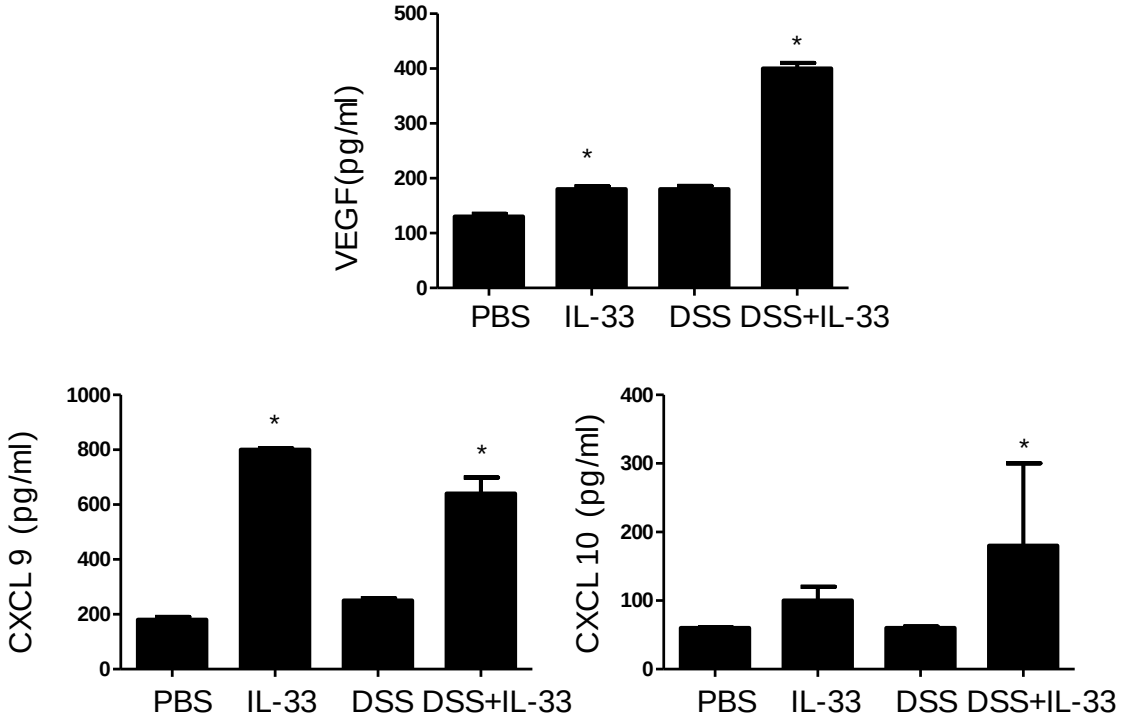



**Fig. 5-19 The serum levels of VEGF, CXCL9 and CXCL10 in mice.**

Serum samples from groups of BALB/c mice given PBS, IL-33 alone, DSS alone or DSS plus IL-33 were harvested on day 20. The VEGF, CXCL9 and CXCL10 concentrations in serum samples were measured using Luminex as described in chapter 5. Data are representative of three experiments, mean ± SEM, n= 5 mice per group, *P < 0.05 compared to IL-33 alone group.

## 5.5 IL-33 exacerbates colitis via IL-4

IL-4, the prototype type II cytokine, is reported to play an essential role in the initiation of DSS-induced colitis. Our results described above demonstrated that IL-33 which exacerbates DSS-colitis also induces IL-4 (Fig. 5-17). To further assess the importance of IL-4 in the IL-33 exacerbated DSS-induced colitis, we investigated the mechanism by which IL-33 exacerbates colitis using IL-$4^{-/-}$ mice. The experimental plan is shown in Fig. 5-20.

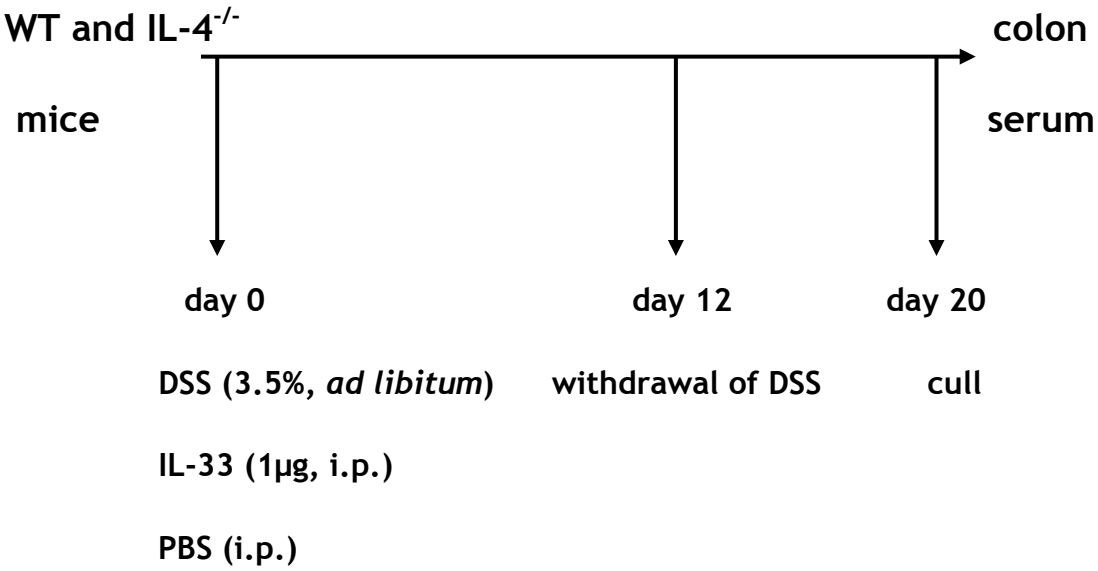



**Fig. 5-20 Experimental plan used to induce colitis in WT and IL-4$^{-/-}$ mice.**

Female WT and IL-4$^{-/-}$ BALB/c mice were given or not given 3.5% (weight/volume) DSS in their drinking water from day 0 for 12 continuous days. Some mice were injected i.p. with 1µg IL-33 or PBS daily from day 0 to day 20. The body weight, stool consistency and bleeding condition were monitored daily. Serum and colon were collected and analysed as described in chapter 2.

### 5.5.1 IL-4 deficiency abolished IL-33-mediated exacerbation of DSS colitis

We next directly evaluate the role of IL-4 in IL-33-enhanced DSS colitis using IL-$4^{-/-}$ mice. WT and IL-$4^{-/-}$ mice were treated with PBS, IL-33, DSS alone, or DSS plus IL-33 as above. The clinical parameters, histological changes in colon tissue and cytokine/chemokine levels were determined.

Consistent with result in Fig. 5-13, control PBS and IL-33 alone caused no diarrhoea and DSS-induced colitis related diarrhoea from day 10 (Fig. 5-13). However, the DSS induced diarrhoea was markedly delayed in IL-$4^{-/-}$ mice and appeared from day 20 (Fig. 5-21). More importantly, similar to $ST2^{-/-}$ mice, IL-33 also failed to exacerbate the clinical change of colitis in the IL-$4^{-/-}$ mice (Fig. 5-21).

Both IL-33 and DSS alone significantly and DSS plus IL-33 synergistically caused the shortness of colon length in WT mice (Fig. 5-14). The synergistic effect of DSS and IL-33 in the pathogenic changes in the colon was lost in IL-$4^{-/-}$ mice compared to WT controls (Fig. 5-21).

Unlike the WT controls, changes in histological score associated with administration of IL-33 were also not apparent in IL-$4^{-/-}$ mice (Fig. 5-21).

Furthermore, IL-$4^{-/-}$ mice also failed to produce IL-13, IL-12, CXCL9 and VEGF in the IL-33-treated group, IL-12 and VEGF in the DSS-treated group and IL-5, IL-13, IL-12, CXCL9 and VEGF in the DSS plus IL-33-treated group in contrast to similarly treated WT mice on day 20. However, the serum IL-10 level remained unchanged (Fig. 5-22).

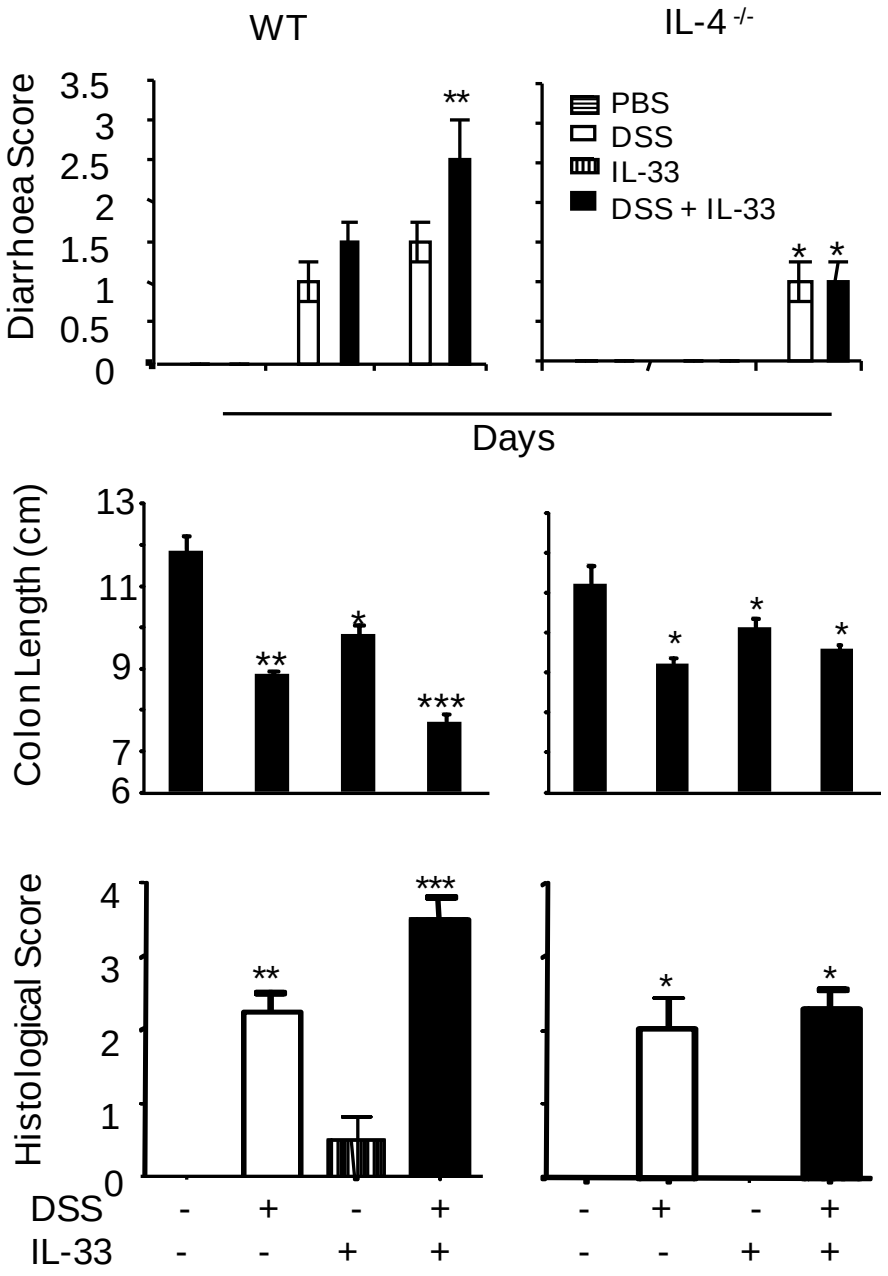



**Fig. 5-21 Diarrhoea score and colon length in WT and IL-4$^{-/-}$ mice.**

The WT and IL4$^{-/-}$ mice were given 3.5% DSS or normal drinking water. Some mice were injected i.p. with 1μg IL-33 or PBS daily for continuous 20 days. The stool consistency and bleeding condition of groups of WT and IL4$^{-/-}$ female mice was scored as follows: 0 (normal, negative hemoccult); 1 (soft but still formed, no blood traces in stool); 2 (very soft, blood traces in stool visible); 3 (diarrhoea, rectal bleeding). The colon tissue samples were collected on day 20, the length was measured, the colon tissue sections were stained with H&E and scored as described in chapter 2. Data are representative of two experiments, mean ± SEM, n= 5 mice per group, *P < 0.05 compared to IL-33 group.

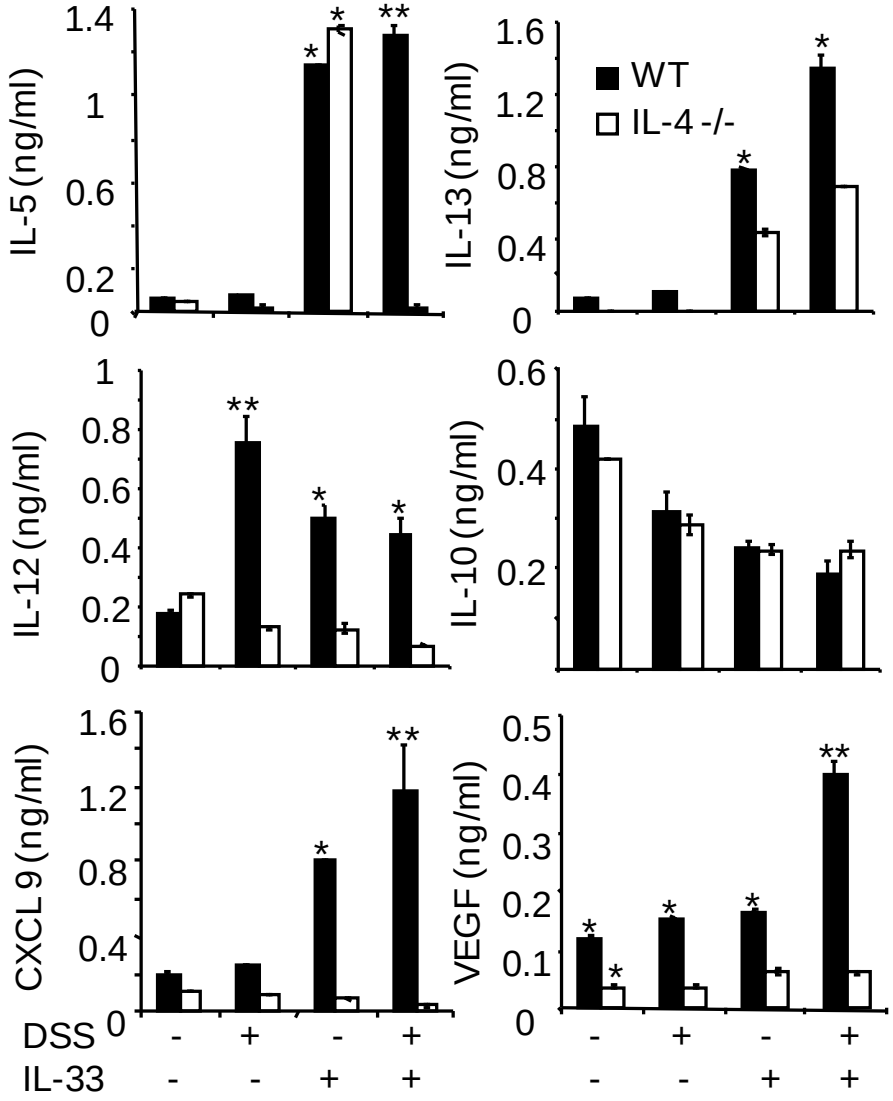



**Fig. 5-22 The levels of IL-5, IL-10, IL-12, IL-13, CXCL9, CXCL10 and VEGF in serum from WT and IL-4$^{-/-}$ mice.**

Serum samples from groups of female WT and IL-4$^{-/-}$ BALB/c mice given or not DSS were harvested on day 20. The IL-5, IL-10, IL-12, IL-13, CXCL9, CXCL10 and VEFG concentrations in serum samples were measured using Luminex as described in chapter 2. Data are representative of two experiments, mean ± SEM, n= 5 mice per group, *P < 0.05 compared to IL-33 alone group.

## 5.6 Chapter Discussion

The main findings from the work described in this chapter are:

i) IL-33 production was induced in colon of mice with DSS-induced colitis
ii) DSS-induced colitis is ameliorated in ST2 deficient mice
iii) IL-33 exacerbated DSS-induced colitis in mice
iv) The effect of IL-33 colitis exacerbation is IL-4 dependent
v) IL-33 also induces angiogenic and inflammatory cytokines in DSS colitis.

Our results showed a pro-inflammatory role for IL-33/ST2 axis played in DSS-induced colitis. This formed the basis of this manuscript (Pushparaj et al., 2013). Briefly we demonstrated that IL-33 is one of the first response genes in acute colitis and IL-33 plays a pathological role in colitis. Increasing evidence suggests that intestinal epithelial barrier dysfunction and unusual angiogenesis contribute to the development of UC, which is also reported to be lymph node independent (Podolsky, 2002, Papadakis and Targan, 2000, Sanchez-Munoz et al., 2008, Bamias et al., 2012, Dieleman et al., 1994). Our results contribute to these concepts and suggest that colon-derived IL-33 may be an important key factor in the initial stage of colitis (Pushparaj et al., 2013).

It is reported that IL-33 is expressed in the early stage of colitis (Kobori et al., 2010, Sedhom et al., 2013). We recently systematically studied the early colon gene expression profile of DSS-induced colitis by analysing the publicly available microarray datasets deposited in the Gene Expression Omnibus (GEO) using a meta analysis method and found that IL-33 is one of the early response genes after epithelium damage in DSS-induced colitis (Pushparaj et al., 2013). Given the direct effect of IL-33 on a wide range of innate immune cells, it may serve as an 'alarmin' molecule which is released from damaged cells in response to infection or inflammation, and thereby triggers an inflammatory response in the intestine (Moussion et al., 2008, Pastorelli et al., 2010, Guabiraba et al., 2014). IL-33 is also capable of inducing other important pathogenic cytokines (IL-4, IL5,

IL-6, IL-13, IL-17, IFN-γ, TNF-α, VEGF) and chemokines (CXCL9, CXCL10) (Guabiraba et al., 2014) while reducing immune suppressive cytokine IL-10 (Fig. 5-18 and 5-19).

It is well documented that Th2 type cytokines, in particular IL-4, play a pathological role in the development of UC (Papadakis and Targan, 2000, Stevceva et al., 2001, Sanchez-Munoz et al., 2008). But how type II cytokines are induced and are involved in the initial stage of colitis is still largely unknown. Our results demonstrated that IL-33 is one of the important IL-4 inducers in colitis and that IL-4 plays an essential role in exacerbation of IL-33-mediated colitis.

How IL-4 is involved in the early stage of pathogenesis of colitis is poorly understood (Papadakis and Targan, 2000, Kmiec, 1998, Iijima et al., 1999, Mizoguchi et al., 1999, Akbari et al., 2003). Our results show for the first time that IL-4 is required for the amplification of IL-33 in DSS-induced colitis and for the induction of VEGF and CXCL9 production. VEGF is a major pro-angiogenesis cytokine which plays a crucial role in the development of colitis. It enhances colon epithelial permeability and sequenced local migration of inflammatory cells (Scaldaferri et al., 2009, Dorward et al., 2007). CXCL9 is an important inflammatory chemokine for the recruitment of monocytes and macrophages (Tokuyama et al., 2005). These cytokines and chemokines are intimately associated with the pathogenesis of colitis (Kmiec, 1998, Baumgart and Carding, 2007) and together may provide a possible mechanism underlying the IL-33/IL-4 pathogenic pathway in colitis.

Th1 and Th17 cytokines including IL-12, IL-17 are also reported to be key cytokines in the development of colitis, especially in the chronic stage of DSS-induced colitis (Obermeier et al., 1999, Papadakis and Targan, 2000, Sanchez-Munoz et al., 2008). Our results showed that IL-33 increased the level of these cytokines in serum at a later stage (20 days after DSS administration) as shown in

Fig. 5-13 and 5-14. These suggested that in addition to the involvement in the initial stage, IL-33 may also contribute to the chronic stage of colitis.

It is unclear why the changes in severity of colitis caused by administration of exogenous IL-33 or ST2 deficiency were not significantly associated with a change in body weight in the mice (Fig. 5-16). However, this is consistent with a report showing unchanged body weight in WT C57BL/6 and IL-33$^{-/-}$ mice when fed with DSS (Oboki et al., 2010). In this report, intriguingly, in contrast to WT mice, the IL-33$^{-/-}$ mice had a delayed recovery in body weight after withdrawal of DSS, suggesting that IL-33 may have a beneficial effect on the recovery of colitis. However, this effect was not observed in ST2$^{-/-}$ mice in our study and the reasons are currently unknown. It could be because of the different genetic backgrounds of mice and the experimental conditions that have been used. Furthermore, since full-length (fl) IL-33 has ST2-independent bioactivity as reported previously (Lefrancais and Cayrol, 2012, Luzina et al., 2012), the different effect in IL-33$^{-/-}$ and ST2$^{-/-}$ mice may be due to the activity of flIL-33. More work is needed to clarify the issue.

The DSS-induced colitis mouse model is well established and widely used for the understanding of human ulcerative colitis (UC). It provides several advantages over other models of ulcerative colitis, such as the atraumatic method of induction, the consistency of colitis disease severity and replication of several key pathogenic features of human disease (Strober et al., 2002, Jurjus et al., 2004, Kawada et al., 2007). However, there are certain limitations of this animal model, mainly because the aetiology of human ulcerative colitis is still unresolved, the DSS model may only mimic the colitis caused by certain environmental factors such as infections and/or exposure to toxic chemicals (Rachmilewitz et al., 2002, O'Hara et al., 2012). Furthermore, there is an emerging literature showing that the intestinal bacterial flora plays a determinant role in human UC and the effects of DSS (Knights et al., 2013, Vermeire et al., 2011). However, the gut microbiota in human is likely to be different from that in animals (Perse and Cerar, 2012, Yin et al., 2013), which makes it's difficult to draw conclusions generated from animal experiments to

human studies in this context. While IL-33 and ST2 genes are expressed in colitis patients, the importance of the IL-33 pathway in clinical colitis is still unknown (Baumgart and Carding, 2007, Liew et al., 2010, Pastorelli et al., 2013, Liew, 2012, Lefrancais and Cayrol, 2012). Results from our animal studies suggest that IL-33 highlights a novel pathogenic pathway in the development and exacerbation of colitis.

# Chapter 6

# General Discussion

# Chapter 6: General Discussion

The results from this thesis demonstrated that IL-33 played a pathological role in two inflammatory diseases which involve the mucosal immune system, at the earlier phase of disease development (ulcerative colitis), and at the later phase of disease remodelling (pulmonary fibrosis), by inducing respectively IL-4 (Chapter 5) and IL-13 and TGF-ß1 (Chapters 3 and 4). In this chapter, I will discuss: i. the role of IL-33 in tissue fibrosis and in inflammatory bowel disease; ii. the function of IL-33 in inflammatory diseases either as a cytokine or as a nuclear factor; and iii. the therapeutic potential of targeting IL-33 in disease.

## 6.1 Is IL-33 a key cytokine in tissue fibrosis?

My results from Chapters 3 and 4 suggest that IL-33 is an important pro-fibrotic cytokine in lung fibrosis. Since several cytokines, including IL-13 and TGF-ß1, are known to contribute to tissue fibrosis (Wynn, 2011) it would be important to identify the relative contribution of IL-33 in the pathogenesis of fibrosis compared to these other cytokines. Furthermore, it is also important to know whether IL-33 is specific for lung fibrosis or if it is involved in other organ fibrosis as described in a search of the literature.

### 6.1.1 Current evidence of IL-33 in fibrosis in different organs in animal models and in humans

Growing evidence suggests that IL-33 is closely associated with fibrotic diseases and tissue remodelling across a variety of organs, including respiratory, alimentary, skin and cardiovascular systems. However, the functions of IL-33 in organ fibrosis can vary depending on the specific organ and the stage of disease, where IL-33 may have either pro-fibrotic or anti-fibrotic activity (Sattler et al., 2013, Pei et al., 2014) depending on specific context.

In the digestive systems, IL-33 is considered as a pro-fibrotic cytokine. In the liver, its expression in hepatic stellate cells and concentration in serum were both increased in patients with hepatic fibrosis (Marvie et al., 2010, Cacopardo et al., 2012) suggesting that IL-33 has a pro-fibrotic role in liver fibrosis. In recent mechanistic studies, it was reported that hepatic expression of IL-33 was both necessary and sufficient for severe hepatic fibrosis in mice using the carbon tetrachloride ($CCL_4$)-induced liver fibrosis model (McHedlidze et al., 2013). IL-33 is also involved in pancreatic fibrosis via regulating proliferation of human pancreatic stellate cells (Masamune et al., 2010).

The IL-33/ST2 nexus could also contribute to systemic sclerosis (SSc). SSc is a multisystem autoimmune disease which is characterized by abnormal growth of connective tissue. IL-33 serum levels were increased in SSc patients and correlated with the extent of skin sclerosis and with the severity of pulmonary fibrosis determined by high-resolution computed tomography. Therefore, IL-33 possibly plays a pro-fibrotic role in cutaneous and pulmonary fibrosis in SSc patients (Yanaba et al., 2011). In animal models of SSc, administration of IL-33 by subcutaneous (s.c.) injection resulted in ST2-dependent accumulation of eosinophils and mononuclear cells, and the development of cutaneous fibrosis. In parallel, the IL-33 also resulted in a significant increase in the number of extracellular matrix-associated genes, including collagen III, collagen IV, and tissue inhibitor of metalloproteases (TIMP)-1 indicating the pro-fibrotic role of IL-33 in skin fibrosis (Rankin et al., 2010).

In patients with other fibrotic conditions e.g. uterine fibroids and endometriosis, serum IL-33 concentrations were higher than normal suggesting that IL-33 may also be related to these disorders (Santulli et al., 2012, Santulli et al., 2013).

IL-33 is reported to be involved in the pathology of remodelling in several clinical and experimental respiratory diseases. Patients with cystic fibrosis

(Roussel et al., 2013), and with idiopathic pulmonary fibrosis (Luzina et al., 2013) had increased expression of IL-33 in their upper airway epithelium. Activating the IL-33/ST2 pathway by stimulating with pro-inflammatory and Th2-type cytokines enhanced lung fibrosis and remodelling in bleomycin-induced lung fibrosis mouse model by increasing the Th2 response and the TGF-ß1 levels, resulting in increased deposition of extracellular matrix (ECM) proteins (Tajima et al., 2007). Furthermore, recombinant adenovirus-mediated *il33* gene delivery exacerbated bleomycin-induced lung fibrosis (Luzina et al., 2013). In human lung tissue, IL-33 expression was enhanced in patients with idiopathic pulmonary fibrosis and in scleroderma lung disease (Luzina et al., 2013). Most of the observed IL-33 expression was intracellular and intranuclear, suggesting involvement of the full-length (fl) protein. In the current thesis we described the potential role for the mature cytokines form of IL-33 and I will discuss the functions of flIL-33 in detail in section 6.3. Together, these observations suggested that IL-33 is a pro-fibrotic factor in lung fibrosis.

In contrast to the previous reports that show a pro-fibrotic role for IL-33 in the lung and skin, the IL-33/ST2 system may protect against fibrosis in the cardiovascular system. IL-33 and ST2 was considered to be beneficial in cardiac hypertrophy. This was first observed by Sanada *et al*. in 2007 using a heart failure model of transverse aortic constriction (TAC)-induced cardiac hypertrophy in mice. They found that recombinant IL-33 treatment reduced hypertrophy and fibrosis and improved survival after TAC (Sanada et al., 2007). In high fat diet (HFD)-induced hypertrophy and fibrosis models, IL-33 also showed protective effects (Willems et al., 2012, Martinez-Martinez et al., 2013). The exact mechanisms by which IL-33 had a protective role are still largely unknown.

In patients with acute myocardial infarctions, the serum concentrations of soluble ST2 (sST2) were higher than normal, which suggests that sST2 could be a potential biomarker to predict future heart failure (Januzzi, 2013). However, the mechanism of increased production of sST2 in this context is still largely unclear; but its potential function is to bind IL-33 and prevent its subsequent

activity. Recently, *in vitro* experiments showed that primary cultured vascular smooth muscle cells (VSMCs) stimulated with sST2 demonstrated an increase in collagen type I and fibronectin production. This study suggested a pathogenic role for sST2 in vascular remodelling associated with vascular hypertrophy and fibrosis potentially by blocking a putative protective effect of IL-33 in cardiovascular systems (Martinez-Martinez et al., 2013).

This potential for IL-33 to modulate fibrosis in different tissues suggests that this diversity will be useful to improve our understanding of the function of IL-33 in future studies comparing and contrasting mechanisms. However, this may provide a potential barrier to therapy if targeting IL-33 to improve fibrosis in one tissue increases the risk in another.

### 6.1.2 Does IL-33 mediate fibrosis in different organs via the same mechanism?

While accumulated evidence suggests that IL-33 is critically involved in the fibrotic process, the detailed mechanisms underlying this effect are still not fully studied. Current evidence suggests that IL-33 may be associated with the fibrotic process via different mechanisms in different organs.

In the case of liver fibrosis, IL-33 exacerbates $CCL_4$-induced liver fibrosis and this is related to the activation and expansion of liver resident innate lymphoid cells (ILC2). ILC2-derived IL-13 then signals through the type-II IL-4 receptor to induce the transcription factor STAT6 and hepatic stellate-cell activation. Thus IL-13 is a critical downstream cytokine of IL-33-dependent pathologic tissue remodelling and fibrosis (McHedlidze et al., 2013).

In skin fibrosis, IL-33 mediates fibrogenesis through its ability to recruit eosinophils and enhance Th2 cytokine (IL-13) production. In this model the IL-33-

mediated fibrosis is attenuated in IL-13 knockout and eosinophil-deficient (ΔdblGATA) mice, suggesting again that in this case eosinophil-derived IL-13 rather than ILC2 cells may promote IL-33-induced cutaneous fibrosis (Rankin et al., 2010).

For BLM-induced lung fibrosis in our study, the mature cytokine form of IL-33 exacerbated fibrosis by attracting and activating ILC2s which then produced IL-13, and also polarized M2 macrophages which in turn produced IL-13 and TGF-β1 which contributed to fibrosis. However, in the model of virus-delivered full-length (fl) IL-33 reported by Luzina *et al*, the mechanism underlying flIL-33 enhanced lung fibrosis is unclear. Intriguingly, it was shown that the effect of flIL-33 was independent of ST2, but instead was associated with increased production of heat shock protein (HSP70) which may contribute to the fibrosis (Luzina et al., 2013). These findings suggest that both mature and flIL-33 are pro-fibrotic factors. They appear to drive lung fibrosis via distinct mechanisms, and a more complete understanding of these potentially parallel pathways will be informative of the fibrotic process.

In cardiovascular diseases, IL-33/ST2 signals may have a beneficial role by antagonizing angiotensin II in phenylephrine-induced cardiomyocyte hypertrophy. NF-κB activity plays a critical role in inflammation and cardiomyocyte hypertrophy and it was suggested that the protective function of IL-33 may be the inhibiting of angiotensin II and phenylephrine-induced phosphorylation of IκBα, an inhibitor of NF-κBα, thereby reducing NF-κB nuclear binding activity. Furthermore, sST2 was shown to block the anti-hypertrophic effects of IL-33, indicating that sST2 functions in the myocardium as a soluble decoy receptor for IL-33, blocking its protective activity (Sanada et al., 2007, Martinez-Martinez et al., 2013).

### 6.1.3 The importance of IL-33 compared to other cytokines in fibrosis

Many fibrotic disorders result from chronic infection and/or inflammation. In sterile inflammation, as discussed in chapter 1, damage-induced cell necrosis results in the release of cytokine IL-33 and other mediators e.g. HMGB1 which give a danger signal for triggering sterile inflammation. It has been shown that in both infection and sterile inflammation, IL-33 can be induced, released and is sufficient to trigger an inflammatory response (Fig. 1-2) (Lukens et al., 2012, Sattler et al., 2013). The ability of IL-33 to prolong acute inflammation into chronic inflammation, and finally to the activation of myofibroblasts demonstrates its importance in the temporal profile of inflammation (Meneghin and Hogaboam, 2007, Wynn and Ramalingam, 2012).

My results show that while it exacerbated bleomycin-induced lung fibrosis, one administration of exogenous IL-33 itself was not sufficient to induce pulmonary fibrosis by day 14 (Fig. 3-18 and 3-19). By collaborating with Professor Zhang in Beijing China, we further investigated the importance of IL-33 on its own in fibrosis. We found that *il33* transgenic (Tg) mice constitutively express low level of mIL-33 (about 80 pg/ml in serum) and spontaneously developed interstitial pulmonary fibrosis by age of 6 months (unpublished results). Thus, mIL-33 at this concentration is sufficient to induce fibrosis and is potentially a key profibrotic factor.

It is widely accepted that profibrogenic cytokines TGF-ß1 and IL-13 are necessary for the development of tissue fibrosis. However, how these cytokines are induced in BLM-induced fibrosis is unresolved. We demonstrated in section 4.5 that IL-33 signalling via ST2 is essential for the optimal induction of both IL-13 and TGF-ß1 expression in BLM-induced lung fibrosis, but in different cells and with different kinetics. IL-33 induces the early production of IL-13 by ILC2 (day 1 to day 3), and the production of TGF-ß1 by macrophages (day 5 onwards) during

the fibrosis process. These findings suggest that mIL-33 is a novel TGF-ß1 inducer, which may further explain its fibrogenic role in tissue fibrosis (Fig. 4-15).

Fibroblasts express receptors for IL-13 and TGF-ß1, and both cytokines have been shown to stimulate fibroblast proliferation and collagen production (Wynn, 2008, Shepherd, 2006). Interestingly, fibroblasts also express ST2 and synthesise and secrete IL-33 (Yanagisawa et al., 1993, Yagami et al., 2010). However, whether the IL-33/ST2 pathway plays a direct role in fibroblast function *in vitro* and *in vivo* is as yet unexplored. To address this issue, I generated primary fibroblasts from explant murine lung tissue and stimulated the cells with IL-33, but did not observe any significant effect of IL-33 on either their proliferation, collagen or cytokine production *in vitro* (data not shown). However, I did observe enhanced proliferation and increased IL-33 production of lung fibroblasts cultured from explanted lung tissue after bleomycin instillation (data not shown). Whether IL-33 is able to directly activate fibroblasts *in vivo,* perhaps together with other factors, needs be confirmed in future studies.

### 6.1.4 IL-33 in tissue fibrosis: conclusions and perspectives

Current evidence suggests that IL-33 is an important factor in the pathogenesis of several fibrotic diseases, and measurement of either IL-33 or sST2 may emerge as a useful biomarker for the diagnosis or prognosis of fibrotic diseases.

In addition, the IL-33/ST2 pathway may be a therapeutic target for a range of fibrotic disorders. Blockade of the IL-33/ST2 pathway by exogenous sST2 may have beneficial effects against progressive liver, pancreas, skin and lung fibrosis.

Compared to animal studies, the role of the IL-33 system in human fibrotic disease and its mechanism are less well understood. Therefore, more detailed

translational research is recommended to confirm the potential profibrotic effect of IL-33 in human disease.

## 6.2 The controversial role of IL-33 in inflammatory bowel disease

The aetiology of inflammatory bowel disease (IBD), such as Crohn's disease (CD) and ulcerative colitis (UC), is unclear. Recent evidence suggests that the IL-33 system may have beneficial or detrimental roles in these disorders (Sponheim et al., 2010, Palmer and Gabay, 2011, Grobeta et al., 2012). The apparently contradictory protective or pro-inflammatory roles of IL-33 in these intestinal disorders suggest that IL-33 may be pivotal in the development and treatment of chronic intestinal diseases. Therefore, understanding the basis of the different functions of IL-33 will be clinically important.

### 6.2.1 The role of IL-33 in colitis in patients and in animal models

The literature provides inconsistent evidence for the role of IL-33 in colitis. Growing evidence suggests that the gene expression of IL-33 and its receptor ST2 are both up-regulated in endoscopic biopsy tissue from IBD patients. In 2010, four research groups independently reported that the expression of IL-33 is up-regulated in active gut tissue lesions from patients with IBD (Beltran et al., 2010, Kobori et al., 2010, Pastorelli et al., 2010, Seidelin et al., 2010), and this expression was reduced after anti-inflammatory, including anti-TNF, treatment (Pastorelli et al., 2010). Furthermore, the expression of IL-33 and ST2 was up-regulated in mucosal colonic biopsies from patients with Crohn's disease (CD) and ulcerative colitis (UC) (Latiano et al., 2013), and the serum concentration of sST2 was also increased in these patients (Beltran et al., 2010, Pastorelli et al., 2010).

This evidence suggests that IL-33 has a pathogenic role in IBD, and this is supported in several animal colitis models (Garcia-Miguel et al., 2013). In our study, using the DSS-induced colitis model in susceptible BALB/c mice, we found that IL-33 can exacerbate experimental UC (Fig. 5-13). The importance of IL-33 in colitis in our study is supported by clinical findings that IL-33 is one of the early response genes found to be up-regulated in gut tissue at the onset of colitis

(Beltran et al., 2010), where its function was suggested to be as an epithelial 'alarmin', similar to HMGB1 and IL-1, and to promote an innate immune response to a range of toxic and infectious agents as discussed in section 5.6. While it is recognised that the type II cytokines, IL-4, IL-5 and IL-13 play a pathogenic role in the development of UC (Fuss et al., 1996, Mizoguchi et al., 1999, Stevceva et al., 2001), until now it was unknown how these typical Th2 cytokines were induced in colitis, and whether these cytokines contributed to the IL-33-mediated effects. Our mechanistic studies suggest that IL-33 can induce type II cytokines, especially IL-4, in order to exacerbate DSS-induced ulcerative colitis. Our results using IL-4 gene deficient mice show for the first time that IL-4 is required for IL-33-mediated exacerbation of colitis, and for the subsequent production of cytokines VEGF, CXCL9 and CXLC10 (Fig 5-21 and 5-22). VEGF is a major pro-angiogenic cytokine and plays an important role in the pathogenesis of colitis by enhancing colonic permeability and facilitating migration of inflammatory cells (Scaldaferri et al., 2009). CXCL9 and CXCL10 are the key chemokines for the recruitment of monocytes and macrophages, and these cells are intimately associated with the pathogenesis of colitis (Tokuyama et al., 2005, Thomas and Baumgart, 2012).

A pathogenic role for IL-33/ST2 in IBD has also been identified by other research groups. Using senescence-prone (SAM) P1/YitFc mice that develop a mixed Th1/Th2 model of IBD, it was shown that IL-33 expression is upregulated in full-thickness ilea that correlated with the disease severity, and that the absence of a functional IL-33/ST2 pathway, as shown in *St2*$^{-/-}$ mice, reduced disease severity (Pastorelli et al., 2010, Pizarro et al., 2011, Sedhom et al., 2013). There are reports showing that IL-33 promoted early-stage disease development of IBD (Imaeda et al., 2011, Grobeta et al., 2012). This finding was validated by demonstrating reduced DSS-induced colitis in IL-33 knockout mice (Oboki et al., 2010).

To further support the hypothesis that IL-33/ST2 has a pathogenic role in human IBD, it was reported recently that common IL-33 and ST2 polymorphisms

contributed to the risk of IBD in an Italian cohort of patients (Latiano et al., 2013).

On the other hand, different studies suggest that IL-33 could have a protective role in IBD (Grobeta et al., 2012, Sedhom et al., 2013). For example, IL-33 treatment increased epithelial barrier permeability *in vivo*, whereas in mice genetically deficient for ST2 the wound healing response following acute mechanical injury in the colon was enhanced (Sedhom et al., 2013). In the established chronic stage of DSS-colitis, the administration of exogenous IL-33 attenuated the pathogenesis of disease as assessed by colon length and body weight loss (Grobeta et al., 2012). Using the same DSS-colitis model, Oboki et al also found that while IL-33 attenuated the development of acute colitis, IL-33 deficient mice of a C57BL/6 background had a delayed return to normal body weight in the recovery stage compared to wild type mice, suggesting that IL-33 may also be involved in the recovery phase after tissue damage (Oboki et al., 2010). Furthermore, recent evidence suggests that injection of IL-33 may have a beneficial effect on trinitrobenzene sulfonic acid (TNBS)–induced colitis, a model of Crohn's disease (CD) in mice, suggesting that IL-33 may play a complex role in different types of IBD and at different times throughout the duration of disease (Duan et al., 2012).

### 6.2.2 What is the reason for the inconsistent findings describing the effects of IL-33 in colitis?

While the reason behind the inconsistencies in the findings that show that IL-33 seems to have both pathogenic and protective functions is still unknown, this may be due to several reasons; the different isoforms of IL-33, the differences in disease models, the genetic background of the mice, colonies of microorganisms and the experimental conditions (Knights et al., 2013).

**A) The controversial effect of IL-33 in different disease models of IBD may be due to the difference in immune-pathogenic mechanisms.**

Current evidence suggests that IL-33 primarily elicits a type II immune response, by enhancing IL-4, IL-13, and IgE production, and eosinophil and mast cell activities. IL-33 does so by directly activating a wide range of innate immune cells, including mast cells, eosinophils, macrophages and NK/NKT cells via their cell membrane ST2. IL-33 could also promote Th2-type adaptive immune responses by directly activating Th2 lymphocytes, but not Th1 or Th17 cells because they do not express ST2 (Xu et al., 1998). The IL-33/ST2 pathway therefore has a dominant role in stimulating type II immune responses, including their functional physiological consequences in allergy, asthma and tissue repair. Since Th2 cytokines are able to counter-regulate the Th1 response, these Th2 cytokines are considered important in the control of Th1-mediated inflammatory diseases (Pastorelli et al., 2011). In human and animal models, CD is generally considered to be mainly a Th1-associated disease, whereas UC is considered to be Th2-mediated. The evidence for this was indicated by purified lamina propria lymphocytes from IBD patients expressing increased level of IFN-γ not IL-4 from patients with CD, and high level of IL-4 and IL-13 but low level of IFN-γ from UC patients (Fuss et al., 1996, Fuss et al., 2004). These findings may help to explain the different role of IL-33 in Th2 (DSS-induced) and Th1 (TNBS-induced) mediated colitis. In the models considered to be Th1 dominated, the IL-33 has a protective role based on its ability to induce Th2 cytokines and thereby reduce Th1 cytokines; whereas in the Th2 dominated model, IL-33 may have an opposite role by increasing Th2 responses thereby decreasing the Th1 response (Grobeta et al., 2012, Pastorelli et al., 2013).

**B) The different roles of IL-33 in the acute and recovery stages of IBD.**

The studies using the colitis models in mice suggest that IL-33 may be involved in the recovery from gut damage after withdrawal of DSS (Oboki et al., 2010). While the mechanism is poorly understood, it is known that the IL-33 and Th2 response is critically involved in tissue repair and remodelling by promoting fibroblasts and epithelial cell proliferation and collagen production (Moussion et al., 2008). These authors reported that IL-33 plays a pathogenic role in intestinal

fibroblast function in senescence-accelerated SAMP1/Fc mice treated with anti-ST2 blocking antibodies, with decreased collagen deposition within the intestinal wall, together with a reduced production of pro-fibrotic molecules, such as TGF-ß1, connective tissue growth factor (CTGF), collagen-1, insulin growth factor (IGF)-1, and matrix metalloproteinase (MMP)-9 (Mattioli et al., 2011). These findings suggest that IL-33 plays a protective role in the recovery phase of IBD. We also demonstrated that IL-33 is a powerful factor in fibroblast functions such as collagen production in lung fibrosis (Chapters 3 and 4). Thus, it is likely that IL-33-mediated type II responses may contribute to the recovery and tissue repair of IBD.

### 6.2.3 Conclusion and perspectives about IL-33 function in inflammatory bowel disease

Inflammatory bowel disease (IBD) is a complicated condition with several clinical phenotypes and unknown aetiology. Animal studies have enhanced our understanding of some of the immune mechanisms of experimental IBD and suggest a role for IL-33 and ST2. While the clinical relevance of the IL-33/ST2 system in IBD is largely unknown, current evidence suggests that the IL-33/ST2 system functions differently in the UC and CD syndromes of IBD, and may represent an important factor for the development and exacerbation specifically of UC. Furthermore, IL-33 signalling may play a different role in CD and chronic UC and may not only be involved in the pathogenesis of IBD but may also contribute to the recovery of IBD. Therefore, more work is needed to understand the precise mechanism of IL-33 in IBD. Because it potentially has dual roles in homeostasis and pathogenesis, therapeutic targeting of the IL-33 pathway must be considered with caution especially with patients with cardiovascular risk factors.

## 6.3 Cytokine IL-33 compared with nuclear IL-33: how important is nuclear IL-33 for inflammatory disease?

The *Il33* gene was first identified in canine vasospastic cerebral artery cells and described as *Dvs 27* which encoded a then unknown nuclear protein (Onda et al., 1999). This protein was subsequently described as a nuclear factor in high endothelial venules and was named NF-HEV (Baekkevold et al., 2003). A smaller molecular isoform of the nuclear IL-33 was later identified as a member of the IL-1 cytokine family and named mature IL-33 (Schmitz et al., 2005, Liew et al., 2010). Current evidence shows that the function of nuclear IL-33 may differ from mature cytokine IL-33 which was considered as a Th2-like cytokine (Martin, 2013). Therefore, IL-33 may possess dual functions as a gene regulator in the nucleus and/or as a cytokine after release.

### 6.3.1 What do we know about the function of nuclear IL-33?

The function of full-length, nuclear (fl)IL-33 is unresolved. After expression, the flIL-33 is translocated immediately into the cell nucleus via the N-terminal of IL-33 which contains a chromatin-binding domain and a nuclear transfer domain. Thus, the flIL-33 is mainly found in the nucleus; mainly in barrier tissue cells including epithelial cells, endothelial cells, fibroblasts and smooth muscle cells. Current reports suggest that flIL-33 is released predominantly when cells sense infection, inflammation, stress or undergo necrosis (Kakkar et al., 2012, Martin, 2013). This suggests that nuclear IL-33 could be considered as a stored stress or damage 'alarmin', with a similar function to IL-1α and HMGB1 that initiate inflammatory responses when released.

Recent findings suggest that flIL-33 may not only be stored in the cell nucleus but may also be actively involved in gene regulation. It is reported that the N-terminal chromatin-binding domain of flIL-33 facilitates nuclear translocation of flIL-33 and the binding to histone H2A-H2B dimer at the surface of the nucleosome and affects chromatin compaction (Carriere et al., 2007, Roussel et

al., 2008). Unlike cytokine IL-33, the mature form of IL33, which can activate NF-κB via its receptor ST2 and MyD88, flIL-33 might down-regulate NF-κB pathway. It is reported that flIL-33 could bind to NF-κB by the interaction between the N-terminus (aa 66-109) of murine IL-33 and N-terminal Rel homology domain of NF-κB p65. This complex reduced NF-κB bioactivity by interfering with NF-κB DNA binding and p65-mediated trans-activation (Ali et al., 2011). Furthermore, over-expression of flIL-33 decreases the IL-1ß-stimulated expression of endogenous NF-κB target genes such as IκBα, TNF-α, and C-REL, suggesting that nuclear flIL-33 may serve as a gene repressor to sequester the pro-inflammatory response (Ali et al., 2011).

However, it is also reported that nuclear flIL-33 can also upregulate the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1) in TNF-α activated human umbilical vein endothelial cells (HUVECs) (Choi et al., 2012). The author further demonstrated that the mechanism by which flIL-33 enhances adhesion molecule expression was through binding to the p65 promoter (Choi et al., 2012). Thus, nuclear flIL-33 may also serve as a transcriptional activator in gene expression. However, the precise conditions for the different effects of flIL-33 on gene regulation particularly in fibrotic disease are still unknown.

In summary, flIL-33 could serve as a nuclear factor with transcriptional regulatory properties. It could decrease the IL-1ß-stimulated responses via binding to NF-κB and increase TNF-α via binding to NF-κB p65 promoter.

### 6.3.2 Does nuclear flIL-33 play a role in inflammatory diseases?

Whereas *in vitro* evidence suggests that nuclear flIL-33 may play both repressor and activator roles in gene regulation, its role in inflammatory diseases *in vivo* is still largely unknown. It is reported that flIL-33 and mIL-33 delivered into the lung by intratracheal instillation in an adenovirus vector resulted in different

effects in mice (Luzina et al., 2012). Both isoforms caused pulmonary infiltration of lymphocytes and neutrophils, whereas the mIL-33 form also caused pulmonary eosinophilia and goblet cell hyperplasia and increased the expression of IL-4, IL-5 and IL-13. Furthermore, the mIL-33-induced Th2-associated effects did not occur in ST2 deficient mice, but this did not affect the flIL-33-mediated response. Thus, this report provided the first *in vivo* evidence that flIL-33 is functionally active in an ST2-independent manner, and its effects partially differ from those of mature IL-33 (Luzina et al., 2012). Recently by using the same approach, this group also showed that over-expression of flIL-33 using an adenovirus vector exacerbated bleomycin-induced lung injury in mice, associated with increased pulmonary lymphocyte infiltration, collagen accumulation and lung fibrosis independent of ST2 (Luzina et al., 2013). These results indicate that flIL-33 which is located in the nucleus may be functional in this disease. However, these results were generated by using a complicated virus flIL-33 gene-delivery system. Therefore more definitive and physiological systems are needed to define the role of flIL-33 *in vivo*.

More recently, the function and importance of nuclear IL-33 was further studied *in vivo* using a mature IL-33 knock-in mouse model (Bessa et al., 2014). This strain of mice was generated by replacing the nuclear domain of flIL-33 with a dsRed fluorescent protein (Bessa et al., 2014). The mice therefore lacked the nuclear domain of flIL-33 and produced higher concentration of mature IL-33 in circulation. Importantly, these mice developed severe multi-organ inflammation in an ST2-dependent manner and died within 3 month of birth. This new finding suggests that the nuclear domain of IL-33 is important at least for the safe storage of IL-33 within the nucleus. However, whether this is the main function of nuclear IL-33 and whether it has further regulatory functions in gene regulation *in vivo* as suggested by the results *in vitro* is still unclear.

### 6.3.3 Nuclear IL-33: conclusion and perspectives

Although the flIL-33 isoform is located in the cell nucleus, the studies of IL-33 function are mainly focused on its role as a cytokine using the mature (m)IL-33 isoform (Klemenz et al., 1989, Tominaga, 1989, Schmitz et al., 2005). I believe this is mainly because the identification of the nuclear function of IL-33 is much more difficult to study because currently there is no definitive method available to identify nuclear IL-33 function *in vivo*. Therefore, it is important to establish a study system. I would suggest, for example, generating flIL-33 transgenic/ST2 deficient mice which would eliminate the potential confounding involvement of cytokine mIL-33. With this system, the physiological and pathological role of nuclear flIL-33 in different conditions could be studied in more detail.

## 6.4 Is the IL-33/ST2 system a likely therapeutic target in disease?

### 6.4.1 Targeting cytokines in inflammatory disease

Inflammatory diseases, in particular the chronic diseases, are still a serious health problem with major unmet clinical needs. The traditional treatment strategy for many inflammatory diseases was mainly based on the use of non-specific anti-inflammatory or cytotoxic drugs for example corticosteroids or methotrexate. With better understanding of disease immune-pathogenesis, it is clear that inflammatory diseases are largely controlled by complex communication networks of cytokines and these cytokines may represent novel therapeutic targets. While cytokines function within a network, the identification of key cytokines and their specific signalling pathways in disease may lead to successful therapy. Clinical evidence demonstrated that cytokines play a critical role in health and disease, and cytokines may therefore be key therapeutic agents or targets in many inflammatory, infectious disorders and cancer (Yoshimoto and Yoshimoto, 2014).

Developing these 'biological' reagents against chronic inflammatory diseases is an important goal for clinical treatment by pharmaceutical companies. The first successful therapy targeting a specific cytokine is anti-TNF therapy. It has become the most valuable drug class with global sales of around 27 billion US dollars in 2013 since its first clinical trial in 1992. TNF inhibition is approved for the treatment of a variety of inflammatory diseases including rheumatoid arthritis (RA), juvenile RA, psoriasis, psoriatic arthritis, CD, UC, and ankylosing spondylitis (Nanchahal et al., 2014). Besides the pro-inflammation property, TNF also has pro-fibrotic properties thus TNF blockade might apply to fibrotic disorders in the future (Harari and Caminati, 2010).

IL-6 is another important pro-inflammatory cytokine, and neutralization using anti-IL-6 antibody and anti-IL-6R antibody represents an excellent target for therapy in inflammatory diseases. It has been approved in some countries for the

treatment of RA; but some studies that used IL-6 inhibitor showed limited efficacy in other chronic diseases such as juvenile idiopathic arthritis (Jones et al., 2011, Schett et al., 2013). The drugs used for targeting other cytokines in inflammatory diseases, including IL-1, IL-12, IL-17A and IL-23 are also in clinical trials or in production pipelines with encouraging results. Inhibition of IL-1 was initially considered as a treatment for RA, CD and psoriasis and it also has positive effect on some genetic syndromes for example gout (Neogi, 2011, Dinarello and van der Meer, 2013). IL-12 and IL-23 combined, or IL-17A inhibition might be beneficial for RA and psoriasis, but it could worsen symptoms of CD (Nickoloff, 2007, Sandborn et al., 2012, Baeten et al., 2013, McInnes et al., 2013).

### 6.4.2 The beneficial vs. detrimental effects of IL-33 in immunity and in disease

Mature IL-33 is a pleiotropic cytokine in immunity and disease. Therefore it can induce either beneficial or detrimental effects depending on disease conditions, dose/time and *in vivo* cytokine milieu.

***Allergic diseases*:** since the main properties of IL-33 appear to be related to the induction of Th2 immune responses, then IL-33 may be an important therapeutic target in these Th2-mediated diseases; including tissue fibrosis, allergy and asthma.

Asthma is an increasingly common disease worldwide, with complex phenotypes and unresolved aetiology. Severe asthma is of considerable clinical and economic importance because of its high morbidity, in part due to a poor therapeutic response to current treatments. Recent findings suggest that IL-33 is one of the earliest cytokines released by airway epithelial cells after contact with allergen or with bacterial products e.g. LPS (Smith, 2010, Liew, 2012). Research results from the author's laboratory have shown that IL-33 instillation into the airways

of mice is sufficient to induce airway inflammation, IgE production and bronchial hyper-reactivity (BHR) (Kurowska-Stolarska et al., 2009). This is due to IL-33 which can directly activate an array of key innate cells in asthma pathogenesis, including basophils, neutrophils, macrophages, dendritic cells, mast cells and Th2 cells (Kurowska-Stolarska et al., 2011).

The clinical relevance of the IL-33/ST2 system in human asthma remains unresolved. Recent reports demonstrated that *IL33* and *ST2* (*IL1RL1*) single nucleotide polymorphisms (SNPs) were major factors associated with both atopic (IgE antibody and eosinophilia) and non-atopic asthma (Reijmerink et al., 2008, Gudbjartsson et al., 2009). Thus, the IL-33/ST2 system is associated with clinical asthma and may be a determining factor for asthma susceptibility. Therefore, the IL-33/ST2 system should be a key therapeutic target in allergy/asthma.

***Inflammatory disease*:** current evidence has demonstrated that IL-33 expression is closely associated with the development of several inflammatory autoimmune diseases, including IBD, systemic lupus erythematosus, multiple neurodegenerative diseases and rheumatoid arthritis (RA) (Martin, 2013, Pastorelli et al., 2013).

RA is a common condition of unknown aetiology characterised by chronic inflammatory cell infiltration into the synovium, leading to cartilage and bone destruction. The pro-inflammatory cytokine milieu in synovial fluid, including TNF-α, IL-6 and IL-1 family members, is thought to play an arthritogenic role in RA. Dr. Xu's lab has demonstrated that, while IL-33 mainly induces a type II response in allergy and asthma, IL-33 also contributes to the pathology of inflammatory joint diseases. IL-33 and its receptor ST2 are expressed in the RA synovial membrane and the dominant synovial source of IL-33 was the fibroblast (Xu et al., 2008). Moreover, administration of IL-33 promoted the development of inflammatory arthritis and bone erosion in DBA/1 mice, whereas ST2 deficiency or soluble ST2 (sST2) suppressed disease (Schmitz et al., 2005, Gadina and Jefferies, 2007, Carriere et al., 2007). The arthritogenic effect of IL-33 is

mainly due to its role in the activation of mast cells in the joints. These data strongly implicate IL-33 as an important mesenchymal derived cytokine that could drive pathogenic immune responses in the synovium.

***Cardiovascular diseases*:** consistent evidence suggests that higher than normal serum sST2 concentrations are closely associated with patients with heart failure or myocardial infarction. Thus sST2 is a potential biomarker for adverse cardiovascular disorders (Januzzi, 2013). Accumulating evidence also demonstrates that the IL-33/ST2 pathway is cardio-protective in mouse models of myocardial infarction, heart transplantation and cardiac hypertrophy and fibrosis. Furthermore, treatment with IL-33 reduced the development of plaques in atherosclerotic mice (Miller et al., 2008, Liew et al., 2010, Miller and Liew, 2011). Therefore, administration but not blockage of IL-33 may be beneficial for cardiovascular disorders.

***Infectious diseases:*** like most other pro-inflammatory cytokines, IL-33 is also involved in host defence against some pathogenic infections (Sattler et al., 2013). IL-33 plays a critical protective role against a range of parasite infections, including *Nippostrongylus brasiliensis*, *Trichuris muris* and *Toxoplasma gondii* (Table 1-1). This is mainly due to its role in the promotion of Th2 response and activating/recruiting immune cells, including eosinophils, macrophages and NKT cells which protect against parasites. However, its role in bacterial and viral infection differs and depends on the type of pathogens (Sattler et al., 2013). For example, IL-33 can protect against Gram-negative bacteria sepsis and *Pseudomonas aeruginosa* infection (Hazlett et al., 2010). However, the IL-33/ST2 system seems less important in protection against *Mycobacterium tuberculosis* infection. Available evidence indicates that IL-33 may also play different roles in viral infections: it protects against lymphocytic choriomeningitis virus (LCMV) (Bonilla et al., 2012) but promotes the infection of influenza (Le Goffic et al., 2011).

Altogether, the IL-33 system may be an important therapeutic target in allergic and inflammatory diseases. However, in cardiovascular and parasite infections, IL-33 may have a beneficial effect.

## 6.4.3 Potential clinical application of IL-33 and ST2

### *How to target the IL-33 system in allergic and inflammatory diseases?*

Research from our and other groups suggests that there are several potential approaches which may be feasible to block IL-33 bioactivity; by using neutralising anti-IL-33 antibodies, anti-ST2, exogenous sST2 protein or blocking the downstream IL-33/ST2 signalling pathways.

### *Anti-IL-33 antibodies*

Anti-IL-33 antibodies should block free IL-33 in blood, body fluids and in the tissues. As I discussed in section 3.4, anti-IL-33 antibody effectively reduced serum IL-33 levels and attenuated bleomycin-induced lung fibrosis. This suggests in principle that it is possible to use anti-IL-33 to block IL-33 function *in vivo*. However, the antibody I used was polyclonal IgG from rabbit. For clinical purpose, humanised monoclonal antibodies are needed and currently no such antibody against human IL-33 is available.

### *Anti-ST2 antibodies*

The IL-33 receptor complex consists of ST2 and IL-1RacP, in which cytokine IL-33 signals via binding to ST2. In addition, the IL-1RacP is also shared by IL-1 and IL-36; therefore, targeting ST2 is more sensible in order to specifically block IL-33 function.

### *Soluble ST2 (sST2)*

There is sST2 naturally occurring in blood which is induced during infection and inflammation. We and others have demonstrated that sST2 is a decoy receptor which binds to IL-33 and blocks IL-33 interaction with cell surface ST2. It has been shown that recombinant sST2-Fc fusion protein can block the activity of IL-33 *in vivo* in inflammatory arthritis (Leung et al., 2004) and intestinal mucositis (Guabiraba et al., 2014) in mice. A similar approach has been applied to develop soluble TNF-receptor-FC fusion protein to block TNF-α in inflammatory diseases (Taylor, 2010).

## *IL-33/ST2 signal pathways*

Targeting the downstream IL-33 signalling pathway is another option to block IL-33 function. IL-33/ST2 signals via the common IL-1/TLR signalling cascade, including MyD88, NF-Kb, IRAKs, p38, p65 *et al* (Liew et al., 2010, Miller, 2011). However, more work is needed to identify the specific signalling element in the IL-33 signalling pathway before selecting a specific signalling target.

However, since IL-33 has beneficial effects on the cardiovascular system and against parasite infections, targeting the IL-33 pathway must be done with caution. The potential therapeutic value and side-effects have to be carefully balanced in any given individual and disease condition.

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# Appendices

# IL-33 promotes ST2-dependent lung fibrosis by the induction of alternatively activated macrophages and innate lymphoid cells in mice

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**Background: The initiation and regulation of pulmonary fibrosis are not well understood. IL-33, an important cytokine for respiratory diseases, is overexpressed in the lungs of patients with idiopathic pulmonary fibrosis.**
**Objectives: We aimed to determine the effects and mechanism of IL-33 on the development and severity of pulmonary fibrosis in murine bleomycin-induced fibrosis.**
**Methods: Lung fibrosis was induced by bleomycin in wild-type or *Il33r* (*St2*)$^{-/-}$ C57BL/6 mice treated with the recombinant mature form of IL-33 or anti–IL-33 antibody or transferred with type 2 innate lymphoid cells (ILC2s). The development and severity of fibrosis was evaluated based on lung histology, collagen levels, and lavage cytology. Cytokine and chemokine levels were quantified by using quantitative PCR, ELISA, and cytometry.**
**Results: IL-33 is constitutively expressed in lung epithelial cells but is induced in macrophages by bleomycin. Bleomycin enhanced the production of the mature but reduced full-length form of IL-33 in lung tissue. ST2 deficiency, anti–IL-33 antibody treatment, or alveolar macrophage depletion attenuated and exogenous IL-33 or adoptive transfer of ILC2s enhanced bleomycin-induced lung inflammation and fibrosis. These pathologic changes were accompanied, respectively, by reduced or increased IL-33, IL-13, TGF-β1, and inflammatory chemokine production in the lung. Furthermore, IL-33 polarized M2 macrophages to produce IL-13 and TGF-β1 and induced the expansion of ILC2s to produce IL-13 *in vitro* and *in vivo*.**
**Conclusions: IL-33 is a novel profibrogenic cytokine that signals through ST2 to promote the initiation and progression of pulmonary fibrosis by recruiting and directing inflammatory cell function and enhancing profibrogenic cytokine production in an ST2- and macrophage-dependent manner. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)**

***Key words:*** *IL-33, lung fibrosis, alternatively activated macrophages, type 2 innate lymphoid cells*

Bleomycin is an important cancer chemotherapeutic agent. However, its cytotoxic activity associated with DNA strand cissation and reactive oxygen species induction can cause severe side effects, including pulmonary fibrosis. This can be recapitulated in experimental models designed to investigate the pathogenesis of pulmonary fibrosis and some aspects of idiopathic pulmonary fibrosis (IPF),[1-3] a devastating treatment-refractory interstitial lung disease of unknown origin.[4,5] A better understanding of the fibrotic process might lead to novel therapeutic approaches for this unmet clinical need. Bleomycin-induced fibrosis in susceptible C57BL/6 mice provides a reliable model to study the underlying mechanisms of fibrosis.[1]

Although the pathogenic mechanisms of bleomycin-induced fibrosis and IPF are not fully understood, both conditions are characterized by alveolar epithelial injury, accumulation of fibroblasts and myofibroblasts, and deposition of collagenous extracellular matrix in the lung, which together compromise functional gas exchange.[1,2,4,5] Lung histology and bronchoalveolar lavage (BAL) show inflammatory cytology, including neutrophils, lymphocytes, and macrophages, which are thought to contribute to fibrogenesis.[1,2,4,5] Macrophages can be polarized into 2 phenotypes: classically activated macrophages (M1 macrophages), which are activated by IFN-γ and LPS, or alternatively activated macrophages (M2 macrophages), which are activated by IL-4 and IL-13.[6,7] M1 macrophages express inducible nitric oxide synthase and proinflammatory cytokines and protect against infection, whereas M2 macrophages express arginase 1 and TGF-β1 and are critically involved in tissue repair and fibrosis.[6,7]

The profibrogenic cytokines TGF-β1 and IL-13 are essential for the development of lung fibrosis by promoting myofibroblast differentiation and stimulating production of extracellular matrix proteins, primarily collagen,[4,5] and thus are important potential therapeutic targets in fibrosis. Similar strategies can be applied to other mediators, including cytokines of the IL-1 family, among which IL-1 and IL-18 have a role in clinical and experimental

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*Abbreviations used*
APC: Allophycocyanin
*Arg1*: Arginase 1
BAL: Bronchoalveolar lavage
BMDM: Bone marrow–derived macrophage
CT: Computed tomography
FITC: Fluorescein isothiocyanate
flIL-33: Full-length IL-33
ICOS: Inducible costimulator
ILC2: Type 2 innate lymphoid cell
IPF: Idiopathic pulmonary fibrosis
M1 macrophage: Classically activated macrophage
M2 macrophage: Alternatively activated macrophage
mIL-33: Mature IL-33
*Nos2*: Inducible nitric oxide synthase 2 gene
PE: Phycoerythrin
qPCR: Quantitative PCR
WT: Wild-type

lung fibrosis.[8] IL-33 is a new member of the IL-1 family and is overexpressed in the lungs of patients with IPF.[9]

IL-33 is a dual-function cytokine: the full-length IL-33 (flIL-33) form serves as an intracellular gene regulator in the nucleus, and the mature IL-33 (mIL-33) form serves as an extracellular cytokine after release when cells sense inflammatory signals or undergo necrosis.[10-15] Once released, flIL-33 can be processed by neutrophil-derived proteases into mIL-33.[13] Although both flIL-33 and mIL-33 are able to bind to and signal through their receptor, ST2, mIL-33 has a 10-fold higher affinity and bioactivity than flIL-33.[13] ST2 is expressed on most innate cells, including macrophages and the newly identified type 2 innate lymphoid cells (ILC2s), and IL-33 plays a direct role in the function of these cells.[16-19] mIL-33 mainly elicits a type 2 immune response and is closely associated with allergic and parasitic diseases.[11,18-22] It has recently been reported that nuclear flIL-33 potentiates bleomycin-induced lung injury in an undefined but ST2-independent manner.[9] The expression of IL-33 mRNA is increased in IPF lung tissue[9]; however, the role of mIL-33 as a cytokine in the fibrotic process is unknown.

We have investigated the effect and mechanism of mIL-33 in the initiation and exacerbation of bleomycin-induced fibrosis in mice. We report here that mIL-33, through ST2, strongly enhances bleomycin-induced pulmonary fibrosis, mainly by promoting inflammatory cell infiltration and function, including polarization of M2 macrophages and ILC2s, and enhancing their IL-13 and TGF-β1 production.

## METHODS

Experimental details are provided in the Methods section in this article's Online Repository at www.jacionline.org.

## RESULTS

### Bleomycin-induced fibrosis is impaired in *St2*$^{-/-}$ mice

Groups of wild-type (WT) and *St2*$^{-/-}$ C57BL/6 mice were given bleomycin or PBS intranasally on day 0. The mice were killed on day 7 or 14 to investigate the role of the cytokine IL-33 in the development of bleomycin-induced fibrosis. WT mice that received bleomycin had progressive lung inflammation (Fig 1, *A*) and fibrosis (Fig 1, *B*) from day 7 compared with PBS control mice. This bleomycin-induced inflammatory and fibrotic response was demonstrated by enhanced inflammatory cell infiltration and collagen deposition in the lung and quantified by using histologic inflammatory and fibrosis scores (Fig 1, *C*). The pathologic changes observed in WT mice given bleomycin were significantly reduced in *St2*$^{-/-}$ mice given bleomycin (Fig 1, *A-C*).

Compared with bleomycin-treated WT mice, bleomycin-treated *St2*$^{-/-}$ mice also had significantly reduced infiltration of neutrophils on day 7 and total leukocytes, including macrophages, neutrophils, and lymphocytes, on day 14 in BAL fluid (Fig 1, *D*). Furthermore, bleomycin-enhanced concentrations of soluble collagen and the expression of collagen 3, which is associated with early-repair fibrosis, were reduced in *St2*$^{-/-}$ compared with WT mice (Fig 1, *E* and *F*), whereas the expression of collagen 1 remained unchanged (data not shown). Moreover, bleomycin-treated *St2*$^{-/-}$ mice have reduced concentrations of IL-33, IL-1, and chemokines (CXCL1, CXCL2 and CCL2) in lung tissue extracts compared with concentrations seen in bleomycin-treated WT mice (see Fig E1, *A*, in this article's Online Repository at www.jacionline.org).

### Neutralizing anti–IL-33 antibody attenuates bleomycin-induced fibrosis

We next assessed the role of endogenous IL-33 in the development of bleomycin-induced fibrosis by treating WT mice with anti–IL-33 antibody. C57BL/6 mice were injected intraperitoneally with anti–IL-33 every fifth day from day 0 of bleomycin administration and killed on day 14. Anti–IL-33 antibody reduced IL-33 and IL-1 levels in the lung tissue of bleomycin-treated mice compared with that seen in control IgG-treated mice (see Fig E1, *B*). The antibody treatment also markedly reduced bleomycin-induced airway inflammation and lung fibrosis (Fig 2, *A-C*) and the number of macrophages, neutrophils, and lymphocytes in BAL fluid on day 14 compared with IgG control values (Fig 2, *D*). Furthermore, the antibody treatment significantly reduced lung tissue soluble collagen (Fig 2, *E*) and collagen 3 mRNA expression (Fig 2, *F*).

### Recombinant mIL-33 exacerbates bleomycin-induced fibrosis in mice

Mice were administered intranasal mIL-33 together with bleomycin on day 0 and lung tissues were analyzed on day 7 to directly assess the role of the cytokine IL-33. Control mice were given either PBS, mIL-33, or bleomycin alone. One administration of exogenous mIL-33 significantly enhanced bleomycin-induced lung inflammation (Fig 3, *A*), collagen deposition (Fig 3, *B*), and pathology score (Fig 3, *C*), compared with controls. The IL-33–enhanced histologic changes were accompanied by significantly increased total numbers of cells in BAL fluid, mainly neutrophils and lymphocytes, compared with control values (Fig 3, *D*). The coadministration of IL-33 did not change the macrophage numbers in BAL fluid at this time point (7 days) compared with bleomycin alone. IL-33 further increased the levels of bleomycin-induced collagen production (Fig 3, *E*)

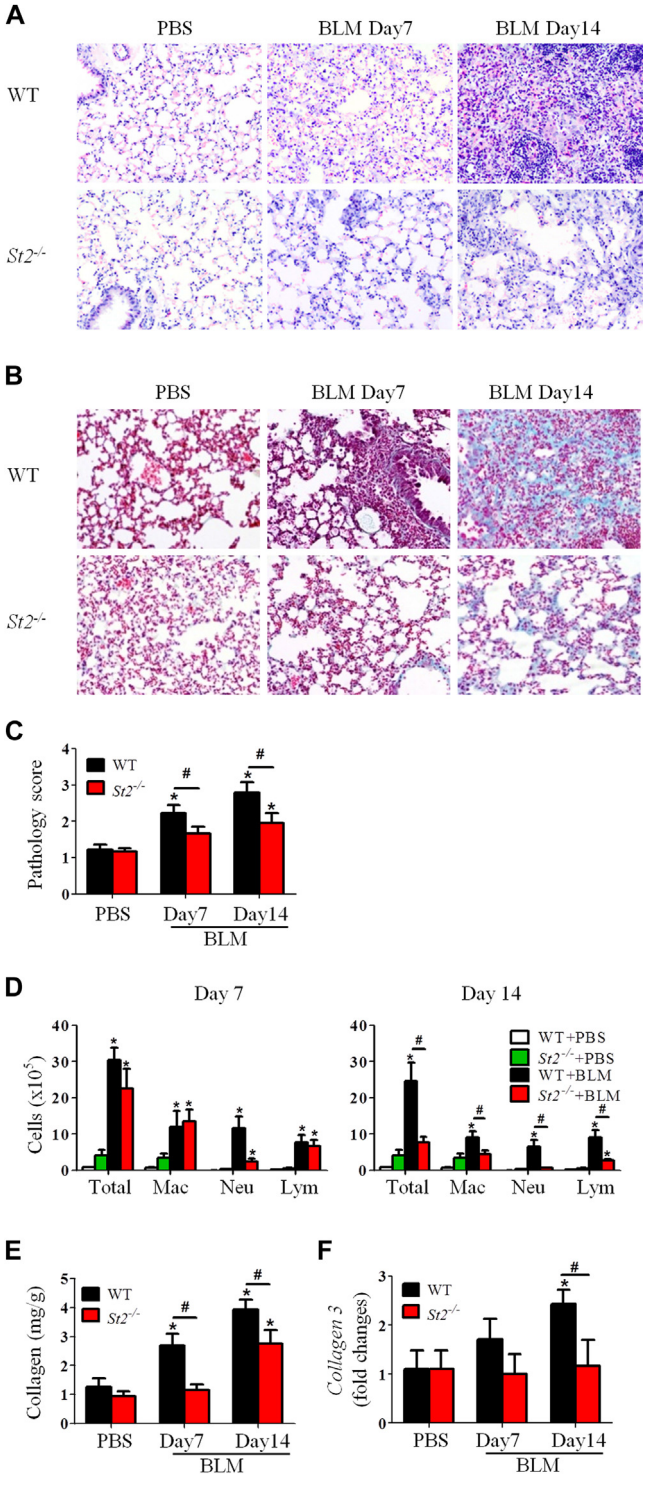


**FIG 1.** *St2*$^{-/-}$ mice have attenuated bleomycin *(BLM)*–induced fibrosis. Lung hematoxylin and eosin staining **(A)**, collagen staining **(B)**, lung pathology score **(C)**, total and differential lung lavage cytology **(D)**, lung tissue collagen content **(E)**, and lung tissue collagen 3 mRNA expression **(F)** are shown. *Vertical bars* = SEMs (n = 5-7 mice per group per experiment). **P* < .05 compared with PBS control and #*P* < .05 compared with WT values. Data are representative of 3 experiments. *Lym*, Lymphocytes; *Mac*, macrophages, *Neu*, neutrophils.

and collagen 3 mRNA expression (Fig 3, *F*). No differences were observed in control groups given one dose of IL-33 compared with the PBS control.

## Bleomycin induces IL-33 production, which promotes lung fibrosis through alveolar macrophages

We next determined the kinetics of bleomycin-induced IL-33 expression in the lung. Mice were given bleomycin as above. Bleomycin administration rapidly enhanced *Il33* expression in lung tissue from day 1 after bleomycin inoculation and lasted for at least 14 days (Fig 4, *A*). Compared with PBS, bleomycin treatment also enhanced lung tissue IL-33 protein production (see Fig E2, *A*, in this article's Online Repository at www.jacionline.org) and *St2* mRNA expression (see Fig E2, *B*). Furthermore, although PBS control WT mice expressed only flIL-33, bleomycin-treated WT mice markedly enhanced mIL-33 concomitant with reduced flIL-33 production (Fig 4, *B*). Bleomycin also increased production of neutrophil cathepsin G (Fig 4, *B*) and myeloperoxidase in lung tissue compared with that seen in PBS control in WT mice (see Fig E2, *C*). The induction of IL-33 isoforms, cathepsin G, and myeloperoxidase in *St2*$^{-/-}$ mice given bleomycin was markedly reduced compared with that seen in WT mice given bleomycin (see Fig E2, *C-E*).

Immunohistochemical analysis of lung tissue sections of PBS- and bleomycin-treated WT mice demonstrated that alveolar epithelial cells constitutively expressed IL-33 (Fig 4, *C*). However, administration of bleomycin induced immunohistochemistry-detectable IL-33 protein in cells located within the alveoli compared that seen in with PBS control mice (Fig 4, *C*). The location and morphologic appearance of these cells suggest that they are alveolar macrophages. The likelihood that these cells were alveolar macrophages is supported by fluorescence-activated cell sorting analysis, showing that bleomycin treatment significantly increased the frequency and number of IL-33–expressing F4/80$^{+}$ macrophages in the lung (approximately 2.5 times the number seen in PBS control mice). This increase was not seen in the *St2*$^{-/-}$ mice given PBS or bleomycin (Fig 4, *D*).

We next evaluated the importance of alveolar macrophages in bleomycin-induced and IL-33 plus bleomycin–exacerbated lung fibrosis based on their depletion.[19,23] Mice were treated with clodronate in liposomes or control liposomes alone administered intranasally on days 2 and 1 before administration of bleomycin or bleomycin plus IL-33. This route of liposome administration depletes alveolar but not lung parenchymal macrophages.[24] Clodronate depleted approximately 80% of alveolar macrophages compared with the control group (Fig 4, *F*) and significantly reduced bleomycin-induced and bleomycin plus IL-33–exacerbated lung fibrosis (Fig 4, *E*) and inflammation (see Fig E3 in this article's Online Repository at www.jacionline.org). Consistent with these observations, macrophage depletion also reduced bleomycin-induced and bleomycin plus IL-33–enhanced neutrophil and lymphocyte numbers in BAL fluid (Fig 4, *F*) and collagen production in lung tissue (Fig 4, *G*) compared with values seen in the control group given PBS.

## IL-33 polarizes M2 macrophages in lung fibrosis

M2 macrophages play a critical role in fibrogenesis.[6] We have shown previously that IL-33, together with IL-13, can polarize alveolar M2 macrophages, but not M1 macrophages, in murine allergic lung remodeling.[19] Therefore we further investigated the effect of bleomycin and IL-33/ST2 signaling on the generation of M2 macrophages in patients with lung fibrosis.

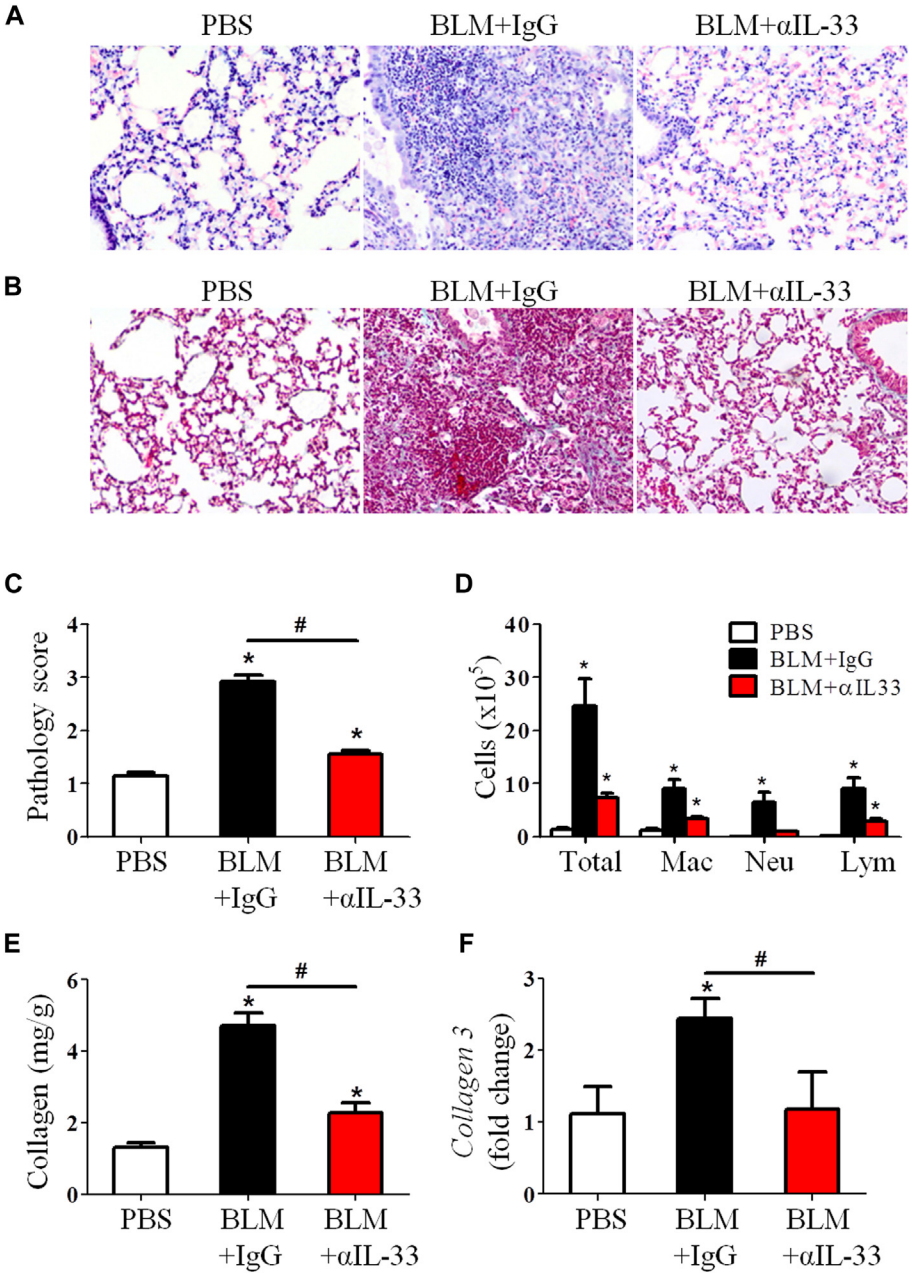


**FIG 2.** Anti–IL-33 antibody treatment attenuates bleomycin *(BLM)*–induced fibrosis. Lung hematoxylin and eosin staining **(A)**, collagen staining **(B)**, lung pathology score **(C)**, total and differential lung lavage cytology **(D)**, lung tissue collagen content **(E)**, and lung tissue collagen 3 mRNA expression **(F)** are shown. *Vertical bars* = SEMs (n = 5 mice per group per experiment). **P* < .05 compared with PBS and #*P* < .05 compared with IgG values. Data are representative of 3 experiments. *Lym*, Lymphocytes; *Mac*, macrophages, *Neu*, neutrophils.

The frequency and number of total macrophages ($CD11b^+F4/80^{high}$) in the lung tissue of bleomycin-treated $St2^{-/-}$ mice was slightly but significantly reduced compared with that of the bleomycin-treated WT mice (Fig 5, *A*, and see Fig E4, *A* and *B*, in this article's Online Repository at www.jacionline.org). Bleomycin markedly enhanced the number and percentage of M2 macrophages ($CD11b^+F4/80^+CD206^+$) in the lungs of WT, but not $St2^{-/-}$, mice on day 7 after bleomycin treatment (Fig 5, *B*, and see Fig E4, *C*). Furthermore, bleomycin also increased IL-$33^+$ M2 macrophage numbers (Fig 5, *C*, and see Fig E4, *D* and *E*) and expression of the gene encoding the M2 macrophage marker arginase 1 *(Arg1)*, but not the M1 macrophage marker inducible nitric oxide synthase 2 *(Nos2)*, in lung tissue in WT mice compared with that seen after control PBS treatment (Fig 5, *D*). The effects of bleomycin administration on macrophage polarization in $St2^{-/-}$ mice given bleomycin showed no increase in *Arg1* expression and increased *Nos2* expression compared with that seen in $St2^{-/-}$ mice given the PBS control (Fig 5, *C* and *D*). However, the expression levels of MHC class II, a common marker on all macrophages, in mice

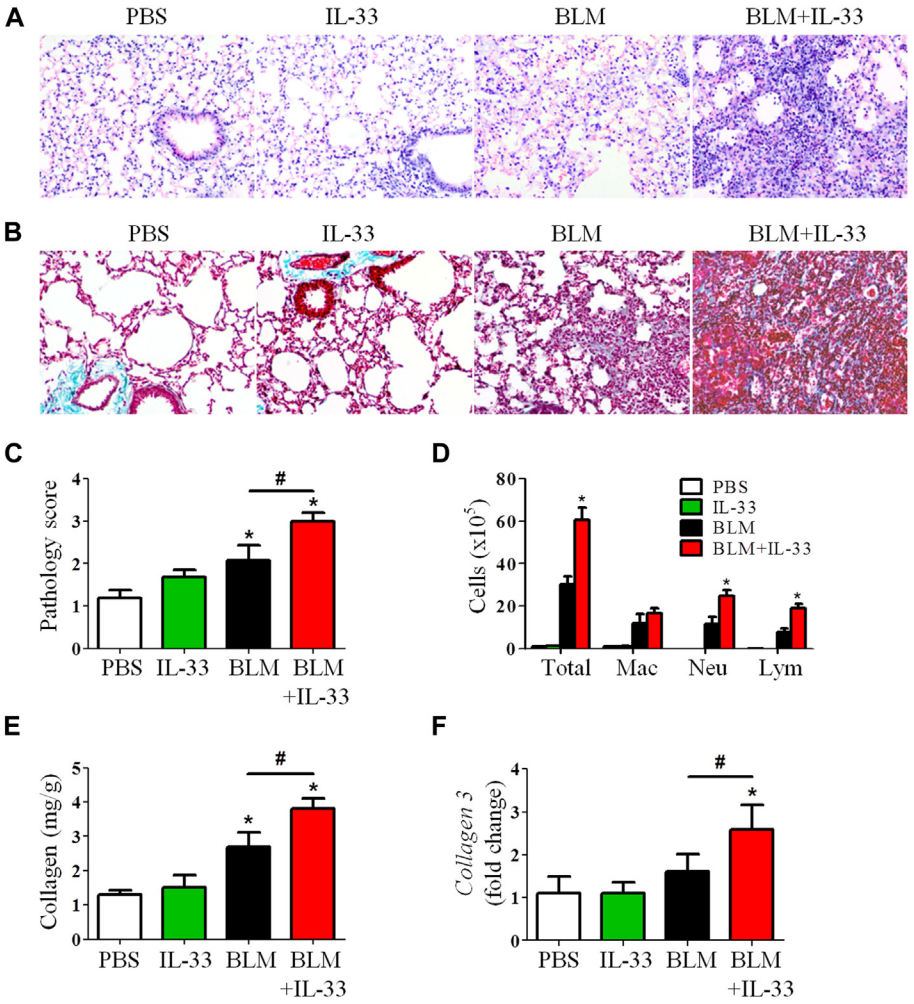


**FIG 3.** Recombinant IL-33 exacerbates bleomycin *(BLM)*–induced fibrosis in mice. Mice were treated with PBS, IL-33, and bleomycin with or without IL-33. Lung hematoxylin and eosin staining **(A)**, collagen staining **(B)**, lung pathology score **(C)**, total and differential lung lavage cytology **(D)**, lung tissue collagen content **(E)**, and lung tissue collagen 3 mRNA expression **(F)** are shown. *Vertical bars* = SEMs (n = 5 mice per group per experiment). **P* < .05 compared with PBS and #*P* < .05 compared with bleomycin values. Data are representative of 3 experiments. *Lym*, Lymphocytes; *Mac*, macrophages, *Neu*, neutrophils.

given bleomycin were not significantly affected by *St2* deficiency compared with that seen in WT control mice (see Fig E4, *F*).

We further assessed the ability of bleomycin, IL-13, and IL-33 to polarize M1 or M2 macrophages *in vitro*. Mouse bone marrow–derived macrophages (BMDMs) were cultured with medium or bleomycin, IL-13, or IL-33 (alone or together), and the induction of *Arg1* and *Nos2* was determined by using quantitative PCR (qPCR). IL-13, but not IL-33, alone significantly induced *Arg1*, but not *Nos2*, expression in macrophages compared with medium control (Fig 5, *E*). The IL-13–induced *Arg1* expression was significantly increased by the presence of IL-33. However, bleomycin alone or in combination with IL-13 or IL-33 had no additional effect on the polarization of M1 or M2 macrophages (Fig 5, *E*).

## Bleomycin and IL-33 induce fibrogenic cytokine and chemokine production

IL-13 and TGF-β1 are key cytokines required for the development of fibrosis.[4,5,25] We next determined how the IL-33/ST2 pathway contributes to bleomycin-induced fibrosis by assessing the cytokine and chemokine profiles induced by bleomycin and IL-33. We first analyzed *Il33*, *Il13*, and *Tgfb1* mRNA expression in the lung tissue of WT or $St2^{-/-}$ mice given bleomycin with or without IL-33. Bleomycin significantly induced expression of these cytokines in WT mice (Fig 6, *A*). Consistent with the attenuated lung fibrosis seen in $St2^{-/-}$ mice given bleomycin compared with that seen in WT mice given bleomycin (Fig 1, *A*), bleomycin did not induce *Il33*, *Il13*, and *Tgfb1* expression in the lungs of $St2^{-/-}$ mice beyond that seen in PBS-treated $St2^{-/-}$ mice (Fig 6, *A*). Furthermore, alveolar macrophage depletion, which abolished bleomycin-induced and IL-33–exacerbated lung fibrosis (Fig 4, *E*), also abrogated the bleomycin and bleomycin plus IL-33–induced expression of these cytokines (Fig 6, *B*).

We then determined the levels of key inflammatory cytokines and chemokines in the BAL fluid of bleomycin-treated mice by using Luminex (Luminex; Biosource, Invitrogen, Carlsbad, Calif) or ELISA (BD Biosciences, San Jose, Calif). Only the fibrogenic cytokines IL-1, IL-33, IL-13, and TGF-β1 and 3 chemokines

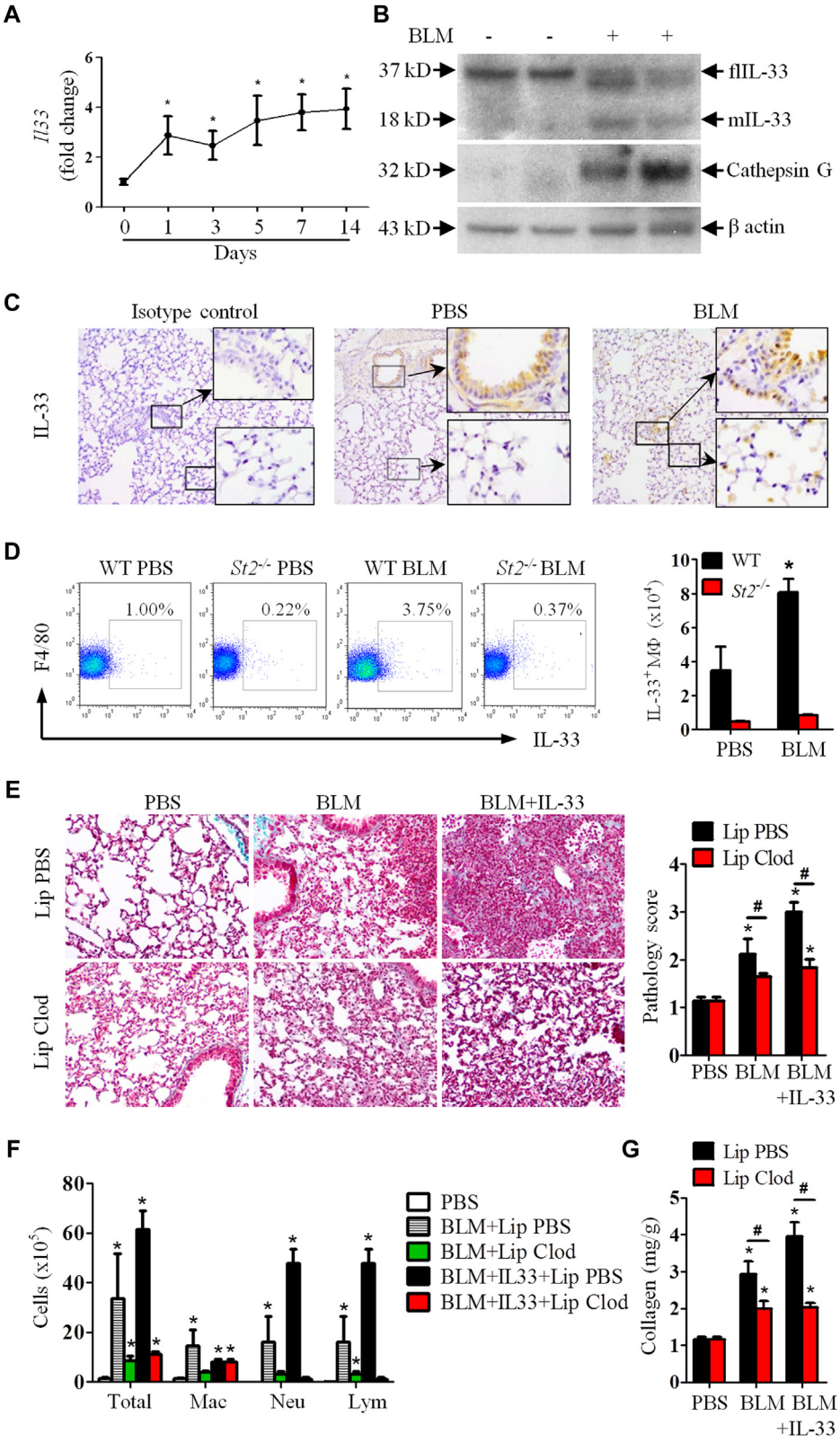


**FIG 4.** Bleomycin *(BLM)* induces IL-33 and IL-33 production and promotes lung fibrosis through alveolar macrophages. WT and *St2*$^{-/-}$ mice were given bleomycin, and lung tissues were analyzed on day 7. **A-D,** *Il33* mRNA expression (Fig 4, *A*); IL-33 isoforms, cathepsin G, and β-actin detected by using western blotting (Fig 4, *B*); immunohistochemical staining of IL-33 (×400 magnification; Fig 4, *C*), and percentage and number of IL-33$^{+}$ macrophages determined by using fluorescence-activated cell sorting (Fig 4, *D*). **E-G,** Mice were given PBS, bleomycin, or bleomycin plus IL-33 and treated with clodronate or control liposomes. Lung collagen staining and pathology score (Fig 4, *E*), total and differential lung lavage cytology (Fig 4, *F*), and lung collagen content (Fig 4, *G*) are shown. *Vertical bars* = SEMs (n = 6 mice per group per experiment) **P* < .05 compared with PBS and #*P* < .05 compared with bleomycin values. Data are representative of 2 experiments. *Lym*, Lymphocytes; *Mac*, macrophages, *Neu*, neutrophils.

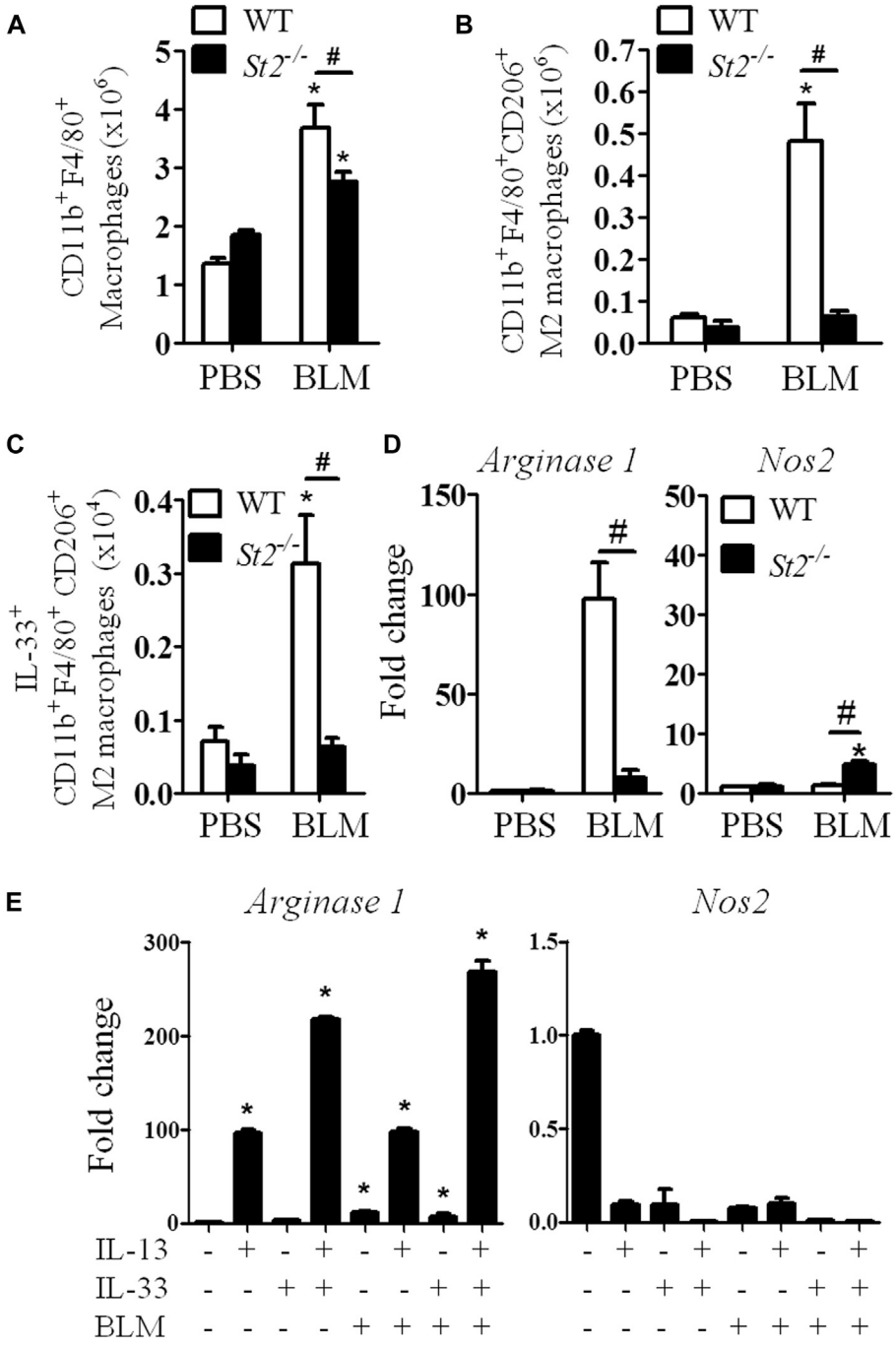


**FIG 5.** IL-33 polarizes M2 macrophages. **A-C,** Numbers of macrophages (Fig 5, *A*), $CD206^+$ M2 macrophages (Fig 5, *B*), and $IL\text{-}33^+$ M2 macrophages (Fig 5, *C*). **D,** Lung tissue *Arg1* and *Nos2* mRNA expression. **E,** BMDMs were stimulated with IL-13, IL-33, and bleomycin (*BLM*; alone or together). *Arg1* and *Nos2* mRNA expression was quantified. *Vertical bars* = SEMs (n = 5 mice per group per experiment). $*P < .05$ compared with PBS and $\#P < .05$ compared with WT values. Data are pooled from 3 experiments.

(CXCL1, CXCL2, and CCL2) were significantly induced by bleomycin and bleomycin plus IL-33 (Fig 6, *C*, and see Fig E1). No other type 2 and inflammatory cytokines were detected at significant levels (data not shown). We also determined the levels of key fibrogenic cytokines in BAL fluid. Bleomycin induced IL-13 production from day 2, and IL-33 plus bleomycin induced IL-13 synthesis from day 1; both returned to baseline by day 5 (Fig 6, *C*). Bleomycin-induced TGF-β1 production appeared from day 3 and increased progressively up to at least day 14 (Fig 6, *C*), and this was further increased by IL-33.

We further confirmed the ability of macrophages to produce IL-13 and TGF-β1 in response to IL-33. BMDMs were stimulated with IL-13, IL-33, or IL-13 plus IL-33. IL-33 stimulated BMDMs to produce a significant amount of IL-13 compared with medium alone (Fig 6, *D*). IL-13 or IL-33 alone stimulated significantly increased levels of TGF-β1 compared with medium alone.

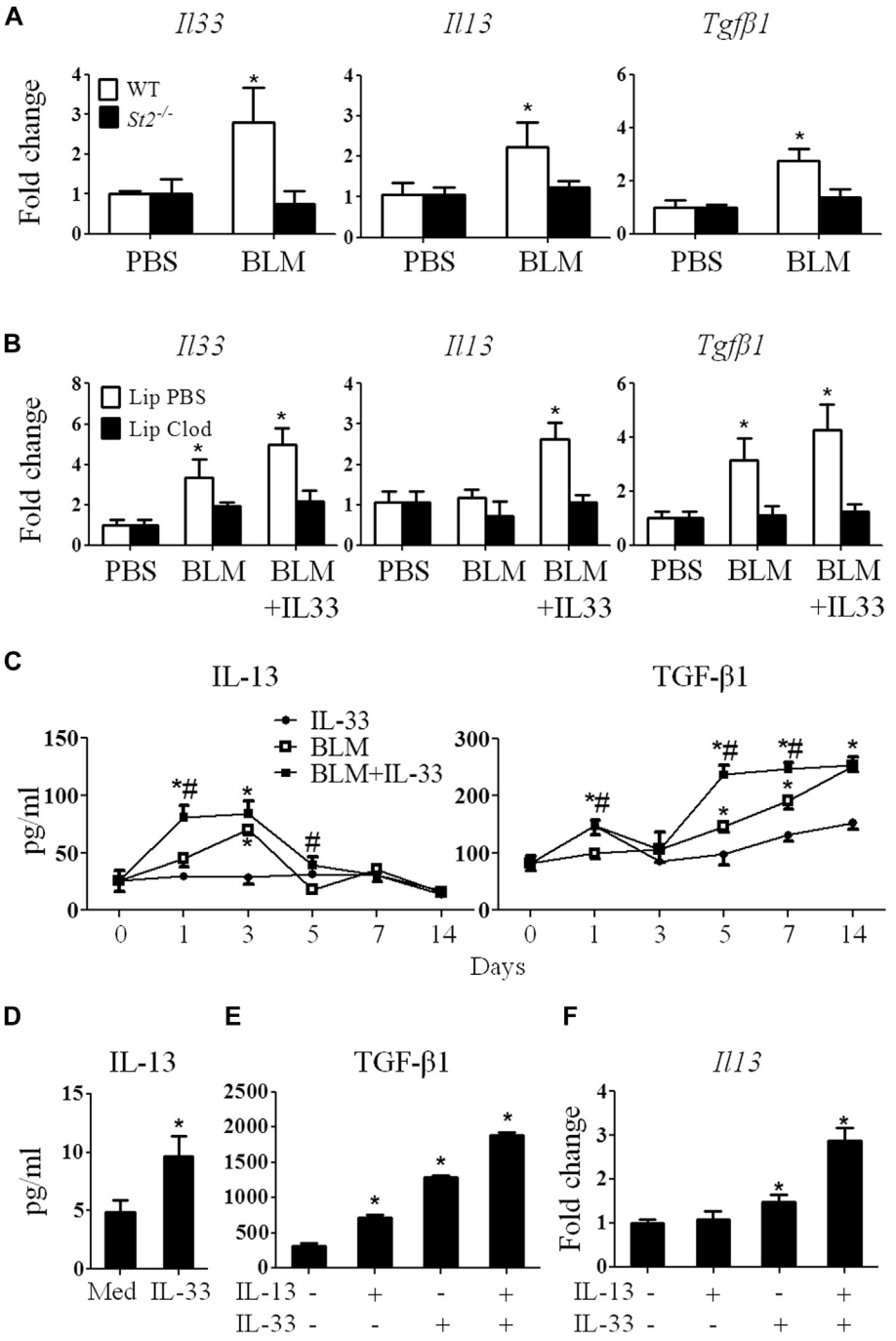


**FIG 6.** Bleomycin *(BLM)* and IL-33 induce fibrogenic cytokine production. **A,** Cytokine mRNA expression in the lungs. **B,** Cytokine mRNA expression in the lungs of clodronate- or liposome-treated mice. **C,** IL-13 and TGF-β1 production in lung lavage fluid. **D** and **E,** IL-13 and TGF-β1 in culture supernatants of BMDMs. **F,** *Il13* mRNA expression in BMDMs. *Vertical bars* = SEMs (n = 5 mice per group per experiment). **P* < .05 and #*P* < .05 compared with control values. Data are representative of 3 experiments.

Furthermore, IL-13 and IL-33 synergized to stimulate even higher levels of TGF-β1 production (Fig 6, *E*) and *Il13* expression in macrophages (Fig 6, *F*).

## IL-33 enhances ILC2 expansion and function in fibrosis through ST2

Recent reports show that ILC2s are a major source of IL-13 *in vivo* and that IL-33 is a key inducer of ILC2s through ST2.[17,26,27] We sought determine whether ILC2s also contribute to the bleomycin plus IL-33–induced IL-13 production and lung fibrosis. WT and $St2^{-/-}$ mice were given bleomycin or PBS control, and the lineage-negative, inducible costimulator, (ICOS)–positive $ST2^{+}$ ILC2s in the lungs were analyzed 3 to 7 days after bleomycin treatment (see Fig E5, *A* and *B*, in this article's Online Repository at www.jacionline.org).[28] Bleomycin treatment markedly enhanced ILC2 numbers in the lungs of WT

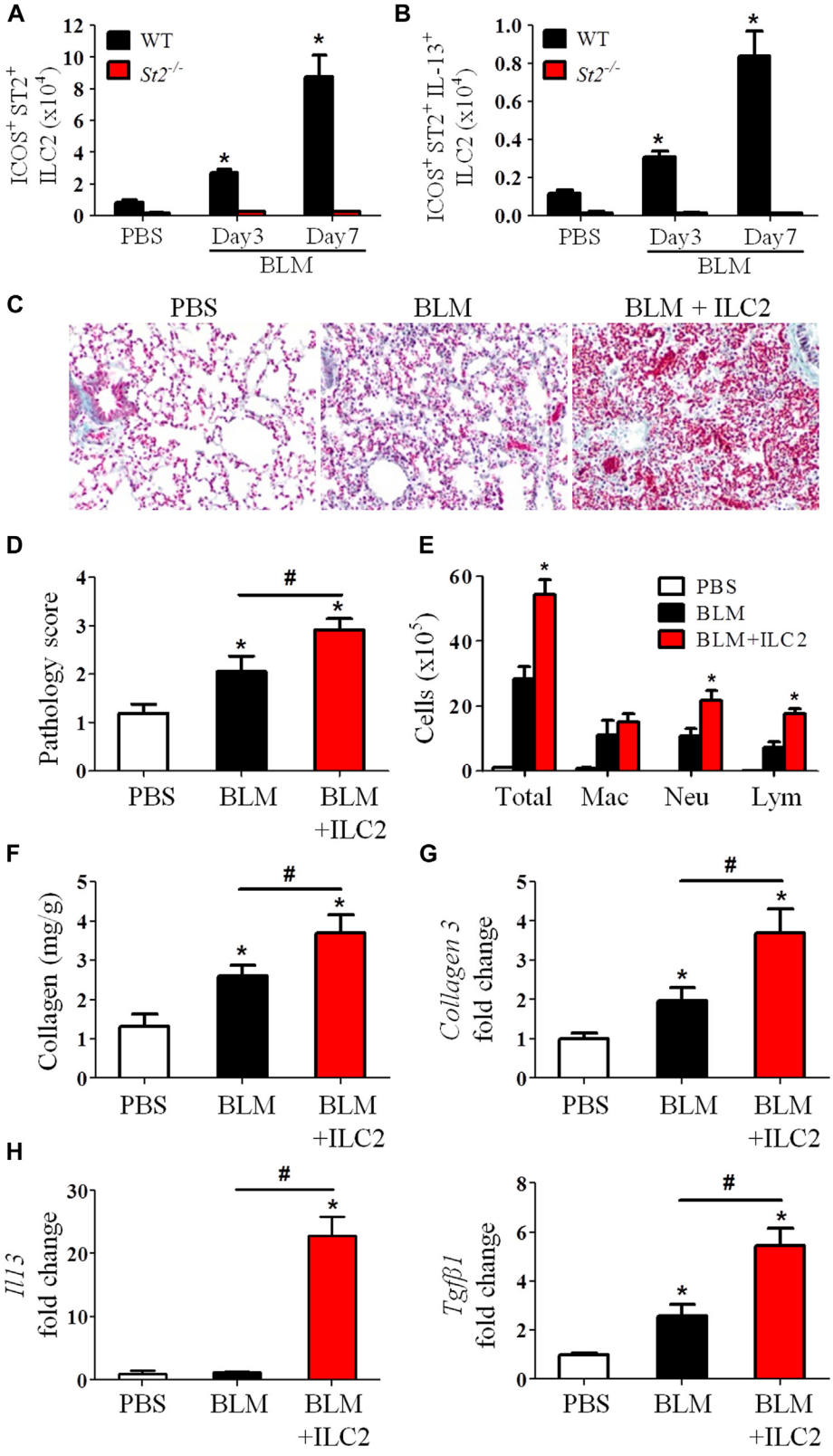


**FIG 7.** Induction and function of ILC2s in the lungs. **A** and **B,** Total number of ILC2s (Fig 7, *A*) and IL-13$^+$ ILC2s (Fig 7, *B*). **C-F,** ILC2s were adoptively transferred into mice after bleomycin *(BLM)* instillation. Collagen staining (Fig 7, *C*), pathology score (Fig 7, *D*), total/differential lung lavage cytology (Fig 7, *E*), and collagen content (Fig 7, *F*) are shown. **G** and **H,** Collagen 3 (Fig 7, *G*) and *Il13* and *Tgfb* (Fig 7, *H*) mRNA expression. *Vertical bars* = SEMs (n = 6 mice per group per experiment). *$P$ < .05 compared with PBS and #$P$ < .05 compared with bleomycin values. Data are representative of 2 experiments.

mice compared with those seen in control PBS-treated mice (Fig 7, *A*). In contrast, ILC2s were almost completely absent in $St2^{-/-}$ mice, irrespective of whether they were treated with bleomycin. By day 7, the number of IL-$13^+$ ILC2s was enhanced 8-fold in WT mice given bleomycin compared with that seen in PBS control mice (Fig 7, *B*, and see Fig E5, *C*). Again, IL-$13^+$ ILC2s were almost completely absent in $St2^{-/-}$ mice (Fig 7, *B*). To understand the role of ILC2s in IL-33– and bleomycin-induced pulmonary fibrosis, we adoptively transferred purified ILC2s into WT mice 1 day after bleomycin and compared this with conditions after bleomycin alone. The ILC2 transfer led to exacerbation of lung inflammation and fibrosis compared with bleomycin alone (Fig 7, *C* and *D*, and see Fig E5, *D*), and this was similar to that observed after bleomycin plus IL-33 instillation (Fig 3). The pathogenic changes were accompanied by increased inflammatory cell infiltration and collagen production and expression of collagen 3, *Il13*, and *Tgfb1* in lung tissue compared with that seen in mice given bleomycin alone (Fig 7, *E-H*, and see Fig E5, *E*).

## DISCUSSION

Data reported in this study reveal a hitherto unrecognized effect and mechanism by which IL-33 exacerbates bleomycin-induced lung fibrosis in mice (see Fig E6 in this article's Online Repository at www.jacionline.org). Bleomycin can elicit early neutrophil infiltration and release of flIL-33 mainly by airway epithelial cells and alveolar macrophages (see Fig E7 in this article's Online Repository at www.jacionline.org). flIL-33 can then be processed into mIL-33 by neutrophil proteases, which subsequently stimulates macrophages and ILC2s to produce IL-13. IL-13 and mIL-33 then synergistically induce the polarization of M2 macrophages and increase production of IL-13 and TGF-β1. These cytokines are powerful activators of fibroblasts, stimulating proliferation and increased collagen synthesis and thereby amplifying pulmonary fibrosis. Thus the IL-33/macrophage/M2 macrophage pathway is centrally involved in the bleomycin-mediated fibrotic process, whereas the IL-33/ILC2 pathway only contributes to the process by providing a proportion of the IL-13 pool in fibrotic tissue (see Fig E6).

Although a single administration of exogenous mIL-33 on its own was not sufficient to induce pulmonary fibrosis by day 14 (Fig 3), we found that *Il33* transgenic mice that constitutively express low mIL-33 levels (approximately 80 pg/mL in serum) spontaneously had lung interstitial fibrosis (see Fig E8 in this article's Online Repository at www.jacionline.org). Thus mIL-33 is likely a key profibrotic factor.

We also show that the profibrogenic effect of mIL-33 is mainly attributed to its role in M2 macrophage polarization. Macrophages are polarized toward an M2 phenotype in a $T_H2$ cytokine–dominant milieu,[6] and local mIL-33, together with IL-13, enhanced M2 macrophage polarization in our study. Furthermore, the specificity of the requirement for IL-33 in this process is demonstrated by the fact that bleomycin alone, which was not able to induce M2 macrophage polarization in $St2^{-/-}$ mice, also did not cause fibrosis in $St2^{-/-}$ mice. We found that macrophages are the predominant cells that express both IL-33 and ST2 in fibrotic lungs (see Figs E7 and E9 in this article's Online Repository at www.jacionline.org). Thus IL-33/ST2 signaling might promote M2 macrophage development and function in bleomycin-induced fibrogenesis in an autocrine and paracrine fashion.

The profibrogenic cytokines TGF-β1 and IL-13 are necessary for the development of tissue fibrosis.[4,5,25] However, how these cytokines are induced in bleomycin-induced fibrosis is unresolved. Here we show that IL-33 signaling through ST2 is essential for optimal induction of both IL-13 and TGF-β1 expression in bleomycin-induced lung fibrosis, although in different cells. IL-33 primarily induces the production of IL-13 by both macrophages and ILC2s and production of TGF-β1 by macrophages. These findings also suggest that mIL-33 is a novel TGF-β1 inducer, which might explain its fibrogenic role in bleomycin-induced fibrosis.

We demonstrated that bleomycin instillation increased the infiltration of leukocytes, mainly neutrophils but also macrophages and lymphocytes, into the airways and lung interstitium. This might be a consequence of IL-33/ST2–dependent and independent production of the key chemokines CXCL1 and CXCL2 in the context. As reported, CXCL1 and CXCL2 determine the migration of neutrophils, and CCL2 is used for the migration of lymphocyte and monocyte/macrophage into the inflamed lung tissue of mice.[29,30] Because these chemokines are mainly produced by macrophages,[29,30] this might explain why macrophage depletion also reduced lung lavage neutrophils and lymphocytes in the setting of bleomycin-induced fibrosis.

The precise role of neutrophils in IL-33–exacerbated pulmonary fibrosis is incompletely understood. We suggest an additional role through which neutrophil proteases contribute to fibrosis. We found that bleomycin simultaneously enhanced the production of mIL-33 but reduced the production of flIL-33 in lung tissue. mIL-33 production was associated with neutrophil cathepsin G production in the same lung tissue, suggesting that flIL-33 was processed to mIL-33, which has greater bioactivity.[31] Because epithelial cells and alveolar macrophages can produce CXCL1 and CXCL2 independent of IL-33/ST2 signals,[29,30,32] these cells might be responsible for the bleomycin-induced early neutrophil infiltration in lung tissue (see Fig E6).

A recent report showed that adenovirus-delivered flIL-33, the form of IL-33 located in the nucleus, promoted lung fibrosis through an undefined but ST2-independent mechanism.[9] This study, together with data in the present report, suggests that both flIL-33 and mIL-33 are fibrogenic and that they might induce fibrosis through distinct mechanisms. These findings are thematically linked with the data presented here; however, there are several important differences between these 2 studies that might together provide deeper understanding of the role of IL-33 in fibrosis. We tested the role of mIL-33 as a secreted cytokine, which acts through the cell-surface IL-33 receptor (ST2), whereas Luzina et al[9] tested the function of intranuclear flIL-33 delivered through a viral vector. We used $St2^{-/-}$ mice of the fibrosis-sensitive C57BL/6 strain, whereas Luzina et al[9] used $St2^{-/-}$ mice of the fibrosis-resistant BALB/c strain.[33] Thus the apparent discrepancies between the 2 studies might be partly due to the different mode of action of mIL-33 versus flIL-33 and the strain of mouse used.

IL-33 is clearly detected in patients with several chronic fibrotic diseases, including IPF, cystic fibrosis, and systemic sclerosis.[9,34,35] Given that inflammation and fibrogenesis are the common pathogenic characteristic of these disorders and can be exacerbated by IL-33, IL-33 might have a general contribution

to a range of fibrotic diseases. Therefore regulation of IL-33 could be a novel therapeutic strategy for these diseases.

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**Key messages**

- **Bleomycin enhanced the production of mIL-33 but reduced flIL-33 production in lung tissue *in vivo*.**
- **ST2 deficiency, anti–IL-33 antibody, or alveolar macrophage depletion attenuated and exogenous mIL-33 or adoptive transfer of ILC2s enhanced bleomycin-induced lung fibrosis in mice.**
- **IL-33 polarized M2 macrophages to produce both IL-13 and TGF-β1 and induced the expansion of ILC2s to produce IL-13 *in vitro* and *in vivo*.**

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# METHODS

## Mice

C57BL/6 mice were purchased from Harlan Olac (Bicester, United Kingdom). *St2*$^{-/-}$ mice were on a C57BL/6 background.[E1] Mice were housed in specific pathogen-free conditions at Glasgow University, and procedures were in accordance with the UK Home Office animal experimentation guidelines. *Il33* transgenic mice were on a C57BL/6 background[E2] and housed in an Association for Assessment and Accreditation of Laboratory Animal Care–accredited facility (Beijing, China). The procedures were approved by the Institutional Animal Care and Use Committee of the Chinese Institute of Laboratory Animal Science (GC-08-2018).

## Bleomycin-induced fibrosis

C57BL/6 mice were lightly anaesthetized with isoflurane gas (4%), and bleomycin sulfate (0.1 U per 25-g mouse in 30 μL of PBS; Sigma, St Louis, Mo) or PBS was administered intranasally. BAL fluid, blood, and lungs were harvested 7 or 14 days later and processed and analyzed, as described previously.[E3,E4] The time points selected in the experiments were determined by our pilot experiments and by a consensus of the various time points used in the literature describing mouse bleomycin-induced lung fibrosis. After bleomycin instillation in mice, lung tissue displays typical acute inflammation from days 1 to 5, and early expression of fibrotic markers and collagen deposition can be seen from day 7 and is more pronounced by day 14. Thus days 7 and 14 were used to investigate whether ST2 signals are involved in early development and overall severity of lung fibrosis in *St2*$^{-/-}$ mice. Day 7 was also used to determine whether exogenous IL-33 instillation could exacerbate bleomycin-induced fibrosis. The mice were inoculated intranasally with a single dose (500 ng per mouse) of recombinant murine mIL-33 (BioLegend, San Diego, Calif, or prepared as described previously[E5]) on day 0 of bleomycin treatment and killed on day 7. Day 7 was also the end point used for the macrophage depletion experiment to test whether this procedure would abolish exogenous IL-33–exacerbated fibrotic effect seen at day 7. Alveolar macrophage depletion was performed by means of intranasal administration of clodronate (ClodLip BV) or control liposomes (40 μL per mouse) at 24 and 72 hours before bleomycin administration, and the mice were killed on day 7.[E3,E6] Day 14 was selected to study the potential therapeutic effect of neutralizing anti–IL-33 on the more severe established fibrosis. The mice were treated intraperitoneally with neutralizing anti–IL-33 antibody[E7] or control normal rabbit IgG (Sigma, 150 μg per mouse) on the day of bleomycin administration and 5 and 10 days thereafter. The mice were killed on day 14. Perfused lung tissue (100 mg) was dispersed in 1 mL of ice-cold RIPA Lysis Buffer with a cocktail of protease inhibitors (Sigma) for 45 minutes. Cell suspensions were centrifuged at 13,000 rpm in an Eppendorf tube for 5 minutes, and the supernatant was collected for cytokine and collagen assay.

## Cell culture

Primary BMDMs were generated with macrophage colony-stimulating factor (10 ng/mL; PeproTech, Rocky Hill, NJ), as described previously.[E3] The subsequent cell preparations contained more than 95% F4/80$^{+}$ macrophages, as determined by using fluorescence-activated cell sorting. The cells ($0.5 \times 10^6$/mL) in culture medium were placed into 24-well plates (Invitrogen) and cultured for 24 or 48 hours. Culture supernatants were stored at −20°C for cytokine analysis, and cells were harvested for mRNA extraction for the qPCR assay.

## Western blotting analysis

Tissue was lysed in RIPA buffer (Thermo Scientific, Uppsala, Sweden) containing protease inhibitors (Roche, Mannheim, Germany). Protein concentrations were estimated by using the BCA protein assay (Pierce, Rockford, Ill). Proteins were then incubated at 70°C for 10 minutes in reducing SDS sample buffer, and 30 μg of protein lysate per lane was run through NuPAGE Novex 4-12% Bis-Tris Protein Gels (Life Technologies, Carlsbad, Calif) and transferred to Hybond ECL membranes (GE Healthcare, Fairfield, Conn). Membranes were blocked for 1 hour in 5% nonfat dried milk in double-distilled PBS and incubated overnight with the appropriate primary antibody at 4°C. Membranes were then washed in double-distilled PBS/Tween 20 and incubated with the appropriate secondary antibody. Detection was performed with ECL Western Blotting Detection Reagents (GE Healthcare). Antibodies against flIL-33 and mIL-33 were obtained from R&D Systems (Minneapolis, Minn; AF3626, goat anti-mouse IL-33 polyclonal antibody); cathepsin G, β-actin, and all secondary antibodies were obtained from Santa Cruz Biotechnology (Dallas, Tex). The intensity of Western blot bands was quantified by means of densitometry with ImageJ software (National Institutes of Health, Bethesda, Md).

## Determination of myeloperoxidase activity

Lung homogenates were prepared in 1 mL of RIPA buffer by using a tissue homogenizer and myeloperoxidase assay performed as previously described.[E8] Results are expressed as relative units (OD, 492 nm) and were corrected for the activity of other peroxidases, which were not inhibited by 3-amino-1,2,4-triazole.

## Flow cytometry

Lungs were harvested on day 3 or 7 after bleomycin administration and digested in 125 μg/mL Liberase TL and 100 μg/mL DNAse 1 (Roche Diagnostics) to characterize the infiltrating leukocytes. Dispersed cells ($1 \times 10^6$ cells per tube) were stained with 4′-6-diamidino-2-phenylindole dihydrochloride, UVE/DEAD fixable Aqua Dead cell stain (Life Technologies), and fluorochrome-conjugated mAbs against F4/80-Pacific blue (eBioscience, San Diego, Calif), cytokeratin 11–fluorescein isothiocyanate (FITC; panepithelial cell marker; Abcam, Cambridge, United Kingdom), ER-TF7–allophycocyanin (APC; panfibroblast marker, Santa Cruz Biotechnology), CD3-PercP, CD11b-FITC or PercP, CD11c-APC, CD49b–phycoerythrin (PE), CD206-APC, Ly6G-APC, Siglec-F–PE, MHC class II–PercP, CD45-AF700, and isotype controls (all from BD Biosciences, unless otherwise indicated). Leukocytes were stained with antibodies against ST2–FITC (MD Biosciences), lineage markers (B220, FcεRI, CD11b, CD3ε, and Siglec F) labeled with PE, CD45-AF700, and ICOS-PerCP/Cy5.5 (eBioscience) to characterize the infiltrating ILC2s. Intracellular IL-33 or IL-13 was detected by staining with anti–IL-33–PE (R&D Systems) or anti–IL-13–APC (eBioscience) after activation with phorbol 12-myristate 13-acetate (50 ng/mL) and ionomycin (1 μg/mL) in the presence of BD GolgiStop and cell permeabilization (BD Cytofix/Cytoperm, BD Biosciences). Cells were analyzed with a Beckman Coulter CyAn ADP Analyzer (Beckman Coulter, Fullerton, Calif). Gating strategy (Fig E4, *A*, and E5, *A*) and analysis were performed with FlowJo software (TreeStar, Eugene, Ore).

## ILC2 amplification and isolation and adoptive cell transfer

For ILC2 amplification *in vivo*, mice were anesthetized with isoflurane and treated with IL-33 (1 μg administered intranasally) daily for 5 days, as described previously.[E9] Lungs were harvested on day 6 and digested in 125 μg/mL Liberase TL and 100 μg/mL DNAse 1 (Roche Diagnostics). Nonadherent cells were stained for ST2, lineage markers, ICOS, CD45, and UVE/DEAD fixable Aqua Dead cell stain (Life Technologies) as above and sorted with a BD FACSAria. For cell transfer, $5 \times 10^5$ ILC2s in 40 μL of PBS were inoculated intranasally 1 day after bleomycin challenge. Mice were culled on day 7 after bleomycin instillation to assess lung inflammation and fibrosis.

## Cytokine measurements

Concentrations of cytokines and chemokines in BAL fluid, cell cultures, and whole-lung homogenates were determined by using Luminex (Luminex, Biosource, Invitrogen) or ELISA (BD Biosciences), according to the manufacturers' instructions.

## qPCR

RNA was purified from tissue samples by using the RNeasy Mini Kit (Qiagen, Manchester, United Kingdom), according to the manufacturer's

instructions. Reverse transcription of RNA into cDNA was carried out with High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, Calif). RT-PCR was performed with Fast SYBR Green master mix on a Prism 7900HT (Applied Biosystems). The primers used were as follows: *Il13* forward 5′-GAA TCC AGG GCT ACA CAG AAC-3′, reverse 5′-AAC ATC ACA CAA GAC CAG ACT C-3′; *Il33* forward 5′-ACT ATG AGT CTC CCT GTC CTG-3′, reverse 5′-ACG TCA CCC CTT TGA AGC-3′; *St2* forward 5′-TCT GTG GAG TAC TTT GTT CAC C-3′, reverse 5′-TCT GCT ATT CTG GAT ACT GCT TTC-3′; *Tgfb1* forward 5′-CCA TGA GGA GCA GGA AGG-3′, reverse 5′-ACA GCA AAG ATA ACA AAC TCC AC-3′; *Arg1* forward 5′-AGT GTT GAT GTC AGT GTG AGC-3′, reverse 5′-GAA TGG AAG AGT CAG TGT GGT-3′; *Nos2* forward 5′-GCC TCG CTC TGG AAA GA-3′, reverse 5′-TCC ATG CAG ACA ACC TT-3′; collagen 1 forward 5′-CAT TGT GTA TGC AGT GAC TTC-3′, reverse 5′-CGC AAA GAG TCT ACA TGT CTA GGC-3′; and collagen 3 forward 5′-TCT CTA GAC TCA TAG GAC TGA CC-3′, reverse 5′-TTC TTC TCA CCC TTC TTC ATC C-3′.

## Collagen assay

The soluble collagen in lung tissues was quantified with the Sircol Collagen Assay (Biocolor, Carrickfergus, United Kingdom), according to the manufacturer's instructions.

## Histologic analysis

The larger left lung lobe was excised, fixed in 4% buffered formalin, and embedded in paraffin. Sections (4 μm) were stained with hematoxylin and eosin (Cellpath, Newtown, United Kingdom) or Gomori Rapid One-Step Trichrome Stain (Sigma) for collagen. The pathology score (from 1-4) was determined by using a method modified from a previous report[E10]: 1, no abnormal fibrosis; 2, occasional small interstitial fibrotic foci; 3, moderate interalveolar septal thickening and fibrotic foci; and 4, continuous interalveolar fibrosis.

## Micro–computed tomographic scanning

*Il33* transgenic mice, which overexpress the mature form of IL-33, were kept in pathogen-free conditions for up to 6 months, and the development of lung fibrosis was determined by using computed tomographic (CT) scans. Scans were performed with a cone-beam micro-CT scanner (Inveon; Siemens Healthcare, Munich, Germany), as previously described.[E11] WT and *Il33* transgenic mice were anesthetized and placed in the prone position on the micro-CT bed without respiratory gating. The tube voltage was 70 kVp, current was 400 mA, and exposure time was 800 ms. The scan field of view was 72.44 mm × 71.31 mm. Projection images were acquired with a single tube/detector over a circular orbit of 360° with a step angle of 1°. Reconstructions were performed by using a commercially available CT reconstruction program (COBRA Exxim, version 6.3), with a filtered back-projection technique. A resolution of approximately 70.74 μm per pixel was achieved.

## Statistical analysis

Data were analyzed by using 1-way ANOVA, followed by Tukey or Newman-Keuls *post hoc* analysis. One-way ANOVA was used to examine mean differences between 2 or more groups to compare every mean with every other mean. Kinetic experiments (ie, cytokine expression over time or *in vitro* data) were analyzed by using repeated-measures ANOVA. The analyses were performed with GraphPad Prism 5.0 statistical software (GraphPad software, San Diego, Calif). All results were presented as means and SEMs from 5 to 7 mice per group per experiment. Data are representative of at least 2 separated experiments. A $P$ value of less than .05 was considered to indicate statistical significance.

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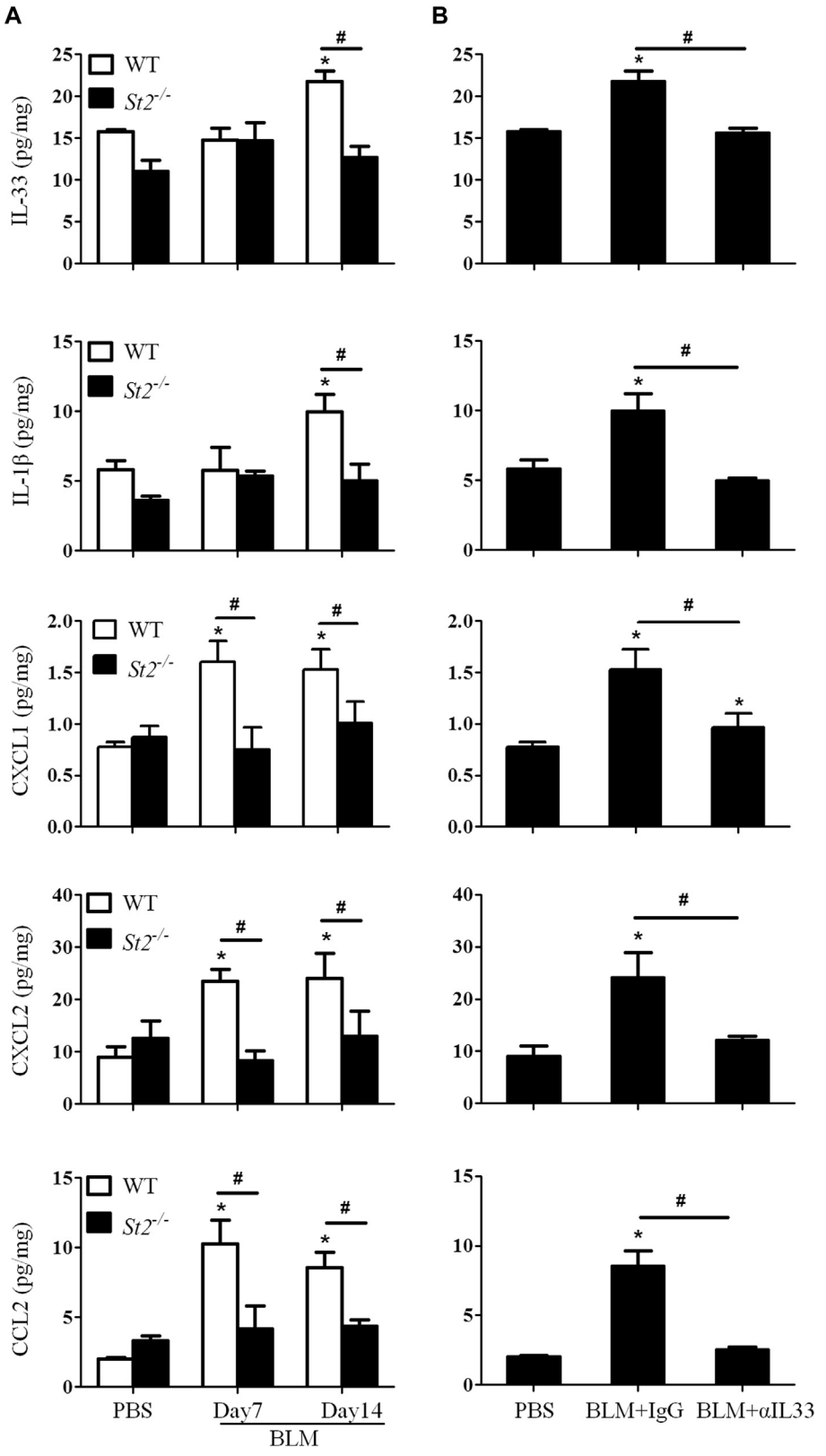


**FIG E1.** Cytokine and chemokine concentrations quantified by means of ELISA in dispersed lung tissue (in milligrams) supernatants of WT and $St2^{-/-}$ mice treated with PBS or bleomycin *(BLM)* at days 7 and 14 **(A)** and WT mice given bleomycin; treated with PBS, anti–IL-33, or control IgG; and killed on day 14 **(B)** are shown. *Vertical bars* = means ± SEMs (n = 5-6 mice per group per experiment) **P* < .05 compared with PBS control and #*P* < .05 compared with $ST2^{-/-}$ mice or IgG control values. Data are representative of 3 experiments.

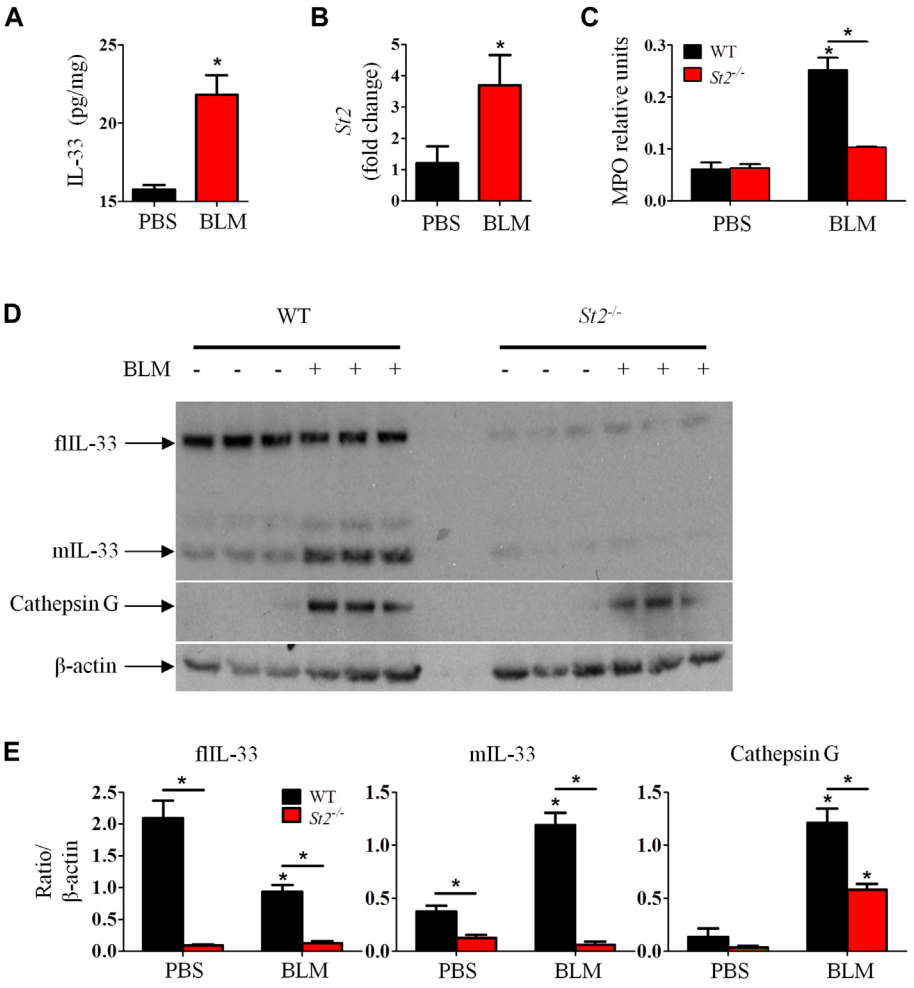


**FIG E2.** Bleomycin *(BLM)* induces IL-33 and ST2 production in fibrotic lung tissue. WT and $St2^{-/-}$ mice were given bleomycin, and lung tissues were analyzed on day 7. IL-33 concentration determined by means of ELISA **(A)**, *St2* mRNA expression **(B)**, myeloperoxidase *(MPO)* activity **(C)**, and IL-33 isoforms, cathepsin G, and β-actin detected by means of Western blotting **(D)** are shown. **E,** The intensity of Western blot bands in Fig E2, *D*, was quantified by means of densitometry. *Vertical bars* = SEMs (n = 6 mice per group per experiment). **P* < .05 compared with PBS values. Data are representative of 3 experiments.

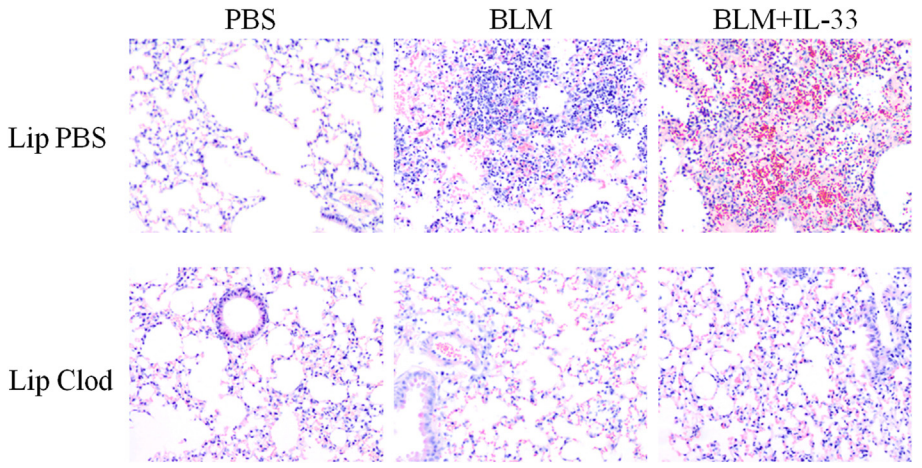


**FIG E3.** Depletion of alveolar macrophages reduces bleomycin *(BLM)*– and bleomycin plus IL-33–induced lung inflammation. Mice were treated with PBS, clodronate in liposome *(Clod)*, or liposomes alone *(Lip)* 1 day before administration of bleomycin or bleomycin plus IL-33. Lungs were harvested on day 7, and lung tissue sections were stained with hematoxylin and eosin (original magnification ×200). Data are representative of 3 experiments (n = 5-6 mice per group per experiment).

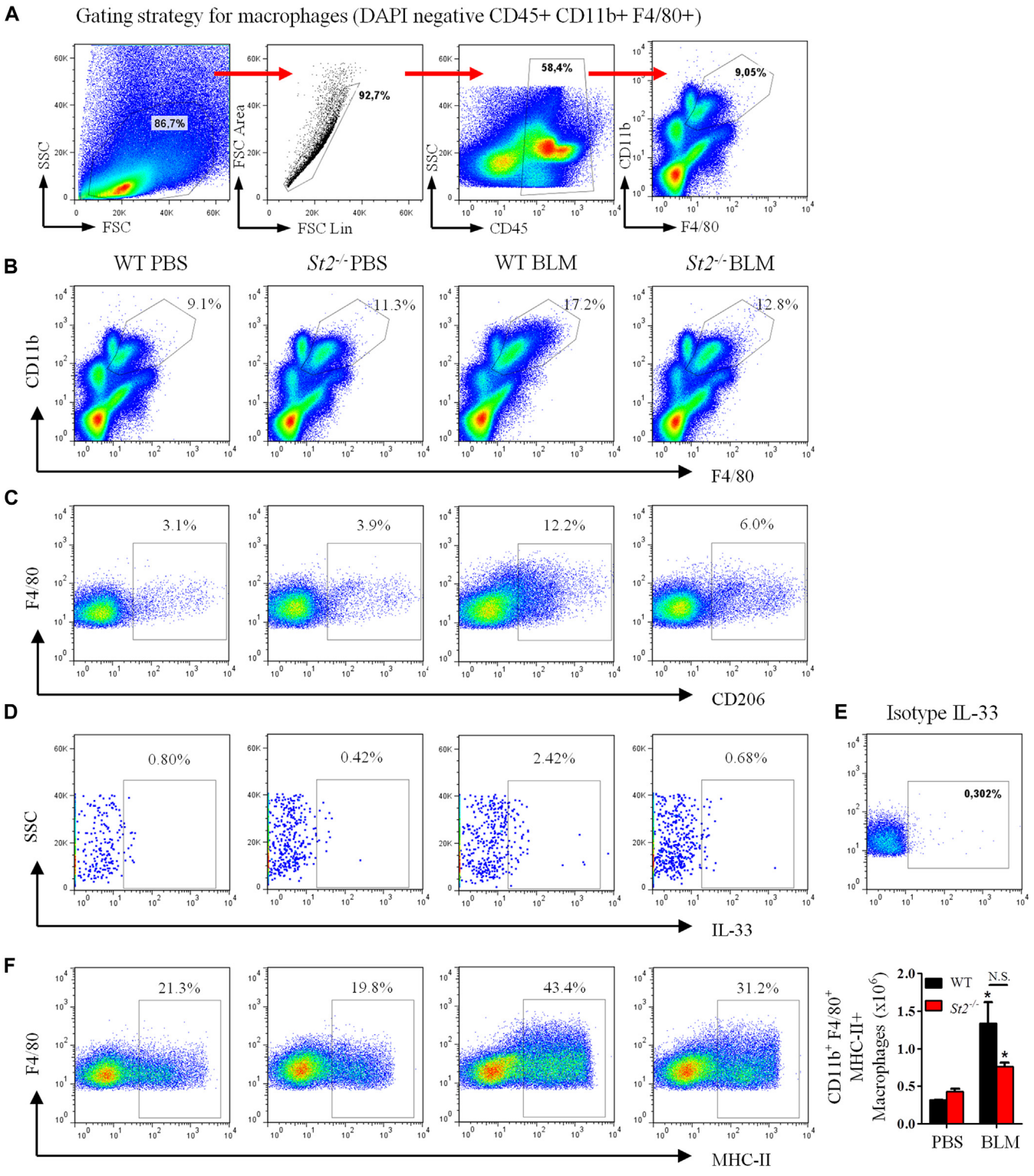


**FIG E4. A,** Flow cytometric gating strategy for analysis of macrophages in dispersed lung cell suspensions. **B-F,** WT or *St2*$^{-/-}$ mice were administered intranasally with PBS or bleomycin, and lung tissue harvested on day 7 was dispersed. The percentage of macrophage subsets in the tissue was determined by using flow cytometry. The percentage of macrophages (Fig E4, *B*), percentage of CD206$^{+}$ (M2) macrophages (Fig E4, *C*), percentage of IL-33$^{+}$ M2 macrophages (Fig E4, *D*), isotype control for the anti–IL-33–PE staining (Fig E4, *E*), and percentage and total number of MHC class II–positive macrophages (Fig E4, *F*) are shown. *Vertical bars* = SEMs (n = 5-6 mice per group per experiment). **P* < .05 compared with control values. Data are representative of 2 experiments. *N.S.*, Not significant.

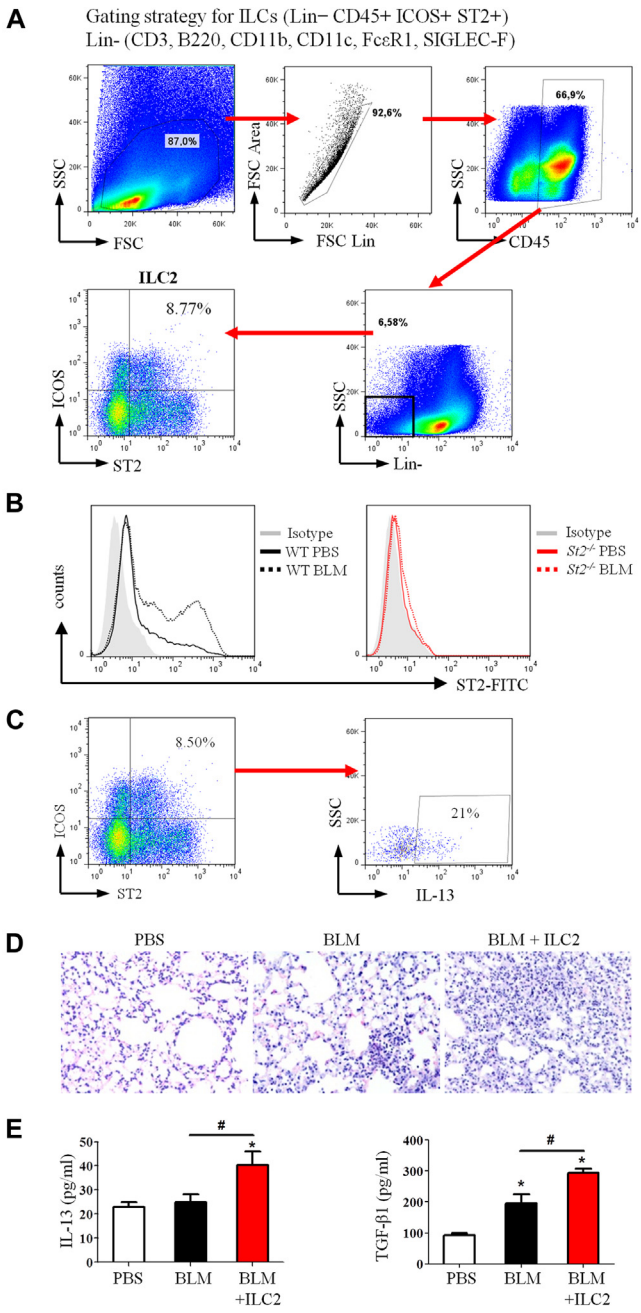


**FIG E5.** Induction and function of ILC2s in lung fibrosis of mice. **A,** Fluorescence-activated cell sorting gating strategy used for identification of ILC2s in murine lung tissue. **B,** ILC2s from WT but not *St2*$^{-/-}$ mice expressed ST2. **C,** Percentage of IL-13$^{+}$ ILC2s in lung tissue of WT mice after bleomycin *(BLM)* instillation. **D** and **E,** Adoptive transfer of ILC2s contributes to lung inflammation in mice. WT mice were instilled with PBS or bleomycin. Sorted ILC2s ($5 \times 10^5$) were adoptively transferred intranasally into mice 1 day after bleomycin instillation, and lung inflammation was assessed on day 7. Fig E5, *D*, Hematoxylin and eosin staining of lung tissues. Fig E5, *E*, Lung lavage fluid IL-13 and TGF-β1 concentrations quantified by means of ELISA. *Vertical bars* = SEMs (n = 5-6 mice per group per experiment). **P* < .05 compared with PBS control and #*P* < .05 compared with bleomycin control values. Data are representative of 2 experiments.

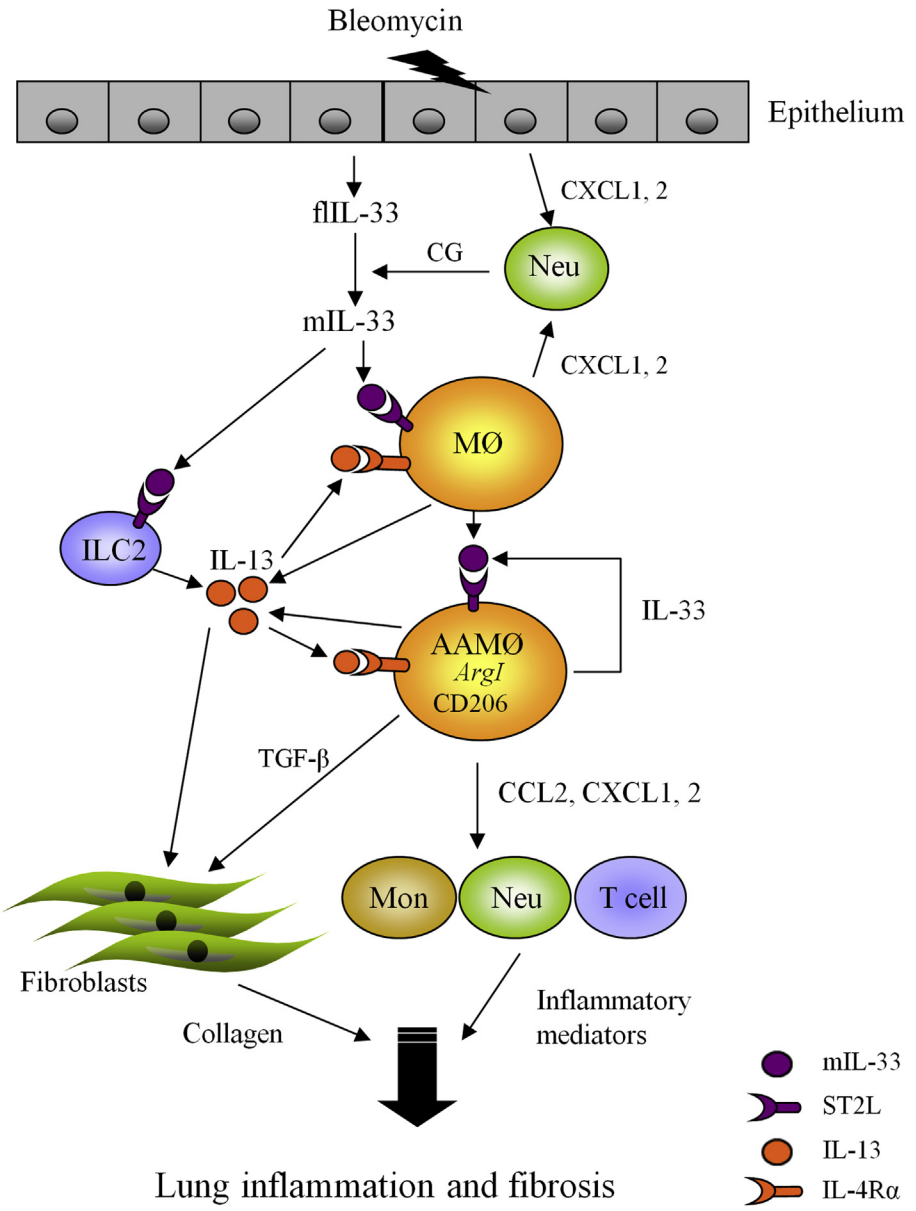


**FIG E6.** Schematic representation of the proposed mechanism of bleomycin *(BLM)*–induced IL-33 synthesis and its contribution to lung fibrosis. Neomycin triggers release of flIL-33 from damaged airway epithelial cells and recruitment of neutrophils. Neutrophil cathepsin G *(CG)* then processes flIL-33 to mIL-33. mIL-33 stimulates alveolar macrophages and ILC2s to produce IL-13. mIL-33 and IL-13 then synergistically polarize macrophages into the M2 macrophage phenotype, which produces more IL-33, IL-13, and TGF-β1 and in turn activates fibroblasts to proliferate and overproduce collagen. IL-33 also enhances macrophage production of chemokines, which induce the infiltration of neutrophils and lymphocytes into the lung and together might exacerbate lung inflammation and development of fibrosis. *Mon*, Monocytes; *Neu*, neutrophils.

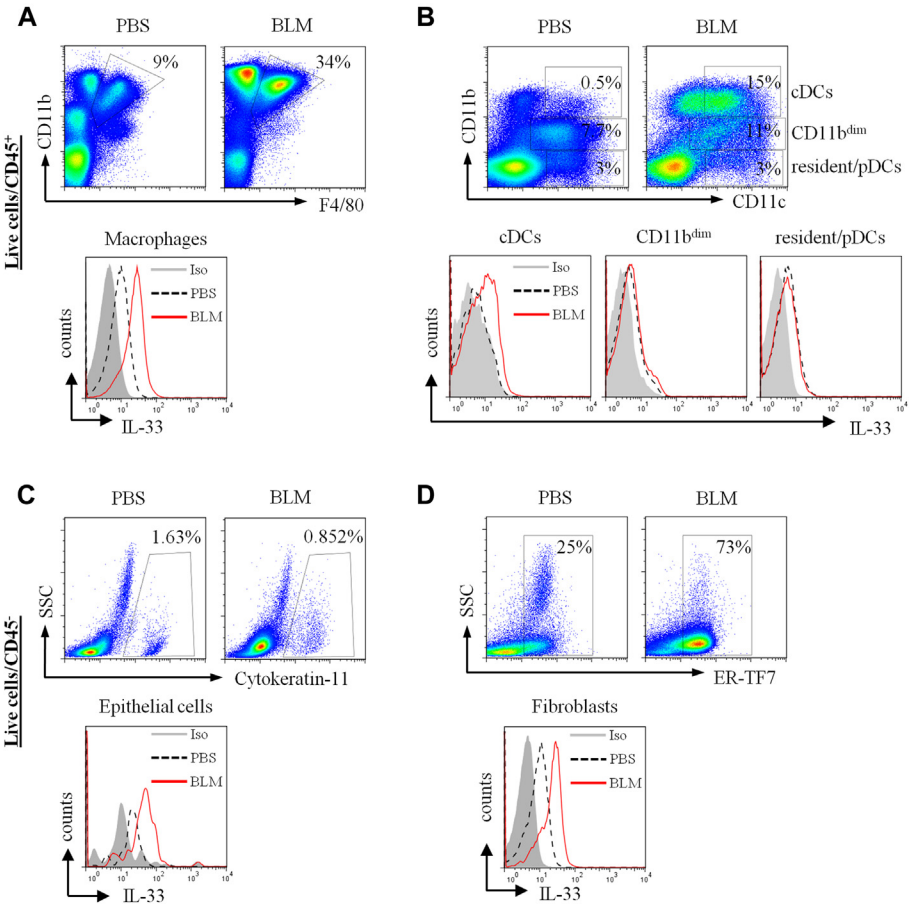


**FIG E7.** Detection of IL-33–producing cell populations in bleomycin-induced fibrotic lung tissue. Mice were given intranasal PBS or bleomycin *(BLM)* on day 0, and lung tissues were harvested on day 7. Tissues were dispersed, and live single-cell preparations were stained with fluorescently labeled antibodies to CD45 to categorize hematopoietic **(A** and **B)** and nonhematopoietic **(C** and **D)** cells. Staining for IL-33 and the different cell linage markers are as described in the Methods section. Fluorescence-activated cell sorting dot plots and histograms are presented. IL-33$^+$ cells were detected in macrophages (CD11b$^+$F4/80$^+$; Fig E7, *A*); conventional DCs (*cDCs*; CD11c$^+$), CD11b$^{dim}$, and resident/plasmacytoid DCs (Fig E7, *B*); epithelial cells (cytokeratin 11$^+$; Fig E7, *C*); and fibroblasts (ER-TF7$^+$; Fig E7, *D*). Data are representative of 3 experiments (n = 5-6 mice per group per experiment).

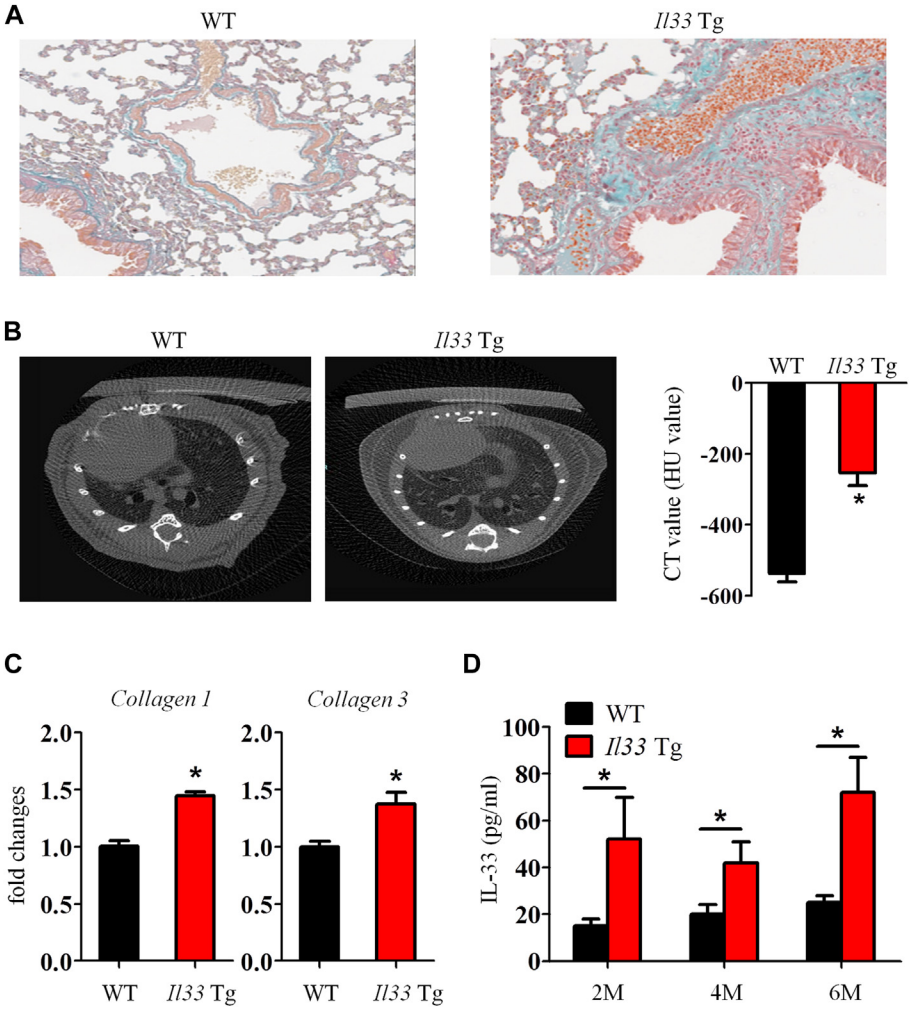


**FIG E8.** *Il33* transgenic mice have spontaneous lung fibrosis. WT and *Il33* transgenic mice were kept in pathogen-free conditions for up to 6 months. **A,** Lung tissues were stained for collagen deposition at 6 months. **B,** CT scan of mouse thorax was performed, and lung radiodensity scale (Hounsfield units *[HU]*) was calculated. **C,** Lung tissue collagen 1 and 3 mRNA expression was quantified by using qPCR. **D,** IL-33 concentration in mouse serum was quantified by means of ELISA at 2, 4, and 6 months of age. *Vertical bars* = SEMs (n = 10 mice per group per experiment). **P* < .05 compared with WT control values. Data are representative of 2 experiments.

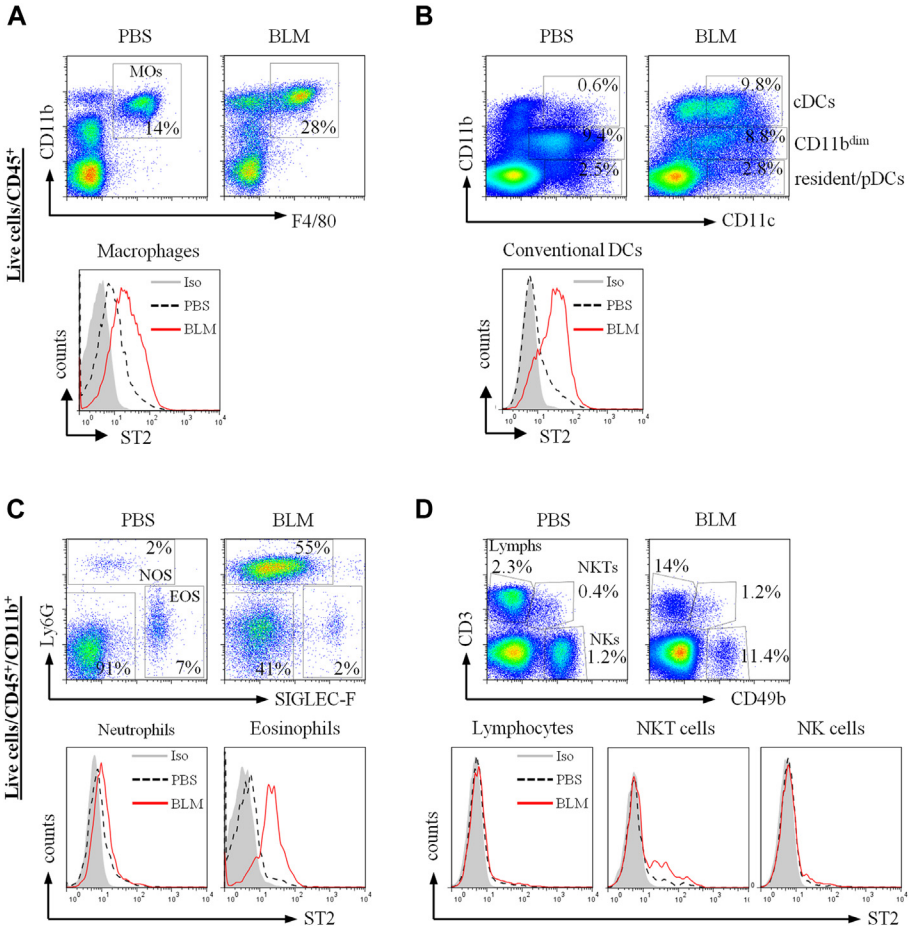


**FIG E9.** Detection of ST2$^+$ cell populations in bleomycin *(BLM)*–induced fibrotic lung tissue. Mice were given intranasal PBS or bleomycin on day 0, and lung tissues were harvested on day 7. Tissues were dispersed, and live single-cell preparations stained with fluorescently labeled antibodies to ST2 and different cell linage markers, as described in the Methods section. Fluorescence-activated cell sorting dot plots and histograms are presented. ST2 expression was detected on macrophages (CD11b$^+$F4/80$^+$; Fig E9, *A*), dendritic cells (CD11c$^+$, conventional DCs *[cDCs]*, CD11b$^{dim}$ and resident/plasmacytoid dendritic cells *[pDCs]*; Fig E9, *B*), granulocytes (neutrophils *[NOS]* and eosinophils *[EOS])*, and lymphocytes *(Lymphs)*, natural killer *(NK)* cells, and natural killer T *(NKT)* cells (Fig E9, *D*). Data are representative of 2 experiments (n = 5-6 mice per group per experiment).

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IMMUNOLOGY ORIGINAL ARTICLE

# Interleukin-33 exacerbates acute colitis via interleukin-4 in mice

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## Summary

**Interleukin-33 (IL-33) and its receptor ST2 are over-expressed in clinical colitis tissue. However, the significance of these observations is at present unknown. Significantly, we demonstrate here that *IL33* and *ST2* are the primary early genes induced in the inflamed colon of BALB/c mice following dextran sulphate sodium (DSS)-induced experimental ulcerative colitis. Accordingly diarrhoea and DSS-induced colon inflammation were impaired in ST2$^{-/-}$ BALB/c mice and exacerbated in wild-type mice by treatment with exogenous recombinant IL-33, associated respectively with reduced and enhanced expression of chemokines (CXCL9 and CXCL10), and inflammatory (IL-4, IL-13, IL-1, IL-6, IL-17) and angiogenic (vascular endothelial growth factor) cytokines *in vivo*. The exacerbation effect of treatment with recombinant IL-33 on DSS-induced acute colitis was abolished in IL-4$^{-/-}$ BALB/c mice. Hence, IL-33 signalling via ST2, by inducing an IL-4-dependent immune response, may be a major pathogenic factor in the exacerbation of ulcerative colitis.**

**Keywords:** colitis; early interleukin-33 expression; interleukin-4 deficiency; ST2 deficiency.

## Introduction

Ulcerative colitis (UC) is an inflammatory disease of the colon associated with recurring inflammation and the formation of ulcers.[1] This leads to clinical symptoms and signs including diarrhoea and serious complications, such as peritonitis and increased risk of colorectal cancer.[1] The aetiology of UC is largely unknown, which is the main reason why current therapeutic options are limited. Environmental and infectious disease factor-mediated barrier dysfunction and abnormal angiogenesis in gut epithelium are thought to play a critical role in the initiation and perpetuation of the disease.[1,2]

Dextran sulphate sodium (DSS) -induced colitis in mice is a well-established model for human UC.[3] Mice fed with DSS polymers develop disease similar to human UC, characterized by diarrhoea, colonic inflammation and ulceration. This is a result of direct toxic effects of DSS on the gut epithelial cells of the basal crypts.[3,4] The induction of acute DSS-induced colitis does not depend on lymphocytes;[4] therefore it is a particularly useful model to study innate immune mechanisms of the intestinal epithelium in the pathogenesis of colitis.

The pathogenesis of ulcerative colitis in humans and animal models is primarily associated with dysregulation of type II cytokines [interleukin-4 (IL-4), IL-5 and IL-13],[2,5–7] whereas type I [interferon-$\gamma$ (IFN-$\gamma$)], and pro-inflammatory [IL-1, IL-6, IL-17 and tumour necrosis factor-$\alpha$ (TNF-$\alpha$)] cytokines may also contribute to the pathogenesis, probably in the chronic phase of UC.[2,8–10] The early innate inflammatory signal(s) that coordinate the engagement of these cytokines are unresolved although IL-33, a new member of the IL-1 family, is a potential candidate.[11]

Interleukin-33 is a pleiotropic cytokine that signals via its receptor ST2 and can elicit different immune responses depending on context.[11,12] It is expressed primarily in the epithelium and endothelium and can be released when cells sense inflammatory signals or undergo necrosis.[11,12] The IL-33 receptor, ST2, is expressed by almost all innate cells but only by selected adaptive immune cells.[11–17] Interleukin-33 signalling via ST2 can induce both antigen-dependent and antigen-independent type II immune responses by directly activating a wide-range of innate immune cells including eosinophils, macrophages, nuocytes, mast cells or T helper type 2 (Th2)


 

and IL-5$^+$ Th cells *in vitro* and *in vivo*.[11–17] In addition, IL-33 can also promote Th1 and/or Th17 type responses in pro-inflammatory disorders in mice, by as yet undefined mechanisms.[18,19] Increasing evidence suggests that IL-33 and ST2 play a pathogenic role in inflammatory bowel disease.[20–23] Interleukin-33 and ST2 expression is increased in inflamed colonic mucosa and in the serum of patients with inflammatory bowel disease.[20–23] Experimental IL-33 gene-deletion impairs pathogenesis of colitis,[24] although the mechanisms by which the IL-33/ST2 system exacerbates colitis are unresolved.

The aims of this study were to elucidate the mechanisms by which IL-33 exacerbates experimental colitis in mice. Our study demonstrated that IL-33 and ST2 are the genes early induced in the colonic tissue during DSS-induced colitis. Furthermore, IL-33 exacerbates acute colitis in association with the induction of pro-inflammatory and angiogenic cytokines as well as chemokine production in an ST2-dependent and IL-4-dependent manner.

## Materials and methods

### *Mice*

BALB/c mice were purchased from Harlan Olac (Bicester, UK), and ST2$^{-/-}$, IL-4$^{-/-}$ and IL-4R$^{-/-}$ mice on a BALB/c background were generated as described previously.[13,17] Mice were housed in specific pathogen-free conditions at the University of Glasgow in accordance with the UK Home Office animal welfare guidelines.

### *The induction of DSS colitis*

For the induction of acute colitis, female mice were given 3·5% (weight/volume) DSS (ICN Biomedicals, Aurora, OH) in their drinking water from day 0 for 12 consecutive days. Some mice received recombinant IL-33 (1 $\mu$g/mouse/day) or PBS intraperitoneally daily from day 0 for 19 days. The IL-33 was produced and purified as previously described.[13] The body weight and stool consistency were monitored daily. Diarrhoea was scored as follows: 0 (normal); 2 (loose stools); 4 (watery diarrhoea).[25] Body weight loss was calculated as the difference between the baseline weight on day 0 and the body weight on a particular day.

### *Cytokine/chemokine measurements*

Colons were opened longitudinally and washed in sterile PBS supplemented with 1% penicillin/streptomycin (Life Technologies, Carlsbad, CA). Three segments from the distal colon of 1 cm in length were placed in 24 flat-bottom well culture plates (Costar, Cambridge, MA) containing fresh RPMI-1640 (Life Technologies) supplemented with 1% penicillin/streptomycin and incubated at 37° for 24 hr. Culture supernatants were then harvested, centrifuged at 13 000 ***g***, and stored at − 20°. Cytokine/chemokine concentrations were detected by a multi-cytokine/chemokine (20-plex) bead fluorescence assay (Invitrogen, Paisley, UK) according to the manufacturer's instructions, using a Luminex platform.

### *Histological analysis*

Colon specimens were fixed in 10% neutral formalin, embedded in paraffin and stained with haematoxylin & eosin. Histological examination was performed on three serial sections at six different sites of the colon and was scored blind using a standard histological scoring system.[25]

### *Meta-analysis of high-throughput transcriptomics data*

Raw RNA microarray (Affymetrix CEL) files in the public domain derived from mouse colon tissue response to DSS induction at days 0, 2, 4 and 6 were downloaded from the Gene Expression Omnibus (GEO, GSE22307 and ref 26) and analysed as previously described.[27] Briefly, the analysis of the differential gene expression patterns used Affymetrix Gene Chip Mouse Genome 430 2.0 Array.[26] The CEL files were normalized with the RMA algorithm and subjected to a highly stringent statistical analysis using one-way analysis of variance, followed by the Tukey honestly significant difference post-hoc test and multiple testing correction by applying the Benjamini–Hochberg false discovery rate ($P < 0{\cdot}05$) and using GENESPRING GX11 software (Agilent, Santa Clara, CA). The significantly expressed genes were selected by a standard cut-off at twofold increased expression compared with the values on day 0. These differentially expressed genes were then classified based on GENE ONTOLOGY (GO) software specifically for genes implicated in the 'regulation of inflammatory response' as well as the 'cytokines and chemokines' in the colonic epithelium of DSS-induced colitis in mice.

### *Statistical analysis*

Analysis using Student's *t*-test was applied to *in vitro* studies. Analysis between individuals in groups *in vivo* was by analysis of variance followed by Student's *t*-test. Results are expressed as mean ± SEM, and are representative of at least two individual experiments. $P < 0{\cdot}05$, was considered significant.

## Results

### IL-33 and ST2 are the major genes early induced in the colonic tissue in DSS colitis

While it has been suggested that *IL33* and *ST2* are expressed in colonic tissue and in epithelial cells in clinical

colitis,[20–23] the kinetics of their expression and relative expression compared with other DSS-induced genes in inflamed colonic tissue is unknown. To understand the inflammatory process associated with the initiation of colitis, we systematically studied the early colon gene expression profile of DSS-induced colitis by analysing the publicly available microarray datasets deposited in the GEO using a meta-analysis approach.[26,27]

We specifically focused on the expression of cytokines and chemokines, and genes implicated in the regulation of inflammation using the Gene Ontology Analysis module in GENESPRING GX11. Hierarchical clustering analysis showed that *IL33* was the strongest of the 40 differentially expressed cytokine and chemokine genes expressed early in the colonic tissue (see Supplementary material, Fig. S1A). Furthermore, *IL33* and its receptor; the ST2 gene (*IL1RL1*) were the most highly induced genes, among the 28 genes, involved in the regulation of the inflammatory response (Fig. S1B). The induced *IL33* message in colonic tissue was detectable from day 4, and *ST2* from day 6 after DSS administration (Fig. 1a and Fig. S1A,B). The expression levels of several other key inflammatory cytokine and chemokines, including IL-1$\beta$, IL-6, CXCL9 and CXCL10 were also significantly up-regulated (> 2-log fold) by DSS in the acute inflamed colonic tissue (Fig. 1a). However, Th2 (IL-4 and IL-5), Th1 (IFN-$\gamma$), IL-17 and the 'alarmin' (IL-1$\beta$ and HMGB1) cytokine genes were not significantly induced (Fig. S1A,B, and data not shown).

We further determined IL-33 protein levels *in vitro* in the cultured colonic tissue from mice that had received DSS or PBS as control as described in the Materials and methods. Consistent with the induction of *IL33* message (Fig. 1a), IL-33 secretion in cultured colonic tissue from mice 5 days after DSS administration was also significantly enhanced compared with that from PBS-administered control mice (Fig. 1b).

These results therefore demonstrated that IL-33 and ST2 are key genes induced early in the inflamed colon of DSS-treated mice, suggesting that this cytokine/receptor system may be associated with the development of acute colitis.

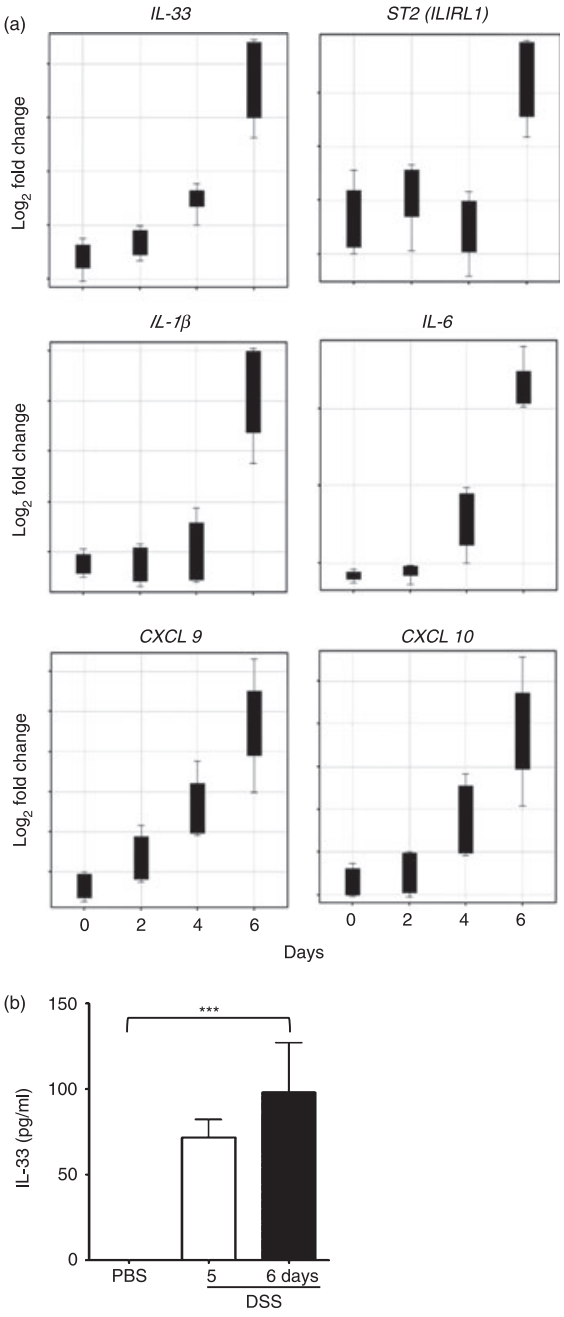


**Figure 1.** Interleukin-33 (*IL-33*) and *ST2* are the early induced genes in colonic tissue of colitic mice. (a) Genespring GX11 analysis of Affymetrix Gene-Chip Expression Data (GSE22307) from the colonic tissue of dextran sulphate sodium (DSS) -induced colitis on Days 0, 2, 4 and 6, respectively, in mice. The differentially expressed cytokines and chemokines and genes implicated in the regulation of inflammation has been obtained from the hierarchical clusters and displayed as Box Plots (Fig. S1). (b) *In vitro* IL-33 protein levels in cultured colonic tissues from mice five or 6 days after DSS or PBS administration, respectively.

### ST2 deficiency impairs, and exogenous IL-33 exacerbates DSS-induced colitis

We next defined the importance of IL-33 and ST2 in the pathogenesis of colitis in wild-type (WT) and ST2$^{-/-}$ mice *in vivo*. Groups of WT and ST2$^{-/-}$ BALB/c mice were given either PBS, DSS, IL-33 alone or DSS plus IL-33 and the development of clinical signs of colitis was monitored up to day 20. As shown in Fig. 2(a), WT mice that received DSS but not PBS or IL-33 alone developed diarrhoea from day 10, which was markedly delayed by 10 days in ST2$^{-/-}$ mice. In addition, exogenous IL-33 significantly exacerbated diarrhoea particularly on day 20 in the WT but not ST2$^{-/-}$ DSS colitis mice (Fig. 2a). However, as reported,[24] the injection of IL-33 or ST2 deficiency had no significant effect on body weight


 

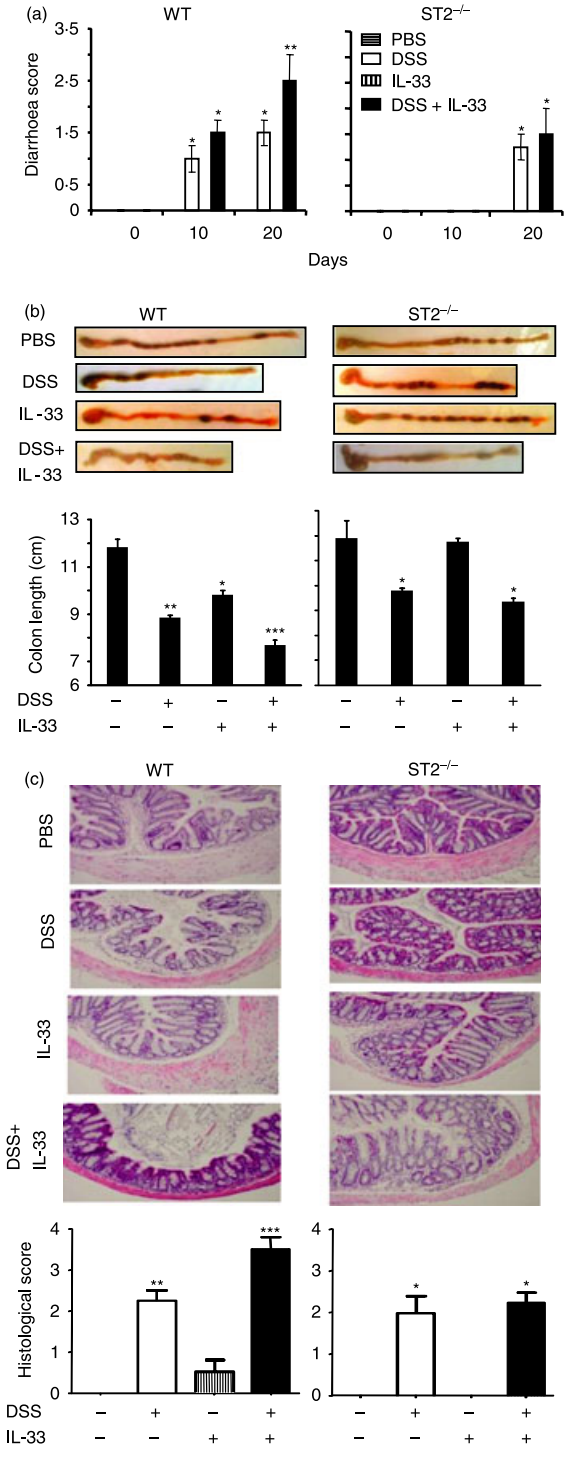


**Figure 2.** ST2 deficiency impairs and interleukin-33 (IL-33) injection exacerbates dextran sulphate sodium (DSS) colitis. Groups of wild-type (WT) and ST2$^{-/-}$ mice were either fed or not fed with DSS and injected with IL-33 (1 $\mu$g/mouse/day) or PBS. (a) Diarrhoea score and (b) colon length (on day 20) in the mice were determined as described in the Materials and methods. (c) The colon sections were stained with haematoxylin & eosin and scored. Data are representative of two experiments, $n = 5$ mice per group, $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$ compared with PBS controls.

changes in the acute stage of colitis in mice (see Supplementary material, Fig. S2A,B).

Consistent with these clinical parameters, compared with PBS control, the IL-33 alone group had slightly shortened, and the DSS, but in particular the DSS plus IL-33-treated group had markedly shortened, colon lengths (Fig. 2b) and colon inflammation (Fig. 2c) that persisted for at least 8 days after DSS was withdrawn. These pathogenic changes examined in groups of similarly treated ST2$^{-/-}$ mice were significantly reduced (Fig. 2b,c).

These results demonstrated that IL-33/ST2 signals have a pathogenic role in the early development and exacerbation of acute colitis.

## IL-33 enhances inflammatory cytokine and chemokine productions in colitis

Pro-inflammatory and angiogenic cytokines and inflammatory chemokines are closely associated with the pathogenesis of colitis.[2,10,28–30] We further assessed the serum cytokine/chemokine profile in colitis mice by 20-plex Luminex (see Materials and methods). Experimental colitis was induced in naive WT and ST2$^{-/-}$ mice, which were then treated with or without IL-33 or PBS as described above. The experiment was terminated on day 20 and serum samples were collected for multi-cytokine/chemokine analysis. Interleukin-33 given alone significantly enhanced IL-13 and CXCL9 but reduced IFN-$\gamma$ and IL-10 production in WT mice but not ST2$^{-/-}$ mice, compared with PBS control serum (Fig. 3). The group treated with DSS alone had no significant effect on serum cytokine concentration, except for increased IL-12 expression in WT and ST2$^{-/-}$ mice at this time-point. However, treatment with DSS plus IL-33 markedly enhanced most of the key pro-inflammatory cytokines and chemokines, including IL-4, IL-13, IL-6, IL-17, vascular endothelial growth factor (VEGF), CXCL9 and CXCL10 but reduced IL-10 and IFN-$\gamma$ production in WT mice but not ST2$^{-/-}$ mice compared with control mice treated with PBS, DSS or IL-33 alone.

Together these results suggest that IL-33 may promote colitis by inducing an ST2-dependent production of inflammatory type II (IL-4, IL-13), type 17 (IL-6, IL-17) and angiogenic (VEGF) cytokines and chemokines (CXCL9 and CXCL10) as well as by reducing type I (IFN-$\gamma$) and immuno-suppressive (IL-10) cytokine production in mice with DSS-induced colitis.

## IL-33 exacerbates colitis via IL-4

Type II cytokines (IL-4 and IL-13), in particular IL-4, have been reported to have a critical role in the initiation of DSS-induced colitis[5,7,28] and we found, above, that IL-33 can induce serum type II cytokines in mice with colitis (Fig. 3). To define the requirement of IL-4 in colitis exac-

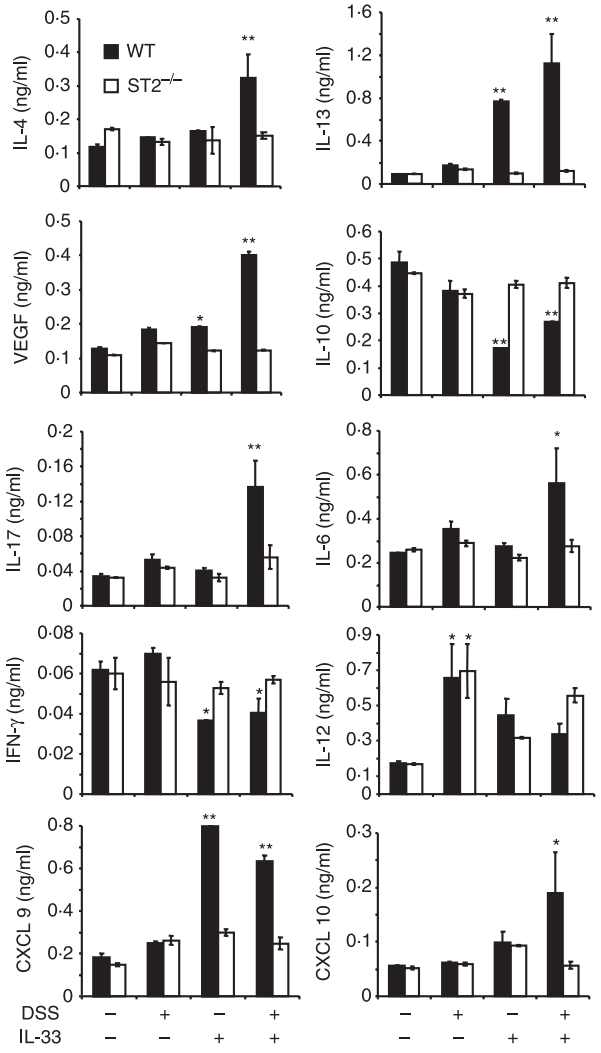


**Figure 3.** Interleukin-33 (IL-33) enhances key inflammatory cytokines and chemokines in colitis. Serum samples were collected on day 20 from the groups of wild-type (WT) and ST2$^{-/-}$ mice in Fig. 2 Total serum cytokine and chemokine concentrations were measured by luminex. Data are representative of two experiments, $n = 5$ mice per group, $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$ compared with the PBS control.

erbation and type II cytokine induction by IL-33, IL-4$^{-/-}$ mice were given the same treatments of PBS, IL-33, DSS or DSS plus IL-33 as described in Fig. 2. As reported,[27] IL-4$^{-/-}$ mice that received DSS to induce colitis showed a delayed appearance of diarrhoea on day 10 and had attenuated pathogenic changes in the colon compared with WT mice (Fig. 4a,b). More importantly, similar to ST2$^{-/-}$ mice, IL-33 failed to exacerbate these clinical and pathological parameters of colitis in the IL-4$^{-/-}$ mice. Compared with WT controls, changes in colon length and histological score associated with administration of IL-33 were also not apparent in IL-4$^{-/-}$ mice (Fig. 4b).

In addition, IL-4 deficiency abolished the production of IL-13, IL-12, CXCL9 and VEGF in the IL-33-treated group, IL-12 and VEGF in the DSS-treated group and IL-5, IL-13, IL-12, CXCL9 and VEGF in the DSS plus IL-33-treated group compared with cytokine and chemokine induction in similarly treated WT mice on day 20 (Fig. 4c). However, the serum concentrations of IL-10 were not affected by IL-4 deficiency.

We further investigated the importance of IL-4 receptor (IL-4R) in the context, which is required for both IL-4 and IL-13 signalling. We found that similar to ST2$^{-/-}$ and IL-4$^{-/-}$ mice, the shortened colon lengths in DSS or DSS plus IL-33 treated WT mice were also prevented in the groups of similarly treated IL-4R$^{-/-}$ mice (see Supplementary material, Fig. S3A). The reduced colon pathogenic change was accompanied by reduced IFN-$\gamma$ and TNF-$\alpha$, but enhanced IL-4 and IL-13 production in colon cultures in IL-4R$^{-/-}$ mice groups compared with the groups of similarly treated WT mice (Fig. S3B). The enhanced IL-4 and IL-13 may be a result of the loss of consumption of these cytokines in the IL-4R$^{-/-}$ mice tissues.

Therefore, these results suggest that IL-33 exacerbates colitis primarily via IL-4.

## Discussion

Data reported in this comprehensive study reveal a hitherto unrecognized effect and mechanism by which the IL-33/ST2 axis exacerbates DSS-induced colitis. Increasing evidence suggests that the development of UC may be attributed to intestinal epithelial barrier dysfunction and abnormal angiogenesis.[1–4] Our results contribute to this evidence and suggest that colon-derived IL-33 may be an additional key pathogenic factor that links epithelial damage and the initiation of colitis for several reasons:

(i) Interleukin-33 may function as a novel epithelial 'alarmin', similar to high-mobility group box 1 (HMGB1) and IL-1$\beta$, that initiate early inflammatory immune responses.[31] Interestingly, we found that IL-33, but not IL-1$\beta$ and HMGB1, is the earliest inflammatory cytokine induced in inflamed colonic epithelium in colitis (Fig. 1 and data not shown). Hence, colon-derived IL-33 may be a critical initiator of pathogenesis of DSS colitis. (ii) ST2$^{-/-}$ mice have impaired colitis (Fig. 2). (iii) IL-33 is capable of specifically inducing the key pathogenic cytokines (IL-4, IL-5, IL-13, IL-6, IL-17, IFN-$\gamma$, TNF-$\alpha$ and VEGF) and chemokines but reducing immunosuppressive (IL-10) cytokines in DSS-induced colitis via ST2 (Fig. 3).

Although it is recognized that type II cytokines, IL-4, IL-5 and IL-13 play a pathogenic role in the development of UC,[5,7,28] until now, it was unknown how these typical Th2 cytokines were induced in the innate context of colitis and whether these cytokines contributed to the IL-33-mediated effect. Our mechanistic studies suggest that IL-33 can induce these type II cytokines and directly via IL-4 and IL-4R in colitis. It is well documented that IL-


 

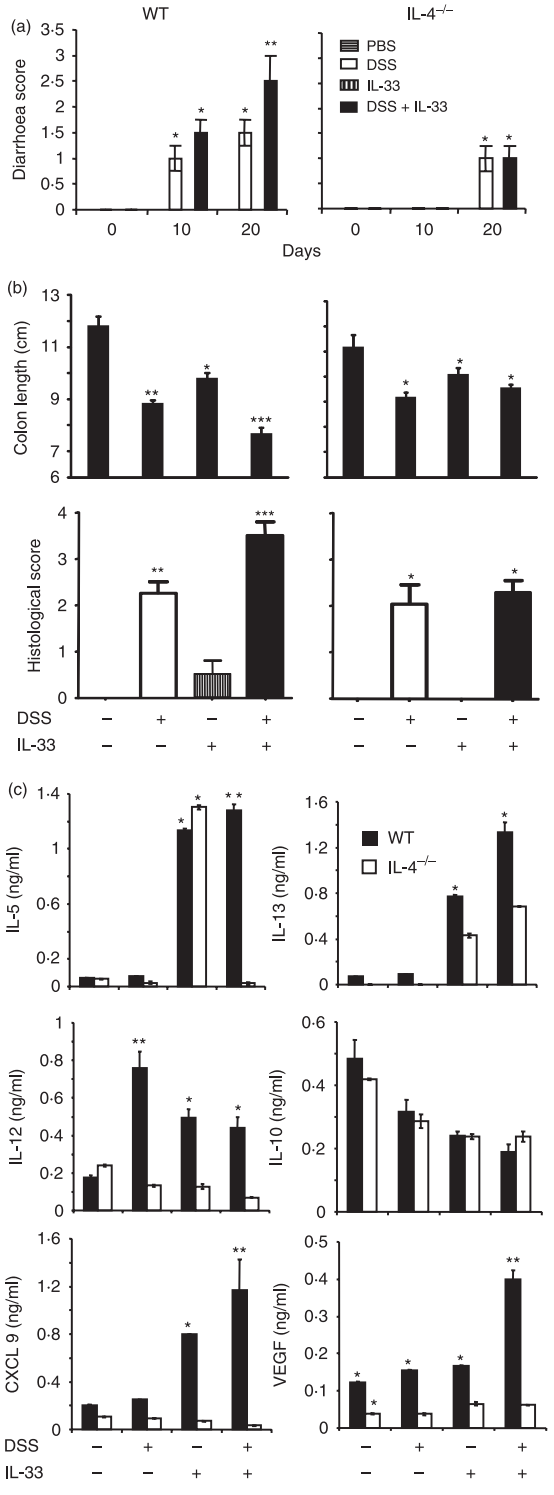


**Figure 4.** Interleukin-33 (IL-33) exacerbates colitis via IL-4. Groups of wild-type (WT) and IL-4$^{-/-}$ mice were injected with IL-33 as above. (a) Diarrhoea score, (b) colon length and histological score in the mice were determined as described in the Materials and methods. (c) The levels of serum cytokine were measured by luminex. Data are representative of two experiments, $n = 5$ mice per group, $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$ compared with the PBS group.

33 can induce all these type II cytokines by an array of innate cells, including eosinophils, basophils, mast cells, but not nuocytes which only produce IL-5 and IL-13, not IL-4[12–17] and data not shown). In contrast, T cells, which are the key cells expressing type II cytokines in allergy and asthma, are not the main IL-4 producers in this innate immune UC model, because naive T cells do not express ST2 in the absence of T-cell receptor activation and are thus unresponsive to IL-33.[14,15]

Our results also show for the first time that IL-4 is required for IL-33-mediated exacerbation of colitis, and for subsequent VEGF and CXCL9 production (Figs. 3 and 4). VEGF is a major pro-angiogenic cytokine and plays an important role in the pathogenesis of colitis by enhancing colonic permeability and facilitating migration of inflammatory cells.[29] CXCL9 and CXCL10 are the key chemokines for the recruitment of monocytes and macrophages, and these are intimately associated with the pathogenesis of colitis.[30,32] Together, these results provide a possible mechanism underlying the IL-33 / IL-4 pathogenic pathway in colitis.

Interleukin-12 and IL-17 are the key cytokines for type I and 17 responses and are also thought to play pathogenic roles in UC, Crohn's disease and the chronic stage of DSS-induced colitis.[2,8,10] We noted in this study that IL-33 can also induce serum IL-12 and IL-17, at the later stages of the disease, 20 days after DSS administration (Fig. 3). This suggests that in addition to its role in the early stages of disease, IL-33 may also contribute to the switching of the early type II to late type I and IL-17 responses in the chronic stages of UC and Crohn's disease. Whereas it is still unclear how IL-33 induces IL-12 and IL-17 in colitis, as Th1 and Th17 cells do not express ST2, it is likely that IL-33 may promote these responses via innate cells.[18,33]

It is noteworthy that changes in the severity of colitis caused by IL-33 injection or ST2 deficiency were not significantly associated with a change in body weight in the mice (Fig. S2A,B). This is consistent with a previous study showing identical body weight loss in WT C57BL/6 and IL-33$^{-/-}$ mice when fed with DSS.[24] Intriguingly, compared with WT mice, the IL-33$^{-/-}$ mice had a delayed recovery in body weight after withdrawal of DSS.[24] However, this was not the case in ST2$^{-/-}$ mice in the present study and the reason is currently unclear. It may be because of the differences in genetic background of the mice and experimental conditions or the ST2-independent bioactivity of full-length IL-33 as previously suggested.[34]

Furthermore, recent evidence suggests that injection of IL-33 may have a beneficial effect on chronic DSS-induced colitis or trinitrobenzene sulphonic acid-induced colitis, a model of Crohn's disease in mice,[35,36] suggesting that IL-33 may play a complex role in different types and throughout the duration of colitis. More studies are needed to clarify this issue.

Interleukin-33 is clearly expressed in the inflamed mucosa of patients with inflammatory bowel disease, particularly in UC, and is reduced after anti-TNF-α therapy.[20–23] In these cases mucosal expression of IL-33 is also mostly localized to intestinal epithelial cells[20,21,23] and in activated sub-epithelial myofibroblasts.[22] However, the clinical relevance of the IL-33/ST2 system in inflammatory bowel disease is unknown. Our results have extended these clinical findings with a putative mechanism and suggest that colon-derived IL-33 may represent an important factor for the development and exacerbation of UC.

## Acknowledgements

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## Disclosure

The authors have no financial conflicts of interest.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** The early gene profile in colonic epithelia in dextran sulphate sodium (DSS) colitis mice. Genespring GX11 analysis of Affymetrix Gene-Chip Expression Data (GSE22307) from the colonic epithelium of DSS-induced mouse colitis on Days 0, 2, 4 and 6, respectively. The differentially expressed genes were then classified and clustered based on Gene Ontology (GO) Analysis to decode the differentially expressed genes in (A) Cytokines and Chemokines and (B) Regulation of Inflammatory Response.

**Figure S2.** Interleukin-33 (IL-33) injection and ST2 deficiency do not significantly affect body weight in dextran sulphate sodium (DSS) colitis mice. Groups of wild-type (WT) and $ST2^{-/-}$ mice were fed with or without 3·5% DSS and/or injected with or without IL-33. (A) Body weight in WT colitis mice with or without IL-33 (B) Body weight in WT and $ST2^{-/-}$ colitic mice. Data are representative of two experiments, $n = 8$ mice per group.

**Figure S3.** Role of interleukin-4 receptor (IL-4R) in IL-33 exacerbates colitis. Groups of wild-type (WT) and $IL\text{-}4R^{-/-}$ mice were either fed or not fed with dextran sulphate sodium (DSS) and injected with IL-33 as above. (A) Colon length score in the mice was determined. (B) The cytokine levels in cultured colonic tissues from PBS, DSS or DSS plus IL-33-treated mice were measured by luminex as described in Materials and methods. $n = 5$ mice per group, $^{*}P < 0{\cdot}05$ compared with PBS group.

**Fig. S1**

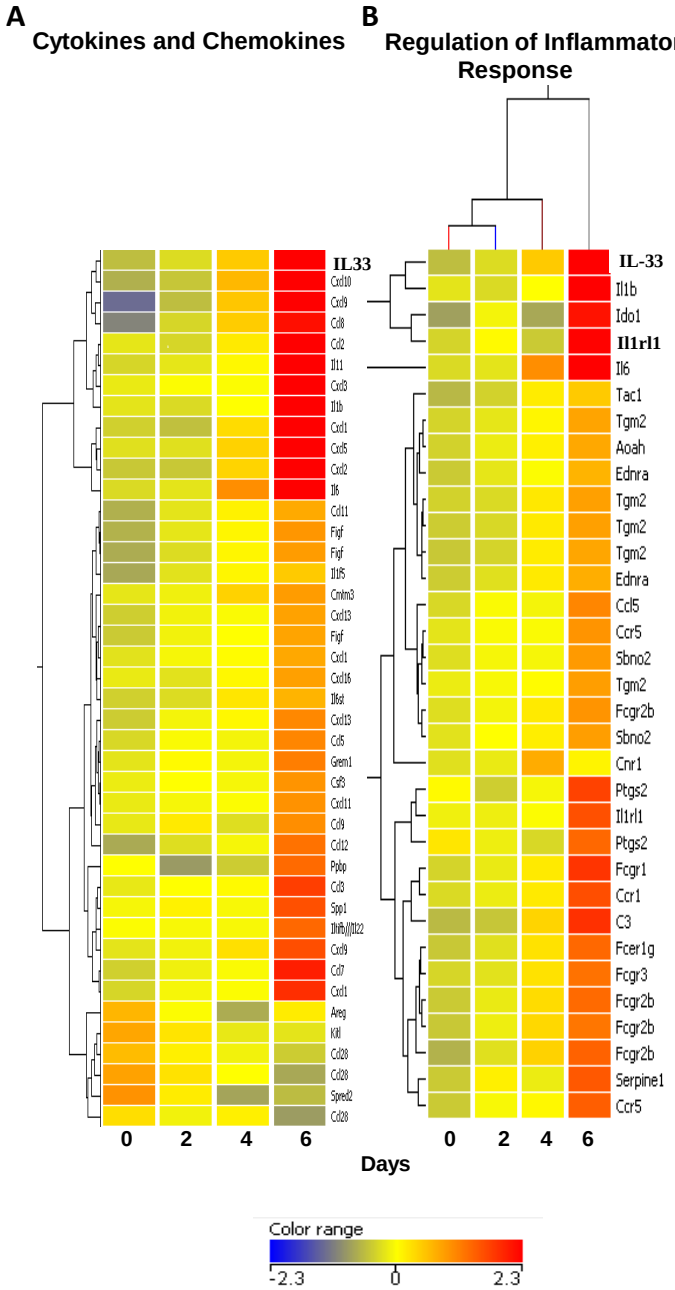

A
Cytokines and Chemokines
IL33
Cxcl10
Cxcl9
Ccl8
Ccl2
Il11
Cxcl3
Il1b
Cxcl1
Cxcl5
Cxcl2
Il6
Ccl11
Figf
Figf
Il1f5
Cmtm3
Cxcl13
Figf
Cxcl1
Cxcl16
Il6st
Cxcl13
Ccl5
Grem1
Csf3
Cxcl11
Ccl9
Ccl12
Pplp
Ccl3
Spp1
Il1rl1/Il122
Cxcl9
Ccl7
Cxcl1
Areg
Kitl
Ccl28
Ccl28
Spred2
Ccl28
B
Regulation of Inflammatory Response
IL-33
Il1b
Ido1
Il1rl1
Il6
Tac1
Tgm2
Aoah
Ednra
Tgm2
Tgm2
Tgm2
Ednra
Ccl5
Ccr5
Sbno2
Tgm2
Fcgr2b
Sbno2
Cnr1
Ptgs2
Il1rl1
Ptgs2
Fcgr1
Ccr1
C3
Fcer1g
Fcgr3
Fcgr2b
Fcgr2b
Fcgr2b
Serpine1
Ccr5
0 2 4 6
0 2 4 6
Days
Color range
-2.3
0
2.3

**Fig. S2**

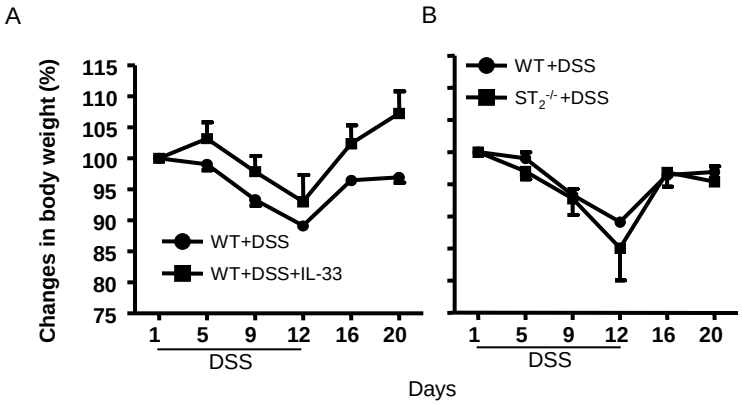
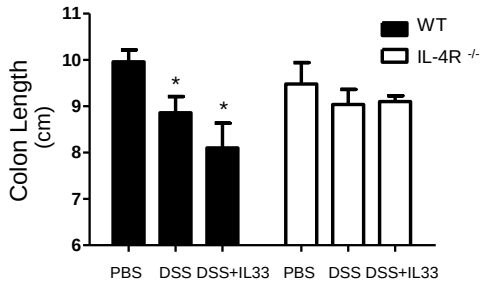
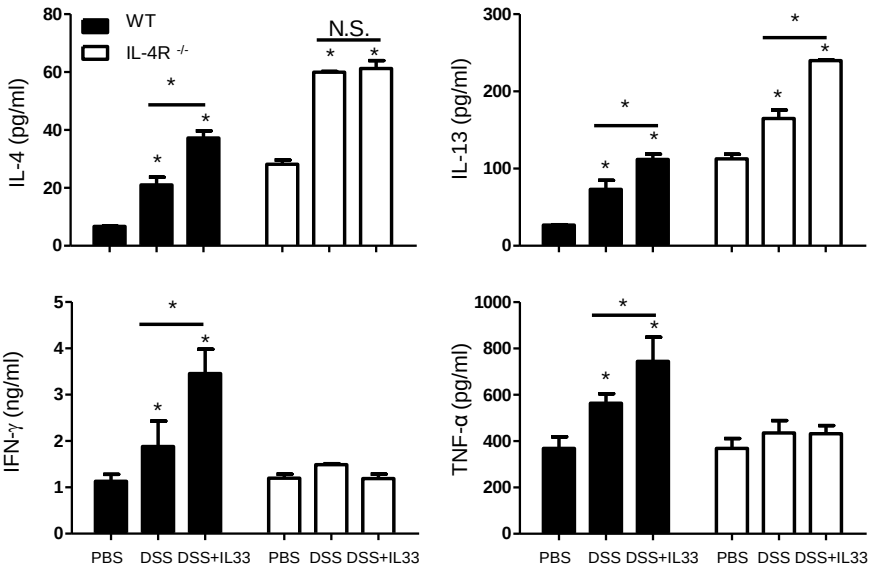

A
B
Changes in body weight (%)
115
110
105
100
95
90
85
80
75
WT+DSS
WT+DSS+IL-33
WT+DSS
ST2-/-+DSS
1
5
9
12
16
20
DSS
DSS
Days

**Fig. S3**

A


Colon Length (cm)
WT
IL-4R -/-
PBS
DSS
DSS+IL33
PBS
DSS
DSS+IL33

B


IL-4 (pg/ml)
WT
IL-4R -/-
N.S.
IL-13 (pg/ml)
IFN-γ (ng/ml)
TNF-α (pg/ml)
PBS
DSS
DSS+IL33
PBS
DSS
DSS+IL33