의학박사 학위논문

The role of airway innate lymphoid cells in respiratory diseases

호흡기 질환에서 기도 내 선천성 림프구 세포의 역할에 대한 연구

2020년 8월

서울대학교 대학원

의과학과 의과학전공

김 지 현

A thesis of the Degree of Doctor of Philosophy

호흡기 질환에서 기도 내 선천성 림프구 세포의 역할에 대한 연구

The role of airway innate lymphoid cells in respiratory diseases

August, 2020

The Department of Biomedical Science

Seoul National University

College of Medicine

Jihyun Kim

Abstract

The role of airway innate lymphoid cells in respiratory diseases

Jihyun Kim

Department of Biomedical Sciences

Seoul National University College of Medicine

Recent studies have emphasized the role of innate lymphoid cells (ILCs) in the development of respiratory diseases including asthma, pulmonary infection, and chronic obstructive pulmonary disease (COPD). The involvement of group 2 ILCs (ILC2s) in asthma is well studied, however, the participation of other types of ILCs in the development of asthma and COPD remains unclear.

In Chapter I, I aimed to understand the role of various ILCs in the patients with asthma, especially their effect on the polarization of macrophages. In patients with asthma, it is reported that ILC2s are increased in peripheral blood, bronchoalveolar lavage fluid (BALF), and induced sputum of the patients compared to healthy controls. However, how each subset of ILCs is related to asthma pathogenesis is largely unknown in asthma patients. To understand the association

of ILCs in patients with asthma, each subset of ILCs in induced sputum from 51 steroid-naïve asthma patients and 18 healthy donors are analyzed by flow cytometry. I also analyzed activated macrophages, which are target immune cells interacting with ILCs, in the induced sputum. In addition to ILC2s, ILC1s and ILC3s were increased in the induced sputum from asthmatics when compared with the healthy controls. The dominance of macrophages in induced sputum was more prominent in asthmatics than healthy controls. According to the inflammatory signature, patients with eosinophilic asthma had more ILC2s and M2 macrophages while those with non-eosinophilic asthma presented a M1 macrophages dominant profile. A positive correlation between ILC2s and M2 macrophages and that of ILC1s/ILC3s and M1 macrophages were observed. Co-culture of ILC2s with alveolar macrophages (AMs) induced the expression of M2 macrophage-related genes, whereas co-culture of ILC1s and ILC3s with AMs induced the expression of M1 macrophage-related genes *via* cytokine secretion as well as cell-cell contact. Taken together, a different subset of ILCs regulates the polarization of macrophages, contributing to developing the distinct phenotype of asthma.

In Chapter II, I aimed to investigate the role of ILC3s in the pathogenesis of emphysema, which is one of main types of COPD, especially their relationship with serum amyloid A (SAA). SAA is one of acute phase proteins, upregulated in response to inflammation. SAA acts as an endogenous ligand for immune cells, which induces the expression of several cytokines such as IL-1β, IL-6, TNFa, IL-33, and IL-10. It has been reported that the level of SAA is increased in plasma and lung tissue in COPD. I observed that the level of SAA and ILC3s were increased in a mouse model of emphysema. When recombinant SAA was injected into the lung,

neutrophilic inflammation and phenotype of emphysema occurred. Moreover, the prevalence of IL-17A production by ILC3s was increased, and IL-1β secretion from neutrophils is responsible for this increase. Depletion of neutrophils in SAA-injected mice ameliorated IL-17A secretion from ILC3s, and emphysema-like phenotype and inflammation was reduced by deletion of ILCs in mouse model. In human sputum, SAA-neutrophils-ILC3s had correlation with each other and increased ILC3s were correlated with decreased lung function. Taken together, SAA activates neutrophils to secrete IL-1β, which induce IL-17A production by ILC3s, and these responses might promote emphysema.

In summary, increased ILC subsets in asthma and emphysema are involved in decision of phenotypes of diseases. In asthma, ILC2s are related to eosinophilic asthma by regulating a polarization of M2 macrophages, while ILC1s and ILC3s are associated with neutrophilic inflammation *via* M1 macrophage polarization. In emphysema model, ILC3s are critical for an emphysema phenotype and activation of ILC3s is regulated by IL-1β secretion from SAA-activated neutrophils. These studies suggest a new insight that ILCs can be a therapeutic target for resolving pathological phenotypes of respiratory diseases.

keywords: Innate lymphoid cells (ILCs), Respiratory disease, Phenotype, Macrophages, Polarization, Serum amyloid A (SAA), Neutrophils

Student Number: 2015-23238

*** Chapter I was previously published in Journal of Allergy and Clinical Immunology (2019)**

Contents

List of Tables and Figures

---

4. Chapter II

Tables

Figures

---

5. Conclusion

List of Abbreviations

AECOPD: acute exacerbation of chronic obstructive pulmonary disease

AHR: airway hyperresponsiveness

Ahr: aryl hydrocarbon receptor

AMs: alveolar macrophages

AR: allergic rhinitis

Areg: amphyregulin

Arg1: arginase 1

BALF: bronchoalveolar lavage fluid

BCR: B cell receptor

BMI: Body mass index

CCR6: C-C chemokine receptor type 6

CGRP: calcitonin gene-related peptide

COPD: chronic obstructive pulmonary disease

CRS: chronic rhinosinusitis

CRTH2: chemoattractant receptor-homologous molecule expressed on Th2 cell

CysLTR1: cysteinyl leukotriene receptor 1

FEV1: forced expiratory volume in 1 second

FPR2: formyl peptide receptor 2

FVC: forced vital capacity

GC: glucocorticoids

GFL: Glial-derived neurotrophic factor family of ligands

GM-CSF: granulocyte macrophage colony stimulating factor

GSEA: gene set enrichment assay

ICOS: Inducible T-cell costimulator

ILCs: innate lymphoid cells

IL-7R: interleukin 7 receptor

Nos2: Nitric oxide synthase 2

LPS: lipopolysaccharide

LT: leukotriene

LTi: Lymphoid tissue inducer

MFI: mean fluorescence intensity

MHC class II: class II major histocompatibility complex

MMP: matrix metalloproteinase

Mrc1: mannose receptor C type 1

NCR: natural cytotoxicity receptor

NLRP3: NOD-, LRR- and pyrin domain-containing protein 3

NMU: neuromedin U

OVA: ovalbumin

PBMC: peripheral blood mononuclear cell

PC20: provocation concentration of methacholine leading a fall of 20% of FEV1

PD-1: Programmed cell death protein 1

PG: prostaglandin

PNECs: pulmonary neuroendocrine cells

PPE: porcine pancreas elastase

Ptgs2: Prostaglandin-endoperoxide synthase 2

Retnla: Resistin-like molecule alpha

SAA: serum amyloid A

SFB: segmented filamentous bacteria

STRA: severe therapy-resistant asthma

TCR: T cell receptor

TGFβ: transforming growth factor beta

TNFα: tumor necrosis factor alpha

TSLP: thymic stromal lymphopoietin

WBC: white blood cell

1. Background

1.1 Innate lymphoid cells

Innate lymphoid cells (ILCs) are innate counterparts of T lymphocytes which mirror several characteristics and functions of T cells[1, 2]. ILCs are largely divided into three groups, group 1, 2, and 3 ILCs (ILC1s, ILC2s, and ILC3s), according to the expression of transcription factors and cytokines[3]. Group 1 ILCs comprise natural killer cells (NK cells) and non-cytotoxic ILC1s which express T-bet and IFNγ. Group 2 ILCs (ILC2s) produce Th2 cytokines, such as IL-5, IL-9, and IL-13 and express GATA3 as a key transcription factor. Group 3 ILCs are divided into 3 cell types, $CCR6^{+}$Lti cells, natural cytotoxicity receptor $(NCR)^{-}$ILC3s, and NCR^{+}ILC3s and RORγt is a key transcription factor of ILC3 lineage commitment. Lti cells are indispensable for formation of secondary lymphoid structures by producing lymphotoxins, while NCR^{-}ILC3s and NCR^{+}ILC3s mainly produce IL-17, IL-22, and GM-CSF, which are important for maintenance of intestinal homeostasis and anti-microbial response. However, recent studies show that ILCs are heterogeneity and plasticity between subpopulations by adaptation to micro-environmental conditions.

ILCs are widely distributed to several tissues, but especially dominant in mucosal tissues, such like lung, skin, and intestine[4, 5]. In mucosal tissues, ILCs play an important role in multiple innate immune responses interacting with other innate immune cells, which induce acute and chronic inflammation, and host defense against pathogens or allergens (Figure 1.1) [6]. By secreting effector cytokines, ILC1s are involved in intracellular anti-viral responses, ILC2s are involved in anti-helminth or anti-fungi response and allergic inflammation, and ILC3s are involved in host

defense against extracellular bacteria and chronic inflammation[7]. Moreover, ILCs act as an immune-regulator which regulate adaptive immune cells both in soluble factor-dependent and direct contact-dependent manners[8]. ILCs activate dendritic cells or macrophages which are important for activation of T lymphocytes[9, 10]. Also, ILCs produce soluble mediators activating T cells or B cells[11, 12] and express regulatory molecules on their surface to interact directly with T cells[13-15]. Therefore, understanding of precise roles of ILCs in specific diseases is important for understanding immune responses in disease conditions and exploring therapeutic targets.

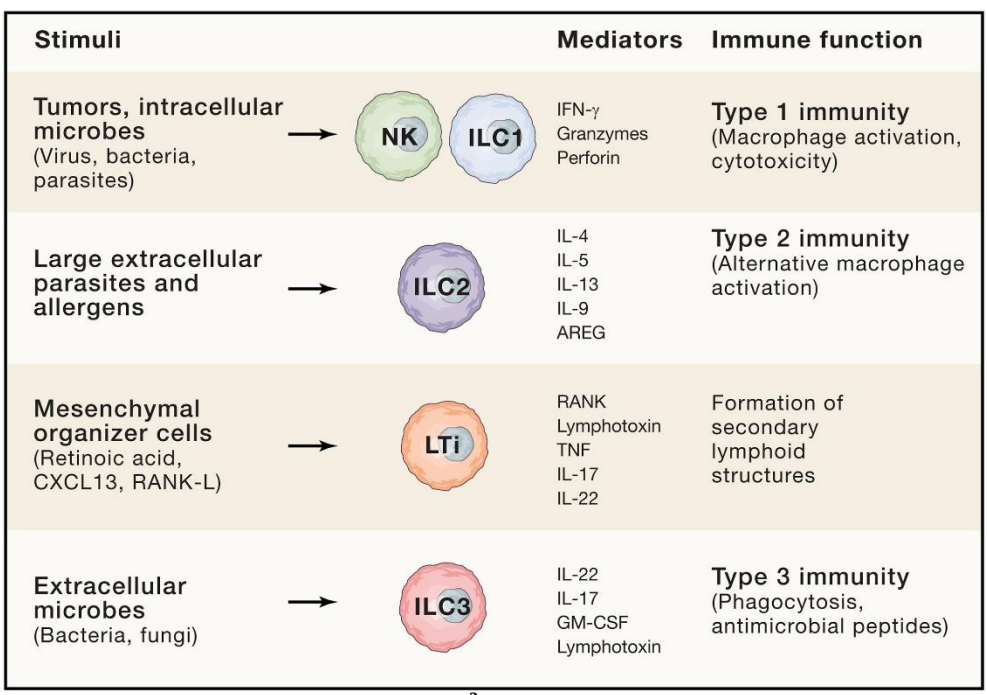

Figure 1.1 Immune functions of ILCs[2]

1.2 The role of ILCs in respiratory inflammatory diseases

1.2.1 Asthma

Asthma is one of the chronic inflammatory diseases, which affects about 300 million

people worldwide[16]. The major symptoms of asthma are difficulty in breathing, coughing and wheezing caused by airway hyperresponsiveness (AHR), mucus hyperproduction and airway remodeling[17]. Asthma is very heterogeneous disease which has many endotypes and has many factors that can trigger the disease, such as allergen exposure, airway infection, genetic background, excessive exercise and stress[18]. Th2 cell-mediated type 2 immune responses are known to be the major immune mechanisms of asthma[19]. Thus, the therapeutic approaches for asthma has been focused on attenuating type 2 cytokines and mediators that can enhance Th2 cell functions. However, there are some patients who do not respond to these therapies. These patients have non-allergic asthma in which neutrophilic inflammation is a main immunological feature[20, 21].

Recently, there are several studies inform us that ILCs are involved in pathogenesis of asthma[22]. Especially, like T cells, most of these studies concentrate on ILC2s which are responsible to similar immune mechanisms with Th2 cells[23]. In mouse model of asthma, it is revealed that ILC2s have a key role in early development of asthma. ILC2s release IL-5 to activate eosinophil recruitment and IL-13 to affect hyper-mucus production and tissue remodeling[24]. Also, ILC2s secrete amphyregulin (Areg), a family of epidermal growth factors, upon influenza virus infection, which is involved in tissue repair[25]. In non-allergic model of asthma, such as obesity-induced asthma, ILC3s are activated by IL-1β released from macrophages and produce IL-17A to enhance neutrophil infiltration and airway hyperresponsiveness (AHR)[26].

In human, it seems that ILC2s also contribute to the pathology of eosinophilic asthma. Type 2 cytokine-producing ILC2s are increased in blood and

sputum of severe asthma patients than mild patients[27]. The severity is only related to the amount of type 2 cytokines produced by ILC2s, but not by Th2 cells and eosinophils. Also, IL-5^{+}IL-13^{+} ILC2s in sputum are significantly increased in patients with sputum eosinophilia. Moreover, there is a research in that ILC2s are increased in sputum of children with severe therapy-resistant asthma[28]. It reveals that ILC2s in human asthma patients also play a key role in development of asthma. However, how several subsets of ILCs are involved in asthma pathophysiology in human remains to be explored.

1.2.2 Chronic obstructive pulmonary disease (COPD)

Chronic obstructive pulmonary disease (COPD) is chronic inflammatory disease in lower respiratory system which is characterized with progressive inflammation, limitation of airflow, and irreversible damage to lung. COPD is one of leading causes of death and more than 3 million patients were died with COPD worldwide in 2010[29]. The major factor inducing COPD is cigarette smoke, but COPD is also occurred by environmental noxious particles, infection, and genetic factors[30]. The main pathological phenotypes of COPD are obstructive bronchiolitis and emphysema, resulting from repeated damage and repair of the lungs and systemic inflammation[31]. Systemic inflammation in COPD is characterized with accumulation of neutrophils, macrophages, $CD8^{+}$ T cells, Th1, and Th17 cells in airway, and these cells produce protease and chemokines that induce epithelial remodeling and recruitment of immune cells, and also inflammatory cytokines which contribute to systemic inflammation[32].

In COPD, the role of ILCs in pathogenesis is poorly understood. In some studies, the conversion of ILC2s to IFNγ-producing ILC1s is occurred in both COPD patients and mouse model of COPD[33, 34]. However, how this plasticity affect immune mechanisms of COPD is unresolved. Moreover, because ILC3s are rare and ILC2s are dominant population in mice lung, there are several studies focused on the role of ILC2s in lung. However, in human lungs, ILC3s are the most dominant population among ILCs and the frequency of ILC3s is increased in lung tissue of COPD patients[35]. Therefore, how ILC3s are increased in COPD patients and the role of ILC3s in pathogenesis of COPD remains to be elucidated.

1.3 Regulation of ILCs in respiratory diseases

As the role of ILCs in several diseases has been revealed, ILCs are becoming a promising target for disease control. Unlike T cells, ILCs do not express antigen-specific receptors, but rather express receptors for environmental factors and respond rapidly through these receptors[36, 37]. Cytokine signaling is an important factor of development and activity of ILCs. IL-12, IL-15, and IL-18 are key cytokines for activation of ILC1s. IL-33, IL-25, and TSLP are important for ILC2 activation. IL-1β and IL-23 are related to ILC3 activation and maintenance. Moreover, ILCs have receptors recognizing metabolites, such as aryl hydrocarbon receptor (Ahr) ligands[38], leukotrienes[39, 40], and prostaglandins[41, 42], and neuropeptide[43, 44], which regulate activity of ILCs and integrate endogenous signals and immune responses (Figure 1.2).

1.3.1 Innate cytokines-induced activation of ILCs

In respiratory diseases, innate cytokines are critical in activation of ILCs. In allergic asthma, IL-25 and IL-33 are released by airway epithelial cells or other innate immune cells with exposure to allergens or helminth infection[45-47]. IL-33 is a IL-1 family cytokine and exist as a pro-form in epithelial cells, endothelial cells, and macrophages[47]. Upon cleaved by neutrophil elastase and cathepsin G[48, 49], IL-33 is released as an active form which binds to a heterodimer of ST-2 and IL-1 receptor (IL-1R) accessory protein [50]. IL-25 is a member of IL-17 family and bind to a heterodimer of IL-17RA and IL-17RB[51]. Both IL-33 and IL-25 activate ILC2s to produce IL-5 and IL-13, but IL-33 is more potent than IL-25 for activation of ILC2s[52]. Moreover, upon IL-33-exposure, ILC2s become expressing IL-17RB and respond to IL-25 signaling[53]. In non-allergic asthma, IL-1β signaling is important for ILC3 activation. IL-1β is a pro-inflammatory cytokine that is crucial for host defense in infection or tissue injury[54]. IL-1β is produced as an inactive precursor and furtherly cleaved into an active form by caspase-1 which activation occurs via recruitment of a multi-protein complex, inflammasome[55, 56]. In obesity-induced asthma exacerbation, IL-1β is released from macrophages dependent in activation of NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome and promote ILC3s to produce IL-17A[26, 57]. In pulmonary infection model with *Streptococcus pneumonia*, IL-23 also activate ILC3s to release IL-17 and IL-22[58]. In this study, *S. pneumonia* infection activates dendritic cells via a MyD88-dependent manner and co-culture of *S. pneumonia*-infected dendritic cells with ILCs enhances the production of IL-22. IL-12 and IL-18 are cytokines promoting production of IFNγ from ILCs upon exposure to various triggers associated with COPD[34]. However, in this study, IL-12R and IL-18R are expressed on "ex-ILC2s" which convert to ILC1-like cells. Therefore, the sole effect of innate cytokines on ILC1s in respiratory

diseases is not fully investigated.

1.3.2 Neuro-endocrine regulation of ILCs

ILCs reside at barriers of mucosal tissues and are located close to neurons and glial cells[44, 59, 60]. Therefore, ILCs might have an interaction with nervous system which allows tissue-specific regulation of ILCs. Recent studies show that ILC2s are regulated by neuronal mediators in lung[61, 62]. Neuromedin U (NMU) acts on ILC2s to produce type 2 cytokines in synergistic manner with IL-25[43, 61]. Mice lacking NMU receptor 1 (NMUR1) shows reduced type 2 immune responses with a decrease of ILC2s in HDM-induced inflammation. Moreover, pulmonary neuroendocrine cells (PNECs) produce calcitonin gene-related peptide (CGRP) to stimulate ILC2 production of cytokines in ovalbumin (OVA)-induced asthma model[62]. Conversely, a sex hormone, testosterone, inhibit the function of ILC2s in lung[63]. 5α-dihydrotestosterone, a downstream molecule of testosterone, reduces the number of lung ILC2s and cytokine production from ILC2s. Also, testosterone attenuates ILC2s-mediated *Alternaria*-induced allergic response. Although IFNγ-producing ILC1s are regulated by glucocorticoids (GC) in liver[64] and ILC3s are regulated by a neuropeptide, Glial-derived neurotrophic factor family of ligands (GFL), in intestine[44], it is unknown whether neuronal mediators regulate ILC1s and ILC3s in lung.

1.3.3 Lipid metabolites mediated regulation of ILCs

Eicosanoids, including leukotrienes and prostaglandins which are derived from arachidonic acids, regulate the functions of ILCs in lung in type 2 immune responses[65, 66]. ILC2s express cysteinyl leukotriene receptor 1 (CysLTR1) and the engagement of leukotriene (LT) C_4 and LTD_4 activates NFAT signaling in ILC2s to promote production of type 2 cytokines[39, 67]. Prostaglandin D_2 (PGD_2) also promote accumulation of ILC2s into inflamed lung by binding to CRTH2 expressed on ILC2s[42, 68]. Also, LTE_4 and PGD_2 have a synergistic effect on ILC2 activation[40]. Conversely, prostaglandin E_2 (PGE_2) and I_2 (PGI_2) has a negative effect on activation of ILC2s[69, 70]. They abrogate cytokine production from ILC2s in IL-33-induced and *Alternaria*-induced allergic lung inflammation. PGE_2 also acts on ILC3s systemically, which enhances IL-22 production from ILC3s to maintain homeostasis[71]. However, whether ILC1s and ILC3s are regulated by lipid mediators in respiratory system remains to be elucidated.

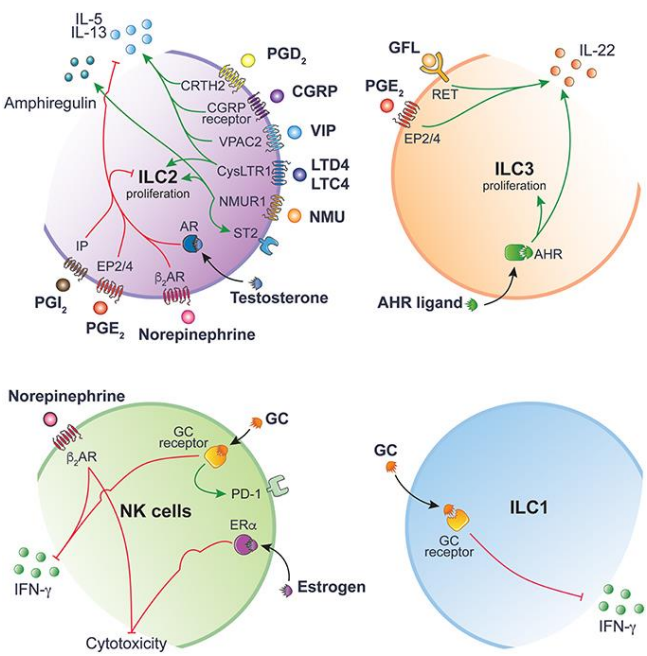

Figure 1.2 ILC function is tightly regulated by ligand-receptor interactions[37]

2. Methods

2.1 Subjects

For Chapter I, 18 healthy donors and 51 asthma patients from Department of Internal Medicine, Seoul National University Hospital (Seoul, South Korea) were recruited from September, 2015 to September, 2016. For Chapter II, 14 healthy donors and 27 patients with chronic obstructive pulmonary disease (COPD) from Department of Internal Medicine, Seoul National University Hospital (Seoul, South Korea) were recruited from December, 2018 to July, 2019.

All subjects recruited in this study provided written informed consent, and the study protocol was approved by the Seoul National University Hospital Institutional Review Board (IRB number 1610-062-799 and 1810-036-977).

2.2 Cell isolation from human subjects

Induced sputum and peripheral blood were obtained from subjects and processed as previously described[72]. In brief, induced sputum was treated with 0.1% of dithiothreitol (Sigma, MO, USA) and incubated at 37℃ for 20 minutes. Then, the mixture was filtered through a 70um strainer (SPL, Gyeonggi-do, South Korea) and resuspended with FACS buffer (PBS + 2% bovine calf serum) for FACS staining and differential counting to analyze immune cells. Differential counting was performed with Diff Quik solution (Sysmex Corporation, Japan). Peripheral blood

was centrifuged and plasma in upper layer was stored in –80℃ for further analysis. The remained part of blood was gently layered on the top of Histopaque® -1119 (Sigma, MO, USA), and centrifuged. Peripheral blood mononuclear cells (PBMCs) were isolated from the upper layer and granulocytes with red blood cells (RBCs) were isolated at the bottom. PBMCs were stored with CELLBANKER® 1 (Amsbio, Abingdon, UK) in deep freezer. Granulocytes with RBCs were treated with RBC lysis solution (BioLegend, CA, USA). Stored PBMCs and freshly isolated granulocytes were analyzed by flow cytometry.

2.3 Mice

For ILC and macrophage co-culture experiment (Chapter I) and emphysema model (Chapter II), C57BL/6 mice were purchased from Orient Company Ltd. (Seoul, South Korea) and KOATECH Ltd. (Gyeonggi-do, South Korea). $Rag1^{-/-}$ mice with C57BL/6 background were purchased from the Jackson Laboratory. All experiments were approved by the Institutional Animal Care and Use Committee in Seoul National University Hospital (SNUH-IACUC, IACUC # 16-0164 and 17-0043) and animals were maintained in the facility accredited AAALAC International (#001169) in accordance with Guide for the Care and Use of Laboratory Animals 8th edition, NRC (2010).

2.4 *In vivo* treatment of mice

For isolation of ILCs in Chapter I, 100ng of recombinant IL-1β (for expansion of

ILC1s and ILC3s) or 500ng of rIL-33 (for expansion of ILC2s) were injected intra-tracheally on three consecutive days. rIL-1β and rIL-33 were purchased from BioLegend (BioLegend, CA, USA).

For emphysema model in Chapter II, a combination of 10ug of lipopolysaccharide (LPS, Merck Millipore, MA, USA) and 0.3U of porcine pancreas elastase (PPE, Sigma, MO, USA) was treated intra-tracheally into C57BL/6 mice and $Rag1^{-/-}$ mice. For acute phase of emphysema model, the combination of LPS and PPE was injected only once and mice were sacrificed at a week after injection. For chronic phase of emphysema model, the combination of LPS and PPE was injected once a week for 4 weeks and mice were sacrificed at a week after last injection. In some experiments, 5ug of serum amyloid A (SAA, PeproTech, NJ, USA) was injected once intra-tracheally and mice were sacrificed at 3 days after injection.

In Chapter II, for depletion of neutrophils from SAA-injected mice, C57BL/6 mice were injected intra-peritoneally with 0.2mg of InVivoMAb anti-mouse Ly6G/Ly6C (Gr-1) (Bioxcell, NH, USA) at a day before and after SAA injection. For ILC depletion, $Rag1^{-/-}$ mice were injected intra-peritoneally with 0.2mg of InVivoMAb anti-mouse Thy1.2 (CD90.2) (Bioxcell, NH, USA) at a day before LPS and PPE injection and at every 2 days after first injection.

2.5 Cell isolation from mice lungs and bone marrows

For isolation of cells from lung, lung tissues were cut into small pieces and digested in RPMI-1640 media with 1mg/ml Collagenase type IV (Worthington Biochemical Corp, UJ, USA) and 0.5mg/ml DNase I (Sigma, MO, USA) at 37℃ for 1 hour. For

isolation of bone marrow cells, mice tibia and femur were obtained. After removing the muscles and tissues surrounding the bones, both end of bones was cut off and bone marrow cells were flushed with media using 1ml syringe. Digested lung cells and flushed bone marrow cells were filtered with a 40um strainer (SPL, Gyeonggi-do, South Korea) and lysed with RBC lysis buffer (BioLegend, CA, USA) to eliminate red blood cells (RBCs).

2.6 Flow cytometry

For staining human cells from induced sputum and blood, the following antibodies were used; Anti-CD45 (HI30), anti-CD3e (UCHT1), anti-CD11c (3.9), anti-CD11b (ICRF44), anti-CD14 (HCD14), anti-CD15 (W6D3), anti-CD16 (3G8) anti-CD19 (HIB19), anti-CD24 (ML5), anti-CD49b (P1E6-C5), anti-CD68 (Y1/82A), anti-CD117 (104D2), anti-CD127 (A019D5), anti-CD206 (15-2), anti-FcεRIα (AER-37), anti-HLA-DR (L243), anti-NKp44 (P44-8), and anti-IL-17A (BL168) were from BioLegend (BioLegend, CA, USA). Anti-ST2L (B4E6) was from MD bioproducts (MD bioproducts, MN, USA).

For staining mice lung cells, the following antibodies were used; Anti-CD3e (145-2C11), anti-CD11b (M1/70), anti-CD11c (HL3), anti-CD19 (ID3), anti-CD31 (MEC13.3), anti-SiglecF (E50-2440), anti-IFNγ (XMG1.2), and anti-RORγt (Q31-378) were purchased from BD bioscience (BD bioscience, NJ, USA). Anti-CD4 (RM4-5), anti-CD25 (PC61), anti-CD45 (30-F11), anti-CD49b (DX5), anti-CD90.2 (30-H12), anti-CD127 (A7R34), anti-EpCAM (G8.8), anti-F4/80 (BM8), anti-FcεRIα (MAR-1), anti-I-Ab (AF6-120.1), anti-Ki67 (16A8), anti-Ly6G (1A8),

and anti-IL-17A (TC11-18H10.1) were from BioLegend (BioLegend, CA, USA). Anti-IL-13 (eBio13A) and anti-IL-1β (NJTEN3) were from Invitrogen (Invitrogen, CA, USA). Anti-ST2 (RMST2-33) were purchased from eBioscience. Anti-SAA polyclonal antibody was purchased from Cloud-Clone Corp. (Cloud-Clone Corp., TX, USA).

Flow cytometry was performed using BD LSR II, BD LSRFortessa™ and BD LSRFortessa™ X-20 and analyzed by FlowJo (V10.2) software.

2.7 *In vitro* co-culture of ILCs and alveolar macrophages

In Chapter I, ILCs and alveolar macrophages were sorted for co-culture experiments by flow cytometry. For ILC sorting, ILCs were enriched with Lineage Cell Depletion Kit (Miltenyi Biotec Inc., CA, USA). Lineage depleted cells were stained with lineage cocktail (anti-CD3e, anti-CD19, anti-CD11c, anti-CD11b, anti-F4/80, anti-FcεRIα and anti-CD49b), anti-CD45, anti-CD127, anti-ST-2, and anti-CD25 antibodies. ILC1s and ILC3s were sorted as $CD45^+Lineage^-CD127^+ST\text{-}2^-CD25^-$ lymphoid cells, while ILC2s were sorted as $CD45^+Lineage^-CD127^+ST\text{-}2^+CD25^+$ lymphoid cells. For alveolar macrophage sorting, $CD11c^+$ cells were enriched with CD11c Microbeads UltraPure (Miltenyi Biotec Inc., CA, USA), and stained with anti-CD45, anti-F4/80 and anti-CD11c antibodies. Alveolar macrophages were sorted as $CD45^+F4/80^+CD11c^+$ cells. ILCs and alveolar macrophages were sorted by BD FACSAria™.

Sorted alveolar macrophages ($2.5x10^5$ cells/well) and ILCs ($5x10^4$ cells/well) were cultured without or with transwell (0.4um pore, Corning, MA, USA).

With transwell, alveolar macrophages were seeded in the lower chamber, and after 24hr, freshly sorted ILC1s and ILC3s or sorted ILC2s were added in the upper chamber. In ILC1s, ILC3s and alveolar macrophages co-culture group, recombinant IL-2 (20U/ml, BioLegend, CA, USA), IL-12 (20ng/ml, BioLegend, CA, USA) and IL-23 (20ng/ml, BioLegend, CA, USA) were added for ILC stimulation. In ILC2s and alveolar macrophages co-culture group, rIL-2 (20U/ml, BioLegend, CA, USA) and rIL-33 (20ng/ml, BioLegend, CA, USA) were added for stimulation. In some experiments, anti-PD-1 (10ug/ml, Bioxcell, NH, USA), anti-ICOS (10ug/ml, Bioxcell, NH, USA), and anti-CD40L blocking antibodies (10ug/ml, Bioxcell, NH, USA) were added to block a direct cell-to-cell contact between alveolar macrophages and ILCs. After 48 hours, the culture supernatant was obtained for analysis cytokine production by ELISA, and alveolar macrophages were lysed with TRIzol® Reagent (Invitrogen, CA, USA) for analysis of genes expression by RT-qPCR.

2.8 *In vitro* stimulation of immune cells with SAA

In Chapter II, neutrophil Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) were used for neutrophil sorting from bone marrow cells. Dendritic cells were differentiated from bone marrow cells. Bone marrow cells were cultured in DMEM media with 20ng/ml recombinant mouse GM-CSF (R&D Systems, MN, USA) and on every 3 days, media with the same concentration of rGM-CSF were added. After 10 days, the non-adherent cells were harvested for further treatment. Sorted neutrophils and differentiated dendritic cells were treated with 1ug/ml of rSAA (PeproTech, NJ, USA).

2.9 Histological analysis

Fixed lung tissues with 4% of paraformaldehyde (Biosesang, Gyeonggi-do, South Korea) were embedded in paraffin and sectioned into 4um thickness. Sectioned tissues were attached on slides and stained with hematoxylin and eosin for histological analysis.

2.10 Quantitative real-time PCR

In Chapter I, sputum cells and alveolar macrophages were homogenized with TRIzol® Reagent (Invitrogen, CA, USA). In Chapter II, lung tissues in TRIzol® Reagent were grinded with BioMasher II (OPTIMA, Tokyo, Japan). Total RNA was extracted, following the manufacturer's instructions and cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad, CA, USA) or SensiFAST™ cDNA Synthesis Kit (Bioline, London, UK). The quantitative RT-qPCR assays were performed using IQ supermix (Bio-Rad, CA, USA), SensiFAST™ SYBR® Lo-ROX Kit or SensiFAST™ Probe Lo-ROX Kit (Bioline, London, UK). Relative expression levels were calculated with normalizing Cq values of target genes to Cq values of *Rplp0* (for human genes) or *Gapdh* (for mice genes). The expression of *Ifng, Il5, Il13, Il17* (for human genes), *Nos2, Cd86, Ptgs2, Arg1, Retnla, Mrc1, Saa3, Il1b, Il23, Il12A, Il18, Il33, Fpr2, Tlr2,* and *Tlr4* (for mice genes) were measured using TaqMan® Gene Expression Assays (Thermo Fisher Scientific, MA, USA). The expression of *Mmp1, Mmp2, Mmp8, Mmp9,* and *Mmp12* were measured using

PrimeTime qPCR Primer Assays (Integrated DNA Technologies, IA, USA).

2.11 ELISA

The level of IFNγ, IL-4, IL-9 (BD bioscience, NJ, USA), IL-5, IL-13, and IL-17 (R&D systems, MN, USA) were analyzed from culture supernatant of ILCs and alveolar macrophages. The level of SAA (R&D systems, MN, USA) was analyzed from supernatant of sputum and plasma, following the manufacturer's instructions.

2.12 Statistical analysis

Normality test was performed with Shapiro-Wilk normality test. Mann-Whitney test and Kruskal-Wallis test followed by a Dunns's post-test were used for comparison of non-parametric data. Unpaired t-test and one-way ANOVA followed by a Bonferroni's posttest were used for comparison of parametric data. Linear regression was performed using Spearman r correlation test (non-parametric) or Pearson correlation test (parametric). Values of measurements were expressed as mean±SD or mean±SEM as indicated. $P < 0.05$ was considered statistically significant. All statistical analysis was performed with GraphPad Prism 7 software.

3. Chapter I

Innate lymphoid cells affect asthma phenotype by crosstalk with lung macrophages

*** Chapter I was previously published in Journal of Allergy and Clinical Immunology (2019)**

3.1 Introduction

Asthma is an immunological disease of the airways characterized by airway hyperresponsiveness and chronic airway inflammation related with intricate network of diverse immune cells[73]. Allergen-specific Th2 cells have long been thought to play critical roles in inducing type 2 cytokine production in the lungs, resulting in the development of allergic asthma, the most common form of the disease[73]. More recently, it has become clear that asthma is mediated by both innate and adaptive immune cells that regulate and shape pulmonary inflammation[18].

As increased awareness of asthma heterogeneity, researchers subdivide patients with asthma into 4-5 phenotypic clusters according to their age, gender, atopy, lung function, health care utilization and body mass index (BMI)[74]. Among the cluster, patients with notable eosinophilia and notable increase of interleukins such as IL-4, IL-5, and IL-13 in the blood called eosinophilic asthma[75, 76]. Another group shows a minimal TH2 response but notable sputum neutrophilia with increase of IL-17A[75]. Therefore, understanding the mechanisms underlying each asthma phenotype will foster the development of improved and more appropriate therapeutic strategies for asthma.

Recently identified innate lymphoid cells (ILCs) have been found to link innate components of immunity with the adaptive immunity[77]. ILCs play critical roles in host defense and tissue homeostasis, particularly in mucosal tissues. Although ILCs lack antigen- specific T-cell and B-cell receptors (TCRs and BCRs, respectively), they rapidly produce a variety of cytokines in response to a wide range of innate signals, that is, interleukin (IL)-25, IL-33, TSLP and IL-1β[78]. Based on

their characteristics and cytokine profiles, ILCs have been divided into three groups – type 1, type 2 and type 3 ILCs (ILC1s, ILC2s and ILC3s) –which may be thought of as the innate counterparts of $CD4^+$ T helper 1 (TH1), TH2, and TH17 cells[79]. ILC1s produce IFN-γ and tumor necrosis factor (TNF)-α[80]. ILC2s produce a set of cytokines similar to that produced by TH2 cells such as IL-5, IL-9, and IL-13[81-83]. ILC3s produce IL-17A, IL-22, granulocyte macrophage colony stimulating factor (GM-CSF) and TNF-α. ILC3s display further heterogeneity and can be subdivided into several subgroups, such as $CCR6^+$ LTi cells and $CCR6^-$ ILC3s[84]. $CCR6^-$ ILC3 cells are further subdivided based on the expression of natural cytotoxicity receptor (NCR)[85]. RAR-related orphan receptor gamma T (RORγt) is a key transcription factor for ILC3 development, however, T-bet is also required for regulation of NKp46 expression on $CCR6^-$ ILC3s[80, 85, 86]. The interactions between ILCs and other immune cells are still under investigation; however, recent studies have shown that ILCs can demonstrate new mechanisms underlying immune responses, especially in mucosal tissues such as the intestines and lungs[25, 26, 82, 87].

Macrophages constitute the foremost controllers of both innate and acquired immunity[88]. They are the most abundant immune cells in the lung (approximately 70% of total immune cells)[89, 90], and polarization of macrophage has been heavily associated with the development of allergic asthma[91]. Macrophages can be polarized following exposure to microenvironmental stimuli and categorized as classically (M1) or alternatively (M2) activated phenotypes[92, 93]. The regulation of macrophage polarization involves a complex interplay between various cytokines, chemokines, and signaling molecules. Interferon-γ (IFN-γ) and lipopolysaccharide (LPS) are known to induce the differentiation of M1 macrophages, whereas IL-4 and

IL-13 upregulate the expression of genes involved the generation of M2 macrophages[94].

The importance of ILCs in the development of asthma is well defined in animal models of asthma[26, 77, 87] and in patients with asthma[27, 95, 96]. Conversely, the interaction between ILCs and other innate immune cells has not been fully understood yet. This study aimed to identify the role of ILCs as mediators in the development of asthma by estimating their population in induced sputum from patients with asthma. In addition, the correlation between ILCs and several innate immune cells, including macrophages, neutrophils, and eosinophils was analyzed. These results suggested that cytokine production from ILCs can coordinate the polarization of macrophages, which participates in the development of different types of asthma.

3.2 Results

3.2.1 All types of ILCs as well as ILC2 were increased in the induced sputum of asthmatics

The presence of ILCs in induced sputum was evaluated in 51 patients with asthma and 18 healthy controls (Table 3.1). Total ILCs were initially gated as $CD45^{+}Lineage^{-}IL\text{-}7R^{+}$ lymphocytes, and subsequently categorized as ILC2s ($CRTH2^{+}$ cells), ILC1s ($CRTH2^{-}$c- $kit^{-}NKp44^{-}$ cells), and ILC3s ($CRTH2^{-}c\text{-}kit^{+}$ cells). Natural cytotoxicity receptor $(NCR)^{+}$ ILC3s and NCR^{-} ILC3s were further categorized on the basis of the expression of NKp44 (Fig. 3.1 A and Fig. 3.2 A). However, it should be noted that the NCR^{-}ILC3 subset remains a heterogeneous population containing LTi-cells. The percentage and absolute number of total ILCs were increased in the induced sputum from patients with asthma when compared with the healthy donors (Fig. 3.1 B and C). In addition to the ILC2s, both ILC1s and NCR^{-}ILC3s were also significantly increased in the asthmatic patients (Fig. 3.2 B). However, the increase in ILCs was not related to pulmonary function index (FEV_1/FVC), airway hypersensitivity (PC_{20} level), and WBC count (data not shown). So far, only ILC2s has been known to be increased in asthmatics, however, this data clearly showed that all types of ILCs were increased in the induced sputum of patients with asthma.

Table 3.1 Characteristics of asthma patients

	Healthy controls	Asthma	p value
No. of patients	18	51	
Age (yr)	52.93±7.57	57.49±14.30	0.0899
Sx durations (yr)	N.D	4.14±5.36	-
FEV1	2803±607.4	2096±665.7	0.0006
FEV1(%)	103.5±12.73	87.69±14.04	0.0003
FVC	3516±785.5	2853±891.6	0.0085
FVC(%)	107.3±13.09	95.61±15.61	0.0129
FEV1/FVC	0.8±0.06	0.73±0.08	0.0040
PC20(mg/ml)	N.D	4.73±3.74	-
Atopy, n(%)	0(0)	18(35.3)	
Allergic rhinitis, n(%)	0(0)	28(54.9)	
Smoking history (never/ex/current), n(%)	18(100)/0(0)/0(0)	42(82.4)/7(13.7)/2(3.9)	
Induced sputum differential count.			
Macrophages (%)	42.07±26.03	57.67±14.09	0.0059
Neutrophils (%)	39.93±28.39	22.59±13.60	0.0508
Eosinophils (%)	0.9±1.57	15.22±13.39	<0.0001
Peripheral blood			
WBC	N.D	6834±1777	-
Eosinophils (%)	N.D	5.45±3.96	-
Total IgE	N.D	238.39±323.7	-

Data are presented as mean ± SDs.

FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; PC_{20}, provocation concentration of methacholine leading a fall of 20% of FEV_1; WBC, white blood cell; N.D, no data

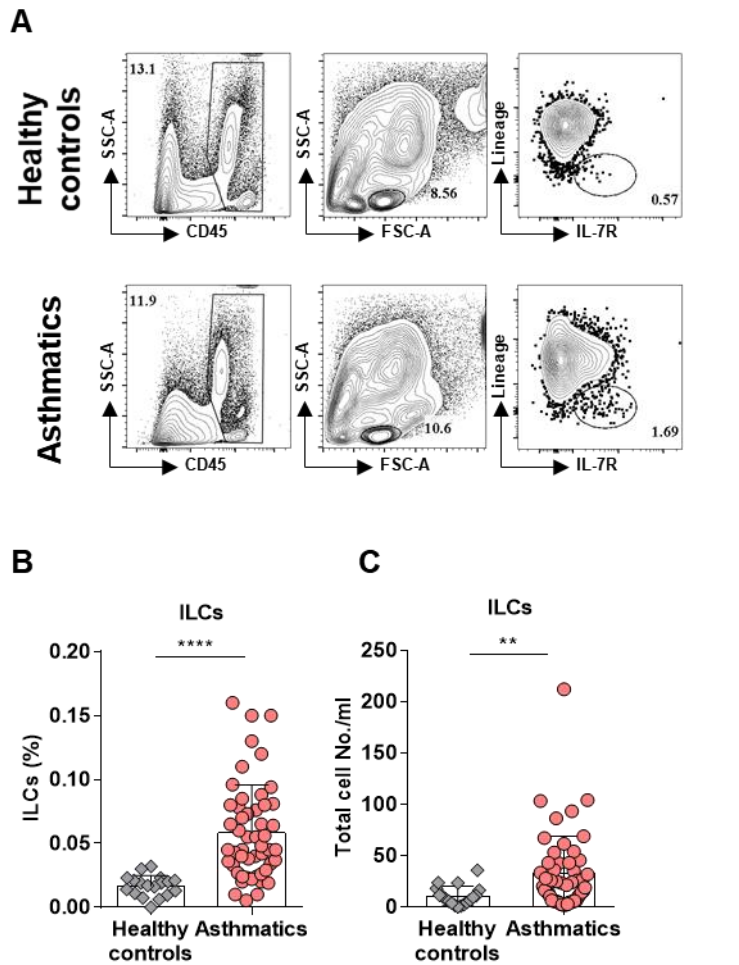

Figure 3.1 Comparison of total innate lymphoid cells between healthy controls and asthmatics

A. Representative ILC gating strategy from induced sputum of healthy donor (n = 18) and asthmatics (n = 51). **B.** Comparison of total ILC frequency (% of $CD45^+$ cells) between healthy donors and asthmatic patients. **C.** Comparison of total ILC cell number (per ml of sputum) between healthy donors and asthmatic patients. Mann–Whitney test was used to compare healthy controls and asthma patients. Values represent the mean ± standard deviation (SD). $^{**}P < 0.01$, $^{****}P < 0.0001$.

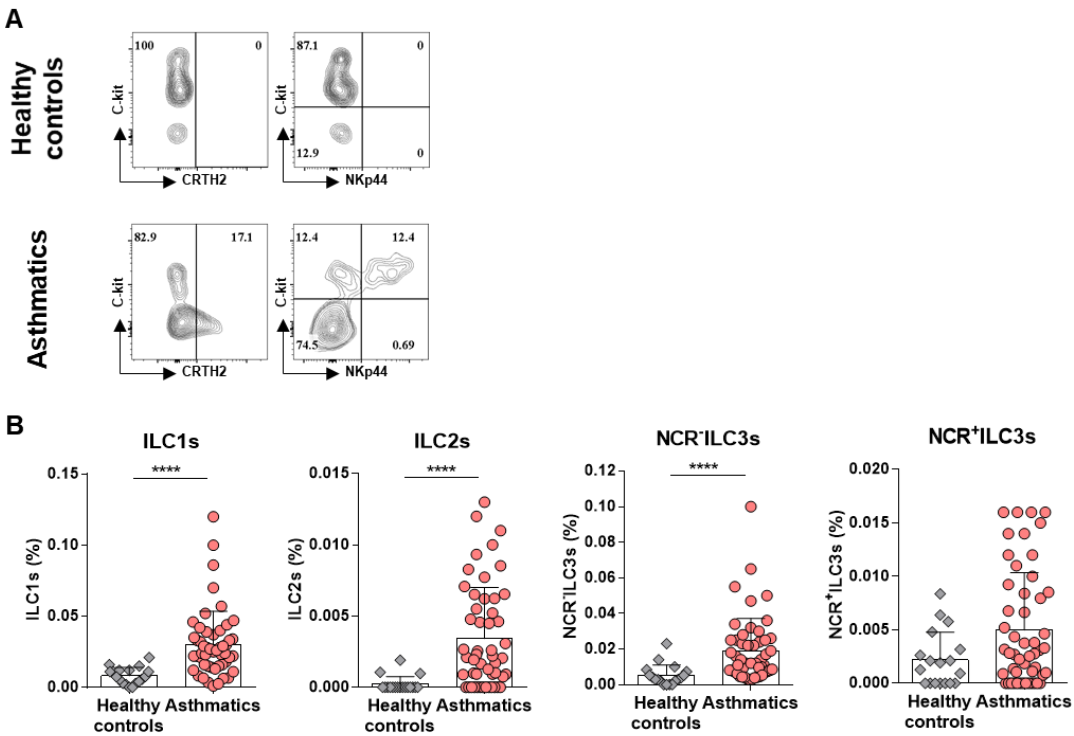

Figure 3.2 All subsets of innate lymphoid cells are increased in induced sputum of asthmatics

A. Representative gating strategy of each subset of ILCs from induced sputum of healthy donor (n = 18) and asthmatics (n = 51). **B**. Comparison of frequency of ILC1s, ILC2s, NCR^{-}ILC3s, and NCR^{+}ILC3s (% of $CD45^{+}$ cells) between healthy donors and asthmatic patients. Mann–Whitney test was used to compare healthy controls and asthma patients. Values represent the mean ± standard deviation (SD). ****P <0.0001.

3.2.2 Increase in the number of ILC2s in induced sputum of patient with eosinophilic asthma

To investigate potential associations between various populations of ILCs and the phenotypes of asthma, the patients were divided into two groups based on the number of eosinophils (Table 3.2). The percentage of ILC2s was significantly increased in induced sputum of eosinophilic asthmatics (Fig. 3.3 A) when compared with that of the non-eosinophilic patients. The percentage of ILC1s or ILC3s were similar in sputum from the eosinophilic and non-eosinophilic asthmatics (Fig. 3.3 A). Also, I tested the various cytokine expression in the pellets from sputum, and found that IL-5 and IL-13 mRNA were upregulated only in the eosinophilic asthmatics but not in the non-eosinophilic asthmatics (Fig. 3.3 B).

Table 3.2 Comparison of characteristics between non-eosinophilic and eosinophilic asthma patients

	Non Eosinophilic	Eosinophilic	p value
No. of patients	21	21	
Age (yr)	60.86±13.53	54.76±14.29	0.0496
Sx durations (yr)	4.105±3.79	3.186±3.79	0.2940
FEV1	1970±567.7	2222±812.1	0.4068
FEV1(%)	91.38±15.57	85.10±13.37	0.1682
FVC	2669±722.9	2973±1096	0.2954
FVC(%)	96.52±20.01	94.24±11.20	0.6503
FEV1/FVC	0.7369±0.07	0.7448±0.08	0.7403
PC20(mg/ml)	6.080±4.49	3.630±3.01	0.0552
Smoking history (never/ex/current), n(%)	17(81.0)/2(9.5)/2(9.5)	16(76.2)/5(23.8)/0(0)	
Induced sputum differential count.			
Macrophages (%)	55.81±12.67	59.61±14.22	0.3786
Neutrophils (%)	31.07±13.49	15.06±7.98	<0.0001
Eosinophils (%)	6.123±6.58	23.48±12.96	<0.0001
Peripheral blood			
WBC	6575±1587	7093±1954	0.3512
Eosinophils (%)	2.490±1.36	8.414±3.44	<0.0001
Total IgE	187.5±321.7	295.8±341.2	0.1977

Data are presented as mean ± SDs.

FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; PC_{20}, provocation concentration of methacholine leading a fall of 20% of FEV_1; WBC, white blood cell; N.D, no data

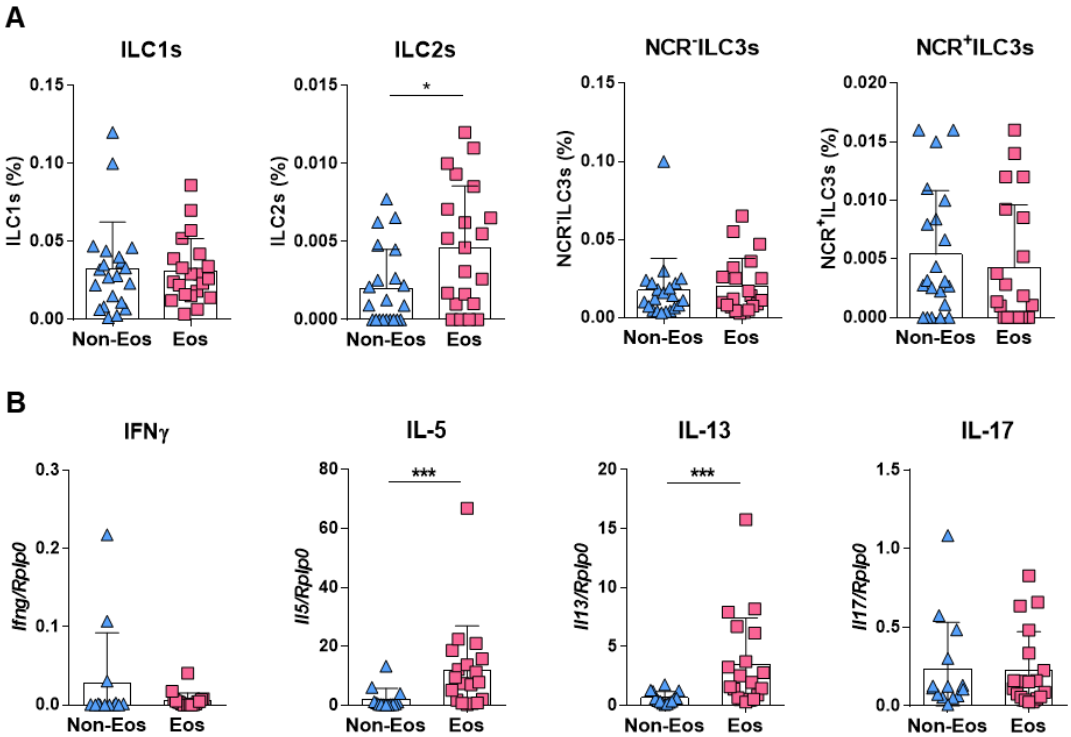

Figure 3.3 Eosinophilic asthmatics show increased type 2 innate lymphoid cells

A. The frequency of each subset of ILCs (% of $CD45^+$ cells) in induced sputum of non-eosinophilic (n = 21) and eosinophilic patients (n = 21). **B.** The gene expression of IFNγ, IL-5, IL-13, and IL-17A in pellets of whole sputum cells. **P* < 0.05, ****P* < 0.001, using Mann–Whitney test to compare between the non-eosinophilic and eosinophilic asthma patients. Values represent the mean ± SD.

3.2.3 $CD68^+$ macrophages were increased in induced sputum from asthmatics

I hypothesized that an increase in the number of ILCs might result in crosstalk between them and other immune cells, especially innate immune cells such as alveolar macrophages. First, I checked the population and composition of macrophages in induced sputum from asthmatics and analyzed a subset of macrophages by flow cytometry. $CD45^+CD68^+$ cells were first gated as macrophages and then categorized as M1 and M2 macrophages based on the expression of CD11c and CD206, respectively (Fig. 3.4 A). The M1 and M2 macrophages presented with different expression levels of HLA-DR (Fig. 3.5 A)[97]. The percentage and absolute number of total macrophages were increased in induced sputum of asthmatics when compared with the healthy controls (Fig. 3.4 B and C); moreover, each subgroup of macrophages was increased in the induced sputum of asthmatics (Fig. 3.5 B) when compared with the controls. Alterations in the number of macrophages were not associated with lung function (FEV_1/FVC), airway hypersensitivity (PC20 level) or the number of WBCs (data not shown). To investigate whether macrophages also have association with the phenotypes of asthma, I compared the frequency of M1 and M2 macrophages between two groups based on the number of eosinophils (Table 3.2). The number of M2 macrophages was higher in eosinophilic asthmatics, whereas an increased number of M1 macrophages was observed in the non-eosinophilic asthmatic patients (Fig. 3.5 C).

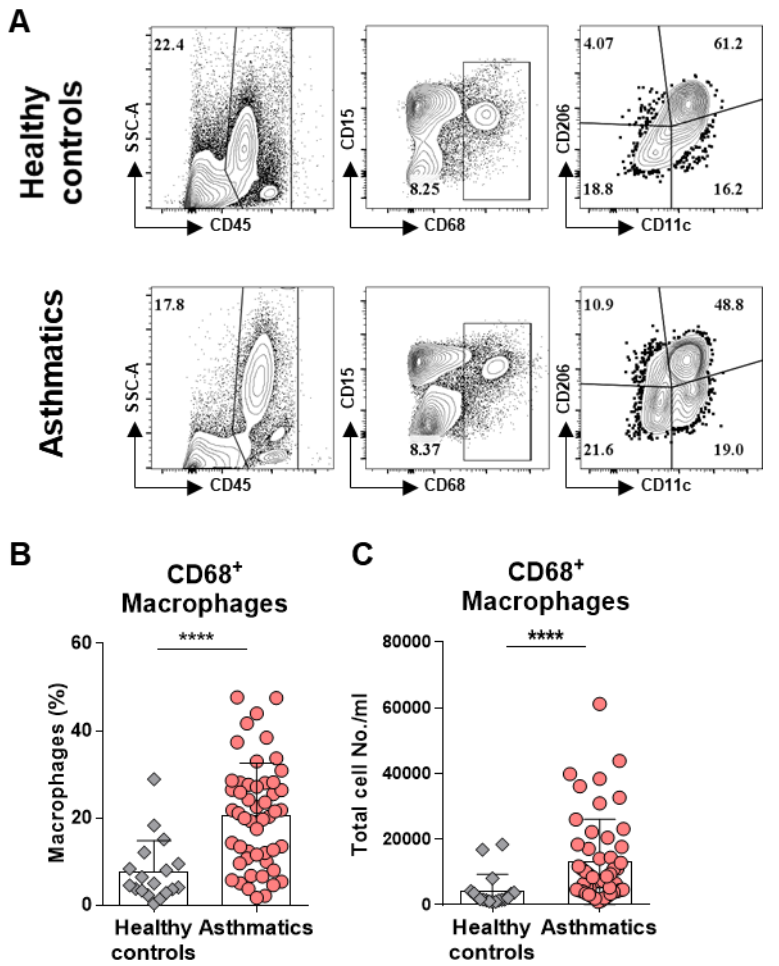

Figure 3.4 Macrophages are increased in induced sputum of asthmatics

A. Representative macrophages gating strategy from induced sputum of healthy donors (n = 18) and asthmatics (n = 51). Total macrophages ($CD68^+$ cells), M1 macrophages ($CD11c^+CD206^-$ cells), and M2 macrophages ($CD11c^-CD206^+$ cells). **B**. Comparison of frequency of total macrophages (% of $CD45^+$ cells) between healthy donors and asthma patients. **C**. Comparison of frequency of total number of macrophages (per ml of sputum) between healthy donors and asthma patients. Mann–Whitney test was used to compare healthy controls and asthma patients. Values represent the mean ± SD. ****$P < 0.0001$.

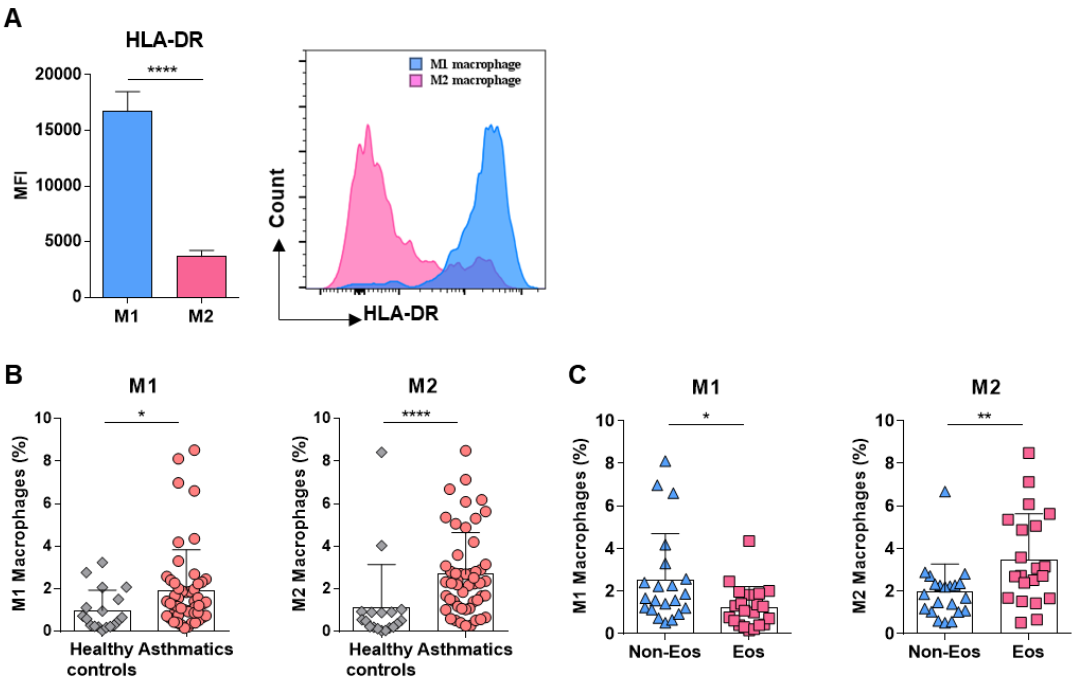

Figure 3.5 Each activated subtype of macrophages are increased in induced sputum of asthmatics

A. Mean fluorescence intensity (MFI) levels and histogram of HLA-DR expression levels in M1 macrophages and M2 macrophages. **B**. Comparison of frequency of M1 and M2 (% of $CD45^+$ cells) in induced sputum of healthy donors (n = 18) and asthmatics (n = 51). **C**. Frequency of M1 and M2 (% of $CD45^+$ cells) in induced sputum of non-eosinophilic (n = 21) and eosinophilic (n = 21) patients. Mann–Whitney test was used to compare healthy controls and asthma patients. Values represent the mean ± SD. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$.

3.2.4 The distribution of innate immune cells in the sputum is different and is related to asthma phenotype

To verify the presence of associations between asthma phenotypes and the distribution of innate immune cells, I compared the relationships of innate immune cells in induced sputum of asthmatics. Interestingly, patients with higher numbers of eosinophils in sputum presented with increased numbers of ILC2s (Fig. 3.6 A). Unlike ILC2s, there were no correlations between ILC1s, NCR^{-}ILC3s or NCR^{+}ILC3s and eosinophils (Fig. 3.6 A), and other subset of ILCs and neutrophils in induced sputum (Fig. 3.6 B). Next, I examined the relationship between macrophages and other innate immune cells. Patients with increased number of eosinophils in induced sputum demonstrated an increase in the percentage of M2 macrophages, whereas M1 macrophages did not show correlation with eosinophils (Fig. 3.7 A). Conversely, neutrophil counts in sputum were positively correlated with M1 macrophages, and negatively correlated with M2 macrophages (Fig. 3.7 B).

I further investigated the presence of associations between ILCs and macrophages because alterations in these two populations are most significant in asthmatics. Interestingly, the percentages of ILC1s and ILC3s were positively correlated with increase in the number of M1 macrophages (Fig. 3.8 A) but not M2 macrophages. On the other hand, asthmatics who had more ILC2s presented with more M2 macrophages in induced sputum (Fig. 3.8 B). However, in healthy donors, no such correlations were observed (data not shown). These data suggest that ILCs and macrophages interact with each other in a cell-specific manner, and some of these interactions could alter the outcome of asthma.

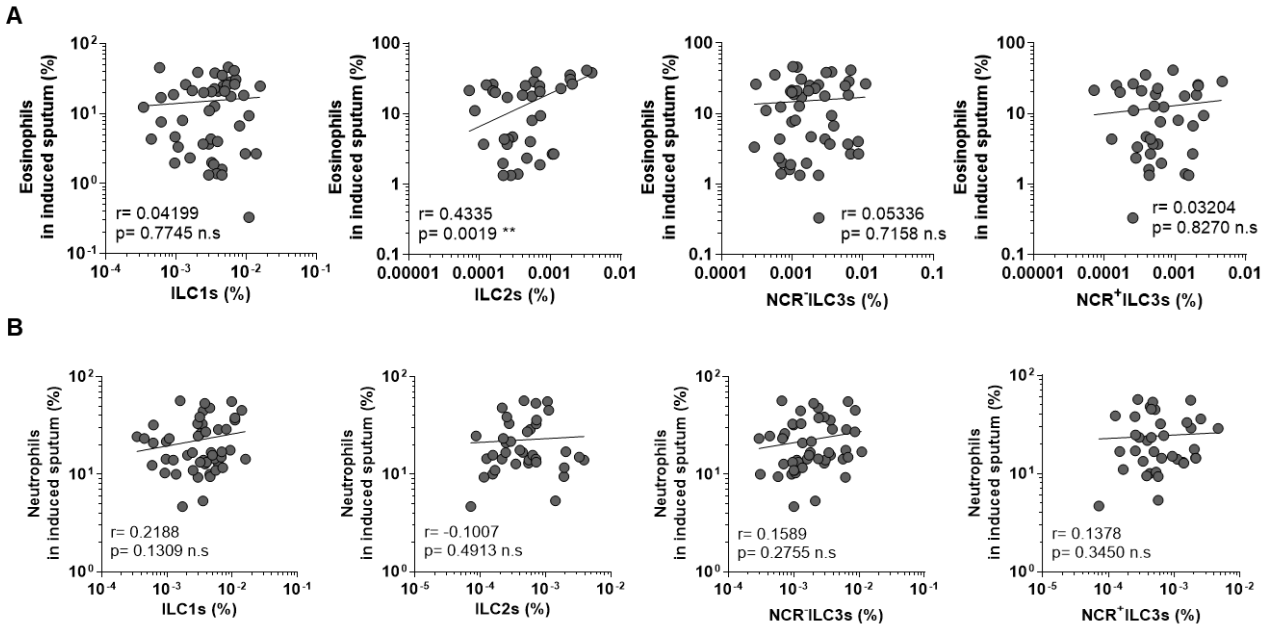

Figure 3.6 ILC2s are correlated with the levels of eosinophils

A. Correlation between ILC1s, ILC2s, NCR$^-$ILC3s or NCR$^+$ILC3s and eosinophils in induced sputum analyzed. **B**. Correlation between ILC1s, ILC2s, NCR$^-$ILC3s or NCR$^+$ILC3s and neutrophils in induced sputum analyzed. $^{**}P < 0.01$, n.s, not significant, using Pearson's correlation test.

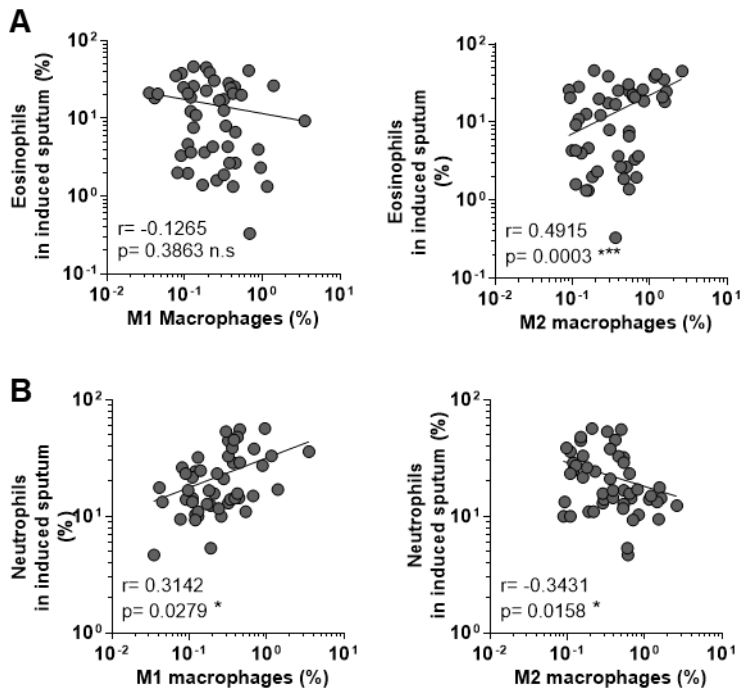

Figure 3.7 Correlations between macrophages and eosinophils or neutrophils in induced sputum

A. Correlation between eosinophils and each subset of macrophages in induced sputum analyzed. **B**. Correlation between neutrophils and each subset of macrophages in induced sputum analyzed. **P* < 0.05, ****P* < 0.001, n.s, not significant, using Pearson's correlation test.

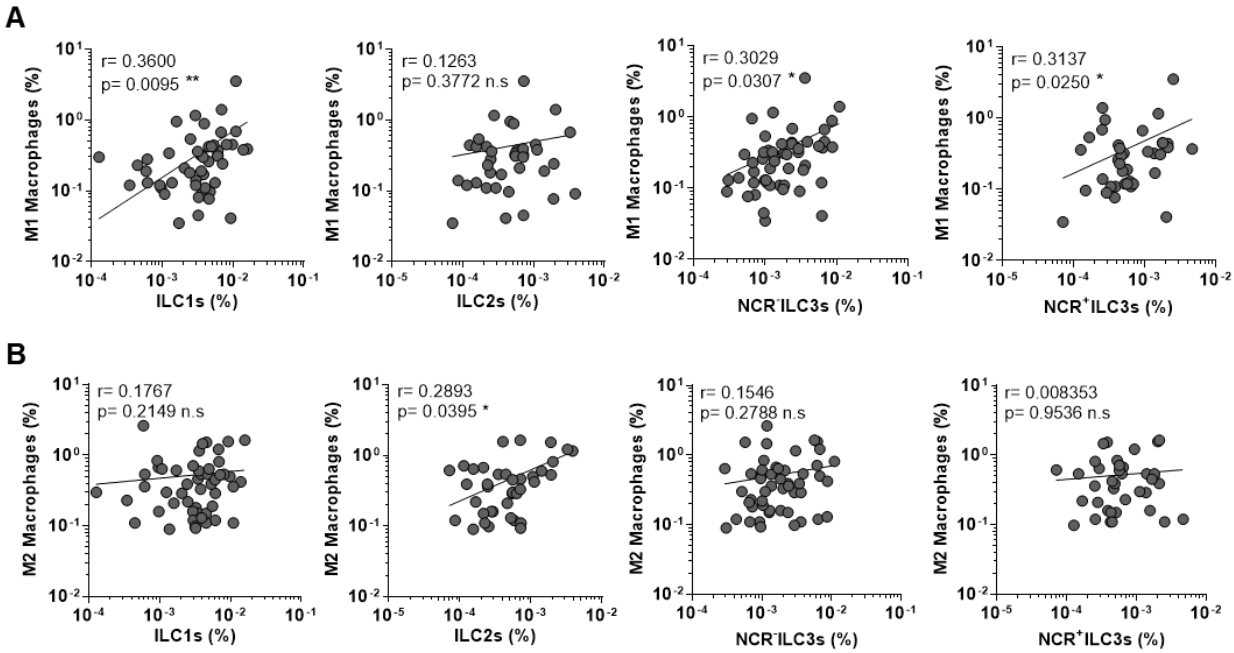

Figure 3.8 Different subset of ILCs is correlated with the levels of macrophages

A. Correlation between M1 macrophages and ILCs in induced sputum of asthmatics.

B. Correlation of M2 macrophages and ILCs in induced sputum of asthmatics. **P* < 0.05, ***P* < 0.01, n.s, not significant, using Pearson's correlation test.

3.2.5 ILCs can promote the polarization of macrophages by secreting cytokines

On the basis of current results, I hypothesized that specific types of ILCs affect the polarization of macrophages. To test this hypothesis, I co-cultured sorted ILCs with AMs (Fig. 3.9). ILC2s were stimulated with recombinant(r) IL-2 and IL-33, whereas ILC1s and ILC3s were stimulated with rIL-2, rIL-12 and rIL-23.

Co-culture of AMs with ILC1s and ILC3s significantly induced the mRNA expressions of M1 macrophage-related genes, such as *Nos2*, *Cd86*, and *ptgs2* (Fig. 3.10 A). It is well known that the polarization of macrophages is regulated by cytokines[94, 97]. Therefore, I presumed that ILCs might be involved in this process by secreting cytokines. To examine cytokine production by the ILCs, I measured IFNγ and IL-17A levels in co-culture supernatants (Fig. 3.10 B). Treatment with rIL-12 and rIL-23 stimulated ILC1s and ILC3s, thereby increasing the levels of IFNγ and IL-17A (Fig. 3.10 B).

In contrast, mRNA expression of M2 macrophage-related genes such as *Arg1* and *Retnla* were increased when AMs were co-cultured with ILC2s (Fig. 3.11 A). Furthermore, stimulation of ILC2s with rIL-33 resulted in increased levels of IL-4 and IL-13 (Fig. 3.11 B). In addition to IL-4 and IL-13, ILC2s produced IL-5 and IL-9 with rIL-33 stimulation (Fig. 3.11 C). These results clearly showed that cytokines from different subsets of ILCs might induce the polarization of macrophages.

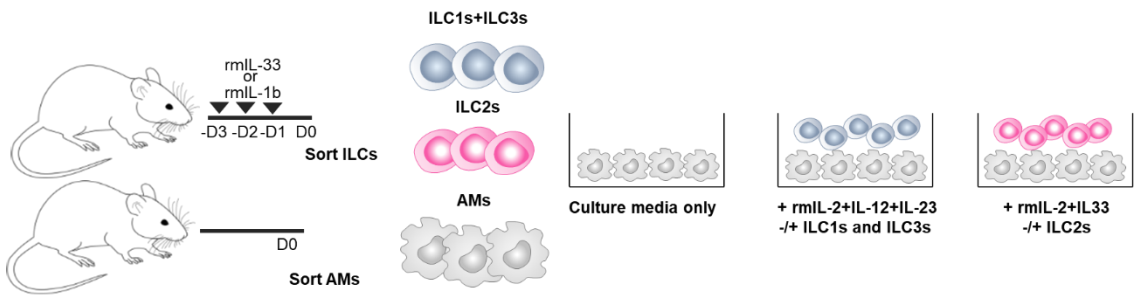

Figure 3.9 Schematic diagram of the co-culture procedure of ILCs and alveolar macrophages

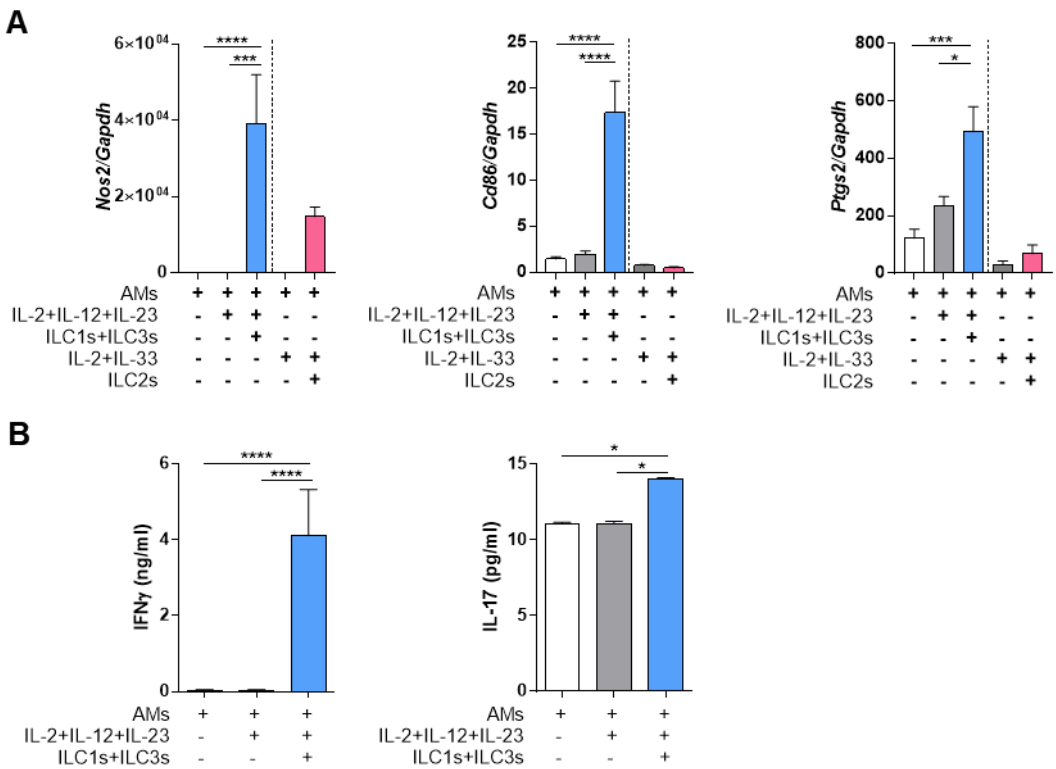

Figure 3.10 ILC1s and ILC3s affect the polarization of alveolar macrophages into M1 macrophages *in vitro*

A. The expression of M1-related genes, *Nos2*, *Cd86*, and *Ptgs2*, in alveolar macrophages co-cultured with ILCs. **B**. IFNγ and IL-17 expression levels in the ILC1s/ILC3s and alveolar macrophages co-cultured group. **P* < 0.05, ****P* < 0.001, *****P* < 0.0001 using Kruskal–Wallis test, followed by Dunn's multiple comparison test. The data are representative of three independent experiments analyzed, and presented as the mean ± SEM.

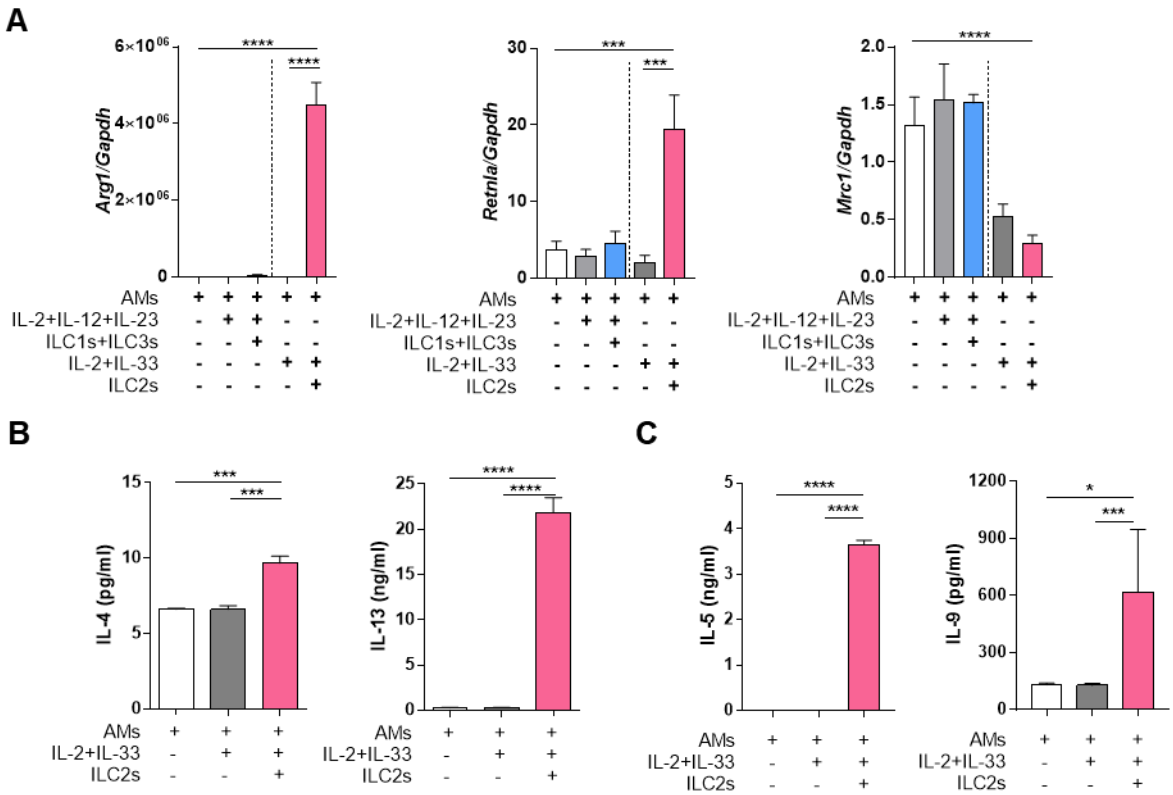

Figure 3.11 ILC2s affect the polarization of alveolar macrophages into M2 macrophages *in vitro*

A. The expression of M2-related genes, *Arg1*, *Retnla*, and *Mrc1*, in alveolar macrophages co-cultured with ILCs. **B**. IL-4 and IL-13 expression levels in the ILC2s and alveolar macrophages co-cultured group. **C**. The protein level of IL-5 and IL-9 in supernatant of co-culture of ILC2s and alveolar macrophages. **P* < 0.05, ****P* < 0.001, *****P* < 0.0001 using Kruskal–Wallis test, followed by Dunn's multiple comparison test. The data are representative of three independent experiments analyzed, and presented as the mean ± SEM.

3.2.6 ILCs can promote the polarization of macrophages via direct interactions

Next, the transwell system was used to investigate whether soluble cytokines from ILCs affected the polarization of macrophages. As shown in Fig. 3.12, AMs were cultured in the lower chamber, and different subsets of ILCs were then added on to the upper chamber. After 48 h, AMs from the lower chamber were harvested and the expression levels of M1 and M2 macrophage-related genes were analyzed.

Unexpectedly, mRNA expression levels of M1 macrophage-related genes (*Nos2* and *Cd86*) were decreased (Fig. 3.13 A) when AMs were co-cultured with ILC1s and ILC3s in the transwell. However, the expression levels of M2-related genes were not affected by co-culture with ILC1s /ILC3s (Fig. 3.13 B). IFNγ and IL-17A levels in culture supernatant from the transwell were significantly lower than that without the transwell (Fig. 3.13 C).

Moreover, the mRNA expression level of one of the M2 macrophage-related gene, *Arg1* was reduced, whereas that of *Mrc1* was increased (Fig. 3.14 A) when AMs were co-cultured with ILC2s in the transwell. However, the expression levels of M1-related genes were not affected by co-culture with ILC2s (Fig. 3.14 B). However, IL-4 and IL-13 levels remained unchanged in the presence of the transwell (Fig. 3.14 C).

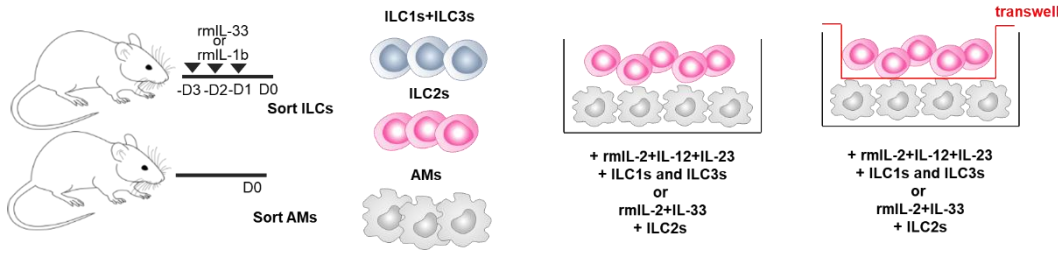

Figure 3.12 Schematic diagram of the co-culture procedure of ILCs and alveolar macrophages using transwell

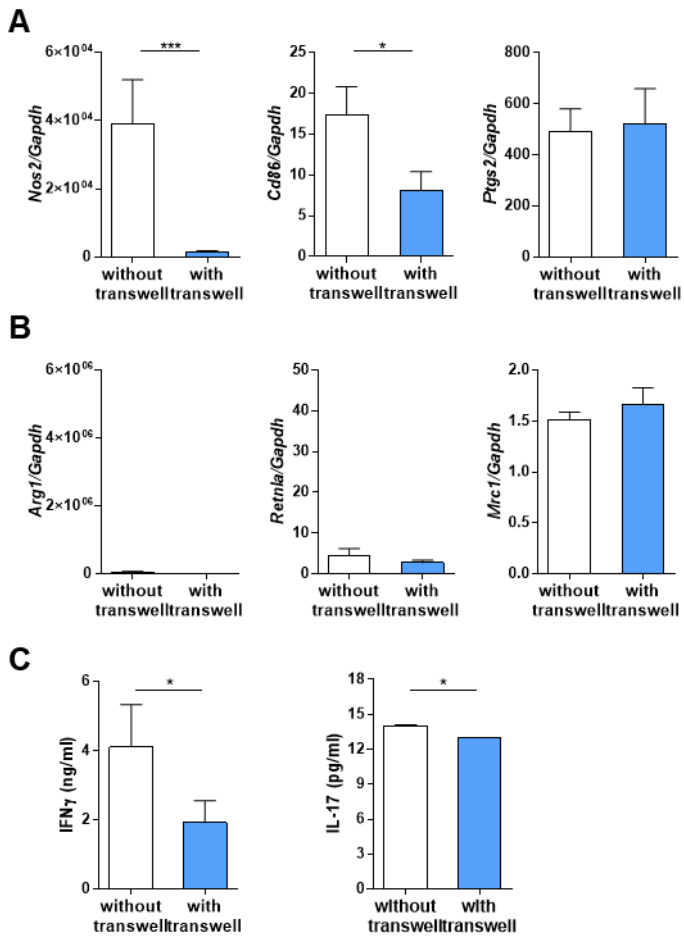

Figure 3.13 ILC1s/ILC3s-macrophage interaction affects polarization of macrophages via cell-cell contact

A-B. Expression of M1-related (*Nos2*, *Cd86*, and *Ptgs2*) (**A**) and M2-related (*Arg1*, *Retnla*, and *Mrc1*) (**B**) genes in alveolar macrophages co-cultured with ILC1s/ILC3s with and without the transwell insert. Each gene expression was relative to *Gapdh* expression. **C**. IFNγ and IL-17 production in the ILC1s/ILC3s and alveolar macrophages co-cultured group. **P* < 0.05, ****P* < 0.001 using Mann–Whitney test. The data are representative of three independent experiments, analyzed, and presented as the mean ± SEM.

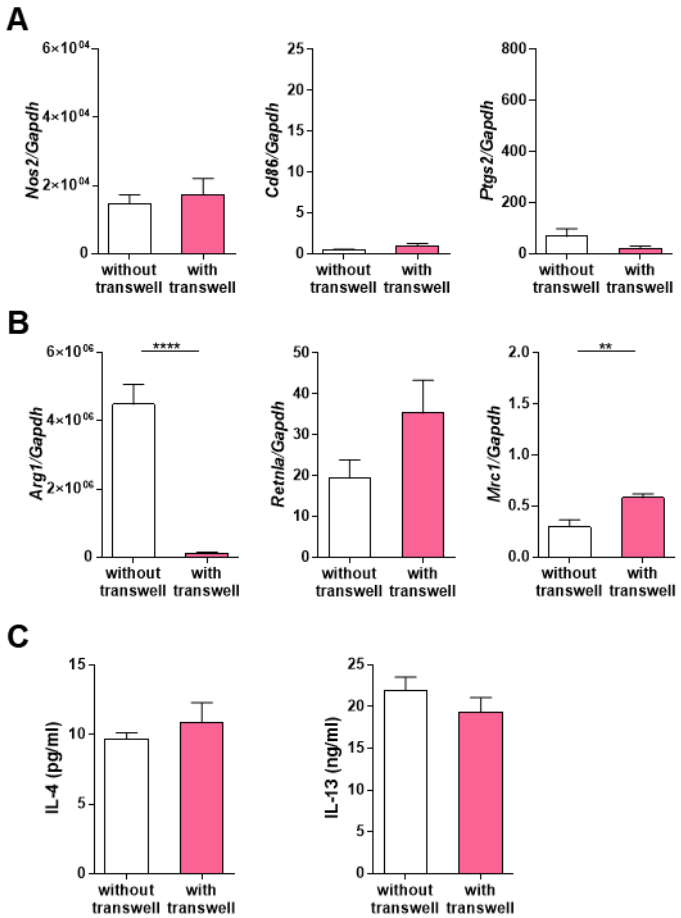

Figure 3.14 ILC2s -macrophage interaction affects polarization of macrophages via cell-cell contact

A-B. Expression of M1-related (*Nos2*, *Cd86*, and *Ptgs2*) (**A**) and M2-related (*Arg1*, *Retnla*, and *Mrc1*) (**B**) genes in alveolar macrophages co-cultured with ILC2s with and without the transwell insert. Each gene expression was normalized by *Gapdh* expression. **C**. IL-4 and IL-13 production in the ILC2s and alveolar macrophages co-cultured group. $^{**}P < 0.01$, $^{****}P < 0.0001$ using Mann–Whitney test. The data are representative of three independent experiments, analyzed, and presented as the mean ± SEM.

To investigate which surface molecules mediate the interaction of ILCs and macrophages, I treated anti-PD-1, anti-ICOS, and anti-CD40L which are co-stimulatory or inhibitory molecule that is expressed by ILCs[98-100]. When I co-cultured AM and ILC1/3s with blocking antibodies, there were no changes on M1 and M2 macrophage-related gene expression (data not shown). On the other hand, blocking antibodies treatment reduced the expression of *Arg1* and *Retnla* in co-culture group of alveolar macrophages and ILC2s (Fig. 3.15 A and B). Although I could not find which molecules specifically regulate the interaction between macrophages and ILC2s, these data mean that ILC2s could induce polarization of macrophages *via* co-stimulatory molecules, such as PD-1, ICOS, or CD40.

Taken together, these data suggest that specific types of ILCs regulate the polarization of AMs by cytokine secretion as well as cell–cell contact. The opposite might also hold true because cytokine secretion from ILCs was decreased when ILCs were separated from AMs. Further studies clarifying the precise mechanisms involved are merited.

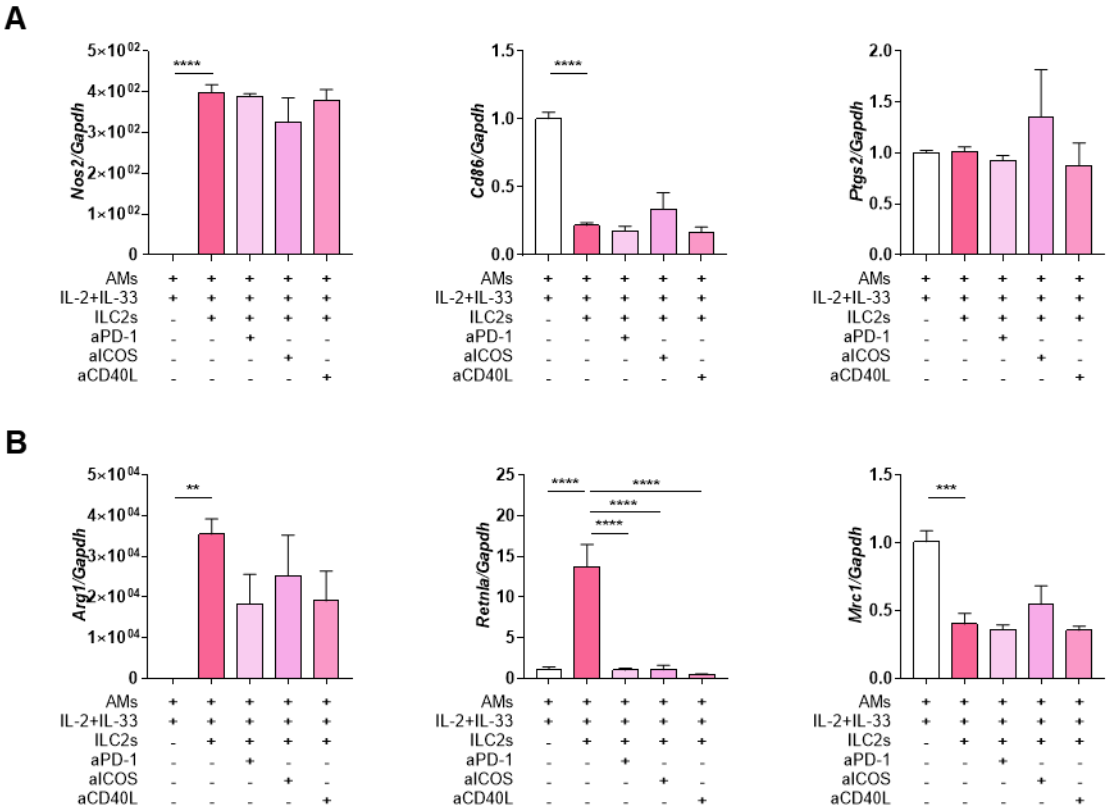

Figure 3.15. The changes of gene expression related to M1 and M2 macrophages in alveolar macrophages co-cultured with ILC2s using anti-PD-1, anti-ICOS, and anti-CD40L blocking antibodies

A. The gene expression of M1-related genes in alveolar macrophages, co-cultured with ILC2s using anti-PD-1, anti-ICOS, and anti-CD40L. **B**. The gene expression of M2-related genes in alveolar macrophages, co-cultured with ILC2s using anti-PD-1, anti-ICOS, and anti-CD40L. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ using one-way ANOVA following by a Bonferroni's post-test. The data are representative of two independent experiments and presented as the mean ± SEM.

3.3 Discussion

This study has demonstrated the distribution of innate immune cells (ILCs) within the airways of subjects with asthma, along with several additional novel findings. First, in addition to ILC2s, other types of ILCs were also increased in the sputum of asthmatics. Second, ILC2s and M2 macrophages were increased in eosinophilic asthmatics, whereas ILC1s, ILC3s, and M1 macrophages were increased in the non-eosinophilic asthmatics. Third, specific types of ILCs could induce the polarization of macrophages differently, thereby affecting the pathogenesis of asthma.

The discovery of ILCs in the lungs has provided a breakthrough in understanding of the cell types and innate pathways underlying the development of asthma[24, 77]. ILCs are an emerging type of immune cells that are lineage-negative but produce large amounts of effector cytokines[78]. Fort *et al.* first identified lineage$^-$ MHC class II^{high} and CD11c^{dull} ILC-like cells in the lungs of mice and demonstrated that IL-25 induces type 2 cytokine production from these cells[46]. Recent studies have intensively examined the role of ILCs following exposure to aero-allergens such as house dust mites, ryegrass or fungal extracts. These aero-allergens were found to cause robust eosinophilic airway inflammation and airway hyperresponsiveness (AHR) and also induce the production of type 2 cytokine from ILC2s in the absence of CD4 T cells in mice[101-103]. Moreover, the involvement of ILCs in non-allergic forms of asthma triggered by environmental factors such as air pollutants[104, 105] or obesity[26] has been reported. For example, CCR6$^+$ ILC3s that produce IL-17A in the lungs of mice fed with a high-fat diet are vital for the development of obesity-related asthma[26]. Moreover, IL-17A in the sputum or peripheral blood of asthmatics is correlated with the severity of the disease[106, 107]. So far, only ILC2s have been

implicated in the development of allergic asthma; however, other types of ILCs also appear to play important roles in different forms of asthma. In support of this hypothesis, the current study showed that the numbers of all types of ILCs were significantly increased in the sputum of patients with asthma when compared with those in the healthy controls. This diverse distribution of ILCs might reflect the heterogeneous nature of asthma.

To the best of my knowledge, this is the first study to report the presence of ILC1s and ILC3s in the sputum of asthmatics, unlike previous studies that focused on ILC2s only[27, 28, 96]. Although the function of ILCs in the development of asthma was originally identified in mice, several human studies have demonstrated their roles in the development of this condition[108, 109]. Bartemes *et al.* reported that the prevalence of ILC2s was increased in PBMC from allergic asthmatics when compared with healthy controls[96]. Nagakumar *et al.* demonstrated the presence of ILC2s in the airways of children with therapy-resistant asthma (STRA). They first identified ILC2s in induced sputum, where the proportion was found to be higher than that in peripheral blood[28]. Although several studies have been reported regarding the role of ILCs in asthma, there are several limitations. First, most studies examined ILCs using PBMC[110-112] rather than BALF or induced sputum. As shown by Gasteiger *et al*., PBMC does not reflect actual lung environments where ILCs are known to reside[113]. In the current study, I demonstrated the presence of ILCs in induced sputum, which might be a more accurate reflection of the profile of ILCs rather than that in blood. Second, there are a limited number of immune cells in the sputum. However, I tried to collect adequate amounts of sputum from the patients to analyze the accurate population of the immune cells and were able to confirm the

interactions between macrophages and ILCs in the present study. Third, previously, all experiments were focused only on ILC2s, and no other cell types were identified; however, in the current study, all types of ILCs were examined and found to be increased in the induced sputum of asthmatics. Moreover, the ILCs were associated with the polarization of macrophages in the sputum.

The proportion of ILC2s in sputum from eosinophilic asthmatics was higher than that from non-eosinophilic asthmatics. A positive correlation between ILC2s and sputum eosinophil counts was observed, whereas no such positive correlations were observed between ILC1s and ILC3s and neutrophils. Alternatively, the number of M1 macrophages in sputum was positively correlated with the number of neutrophils, whereas M2 macrophages proportions were positively correlated with eosinophil counts. These findings suggest that ILCs might directly affect the polarization of macrophages, which could regulate the infiltration of eosinophils or neutrophils into the airways. To confirm this hypothesis, I analyzed other immune cells from sputum and found that increase in the number of macrophages was correlated with the number of ILCs. In other words, ILC2s had a positive correlation with M2 macrophages, whereas both ILC1s and ILC3s demonstrated positive correlations with M1 macrophages. Co-culture experiments using mouse ILCs and AMs showed that activated ILCs could regulate the polarization of macrophages by secreting cytokines as well as cell– cell contact. M1 macrophages can be differentiated by IFNγ, whereas the key cytokines that activate M2 macrophages are IL-4 and IL-13[91, 114]. Therefore, IL-4 and IL-13 from ILC2s could induce the polarization of M2 macrophages, whereas IFNγ or IL-17A from ILC1s and ILC3s could induce the polarization of M1 macrophages. Among markers for M2

macrophages, the gene expression of mannose receptor C-type 1 (*Mrc1*) was down-regulated in alveolar macrophages co-cultured with ILC2s (**Figure 3.11 A**). *Mrc1* is one of typical markers for M2 macrophages, which is up-regulated by IL-4 and IL-13 stimulation[91]. However, in tissue-resident macrophages, the expression of *Mrc1* is independent of IL-4 and IL-13[115]. Alveolar macrophages are lung-resident macrophages, so IL-4 and IL-13 released from ILC2s might not affect the expression of *Mrc1*. Also, because alveolar macrophages highly expressed *Mrc1* in steady states (**Figure 3.4 A**; $CD11c^{+}CD206^{+}$ cells), I could know that the expression of Mrc1 in alveolar macrophages was regulated independent of IL-4 and IL-13. In addition, M2 macrophages do not constitute a uniform population and are further subdivided into M2a, M2b and M2c. MRC1 is expressed in M2a and M2c but not in M2b[116]. Therefore, decrease of MRC1 might indicate a change in the M2 subtype although further analysis need to be done. The roles of M1 and M2 macrophages in the development of asthma differ from each other. M1 macrophages are the major effector cells in non-allergic forms of asthma, while M2 macrophages are predominant in the allergic forms of asthma[117]. Although the current M1/M2 paradigm with its polarized extremes is oversimplified, these data showed that certain subsets of ILCs could regulate the polarization of macrophages. More interestingly, the polarization of macrophages was only induced when macrophages were co-cultured with ILCs. The combination of recombinant cytokines did not affect the polarization of macrophages indicating the importance of ILCs during this process. Thus, the ILCs-macrophages axis might be important to induce the development of different types of asthma.

It is worthy to note that polarized M1/M2 macrophages could induce the

activation of ILCs. M1 macrophages induce the activation of Th1 cells via TNFα and IL-12 production and by interacting with CD86 and MHCII molecules, thereby affecting the development of non-allergic asthma[118, 119]. Similarly, M2 macrophages activate Th2 cells via IL-4 and IL-13 production and by interacting with CCL17 and MRC1 and lead to the development of allergic asthma[91]. There is a possibility that M1 or M2 macrophages, polarized by ILCs, regulate ILCs in return in a cell contact manner, and the inhibition of this interaction in the transwell system may have reduced cytokine production by the ILCs. In **Figure 3.13 C**, IFNγ and IL-17A production from ILC1/3s was reduced by separating alveolar macrophages and ILCs. However, IL-4 and IL-13 level released by ILC2s was not changed by the transwell system (**Figure 3.14 C**). These data suggest that surface molecules on M1 or M2 macrophages differ from alveolar macrophages by polarization. Differences in surface molecules between M1 and M2 macrophages might affect activation of each subtypes of ILCs. However, it is also possible that cytokine production from ILC1/3s was reduced by alleviated polarization of macrophages. In **Figure 3.13 A**, gene expression related to M1 macrophages was reduced by using transwell. M1 macrophages can produce IL-6, IL-12, and IL-1β, which activate ILC1s or ILC3s[120, 121]. Thus, these cytokine productions could be also reduced as M1 macrophage polarization was relieved by transwell, which, in turn, reduced ILC1/3s activation. On the other hand, M2 macrophages do not seem to produce cytokines which activate ILC2s. ILC2s activated by IL-33, IL-25, and TSLP released by epithelial cells, structural cells or dendritic cells[122]. In this regard, although M2 macrophage polarization was relieved in transwell system (**Figure 3.14 B**), innate cytokines activating ILC2s might not be changed. Therefore, IL-4 and IL-13 production from ILC2s was not changed by using transwell in **Figure 3.14 C**.

Recently, it has been reported that ILCs also expresses co-stimulatory or inhibitory molecules such as PD-1, ICOS, and CD40L on their surfaces[98-100, 123]. Activated alveolar macrophages also express high level of PD-L1 and CD40[124, 125]. Therefore, I hypothesized that ILCs and macrophage could interact through these molecules. When I blocked the ILC1/3s-macrophage interaction using anti-PD-1, anti-ICOS, and anti-CD40L antibodies, M1 and M2 macrophage-related genes were not changed (data not shown). That means unknown molecules, not PD-1, ICOS, and CD40L, might mediate cell-cell interaction between ILC1/3s and alveolar macrophages. In case of ILC2s-macrophage interaction, the expression of *Arg1* and *Retnla* were down-regulated by these blocking antibodies (**Figure 3.15 B**). PD-1 is originally inhibitory molecules in T cells[126, 127]. Ligation with PD-L1, PD-1 signaling inhibits TCR signaling and co-stimulatory signals by CD28 and B7 molecules, which reduces cytokine production from T cells. The function of ILC2s is also reduced by PD-l blockade[128]. However, in ILC2s, activation of ILC2s with IL-33 stimulation or helminth infection enhances the expression of PD-1 on ILC2s, which activates Th2 cells and promotes type 2 effector function[129]. Therefore, how PD-1 signaling in ILC2s and PD-1/PD-L1 interaction between macrophages and ILC2s affect M2 macrophage polarization should be further studied. ICOS and ICOS-L are both expressed in ILC2s and ICOS signaling is important for ILC2 activation[130, 131]. ICOS-L expression is also important for macrophage functions. ICOS-L deficient-mice shows decreased IL-4, IL-6, and IL-10 expression and less wound healing upon skin injury[132]. Theses cytokines are majorly produced by Th2 cells, but also from M2 macrophages. Moreover, ICOS-L depletion enhances fibrosis in bleomycin-induced fibrosis model in which M2 macrophages are increased and related to myofibroblast differentiation[133-135]. In this study, the major cell population which express ICOS-L

is macrophages. Although both ILC2s and ILC3s express CD40L in response to stimulation[100, 123], how CD40L-CD40 interaction affects polarization of M2 macrophages should be elucidated. Although I could not show specific surface molecules that mediate the interaction between ILCs and macrophages in current study, I believe that identifying these interactions will be important for understanding the features of ILCs and macrophages.

Although I have found a close relationship between ILCs and macrophages according to the phenotypes of asthma, I failed to see the correlation between ILCs and macrophages with AHR (data not shown). As far as I know, there was no report about the correlation between ILCs and AHR in human asthmatics. One reason why I couldn't see the positive relationship might due to the sample I obtained. In this study, I recruited newly diagnosed, and treatment-naïve patients to exclude the interference effects of drugs on immune cells. Therefore, most patients I recruited belong to mild asthmatics with the level of FEV_1 % was more than 80%. Since AHR was caused by severe inflammation and airway damage, it is hard to see the correlation between AHR and immune cells in the subjects of this study which are mostly from mild asthmatics. Also, it is possible that ILCs and macrophages are not direct mediators of AHR. The inflammatory factors that known to induce AHR are histamine and leukotrienes, and these proteins are mostly released from eosinophils and mast cells[136]. I could find negative correlation between AHR and eosinophils (data not shown). Perhaps increased ILCs or macrophages could regulate AHR through interaction with other immune cells such as eosinophils and mast cells.

In conclusion, this study shows, for the first time, that in addition to ILC2s various other ILCs are also increased in the airway of subjects with asthma. These

ILCs might be essential sources of cytokines that induce the polarization of macrophages and control the development of airway eosinophilia or non-eosinophilic inflammation. Understanding the factors that regulate the activation of ILCs may be important for the treatment of subjects with difficult-to-control asthma because ILCs can crosstalk with several innate immune cells. ILCs might play key roles in controlling allergic or non-allergic inflammation in asthmatics during the early stages of development of asthma. However, more experiments evaluating the relationships between ILCs, macrophages, and other immune cells in the lungs are warranted.

4. Chapter II

Serum amyloid A is associated with emphysema through group 3 innate lymphoid cells

4.1 Introduction

Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory disease of the lower respiratory tract. Obstructive bronchiolitis and emphysema, induced by continuous damage and repair and chronic inflammation, are two representative pathological phenotypes of COPD[31]. Cigarette smoke is a well-known cause of COPD, and environmental noxious particles, viral or bacterial infection, and genetic factors also affect the development of COPD[137]. Accumulation of inflammatory cells, such as neutrophils, macrophages, $CD8^+$ T cells, Th1, and Th17 cells[138-140], and production of protease, such as metalloproteinase and neutrophil elastase[141, 142], contribute to systemic inflammation in COPD. In COPD, acute exacerbation of COPD (AECOPD) causes serious progress of the disease, which is occurred by infections or exposure to air pollutants[143]. The onset frequency of AECOPD and severity are closely associated with total disease burden and survival outcome[144]. Therefore, the clinical diagnosis and assessment of AECOPD are significant in prognosis of patients.

Serum amyloid A (SAA) is one of acute-phase protein which level in blood is 1000-fold upregulated after acute inflammatory responses[145]. SAA is released mainly from liver, but also from macrophages and epithelial cells in inflammatory sites[146, 147]. SAA is increased also in several persistent inflammatory diseases, such as atherosclerosis, rheumatoid arthritis, and cancer[148-150], which suggest SAA is used as a biomarker for certain diseases. In COPD, serum level of SAA is elevated both in stable patients and acute exacerbation of COPD[151, 152], and SAA is an effective biomarker for diagnosis and treatment of AECOPD[153]. SAA acts as an endogenous ligand binding to formyl peptide receptor 2 (FPR2) or toll-like receptors (TLRs) to

induce immunologic responses in several diseases[154]. However, whether and how SAA increased in COPD patients affects immune systems, especially innate lymphoid cells, is not fully understood.

Innate lymphoid cells (ILCs) are one of innate lymphocytes which share many characteristics with helper T cells[155]. Like helper T cells, ILCs are divided into three groups, ILC1s, ILC2s, and ILC3s, by expression of transcription factors and cytokines. However, ILCs do not express antigen-specific receptors but express several receptors for environmental factors that make ILCs respond rapidly to environmental change[78]. In respiratory system, the role of ILCs are mostly studied in asthma. ILC2s are related to both pathogenesis of asthma by producing type 2 cytokines such as IL-5 and IL-13 in allergen-induced asthma[82], and tissue repair by secreting epidermal-growth-factor-like molecule amphiregulin in virus-induced asthma[25]. ILC3s are also related to non-allergic asthma, such as obesity-induced asthma, where they produce IL-17 to enhance airway hyperresponsiveness[26]. In COPD, the role of ILCs in pathogenesis is less investigated. Recent studies show that the conversion of ILC2s to IFNγ-producing ILC1s both in COPD patients and a mouse model of COPD[33, 34]. Because ILC2s are the most abundant ILCs and other subsets of ILCs are relatively rare in mice lung[156], the most studies about the association of ILCs in COPD pathogenesis have focused on ILC2s. However, ILC3s are the most prevalent subsets of ILCs in human lung, and COPD patients have a larger number of ILC3s compared to healthy donors[35]. Moreover, IL-17A is elevated in bronchial mucosa of COPD patients and also associated with emphysema in chronic COPD induced by smoking[157, 158],which suggests ILC3s have a potency to be related to inflammation seen in COPD. However, it is unclear how ILC3s are

increased in COPD and whether they are involved in pathogenesis of COPD.

In this study, I found that IL-17A-producing ILC3s were increased in emphysema mice model, independent with adaptive immune system. In emphysema condition, the expression of SAA from myeloid cells was upregulated. SAA administration into murine lung led to IL-1β secretion from neutrophils, which reinforced the proliferation of ILC3s and production of IL-17A. Moreover, depletion of ILC3s impaired emphysema-like phenotypes. The SAA-neutrophils-ILC3s axis was confirmed in patients with COPD and the frequency of ILC3s was correlated with decrease of lung function of the patients. Collectively, these results suggested that ILC3s are critical to emphysema pathogenesis and should be considered as a therapeutic target for emphysema dominant COPD.

4.2 Results

4.2.1 A combination model of lipopolysaccharide (LPS) and porcine pancreas elastase (PPE) to induce emphysema

To induce chronic obstructive pulmonary disease (COPD), a combination of lipopolysaccharide (LPS) and porcine pancreas elastase (PPE) was administrated into the mice by intra-tracheal injection (Fig. 4.1 A and B). Emphysema phenotype, which is major pathological types of COPD, was dominant in this model, so I called it as an emphysema model. Only single dose of LPS and PPE induced an emphysema-like histologic change in acute model, which was enhanced with continuous treatment of LPS and PPE in chronic model (Fig. 4.1 C). Several proteases, such as matrix metalloproteinase (MMP), are produced by inflammatory cells and these proteases lead to degradation of elastin and collagen to induce emphysema-like phenotype in COPD[159]. Among several types of MMP, the expression of *Mmp12* was increased both in acute and chronic emphysema model (Fig. 4.1 D). The mRNA expression of *Mmp1*, *Mmp2*, *Mmp8*, and *Mmp9* were not changed with induction of emphysema (data not shown). Serum amyloid A (SAA) is known as a biomarker for COPD[152] and related to disease severity of the patients[160]. The mRNA level of *Saa3*, an isoform of SAA observed in inflammatory tissues[146], was increased in lung tissue after induction of emphysema (Fig. 4.1 E).

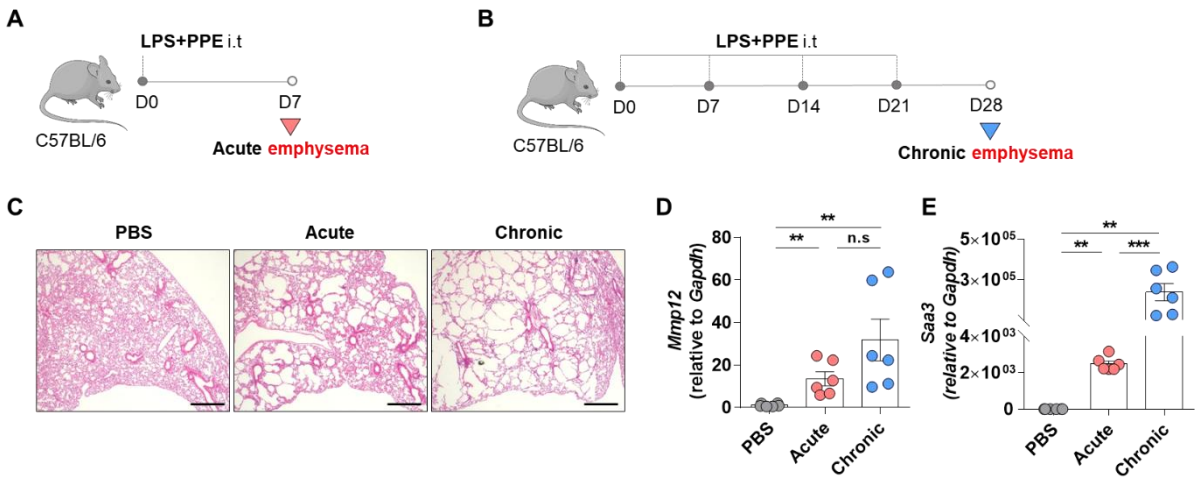

Figure 4.1 The expression of serum amyloid A was increased in emphysema-induced mice

A-B. Schematic representation of the experimental protocol. A combination of LPS and PPE was injected intra-tracheally (i.t) to C57BL/6 mice at day 0 and sacrificed 7 days after treatment (acute model) (**A**), or treated at day0, 7, 14, and 21 then sacrifice at 7 days after last treatment (chronic model) (**B**). **C**. Representative images of lungs from PBS or emphysema-induced mice. Scale bars, 500 µm. **D-E**. The expression of *Mmp12* (**D**) and *Saa3* (**E**) from lung tissue of emphysema-induced mice was quantitatively analyzed relative to the expression *Gapdh*. n.s; non-significant, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ using one-way ANOVA followed by a Bonferroni's post-test. The data are presented as the mean ± SEM.

4.2.2 Pulmonary IL-17A-producing ILC3s are increased in emphysema model

To investigate the relevance of innate lymphoid cells (ILCs) in pathogenesis of emphysema, ILCs were analyzed by flow cytometry after induction of emphysema (Fig. 4.2) and IL-17A-producing ILC3s were increased both in acute and chronic model of emphysema (Fig. 4.3 A and B). In lung, ILC2s also produce IL-17A after activation and play a pathologic role as they express IL-5, IL-13, and IL-17 simultaneously[161]. Therefore, I checked if IL-17A was produced by ILC2s, but IL-17A was secreted independently with IL-13 (data not shown). IFNγ-producing ILC1s or IL-13-producing ILC2s were not changed in acute model of emphysema, while ILC1s were slightly increased in chronic model of emphysema (Fig. 4.3 A and B). IFNγ, IL-13, and IL-17A$^+$ CD4$^+$ T cells were also not changed in acute model of emphysema, even though IFNγ$^+$ and IL-17A$^+$ CD4$^+$ T cells were increased in chronic emphysema model (Fig. 4.4 A and B). These data suggest that ILC3s might be involved in initiation of pathogenesis of emphysema.

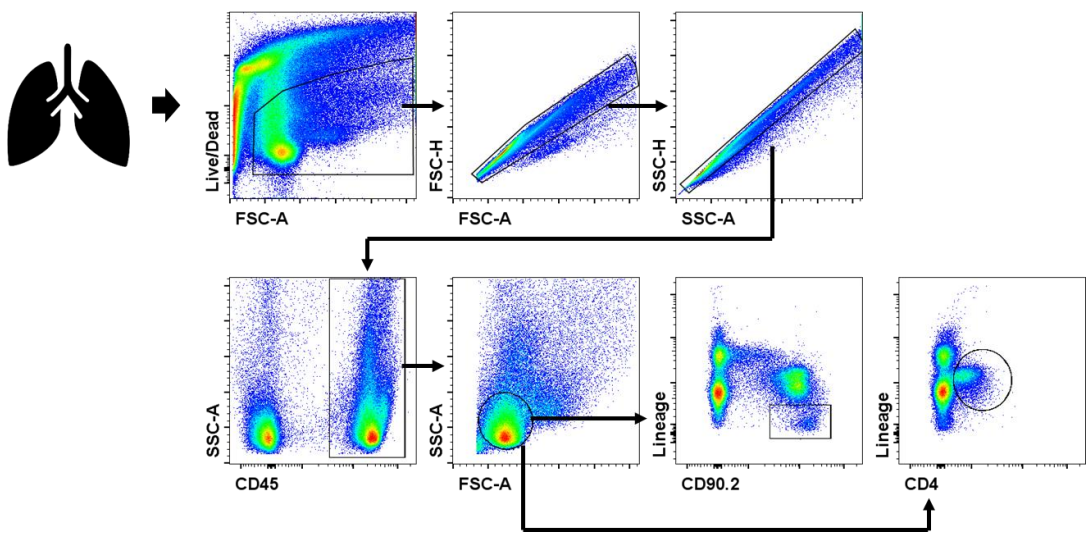

Figure 4.2 Gating strategy of ILCs and CD4$^+$ T cells from murine lung

ILCs were gated as live single cells, expressing CD45 and CD90.2 and not expressing lineage markers. CD4$^+$ T cells were gated as CD45$^+$lineage$^+$CD4$^+$ live single cells.

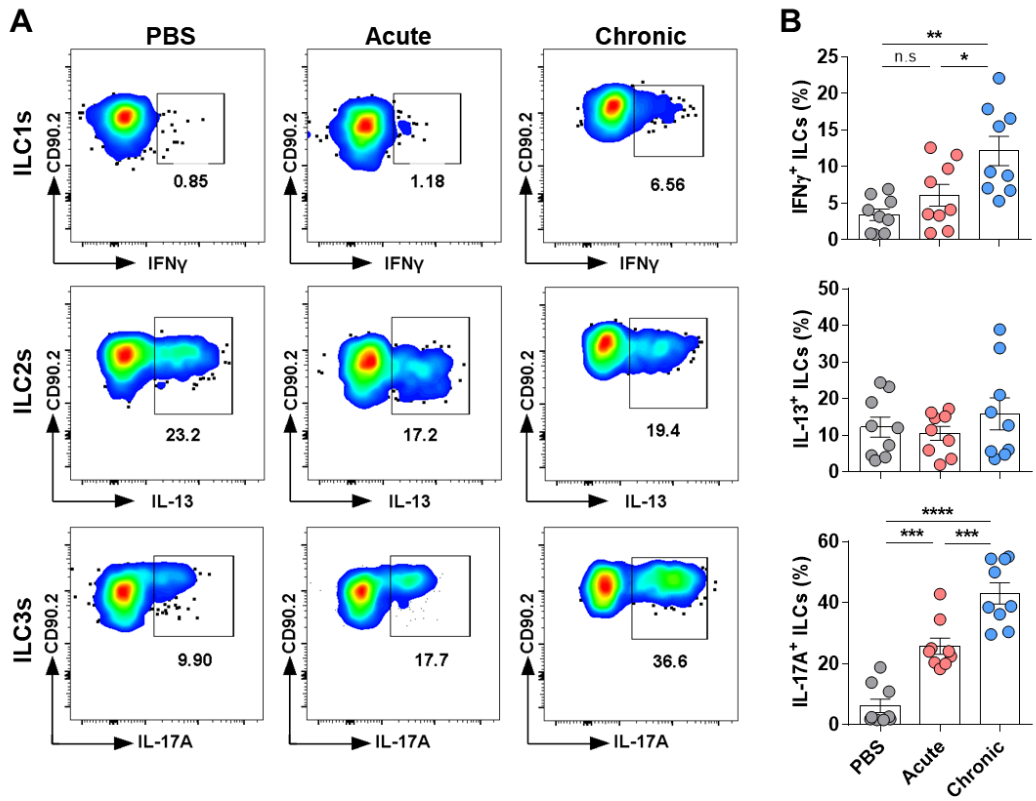

Figure 4.3 Type 3 innate lymphoid cells were increased in emphysema-induced mice

A. Representative flow cytometry dot plots IFNγ, IL-13, and IL-17A production from innate lymphoid cells ($CD45^+Lineage^-CD90.2^+$ cells). The quantification results are shown in **B**. n.s; non-significant, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$, using one-way ANOVA followed by a Bonferroni's post-test. The data are presented as the mean ± SEM.

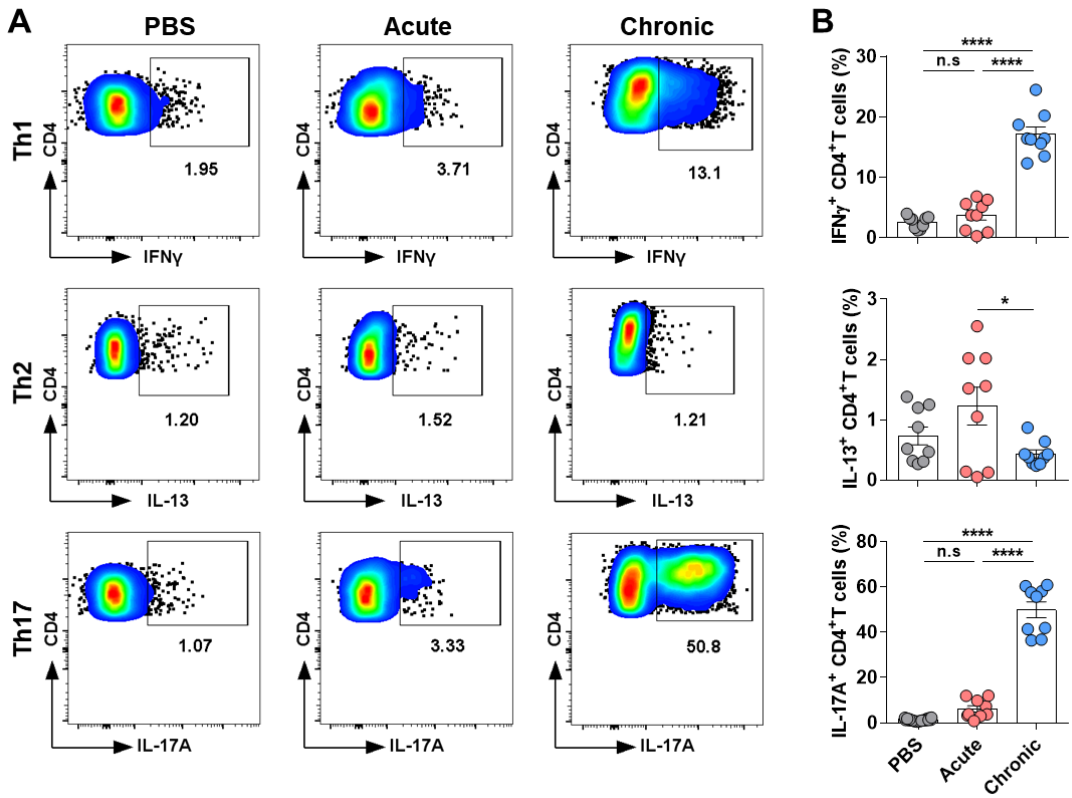

Figure 4.4 Cytokine production of $CD4^+$ T cells were only increased in chronic emphysema

A. Representative flow cytometry dot plots IFNγ, IL-13, and IL-17A production from $CD4^+$ T cells ($CD45^+Lineage^+CD4^+$ cells). The quantification results are shown in **B**. n.s; non-significant, *$P \leq 0.05$, ****$P \leq 0.0001$ using one-way ANOVA followed by a Bonferroni's post-test. The data are presented as the mean ± SEM.

4.2.3 Type 3 innate lymphoid cells are increased in emphysema model independently with adaptive immune cells

To confirm the involvement of ILCs in emphysema pathogenesis in T cell-independent manner, LPS and PPE were administrated into Rag1 knockout ($Rag1^{-/-}$) mice which have a defect in T cells and B cells, with the same scheme in Fig. 4.1 A and B. As in wild type mice, emphysema-like phenotypes were observed in $Rag1^{-/-}$ mice (Fig. 4.5 A), and the expression of *Mmp12* and *Saa3* were increased both in acute and chronic model of emphysema (Fig. 4.5 B and C). However, the expression of *Mmp12* and *Saa3* were tended to decrease in chronic model compared to in acute model. Moreover, like in wild type, IL-$17A^{+}$ ILCs also showed increase in emphysema model in the absence of T cells (Fig. 4.6 A and B). IFNγ^{+} ILCs and IL-13^{+} ILCs were also changed by LPS and PPE treatment, while the most affected ILCs were type 3 ILCs, also in $Rag1^{-/-}$ mice. These results suggest that type 3 ILCs might affect early phase of pathogenesis of emphysema, although T cells are needed to induce inflammation and recruit inflammatory cells in chronic phase of emphysema.

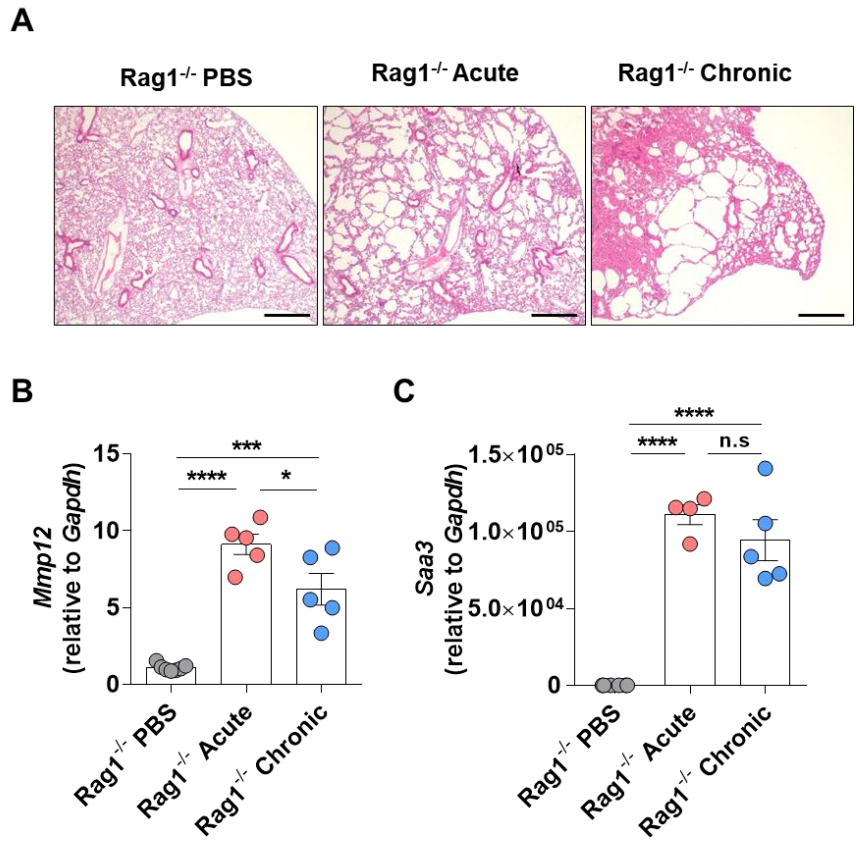

Figure 4.5 Increased expression of serum amyloid A in acute phase of emphysema is T cell-independent

A. Representative images of lungs from Rag1$^{-/-}$ mice. Scale bars, 500 µm. **B-C**. The expression of *Mmp12* (**B**) and *Saa3* (**C**) in the lungs from emphysema-induced Rag1$^{-/-}$ mice was quantitatively analyzed relative to the expression of *Gapdh*. n.s; non-significant, *$P \leq 0.05$, ***$P \leq 0.001$, ****$P \leq 0.0001$, using one-way ANOVA followed by a Bonferroni's post-test. The data are presented as the mean ± SEM.

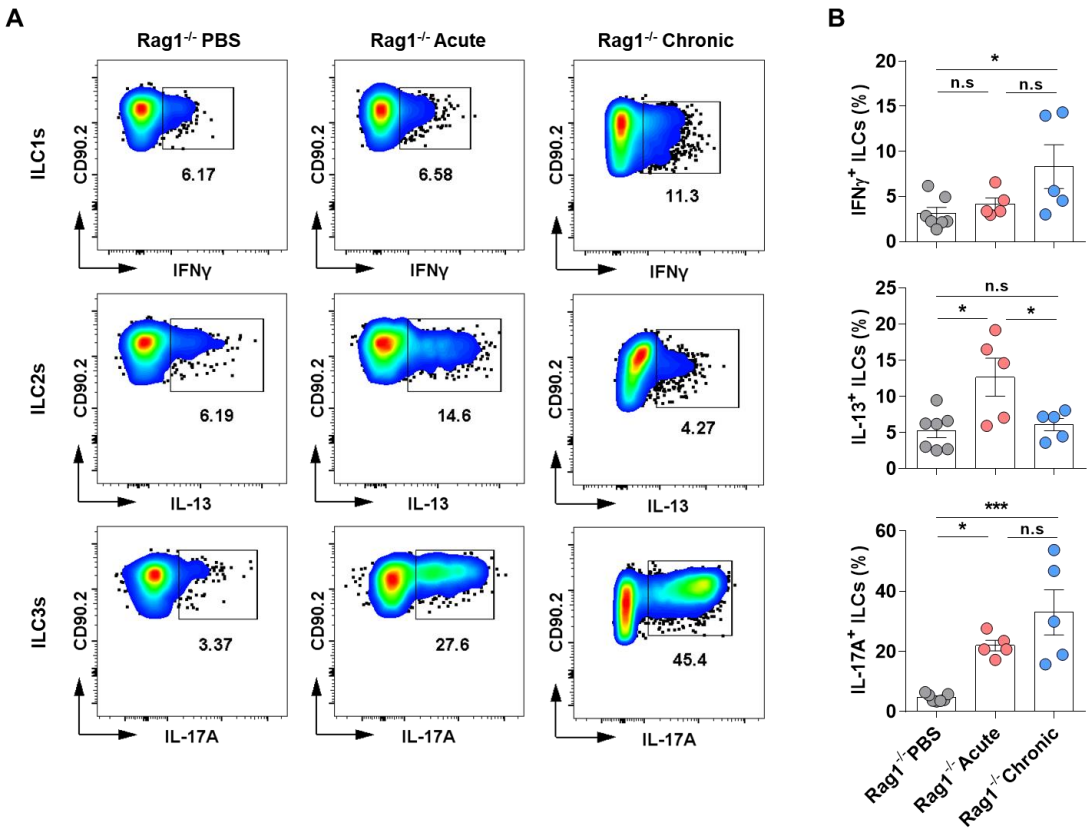

Figure 4.6 Increase of type 3 innate lymphoid cells both in acute and chronic emphysema is T cell-independent

A. Representative flow cytometry dot plots of IFNγ, IL-13, and IL-17A production from ILCs ($CD45^+Lineage^-CD90.2^+$ cells) of $Rag1^{-/-}$ mice. **B.** Comparison of IFNγ, IL-13, and IL-17A production from ILCs in $Rag1^{-/-}$ mice between control and emphysema-induced mice. n.s; non-significant, $*P \leq 0.05$, $***P \leq 0.001$ using one-way ANOVA followed by a Bonferroni's post-test. The data are presented as the mean ± SEM.

4.2.4 Inflammatory monocytes are major source of SAA in emphysema

SAA is known as an acute phase protein released by hepatocytes in liver and epithelial cells and macrophages in inflammatory tissues[154]. To identify which cells mainly produce SAA in emphysema model, I examined cellular sources of SAA in the lung by flow cytometry. SAA^+ cells were increased in emphysema model of WT and $Rag1^{-/-}$ mice (Fig. 4.7 A and B). In model of emphysema in wild type mice, SAA were mainly produced by epithelial cells and alveolar macrophages in control mice, while inflammatory monocytes and dendritic cells became major cell sources of SAA after LPS and PPE injection (Fig. 4.8 A and B). Monocytes and dendritic cells were also major sources of SAA in emphysema in $Rag1^{-/-}$ mice (data not shown). These results were confirmed with comparison of expression of SAA after gating of monocytes and dendritic cells (Fig. 4.9 A and B).

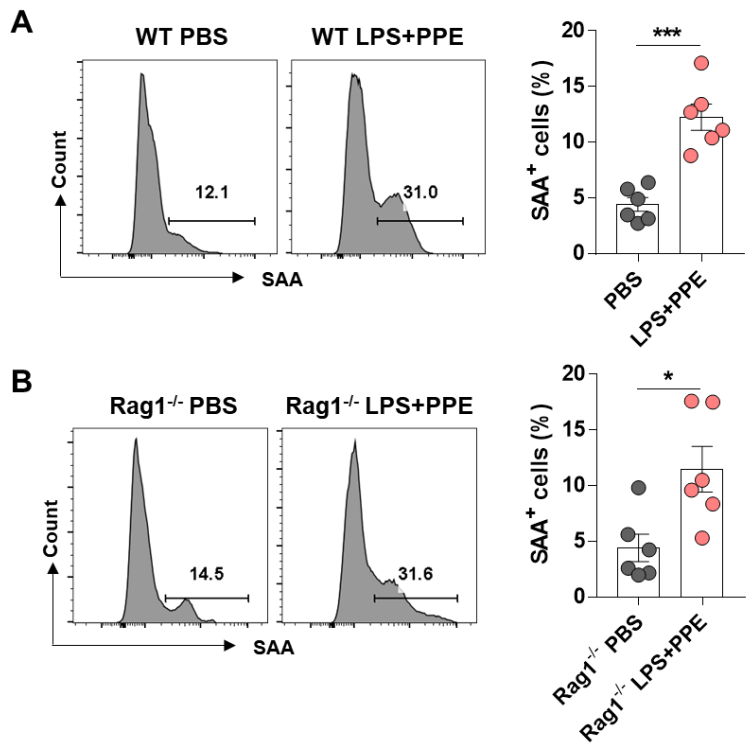

Figure 4.7 Release of SAA in wild type (WT) and Rag1$^{-/-}$ mice was upregulated in emphysema model

A. SAA^{+} cells from total cells of wild type (WT) mice. **B**. SAA^{+} cells from total cells of Rag1$^{-/-}$ mice. *$P \leq 0.05$, ***$P \leq 0.001$ using unpaired Mann-Whitney test. The data are presented as the mean ± SEM.

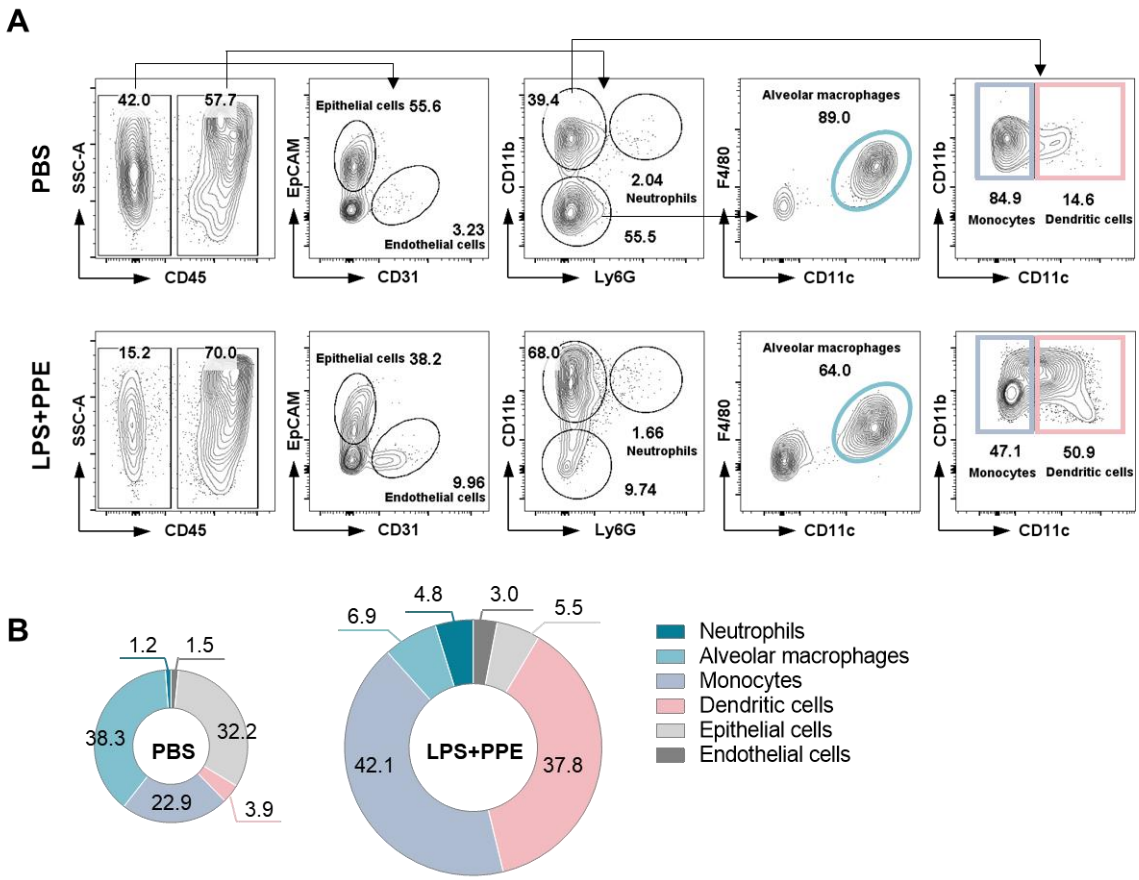

Figure 4.8 Serum amyloid A was released by myeloid cells after emphysema model induction

A. The source of SAA from wild type (WT) mice after acute emphysema model induction. **B**. Pie chart of cells secreting SAA both in PBS and emphysema group of WT mice lung.

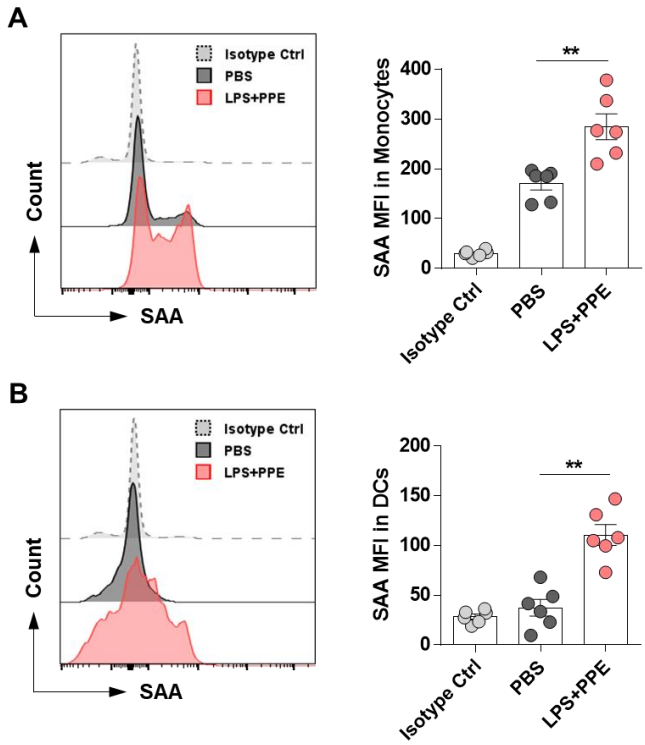

Figure 4.9 Serum amyloid A were released by monocytes and dendritic cells in emphysema model

A. Comparison of SAA expression from monocytes ($CD45^{+}CD11b^{+}Ly6G^{-}CD11c^{-}$). **B**. Comparison of SAA expression from dendritic cells ($CD45^{+}CD11b^{+}Ly6G^{-}CD11c^{+}$). $^{**}P \leq 0.01$ using Kruskal-Wallis test followed by a Dunn's post-test or one-way ANOVA followed by a Bonferroni's post-test. The data are presented as the mean ± SEM.

4.2.5 Serum amyloid A induce emphysema-like inflammation with the increase of IL-17A-producing ILC3s

SAA induced by segmented filamentous bacteria colonization acts as an enhancer to IL-17 production of T cells in intestine[162]. I hypothesized that SAA might affect the increase of type 3 ILCs in early phase of emphysema model. To determine whether SAA affect increase of ILC3s, I administrated human SAA1 recombinant protein by intra-tracheal injection. Because SAA3 is pseudogene in human, I used SAA1 protein and this isoform in human is the most similar to mouse SAA3 in amino acid sequence[163]. I sacrificed mice at 3 days after SAA injection to identify the role of SAA in induction of ILC3s in early phase of inflammation (Fig. 4.10 A). Only single dose of SAA induced acute inflammation and histologic change in lung (Fig. 4.10 B). Moreover, administration of SAA in lung induced IL-17A production from ILCs, not IFNγ or IL-13 production (Fig. 4.11 A). IL-17A secretion from ILCs were gradually increased after SAA injection (Fig. 4.11 B). IL-17A produced by T cells were also increased after SAA injection, however, the extent of IL-17A production from T cells was less than from ILCs (Fig. 4.12 A and B).

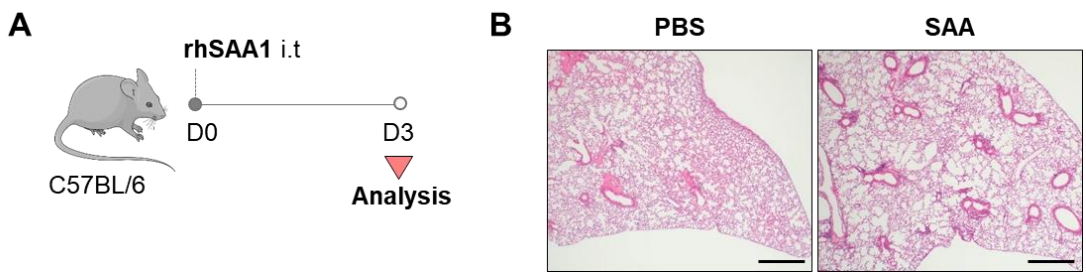

Figure 4.10 Inoculation of serum amyloid A induced inflammation in lung

A. Schematic diagram of SAA-induced emphysema model. Recombinant SAA (10μg) was injected intra-tracheally (i.t). **B**. Microscopic analysis of lungs of SAA-induced emphysema model with H&E staining. Scale bars, 500μm.

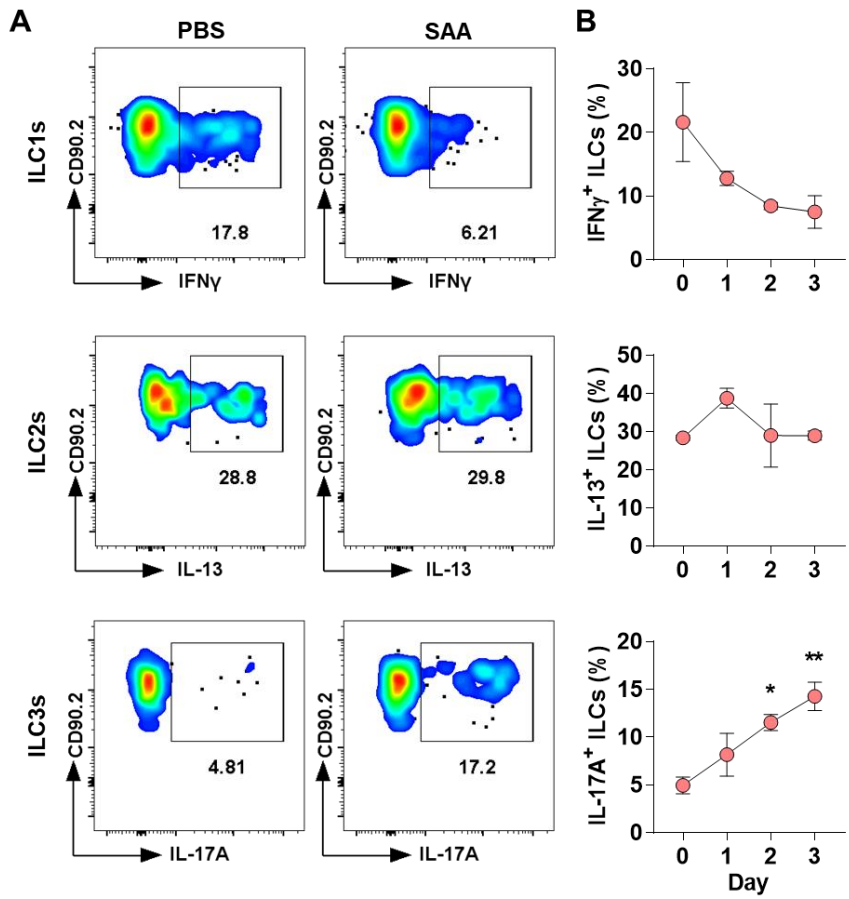

Figure 4.11 Type 3 innate lymphoid cells were increased in SAA-induced emphysema model

A. Plot of flow cytometry of IFNγ, IL-13, and IL-17A production from ILCs ($CD45^+Lineage^-CD90.2^+$ cells) at 3 days after SAA injection. **B**. Kinetic analysis of cytokine production from ILCs after SAA injection. *$P \leq 0.05$, **$P \leq 0.01$, using Kruskal-Wallis test followed by a Dunn's post-test or one-way ANOVA followed by a Bonferroni's post-test. The data are representative of 2 or 3 independent experiments and presented as the mean ± SEM.

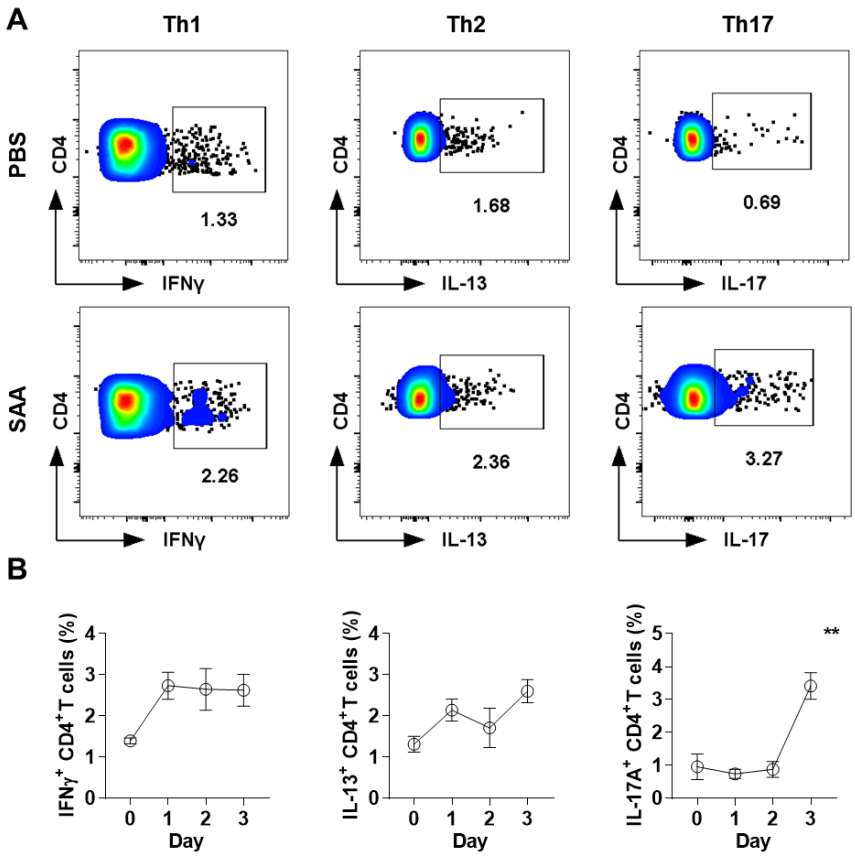

Figure 4.12 Cytokine production from CD4$^+$ T cells

A. Plot of flow cytometry of IFNγ, IL-13, and IL-17A production CD4$^+$ T cells (CD45$^+$Lineage$^+$CD4$^+$ cells) at 3 days after SAA injection. **B**. Kinetic analysis of cytokine production from CD4$^+$ T cells after SAA injection. $^{**}P \leq 0.01$, using Kruskal-Wallis test followed by a Dunn's post-test or one-way ANOVA followed by a Bonferroni's post-test. The data are representative of 2 or 3 independent experiments and presented as the mean ± SEM.

4.2.6 Serum amyloid A promote the expression of IL-1β to activate proliferation of ILC3s

After SAA inoculation, IL-17A$^+$ ILCs were proliferated with the expression of proliferation marker, Ki67 (Fig. 4.13 A). To determine whether SAA directly induce IL-17A production from ILCs, SAA were treated to lineage depleted cells *in vitro*. Although IL-17A$^+$ ILCs were increased in SAA-treated group (Fig. 4.13 B) *in vitro*, there was more increase of IL-17A production from ILCs *in vivo* (Fig. 4.11 A and B). Therefore, it is possible that there are other mechanisms in IL-17A production from ILCs after SAA administration. ILCs are activated by innate cytokines, and IL-1β and IL-23 are key cytokines that induce IL-17 secretion from ILC3s[155]. Thus, I analyzed the mRNA expression of IL-1β and IL-23 in lung tissue after SAA administration. The expression of IL-1β was acutely increased after SAA injection, while the expression of IL-23 was not changed significantly (Fig. 4.14 A). Other innate cytokines, IL-12 and IL-18, which are key cytokines to induce IFNγ from ILC1s, and IL-33, which activates ILC2s to produce type 2 cytokines, were not changed significantly after SAA inhalation (Fig. 4.14 B and C). These results suggest that SAA induce IL-1β secretion that activates IL-17A production and proliferation of ILC3s.

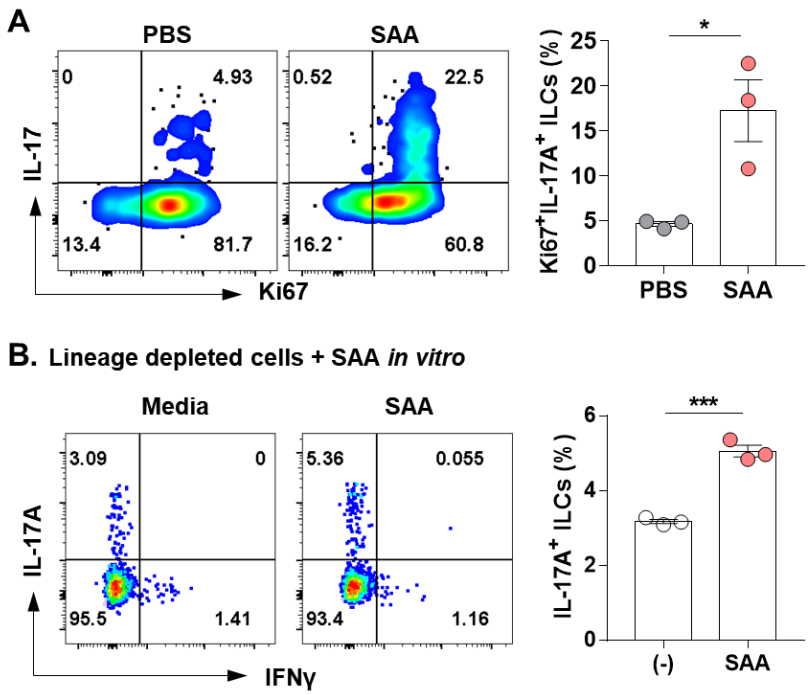

Figure 4.13 Serum amyloid A promoted the proliferation of IL-17A$^+$ ILC3s

A. Comparison of Ki67 expressing IL-17$^+$ innate lymphoid cells between PBS and SAA-injected mice. **B**. *In vitro* treatment of SAA to lineage depleted cells and comparison of IL-17A production from ILCs. *$P \leq 0.05$, ***$P \leq 0.001$, using Mann-Whitney comparison test. The data are representative of 2 or 3 independent experiments and presented as the mean ± SEM.

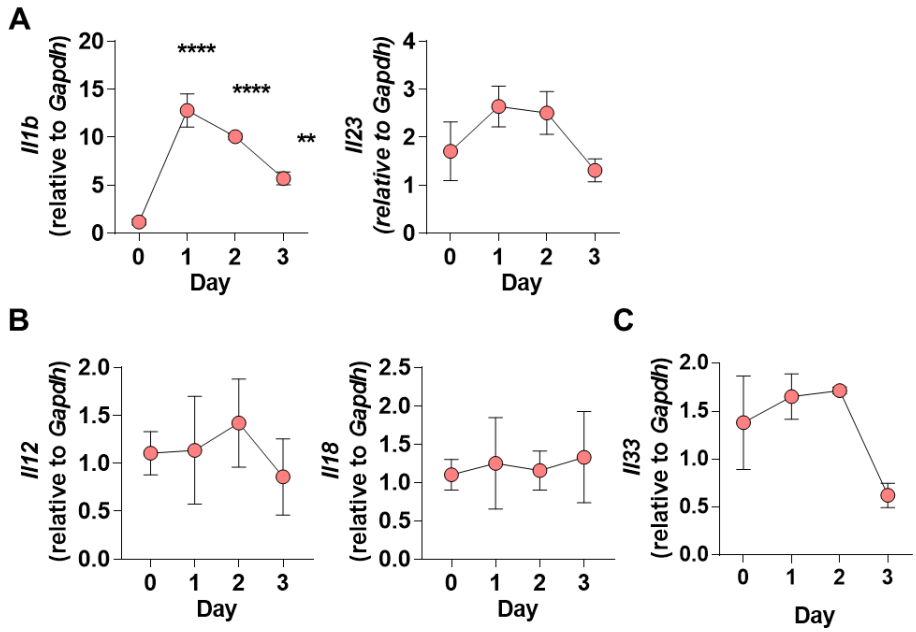

Figure 4.14 Gene expression of ILC-stimulating cytokines in SAA-induced emphysema model

A-C. Gene expression of cytokines stimulating different subsets of ILC. Gene expression of *Il1b* and *Il23* (**A**), *Il12* and *Il18* (**B**), and *Il33* (**C**) from lung tissue of SAA-induced emphysema model. The expression was relative to *Gapdh*. ***P*≤0.01, *****P*≤0.0001, using one-way ANOVA followed by a Bonferroni's post-test. The data are representative of 2 or 3 independent experiments and presented as the mean ± SEM.

4.2.7 SAA promotes IL-1β production from neutrophils directly

To identify cellular sources of IL-1β for ILC3 activation, I analyzed IL-1β$^+$ cells by flow cytometry at 3 days after SAA injection (Fig. 4.10 A). IL-1β-expressing cells were increased after SAA administration (Fig. 4.15 A and B). In naïve mice, neutrophils were major cell types of IL-1β-expressing cells in lung, and after SAA injection, neutrophils comprised more proportion of IL-1β$^+$ cells (Fig. 4.15 A and C). The most promised cell type producing IL-1β, next to neutrophils, was dendritic cells (Fig. 4.15 C). To confirm whether SAA directly activate neutrophils or dendritic cells, I treated recombinant protein SAA1 to bone-marrow derived neutrophils and dendritic cells *in vitro*. The expression of IL-1β were upregulated at 12 hours after SAA treatment (Fig. 4.16 A and C) and the expression of IL-1β was larger in neutrophils than in dendritic cells. Moreover, the expression of formyl peptide receptor 2 (FPR2), toll-like receptor 2 (TLR2), and TLR4, which are known as receptors for SAA[154], were increased in neutrophils after SAA treatment *in vitro* (Fig. 4.16 B). The expression of FPR2 and TLR2 were also increased in dendritic cells, but not the expression of TLR4 (Fig. 4.16 D).

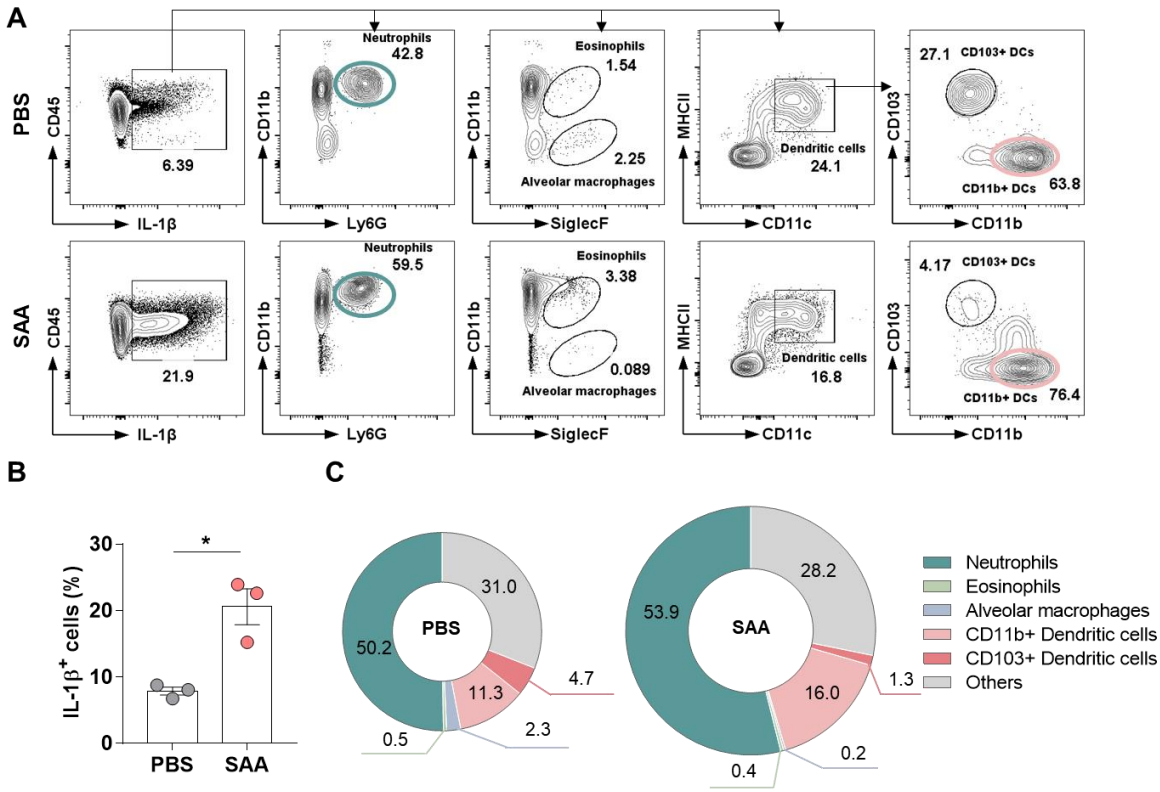

Figure 4.15 IL-1β secreted by neutrophils is increased in SAA-induced emphysema model

A. Cellular source of IL-1β in lung of SAA-induced emphysema model. **B**. Comparison of IL-1β^{+} cells between PBS- and SAA-injected mice. **C**. Pie chart of IL-1β secreting immune cells in lung. *$P \leq 0.05$, using Mann-Whitney test. The data are presented as the mean ± SEM.

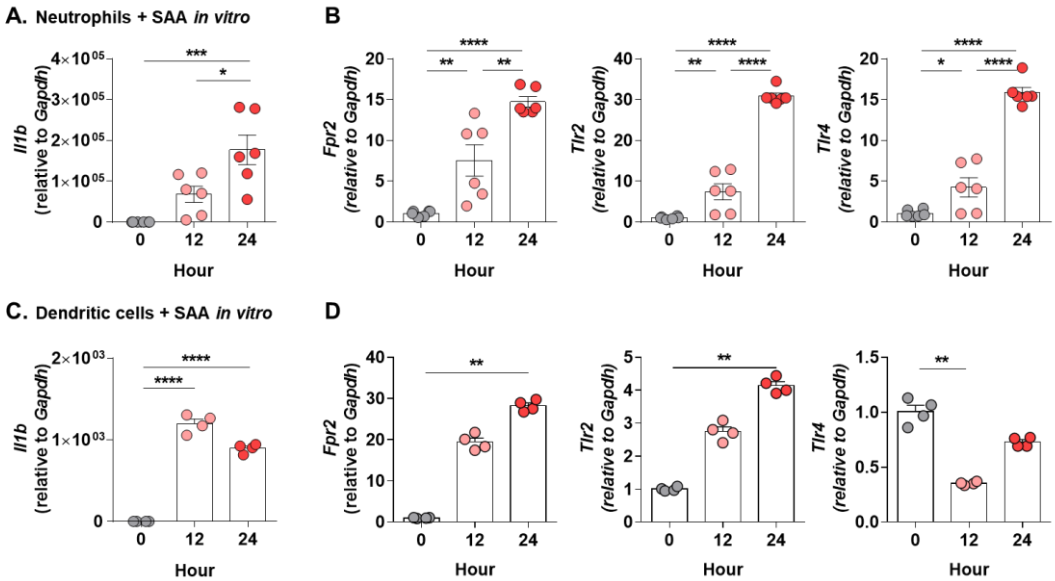

Figure 4.16 SAA directly activate neutrophils to produce IL-1β

A. Expression of *Il1b* in bone marrow-derived neutrophils after SAA treatment *in vitro*. **B**. Expression of SAA receptors, *Fpr2*, *Tlr2*, and *Tlr4*, in bone marrow-derived neutrophils after SAA treatment *in vitro*. **C**. Expression of *Il1b* in bone marrow-derived dendritic cells after SAA treatment *in vitro*. **D**. Expression of SAA receptors, *Fpr2*, *Tlr2*, and *Tlr4*, in bone marrow-derived dendritic cells after SAA treatment *in vitro*. **P*≤0.05, ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001, using one-way ANOVA followed by a Bonferroni's post-test. The data are presented as the mean ± SEM.

4.2.8 Neutrophils are critical for the proliferation of ILC3s in SAA-induced inflammation

To ensure that neutrophils are related to activation of ILC3s and inflammation, anti-Gr-1 blocking antibodies were administrated by intra-peritoneal injection at a day before and after the SAA injection (Fig. 4.17 A). Neutrophils were effectively deficient in anti-Gr-1-treated mice compared to isotype control-treated mice after SAA injection (Fig. 4.17 B and C). IL-17A production from ILCs were decreased after neutrophil depletion (Fig. 4.18 A and B), and recruitment of inflammatory cells were reduced in lung (Fig. 4.18 C). These results suggest that neutrophils are essential to induce IL-17A production from ILC3s after SAA administration.

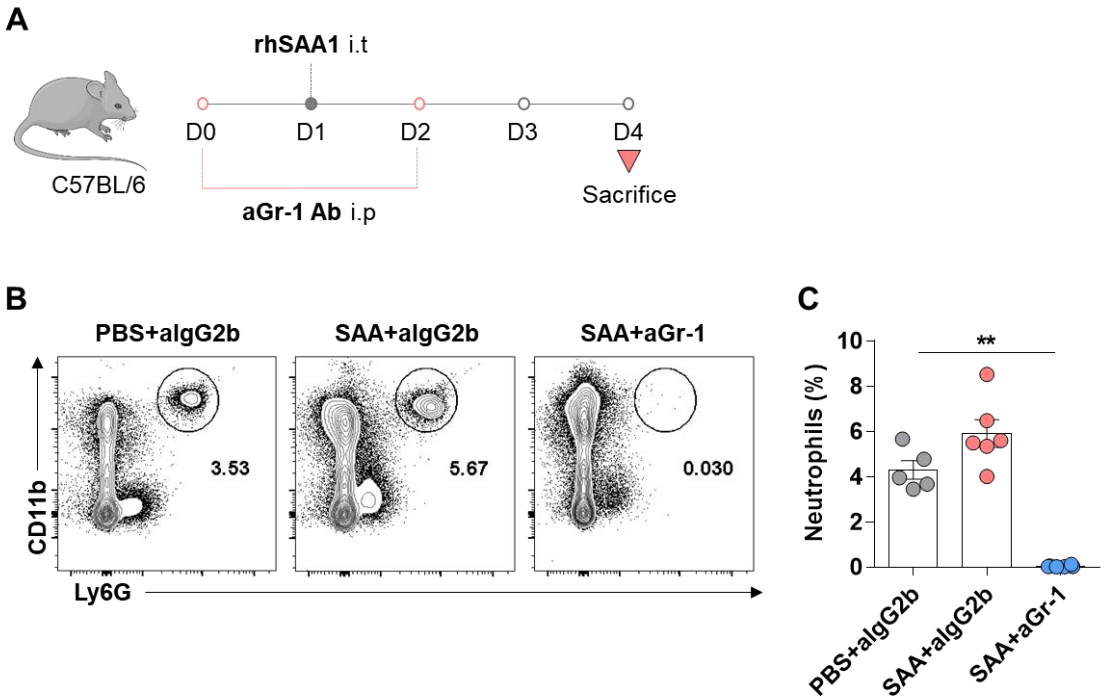

Figure 4.17 Depletion of neutrophils in SAA-induced inflammation

A. Schematic diagram of anti-Gr-1 antibody treatment in SAA-induced emphysema model. **B**. Plot of neutrophils after aGr-1 antibody treatment in SAA-induced emphysema model. **C**. Comparison of frequency of neutrophils after aGr-1 antibody treatment. $^{**}P \leq 0.01$, using one-way ANOVA followed by a Bonferroni's post-test. The data are presented as the mean ± SEM.

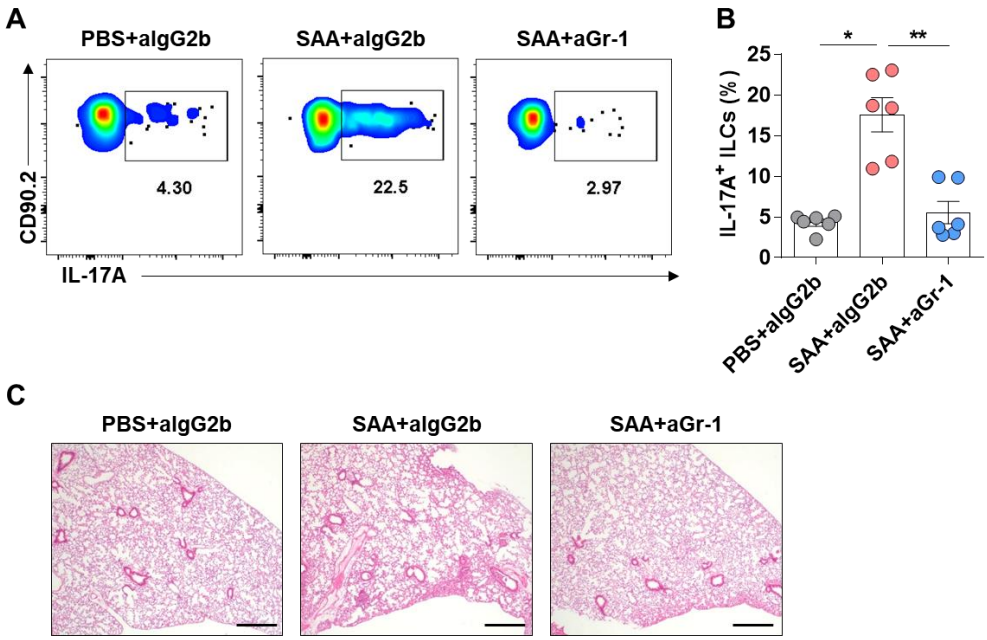

Figure 4.18 Neutrophils are essential to activate IL-17A$^+$ ILC3s in SAA-induced emphysema model

A. Plot of IL-17A$^+$ ILC3s after aGr-1 antibody treatment in SAA-induced emphysema model. **B**. Comparison of frequency of IL-17A$^+$ ILC3s after aGr-1 antibody treatment. **C**. Microscopic analysis of lungs after aGr-1 antibody treatment in SAA-induced emphysema model with H&E staining. Scale bars, 500μm. *P≤0.05, **P≤0.01, using one-way ANOVA followed by a Bonferroni's post-test. The data are presented as the mean ± SEM.

4.2.9 ILC3s are indispensable for initiation of emphysema-like phenotype

I further investigated whether IL-17A-producing ILC3s are needed for pathogenesis of emphysema. To exclude the effect of T cells, LPS and PPE were injected to $Rag1^{-/-}$ mice and anti-Thy1.2 blocking antibody was administrated by intra-peritoneal injection to remove ILCs (Fig. 4.19 A). Total ILCs (Fig. 4.19 B and C) and $IL\text{-}17A^{+}$ ILCs were completely disappeared (Fig. 4.19 D and E). Neutrophils were not changed after anti-Thy1.2 antibody injection (Fig. 4.19 F and G). Emphysema-like features were relieved and the expression of *Mmp12* was reduced after anti-Thy1.2 antibody treatment in emphysema model (Fig. 4.20 A and B). These results show that neutrophils regulate the function of ILC3s and ILC3s are required for induction of emphysema phenotypes in COPD.

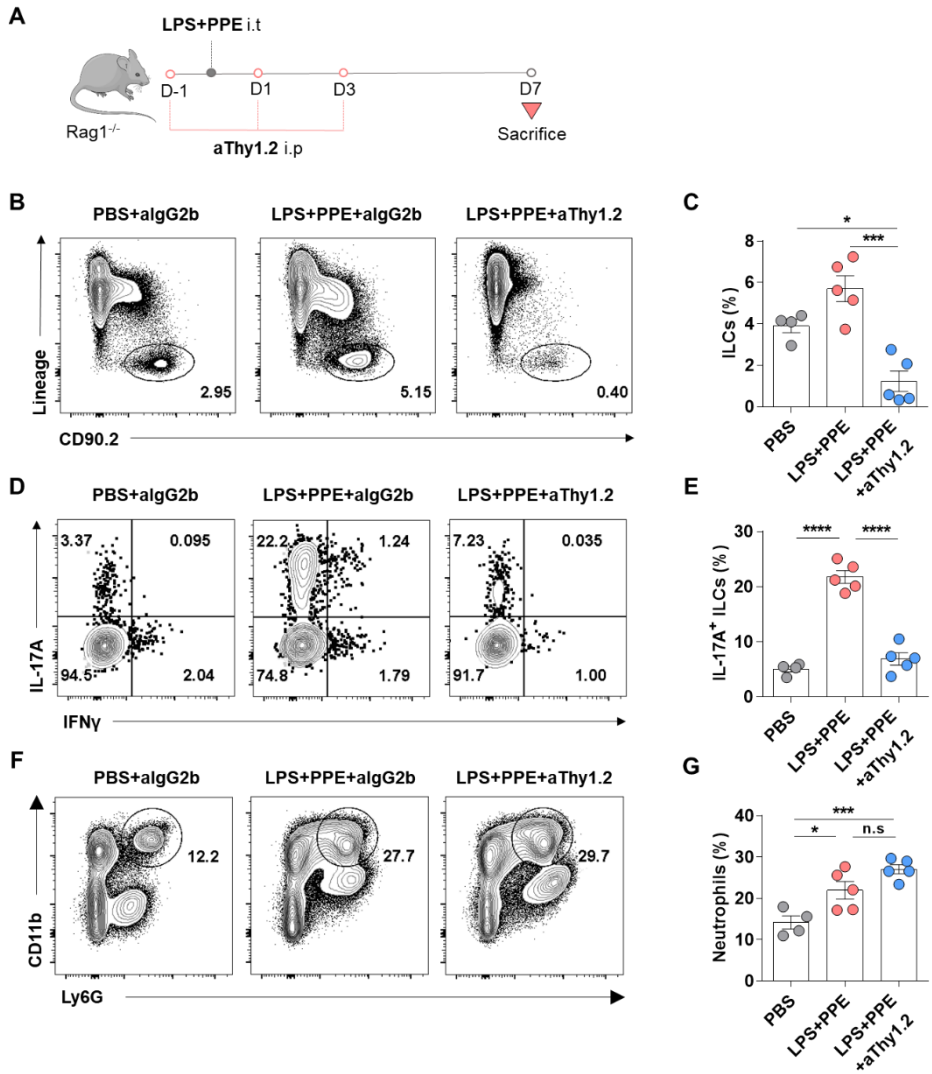

Figure 4.19 Depletion of ILCs in emphysema model

A. Schematic diagram of anti-Thy1.2 antibody treatment in acute LPS+PPE-induced emphysema model. **B-C**. Dot plots and frequency of total ILCs ($CD45^+Lineage^-CD90.2^+$) after aThy1.2 antibody treatment. **D-E**. Dot plots and frequency of IL-$17A^+$ ILC3s after aThy1.2 antibody treatment. **F-G**. Dot plots and frequency of neutrophils after Thy1.2 antibody treatment. *$P \leq 0.05$, ***$P \leq 0.001$, ****$P \leq 0.0001$, using one-way ANOVA followed by a Bonferroni's post-test. The data are presented as the mean ± SEM.

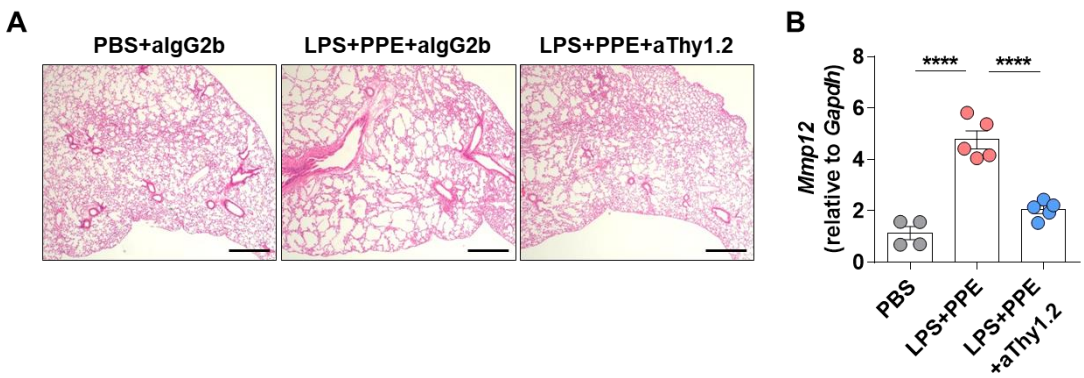

Figure 4.20 IL-17^{+} ILC3s were critical for initiation of emphysema pathogenesis

A. Microscopic analysis of lungs after aThy1.2 antibody treatment in LPS+PPE-induced emphysema model with H&E staining. Scale bars, 500μm. **B**. The expression of *Mmp12* in lung tissue after aThy1.2 antibody treatment. ****$P \leq 0.0001$, using one-way ANOVA followed by a Bonferroni's post-test. The data are presented as the mean ± SEM.

4.2.10 The SAA-neutrophils-ILC3s axis is related to the lung function of COPD patients with emphysema

So far, these results suggested that IL-17A$^+$ILC3s are involved in the development of COPD in murine model. Finally, to ensure that ILC3s activated by SAA-neutrophils axis are needed to pathogenesis of COPD in human patients, I examined the protein level of SAA1 and proportion of neutrophils and ILC3s both in sputum from healthy controls and patients with COPD (Table 4.1). Although SAA1 level was not changed (Fig. 4.21 A), the proportion of neutrophils and ILC3s in sputum were increased in COPD patients compared to healthy controls (Fig. 4.21 B and C). SAA1 level had a positive correlation with proportion of neutrophils in sputum (Fig. 4.21 D). Also, proportion of neutrophils was positively correlated with proportion of ILC3s in sputum (Fig. 4.21 E). As ILC3s were increased in sputum of COPD patients, FEV_1/FVC ratio was decreased (Fig. 4.21 F).

In COPD patients, 19 patients had an emphysema phenotype as a result from computed tomography scan (Table 4.1). SAA level in induced sputum was higher in patients with emphysema than in patients without emphysema (Fig. 4.22 A). Also, patients with emphysema had more neutrophils and ILC3s in induced sputum (Fig. 4.22 B and C). Correlation between the level of SAA and neutrophils, neutrophils and ILC3s, and ILC3s and FEV_1/FVC ratio (Fig. 4.22 D-F) were observed in COPD patients with emphysema. However, SAA level, neutrophils, ILC3s, or FEV_1/FVC ratio were not correlated with each other in patients without emphysema (Fig. 4.22 G-I). These data suggest that the SAA-neutrophils-ILC3s axis in lung is also applicable to the COPD patients with emphysema, and IL-17A$^+$ ILC3s could be targets for therapy of COPD with emphysema.

Table 4.1 Characteristics of COPD patients

	Healthy controls	COPD	p value
No. of patients	14	27	
Age (yr)	66.67±1.23	68.11±8.28	0.7248
Sx durations (yr)	N.D	5.05±4.98	-
FEV_1	2136±341.2	1827±556	0.0936
FEV_1(%)	103.4±25.7	65.85±16.86	<0.0001
FVC	2860±568.8	2906±823.2	0.8674
FVC(%)	111.3±26.45	78.85±17.23	<0.0001
FEV_1/FVC	0.75±0.01	0.63±0.08	0.0007
Emphysema, n(%)	N.D	19(70.3)	
Atopy, n(%)	1(7.1)	1(3.7)	
Allergic rhinitis, n(%)	1(7.1)	5(18.5)	
Smoking history (never/ex/current), n(%)	14(100)/0(0)/0(0)	3(11.1)/13(48.1)/11(40.7)	

Data are presented as mean ± SDs.

FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; N.D, no data

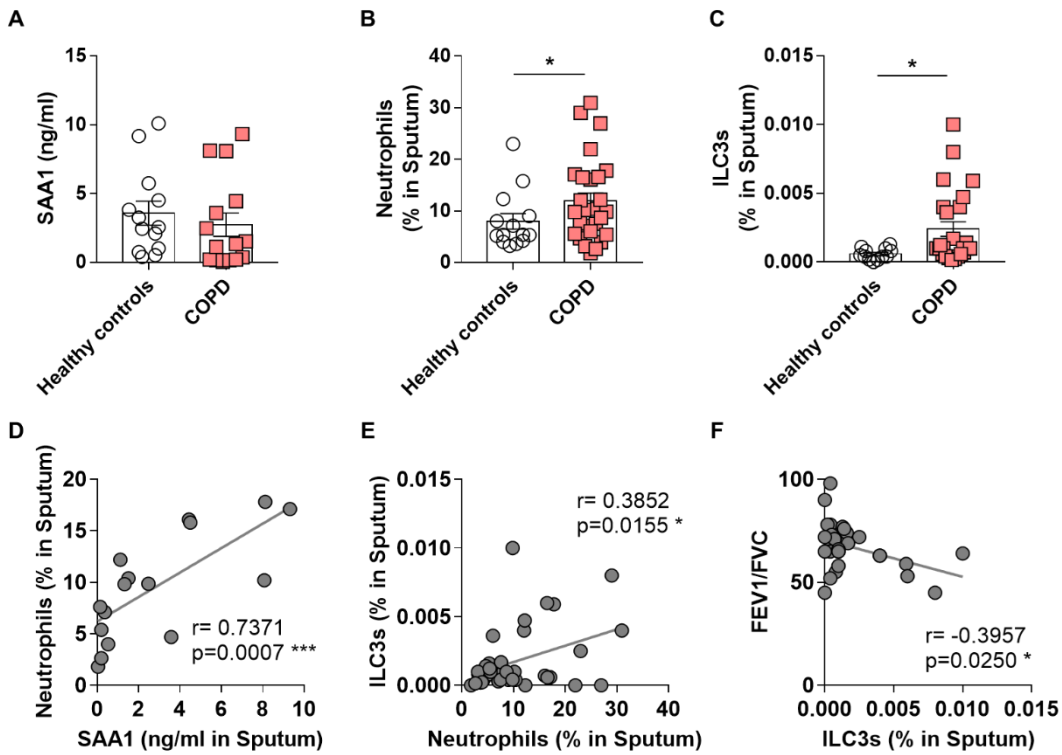

Figure 4.21 The SAA-Neutrophils-ILC3s axis was applicable to COPD patients

A. SAA1 level in induced sputum of healthy controls (n=14) and COPD patients (n=27) measured by ELISA. **B**. Proportion of neutrophils in induced sputum of healthy controls and COPD patients. **C**. Proportion of ILC3s in induced sputum of healthy controls and COPD patients. **D**. Correlation between SAA1 level and neutrophils in induced sputum. **E**. Correlation between neutrophils and ILC3s in induced sputum. **F**. Correlation between ILC3s in induced sputum and FEV_1/FVC ratio. *$P \leq 0.05$, ***$P \leq 0.001$, using Mann-Whitney test (A-C) and Spearman r correlation test (D-F). The data are presented as the mean ± SD.

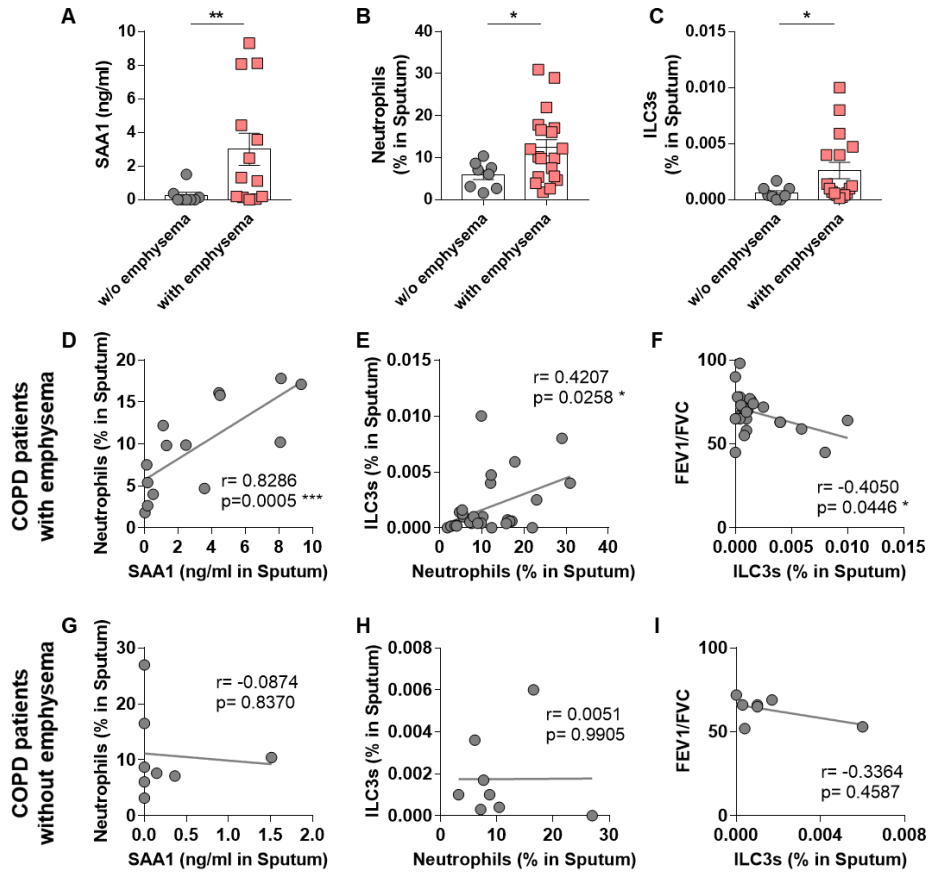

Figure 4.22 The SAA level, neutrophils, ILC3s, and lung function became more correlated in COPD patients with emphysema phenotype.

A-C. Comparison of SAA1 level (**A**), neutrophils (**B**), and ILC3s (**C**) in induced sputum between COPD patients without emphysema (n=8) and COPD patients with emphysema (n=19). **D-F**. Correlation between SAA1 level and neutrophils (**D**), neutrophils and ILC3s (**E**), and ILC3s and FEV1/FVC ratio (**F**) in induced sputum of COPD patients with emphysema. **G-I**. Correlation between SAA1 level and neutrophils (**G**), neutrophils and ILC3s (**H**), and ILC3s and FEV1/FVC ratio (**I**) in induced sputum of COPD patients without emphysema. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, using Mann-Whitney test (A-C) and Spearman r correlation test (D-I). The data are presented as the mean ± SD.

4.3 Discussion

The identification of ILCs in various tissues has provided numerous insights into the understanding of innate immune mechanisms in several diseases, especially in mucosal tissues. In lung, ILCs have unique roles in pathogenesis and regulation of respiratory diseases[2]. In asthmatic conditions, ILC2s produce IL-4, IL-5, IL-9 and IL-13 not only to enhance innate immune responses such as eosinophilic inflammation, activation of M2 macrophages, and mucus hyper-production, but also to promote adaptive immune responses such as Th2 cell differentiation and antibody production from B cells[9, 81, 164]. Moreover, ILC2s produce amphiregulin (Areg) which promote the repair of damaged epithelial cells after infection[25]. ILC3s in respiratory system also promote and regulate pathogenesis of asthma by producing IL-17 and IL-22[26, 165]. In COPD, the role of ILCs is poorly understood. Although the conversion of ILC2s into IFNγ^+ ILC1s is observed in mouse model of COPD and human patients[33, 34], the major population of ILCs in steady state of human lung is ILC3s and ILC3s are increased in lung of COPD patients[35]. Also, recent study shows that gene set enrichment assay (GSEA) revealed that gene signature of ILC3s and NK cells are enriched in lymphocytes in lungs of severe COPD patients[166]. However, the role of ILC3s in COPD is not fully understood.

In this study, I identified the first evidence of the role of ILC3s in pathogenesis of emphysema, which is one of major types of COPD. As I depleted ILCs in emphysema model of mice, emphysema-like features were dampened by the decreased expression of *Mmp12* in lung. IL-17A production from ILCs, which was majorly increased in emphysema model, was down-regulated by treatment of anti-Thy1.2. Also, ILC3s in sputum of COPD patients had a negative correlation with

FEV_1/FVC ratio. Although I could not show the secretion of IL-17A from ILCs in human sputum, because of the limitation of cell number of ILCs, this data suggests that IL-17A released from ILC3s has a pathologic impact on COPD, especially emphysema dominant COPD.

COPD is a generic term of chronic bronchitis and emphysema[167]. Both types of COPD are occurred commonly in patients, but in the mouse model of this study, emphysema-like phenotype was dominantly observed. Emphysema is a destruction of the alveolar wall which is caused by several proteases, such as elastase and matrix metalloproteinase (MMP)[168]. In the results of this study, the expression of *Mmp12* was up-regulated in LPS and PPE-induced emphysema model, while other MMP genes were not changed (**Figure 4.1 D**). Previous reports suggest that MMP12 concentration is increased in bronchoalveolar lavage fluid (BALF) and sputum of COPD patients and related to pulmonary function of the patients[169, 170]. MMP12 is a macrophage elastase which is able to degrade extracellular matrix components and induce emphysema[171]. Moreover, MMP12 activates latent form of TGFβ or TNFα, which affect tissue remodeling in COPD[172]. I observed the decrease of *Mmp12* gene expression after anti-Thy1.2 treatments, which means ILCs affect the expression of *Mmp12* (**Figure 4.20 B**). Whether ILCs directly secrete MMPs is not clear. However, there is an evidence which ILC3s affect other immune cells to produce several types of MMPs[173]. RORγt$^+$ ILC3s in isolated lymphoid follicle of cyptopatches produce TNFα, which activate stromal cells and macrophages to release MMPs. Moreover, ILC3s might affect recruitment of macrophages, a major cell sources of proteases, into inflamed tissues by producing IL-17A in lung injury[174].

The role of SAA in immune system is reported by recent studies. In

intestine, SAA is released from epithelial cells by commensal bacteria, segmented filamentous bacteria (SFB), colonization and this reaction is related to induction of Th17 cells[162]. More recently, it is reported that SFB accumulation induces IL-23 production from dendritic cells, which activate ILC3s to produce IL-22, and then, IL-22 activates intestinal epithelial cells to release SAA1/2[175]. This reaction affects SFB-specific Th17 differentiation in ileum. Therefore, ILC3s are responsible for induction of SAA in intestine. However, this study showed that SAA inoculation activated neutrophils to produce IL-1β, which in turn affected activation and proliferation of ILC3s. SAA activated neutrophils acutely, as shown in *in vitro* and *in vivo* kinetics experiments. Also, the expression of *Il1b* in lung was acutely up-regulated at 24 hours after SAA injection, while IL-17A^{+} ILCs was gradually increased (**Figure 4.11 B and 4.14 A**). Moreover, depletion of ILC3s using anti-Thy1.2 did not affect the population of neutrophils (**Figure 4.20 C and D**). These data suggest that the SAA-neutrophils-ILC3s axis is important in the model of emphysema in this study. In lung, SAA activates NLRP3 inflammasome, which is important for IL-1β secretion, and affects differentiation of Th17 cells in asthma[176]. SAA also activate γδ T cells to produce IL-17, but reversely, neutralizing of IL-17 inhibits SAA-induced recruitment of neutrophils in COPD[177]. Therefore, it is also possible that ILC3s, activated by SAA, might affect SAA induction reversely through IL-17 production and increased ILC3s and SAA act synergistically to enhance pathogenesis of emphysema in COPD.

SAA has multi-functions in several diseases, binding to various receptors[154]. Major role of SAA in blood stream is a carrier for high-density lipoproteins (HDL)[178, 179]. SAA also recognize outer membrane protein of gram-positive bacteria and

opsonize them for clearance[180]. Moreover, SAA acts as a cytokine to activate macrophages or monocytes, and as a chemokine for neutrophils and macrophages[181-183]. This multi-functional SAA is due to ability of SAA binding to several receptors by their polymeric structures. Mature SAA proteins consist of 4 antiparallel α-helix bundles and C-terminal tail[184]. Each α-helix bundles and C-terminal tail interact with each other by hydrogen bonds to regulate various surface structures and stability of SAA[185]. *In vivo*, native SAA adopts a hexamer structure, but changes of physiological condition could induce SAA dissociation into dimer or monomer or cleavage of C-terminal tail[186, 187]. Moreover, a recent study shows that SAA binds to allergen from house dust mite (HDM), as a pattern recognition receptor[188]. In this study, monomer or dimer structure of SAA induced more IL-33 expression from airway epithelial cells than hexamer structure of SAA did. Therefore, the function of SAA might be different in normal condition and inflammatory condition. I showed a significantly positive correlation between SAA1 level and neutrophils in induced sputum of COPD patients (**Figure 4.21 D**). However, SAA1 level in sputum of healthy controls did not show any correlation with the frequency of neutrophils of healthy controls (data not shown). Moreover, in COPD patients with emphysema phenotype, the correlation between SAA1 and neutrophils became higher than in COPD patients without emphysema (**Figure 4.22 D**). These results suggest that SAA could convert to other conformations, such as monomer or dimer, according to changes of physiological condition in COPD and emphysema. However, whether SAA conformational changes occurs and which structure of SAA mediates the recruitment and activation of neutrophils in COPD condition need to be further studied.

In summary, this study shows a first evidence of the role of ILC3s in pathologic mechanisms of emphysema dominant COPD, especially in the context of interaction with neutrophils and SAA. ILC3s might be essential for induction of the emphysema phenotype in COPD condition, which suggests that ILC3s could be a therapeutic target for COPD. Since SAA inoculation activated ILC3s to produce IL-17A, regulation of SAA in emphysema-dominant COPD could be considered as an approach for regulating activity of ILC3s and reducing neutrophilic inflammation. Also, I found that neutrophils were responsible for activation of ILC3s. SAA activated neutrophils to produce IL-1β and ILC3s to produce IL-17A, and blocking neutrophils reduced the secretion of IL-17A from ILC3s. These data suggest that IL-1β-expressing neutrophils might activate IL-17A production from ILC3s, however, there are possibilities that neutrophils affect ILC3s through other cytokines or direct interaction. Therefore, more studies validating the interaction between neutrophils and ILC3s in lungs are required for further understanding in regulation of ILC3s in COPD.

5. Conclusion

The biology of ILCs has been investigated for years and there are tremendous advances in understanding the functions of ILCs in various diseases. Because ILCs are abundant in mucosal tissues such as lung, intestine, and skin, involvement of ILCs in respiratory diseases, especially the role of ILC2s, has been heavily studied using murine model of asthma. However, in human patients, the association of ILCs in pathogenesis of respiratory diseases remains to be explored.

In the first part of this study, I focused on the role of all subsets of ILCs in asthmatics. Each subset of ILCs in lung affects a polarization of pulmonary macrophages both in cytokine-mediated and cell contact manners, which determines certain phenotypes of asthma. ILC2s enhance M2 macrophage polarization in eosinophilic asthma, whereas ILC1s and ILC3s promote M1 macrophage polarization in non-eosinophilic asthma.

In the second part of this study, I tried to elucidate the association of ILC3s in pathogenesis of emphysema. ILC3s are increased both in murine emphysema model and induced sputum of COPD patients. In emphysema, IL-17A-producing ILC3s are upregulated with IL-1β secretion from neutrophils which are activated by SAA. Increase of ILC3s affects an emphysema phenotype and lung function of COPD.

To sum up, ILCs are crucial for decision of pathological phenotypes in respiratory diseases (Figure 5.1). These results suggest that the regulation of ILCs in asthma and COPD should be considered as a novel target for therapy. Within the context of cytokine secretion, inhibition of the functions of ILCs could lead to

substantial improvements to immunotherapy in various respiratory disorders. However, this approach has a limitation that there is no specific inhibitor of ILCs because the cytokines released from ILCs are also produced by other immune cells. Moreover, the inhibition of those cytokines has side effects such as increase of susceptibility to infections and systemic deregulation of immunity. Therefore, approaches for targeting ILCs such as local administration or timing of the treatment should be further investigated. Also, regulation of ILCs through several molecules which mediate the interaction of ILCs and other immune cells could be a possible strategy for treatment of respiratory diseases. As I could not find the molecules regulating the interaction of ILCs and macrophages, future studies are needed to define regulatory molecules expressed on ILCs and validate whether the inhibition of these molecules is effective for treatment of respiratory diseases.

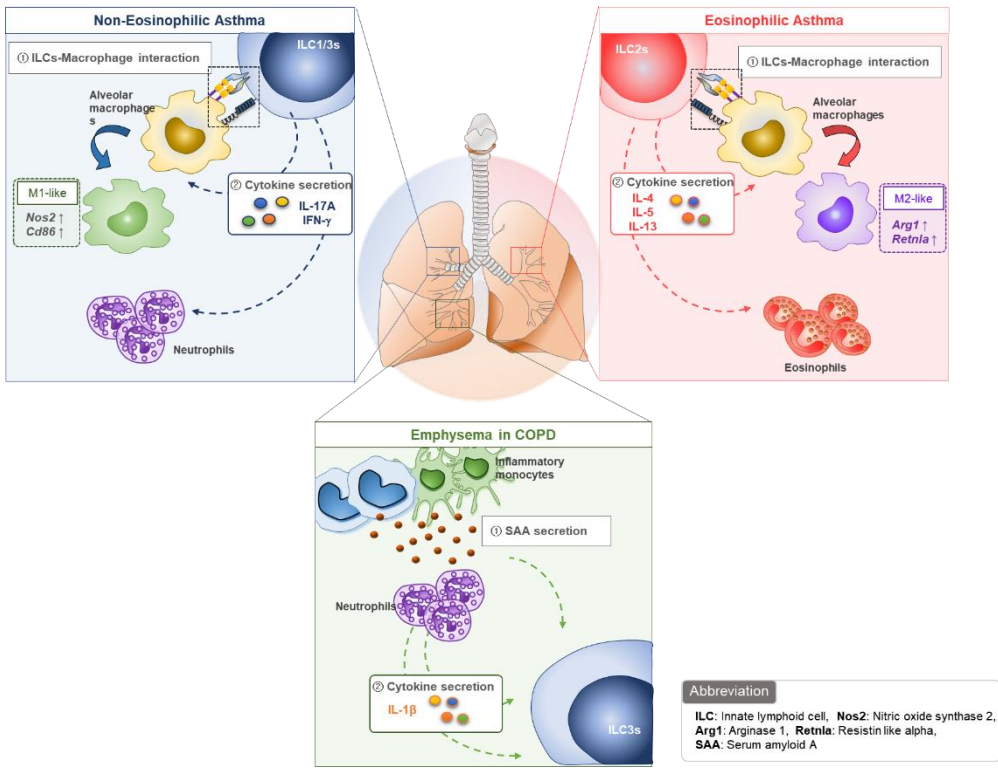

Figure 5.1 Summary of the study

6. References

1. Spits H, Cupedo T. Innate lymphoid cells: emerging insights in development, lineage relationships, and function. Annu Rev Immunol 2012; 30:647-75.
2. Vivier E, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate Lymphoid Cells: 10 Years On. Cell 2018; 174:1054-66.
3. Tanriver Y, Diefenbach A. Transcription factors controlling development and function of innate lymphoid cells. Int Immunol 2014; 26:119-28.
4. Bando JK, Liang HE, Locksley RM. Identification and distribution of developing innate lymphoid cells in the fetal mouse intestine. Nat Immunol 2015; 16:153-60.
5. Kim CH, Hashimoto-Hill S, Kim M. Migration and Tissue Tropism of Innate Lymphoid Cells. Trends Immunol 2016; 37:68-79.
6. Morita H, Moro K, Koyasu S. Innate lymphoid cells in allergic and nonallergic inflammation. J Allergy Clin Immunol 2016; 138:1253-64.
7. Branzk N, Gronke K, Diefenbach A. Innate lymphoid cells, mediators of tissue homeostasis, adaptation and disease tolerance. Immunological Reviews 2018; 286:86-101.
8. Sonnenberg GF, Hepworth MR. Functional interactions between innate lymphoid cells and adaptive immunity. Nat Rev Immunol 2019; 19:599-613.
9. Halim TY, Steer CA, Matha L, Gold MJ, Martinez-Gonzalez I, McNagny KM, et al. Group 2 innate lymphoid cells are critical for the initiation of adaptive T helper 2 cell-mediated allergic lung inflammation. Immunity 2014; 40:425-35.
10. Weizman OE, Adams NM, Schuster IS, Krishna C, Pritykin Y, Lau C, et al. ILC1 Confer Early Host Protection at Initial Sites of Viral Infection. Cell 2017; 171:795-808.e12.
11. Zhou L, Chu C, Teng F, Bessman NJ, Goc J, Santosa EK, et al. Innate lymphoid cells support regulatory T cells in the intestine through interleukin-2. Nature 2019; 568:405-9.
12. Noval Rivas M, Burton OT, Oettgen HC, Chatila T. IL-4 production by group 2 innate lymphoid cells promotes food allergy by blocking regulatory T-cell function. J Allergy Clin Immunol 2016; 138:801-11.e9.

13. Mebius RE, Rennert P, Weissman IL. Developing lymph nodes collect CD4+CD3- LTbeta+ cells that can differentiate to APC, NK cells, and follicular cells but not T or B cells. Immunity 1997; 7:493-504.
14. Hepworth MR, Monticelli LA, Fung TC, Ziegler CG, Grunberg S, Sinha R, et al. Innate lymphoid cells regulate CD4+ T-cell responses to intestinal commensal bacteria. Nature 2013; 498:113-7.
15. Halim TYF, Rana BMJ, Walker JA, Kerscher B, Knolle MD, Jolin HE, et al. Tissue-Restricted Adaptive Type 2 Immunity Is Orchestrated by Expression of the Costimulatory Molecule OX40L on Group 2 Innate Lymphoid Cells. Immunity 2018; 48:1195-207.e6.
16. Oro AS, Guarino TJ, Driver R, Steinman L, Umetsu DT. Regulation of disease susceptibility: decreased prevalence of IgE-mediated allergic disease in patients with multiple sclerosis. J Allergy Clin Immunol 1996; 97:1402-8.
17. Lambrecht BN, Hammad H. The immunology of asthma. Nat Immunol 2015; 16:45-56.
18. Kim HY, DeKruyff RH, Umetsu DT. The many paths to asthma: phenotype shaped by innate and adaptive immunity. Nature immunology 2010; 11:577-84.
19. Barnes PJ. Th2 cytokines and asthma: an introduction. Respiratory research 2001; 2:64-5.
20. Novak N, Bieber T. Allergic and nonallergic forms of atopic diseases. Journal of Allergy and Clinical Immunology 2003; 112:252-62.
21. Fahy JV. Eosinophilic and Neutrophilic Inflammation in Asthma. Proceedings of the American Thoracic Society 2009; 6:256-9.
22. Yu S, Kim HY, Chang YJ, DeKruyff RH, Umetsu DT. Innate lymphoid cells and asthma. J Allergy Clin Immunol 2014; 133:943-50; quiz 51.
23. Vercelli D, Gozdz J, von Mutius E. Innate lymphoid cells in asthma: when innate immunity comes in a Th2 flavor. Curr Opin Allergy Clin Immunol 2014; 14:29-34.
24. McKenzie AN. Type-2 innate lymphoid cells in asthma and allergy. Ann Am Thorac Soc 2014; 11 Suppl 5:S263-70.
25. Monticelli LA, Sonnenberg GF, Abt MC, Alenghat T, Ziegler CG, Doering TA, et al. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. Nat Immunol 2011; 12:1045-54.
26. Kim HY, Lee HJ, Chang YJ, Pichavant M, Shore SA, Fitzgerald KA, et al.

Interleukin-17-producing innate lymphoid cells and the NLRP3 inflammasome facilitate obesity-associated airway hyperreactivity. Nat Med 2014; 20:54-61.

27. Smith SG, Chen R, Kjarsgaard M, Huang C, Oliveria JP, O'Byrne PM, et al. Increased numbers of activated group 2 innate lymphoid cells in the airways of patients with severe asthma and persistent airway eosinophilia. J Allergy Clin Immunol 2016; 137:75-86 e8.
28. Nagakumar P, Denney L, Fleming L, Bush A, Lloyd CM, Saglani S. Type 2 innate lymphoid cells in induced sputum from children with severe asthma. J Allergy Clin Immunol 2016; 137:624-6 e6.
29. Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet 2012; 380:2095-128.
30. Barnes PJ, Burney PG, Silverman EK, Celli BR, Vestbo J, Wedzicha JA, et al. Chronic obstructive pulmonary disease. Nat Rev Dis Primers 2015; 1:15076.
31. Hogg JC, Timens W. The pathology of chronic obstructive pulmonary disease. Annu Rev Pathol 2009; 4:435-59.
32. Chung KF, Adcock IM. Multifaceted mechanisms in COPD: inflammation, immunity, and tissue repair and destruction. Eur Respir J 2008; 31:1334-56.
33. Bal SM, Bernink JH, Nagasawa M, Groot J, Shikhagaie MM, Golebski K, et al. IL-1beta, IL-4 and IL-12 control the fate of group 2 innate lymphoid cells in human airway inflammation in the lungs. Nat Immunol 2016; 17:636-45.
34. Silver JS, Kearley J, Copenhaver AM, Sanden C, Mori M, Yu L, et al. Inflammatory triggers associated with exacerbations of COPD orchestrate plasticity of group 2 innate lymphoid cells in the lungs. Nat Immunol 2016; 17:626-35.
35. De Grove KC, Provoost S, Verhamme FM, Bracke KR, Joos GF, Maes T, et al. Characterization and Quantification of Innate Lymphoid Cell Subsets in Human Lung. PLoS One 2016; 11:e0145961.
36. Cording S, Medvedovic J, Aychek T, Eberl G. Innate lymphoid cells in defense, immunopathology and immunotherapy. Nat Immunol 2016; 17:755-7.

37. Jacquelot N, Luong K, Seillet C. Physiological Regulation of Innate Lymphoid Cells. Frontiers in Immunology 2019; 10.
38. Qiu J, Heller Jennifer J, Guo X, Chen Z-ming E, Fish K, Fu Y-X, et al. The Aryl Hydrocarbon Receptor Regulates Gut Immunity through Modulation of Innate Lymphoid Cells. Immunity 2012; 36:92-104.
39. Doherty TA, Khorram N, Lund S, Mehta AK, Croft M, Broide DH. Lung type 2 innate lymphoid cells express cysteinyl leukotriene receptor 1, which regulates TH2 cytokine production. J Allergy Clin Immunol 2013; 132:205-13.
40. Salimi M, Stoger L, Liu W, Go S, Pavord I, Klenerman P, et al. Cysteinyl leukotriene E4 activates human group 2 innate lymphoid cells and enhances the effect of prostaglandin D2 and epithelial cytokines. J Allergy Clin Immunol 2017; 140:1090-100.e11.
41. Zhou Y, Wang W, Zhao C, Wang Y, Wu H, Sun X, et al. Prostaglandin E2 Inhibits Group 2 Innate Lymphoid Cell Activation and Allergic Airway Inflammation Through E-Prostanoid 4-Cyclic Adenosine Monophosphate Signaling. Front Immunol 2018; 9:501.
42. Wojno ED, Monticelli LA, Tran SV, Alenghat T, Osborne LC, Thome JJ, et al. The prostaglandin D(2) receptor CRTH2 regulates accumulation of group 2 innate lymphoid cells in the inflamed lung. Mucosal Immunol 2015; 8:1313-23.
43. Klose CSN, Mahlakoiv T, Moeller JB, Rankin LC, Flamar AL, Kabata H, et al. The neuropeptide neuromedin U stimulates innate lymphoid cells and type 2 inflammation. Nature 2017; 549:282-6.
44. Ibiza S, García-Cassani B, Ribeiro H, Carvalho T, Almeida L, Marques R, et al. Glial-cell-derived neuroregulators control type 3 innate lymphoid cells and gut defence. Nature 2016; 535:440-3.
45. Hurst SD, Muchamuel T, Gorman DM, Gilbert JM, Clifford T, Kwan S, et al. New IL-17 Family Members Promote Th1 or Th2 Responses in the Lung: In Vivo Function of the Novel Cytokine IL-25. The Journal of Immunology 2002; 169:443-53.
46. Fort MM, Cheung J, Yen D, Li J, Zurawski SM, Lo S, et al. IL-25 Induces IL-4, IL-5, and IL-13 and Th2-Associated Pathologies In Vivo. Immunity 2001; 15:985-95.
47. Lloyd CM. IL-33 family members and asthma – bridging innate and

adaptive immune responses. Current Opinion in Immunology 2010; 22:800-6.

48. Carriere V, Roussel L, Ortega N, Lacorre D-A, Americh L, Aguilar L, et al. IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo. Proceedings of the National Academy of Sciences 2007; 104:282-7.

49. Lefrançais E, Roga S, Gautier V, Gonzalez-de-Peredo A, Monsarrat B, Girard J-P, et al. IL-33 is processed into mature bioactive forms by neutrophil elastase and cathepsin G. Proceedings of the National Academy of Sciences 2012; 109:1673-8.

50. Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK, et al. IL-33, an Interleukin-1-like Cytokine that Signals via the IL-1 Receptor-Related Protein ST2 and Induces T Helper Type 2-Associated Cytokines. Immunity 2005; 23:479-90.

51. Gu C, Wu L, Li X. IL-17 family: Cytokines, receptors and signaling. Cytokine 2013; 64:477-85.

52. Barlow JL, Peel S, Fox J, Panova V, Hardman CS, Camelo A, et al. IL-33 is more potent than IL-25 in provoking IL-13–producing nuocytes (type 2 innate lymphoid cells) and airway contraction. Journal of Allergy and Clinical Immunology 2013; 132:933-41.

53. Martinez-Gonzalez I, Mathä L, Steer Catherine A, Ghaedi M, Poon Grace FT, Takei F. Allergen-Experienced Group 2 Innate Lymphoid Cells Acquire Memory-like Properties and Enhance Allergic Lung Inflammation. Immunity 2016; 45:198-208.

54. Lopez-Castejon G, Brough D. Understanding the mechanism of IL-1β secretion. Cytokine & growth factor reviews 2011; 22:189-95.

55. Thornberry NA, Bull HG, Calaycay JR, Chapman KT, Howard AD, Kostura MJ, et al. A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. Nature 1992; 356:768-74.

56. Kim RY, Pinkerton JW, Essilfie AT, Robertson AAB, Baines KJ, Brown AC, et al. Role for NLRP3 Inflammasome-mediated, IL-1beta-Dependent Responses in Severe, Steroid-Resistant Asthma. Am J Respir Crit Care Med 2017; 196:283-97.

57. Everaere L, Ait-Yahia S, Molendi-Coste O, Vorng H, Quemener S, LeVu P, et al. Innate lymphoid cells contribute to allergic airway disease

exacerbation by obesity. J Allergy Clin Immunol 2016; 138:1309-18.e11.
58. Van Maele L, Carnoy C, Cayet D, Ivanov S, Porte R, Deruy E, et al. Activation of Type 3 Innate Lymphoid Cells and Interleukin 22 Secretion in the Lungs During Streptococcus pneumoniae Infection. The Journal of Infectious Diseases 2014; 210:493-503.
59. Veiga-Fernandes H, Artis D. Neuronal–immune system cross-talk in homeostasis. Science 2018; 359:1465-6.
60. Quatrini L, Vivier E, Ugolini S. Neuroendocrine regulation of innate lymphoid cells. Immunological reviews 2018; 286:120-36.
61. Wallrapp A, Riesenfeld SJ, Burkett PR, Abdulnour R-EE, Nyman J, Dionne D, et al. The neuropeptide NMU amplifies ILC2-driven allergic lung inflammation. Nature 2017; 549:351-6.
62. Sui P, Wiesner DL, Xu J, Zhang Y, Lee J, Van Dyken S, et al. Pulmonary neuroendocrine cells amplify allergic asthma responses. Science (New York, N.Y.) 2018; 360:eaan8546.
63. Cephus JY, Stier MT, Fuseini H, Yung JA, Toki S, Bloodworth MH, et al. Testosterone Attenuates Group 2 Innate Lymphoid Cell-Mediated Airway Inflammation. Cell Rep 2017; 21:2487-99.
64. Quatrini L, Wieduwild E, Guia S, Bernat C, Glaichenhaus N, Vivier E, et al. Host resistance to endotoxic shock requires the neuroendocrine regulation of group 1 innate lymphoid cells. J Exp Med 2017; 214:3531-41.
65. Boyce JA. Mast cells and eicosanoid mediators: a system of reciprocal paracrine and autocrine regulation. Immunological Reviews 2007; 217:168-85.
66. Doherty TA, Broide DH. Lipid regulation of group 2 innate lymphoid cell function: Moving beyond epithelial cytokines. The Journal of allergy and clinical immunology 2018; 141:1587-9.
67. von Moltke J, O'Leary CE, Barrett NA, Kanaoka Y, Austen KF, Locksley RM. Leukotrienes provide an NFAT-dependent signal that synergizes with IL-33 to activate ILC2s. Journal of Experimental Medicine 2017; 214:27-37.
68. Chang JE, Doherty TA, Baum R, Broide D. Prostaglandin D2 regulates human type 2 innate lymphoid cell chemotaxis. J Allergy Clin Immunol 2014; 133:899-901.e3.
69. Maric J, Ravindran A, Mazzurana L, Bjorklund AK, Van Acker A, Rao A, et

al. Prostaglandin E2 suppresses human group 2 innate lymphoid cell function. J Allergy Clin Immunol 2018; 141:1761-73.e6.
70. Zhou W, Toki S, Zhang J, Goleniewksa K, Newcomb DC, Cephus JY, et al. Prostaglandin I2 Signaling and Inhibition of Group 2 Innate Lymphoid Cell Responses. Am J Respir Crit Care Med 2016; 193:31-42.
71. Duffin R, O'Connor RA, Crittenden S, Forster T, Yu C, Zheng X, et al. Prostaglandin E(2) constrains systemic inflammation through an innate lymphoid cell-IL-22 axis. Science 2016; 351:1333-8.
72. Sohn SW, Lee HS, Park HW, Chang YS, Kim YK, Cho SH, et al. Evaluation of cytokine mRNA in induced sputum from patients with allergic rhinitis: relationship to airway hyperresponsiveness. Allergy 2008; 63:268-73.
73. Busse WW, Lemanske RF, Jr. Asthma. N Engl J Med 2001; 344:350-62.
74. Moore WC, Meyers DA, Wenzel SE, Teague WG, Li HS, Li XN, et al. Identification of Asthma Phenotypes Using Cluster Analysis in the Severe Asthma Research Program. American Journal of Respiratory and Critical Care Medicine 2010; 181:315-23.
75. Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. Nature Medicine 2012; 18:716-25.
76. Robinson DS, Hamid Q, Ying S, Tsicopoulos A, Barkans J, Bentley AM, et al. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. N Engl J Med 1992; 326:298-304.
77. Kim HY, Umetsu DT, Dekruyff RH. Innate lymphoid cells in asthma: Will they take your breath away? Eur J Immunol 2016; 46:795-806.
78. Artis D, Spits H. The biology of innate lymphoid cells. Nature 2015; 517:293-301.
79. Spits H, Di Santo JP. The expanding family of innate lymphoid cells: regulators and effectors of immunity and tissue remodeling. Nat Immunol 2011; 12:21-7.
80. Klose CSN, Flach M, Mohle L, Rogell L, Hoyler T, Ebert K, et al. Differentiation of Type 1 ILCs from a Common Progenitor to All Helper-like Innate Lymphoid Cell Lineages. Cell 2014; 157:340-56.
81. Moro K, Yamada T, Tanabe M, Takeuchi T, Ikawa T, Kawamoto H, et al. Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. Nature 2010; 463:540-4.
82. Neill DR, Wong SH, Bellosi A, Flynn RJ, Daly M, Langford TKA, et al.

Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. Nature 2010; 464:1367-U9.

83. Wilhelm C, Hirota K, Stieglitz B, Van Snick J, Tolaini M, Lahl K, et al. An IL-9 fate reporter demonstrates the induction of an innate IL-9 response in lung inflammation. Nat Immunol 2011; 12:1071-7.

84. Montaldo E, Juelke K, Romagnani C. Group 3 innate lymphoid cells (ILC3s): Origin, differentiation, and plasticity in humans and mice. Eur J Immunol 2015; 45:2171-82.

85. Klose CS, Kiss EA, Schwierzeck V, Ebert K, Hoyler T, d'Hargues Y, et al. A T-bet gradient controls the fate and function of CCR6-RORgammat+ innate lymphoid cells. Nature 2013; 494:261-5.

86. Rankin LC, Groom JR, Chopin M, Herold MJ, Walker JA, Mielke LA, et al. The transcription factor T-bet is essential for the development of NKp46(+) innate lymphocytes via the Notch pathway. Nature Immunology 2013; 14:389-95.

87. Chang YJ, Kim HY, Albacker LA, Baumgarth N, McKenzie AN, Smith DE, et al. Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity. Nat Immunol 2011; 12:631-8.

88. Arora S, Dev K, Agarwal B, Das P, Syed MA. Macrophages: Their role, activation and polarization in pulmonary diseases. Immunobiology 2018; 223:383-96.

89. Cai Y, Sugimoto C, Arainga M, Alvarez X, Didier ES, Kuroda MJ. In vivo characterization of alveolar and interstitial lung macrophages in rhesus macaques: implications for understanding lung disease in humans. J Immunol 2014; 192:2821-9.

90. Hussell T, Bell TJ. Alveolar macrophages: plasticity in a tissue-specific context. Nat Rev Immunol 2014; 14:81-93.

91. Saradna A, Do DC, Kumar S, Fu QL, Gao P. Macrophage polarization and allergic asthma. Transl Res 2018; 191:1-14.

92. Aggarwal NR, King LS, D'Alessio FR. Diverse macrophage populations mediate acute lung inflammation and resolution. Am J Physiol Lung Cell Mol Physiol 2014; 306:L709-25.

93. Jiang Z, Zhu L. Update on the role of alternatively activated macrophages in asthma. J Asthma Allergy 2016; 9:101-7.

94. Sica A, Mantovani A. Macrophage plasticity and polarization: in vivo veritas. J Clin Invest 2012; 122:787-95.
95. Christianson CA, Goplen NP, Zafar I, Irvin C, Good JT, Jr., Rollins DR, et al. Persistence of asthma requires multiple feedback circuits involving type 2 innate lymphoid cells and IL-33. J Allergy Clin Immunol 2015; 136:59-68 e14.
96. Bartemes KR, Kephart GM, Fox SJ, Kita H. Enhanced innate type 2 immune response in peripheral blood from patients with asthma. J Allergy Clin Immunol 2014; 134:671-8 e4.
97. Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. F1000Prime Rep 2014; 6:13.
98. Taylor S, Huang YF, Mallett G, Stathopoulou C, Felizardo TC, Sun MA, et al. PD-1 regulates KLRG1(+) group 2 innate lymphoid cells. Journal of Experimental Medicine 2017; 214:1663-78.
99. Paclik D, Stehle C, Lahmann A, Hutloff A, Romagnani C. ICOS regulates the pool of group 2 innate lymphoid cells under homeostatic and inflammatory conditions in mice. European Journal of Immunology 2015; 45:2766-72.
100. Maggi L, Montaini G, Mazzoni A, Rossettini B, Capone M, Rossi MC, et al. Human circulating group 2 innate lymphoid cells can express CD154 and promote IgE production. Journal of Allergy and Clinical Immunology 2017; 139:964-+.
101. Iijima K, Kobayashi T, Hara K, Kephart GM, Ziegler SF, McKenzie AN, et al. IL-33 and thymic stromal lymphopoietin mediate immune pathology in response to chronic airborne allergen exposure. J Immunol 2014; 193:1549-59.
102. Kouzaki H, Iijima K, Kobayashi T, O'Grady SM, Kita H. The Danger Signal, Extracellular ATP, Is a Sensor for an Airborne Allergen and Triggers IL-33 Release and Innate Th2-Type Responses. Journal of Immunology 2011; 186:4375-87.
103. Doherty TA, Khorram N, Chang JE, Kim HK, Rosenthal P, Croft M, et al. STAT6 regulates natural helper cell proliferation during lung inflammation initiated by Alternaria. Am J Physiol Lung Cell Mol Physiol 2012; 303:L577-88.
104. Yang Q, Ge MQ, Kokalari B, Redai IG, Wang X, Kemeny DM, et al. Group

2 innate lymphoid cells mediate ozone-induced airway inflammation and hyperresponsiveness in mice. J Allergy Clin Immunol 2016; 137:571-8.

105. Kumagai K, Lewandowski R, Jackson-Humbles DN, Li N, Van Dyken SJ, Wagner JG, et al. Ozone-Induced Nasal Type 2 Immunity in Mice Is Dependent on Innate Lymphoid Cells. American Journal of Respiratory Cell and Molecular Biology 2016; 54:782-91.

106. Sun YC, Zhou QT, Yao WZ. Sputum interleukin-17 is increased and associated with airway neutrophilia in patients with severe asthma. Chinese Medical Journal 2005; 118:953-6.

107. Agache I, Ciobanu C, Agache C, Anghel M. Increased serum IL-17 is an independent risk factor for severe asthma. Respiratory Medicine 2010; 104:1131-7.

108. Chen R, Smith SG, Salter B, El-Gammal A, Oliveria JP, Obminski C, et al. Allergen-induced Increases in Sputum Levels of Group 2 Innate Lymphoid Cells in Subjects with Asthma. Am J Respir Crit Care Med 2017; 196:700-12.

109. Barlow JL, McKenzie AN. Type-2 innate lymphoid cells in human allergic disease. Curr Opin Allergy Clin Immunol 2014; 14:397-403.

110. Zhu M, Liang ZA, Wang T, Chen RZ, Wang G, Ji YL. Th1/Th2/Th17 cells imbalance in patients with asthma with and without psychological symptoms. Allergy and Asthma Proceedings 2016; 37:148-56.

111. Shirai T, Suzuki K, Inui N, Suda T, Chida K, Nakamura H. Th1/Th2 profile in peripheral blood in atopic cough and atopic asthma. Clinical and Experimental Allergy 2003; 33:84-9.

112. Magnan AO, Mely LG, Camilla CA, Badier MM, Montero-Julian FA, Guillot CM, et al. Assessment of the Th1/Th2 paradigm in whole blood in atopy and asthma - Increased IFN-gamma-producing CD8(+) T cells in asthma. American Journal of Respiratory and Critical Care Medicine 2000; 161:1790-6.

113. Gasteiger G, Fan X, Dikiy S, Lee SY, Rudensky AY. Tissue residency of innate lymphoid cells in lymphoid and nonlymphoid organs. Science 2015; 350:981-5.

114. Murray PJ. Macrophage Polarization. Annu Rev Physiol 2017; 79:541-66.

115. Dupasquier M, Stoitzner P, Wan H, Cerqueira D, van Oudenaren A, Voerman JS, et al. The dermal microenvironment induces the expression

of the alternative activation marker CD301/mMGL in mononuclear phagocytes, independent of IL-4/IL-13 signaling. J Leukoc Biol 2006; 80:838-49.

116. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol 2004; 25:677-86.

117. Robbe P, Draijer C, Borg TR, Luinge M, Timens W, Wouters IM, et al. Distinct macrophage phenotypes in allergic and nonallergic lung inflammation. Am J Physiol Lung Cell Mol Physiol 2015; 308:L358-67.

118. Denning TL, Wang YC, Patel SR, Williams IR, Pulendran B. Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17-producing T cell responses. Nat Immunol 2007; 8:1086-94.

119. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. Nat Rev Immunol 2008; 8:958-69.

120. Jablonski KA, Amici SA, Webb LM, Ruiz-Rosado JdD, Popovich PG, Partida-Sanchez S, et al. Novel Markers to Delineate Murine M1 and M2 Macrophages. PLOS ONE 2015; 10:e0145342.

121. Orecchioni M, Ghosheh Y, Pramod AB, Ley K. Macrophage Polarization: Different Gene Signatures in M1(LPS+) vs. Classically and M2(LPS–) vs. Alternatively Activated Macrophages. Frontiers in Immunology 2019; 10.

122. Mindt BC, Fritz JH, Duerr CU. Group 2 Innate Lymphoid Cells in Pulmonary Immunity and Tissue Homeostasis. Frontiers in Immunology 2018; 9.

123. Komlosi ZI, Kovacs N, van de Veen W, Kirsch AI, Fahrner HB, Wawrzyniak M, et al. Human CD40 ligand-expressing type 3 innate lymphoid cells induce IL-10-producing immature transitional regulatory B cells. J Allergy Clin Immunol 2018; 142:178-94 e11.

124. Dong L, Wang S, Chen M, Li H, Bi W. The activation of macrophage and upregulation of CD40 costimulatory molecule in lipopolysaccharide-induced acute lung injury. J Biomed Biotechnol 2008; 2008:852571.

125. Morrell ED, Wiedeman A, Long SA, Gharib SA, West TE, Skerrett SJ, et al. Cytometry TOF identifies alveolar macrophage subtypes in acute respiratory distress syndrome. JCI Insight 2018; 3.

126. Mizuno R, Sugiura D, Shimizu K, Maruhashi T, Watada M, Okazaki I-M, et al. PD-1 Primarily Targets TCR Signal in the Inhibition of Functional T Cell Activation. Frontiers in immunology 2019; 10:630-.

127. Riley JL. PD-1 signaling in primary T cells. Immunological reviews 2009; 229:114-25.
128. Taylor S, Huang Y, Mallett G, Stathopoulou C, Felizardo TC, Sun M-A, et al. PD-1 regulates KLRG1+ group 2 innate lymphoid cells. Journal of Experimental Medicine 2017; 214:1663-78.
129. Schwartz C, Khan AR, Floudas A, Saunders SP, Hams E, Rodewald H-R, et al. ILC2s regulate adaptive Th2 cell functions via PD-L1 checkpoint control. Journal of Experimental Medicine 2017; 214:2507-21.
130. Maazi H, Patel N, Sankaranarayanan I, Suzuki Y, Rigas D, Soroosh P, et al. ICOS:ICOS-ligand interaction is required for type 2 innate lymphoid cell function, homeostasis, and induction of airway hyperreactivity. Immunity 2015; 42:538-51.
131. Paclik D, Stehle C, Lahmann A, Hutloff A, Romagnani C. ICOS regulates the pool of group 2 innate lymphoid cells under homeostatic and inflammatory conditions in mice. Eur J Immunol 2015; 45:2766-72.
132. Maeda S, Fujimoto M, Matsushita T, Hamaguchi Y, Takehara K, Hasegawa M. Inducible costimulator (ICOS) and ICOS ligand signaling has pivotal roles in skin wound healing via cytokine production. The American journal of pathology 2011; 179:2360-9.
133. Tanaka C, Fujimoto M, Hamaguchi Y, Sato S, Takehara K, Hasegawa M. Inducible costimulator ligand regulates bleomycin-induced lung and skin fibrosis in a mouse model independently of the inducible costimulator/inducible costimulator ligand pathway. Arthritis & Rheumatism 2010; 62:1723-32.
134. Braga TT, Agudelo JSH, Camara NOS. Macrophages During the Fibrotic Process: M2 as Friend and Foe. Frontiers in immunology 2015; 6:602-.
135. Hou J, Shi J, Chen L, Lv Z, Chen X, Cao H, et al. M2 macrophages promote myofibroblast differentiation of LR-MSCs and are associated with pulmonary fibrogenesis. Cell Communication and Signaling 2018; 16:89.
136. Chapman DG, Irvin CG. Mechanisms of airway hyper-responsiveness in asthma: the past, present and yet to come. Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology 2015; 45:706-19.
137. MacNee W. Pathogenesis of chronic obstructive pulmonary disease. Proceedings of the American Thoracic Society 2005; 2:258-91.

138. Noguera A, Batle S, Miralles C, Iglesias J, Busquets X, MacNee W, et al. Enhanced neutrophil response in chronic obstructive pulmonary disease. Thorax 2001; 56:432-7.
139. Eapen MS, Hansbro PM, McAlinden K, Kim RY, Ward C, Hackett TL, et al. Abnormal M1/M2 macrophage phenotype profiles in the small airway wall and lumen in smokers and chronic obstructive pulmonary disease (COPD). Sci Rep 2017; 7:13392.
140. Di Stefano A, Caramori G, Ricciardolo FL, Capelli A, Adcock IM, Donner CF. Cellular and molecular mechanisms in chronic obstructive pulmonary disease: an overview. Clin Exp Allergy 2004; 34:1156-67.
141. Churg A, Zhou S, Wright JL. Matrix metalloproteinases in COPD. European Respiratory Journal 2012; 39:197-209.
142. Hoenderdos K, Condliffe A. The Neutrophil in Chronic Obstructive Pulmonary Disease. Too Little, Too Late or Too Much, Too Soon? American Journal of Respiratory Cell and Molecular Biology 2013; 48:531-9.
143. Viniol C, Vogelmeier CF. Exacerbations of COPD. European Respiratory Review 2018; 27:170103.
144. Soler-Cataluña JJ, Martínez-García MÁ, Román Sánchez P, Salcedo E, Navarro M, Ochando R. Severe acute exacerbations and mortality in patients with chronic obstructive pulmonary disease. Thorax 2005; 60:925-31.
145. Zhang N, Ahsan MH, Purchio AF, West DB. Serum amyloid A-luciferase transgenic mice: response to sepsis, acute arthritis, and contact hypersensitivity and the effects of proteasome inhibition. J Immunol 2005; 174:8125-34.
146. Meek RL, Benditt EP. Amyloid A gene family expression in different mouse tissues. J Exp Med 1986; 164:2006-17.
147. Chiba T, Han CY, Vaisar T, Shimokado K, Kargi A, Chen MH, et al. Serum amyloid A3 does not contribute to circulating SAA levels. J Lipid Res 2009; 50:1353-62.
148. Shridas P, Tannock LR. Role of serum amyloid A in atherosclerosis. Curr Opin Lipidol 2019; 30:320-5.
149. Agilli M, Aydin FN, Cayci T, Kurt YG. Evaluation of Serum Amyloid A as a Marker of Persistent Inflammation in Patients with Rheumatoid Arthritis. Mediators of Inflammation 2015.

150. Cho WCS, Yip TT, Cheng WW, Au JSK. Serum amyloid A is elevated in the serum of lung cancer patients with poor prognosis. British Journal of Cancer 2010; 102:1731-5.
151. Maskey-Warzęchowska M, Rubinsztajn R, Przybyłowski T, Karwat K, Nejman-Gryz P, Chazan R. Serum amyloid A in patients with stable chronic obstructive pulmonary disease. European Respiratory Journal 2018; 52:PA2012.
152. Bozinovski S, Hutchinson A, Thompson M, Macgregor L, Black J, Giannakis E, et al. Serum amyloid a is a biomarker of acute exacerbations of chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2008; 177:269-78.
153. Lin T-L, Chen W-W, Ding Z-R, Wei S-C, Huang M-L, Li C-H. Correlations between serum amyloid A, C-reactive protein and clinical indices of patients with acutely exacerbated chronic obstructive pulmonary disease. Journal of Clinical Laboratory Analysis 2019; 33:e22831.
154. Ye RD, Sun L. Emerging functions of serum amyloid A in inflammation. Journal of Leukocyte Biology 2015; 98:923-9.
155. Klose CSN, Artis D. Innate lymphoid cells as regulators of immunity, inflammation and tissue homeostasis. Nature Immunology 2016; 17:765-74.
156. Mindt BC, Fritz JH, Duerr CU. Group 2 Innate Lymphoid Cells in Pulmonary Immunity and Tissue Homeostasis. Frontiers in immunology 2018; 9:840-.
157. Di Stefano A, Caramori G, Gnemmi I, Contoli M, Vicari C, Capelli A, et al. T helper type 17-related cytokine expression is increased in the bronchial mucosa of stable chronic obstructive pulmonary disease patients. Clin Exp Immunol 2009; 157:316-24.
158. Chen K, Pociask DA, McAleer JP, Chan YR, Alcorn JF, Kreindler JL, et al. IL-17RA Is Required for CCL2 Expression, Macrophage Recruitment, and Emphysema in Response to Cigarette Smoke. Plos One 2011; 6.
159. Bourdin A, Burgel PR, Chanez P, Garcia G, Perez T, Roche N. Recent advances in COPD: pathophysiology, respiratory physiology and clinical aspects, including comorbidities. Eur Respir Rev 2009; 18:198-212.
160. Mihailichenko D, Pertseva T. Levels of serum amyloid A (SAA) and C-reactive protein (CRP) in stable COPD patients and its relationship with disease severity. European Respiratory Journal 2017; 50.

161. Cai T, Qiu JX, Ji Y, Li WJ, Ding ZY, Suo CX, et al. IL-17-producing ST2(+) group 2 innate lymphoid cells play a pathogenic role in lung inflammation. Journal of Allergy and Clinical Immunology 2019; 143:229-+.
162. Ivanov II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, et al. Induction of Intestinal Th17 Cells by Segmented Filamentous Bacteria. Cell 2009; 139:485-98.
163. Reigstad CS, Lunden GO, Felin J, Backhed F. Regulation of Serum Amyloid A3 (SAA3) in Mouse Colonic Epithelium and Adipose Tissue by the Intestinal Microbiota. Plos One 2009; 4.
164. Monticelli LA, Sonnenberg GF, Artis D. Innate lymphoid cells: critical regulators of allergic inflammation and tissue repair in the lung. Curr Opin Immunol 2012; 24:284-9.
165. Taube C, Tertilt C, Gyulveszi G, Dehzad N, Kreymborg K, Schneeweiss K, et al. IL-22 is produced by innate lymphoid cells and limits inflammation in allergic airway disease. PLoS One 2011; 6:e21799.
166. Suzuki M, Sze MA, Campbell JD, Brothers JF, 2nd, Lenburg ME, McDonough JE, et al. The cellular and molecular determinants of emphysematous destruction in COPD. Sci Rep 2017; 7:9562.
167. Lange P, Halpin DM, O'Donnell DE, MacNee W. Diagnosis, assessment, and phenotyping of COPD: beyond FEV(1). Int J Chron Obstruct Pulmon Dis 2016; 11 Spec Iss:3-12.
168. Spurzem JR, Rennard SI. Pathogenesis of COPD. Semin Respir Crit Care Med 2005; 26:142-53.
169. Chaudhuri R, McSharry C, Brady J, Donnelly I, Grierson C, McGuinness S, et al. Sputum matrix metalloproteinase-12 in patients with chronic obstructive pulmonary disease and asthma: relationship to disease severity. J Allergy Clin Immunol 2012; 129:655-63 e8.
170. Molet S, Belleguic C, Lena H, Germain N, Bertrand CP, Shapiro SD, et al. Increase in macrophage elastase (MMP-12) in lungs from patients with chronic obstructive pulmonary disease. Inflamm Res 2005; 54:31-6.
171. Parks WC, Wilson CL, Lopez-Boado YS. Matrix metalloproteinases as modulators of inflammation and innate immunity. Nat Rev Immunol 2004; 4:617-29.
172. Demedts IK, Brusselle GG, Bracke KR, Vermaelen KY, Pauwels RA. Matrix metalloproteinases in asthma and COPD. Curr Opin Pharmacol 2005;

5:257-63.

173. Tsuji M, Suzuki K, Kitamura H, Maruya M, Kinoshita K, Ivanov, II, et al. Requirement for lymphoid tissue-inducer cells in isolated follicle formation and T cell-independent immunoglobulin A generation in the gut. Immunity 2008; 29:261-71.

174. Hams E, Bermingham R, Fallon PG. Macrophage and Innate Lymphoid Cell Interplay in the Genesis of Fibrosis. Front Immunol 2015; 6:597.

175. Sano T, Huang W, Hall JA, Yang Y, Chen A, Gavzy SJ, et al. An IL-23R/IL-22 Circuit Regulates Epithelial Serum Amyloid A to Promote Local Effector Th17 Responses. Cell 2015; 163:381-93.

176. Ather JL, Ckless K, Martin R, Foley KL, Suratt BT, Boyson JE, et al. Serum amyloid A activates the NLRP3 inflammasome and promotes Th17 allergic asthma in mice. J Immunol 2011; 187:64-73.

177. Anthony D, Seow HJ, Uddin M, Thompson M, Dousha L, Vlahos R, et al. Serum amyloid A promotes lung neutrophilia by increasing IL-17A levels in the mucosa and gammadelta T cells. Am J Respir Crit Care Med 2013; 188:179-86.

178. Benditt EP, Eriksen N. Amyloid protein SAA is associated with high density lipoprotein from human serum. Proc Natl Acad Sci U S A 1977; 74:4025-8.

179. Banka CL, Yuan T, de Beer MC, Kindy M, Curtiss LK, de Beer FC. Serum amyloid A (SAA): influence on HDL-mediated cellular cholesterol efflux. J Lipid Res 1995; 36:1058-65.

180. Shah C, Hari-Dass R, Raynes JG. Serum amyloid A is an innate immune opsonin for Gram-negative bacteria. Blood 2006; 108:1751-7.

181. Patel H, Fellowes R, Coade S, Woo P. Human serum amyloid A has cytokine-like properties. Scand J Immunol 1998; 48:410-8.

182. Su SB, Gong W, Gao JL, Shen W, Murphy PM, Oppenheim JJ, et al. A seven-transmembrane, G protein-coupled receptor, FPRL1, mediates the chemotactic activity of serum amyloid A for human phagocytic cells. J Exp Med 1999; 189:395-402.

183. Badolato R, Wang JM, Murphy WJ, Lloyd AR, Michiel DF, Bausserman LL, et al. Serum amyloid A is a chemoattractant: induction of migration, adhesion, and tissue infiltration of monocytes and polymorphonuclear leukocytes. J Exp Med 1994; 180:203-9.

184. Lu J, Yu Y, Zhu I, Cheng Y, Sun PD. Structural mechanism of serum amyloid A-mediated inflammatory amyloidosis. Proceedings of the National Academy of Sciences of the United States of America 2014; 111:5189-94.
185. Lu J, Yu Y, Zhu I, Cheng Y, Sun PD. Structural mechanism of serum amyloid A-mediated inflammatory amyloidosis. Proc Natl Acad Sci U S A 2014; 111:5189-94.
186. Wang L, Colón W. Urea-induced denaturation of apolipoprotein serum amyloid A reveals marginal stability of hexamer. Protein Sci 2005; 14:1811-7.
187. De Buck M, Gouwy M, Wang JM, Van Snick J, Opdenakker G, Struyf S, et al. Structure and Expression of Different Serum Amyloid A (SAA) Variants and their Concentration-Dependent Functions During Host Insults. Current medicinal chemistry 2016; 23:1725-55.
188. Smole U, Gour N, Phelan J, Hofer G, Köhler C, Kratzer B, et al. Serum amyloid A is a soluble pattern recognition receptor that drives type 2 immunity. Nature Immunology 2020; 21:756-65.

국문초록

호흡기 질환에서 기도 내 선천성 림프구 세포의 역할에 대한 고찰

서울대학교 대학원

의과학과 의과학전공

김지현

선천성 림프구 세포(innate lymphoid cells; ILCs)는 도움 T 세포에 대응하는 선천면역세포 중 하나로, 다양한 호흡기 질환에서의 역할이 보고되어 있다. 특히, 천식 발병에서 제 2형 선천성 림프구 세포(group 2 innate lymphoid cells; ILC2s)의 연관성에 대해서는 연구가 활발히 되고 있는 추세이다. 그러나 다른 종류의 선천성 림프구 세포가 천식이나 만성 폐쇄성 폐질환(Chronic obstructive pulmonary disease; COPD) 등의 호흡기 질환의 발병에 미치는 영향에 대해서는 잘 알려지지 않았다.

제 1장에서는 다양한 종류의 선천성 림프구 세포가 천식 발병에

미치는 영향을 대식 세포(macrophages)의 극화(polarization) 현상을 중심으로 규명하였다. 기존 연구에 따르면, 제 2형 선천성 림프구 세포가 천식 환자의 혈액, 기관지세척액(bronchoalveolar lavage fluid; BALF), 그리고 객담에서 증가되어 있음이 보고 되었다. 본 연구에서는, 천식 환자에서 선천성 림프구 세포의 역할을 확인하기 위해 51명의 스테로이드 제제를 사용하지 않은 환자와 18명의 정상 대상군의 객담에서 유세포 분석 기법을 사용하여 각 아형의 선천성 림프구 세포의 변화를 확인하였다. 또한 선천성 림프구 세포와 상호작용할 수 있는 선천 면역 세포로 극화된 대식 세포의 아형 또한 확인하였다. 기존에 보고된 대로 제 2형 선천성 림프구 세포가 천식환자의 객담에서 증가하였을 뿐만 아니라, 제 1형과 제 3형의 선천성 림프구 세포(각각 group 1 innate lymphoid cells; ILC1s, group 3 innate lymphoid cells; ILC3s)도 천식 환자의 기도 내에 증가되어 있는 것을 확인하였다. 또한 대식 세포 역시 천식 환자의 객담에서 증가되어 있었다. 혈액 내 호산구 수에 따라 천식 환자를 호산구성(eosinophilic) 천식과 비 호산구성(non-eosinophilic) 천식으로 분류하였을 때, 호산구성 천식 환자에서는 제 2형 선천성 림프구 세포와 M2 극화 대식세포가 증가되어 있고 비 호산구성 천식 환자에서는 M1 극화 대식세포가 증가되어 있었다. 천식 환자의 기도 내에서 제 2형 선천성 림프구 세포는 M2 극화 대식 세포와, 제 1형과 제 3형 선천성 림프구 세포는 M1 극화 대식 세포와 양의 상관관계를 가지는 것을 확인하였다. 실제로, 제 2형 선천성 림프구 세포와 공생배양한 폐포 대식 세포(alveolar macrophages; AMs)에서 M2 극화와 관련된 유

전자의 발현이 증가되었고, 제 1형과 제 3형 선천성 림프구 세포와 공생배양한 폐포 대식 세포에서는 M1 극화와 관련된 유전자 발현이 증가되었다. 이러한 현상은 사이토카인과 세포간 직접적인 상호작용에 의해 유도된 것을 확인할 수 있었다. 이러한 결과는 서로 다른 종류의 선천성 림프구 세포가 대식세포의 극화를 조절함으로써 천식의 아형에 영향을 줌을 시사하였다.

제 2장에서는 혈청아밀로이드 A(serum amyloid Al; SAA)에 의한 제 3형 선천성 림프구 세포의 활성화와 제 3형 선천성 림프구 세포의 만성 폐쇄성 폐질환 중 하나인 폐 기종(emphysema)에서의 역할에 대한 연구를 진행하였다. 혈청아밀로이드 A는 염증 반응의 급성기(acute phase)에 분비되는 단백질 중 하나이다. 이러한 혈청아밀로이드 A는 다양한 면역 세포의 염증성 사이토카인 분비를 증가시킬 수 있는 내인성(endogenous) 리간드로서 작용한다고 알려져 있다. 또한 만성 폐쇄성 폐질환 환자의 혈장과 폐 조직에서 혈청아밀로이드 A가 증가되어 있다고 보고되어 있다. 본 연구에서는, 마우스를 이용한 폐 기종 동물 모델에서 혈청아밀로이드 A와 제 3형 선천성 림프구 세포가 폐 조직에서 증가되어 있음을 확인하였다. 혈청아밀로이드 A를 직접적으로 폐에 주입해주었을 때, 호중구(neutrophils)성 염증반응과 폐 기종의 표현형을 관찰할 수 있었다. 또한 혈청아밀로이드 A를 주입해 주었을 때, IL-17A 사이토카인을 분비하는 선천성 림프구 세포가 증가하는 것을 확인하였다. 이러한 제 3형 선천성 림프구 세포의 증가는 혈청아밀로이

드 A에 의해 활성화된 호중구에 의해 매개되는 것을 확인하였다. 폐 기종 모델에서 선천성 림프구 세포를 제거하였을 때, 만성 폐쇄성 폐질환의 주요 증상으로 나타나는 폐 기종이 완화되는 것을 확인하였고 염증 반응이 감소하는 것을 확인하였다. 또한 만성 폐쇄성 폐질환 환자의 객담에서 혈청아밀로이드 A과 호중구, 호중구와 제 3형 선천성 림프수 세포 사이의 양의 상관관계를 확인함으로써 이러한 반응이 마우스 모델뿐만 아니라 환자에서도 작용함을 확인하였다. 만성 폐쇄성 폐질환 환자의 기도 내 제 3형 선천성 림프구 세포 증가는 환자의 폐 기능 감소와 연관성이 있음을 확인하였다. 이러한 결과는 혈청아밀로이드 A가 호중구에서의 사이토카인 분비를 촉진시킴으로써 제 3형 선천성 림프구 세포를 증가시키는 기전을 보여주고 있고, 이러한 반응이 폐 기종의 발병에 기여할 수 있는 가능성을 제시하고 있다.

요약하면, 위의 연구를 통하여 천식과 만성 폐쇄성 폐질환 중 하나인 폐 기종에서 증가된 선천성 림프구 세포가 질환의 아형을 결정하는데 관여함을 확인하였다. 천식에서 제 2형 선천성 림프구 세포는 대식세포의 M2 극화를 통해 호산구성 천식을 유도하고, 제 1형과 제 3형 선천성 림프구 세포는 대식세포의 M1 극화를 통해 호중구성 천식을 유도하였다. 만성 폐쇄성 폐질환에서는 제 3형 선천성 림프구 세포가 폐 기종 증상에 기여함을 확인하였고, 이러한 제 3형 선천성 림프구 세포의 활성화는 혈청아밀로이드 A에 의해 활성화된 호중구에 의해 유도됨을 확인하였다. 본 연구 결과는 호흡기 질환의 병리학적 표현형을 완화시키

는 치료를 위한 새로운 타겟으로 선천성 림프구 세포의 중요성에 대해 시사하고 있다.

주요어: 선천성 림프구 세포, 호흡기 질환, 아형, 대식세포, 극화, 혈청아밀로이드 A, 호중구

학번: 2015-23238