의학박사 학위논문

A study on memory-like characteristics of innate lymphoid cells in asthma

천식 상황에서
기억유사 선천성 림프구세포의
특성에 대한 연구

2023년 2월

서울대학교 대학원
의과학과 의과학전공

함 종 호

Ph.D. Dissertation of Medical Science

천식 상황에서 기억유사 선천성림프구세포의 특성에 대한 연구

A study on memory-like characteristics of innate lymphoid cells in asthma

February 2023

Seoul National University Graduate School
Department of Biomedical Sciences

Jongho Ham

A study on memory-like characteristics of innate lymphoid cells in asthma

지도 교수 김 혜 영

이 논문을 의학박사 학위논문으로 제출함
2022년 10월

서울대학교 대학원
의과학과 의과학전공
함 종 호

함종호의 의학박사 학위논문을 인준함
2023년 1월

위 원 장 _____ (인)

부위원장 _____ (인)

위 원 _____ (인)

위 원 _____ (인)

위 원 _____ (인)

ABSTRACT

A study on memory-like characteristics of innate lymphoid cells in asthma

Jongho Ham
Department of Biomedical Sciences
Seoul National University Graduate School

Innate lymphoid cells (ILCs) are the recently discovered innate immune cells located in various mucosal tissues and potent to secrete diverse effector cytokines. As innate immune cells, it has been thought that the roles of ILCs are confined only to acute inflammation or homeostatic conditions. Besides, it also has been considered that the ILCs only shows effector phenotype. However, a series of studies observed the participation of ILCs in many chronic inflammatory diseases and, under repetitive stimulation, some portion of ILCs is showed memory-like phenotypes. Those memory-like ILCs participate in worsening diseases by producing vast cytokines. Thus, understanding the characteristics and the roles of memory-like ILCs in chronic inflammations is critical to comprehend the pathophysiology and help to developing new treatment approaches.

In this study, I analyzed the blood and induced sputum of healthy or

asthmatics to investigate the changes and the roles of ILCs by cigarette smoking. Peripheral blood and induced sputum from a non-smoking and smoking asthmatic patient were analyzed by flow cytometry and found that the ILC3s from induced sputum and circulating $CD4^{+}CD45RO^{+}$ILC3s were significantly increased in smoking asthma patients. *In vitro* study also showed that the CD45RO expression from ILC3s was directly increased by cigarette smoke. Especially, as CD45RO-expressing ILC3s, showed more potent to producing effector cytokine and lasted even after quitting smoke, $CD45RO^{+}$ ILC3s could be considered as memory-like ILCs. Besides, those ILC3 subsets were negatively correlated with pulmonary functionalities represented by FEV1 and FVC. Inflammatory M1 macrophages, in induced sputum, and circulating neutrophils were also positively associated with increased ILC3 subsets. Those results imply that smoking-induced non-allergic inflammations were generated via memory-like ILC3 formation that further stimulated neutrophils and M1 macrophages. Collectively, my data suggested that smoking induces the transition of ILC3s into a memory-like form that is associated with asthmatic severity.

In summary, smoking with asthma directly induces memory-like ILC3 subsets that govern the non-allergic inflammation and aggravation of asthma. These results widen the understanding of the roles and characters of ILCs in chronic airway inflammation, which can be a novel therapeutic target for controlling asthma severity.

Keywords: Chronic inflammation, Innate lymphoid cell, Innate memory, Asthma, Cigarette smoke, Neutrophilic inflammation

Student Number: 2016-29578

* This study included data previously published in Nature Communications (2022).

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ACQ: Asthma control questionnaire

ACT: Asthma control test

AHR: Airway hyperresponsiveness

COPD: Chronic obstructive pulmonary disease

CSE: Cigarette smoke extract

FEV1: Forced expiration volume of 1 second

FVC: Forced vital capacity

GINA: Global initiative for asthma

IF: Immunofluorescence

IFN: Interferon

Ig: Immunoglobulin

IL: Interleukin

ILC: Innate lymphoid cell

LT: Lymphotoxin

LTi: Lymphoid tissue inducer

Mac: Macrophage

MCP-1: Monocyte chemoattractant preotin-1

n.d: not determined

NCR: Natural cytotoxicity receptor

NLRP3: NLR family pyrin domain-containing-3

OCS: Oral corticosteroid

PB: Peripheral blood

PBMC: Peripheral blood mononuclear cell

PBST: Tween 20 diluted in PBS

PC20: Provocative concentration of methacholine required to decrease FEV1 by 20%

PMA: Phorbol 12-myristate 13-acetate

PRR: Pattern recognition receptors

PY: Pack/Year

SD: Standard deviation

SEM: Standard error mean

TEER: Transepithelial electrical resistance

TNF: Tumor necrosis factor

WBC: White blood cell

INTRODUCTION

1. Innate lymphoid cells

I. Innate lymphoid cells

Innate lymphoid cells (ILCs) are the recently discovered innate immune cells that mostly reside and self-renew in mucosal tissues (although they can also be found in the blood)[1]. Those cells are differentiated from common lymphoid progenitor cells, like T cells, but divided into ILC progenitor cells by expressing *Id2*[2] and *Tox*[3]. After passing the several differential sequences, ILCs are divided into three groups, namely, group 1, 2, and 3 ILCs (**Figure 1**)[4, 5]. Especially, group 1 and 3 ILCs are further divided into each subset; NK cells and ILC1s of group 1, lymphoid tissue inducer (LTi) cells, natural cytotoxicity receptor $(NCR)^+$ ILC3s, and NCR^- ILC3s of group 3 ILCs.

Although ILCs are developmentally distinct from T cells, the functions of ILC1s, ILC2s, and ILC3s have been found to mirror those of the adaptive $CD4^+$ T cell subsets known as T_H1, T_H2, and T_H17 cells, respectively[6]. Specifically, in response to IL-12, IL-15, and IL-18, ILC1s are highly expressing *Tbet* and producing interferon (IFN)-γ and tumor necrosis factor (TNF)[1, 4]. ILC1s are activated and initiate immune responses against intracellular pathogens[7]. On the other hand, NK cells show cytotoxic characteristics by producing granzymes and perforins along with *EOMES* expressions, which are considered the counterpart cells of $CD8^+$ T cells[1, 8].

ILC2s can be stimulated by alarmins and secrete type-2 cytokines (*e.g.,* IL-4, IL-5, IL-9, and IL-13) in allergic inflammations. Allergen-mediated IL-25, IL-33, and TSLP activate ILC2s with increasing *GATA3* expressions[1, 4]. In many allergic

diseases, such as atopic dermatitis and asthma, or parasite infections, tissue-resident ILC2s produce a significant amount of cytokines and activate eosinophils, and mast cells, and inducing alternative polarization into M2 macrophages[1, 4].

Group ILC3s respond to IL-1β and IL-23 and produce IL-22 and IL-17 by expressing *RORγt* as a key transcription factor[1, 4, 9]. ILC3s in various mucosae participate in microbial infection, such as bacterial or fungal infections[4, 10]. IL-17A and GM-CSF from activated ILC3s recruit and stimulate neutrophils so which helps clear infection[11]. Especially, NCR^- ILC3s are more prone to produce IL-17A and exert pro-inflammatory phenotypes, however, NCR^+ ILC3s are preferring to secrete IL-22 which is an important cytokine for protecting epithelial barrier[12]. IL-22 directly induces mucin production and supports cell survival of epithelial cells through the STAT3 signaling pathway[13, 14, 15]. Therefore, NCR^+ ILC3s serve protective roles in mucosal tissue. Anther ILC3 subsets, LTi cells, are essential for the lymphoid organogenesis by lymphotoxin (LT) secretion[16].

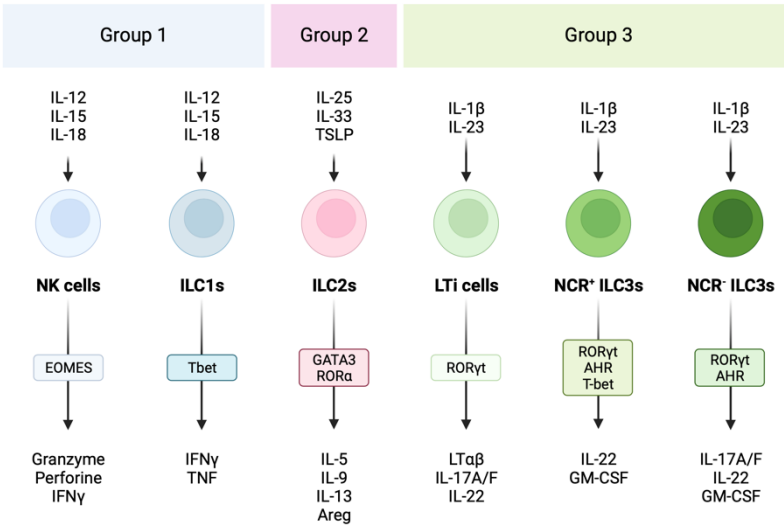

Figure 1. Characteristic of ILCs

Three groups of ILCs have been defined by their developmental differences and key transcription factors. NK cells and ILC1s comprise group 1 ILCs. IL-12, IL-15, and IL-18 induce *EOMES* or *Tbet* expressions from NK cells or ILC1s and promote effector cytokine productions. ILC2s are the lone subset of group 2 ILCs that involve type-2 immunity. LTi cells, $NCR^{+/-}$ ILC3s are in group 3 ILCs. LTi cells produced lymphotoxins (LTs) and IL-17, NCR^{+} ILC3s prefer secreting IL-22, while NCR^{-} ILC3s are dominantly producing IL-17.

II. Memory-like phenotypes of ILCs

Immunological memory formation has been considered as the key differences between innate and adaptive immunity. In repetitive and chronic inflammation, effector T cells differentiate into memory T cells[17, 18]. Memory T cells are defined as a cell subset that can recognize and respond against a specific antigen, that was previously encountered, effetely[19, 20]. Although the origin and mechanism of generating memory T cells are unclear, these cells are derived and participate in numerous chronic inflammations like viral infections[21], tumors[22], and asthma[23].

It has been long believed that the concept of the memory immune cells is confined to adaptive immunity, however, the notion of immunological memory is widened by the findings of innate memory characteristics from monocytes, macrophages, and NK cells[24, 25]. These findings suggested the possibility that the ILCs may also show memory-like phenotypes like other innate immune cells. At the very beginning of the ILC studies, ILCs are only regarded as an initiator of inflammation because (i) ILCs do not undergo clonal selection, (ii) they have vast cytokine-producing ability than $CD4^+$ T cells, (iii) ILCs are tissue-resident and can respond rapidly at the target site. However, a series of studies have observed the importance of ILCs in miscellaneous chronic inflammations (*e.g.,* tumor, asthma, obesity, and inflammatory bowel disease)[26, 27, 28, 29]. Besides, according to recent studies, the notion of memory-like ILCs is also introduced.

Although ILCs do not express specific antigen receptors, ILCs can recognize antigens through pattern recognition receptors (PRRs; such as the toll-like receptor family)[30]. As ILCs also respond to cytokine signaling sensitively, memory-like formation of ILCs is induced via PRR and cytokine signals[31, 32].

Repetitive and perpetuate inflammation generate memory-like phenotypes from ILCs via promoting epigenetic modifications[33] or *c-Maf* expression which is suggested as a key transcription factor of ILC memory formation[34]. Besides, markers for memory-like ILCs have been found along with *c-Maf* and one of the markers is CD45RO. CD45RO is a firstly introduced as a marker of memory T cells while CD45RA are highly expressed on naïve T cells[35, 36]. However, a recent data discovered CD45RO expressions from human inflamed mucosal tissue ILC2s. Additionally, alarmins stimulation induces $CD45RA^+$ ILC2s to differentiate into $CD45RO^+$ ILC2s and the CD45RO expressing ILC2s produce more cytokines than $CD45RA^+$ ILC2s[37].

As cytokine signals are the major pathway of activating ILCs, cytokine signaling is also an important source of generating memory-like responses. It has been found that memory-like ILC2s express higher levels of IL-25R than naïve lung ILC2s. Besides, without allergens but only repetitive administration of IL-33 can also induce memory-like ILC2s (**Figure 2**)[38]. Therefore, not only infectious diseases but also chronic inflammations, such as asthma and chronic obstructive pulmonary disease (COPD) can be the cause of generation of the memory-like ILCs.

Based on those findings, I tried to elucidate the roles of ILCs in the chronic inflammatory disease, asthma. In this study, I investigated human asthma patients' blood and induced sputum to elucidate the changes in ILC subsets. In addition, I found memory-like ILC subsets and its roles in asthma exacerbation.

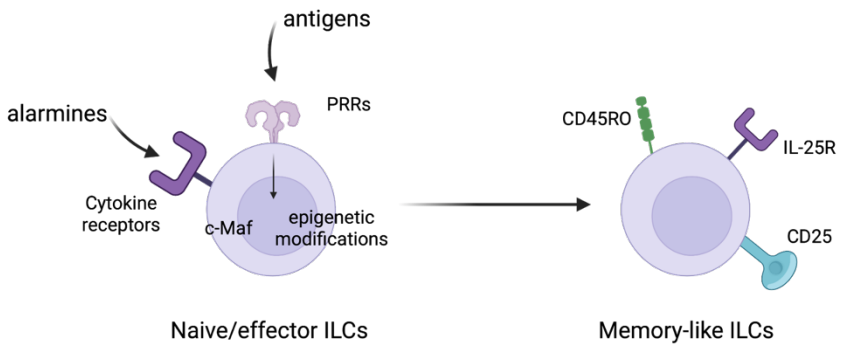

Figure 2. Differentiation of memory-like ILCs.

Repetitive and chronic PRR signaling or alarmin activations induce epigenetic modifications and generate memory-like ILC formation.

2. ILCs in asthma

Asthma is one of the most prevalent chronic lung diseases with clinical features of airway obstruction and excessive mucus production, affecting more than 300 million people worldwide[39, 40]. Asthma has long been considered an allergic disease mediated by type-2 dominant immune responses. Inhaled foreign antigens irritate airway epithelial cells and promote alarmin secretions, IL-25, IL-33, and TSLP, which then increase the production of IL-4, IL-5, IL-9, and IL-13. These cytokines typically associate with immunoglobulin(Ig)-E production, eosinophilia, mast cell activation, and mucus secretion by airway epithelial cells[39]. Besides, type-2 cytokines also induce alternative polarization of macrophages (M2 macrophages) and degranulation of granulocytes, which worsen bronchial obstruction (**Figure 3**). However, asthma is a complex disease that is now known to comprise a variety of disease phenotypes and endotypes[41].

10–33% of asthmatics have a non-allergic asthma phenotype triggered by ozone exposure, obesity, and air pollutants[42]; it is also typically more severe than allergic asthma[43]. Besides, hitherto the most universal medication for asthma is the corticosteroid inhalers[44], however, non-allergic asthma shows steroid resistance[45]. Unlike allergic asthma, non-allergic asthma is governed by type1/17-immune responses. Various environmental factors induce epithelial damage and recruit IL-6, IL-8, and IL-17A-producing cells[46] and those cytokines further promote activation of neutrophils[47, 48] and M1 macrophage polarization[49]. Activated neutrophils and macrophages secrete assorted proteases and chemokines that damage the airway and leading severe inflammations[50, 51, 52] (**Figure 3**).

Like other immune cells, pulmonary ILCs are also involved in both allergic and non-allergic asthma. In allergic asthma, IL-33 (and the other epithelial alarmins, IL-25 and TSLP) is a major stimulator of ILC2s. Clinical studies observed that the IL-33 levels and ILC2 frequencies in the blood[53] and lung lavage fluid[54] of asthma patients are correlated positively. Also, the increased number of ILC2s in animal asthma models is accompanied by eosinophil infiltration, airway hyperresponsiveness (AHR), and mucus production that characterizes allergic asthma[55, 56, 57, 58]. ILC2s also induce the polarization of alveolar macrophages into the alternatively activated macrophages (M2 macrophages), which is known to induce eosinophilia[59]. Not only innate immunity but also adaptive immune cells can be regulated by lung ILC2s. *In vitro* experiment suggests that ILC2s directly upregulate T_H2 cell responses by expressing MHC class II[60]. Together, these observations demonstrate the importance of ILC2s in allergic asthma (**Figure 4**).

The other type of asthma, non-allergic asthma, also associate with ILCs in its pathophysiology. In obesity-induced asthma, augmented NLR family Pyrin domain-containing (NLRP)3 inflammasome derived IL-1β from classically activated (M1) macrophages and upregulated of IL-1β in the obese lung stimulates ILC3s[61, 62]. As ILC3s actively produce IL-17A which is a potent neutrophil chemotactic molecule, the association between obesity-induced asthma and neutrophilia is mediated via activated ILC3s[63]. Moreover, environmental pollutants can also cause non-allergic asthma[64, 65, 66]. Air pollutants, such as ozone prompt to secrete IL-1β, IL-23 that leads to ILC3-mediated type-17 immune responses[67, 68] (**Figure 4**). Thus, the lung ILCs work as a key player in both asthmatic inflammations.

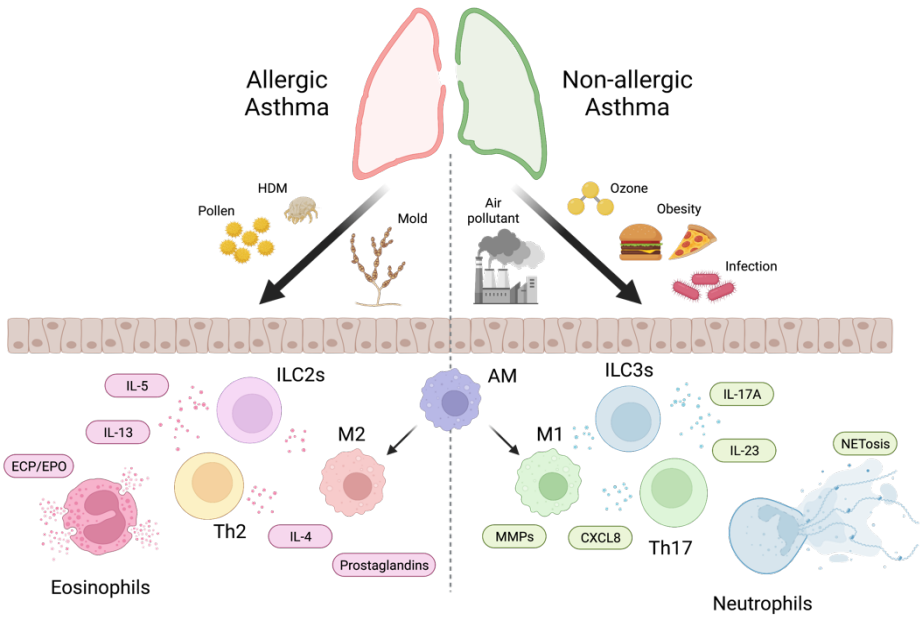

Figure 3. Diversity of asthma.

In allergic asthma, inhaled allergens disturb airway epithelium and induce alarmins secretion. Next, alarmins recruit and stimulate eosinophils, T_H2 cells, and ILC2s, which further promoting type-2 mediated inflammations. However, non-allergic asthma is induced via diverse environmental factors. Various environmental factors directly or indirectly activate ILC3s and T_H17 cells that secreting IL-17A and IL-23. Besides, protease and chemokines from neutrophils and M1 macrophages exaggerate airway inflammation.

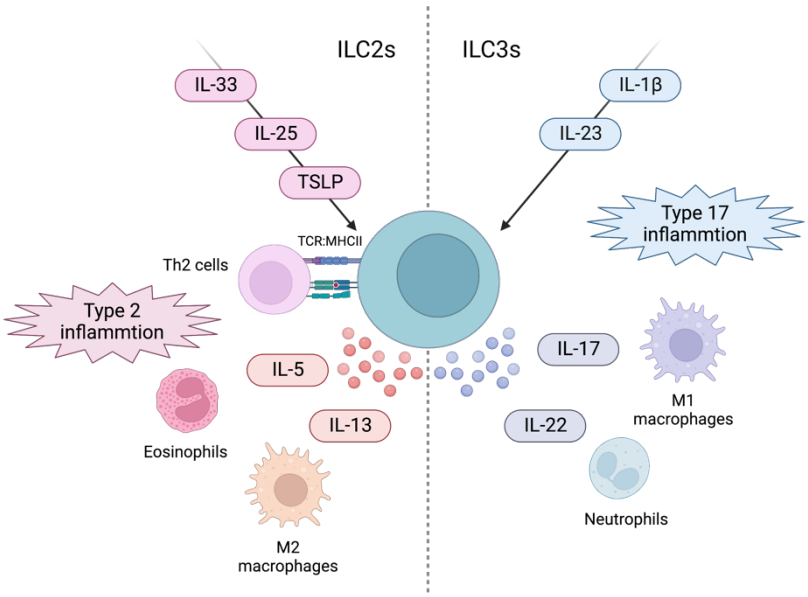

Figure 4. Roles of ILCs in asthma.

ILC2s are activated by allergen-mediated alarmins (*e.g.*, IL-25, IL-33, and TSLP) and stimulated ILC2s to secrete IL-5 and IL-13 that increase type-2 inflammations through eosinophils and M2 macrophages. In non-allergic asthma, various environmental factors prompt the secretion of IL-1β and IL-23. These cytokines induce type-17 cytokine productions from ILC3s and further promote M1 macrophage differentiation and neutrophilic inflammation.

MATERIALS AND METHODS

Participants

I recruited 33 non-smokers with asthma (non-smoker; who have never smoked), 58 smokers with asthma who are currently smoking or have smoked previously, 13 non-smoking healthy controls, and 11 healthy smoking controls from Seoul National University Hospital (Seoul, South Korea) and Chung-Ang University Hospital (Seoul, South Korea) between December 2016 and June 2017. All patients with asthma met one of the following criteria: FEV_1 changed over 12 % and 200 mL after bronchodilator response, and/or there was significant airway hyperresponsiveness to methacholine or mannitol provocation. Patients with cancer, severe medical conditions, or other pulmonary diseases were excluded along with patients on medications such as antibiotics, antifungal agents, antiviral drugs, probiotics, or any systemic steroids. Patients treated with immunotherapy were also excluded. I checked and followed the Strengthening the Reporting of Observational studies in Epidemiology (STROBE) guidelines.

Study approval

All participants enrolled in this study provided written informed consent. The study protocol was approved by the Chung-Ang University Hospital Institutional Review Board (IRB number 1600-002-253) and the Seoul National University Hospital Institutional Review Board (IRB number 1608-163-788).

Immune cell isolation from induced sputum and peripheral blood

To eliminate mucus from sputum samples, the same volume of 0.1% dithiothreitol (Sigma, MO, USA) was added, the tube was shaken for 20 minutes at 37°C, and the mixture was filtered through a 70 μm strainer. After centrifugation at 1400 rpm for 6 minutes, the cell pellet was resuspended with 100 μL FACS buffer (PBS + 2% fetal bovine serum) for staining. Peripheral blood (PB) mononuclear cells (PBMC) were isolated by using Ficoll-Paque PLUS density gradient media (GE Healthcare, IL, USA). Briefly, PB was centrifuged at 2000 rpm for 10 minutes at 4°C to separate the plasma from the cells. After removing the plasma, the remaining cell pellet was resuspended with PBS and loaded onto a Ficoll-Paque layer. After centrifuging at 1800 rpm for 30 minutes, the PBMC layer was collected and washed with PBS.

PBMC stimulation

To stimulate PBMCs, they were cultured in RPMI 1640 containing 10% FBS and 10 μg/mL gentamycin. 1 μg/mL ionomycin and 100 ng/mL phorbol 12-myristate 13-acetate (PMA) were added to the culture media with 0.01% Protein Transport Inhibitor (BD GolgiStopTM, BD biosciences, NJ, USA). After 3 hours at 37°C, the PBMCs were harvested.

Flow Cytometry Analysis

Cells isolated from induced sputum and PBMCs were blocked with anti-CD16/CD32 and stained with the following fluorochrome-labeled monoclonal antibodies: anti-CD45 (HI30; BD Biosciences, NJ, USA); anti-CD3ε (UCHT1),

anti-CD11c (3.9), anti-CD11b (ICRF44), anti-CD14 (HCD14), anti-CD19 (HIB19), anti-CD49b (P1E6-C5), and anti-FcεRIα (AER-37), which were used as lineage markers and were from BioLegend (CA, USA); anti-CD68 (Y1/82A), anti-CD117 (C-Kit, 104D2), anti-CD127 (IL-7R, A019D5), anti-CD206 (15-2), anti-HLA-DR (L243), anti-CD4 (OKT4), anti-CD45RO (UCHL1), anti-CD45RA (HI100), anti-CD56 (HCD56), anti-CD16 (3G8), anti-NKp44 (P44-8), anti-IFNγ (4S.B3), anti-IL-5 (TRFK5), anti-IL-17A (BL168), and anti-IL-1β (H1b-98), which were from BioLegend (CA, USA); and anti-ST2 (B4E6; MD Bioproducts, MN, USA). To analyze cytokine production, PBMCs were fixed and permeabilized with the Fixation/Permeabilization Solution Kit (BD CytoFix/CytoPermTM, BD biosciences, NJ, USA). Flow cytometry was performed using BD LSRFortessa™ and BD LSRFortessa™ X-20 (BD, NJ, USA) and analyzed by FlowJo (V10) software (BD, NJ, USA).

Preparation of cigarette smoke extract

Cigarette smoke extract (CSE) was generated from a single pack of Marlboro Red cigarettes (Philip Morris, VA, USA) containing 8 mg tar and 0.7 mg nicotine. The smoke from this pack of cigarettes was dissolved in 20 mL PBS (considered to be 100% CSE) by using a vacuum pump. After dissolving the cigarette smoke, CSE was sterilized by filtration through a 0.2 μm filter.

Cigarette smoke extract treatment

To analyze the direct effects of CSE on ILCs, PBMCs isolated from healthy controls and asthma patients were cultured for 48 hours with RPMI1640 containing

10% FBS and 0.1% of CSE. The cells were then harvested and analyzed by flow cytometry.

The effect of CSE treatment on epithelial cells was assessed with the human alveolar basal epithelial cell line A549, the human nasal epithelial cell line RPMI2650, the human bronchial epithelial cell line BEAS-2B, or the mouse lung epithelial cell line MLE12. Thus, 1×10^5–5×10^5 cells were seeded for 48 hours and then cultured with 1, 5, or 10% CSE for 6–48 hours. To analyze IL-1β secretion, 5 mM ATP (Sigma, MO, USA) was added to the culture media for the last 6 hours before harvest.

Confocal microscopy to measure airway epithelial cell layer integrity

To determine the effect of CSE on epithelial intercellular adhesion, coverslips were coated with poly-L-lysine solution (Sigma, MO, USA) for 5 minutes at RT, washed with PBS, and dried for 1 hour at 60°C. Coated coverslips were loaded into a culture plate and cultured with A549 cells as described above for 48 hours. The coverslips were then washed with PBS and the cells were fixed with 4% PFA for 15 minutes, washed with 0.05% Tween 20 (Promega, WI, USA) diluted in PBS (PBST), permeabilized with 0.2% Triton X-100 (Promega, WI, USA) for 10 minutes, and blocked with 3% BSA in PBST for 3 hours. The cells were stained for 2 hours at RT with anti-human E-cadherin antibody (Invitrogen, CA, USA) that was diluted in PBST containing 1% BSA. The cells were then incubated with Alexa Fluor 488-labeled anti-mouse IgG antibody (Thermofisher, MA, USA) for 1 hour. The coverslips were flipped over onto ProLong diamond antifade mountant with DAPI (Invitrogen, CA, USA)-loaded slide glasses and

incubated overnight at 4°C. The slides were imaged with a Confocal-A1 (Nikon, Tokyo, Japan) confocal microscope and analyzed by Image J software (NIH, MD, USA).

TEER measurements of airway epithelial cell layer integrity

TEER measurements were conducted with an Epithelial Voltohmmeter (World Precision Instruments, FL, USA). A549, RPMI2650, BEAS-2B, or MLE12 cells were seeded on 12 well-transwell inserts (Corning, ME, USA) for 24 hours before CSE treatment and then, cells were cultured with 10% CSE-containing media for 48 hours, rinsed with PBS. The electrodes were swiped with 70% ethanol and rinsed with PBS prior to use. TEER was calculated by using the following equation[69]: TEER $(\Omega \cdot cm^2) = (R_{total}\ (\Omega) - R_{blank}\ (\Omega)) \times$ membrane area (cm^2).

RT-qPCR

The total RNA of cultured epithelial cell lines was extracted by using TRIzol (Invitrogen, CA, USA) reagent according to the manufacturer's protocol. cDNA was synthesized with the iScript cDNA Synthesis Kit (Bio-Rad, CA, USA). RT-qPCR assay was performed with IQ Supermix (Bio-Rad, CA, USA). Primers of IL1B, IL23A, and IL33 were purchased from Thermofisher Scientifics (TaqMan Gene Expression Assay; Thermofisher, CA, USA).

ELISA

ELISA of supernatant IL-1β (BD Biosciences, NJ, USA) was conducted according to the instructions of the manufacturer.

Statistics

The data are presented as mean ± standard deviation (SD). To determine the normality of data, Shapiro-Wilk normality tests were conducted. Two groups were compared by using two-tailed Mann-Whitney U test or two-tailed unpaired *t*-test. Three or more groups were compared with one-way ANOVA following by a Bonferroni's post-test or two-way ANOVA. All correlation analyses were conducted with Spearman r correlation test. *P*-values of < 0.05 were considered to indicate statistical significance. GraphPad Prism 7 was used for statistical analysis.

RESULTS

Cigarette smoking in asthma associates with higher ILC3 frequencies in induced sputum

To investigate the effect of cigarette smoking on ILCs in patients with asthma, I recruited 58 current or previous smokers with asthma, 33 non-smokers with asthma, 11 healthy smokers, and 13 healthy non-smokers (**Table 1**). Compared to the non-smokers with asthma, the smokers with asthma had significantly impaired lung function, as shown by (i) lower forced expiration volume of 1 second % (FEV_1 %), ratio of FEV_1/FVC, and asthma control test (ACT) scores, and (ii) higher asthma control questionnaire (ACQ) scores. As indicated by the PC_{20} value, the two groups did not differ in airway hyperresponsiveness (**Table 2**).

Table 1. Characteristics of the study subjects

	Healthy Control		Asthma	
	Non-smoker	**Smoker**	**Non-smoker**	**Smoker**
n	13	11	33	58
Age (Year)	55.1±5.7	59.0±13.3	52.1±14.0	55.1±11.4
Sex (M/F)	6/7	8/3	6/27	49/9
Body mass index (kg/m^2)	23.0±2.6	26.3±2.7	23.6±3.1	25.8±4.9
Allergic rhinitis, n (%)	0 (0)	0 (0)	21 (63.6)	32 (55.2)
Atopic dermatitis, n (%)	0 (0)	0 (0)	3 (9.1)	6 (10.4)

The data are presented as mean ± standard deviation of *n* (%).

Table 2. Lung functions and complete blood count of the study subjects

	Healthy Control		Asthma		
	Non-smoker	**Smoker**	**Non-smoker**	**Smoker**	*P-value*
FVC (mL)	3465.0±837.0	4002.0±732.4	3180.0±1014.0	3646.0±878.3	0.1037
FVC (%)	107.3±12.6	100.7±19.1	103.5±13.8	88.2±15.1	<0.0001
FEV1 (mL)	2749.0±639.6	3177.0±595.3	2542.0±657.5	2444.0±786.6	0.0747
FEV1 (%)	103.5±12.6	104.8±14.1	95.6±10.5	76.1±16.7	<0.0001
FEV_1/FVC (%)	79.8±6.4	79.6±7.7	77.0±5.4	65.9±11.2	<0.0001
ACQ	0	0.1±0.1	5.8±4.4	7.0±5.3	0.0271
ACT	25	25	21.8±3.1	20.2±3.4	0.0061
PC_{20} (mg/ml)	n.d	n.d	10.9±9.0	5.5±6.2	0.1364
OCS, n (%)	n.d	n.d	5 (15.2)	13 (22.4)	
Hemoglobin (g/dL)	n.d	n.d	13.3±2.4	15.0±1.7	0.0003
WBC (/μL)	n.d	n.d	7055.0±2830.0	7948.0±3650.0	0.2978
Lymphocytes (/μL)	n.d	n.d	1958.0±740.0	2317.0±1310.0	0.2548
Monocytes (/μL)	n.d	n.d	428.3±153.1	576.8±365.5	0.1301
Eosinophils (/μL)	n.d	n.d	317.3±349.3	318.6±314.5	0.6822
Neutrophils (/μL)	n.d	n.d	4174.0±2510.0	4655.0±3514.0	0.6754
Basophils (/μL)	n.d	n.d	39.7±23.4	39.0±22.2	0.6179

The data are presented as mean ± standard deviation of *n* (%).

*P values were determined by comparing the non-smoker asthma patients with the smoking asthma patients by Mann-Whitney U test. The healthy non-smoker and smoker groups did not differ significantly in in lung function indices (P values not shown).

ACT: asthma control test; ACQ: asthma control questionnaire; FEV_1: Forced expiratory volume in one second; FVC: forced vital capacity; n.d, not determined; OCS: oral corticosteroid; PC_{20}: provocative concentration of methacholine required to decrease

FEV_1 by 20%; WBC: white blood cells

I first analyzed the ILC subsets in induced sputum. Total ILCs were defined as $CD45^{+}Lineage^{-}IL\text{-}7R^{+}$ lymphocytes, after which the ILC1s ($ST\text{-}2^{-}C\text{-}kit^{-}$ cells), ILC2s ($ST\text{-}2^{+}$ cells), and ILC3s ($ST\text{-}2^{-}C\text{-}kit^{+}$ cells) were identified (**Figure 5a**). Smoking in both the asthma and healthy groups tended to associate with higher frequencies of total airway ILCs but these differences did not achieve statistical significance (**Figure 5b**). Smoking in the healthy group associated with higher ILC1 frequencies (**Figure 6a**), but this was not observed for the asthma group (**Figure 6b**). The four groups had similar ILC2 frequencies (**Figure 6a and 6c**). By contrast, smoking associated with significantly greater sputum ILC3 frequencies in the asthma group (**Figure 6d**) but not in the healthy group (**Figure 6a**).

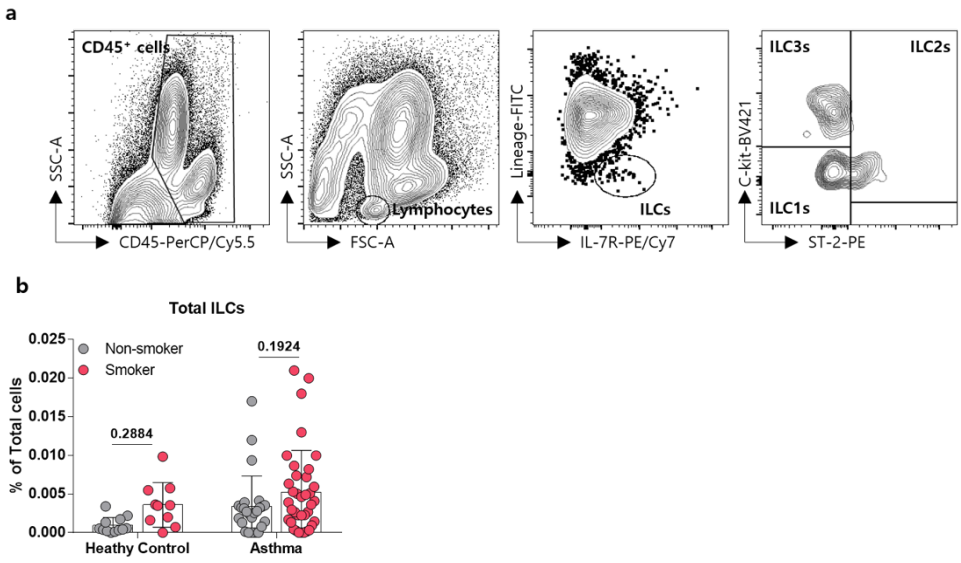

Figure 5. Comparison of ILCs from non-smoking and smoking asthma patients in induced sputum.

a. Gating strategy for the three subsets of innate lymphoid cells (ILCs) in induced sputum. Total ILCs were gated as Lineage (CD3ε, CD11c, CD11b, CD14, CD19, CD49b, and FcεRIα)-negative and IL-7R-positive cells. ST-2 and C-kit expression was used to distinguish ILC1s (ST-2$^-$C-kit$^-$), ILC2s (ST-2$^+$), and ILC3s (ST-2$^-$C-kit$^+$). **b**. Comparison of non-smoking and smoking asthma patients and non-smoking and smoking healthy individuals in terms of total ILC frequencies in induced sputum. Each dot represents individual subjects. The non-smokers and smokers in the asthma patients or healthy individuals were compared by two-way ANOVA **(b)**. All data are presented as mean ± standard deviation. $p < 0.05$ is considered as significant.

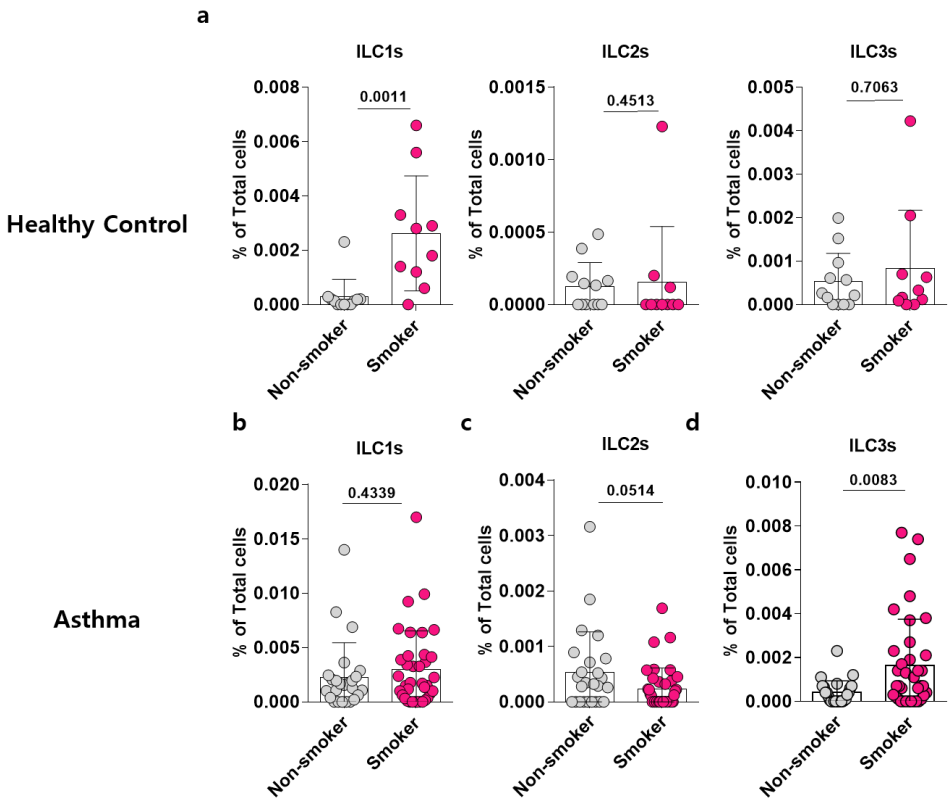

Figure 6. Differences of ILC subsets from healthy control and asthmatics.

a. Comparison of non-smoking and smoking healthy controls in terms of sputum ILC1, ILC2, and ILC3 frequencies. **b-d.** Comparison of non-smoking and smoking asthmatics in terms of sputum ILC1 **(b)**, ILC2 **(c)**, and ILC3 **(d)** frequencies. Each dot represents individual subjects. The non-smokers and smokers in the asthma patients or healthy individuals were compared by two-tailed Mann-Whitney U test **(a-d)**. All data are presented as mean ± standard deviation. $p < 0.05$ is considered as significant.

ILC3s can be further subdivided according to their expression of natural cytotoxicity receptors (NCRs): NCR^{+}ILC3s secrete IL-22 to maintain epithelial barrier integrity whereas NCR^{-}ILC3s mainly produce IL-17A and IL-17F, which are known to induce pathogenic inflammation[70]. I observed that smoking in asthma associated with a greater frequency of NCR negative sputum ILC3s (**Figure 7a**). By contrast, smoking did not have this effect in the healthy controls (**Figure 7b**). These data suggest that cigarette smoking may provoke harmful changes in the ILC3 subset in the asthmatic airways.

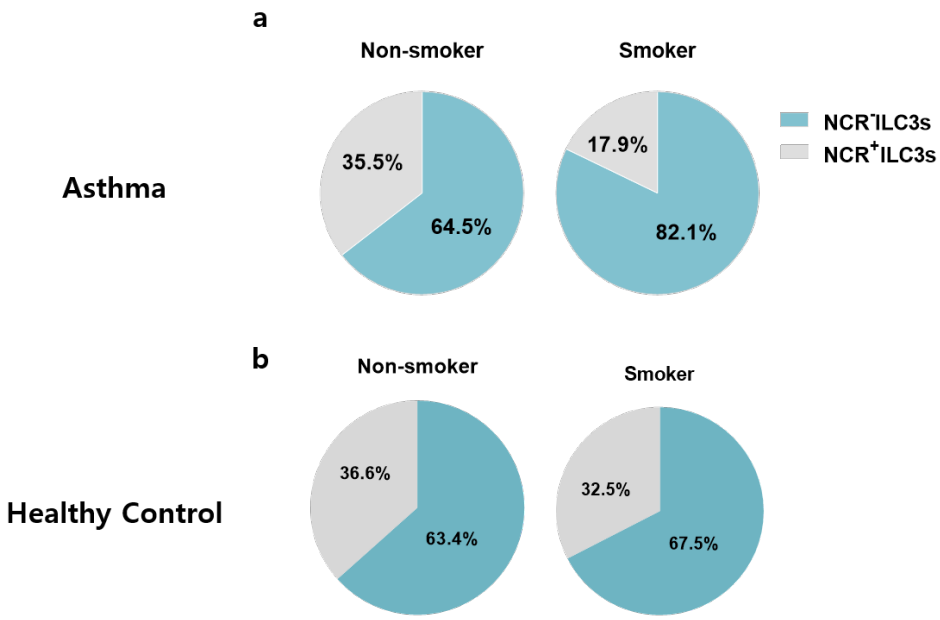

Figure 7. Ratio of NCR expressions from sputum ILC3s.

a-b. The proportion of NCR^- ($NKp44^-$) and NCR^+ ($NKp44^+$) ILC3s in the induced sputum of non-smoking and smoking asthma patients **(a)** and healthy controls **(b)**.

To exclude the possibility that the smoking-related changes in ILC subset frequencies were merely due to the severity of asthma, I selected the 32 non-smokers and 34 smokers in the asthma group who had normal lung function (defined as FEV_1 >80%). Again, smoking associated with higher total ILC and ILC3 frequencies in the asthmatic airway (**Figure 8a and b**).

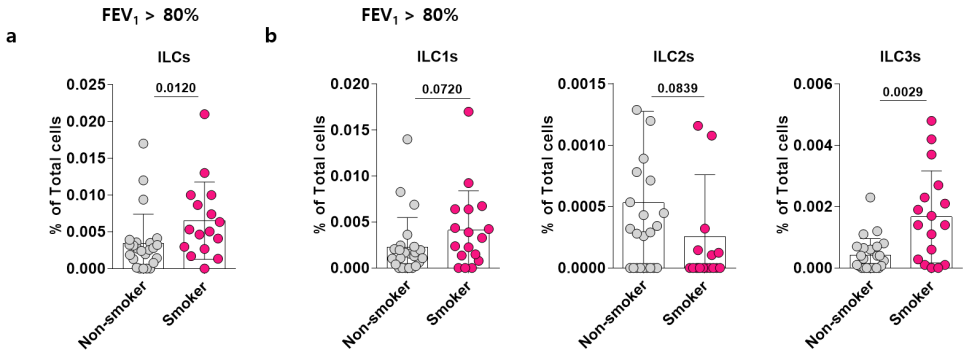

Figure 8. Smokers with normal FEV_1 (%) have higher ILC3s in induced sputum than non-smokers with normal FEV_1 (%) from asthma patients.

Asthmatics with normal FEV_1 (%) have more than 80% of FEV_1 (%). **a.** Comparison of frequency of total ILCs between non-smoking and smoking patients from asthmatics. **b.** Comparison of ILC1s, ILC2s, and ILC3s between non-smokers and smokers from asthmatics with normal FEV_1 (%). Each dot represents individual subjects. The non-smokers and smokers in asthma patients with normal FEV_1(%) were compared by two-tailed Mann-Whitney U test. The data are presented as mean ± standard deviation. $p < 0.05$ is considered as significant.

Smoking in asthma associates with CD45RO-expressing ILC3s in the peripheral blood

The effect of smoking on the ILCs in the peripheral blood mononuclear cells (PBMCs) from the four groups was analyzed in the same way as for the sputum ILCs (**Figure 9a**). Smoking associated with a modest (statistically insignificant) increase in total ILC frequencies in the PBMCs from asthma patients but significantly lower total ILC frequencies in the healthy group (**Figure 9b**). ILC subset analyses then showed that smoking slightly increased ILC1 and ILC3 frequencies and had no effect on ILC2s in the asthma group (**Figure 9c**) but significantly decreased ILC1 and ILC2 frequencies and had no effect on the ILC3s in the healthy group (**Figure 10**). Thus, at first glance, smoking in asthma did not appear to have a marked effect on the ILC3 frequencies in the peripheral blood, unlike in the sputum.

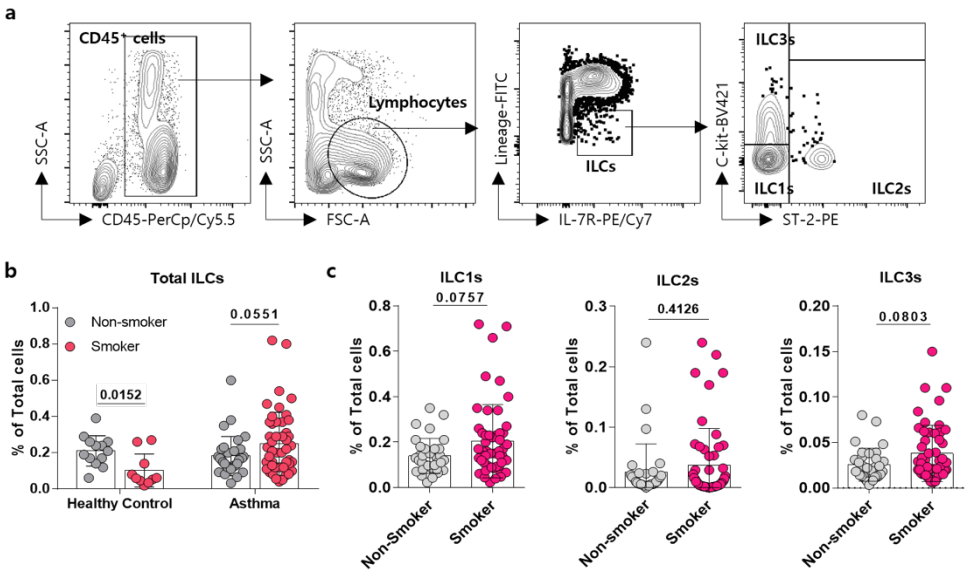

Figure 9. Analysis of blood circulating ILCs.

a. Gating strategy for the three subsets of innate lymphoid cells (ILCs; Lineage$^-$IL-7R$^+$) in the blood. **b.** Comparison of non-smoking and smoking asthma patients and non-smoking and smoking healthy individuals in terms of total ILC frequencies in the peripheral blood. **c.** Comparison of non-smoking and smoking asthma patients in terms of peripheral blood ILC1 (ST-2$^-$C-kit$^-$), ILC2 (ST-2$^+$), and ILC3 (ST-2$^-$C-kit$^+$) frequencies. Each dot represents individual subject. The non-smokers and smokers in the asthma patients or healthy individuals were compared by multiple t-tests **(b)** or two-tailed Mann-Whitney U test **(c)**. All data are presented as mean ± standard deviation. $p < 0.05$ is considered as significant.

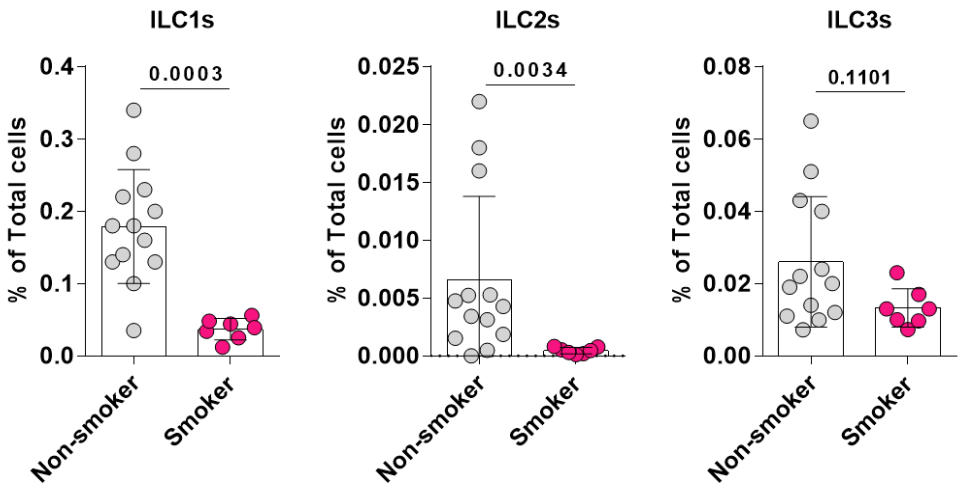

Figure 10. Peripheral blood ILCs decreases in smokers among healthy controls.

Comparison of each type of ILCs from healthy controls between non-smokers and smokers. Each dot represents individual subjects. The non-smokers and smokers in healthy controls were compared by two-tailed Mann-Whitney U test. The data are presented as mean ± standard deviation. $p < 0.05$ is considered as significant.

Nonetheless, I decided to explore the PBMCs further. Since smoking significantly increased NCR^{-}ILC3s in the airway of asthma patients (**Figure 7a**) but circulating ILC3s do not express NCR[71], I asked whether the PBMCs of the smoking asthma patients contained higher frequencies of an airway disease-associated ILC3 subset, namely, CD45RO-expressing ILC3s. These cells are elevated in the lung and tonsils of patients with chronic obstructive pulmonary disease (COPD)[72]. Moreover, CD45RO-expressing blood ILC2s are elevated in steroid-resistant asthmatic patients[73]. While it is not yet clear what CD45RO expression signifies in terms of ILC functions, it is well-known that in T cells (the adaptive immunity equivalents of ILCs), CD45RO is a marker of activated/memory T cells while CD45RA is expressed on naïve T cells[74]. Indeed, when I assessed the CD45RA and CD45RO expression of the blood ILCs (**Figure 11a**), I observed that smoking in the asthma group, but not the healthy group, associated with a modest increase of CD45RO^{+}ILC3s in the PBMCs (**Figure 11b and c**). By contrast, the four groups did not differ significantly in terms of circulating CD45RA^{+}ILC3s (data not shown).

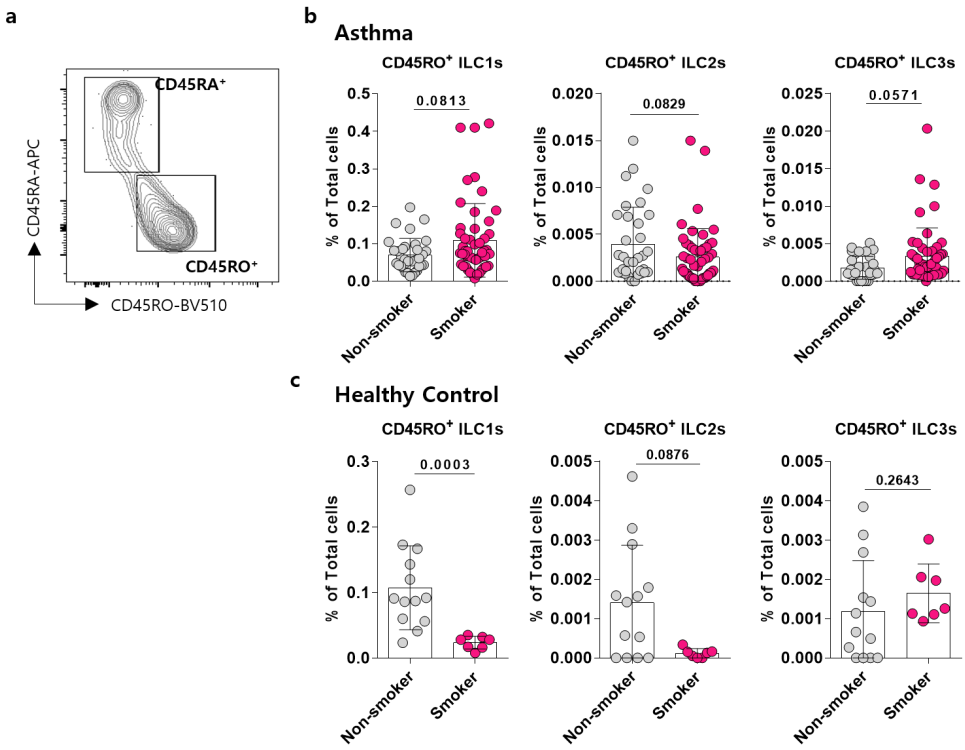

Figure 11. CD45RA and CD45RO expressing ILCs.

a. Representative plot of CD45RA and CD45RO expressing ILCs. **b-c.** Comparison of non-smoking and smoking asthma patients **(b)** and healthy controls **(c)** in terms of peripheral blood CD45RO^{+} ILC1, ILC2, and ILC3 frequencies. Each dot represents individual subject. The non-smokers and smokers in the asthma patients or healthy individuals were compared by two-tailed Mann-Whitney U test. All data are presented as mean ± standard deviation. $p < 0.05$ is considered as significant.

Retinoic acid induces RORγt expression in NCR^{-} $CD4^{-}$ ILC3 precursors, and RORγt differentiates them into NCR^{-} $CD4^{+}$ ILC3s, in mice[75]. Assuming that the CD4-expressing NCR^{-}ILC3s would be in a more differentiated form, I further confirmed CD4 expression. I noted that the $CD45RO^{+}$ILCs could be further divided into $CD4^{+}$ and $CD4^{-}$ subsets (**Figure 12a**). Analysis of these $CD4^{+}$ expressing subsets showed that smoking in the asthma group, but not the healthy group, associated with significantly increased blood frequencies of $CD4^{+}CD45RO^{+}$ILC3s (**Figure 12b and c**). Thus, smoking in asthma specifically increased the frequencies of $CD4^{+}CD45RO^{+}$ILC3s in the blood. Moreover, the blood $CD4^{+}CD45RO^{+}$ILCs were significantly more likely to express IL-17A, a major cytokine released from ILC3s, than the $CD4^{-}CD45RO^{+}$ILCs or $CD45RA^{+}$ILCs (**Figure 12d**), which suggests that they are more active than the other subsets. Similar analyses with the asthma patients with normal lung function showed again that although smoking did not significantly alter the blood frequencies of total ILCs or the three ILC subsets (**Figure 13a and b**), it did associate with significantly higher circulating $CD4^{+}CD45RO^{+}$ILC3 frequencies (**Figure 13c**).

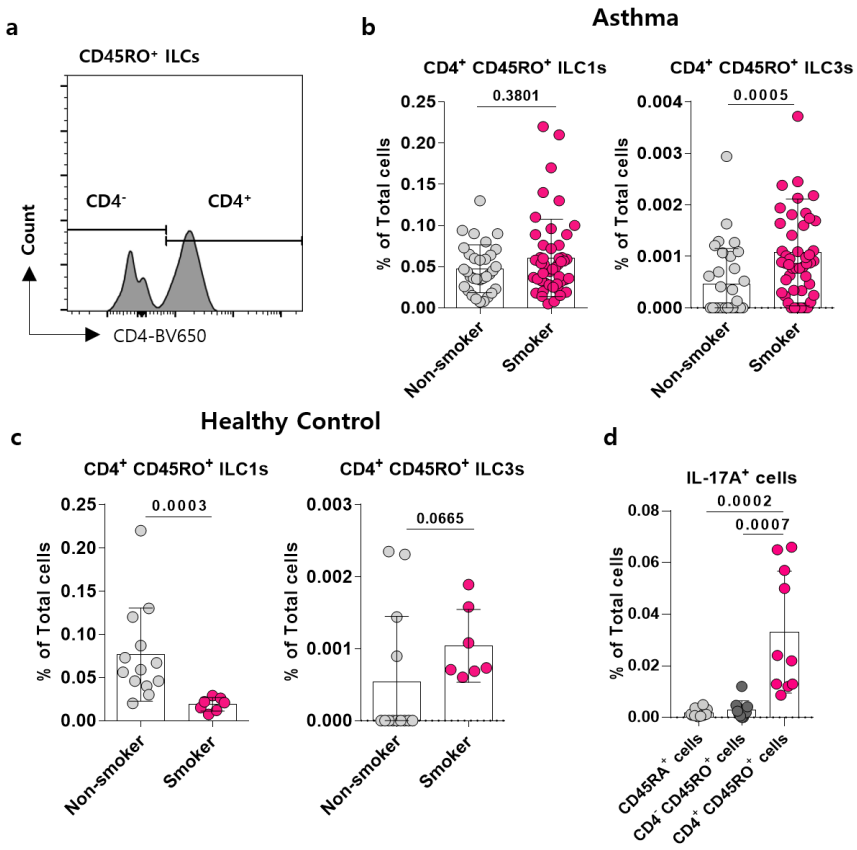

Figure 12. Characteristics of CD45RO expressing ILCs.

a. Representative histogram of CD4 expression of CD45RO$^+$ILCs. **b.** Comparison of non-smoking and smoking asthma patients in terms of peripheral blood CD4$^+$CD45RO$^+$ILC1 and CD4$^+$CD45RO$^+$ILC3 frequencies. **c.** Comparison of non-smoking and smoking healthy controls in terms of peripheral blood CD4$^+$CD45RO$^+$ILC1 and CD4$^+$CD45RO$^+$ILC3 frequencies. **d.** Frequencies of CD45RA$^+$, CD4$^-$CD45RO$^+$, and CD4$^+$CD45RO$^+$ total ILCs that co-express IL-17A. Each dot represents individual subject. The non-smokers and smokers in the asthma patients or healthy individuals were compared by two-tailed Mann-Whitney U test **(b and c)**. One-way ANOVA was conducted to compare the frequencies of IL-17A$^+$ cells among CD45RA$^+$, CD4$^-$CD45RO$^+$, and CD4$^+$CD45RO$^+$ ILCs **(d)**. All data are presented as mean ± standard deviation. $p < 0.05$ is considered as significant.

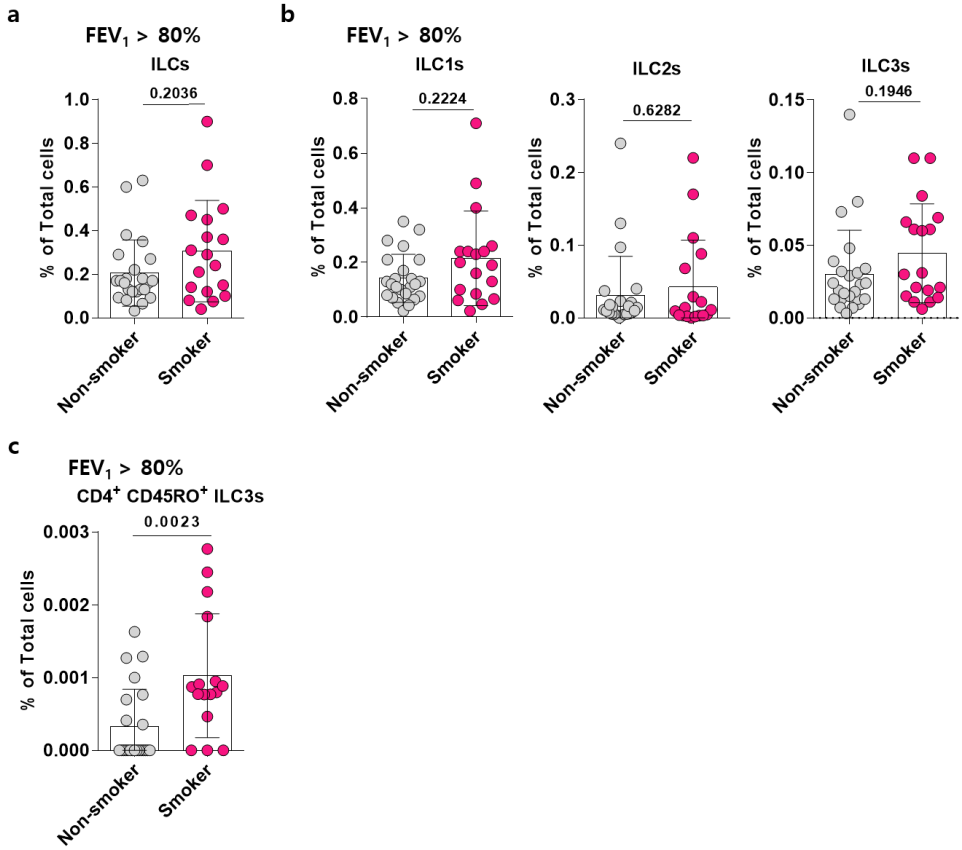

Figure 13. CD4⁺CD45RO⁺ILC3s in blood are increased in smokers from asthmatics with normal FEV_1 (%).

Asthmatics with normal FEV_1 (%) have more than 80% of FEV_1 (%). **a-b.** Comparison of frequency of circulating total ILCs **(a)**, ILC1s, ILC2s, and ILC3s **(b)** between non-smokers and smokers from asthmatics with normal FEV_1 (%). **c.** Comparison of frequency of $CD4^+CD45RO^+$ILC3 subsets in blood between non-smokers and smokers in asthmatics with normal FEV_1 (%). Each dot represents individual subjects. The non-smokers and smokers in asthma patients were compared by two-tailed Mann-Whitney U test. The data are presented as mean ± standard deviation. $p < 0.05$ is considered as significant.

It should be noted that smoking in the asthma group also associated with slightly higher circulating ILC1 (**Figure 9c**) and $CD45RO^+$ ILC1 (**Figure 11b**) frequencies; however, it did not associate with higher $CD4^+CD45RO^+$ILC1 frequencies (**Figure 12b**). By contrast, smoking in the healthy group associated with significantly fewer ILC1s, $CD45RO^+$ILC1s, and $CD4^+CD45RO^+$ILC1s (**Figure 10, 11c, and 12c**). Thus, unlike the equivalent subset in ILC3s, $CD4^+CD45RO^+$ILC1s are not specifically expanded in the blood of smoking patients with asthma. This suggests that the expansion of ILC1s in the blood of smoking patients with asthma may be secondary to decreased pulmonary function rather than playing a pathogenic role.

I also examined the $CD4^+$ T cells in the peripheral blood of the four groups. While smoking associated with significantly reduced $CD4^+$ T cell frequencies in the healthy group, this was not observed in the asthma patients. The frequencies of $CD4^+$ T cells that expressed IFNγ, IL-5, or IL-17A were also similar between smoking and non-smoking asthmatics (**Figure 14a-c**). Moreover, smokers in the asthma group did not have higher frequencies of memory $CD45RO^+$ T cells than non-smokers (**Figure 14d**). Thus, memory T cells do not appear to be augmented by smoking in asthma.

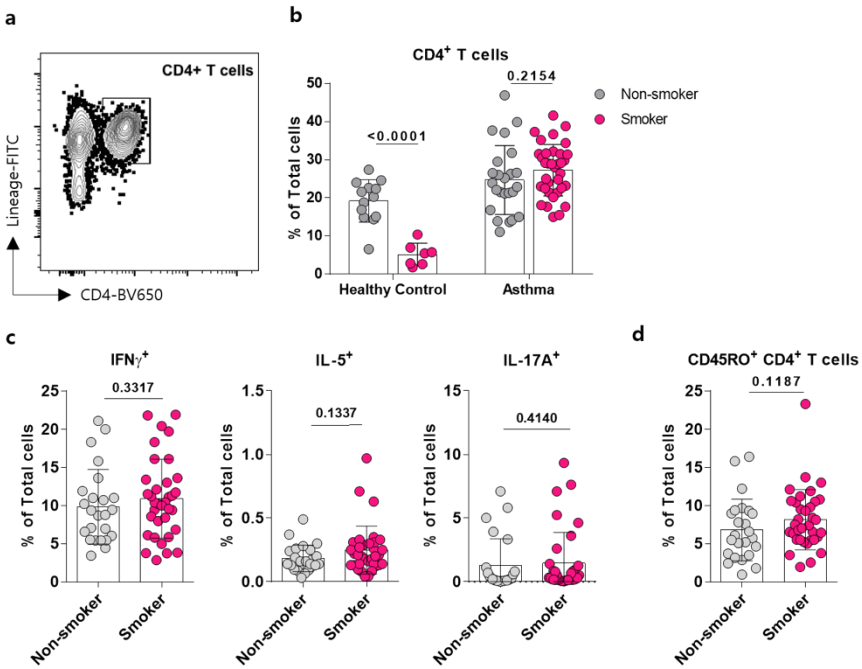

Figure 14. Circulating CD4$^+$ T cells have no differences between non-smokers and smokers from asthmatics.

a. Gating strategy of CD4$^+$ T cells from peripheral blood. **b.** Comparison of frequency of CD4$^+$ T cells in blood between non-smoking and smoking patients with asthma; non-smoking and smoking individuals in healthy controls. **c.** Comparison of proportion of IFNγ, IL-5, and IL-17A$^+$ T cells in blood between non-smokers and smokers in asthmatics. **d.** Comparison of frequency of CD45RO$^+$CD4$^+$ T cells in blood between non-smokers and smokers in asthmatics. Each dot represents individual subjects. The non-smokers and smokers in the asthma patients or healthy individuals were compared by multiple t-tests **(b)**. The non-smokers and smokers in asthma patients were compared by two-tailed Mann-Whitney U test **(c and d)**. The data are presented as mean ± standard deviation. $p < 0.05$ is considered as significant.

In vitro cigarette smoke exposure induces memory-like ILC3s in PBMCs from asthma patients but not healthy individuals

Since cigarette smoke appeared to augment sputum ILC3s and circulating $CD4^{+}CD45RO^{+}$ILC3s in patients with asthma (but not healthy individuals), I assessed whether the smoking amount, as expressed by Pack/Year (PY), correlated with the frequencies of these ILC3 populations in asthma patients. Indeed, both populations in the asthma group correlated positively and significantly with PY (**Figure 15a and b**).

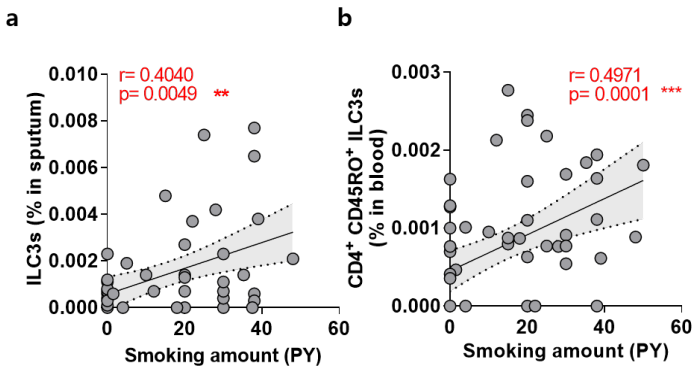

Figure 15. Smoking amounts are positively correlated with blood and sputum ILC3 subsets.

a–b. Correlation between smoking amount (PY) and (**a**) ILC3 frequencies in induced sputum and (**b**) $CD4^{+}CD45RO^{+}ILC3$ frequencies in peripheral blood, as determined by Spearman r correlation test. Each dot represents individual subjects. Correlation analyses were conducted with Spearman r correlation test, dotted line represents 95% confidence interval **(a and b)**. All data are presented as mean ± standard deviation. $p < 0.05$ is considered as significant.

Since CD45RO is a key marker for primed memory T cells[74] and I observed that the circulating $CD4^+CD45RO^+$ILCs from asthma patients were more likely to express IL-17A than the corresponding $CD4^-CD45RO^+$ILCs or $CD45RA^+$ILCs (**Figure 12d**), I speculated that when asthma patients (but not healthy individuals) are exposed to cigarette smoke, the biological properties of ILC3s may change, including acquiring an activated memory-like phenotype ($CD4^+CD45RO^+$). To test whether the $CD4^+CD45RO^+$ circulating ILC3s is maintained after quitting smoking (*i.e.,* whether they display a memory function), I divided the asthma patients according to their current smoking status (non-smokers, former-smokers, and current-smokers). Indeed, both former and current smokers had significantly higher frequencies of $CD4^+CD45RO^+$ILC3s in the blood than non-smoker asthma patients (**Figure 16**). These data suggested that smoking induces the $CD4^+CD45RO^+$ILC3 subset that persists even after quitting smoking like as memory immune cells (memory-like ILC3s).

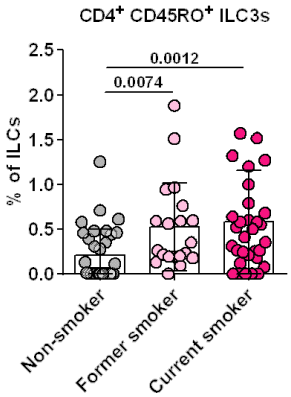

Figure 16. Blood CD4$^+$CD45RO$^+$ILC3s are increased both from the former and current smoking asthmatics.

Comparison of non-smokers, former-smokers, and current-smokers in asthma patients in terms of CD4$^+$CD45RO$^+$ILC3s in the peripheral blood. Each dot represents individual subjects. Comparison of the CD4$^+$CD45RO$^+$ILC3s frequencies among non-smokers, former-smokers, and current-smokers in asthma patients was conducted by one-way ANOVA. All data are presented as mean ± standard deviation. $p < 0.05$ is considered as significant.

Next, I asked whether *in vitro* culture of PBMCs from the non-smoking healthy and asthma groups with 0.1% cigarette smoke extract (CSE) directly altered the phenotype of their ILC populations (**Figure 17a**). Treatment with CSE did not alter the CD45RA or CD45RO expression of the circulating ILC1s from any of the groups (**Figure 17b and c**). By contrast, CSE treatment significantly increased the expression of CD45RO by the ILC3s from asthma patients but this was not observed in the healthy individuals (**Figure 17d**). CSE treatment did not alter the CD45RA expression of the ILC3s of any group (**Figure 17e**). Thus, in asthma patients but not healthy controls, CSE treatment readily activated ILC3s in the blood into memory-like $CD45RO^{+}$ILC3s. These data together suggest that cigarette smoke directly induces memory-like CD45RO expressingILC3s (but not ILC1s) only in patients with asthma.

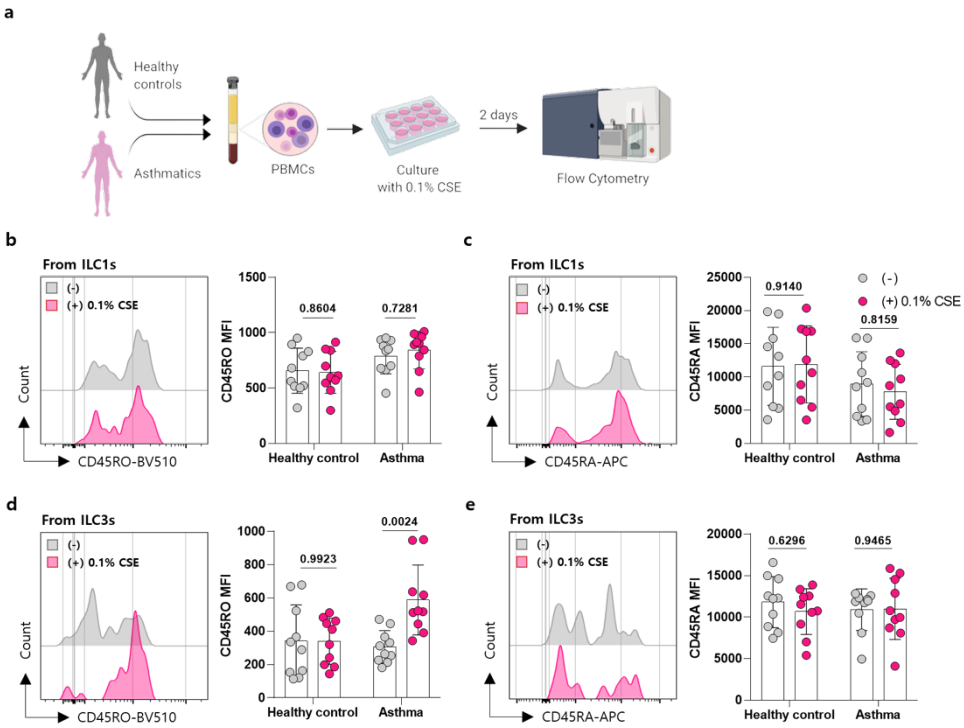

Figure 17. *In vitro* cigarette smoke exposure elevates the CD45RO expression of ILC3s from asthma patients but not healthy individuals.

a-e. PBMCs were treated *in vitro* with cigarette smoke extract (CSE) as shown schematically in **(a)**. Healthy individuals and asthma patients were compared in terms of the CD45RO **(b and d)** and CD45RA **(c and e)** mean fluorescence intensity (MFI) of ILC1s **(b and c)** and ILC3s **(d and e)** that were and were not treated with 0.1% CSE. Each dot represents individual subjects. CSE-treated and untreated cultures were compared by multiple t-tests **(b-e)**. All data are presented as mean ± standard deviation. $p < 0.05$ is considered as significant.

Cigarette smoke-induced damage to airway epithelial cells generate an ILC3-activating environment

Cigarette smoke can damage airway epithelial cells, thereby increasing mucosal permeability and the infiltration of inflammatory immune cells[76, 77]. To examine whether CSE affects the barrier function of airway epithelial cells, I treated the A549 human alveolar basal epithelial cell line with CSE. The treatment decreased A549 expression of E-cadherin (which associates with epithelial tight junction functions[78]) and destroyed the close-knit epithelial layer (**Figure 18a**). To further assess the effect of CSE on epithelial barrier function, I treated the A549 cell line, the RPMI2650 human nasal epithelial cell line, the BEAS-2B human bronchial epithelial cell line, and the MLE12 mouse lung epithelial cell line with CSE and measured their transepithelial electrical resistance (TEER). CSE significantly decreased TEER in all lines (**Figure 18b**).

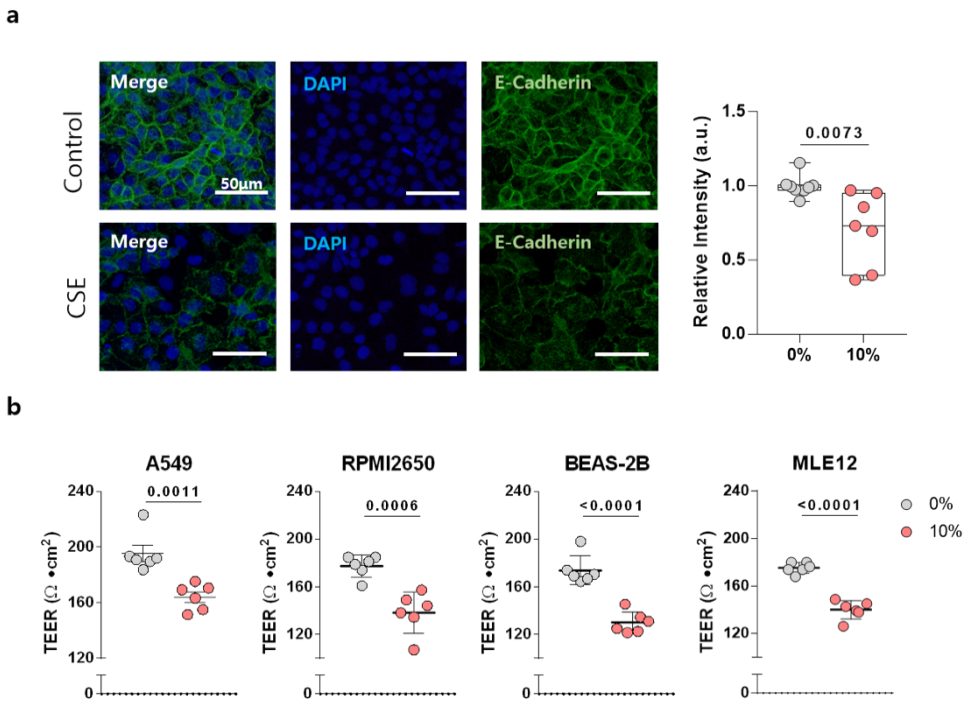

Figure 18. Cigarette smoke exposure destruct airway epithelial lining.

a. The A549 was treated with 10% cigarette smoke extract (CSE) for 48 hours and then subjected to anti-E-cadherin immunostaining (AF488; green) and DAPI staining (blue). Scale bar, 50 μm. The plot shows the relative intensity of E-Cadherin. **b.** A549 cells, RPMI2650, BEAS-2B, and MLE12 were treated with 10% CSE for 48 hours and their transepithelial electrical resistance (TEER) was measured. Each of the data was the representative data from more than twice replications. For the box plots, lower and upper box boundaries 25th and 75th percentiles, respectively, line inside box median, lower and upper error lines 10th and 90th percentiles, dots indicate data points, respectively. Relative intensity of E-cadherin, TEERs from CSE-treated and untreated cell lines were compared by two-tailed unpaired t test **(a and b)**. All data are presented as mean ± standard deviation. $p < 0.05$ is considered as significant.

Since damaged epithelial cells can release alarmins such as IL-1, IL-23, and IL-33, all of which are essential for ILC activation[1, 79], I examined the effect of CSE treatment on A549 expression of these innate cytokines. CSE treatment elevated *IL1B* expression in a dose-dependent manner, decreased *IL23A* expression at the highest dose, and had no effect on *IL33* expression (**Figure 19a**). Increasing the duration of CSE treatment also augmented the IL-1β mRNA and protein levels in the A549 cells (**Figure 19b**). In addition, CSE-treatment elevated *IL1B* expression in the other epithelial cell lines (**Figure 20a**). However, the expression of *IL23A* and *IL33* did not differ according to CSE-treatment with the exception of RPMI2650 (**Figure 20b and c**).

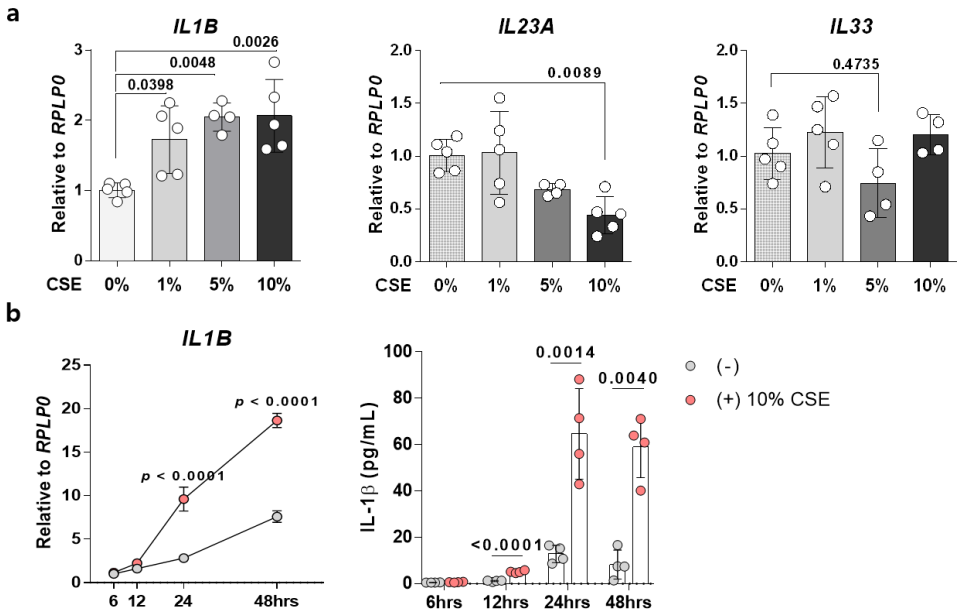

Figure 19. Cigarette smoke exposure promotes IL-1β secretion.

a. A549 cells were treated with 0–10% CSE for 48 hours and their *IL1B*, *IL23A*, and *IL33* mRNA expression were measured. **b.** Comparison of CSE-treated and untreated A549 cells *IL1B* genes and protein expressions. The CSE concentrations ranged from 0% to 10% and cells were cultured for 6 to 48 hours. Each of the data was the representative data from more than twice replications. The CSE-treated cells were compared to the untreated cells in different CSE concentrations by one-way ANOVA **(a)**. The CSE-treated A549 cells were compared to the untreated cells at the same time-point by multiple t test **(b)**. All data are presented as mean ± standard deviation. $p < 0.05$ is considered as significant.

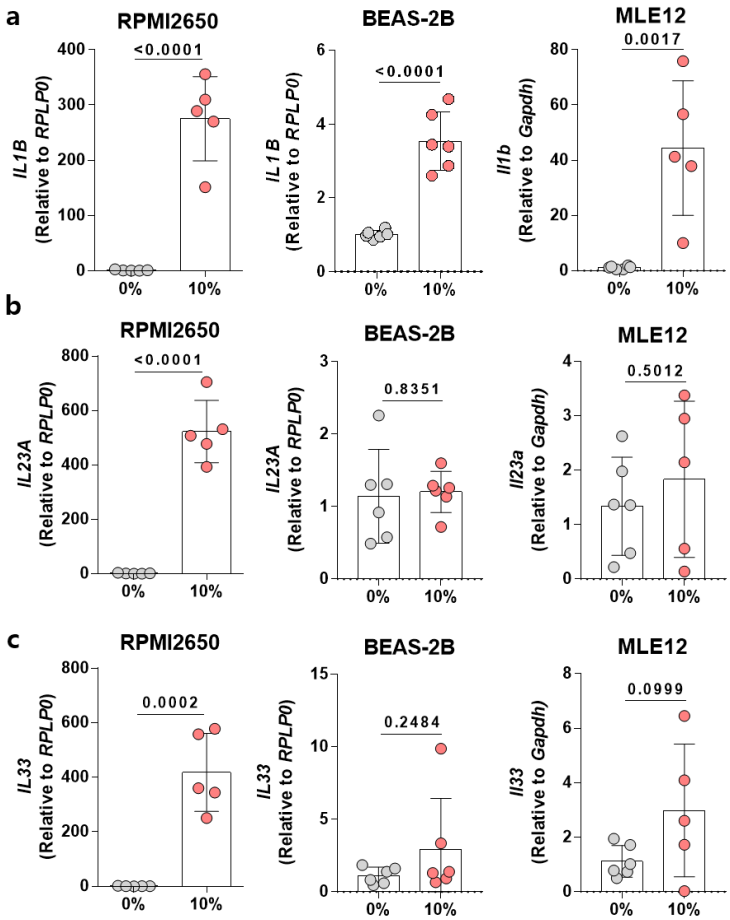

Figure 20. Smoking induced stimulatory cytokine expressions.

a-c. Comparison of *IL1B* **(a)**, *IL23A* **(b)**, and *IL33* **(c)** expressions from CSE treated and untreated RPMI2650, BEAS-2B, and MLE12. Each of the data was the representative data from more than twice replications. The gene expressions from CSE-treated and untreated cell lines were compared by two-tailed unpaired t test **(a-c)**. All data are presented as mean ± standard deviation. $p < 0.05$ is considered as significant.

Since IL-1β engages an IL-1 receptor on ILC3s and promotes their proliferation and IL-17A production[79], these findings suggest that the smoking-induced sputum ILC3s and circulating memory-like $CD4^{+}CD45RO^{+}$ILC3 subset in asthma patients could be generated by smoke-damaged airway epithelial cells rather than circulating immune cells. Notably, the whole PBMCs or circulating immune cell subsets from the four healthy and asthmatic smoker/never-smoker groups did not differ in terms of IL-1β-producing cell frequencies (**Figure 21a-c**): this analysis supports the notion that the IL-1β potentially driving the smoking-induced generation of ILC3s in asthma is produced locally rather than systemically.

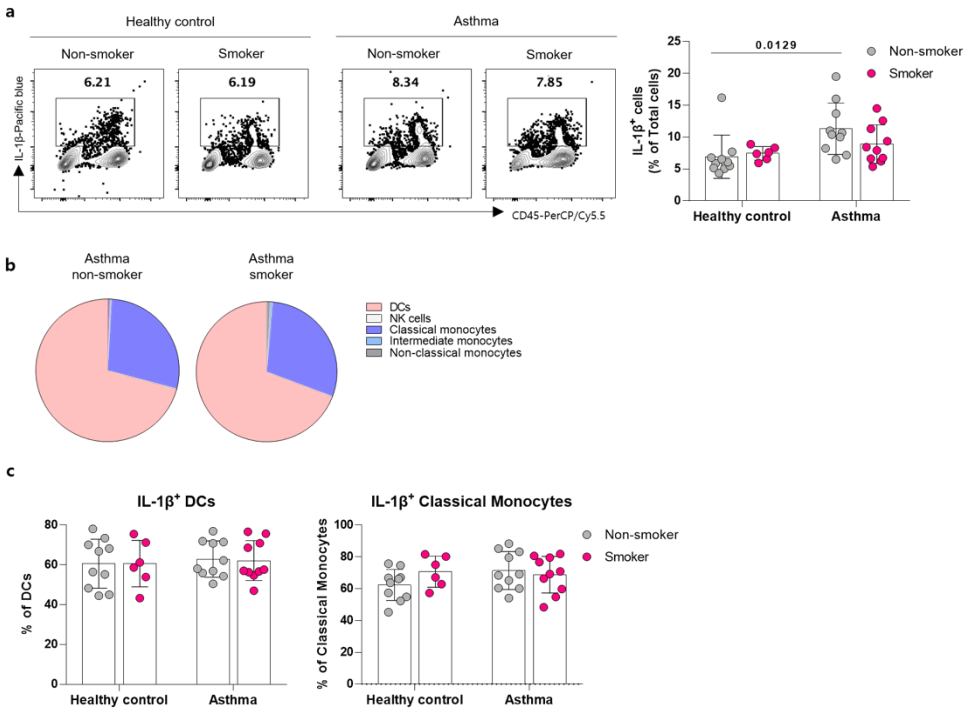

Figure 21. Effect of smoking in IL-1β production from PBMCs.

a. Comparison of IL-1β^{+} cells between non-smokers and smokers from healthy controls or asthma patients. **b.** Composition of IL-1β^{+} immune cells from PBMCs of asthmatic non-smokers and asthmatic smokers. Each cell was primary gated as IL-1β^{+} cells and further divided as described. Dendritic cells (DC): CD45^{+}CD11c^{+}HLA-DR^{+} cells; natural killer (NK) cells: CD45^{+}CD11c^{-}HLA-DR^{-}CD56^{+} cells; classical monocytes: CD45^{+}CD11c^{-}HLA-DR^{+}CD56^{-}CD14^{+}CD16^{-} cells; intermediate monocytes: CD45^{+}CD11c^{-}HLA-DR^{+}CD56^{-}CD14^{+}CD16^{+} cells; and non-classical monocytes: CD45^{+}CD11c^{-}HLA-DR^{+}CD56^{-}CD14^{-}CD16^{+} cells. **c.** Comparison of proportion of IL-1β^{+} cells from DCs or classical monocytes in between non-smokers and smokers from healthy controls or asthma patients. Each dot represents individual subjects. The smokers and non-smokers from healthy controls and asthma patients were compared by Two-way ANOVA **(a and c)**. The data are presented as mean ± standard deviation. $p < 0.05$ is considered as significant.

The increased ILC3 frequencies in asthma patients correlate with asthma severity

To understand the clinical significance of the increased ILC3 frequencies in smoking asthma patients, I analyzed the relationships between the frequencies of sputum ILC3s and blood $CD4^+CD45RO^+$ILC3s and asthma severity, as measured by (i) the pulmonary function indices FEV_1 and FEV_1/FVC ratio or (ii) asthma control, as measured by the ACQ and ACT scores. ACQ and ACT quantify asthma control and play a prominent role in Global Initiative for Asthma (GINA) guidelines:[80] patients with well-controlled asthma have higher ACT and lower ACQ values. ILC3 frequencies in the sputum of asthma patients correlated negatively with FEV_1 (**Figure 22a**), FEV_1/FVC ratio (**Figure 22b**), and ACT score (**Figure 22c**) and positively with ACQ score (**Figure 22d**). The $CD4^+CD45RO^+$ILC3 frequencies in the peripheral blood also correlated negatively with the pulmonary function indices but not the asthma control indices (**Figure 23a-d**). Thus, the frequencies of ILC3 subsets in the induced sputum and blood of asthma patients correlated with their disease severity.

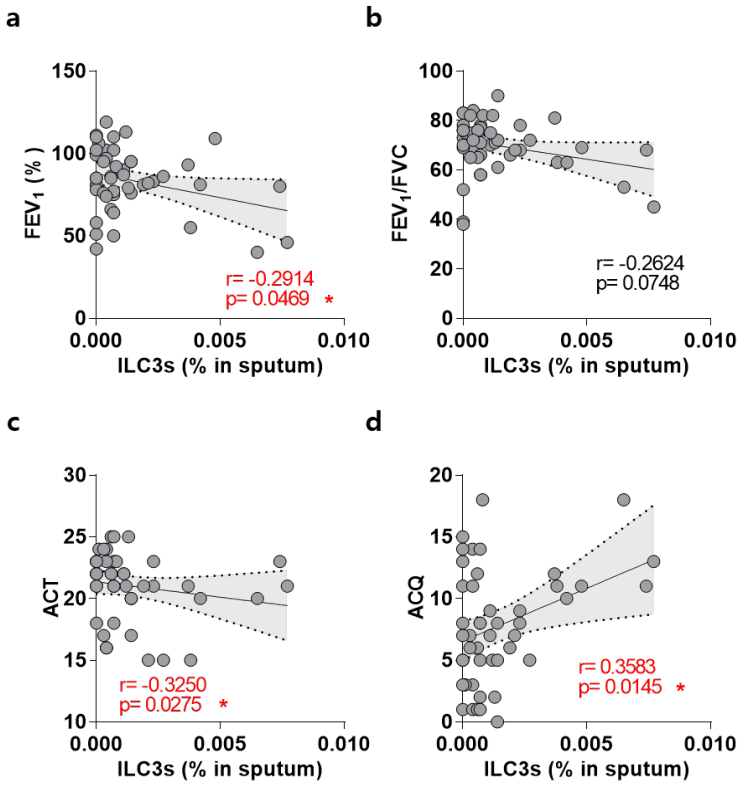

Figure 22. ILC3s in sputum correlate positively with asthma severity.

a-d. Correlation between ILC3 frequencies in induced sputum and four clinical indices of asthma severity, namely, FEV_1 (%) **(a)**, FEV_1/FVC **(b)**, ACT **(c)**, and ACQ **(d)**. Each dot represents individual subjects. Correlation analyses were conducted with Spearman r correlation test, dotted line represents 95% confidence interval. $^*P < 0.05$.

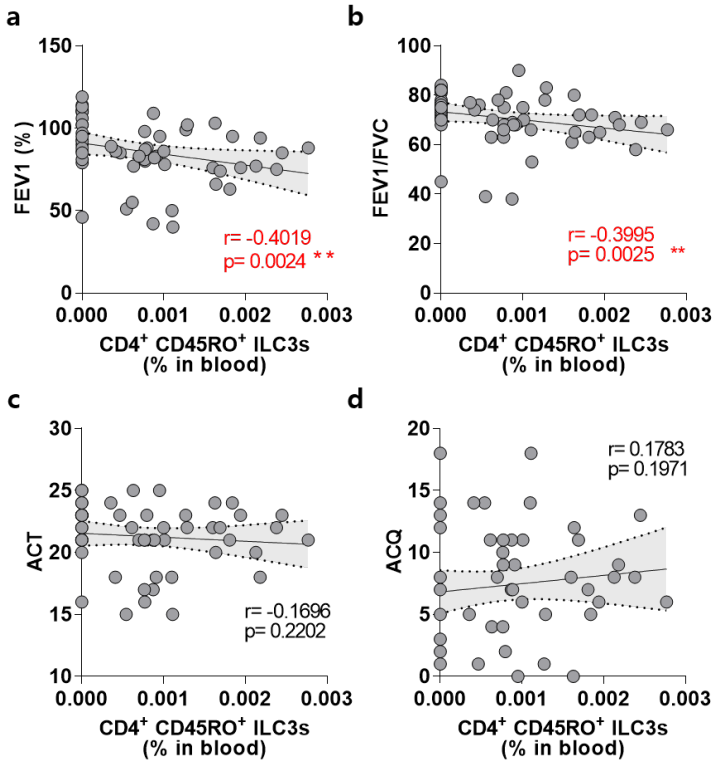

Figure 23. CD4$^+$CD45RO$^+$ILC3s in blood correlate positively with asthma severity.

a-d. Correlation between CD4$^+$CD45RO$^+$ILC3 frequencies in the blood and FEV_1 (%) **(a)**, FEV_1/FVC **(b)**, ACT **(c)**, and ACQ **(d)**. Each dot represents individual subjects. Correlation analyses were conducted with Spearman r correlation test, dotted line represents 95% confidence interval. $^{**}P < 0.01$.

The increased ILC3 frequencies in smoking asthma patients associate with a non-T_H2 asthma phenotype

Asthma is a heterogeneous disease that can be divided into eosinophilic (type-2 immunity-dominant) and non-eosinophilic (type-1/3 immunity-dominant) asthma. Some forms of non-eosinophilic asthma also associate with neutrophilia[81]. Therefore, I asked whether the increased ILC3 frequencies in asthma patients correlated with their circulating eosinophil and neutrophil counts. The sputum ILC3s and circulating $CD4^+CD45RO^+$ILC3s correlated positively with circulating neutrophils (**Figure 24a and b**). Despite the fact that the smoker and non-smoker asthma patients had similar numbers of circulating neutrophils, circulating neutrophils correlated positively with smoking amount (PY) (**Figure 24c and d**). By contrast, circulating eosinophil counts did not correlate with either blood/sputum ILC3 frequencies and negatively correlated with smoking amount (PY) (**Figure 25a-d**). Since blood/sputum ILC3 frequencies also correlated with smoking amount (**Figure 25a and b**), impaired lung function (**Figure 22 and 23**), and neutrophil counts (**Figure 24a and b**), these findings suggest that smoking-upregulated ILC3 subsets in asthma patients associate with neutrophilic but not eosinophilic immune responses that are harmful to lung function.

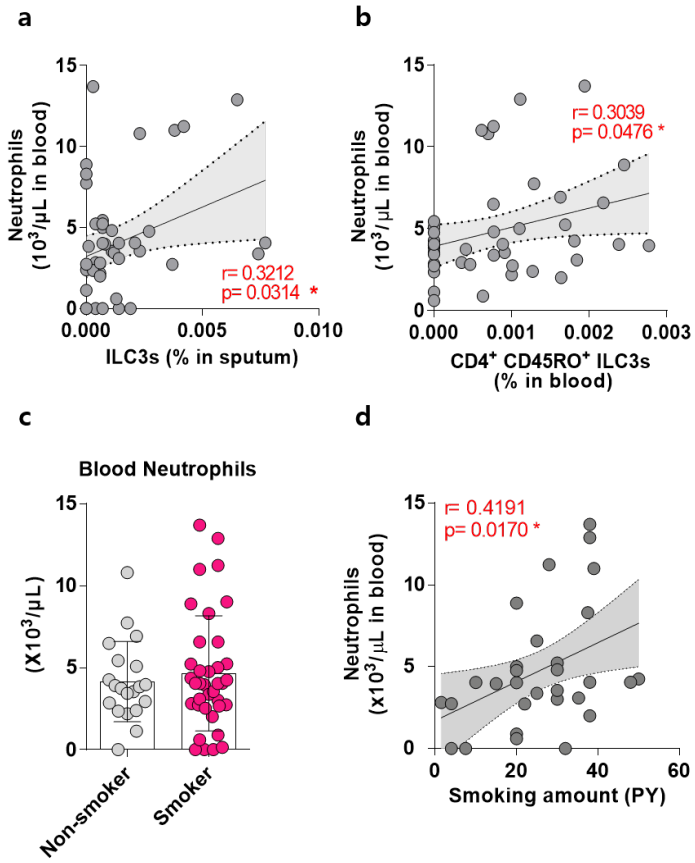

Figure 24. Sputum ILC3 and circulating CD4⁺CD45RO⁺ILC3 frequencies in asthma patients correlate with circulating neutrophil counts.

a-b. Correlations between ILC3 frequencies in induced sputum **(a)** or CD4⁺CD45RO⁺ILC3 frequencies in the blood **(b)** and circulating neutrophil counts. **c.** Comparison of circulating neutrophils in peripheral blood between non-smoking and smoking individuals in asthma patients. **d.** Correlation between smoking amount (Pack-Year; PY) and circulating neutrophil counts in blood. Each dot represents individual subjects. Correlation analyses were conducted with Spearman r correlation test, dotted line represents 95% confidence interval. The blood neutrophil counts from smokers and non-smokers of asthmatics were compared by two-tailed Mann-Whitney U test **(c)**. All data are presented as mean ± standard deviation. $p < 0.05$ is considered as significant. $*P < 0.05$.

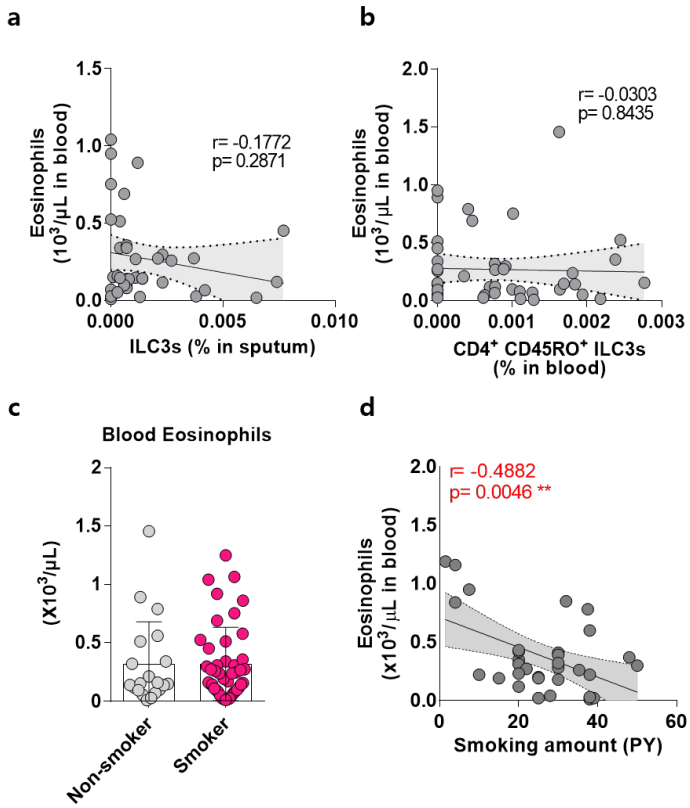

Figure 25. Correlation analysis of sputum ILC3 and circulating CD4⁺CD45RO⁺ILC3 frequencies in asthma patients and circulating eosinophil counts.

a-b. Correlations between ILC3 frequencies in induced sputum **(a)** or CD4⁺CD45RO⁺ILC3 frequencies in the blood **(b)** and circulating eosinophil counts. **c.** Comparison of circulating eosinophils in peripheral blood between non-smoking and smoking individuals in asthma patients. **d.** Correlation between smoking amount (Pack-Year; PY) and circulating eosinophil count in blood. Each dot represents individual subjects. Correlation analyses were conducted with Spearman r correlation test, dotted line represents 95% confidence interval. The blood eosinophil counts from smokers and non-smokers of asthmatics were compared by two-tailed Mann-Whitney U test **(c)**. All data are presented as mean ± standard deviation. $p < 0.05$ is considered as significant. $*P < 0.05$.

I previously reported that ILCs regulate macrophage polarization in patients with asthma: ILC1 and ILC3 associate with classical macrophage (M1) polarization and non-eosinophilic asthma whereas ILC2 associates with alternative macrophage (M2) polarization and eosinophilic asthma[82]. Therefore, I also analyzed the macrophages in the induced sputum of the smoking and non-smoking asthma patients by flow cytometry and then determined their relationship with ILC3 frequencies. Thus, total macrophages were gated as $CD45^{+}CD68^{+}$ cells and then divided into M1 ($CD11c^{+}CD206^{-}$) and M2 ($CD11c^{-}CD206^{+}$) macrophages (**Figure 26a**). Smoking in asthma associated with elevated total and M1 type macrophage frequencies but a similar association with M2 type macrophage frequencies was not observed (**Figure 26b and c**). Moreover, sputum ILC3 frequencies correlated positively with M1 (**Figure 27a**) but not M2 (**Figure 27b**) macrophage frequencies. Blood $CD4^{+}CD45RO^{+}$ILC3 frequencies did not correlate with either M1 or M2 macrophage frequencies (**Figure 27c and d**). Notably, sputum M1 macrophage frequencies did not associate with impaired lung function, unlike neutrophil counts (**Figure 28a-d**). This suggests that neutrophils interact more closely with ILC3 subsets in the pathogenesis of smoking asthma than M1 macrophages.

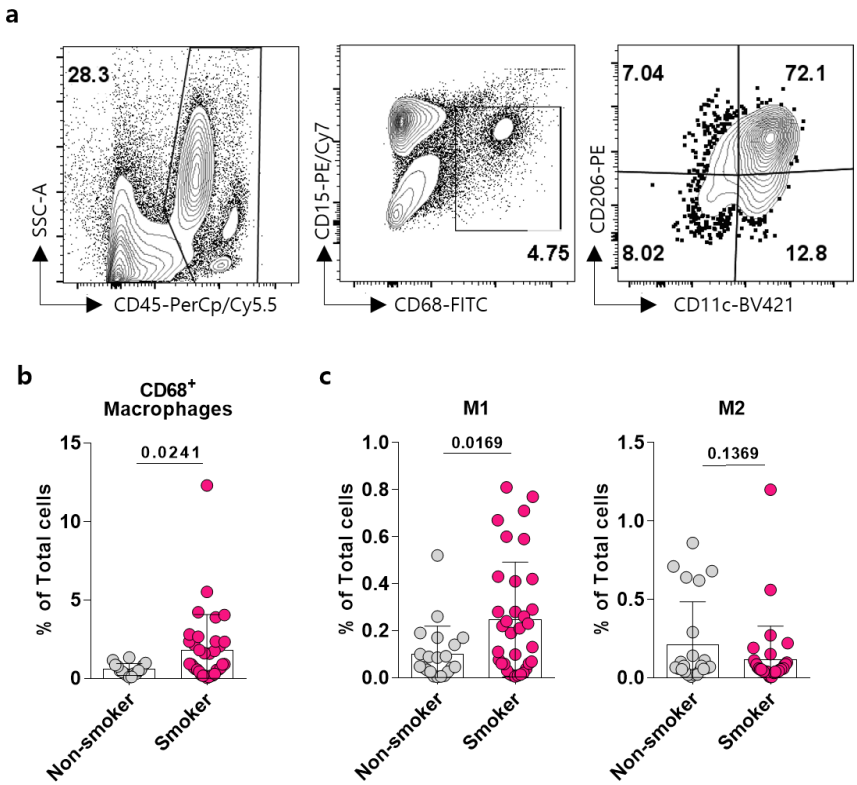

Figure 26. M1 macrophages increase in induced sputum of smokers from asthma patients.

a. Gating strategy of macrophages in induced sputum. $CD45^{+}CD68^{+}$ cells are total macrophage. Among macrophages, $CD11c^{+}CD206^{-}$ cells are M1 macrophages and $CD11c^{-}CD206^{+}$ cells are M2 macrophages. **b.** Comparison of total macrophages in induced sputum between non-smokers and smokers in asthma patients. **c.** Comparison of frequency of M1 and M2 macrophages in induced sputum between non-smokers and smokers in asthmatics. Each dot represents individual subjects. The non-smokers and smokers in asthma patients were compared by two-tailed Mann-Whitney U test **(b and c)**. The data are presented as mean ± standard deviation. $p < 0.05$ is considered as significant.

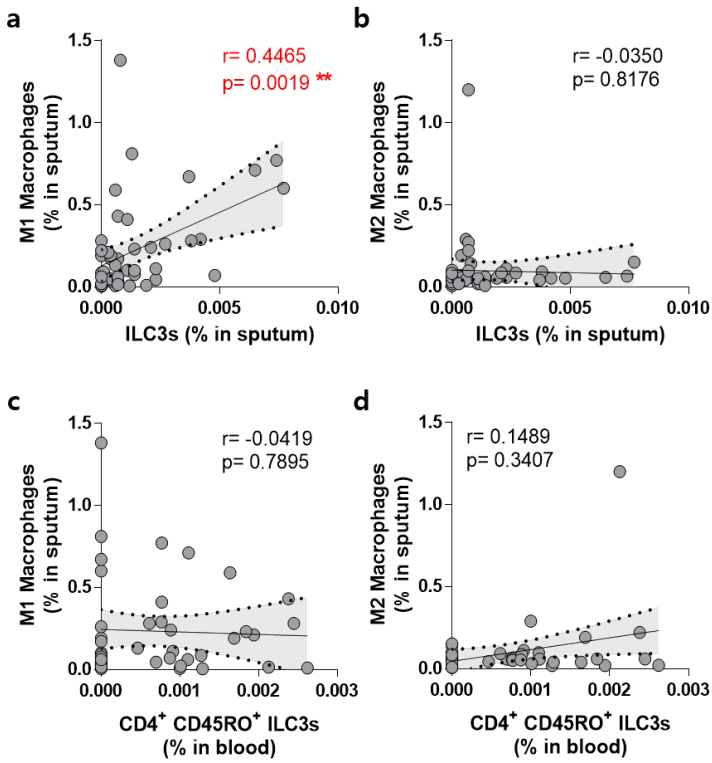

Figure 27. Correlation between macrophage subsets and ILC3s.

a-d. Correlations between ILC3 frequencies in induced sputum **(a and b)** or $CD4^{+}CD45RO^{+}ILC3$ frequencies in the blood **(c and d)** and sputum M1 macrophage frequencies **(a and c)**, or sputum M2 macrophage frequencies **(b and d)**. Each dot represents individual subjects. Correlation analyses were conducted with Spearman r correlation test, dotted line represents 95% confidence interval. $^{**}P < 0.01$.

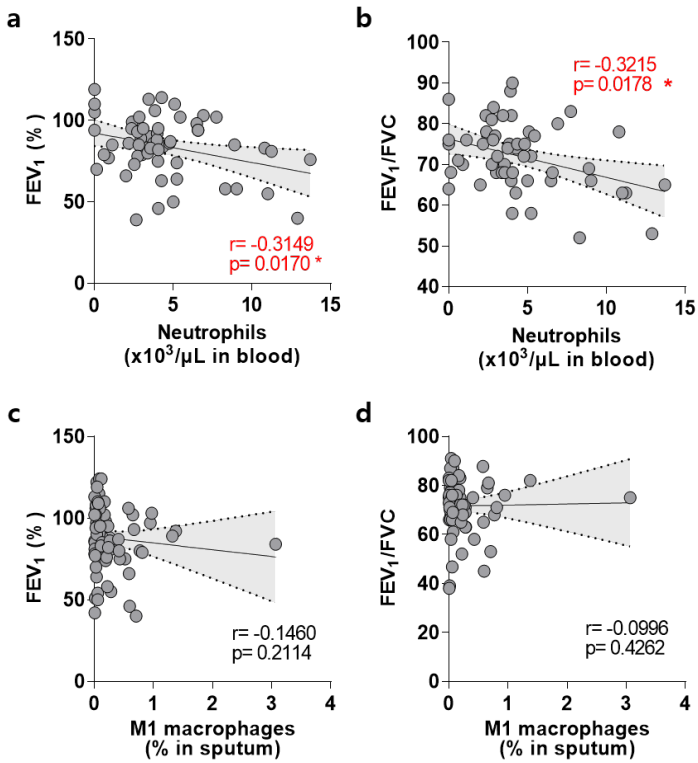

Figure 28. Blood neutrophils and sputum M1 macrophage have less correlation with asthma severity. a-b. Correlation between the number of circulating neutrophils and clinical indices of asthma severity, FEV_1 (%) **(a)** and FEV_1/FVC **(b)**. **c-d.** Correlation between the frequency of M1 macrophages in sputum and FEV_1 (%) **(c)** and FEV_1/FVC **(d)**. Each dot represents individual subjects. Correlation analyses were conducted with Spearman r correlation test, dotted line represents 95% confidence interval. $p < 0.05$ is considered as significant.

Taken together, these data suggest that the cigarette smoking-related increase in ILC3 frequencies that are seen in asthma patients may reflect a non-eosinophilic phenotype of asthma that is characterized by non-T_H2 type inflammation such as M1 macrophages and neutrophils.

DISCUSSION

Here, I focused on changes in ILC3s from asthmatic patients with smoking. Cigarette smoke is a well-known cause of inflammatory lung diseases such as COPD, and lung cancers[83, 84, 85]. Moreover, in asthma, cigarette smoke has been suggested that inducing asthma exacerbation and increasing diseases severity[86, 87]. However, the mechanisms, how cigarette smoke worsens asthma are unclear so that hampers the development of therapeutic targets for smoking-related inflammation in asthma. I demonstrate here for the first time that smokers with asthma have increased numbers of ILC3s in the airway and increased numbers of memory-like ILC3s ($CD4^{+}CD45RO^{+}$ ILC3s) in the peripheral blood compared with non-smoking asthmatics. Besides, these changes correlated with impaired lung functions and smoking amounts. Therefore, the increased ILC3s in the airway and peripheral blood may participate as a critical modulator of asthma aggravation by smoking. These findings are supported by several studies that suggest ILC3s are linked to smoking. COPD patients have increased $NKp44^{-}$ILC3(NCR^{-}ILC3) frequencies in their blood compared to control groups[88, 89]. Besides, IL-17A or IL-22 producing ILC3s were elevated in the lungs of cigarette smoke-exposed mice[90]. Those studies showed that the smoking induces and stimulates airway ILC3 and activated ILC3s are modulate T_H17 dominant immunity.

Accumulating evidence suggests that cigarette smoke causes critical changes in macrophages and neutrophils in asthmatics[91, 92]. For example, cigarette smoke-induced AMs and neutrophils produce pro-inflammatory cytokines, reactive oxygen species, matrix metalloproteinase, and various chemokines[93]. Indeed, my data also presented that smoking in asthma associated with increased M1

macrophages. In addition, there existed a positive correlation between smoking amounts and neutrophil counts. Although M1 macrophages did not associate with decreased lung function, I observed that circulating neutrophils correlated with both smoking amount and decreased pulmonary function. Thus, the elevated ILC3 frequencies in smoking asthmatics correlated positively with not only the smoking amount and decreased lung function but also M1 macrophage and circulating neutrophil frequencies, ILC3s may play a key role in the pathogenesis of smoking-related asthma.

It should be noted that the other type of ILCs, ILC2s, has been thought that participate in the central roles in asthma exacerbation; ILC2s help to modulate and induce type-2 inflammation in the airway[94]. However, some studies have suggested that smoking cigarette directly suppresses the function of ILC2s, thereby affecting the pathogenesis of two respiratory diseases, asthma and COPD. One study showed that murine and human ILC2s express the α7 nicotinic acetylcholine receptor, a receptor for nicotine, which is a component of tobacco[95]. Administration of nicotine agonists also reduces cytokine production from ILC2s via decreasing the expression of Gata3 and blocking the NF-kB pathway[95]. Moreover, another study also reported that chronic exposure to cigarette smoke silences ILC2s by regulating IL-33-ST2 axis[96]. Evidence from these two studies shows that cigarette smoke directly alters ILC2s, which can affect the pathogenesis of respiratory diseases including asthma and COPD. Thus, these results are inconsistent with the general notion that asthma is exacerbated by type-2 cytokines secretion from ILC2s.

Rather, in the current study, I elucidated that cigarette smoke induces changes in ILC3s and suggested that ILC3-mediated aggravation of asthma. In this

study, I found that cigarette smoke affects ILC3s both directly and indirectly. *In vitro* administration of cigarette smoke extract (CSE) on various airway epithelial cell lines increased the release of IL-1β but not IL-23A and IL-33. IL-1β is a key cytokine that promotes differentiation and activation of ILC3s[97]. Moreover, other studies also observed that exposure to smoke induces epithelial cells to produce CCL20, a chemoattractant for CCR6-expressing cells[98]. Since ILC3s reside in the lung and intestine and express CCR6 on their surface[99], smoking can promote a lung microenvironment that favors the recruitment and activation of ILC3s. Therefore, cigarette smoke indirectly affects ILC3s via IL-1β and CCL20 secretion from airway epithelium. On the other hand, cigarette smoke can also act directly on ILC3s. In the present study, *in vitro* treatment of CSE to PBMCs up-regulated the expression of CD45RO on ILC3s. Cigarette smoke is a complex mixture of chemicals including tar, nicotine, and carbon monoxide[100, 101], and these compounds may bind directly to ILC3s through toll-like receptors and aryl hydrocarbon receptors, which are known to be expressed on ILC3s[102]. However, it should be confirmed whether ILC3s express nicotine receptors and examined the mechanisms of cigarette smoke-mediated changes in ILC3s.

I also noted that the numbers of ILC3s expressing CD45RO increased in the peripheral blood of smoking asthmatics. CD45 is abundant glycoproteins on the surface of hematopoietic-lineage cells, such as immune cells; it also has phosphatase activity that represses signaling pathways through JAK and LCK[103, 104]. Since CD45RO has less phosphatase activity than CD45RA[105], the transition of CD45RA to CD45RO suggests that immune cells have become more sensitive to external stimuli[106]. This isoform switch is well examined in T cell activation: naïve T cells express CD45RA while, activated/memory T cells express

CD45RO[74]. Recently, it was reported that ILCs can also express, which suggests they have been primed[72, 73]. Especially, a study by Shikhagie *et al.* showed increased frequencies of NRP1^{+}ILC3s in smokers' and COPD patients' lungs than the control group. Besides, those NRP1^{+}ILC3s expressed greater level of CD45RO^{+} and their cytokine productions were higher than NRP^{-}ILC3s which have lesser CD45RO expressions[72]. I also found that the IL-17A production of peripheral blood CD45RO^{+}ILCs was higher than other ILC subsets including CD45RA-expressing ILCs. Therefore, I speculate that blood CD45RO^{+}ILC3s have memory-like features; greater cytokine production capacities, generated and remained systemically after stimulation. Thus, cigarette smoke may increase the peripheral blood frequencies of CD45RO^{+}ILC3 cells that are highly primed to produce inflammatory cytokines that further induces neutrophilic inflammations.

In this study, I observed the elevation of ILC3 subsets in smoking asthmatics. As ILC3s are the most dominant ILC subsets in healthy human lung, understanding the roles of ILC3s are important[89]. Also, the ILC3-related gene signatures were highly enriched in adult-onset asthmatics. Adult-onset asthma usually shows more severe symptoms and associate with a worse prognosis than childhood-onset asthma[107]. Thus, this data suggested that the ILC3s are involved in asthma severity. Moreover, there are several other reports that the IL-17A level, the key cytokine of ILC3s, is increased in the sputum and lung tissue of severe asthmatics and steroid-resistant asthmatics[108, 109]. According to those findings, the elevated ILC3 subsets by smoking are critical in asthma exacerbation and developing a drug to control pulmonary ILC3s may help to manage the severity of asthma. Although my study mainly focused on the association of immune cells in human asthmatics and lacked the specific

mechanisms of immune cells' interactions, however, I provided quantitative and novel observational data that address clinically important needs. First, my results provide a cornerstone for immunological understanding in patients with asthma who smoke. I elucidated for the first time that smoking specifically increases ILC3s in both the airways and peripheral blood. I also showed that the elevated ILC3s are significantly correlated with decreased lung function in smoking asthmatics. Besides, the increased ILC3 subsets in the blood of smoking asthmatics showed memory-like features; induced via additional stimuli (cigarette smoke along with asthma), showed more activated to produce inflammatory cytokines, and persisted even after the stimuli disappeared.

Collectively, my data proposed that cigarette smoke adversely affects asthma by altering airway immunity especially by generating memory-like ILC3s and their effector functions. Therefore, sputum ILC3s and blood memory-like ILC3s could be used as a novel biomarker for asthma severity in smokers. In addition, the strategy of targeting memory-like ILC3s may provide efficient and personalized treatment regimens to ameliorate and inhibit the progression of asthma.

CONCLUSION

In this study, I discovered the novel phenotype of ILCs, memory-like ILC3s from smoking asthmatics. The memory-like ILC3s were generated only when the cells from asthma patients were treated with cigarette smoking. This result implied that the memory formation of ILCs also required repetitive stimulation; asthma and smoking as primary and secondary stimulation respectively. This memory-like ILC3s expresses CD45RO, like memory T cells, and produces IL-17A significantly more than other ILCs. As elevated neutrophils are the major feature of non-allergic asthma, memory-like ILC3s can be the possible inducer of non-allergic asthma. Moreover, I found a negative correlation between $CD45RO^{+}$ ILC3s and asthma severity that also reinforces the pathogenic role of memory-like ILC3s in asthma. Therefore, targeting memory-like ILC3s would be practical to regulate immune cells that associate asthma severity.

Collectively, I demonstrated the pathogenic roles of memory-like ILCs in asthma **(Figure 29)**. Thus, this study proposed and added the evidence of the new concepts of ILCs' character; the memory phenotype of ILCs, which are associated with aggravating asthma, and potentially, a novel therapeutic target for non-allergic/severe asthma. Although my study only focused on the changes of ILC3 subset from asthma, the notion about ILC memory formation can be applied to other types of chronic inflammatory diseases (*e.g.*, tumors, COPD, and chronic infections) and help to understand their pathophysiology.

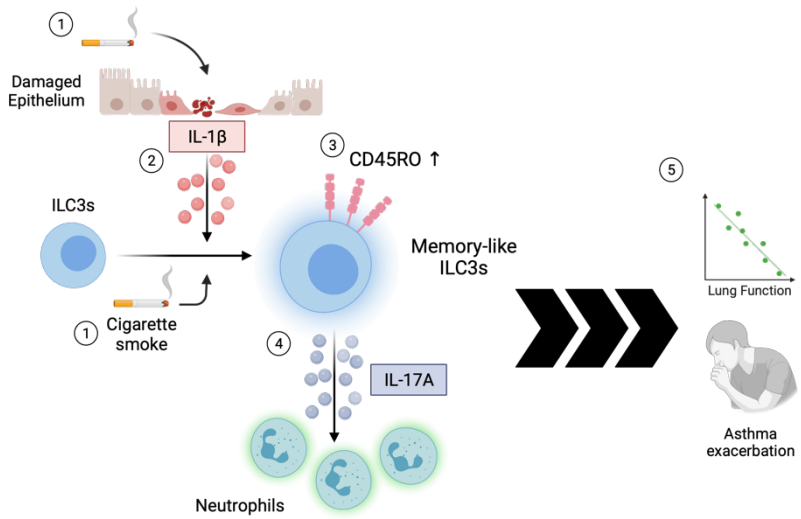

Figure 29. Graphical summary of the generation of memory-like ILC3s in asthma.

Cigarette smoke both directly induces CD45RO expression from ILC3s and indirectly increases IL-1β from epithelial cells that further activates ILC3s. CD45RO expressing memory-like ILC3s remains even after quitting smoke and promotes neutrophilic inflammations, then exacerbates asthma.

REFERENCES

1. Vivier, E. *et al.* Innate Lymphoid Cells: 10 Years On. *Cell* **174**, 1054-1066 (2018).

2. Harly, C., Cam, M., Kaye, J. & Bhandoola, A. Development and differentiation of early innate lymphoid progenitors. *J Exp Med* **215**, 249-262 (2018).

3. Seehus, C.R. *et al.* The development of innate lymphoid cells requires TOX-dependent generation of a common innate lymphoid cell progenitor. *Nat Immunol* **16**, 599-608 (2015).

4. Eberl, G., Colonna, M., Di Santo, J.P. & McKenzie, A.N. Innate lymphoid cells. Innate lymphoid cells: a new paradigm in immunology. *Science (New York, N.Y.)* **348**, aaa6566 (2015).

5. Kansler, E.R. & Li, M.O. Innate lymphocytes-lineage, localization and timing of differentiation. *Cell Mol Immunol* **16**, 627-633 (2019).

6. Robinette, M.L. & Colonna, M. Immune modules shared by innate lymphoid cells and T cells. *J Allergy Clin Immunol* **138**, 1243-1251 (2016).

7. Fuchs, A. ILC1s in Tissue Inflammation and Infection. *Front Immunol* **7**, 104 (2016).

8. Vivier, E., Tomasello, E., Baratin, M., Walzer, T. & Ugolini, S. Functions of natural killer cells. *Nat Immunol* **9**, 503-510 (2008).

9. Eberl, G., Di Santo, J.P. & Vivier, E. The brave new world of innate lymphoid cells. *Nat Immunol* **16**, 1-5 (2015).

10. Yang, D., Guo, X., Huang, T. & Liu, C. The Role of Group 3 Innate Lymphoid Cells in Lung Infection and Immunity. *Front Cell Infect Microbiol* **11**, 586471 (2021).

11. Croxatto, D. *et al.* Group 3 innate lymphoid cells regulate neutrophil migration and function in human decidua. *Mucosal immunology* **9**, 1372-1383 (2016).

12. Zeng, B. *et al.* ILC3 function as a double-edged sword in inflammatory bowel diseases. *Cell Death Dis* **10**, 315 (2019).

13. Sugimoto, K. *et al.* IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. *J Clin Invest* **118**, 534-544 (2008).

14. Aparicio-Domingo, P. *et al.* Type 3 innate lymphoid cells maintain intestinal epithelial stem cells after tissue damage. *J Exp Med* **212**, 1783-1791 (2015).

15. Keir, M., Yi, Y., Lu, T. & Ghilardi, N. The role of IL-22 in intestinal health and disease. *J Exp Med* **217**, e20192195 (2020).

16. Strober, W. The LTi cell, an immunologic chameleon. *Immunity* **33**, 650-652 (2010).

17. Hope, J.L., Stairiker, C.J., Bae, E.A., Otero, D.C. & Bradley, L.M. Striking a Balance-Cellular and Molecular Drivers of Memory T Cell Development and

Responses to Chronic Stimulation. *Front Immunol* **10**, 1595 (2019).

18. Raphael, I., Joern, R.R. & Forsthuber, T.G. Memory CD4(+) T Cells in Immunity and Autoimmune Diseases. *Cells* **9** (2020).

19. Omilusik, K.D. & Goldrath, A.W. The origins of memory T cells. *Nature* **552**, 337-339 (2017).

20. Martin, M.D. & Badovinac, V.P. Defining Memory CD8 T Cell. *Front Immunol* **9**, 2692 (2018).

21. Wherry, E.J. & Ahmed, R. Memory CD8 T-cell differentiation during viral infection. *J Virol* **78**, 5535-5545 (2004).

22. Okla, K., Farber, D.L. & Zou, W. Tissue-resident memory T cells in tumor immunity and immunotherapy. *J Exp Med* **218** (2021).

23. Mojtabavi, N., Dekan, G., Stingl, G. & Epstein, M.M. Long-lived Th2 memory in experimental allergic asthma. *J Immunol* **169**, 4788-4796 (2002).

24. O'Leary, J.G., Goodarzi, M., Drayton, D.L. & von Andrian, U.H. T cell- and B cell-independent adaptive immunity mediated by natural killer cells. *Nat Immunol* **7**, 507-516 (2006).

25. Netea, M.G. *et al.* Defining trained immunity and its role in health and disease. *Nat Rev Immunol* **20**, 375-388 (2020).

26. Schulz-Kuhnt, A., Wirtz, S., Neurath, M.F. & Atreya, I. Regulation of Human Innate Lymphoid Cells in the Context of Mucosal Inflammation. *Front Immunol* **11**, 1062 (2020).

27. Saez, A. *et al.* Innate Lymphoid Cells in Intestinal Homeostasis and Inflammatory Bowel Disease. *Int J Mol Sci* **22** (2021).

28. Hsu, A.T., Gottschalk, T.A., Tsantikos, E. & Hibbs, M.L. The Role of Innate Lymphoid Cells in Chronic Respiratory Diseases. *Front Immunol* **12**, 733324 (2021).

29. Sasaki, T. *et al.* Innate Lymphoid Cells in the Induction of Obesity. *Cell Rep* **28**, 202-217 e207 (2019).

30. Sonnenberg, G.F. & Artis, D. Innate lymphoid cell interactions with microbiota: implications for intestinal health and disease. *Immunity* **37**, 601-610 (2012).

31. Sun, J.C., Beilke, J.N. & Lanier, L.L. Adaptive immune features of natural killer cells. *Nature* **457**, 557-561 (2009).

32. Wang, X., Peng, H. & Tian, Z. Innate lymphoid cell memory. *Cell Mol Immunol* **16**, 423-429 (2019).

33. Verma, M. *et al.* The molecular and epigenetic mechanisms of innate lymphoid cell (ILC) memory and its relevance for asthma. *J Exp Med* **218** (2021).

34. Trabanelli, S. *et al.* c-Maf enforces cytokine production and promotes memory-

like responses in mouse and human type 2 innate lymphoid cells. *EMBO J* **41**, e109300 (2022).

35. Wills, M.R. *et al.* Identification of naive or antigen-experienced human CD8(+) T cells by expression of costimulation and chemokine receptors: analysis of the human cytomegalovirus-specific CD8(+) T cell response. *J Immunol* **168**, 5455-5464 (2002).

36. Caccamo, N., Joosten, S.A., Ottenhoff, T.H.M. & Dieli, F. Atypical Human Effector/Memory CD4(+) T Cells With a Naive-Like Phenotype. *Front Immunol* **9**, 2832 (2018).

37. van der Ploeg, E.K. *et al.* Steroid-resistant human inflammatory ILC2s are marked by CD45RO and elevated in type 2 respiratory diseases. *Sci Immunol* **6** (2021).

38. Martinez-Gonzalez, I. *et al.* Allergen-Experienced Group 2 Innate Lymphoid Cells Acquire Memory-like Properties and Enhance Allergic Lung Inflammation. *Immunity* **45**, 198-208 (2016).

39. Lambrecht, B.N. & Hammad, H. The immunology of asthma. *Nat Immunol* **16**, 45-56 (2015).

40. Whetstone, C.E., Ranjbar, M., Omer, H., Cusack, R.P. & Gauvreau, G.M. The Role of Airway Epithelial Cell Alarmins in Asthma. *Cells* **11** (2022).

41. Kuruvilla, M.E., Lee, F.E. & Lee, G.B. Understanding Asthma Phenotypes, Endotypes, and Mechanisms of Disease. *Clin Rev Allergy Immunol* **56**, 219-233

(2019).

42. Peters, S.P. Asthma phenotypes: nonallergic (intrinsic) asthma. *J Allergy Clin Immunol Pract* **2**, 650-652 (2014).

43. Kim, H.Y., DeKruyff, R.H. & Umetsu, D.T. The many paths to asthma: phenotype shaped by innate and adaptive immunity. *Nat Immunol* **11**, 577-584 (2010).

44. Barnes, P.J. Inhaled Corticosteroids. *Pharmaceuticals (Basel)* **3**, 514-540 (2010).

45. Green, R.H. *et al.* Analysis of induced sputum in adults with asthma: identification of subgroup with isolated sputum neutrophilia and poor response to inhaled corticosteroids. *Thorax* **57**, 875-879 (2002).

46. Siew, L.Q.C., Wu, S.Y., Ying, S. & Corrigan, C.J. Cigarette smoking increases bronchial mucosal IL-17A expression in asthmatics, which acts in concert with environmental aeroallergens to engender neutrophilic inflammation. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* **47**, 740-750 (2017).

47. Ham, J., Kim, J., Ko, Y.G. & Kim, H.Y. The Dynamic Contribution of Neutrophils in the Chronic Respiratory Diseases. *Allergy Asthma Immunol Res* **14**, 361-378 (2022).

48. Crisford, H. *et al.* Neutrophils in asthma: the good, the bad and the bacteria. *Thorax* (2021).

49. Chen, X. *et al.* IL-6 induced M1 type macrophage polarization increases radiosensitivity in HPV positive head and neck cancer. *Cancer Lett* **456**, 69-79 (2019).

50. Murphy, E.A., Danna-Lopes, D., Sarfati, I., Rao, S.K. & Cohen, J.R. Nicotine-stimulated elastase activity release by neutrophils in patients with abdominal aortic aneurysms. *Ann Vasc Surg* **12**, 41-45 (1998).

51. Vlahos, R. *et al.* Differential protease, innate immunity, and NF-kappaB induction profiles during lung inflammation induced by subchronic cigarette smoke exposure in mice. *American journal of physiology. Lung cellular and molecular physiology* **290**, L931-945 (2006).

52. Barnes, P.J. The cytokine network in chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol* **41**, 631-638 (2009).

53. Liu, T. *et al.* Type 2 innate lymphoid cells: A novel biomarker of eosinophilic airway inflammation in patients with mild to moderate asthma. *Respiratory medicine* **109**, 1391-1396 (2015).

54. Christianson, C.A. *et al.* Persistence of asthma requires multiple feedback circuits involving type 2 innate lymphoid cells and IL-33. *J Allergy Clin Immunol* **136**, 59-68 e14 (2015).

55. Morita, H. *et al.* An Interleukin-33-Mast Cell-Interleukin-2 Axis Suppresses Papain-Induced Allergic Inflammation by Promoting Regulatory T Cell Numbers. *Immunity* **43**, 175-186 (2015).

56. Halim, T.Y., Krauss, R.H., Sun, A.C. & Takei, F. Lung natural helper cells are a critical source of Th2 cell-type cytokines in protease allergen-induced airway inflammation. *Immunity* **36**, 451-463 (2012).

57. Miller, J.E. *et al.* IL-33 activates group 2 innate lymphoid cell expansion and modulates endometriosis. *JCI insight* **6** (2021).

58. Takai, T. & Ikeda, S. Barrier dysfunction caused by environmental proteases in the pathogenesis of allergic diseases. *Allergol Int* **60**, 25-35 (2011).

59. Abdelaziz, M.H. *et al.* Alternatively activated macrophages; a double-edged sword in allergic asthma. *J Transl Med* **18**, 58 (2020).

60. Mirchandani, A.S. *et al.* Type 2 innate lymphoid cells drive CD4+ Th2 cell responses. *J Immunol* **192**, 2442-2448 (2014).

61. Bing, C. Is interleukin-1beta a culprit in macrophage-adipocyte crosstalk in obesity? *Adipocyte* **4**, 149-152 (2015).

62. Kim, H.Y. *et al.* Interleukin-17-producing innate lymphoid cells and the NLRP3 inflammasome facilitate obesity-associated airway hyperreactivity. *Nature medicine* **20**, 54-61 (2014).

63. Telenga, E.D. *et al.* Obesity in asthma: more neutrophilic inflammation as a possible explanation for a reduced treatment response. *Allergy* **67**, 1060-1068 (2012).

64. Nightingale, J.A. *et al.* Airway inflammation after controlled exposure to diesel exhaust particulates. *American journal of respiratory and critical care medicine* **162**, 161-166 (2000).

65. Paulin, L. & Hansel, N. Particulate air pollution and impaired lung function. *F1000Res* **5** (2016).

66. Kim, J. *et al.* The effect of air pollutants on airway innate immune cells in patients with asthma. *Allergy* **75**, 2372-2376 (2020).

67. Che, L. *et al.* Ozone-induced IL-17A and neutrophilic airway inflammation is orchestrated by the caspase-1-IL-1 cascade. *Sci Rep* **6**, 18680 (2016).

68. Enweasor, C., Flayer, C.H. & Haczku, A. Ozone-Induced Oxidative Stress, Neutrophilic Airway Inflammation, and Glucocorticoid Resistance in Asthma. *Front Immunol* **12**, 631092 (2021).

69. Srinivasan, B. *et al.* TEER measurement techniques for in vitro barrier model systems. *J Lab Autom* **20**, 107-126 (2015).

70. Pantazi, E. & Powell, N. Group 3 ILCs: Peacekeepers or Troublemakers? What's Your Gut Telling You?! *Front Immunol* **10**, 676 (2019).

71. Simoni, Y. *et al.* Human Innate Lymphoid Cell Subsets Possess Tissue-Type Based Heterogeneity in Phenotype and Frequency. *Immunity* **46**, 148-161 (2017).

72. Shikhagaie, M.M. *et al.* Neuropilin-1 Is Expressed on Lymphoid Tissue Residing LTi-like Group 3 Innate Lymphoid Cells and Associated with Ectopic Lymphoid Aggregates. *Cell Rep* **18**, 1761-1773 (2017).

73. van der Ploeg, E.K. *et al.* Steroid-resistant human inflammatory ILC2s are marked by CD45RO and elevated in type 2 respiratory diseases. *Science Immunology* **6**, eabd3489 (2021).

74. Michie, C.A., McLean, A., Alcock, C. & Beverley, P.C. Lifespan of human lymphocyte subsets defined by CD45 isoforms. *Nature* **360**, 264-265 (1992).

75. van de Pavert, S.A. & Vivier, E. Differentiation and function of group 3 innate lymphoid cells, from embryo to adult. *Int Immunol* **28**, 35-42 (2016).

76. Heijink, I.H., Brandenburg, S.M., Postma, D.S. & van Oosterhout, A.J. Cigarette smoke impairs airway epithelial barrier function and cell-cell contact recovery. *Eur Respir J* **39**, 419-428 (2012).

77. van der Vaart, H., Postma, D.S., Timens, W. & ten Hacken, N.H. Acute effects of cigarette smoke on inflammation and oxidative stress: a review. *Thorax* **59**, 713-721 (2004).

78. Bhat, A.A. *et al.* Tight Junction Proteins and Signaling Pathways in Cancer and Inflammation: A Functional Crosstalk. *Front Physiol* **9**, 1942 (2018).

79. Klose, C.S. & Artis, D. Innate lymphoid cells as regulators of immunity, inflammation and tissue homeostasis. *Nat Immunol* **17**, 765-774 (2016).

80. Korn, S. *et al.* Prospective evaluation of current asthma control using ACQ and ACT compared with GINA criteria. *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology* **107**, 474-479 (2011).

81. Haldar, P. & Pavord, I.D. Noneosinophilic asthma: a distinct clinical and pathologic phenotype. *J Allergy Clin Immunol* **119**, 1043-1052; quiz 1053-1044 (2007).

82. Kim, J. *et al.* Innate immune crosstalk in asthmatic airways: Innate lymphoid cells coordinate polarization of lung macrophages. *J Allergy Clin Immunol* **143**, 1769-1782 e1711 (2019).

83. Tiotiu, A., Ioan, I., Wirth, N., Romero-Fernandez, R. & Gonzalez-Barcala, F.J. The Impact of Tobacco Smoking on Adult Asthma Outcomes. *Int J Environ Res Public Health* **18** (2021).

84. Walser, T. *et al.* Smoking and lung cancer: the role of inflammation. *Proc Am Thorac Soc* **5**, 811-815 (2008).

85. Laniado-Laborin, R. Smoking and chronic obstructive pulmonary disease (COPD). Parallel epidemics of the 21 century. *Int J Environ Res Public Health* **6**, 209-224 (2009).

86. Patel, S.N. *et al.* Multicenter study of cigarette smoking among patients presenting to the emergency department with acute asthma. *Annals of allergy, asthma &*

immunology : official publication of the American College of Allergy, Asthma, & Immunology **103**, 121-127 (2009).

87. Ulrik, C.S. & Frederiksen, J. Mortality and markers of risk of asthma death among 1,075 outpatients with asthma. *Chest* **108**, 10-15 (1995).

88. Bal, S.M. *et al.* IL-1β, IL-4 and IL-12 control the fate of group 2 innate lymphoid cells in human airway inflammation in the lungs. *Nature Immunology* **17**, 636 (2016).

89. De Grove, K.C. *et al.* Characterization and Quantification of Innate Lymphoid Cell Subsets in Human Lung. *PLoS One* **11**, e0145961 (2016).

90. Starkey, M.R. *et al.* IL-22 and its receptors are increased in human and experimental COPD and contribute to pathogenesis. *The European respiratory journal* **54** (2019).

91. Boulet, L.P. *et al.* Smoking and asthma: clinical and radiologic features, lung function, and airway inflammation. *Chest* **129**, 661-668 (2006).

92. Chalmers, G.W. *et al.* Smoking and airway inflammation in patients with mild asthma. *Chest* **120**, 1917-1922 (2001).

93. Polosa, R. & Thomson, N.C. Smoking and asthma: dangerous liaisons. *Eur Respir J* **41**, 716-726 (2013).

94. Karta, M.R., Broide, D.H. & Doherty, T.A. Insights into Group 2 Innate Lymphoid

Cells in Human Airway Disease. *Current allergy and asthma reports* **16**, 8-8 (2016).

95. Galle-Treger, L. *et al.* Nicotinic acetylcholine receptor agonist attenuates ILC2-dependent airway hyperreactivity. *Nat Commun* **7**, 13202 (2016).

96. Kearley, J. *et al.* Cigarette smoke silences innate lymphoid cell function and facilitates an exacerbated type I interleukin-33-dependent response to infection. *Immunity* **42**, 566-579 (2015).

97. Klose, C.S.N. & Artis, D. Innate lymphoid cells as regulators of immunity, inflammation and tissue homeostasis. *Nature Immunology* **17**, 765 (2016).

98. Wang, G.-Z. *et al.* Tobacco smoke induces production of chemokine CCL20 to promote lung cancer. *Cancer Letters* **363**, 60-70 (2015).

99. Kim, C.H., Hashimoto-Hill, S. & Kim, M. Migration and Tissue Tropism of Innate Lymphoid Cells. *Trends in immunology* **37**, 68-79 (2016).

100. Kasai, A. *et al.* High levels of dioxin-like potential in cigarette smoke evidenced by in vitro and in vivo biosensing. *Cancer research* **66**, 7143-7150 (2006).

101. Stedman, R.L. The chemical composition of tobacco and tobacco smoke. *Chemical reviews* **68**, 153-207 (1968).

102. Qiu, J. *et al.* The aryl hydrocarbon receptor regulates gut immunity through modulation of innate lymphoid cells. *Immunity* **36**, 92-104 (2012).

103. Irie-Sasaki, J. *et al.* CD45 is a JAK phosphatase and negatively regulates cytokine receptor signalling. *Nature* **409**, 349-354 (2001).

104. McNeill, L. *et al.* The Differential Regulation of Lck Kinase Phosphorylation Sites by CD45 Is Critical for T Cell Receptor Signaling Responses. *Immunity* **27**, 425-437 (2007).

105. Hermiston, M.L., Xu, Z. & Weiss, A. CD45: A Critical Regulator of Signaling Thresholds in Immune Cells. *Annual Review of Immunology* **21**, 107-137 (2003).

106. Dornan, S. *et al.* Differential Association of CD45 Isoforms with CD4 and CD8 Regulates the Actions of Specific Pools of p56lck Tyrosine Kinase in T Cell Antigen Receptor Signal Transduction*. *Journal of Biological Chemistry* **277**, 1912-1918 (2002).

107. Hekking, P.P. *et al.* Pathway discovery using transcriptomic profiles in adult-onset severe asthma. *J Allergy Clin Immunol* **141**, 1280-1290 (2018).

108. Al-Ramli, W. *et al.* T(H)17-associated cytokines (IL-17A and IL-17F) in severe asthma. *J Allergy Clin Immunol* **123**, 1185-1187 (2009).

109. Barczyk, A., Pierzchala, W. & Sozanska, E. Interleukin-17 in sputum correlates with airway hyperresponsiveness to methacholine. *Respiratory medicine* **97**, 726-733 (2003).

국문초록

천식 상황에서 기억유사 선천성림프구세포의 특성에 대한 연구

서울대학교 대학원

의과학과 의과학전공

함종호

선천성 림프구세포는 최근 발견된 선천 면역세포로 점막 조직에 존재하여 다양한 사이토카인을 분비하는 세포임. 선천성 림프구세포는 선천 면역세포이기 때문에 급성 면역 반응 혹은 면역학적 항상성을 유지하는 기능만을 한다고 생각되었음. 또한 선천성 림프구세포는 적응면역세포들과 달리 활성화 상태의 세포로 존재한다고 여겨짐. 하지만 최근 연구들에서 선천성 림프구세포 또

한 다양한 만성 염증성 질환의 면역반응에 참여하며, 반복적이고 지속적인 자극에 의하여 일부 선천성 림프구세포는 기억 유사 세포의 특성일 보인다는 것이 밝혀짐. 이러한 기억 유사 선천성 림프구세포는 사이토카인을 활발히 분비하며 질병의 악화에 참여하고 있음. 따라서 만성 염증 상황에서 나타나는 기억 유사 선천성 림프구세포의 특성과 기능을 이해할 수 있다면, 선천성 림프구세포를 통한 새로운 치료제의 개발에 도움이 될 수 있음. 따라서 본 연구자는 천식이라는 만성 염증성 질환 상황에서 흡연에 의하여 변화되는 선천성 림프구세포의 기능과 상태에 초점을 맞추어 연구를 진행하였음.

본 연구에서 정상군과 천식환자의 혈액과 유도객담을 분석하여 선천성 림프구세포의 변화를 확인하고자 하였음. 말초 혈액과 유도객담의 선천성 림프구세포를 분석하였을 때, 흡연을 하는 천식환자군의 객담에서 제 3형 선천성 림프구세포가 증가 됨을 확인할 수 있었으며, 혈액에 존재하는 제 3형 선천성 림프구세포 중 CD4와 CD45RO를 발현하는 아형이 증가 되어 있음을 발견함. 또한 in vitro 실험을 통하여 흡연이 직접적으로 제 3형 선천성 림프구세포에서 CD45RO의 발현을 증가시킴을 확인할 수 있었음. CD45RO를 발현하는 제 3형 선천성 림프구세포는 IL-17A와 같은 사이토카인을 다른 선천성 림프구세포에 비하여 더 활발히 분비하고 있었으며, 과거에는 흡연을 하였지만 현재는 흡연을 중단한 환자의 혈액 에서도 높게 유지 되고 있었음.

따라서 CD45RO를 발현하는 제 3형 선천성 림프구세포는 기억유사 선천성 림프구세포라고 생각할 수 있었음. 한편 이러한 선천성 림프구세포는 FEV1과 FVC로 측정되는 폐기능과 음의 상관관계를 보였으며, 염증성 대식세포 (M1 대식세포) 및 호중구와 양의 상관관계를 보였음. 따라서 흡연에 의하여 발생하는 비알러지성 염증상황 (호중구와 M1 대식세포의 증가)은 기억 유사 제 3형 선천성 림프구세포의 형성에 의하여 야기된다고 정리할 수 있었음. 따라서 흡연에 의하여 형성되는 기억 유사 제 3형 선천성 림프구세포는 천식의 중증도를 악화시킴.

본 연구자는 천식 상황에 존재하는 제 3형 선천성 림프구세포를 중심으로 연구를 진행하였으며, 제 3형 선천성 림프구세포는 흡연에 의하여 직접적으로 기억 유사 세포로 분화됨을 확인하였음. 기억 유사 제 3형 선천성 림프구세포는 비알러지성 염증과 천식의 악화를 촉진하는 작용을 하였음. 따라서 본 연구를 통하여 선천성 림프구세포의 다양한 역할과 상태에 대한 폭넓은 이해가 가능하게 되었으며, 만성 질환 상황에서 변화되는 선천성 림프구세포의 상태 조절을 통한 새로운 치료 전략을 제공하였음.

주요어: 만성염증, 선천성 림프구세포, 선천성 기억, 천식, 흡연, 호중구성 염증

학번: 2016-29578