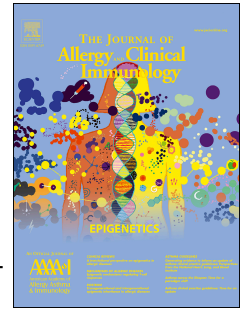


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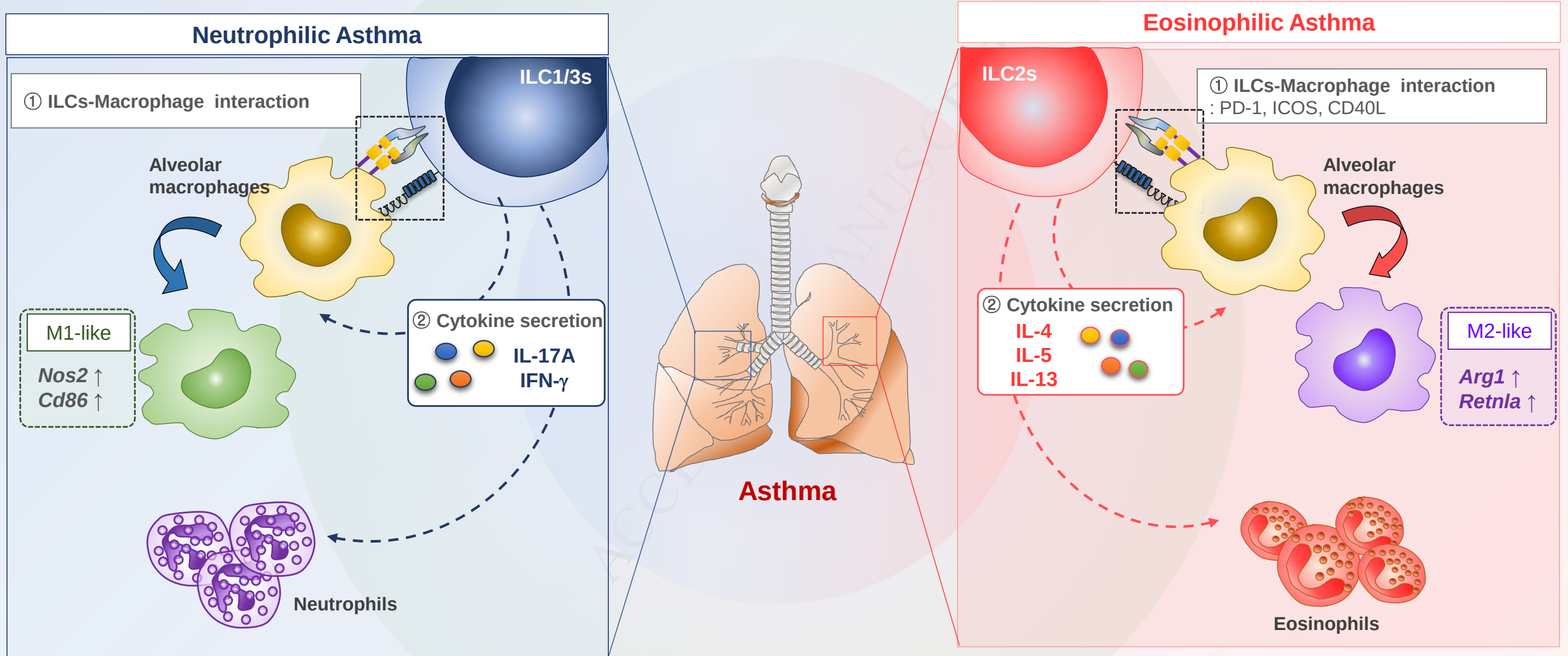
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Abbreviations

ILC: innate lymphoid cell, **Nos2:** Nitric oxide synthase 2, **Arg1:** Arginase 1, **Retnla:** Resistin like alpha, **PD-1:** Programed cell death-1, **ICOS** ; Inducible T-cell costimulator

Innate immune crosstalk in asthmatic airways: innate lymphoid cells coordinate the polarization of lung macrophage

Jihyun Kim¹, B.S, Yuna Chang¹, B.S, Boram Bae², B.S, Kyoung-Hee Sohn^{2,3}, M.D., Sang-Heon Cho^{2,3}, M.D., Ph.D., Doo Hyun Chung^{4,5}, M.D., Ph.D., Hye Ryun Kang^{2,3}, M.D, Ph.D, Hye Young Kim, Ph.D^{1,2}

¹Laboratory of mucosal immunology in Department of Biomedical Sciences, Seoul National University College of Medicine, Seoul, Korea

²Institute of Allergy and Clinical Immunology, Seoul National University Medical Research Center, Seoul, Korea

³Department of Internal Medicine, Seoul National University College of Medicine, Seoul, Korea,

⁴Department of Pathology Seoul National University College of Medicine, Seoul, Korea

⁵Laboratory of Immune Regulation in Department of Biomedical Sciences, Seoul National University College of Medicine, Seoul, Korea

Corresponding Author:

Hye-Ryun Kang

Department of Internal Medicine, Seoul National University College of Medicine, 101

Daehak-ro, Jongno-gu, Seoul 03080, Korea

Tel: +82-2-2072-0820

E-mail: helenmed@snu.ac.kr

Hye Young Kim, Ph.D.

Laboratory of mucosal immunology, Department of Biomedical Sciences, Seoul National University College of Medicine

Address: 103 Daehak-ro, Jongno-gu, Seoul 03080, Korea

Tel: 82-2-740-8970

Fax: 82-2-743-5530

E-mail: hykim11@snu.ac.kr.

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Abstract

Background: Recent studies have emphasized the role of innate lymphoid cells (ILCs) in the development of asthma. The involvement of group 2 ILCs (ILC2s) in asthma is well studied, however, the participation of other types of ILCs in the development of asthma remains unclear.

Objective: This study aims to understand the role of various ILCs in the patients with asthma, especially their effect on the polarization of macrophages.

Methods: Each subset of ILCs and macrophages in induced sputum from 51 steroid-naïve asthma patients and 18 healthy donors analyzed by flow cytometry. To determine whether the polarization of macrophages could be regulated by ILCs, alveolar macrophages (AM) were sorted and co-cultured with each subset of ILCs.

Results: In addition to ILC2s, ILC1s and ILC3s were increased in the induced sputum from asthmatics when compared with the healthy controls. The dominance of macrophages in induced sputum was more prominent in asthmatics than healthy controls. A positive correlation between ILC2s and M2 macrophages and that of ILC1s/ILC3s and M1 macrophages observed. Co-culture of ILC2s with AMs induced the expression of M2 macrophage-related genes, whereas co-culture of ILC1s and ILC3s with AMs induced the expression of M1 macrophage-related genes via cytokine secretion as well as cell-cell contact. According to the inflammatory signature, patients with eosinophilic asthma have more ILC2s

and M2 macrophages while those with non-eosinophilic asthma present are M1 macrophages dominant profile.

Conclusion: A different subset of ILCs regulates the polarization of macrophages, contributing to developing the distinct phenotype of asthma.

Key Messages

- Asthma patients have more significant number of ILCs in induced sputum than healthy donors.
- ILC2s-M2 macrophage numbers are increased in eosinophilic asthma, whereas ILC1s/ILC3s-M1 macrophages are increased in non-eosinophilic asthma.
- ILC2s induce the polarization of M2 macrophages, and ILC1s/ILC3s induce the polarization of M1 macrophage via cytokine secretion as well as cell–cell contact.

Capsule summary

Increased numbers of all types of ILCs, including ILC2s, were observed in induced sputum of asthma patients when compared with healthy controls. ILCs affect the polarization of macrophages and contribute to the development of different phenotypes of asthma.

Keywords: Asthma, Innate lymphoid cells (ILCs), macrophage, polarization, induced sputum, asthma phenotype

Abbreviations

AHR: airway hyperresponsiveness

AMs: alveolar macrophages

AR: allergic rhinitis

Arg1: arginase 1

BALF: bronchoalveolar lavage fluid

BCR: B cell receptor

BMI: Body mass index

COPD: chronic obstructive pulmonary disease

CRS: chronic rhinosinusitis

CRTH2: chemoattractant receptor-homologous molecule expressed on Th2 cell

FEV1: forced expiratory volume in 1 second

FVC: forced vital capacity

GM-CSF: granulocyte macrophage colony stimulating factor

ICOS: Inducible T-cell costimulator

ILCs: innate lymphoid cells IL-7R: interleukin 7 receptor

Nos2: Nitric oxide synthase 2

LPS: lipopolysaccharide

LTi: Lymphoid tissue inducer

MFI: mean fluorescence intensity

MHC class II: class II major histocompatibility complex

NCR: natural cytotoxicity receptor

Mrc1: mannose receptor C type 1

PBMC: peripheral blood mononuclear cell

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

PD-1: Programmed cell death protein 1

Ptgs2: Prostaglandin-endoperoxide synthase 2

Retnla: Resistin-like molecule alpha

STRA: severe therapy-resistant asthma

TCR: T cell receptor

TNF α : tumor necrosis factor alpha

TSLP: thymic stromal lymphopoietin

WBC: white blood cell

Introduction

Asthma is an immunological disease of the airways characterized by airway hyperresponsiveness and chronic airway inflammation related with intricate network of diverse immune cells (1). Allergen-specific Th2 cells have long been thought to play critical roles in inducing type 2 cytokine production in the lungs, resulting in the development of allergic asthma, the most common form of the disease (1). More recently, it has become clear that asthma is mediated by both innate and adaptive immune cells that regulate and shape pulmonary inflammation (2).

As increased awareness of asthma heterogeneity, researchers subdivide patients with asthma into 4-5 phenotypic clusters according to their age, gender, atopy, lung function, health care utilization and body mass index (BMI) (3). Among the cluster, patients with notable eosinophilia and notable increase of interleukins such as IL-4, IL-5, and IL-13 in the blood called eosinophilic asthma (4, 5). Another group shows a minimal T_H2 response but notable sputum neutrophilia with increase of IL-17A (4). Therefore, understanding the mechanisms underlying each asthma phenotype will foster the development of improved and more appropriate therapeutic strategies for asthma.

Recently identified innate lymphoid cells (ILCs) have been found to link innate components of immunity with the adaptive immunity (6). ILCs play critical roles in host defense and tissue homeostasis, particularly in mucosal tissues. Although ILCs lack antigen-specific T-cell and B-cell receptors (TCRs and BCRs, respectively), they rapidly produce a variety of cytokines in response to a wide range of innate signals, that is, interleukin (IL)-25, IL-33, TSLP and IL-1 β (7). Based on their characteristics and cytokine profiles, ILCs have been divided into three groups – type 1, type 2 and type 3 ILCs (ILC1s, ILC2s and ILC3s) –

which may be thought of as the innate counterparts of CD4⁺ T helper 1 (TH1), TH2, and TH17 cells (8). ILC1s produce IFN- γ and tumor necrosis factor (TNF)- α (9). ILC2s produce a set of cytokines similar to that produced by TH2 cells such as IL-5, IL-9, and IL-13 (10-12). ILC3s produce IL-17A, IL-22, granulocyte macrophage colony stimulating factor (GM-CSF) and TNF- α . ILC3s display further heterogeneity and can be subdivided into several subgroups, such as CCR6⁺ LTis and CCR6⁻ ILC3s (13). CCR6⁻ ILC3 cells are further subdivided based on the expression of natural cytotoxicity receptor (NCR) (14). RAR-related orphan receptor gamma T (ROR γ t) is a key transcription factor for ILC3 development, however, T-bet is also required for regulation of NKp46 expression on CCR6⁻ ILC3s (9, 14, 15). The interactions between ILCs and other immune cells are still under investigation; however, recent studies have shown that ILCs can demonstrate new mechanisms underlying immune responses, especially in mucosal tissues such as the intestines and lungs (11, 16-18).

Macrophages constitute the foremost controllers of both innate and acquired immunity (19). They are the most abundant immune cells in the lung (approximately 70% of total immune cells) (20, 21), and polarization of macrophage has been heavily associated with the development of allergic asthma (22). Macrophages can be polarized following exposure to microenvironmental stimuli and categorized as classically (M1) or alternatively (M2) activated phenotypes (23, 24). The regulation of macrophage polarization involves a complex interplay between various cytokines, chemokines, and signaling molecules. Interferon- γ (IFN- γ) and lipopolysaccharide (LPS) are known to induce the differentiation of M1 macrophages, whereas IL-4 and IL-13 upregulate the expression of genes involved the generation of M2 macrophages (25).

The importance of ILCs in the development of asthma is well defined in animal

models of asthma (6, 16, 17) and in patients with asthma (26-28). Conversely, the interaction between ILCs and other innate immune cells has not been fully understood yet. This study aimed to identify the role of ILCs as mediators in the development of asthma by estimating their population in induced sputum from patients with asthma. In addition, the correlation between ILCs and several innate immune cells, including macrophages, neutrophils, and eosinophils was analyzed. Our data suggested that cytokine production from ILCs can coordinate the polarization of macrophages, which participates in the development of different types of asthma.

Methods

Subjects

We recruited 51 asthma patients from Department of Internal Medicine, Seoul National University Hospital (Seoul, South Korea) from September, 2015 to September, 2016. All patients had a history of repetitive respiratory symptoms such as cough, dyspnea, or wheezing and showed significant airway hyperresponsiveness in methacholine challenge ($PC_{20} < 16$ mg/mL) (29). Eighteen healthy donors (non-smoker or ex-smokers < 10 pack-year) who were free of any respiratory symptoms and had normal lung function served as controls. All asthmatics were treatment-naïve. Sputum induction and sample processing were performed as previously described (30). To distinguish non-eosinophilic and eosinophilic asthma patients, blood eosinophil was counted in whole blood sample from patients and if eosinophils exceed 300/uL, patients were classified as eosinophilic asthma.

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

Cell isolation from induced sputum

For eliminating mucus, we processed induced sputum with the same volume of 0.1% dithiothreitol (Sigma, MO, USA), shaking for 20 minutes at 37°C. Then we filtered it through a 70um strainer and centrifuged at 1400rpm for 6minutes. The pellet of sputum cell was resuspended with 100ul FACS buffer (PBS+2% fetal bovine serum) for staining and differential counting. Diff Quik solution (Sysmex Corporation, Japan) was used to distinguish myeloid cells from induced sputum following the manufacturer's instructions.

Flow cytometry

Single cells from sputum were blocked with anti-CD16/CD32 (BD bioscience) and stained with fluorochrome-labeled monoclonal antibodies directed against cell surface marker for 1 hour at 4°C. For analysis of human induced sputum, following antibodies were used. Anti-CD45 (HI30) was purchased from BD bioscience. Anti-CD3e (UCHT1), anti-CD11c (3.9), anti-CD11b (ICRF44), anti-CD14 (HCD14), anti-CD15 (W6D3), anti-CD19 (HIB19), anti-CD49b (P1E6-C5), anti-CD68 (Y1/82A), anti-CD117 (104D2), anti-CD127 (A019D5), anti-CD206 (15-2), anti-FcεRIα (AER-37), anti-HLA-DR (L243) and anti-NKp44 (P44-8) were from BioLegend. Anti-ST2L (B4E6) was from MD bioproducts. For FACS sorting of ILC and alveolar macrophage from mouse lung, the following antibodies were used. Anti-CD45 (30-F11) and anti-ST2 (RMST2-33) were purchased from eBioscience. Anti-CD11c (HL3), anti-CD11b (M1/70), anti-CD3e (145-2C11), anti-CD19 (ID3) and anti-CD49b (DX5) were from BD bioscience. Anti-F4/80 (BM8), anti-FcεRIα (MAR-1), anti-CD127 (A7R34) and anti-CD25 (PC61) were from BioLegend. Flow cytometry was performed using BD LSR II, BD LSRFortessa™ and BD LSRFortessa™ X-20 and analyzed by FlowJo (V10.2) software.

Mice

For ILCs and macrophage co-culture experiment, female C57BL/6 mice were purchased from the Orient Company Ltd. (Seoul, South Korea). All experiments were approved by the Institutional Animal Care and Use Committee in Seoul National University Hospital (SNUH-IACUC 17-0043-S1A1) and animals were maintained in the facility

accredited AAALAC International (#001169) in accordance with Guide for the Care and Use of Laboratory Animals 8th edition, NRC (2010).

Sorting of ILCs and alveolar macrophages

For expansion of ILC1s and ILC3s *in vivo*, 6-8-wk-old mice were given intratracheal injection of 100ng recombinant IL-1 β (BioLegend, CA, USA) on three consecutive days. For expansion of ILC2s *in vivo*, 6-8-wk-old mice were inoculated intratracheally with 500ng recombinant IL-33 (BioLegend, CA, USA) on three consecutive days. Alveolar macrophages were sorted from naïve 6-8-wk-old mice. Lung tissue was digested with 1mg/ml collagenase type IV (Worthington Biochemical Corp, UJ, USA) and 0.5mg/ml DNase I (Sigma, MO, USA) for 1 hour at 37°C. For ILCs sorting, lineage negative cells were sorted by lineage cell depletion kit (Miltenyi Biotec Inc., CA, USA), following the protocol. Lineage negative cells were stained with lineage cocktail antibodies (anti-CD3e, anti-CD19, anti-CD11c, anti-CD11b, anti-F4/80, anti-Fc ϵ RI α and anti-CD49b), anti-CD45, anti-CD127, anti-ST-2, and anti-CD25 antibodies for 30 minutes. ILC1 and ILC3 were defined as CD45⁺Lineage⁻CD127⁺ST-2⁻CD25⁻ lymphoid cells. ILC2 were defined as CD45⁺Lineage⁻CD127⁺ST-2⁺CD25⁺ lymphoid cells. For alveolar macrophage sorting, CD11c⁺ cells were sorted by CD11c Microbeads UltraPure (Miltenyi Biotec Inc., CA, USA), following the protocol. CD11c⁺ cells were stained with anti-CD45, anti-F4/80 and anti-CD11c antibodies for 30 minutes. Alveolar macrophages were defined as CD45⁺F4/80⁺CD11c⁺ cells. Each subset of ILCs and alveolar macrophages were sorted by BD FACSAria™.

Co-culture of ILCs and macrophages

Sorted alveolar macrophages (2.5×10^5 cells) were cultured in the lower chamber of a 24-well transwell plate (0.4 μ m pore, Corning, MA, USA). After 24hr, freshly sorted ILC1s and ILC3s or sorted ILC2s (5×10^4 cells) were added in the upper chamber of the transwell. In some experiments, ILCs were added in the same chamber of macrophages.

Recombinant IL-2 (20U/ml, BioLegend, CA, USA), IL-12 (20ng/ml, BioLegend, CA, USA) and IL-23 (20ng/ml, BioLegend, CA, USA) or IL-2 (20U/ml) and IL-33 (20ng/ml, BioLegend, CA, USA) were added for ILC1s/ILC3s or ILC2s stimulation, respectively. In some experiments, anti-PD-1 (10ug/ml, Bioxcell, NH, USA), anti-ICOS (10ug/ml, Bioxcell, NH, USA), and anti-CD40L blocking antibodies (10ug/ml, Bioxcell, NH, USA) were used to block cell-cell contact between alveolar macrophages and ILCs. After 48 hours, supernatant was harvested for analysis cytokine production by ELISA, and alveolar macrophages were harvested using TRIzol® Reagent (Invitrogen, CA, USA) for analysis of M1 and M2 genes expression by RT-qPCR.

Quantitative real-time PCR

Harvested alveolar macrophages were homogenized with TRIzol® Reagent. After homogenization, total RNA was extracted by manufacturer's instructions. cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad, CA, USA). The quantitative RT-qPCR assays were performed using IQ supermix (Bio-Rad, CA, USA). Relative expression levels were calculated with normalizing Cq values of target genes to Cq values of Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). All primers were purchased from Thermo Fisher Scientific (TaqMan® Gene Expression Assays, MA, USA)

ELISA

ELISA for supernatant IFN γ , IL-4, IL-9 (BD bioscience, NJ, USA) and IL-5, IL-13, IL-17 (R&D systems, MN, USA) were performed following the manufacturer's instructions.

Statistical analysis

Before comparison, normality test was performed with Shapiro-Wilk normality test. Comparisons of 2 groups were performed using Mann-Whitney test if the data was non-parametric or using unpaired t-test if the data was parametric. Multiple groups were compared using Kruskal-Wallis test followed by a Dunns's post-test if the data was non-parametric or using one-way ANOVA following by a Bonferroni's post-test.

For analysis correlation between the frequency of macrophages and ILCs in induced sputum, Spearman r correlation test was performed if the data was non-parametric or Pearson correlation test were performed if the data was parametric. Values of measurements were expressed as mean $\pm$ SD or mean $\pm$ SEM. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using GraphPad Prism 7.

Results

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

The presence of ILCs in induced sputum was evaluated in 51 patients with asthma and 18 healthy controls (Table 1). Total ILCs were initially gated as CD45⁺Lineage⁻IL-7R⁺ lymphocytes, and subsequently categorized as ILC2s (CRTH2⁺ cells), ILC1s (CRTH2⁻c-kit⁻NKp44⁻ cells), and ILC3s (CRTH2⁻c-kit⁺ cells). Natural cytotoxicity receptor (NCR)⁺ ILC3s and NCR⁻ ILC3s were further categorized on the basis of the expression of NKp44 (Fig. 1A). However, it should be noted that the NCR-ILC3 subset remains a heterogeneous population containing LT_i-cells. Total frequency of CD45⁺ cells was increased in induced sputum of asthmatics compared with healthy controls ($P < 0.001$; Fig. E1A). The percentage and absolute number of total ILCs were increased in the induced sputum from patients with asthma when compared with the healthy donors ($P < 0.01$; Fig. 1A and B, Fig. E1B). In addition to the ILC2s, both ILC1s and NCR⁻ILC3s were also significantly increased in the asthmatic patients ($P < 0.0001$; Fig. 1C). However, the increase in ILCs was not related to pulmonary function index (FEV1/FVC), airway hypersensitivity (PC20 level), and WBC count (Fig. E2A–C). Negative correlations were observed between ILC1s and NCR⁻ILC3s ($P < 0.0001$; Fig. 1D) and NCR⁺ILC3s ($P < 0.05$; Fig. 1E), which might be due to the transition between ILC1s and ILC3s reported in humans (31). However, we could not see any correlations between other subset of ILCs (Fig. E2D). So far, only ILC2s has been known to be increased in asthmatics, however, our data clearly showed that all types of ILCs were increased in the induced sputum of patients with asthma.

CD68⁺ macrophages were increased in induced sputum from asthmatics

We hypothesized that an increase in the number of ILCs might result in crosstalk between them and other immune cells, especially innate immune cells such as alveolar macrophages. First, we checked the population and composition of macrophages in induced sputum from asthmatics and analyzed a subset of macrophages by flow cytometry. CD45⁺CD68⁺ cells were first gated as macrophages and then categorized as M1 and M2 macrophages based on the expression of CD11c and CD206, respectively (Fig. 2A). The M1 and M2 macrophages presented with different expression levels of HLA-DR (Fig. E3B and C) (32). Macrophages that expressed both CD11c and CD206 were considered as alveolar macrophages (AMs) (33). The percentage and absolute number of total macrophages were increased in induced sputum of asthmatics when compared with the healthy controls ($P < 0.0001$; Fig. 2B, Fig. E3A); moreover, each subgroup of macrophages was increased in the induced sputum of asthmatics (M1, $P < 0.05$; M2, $P < 0.0001$; AMs, $P < 0.0001$; Fig. 2C) when compared with the controls. Alterations in the number of macrophages were not associated with lung function (FEV1/FVC), airway hypersensitivity (PC20 level) or the number of WBCs (Fig. E4A–C). Among the population of macrophages, AMs had a strong negative correlation with M1 ($P = 0.0001$; Fig. 2D) and M2 ($P < 0.0001$; Fig. 2E) macrophages indicating that AMs might be differentiated into the M1 or M2 phenotypes based on the type of stimulus in asthmatics.

Increase in the number of ILC2s and M2 macrophages in induced sputum of patient with eosinophilic asthma

To investigate potential associations between various populations of ILCs and

macrophages and the phenotypes of asthma, the patients were divided into two groups based on the number of eosinophils (Table E1). The percentage of ILC2s was significantly increased in induced sputum of eosinophilic asthmatics ($P < 0.05$; Fig. 3A) when compared with that of the non-eosinophilic patients. The percentage of AMs was similar in sputum from the eosinophilic and non-eosinophilic asthmatics (Fig. 3B). However, the number of M2 macrophages was higher in eosinophilic asthmatics, whereas an increased number of M1 macrophages was observed in the non-eosinophilic asthmatic patients (M1, $P < 0.05$; M2, $P < 0.01$; Fig. 3B). Also, we tested the various cytokine expression in the pellets from sputum, and found that IL-5 and IL-13 mRNA were upregulated only in the eosinophilic asthmatics but not in the non-eosinophilic asthmatics ($P < 0.001$; Fig. 3C).

Respiratory diseases are known to influence asthma outcome and phenotypes (34-36). In order to assess whether the comorbidity of upper airway disease in asthmatics affects the distribution of ILCs and other immune cells, patients were classified into three different groups based on the presence or absence of allergic rhinitis (AR) and chronic rhinosinusitis (CRS; Table E2). No differences between the subtypes of ILCs and macrophages (Fig. E5A–D) were noted. However, the number of NCR⁺ ILC3s was decreased in the sputum from asthmatics with AR and CRS ($P < 0.01$; Fig. E5B).

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

To verify the presence of associations between asthma phenotypes and the distribution of innate immune cells, we compared the relationships of innate immune cells in induced sputum of asthmatics. Interestingly, patients with higher numbers of eosinophils in

sputum presented with increased numbers of ILC2s ($P < 0.05$; Fig. 4A). Unlike ILC2s, there were no correlations between ILC1s, NCR⁻ILC3s or NCR⁺ILC3s and eosinophils (Fig. 4A), and other subset of ILCs and neutrophils in induced sputum (Fig. E6A).

Next, we examined the relationship between macrophages and other innate immune cells. Patients with increased number of eosinophils in induced sputum demonstrated an increase in the percentage of M2 macrophages ($P < 0.01$), whereas M1 macrophages did not show correlation with eosinophils (Fig. E6B). Conversely, neutrophil counts in sputum were positively correlated with M1 macrophages ($P < 0.05$), and negatively correlated with M2 macrophages ($P < 0.001$; Fig. E6C).

We further investigated the presence of associations between ILCs and macrophages because alterations in these two populations are most significant in asthmatics. Interestingly, the percentages of ILC1s and ILC3s were positively correlated with increase in the number of M1 macrophages ($P < 0.01$ and $P < 0.05$, respectively; Fig. 4B) but not M2 macrophages. On the other hand, asthmatics who had more ILC2s presented with more M2 macrophages in induced sputum ($P < 0.01$; Fig. 4C). However, in healthy donors, no such correlations were observed (data not shown). These data suggest that ILCs and macrophages interact with each other in a cell-specific manner, and some of these interactions could alter the outcome of asthma.

ILCs can promote the polarization of macrophages by secreting cytokines and via direct interactions

On the basis of current results, we hypothesized that specific types of ILCs affect the polarization of macrophages. To test this hypothesis, we co-cultured sorted ILCs with AMs

(Fig. E7A–C). ILC2s were stimulated with recombinant(r) IL-2 and IL-33, whereas ILC1s and ILC3s were stimulated with rIL-2, rIL-12 and rIL-23 (Fig. 5A). Co-culture of AMs with ILC1s and ILC3s significantly induced the mRNA expressions of M1 macrophage-related genes, such as *Nos2*, *Cd86*, and *ptgs2* ($P < 0.001$, $P < 0.0001$, and $P < 0.05$, respectively; Fig. 5B). In contrast, mRNA expression of M2 macrophage-related genes such as *Arg1* and *Retnla* were increased when AMs were co-cultured with ILC2s ($P < 0.0001$ and $P < 0.001$, respectively; Fig. 5C). It is well known that the polarization of macrophages is regulated by cytokines (27, 28). Therefore, we presumed that ILCs might be involved in this process by secreting cytokines. To examine cytokine production by the ILCs, we measured IFN γ , IL-17A, IL-4, and IL-13 levels in co-culture supernatants (Fig. 5D and E). Treatment with rIL-12 and rIL-23 stimulated ILC1s and ILC3s, thereby increasing the levels of IFN γ ($P < 0.0001$) and IL-17A ($P < 0.05$; Fig. 5D). Furthermore, stimulation of ILC2s with rIL-33 resulted in increased levels of IL-4 and IL-13 ($P < 0.001$ and $P < 0.0001$, respectively; Fig. 5E). In addition to IL-4 and IL-13, ILC2s produced IL-5 and IL-9 with rIL-33 stimulation ($P < 0.0001$ and $P < 0.5$, respectively; Fig. E8A and E8B). These results clearly showed that cytokines from different subsets of ILCs might induce the polarization of macrophages.

Next, the transwell system was used to investigate whether soluble cytokines from ILCs affected the polarization of macrophages. As shown in Fig. 6A, AMs were cultured in the lower chamber, and different subsets of ILCs were then added on to the upper chamber. After 48 h, AMs from the lower chamber were harvested and the expression levels of M1 and M2 macrophage-related genes were analyzed. Unexpectedly, mRNA expression levels of M1 macrophage-related genes (*Nos2* and *Cd86*) were decreased ($P < 0.001$ and $P < 0.05$, respectively; Fig. 6B) when AMs were co-cultured with ILC1s and ILC3s in the transwell.

Moreover, the mRNA expression level of one of the M2 macrophage-related gene, *Arg1* was reduced, whereas that of *Mrc1* was increased ($P < 0.0001$ and $P < 0.01$; Fig. 6E) when AMs were co-cultured with ILC2s in the transwell. However, the expression levels of M1- and M2-related genes were not affected by co-culture with ILC2s, and ILC1s /ILC3s, respectively (Fig. 6C and D). IFN γ and IL-17A levels in culture supernatant from the transwell were significantly lower than that without the transwell (IFN γ , $P < 0.05$; IL-17A, $P < 0.05$; Fig. 6F). However, IL-4, IL-5, IL-9, and IL-13 levels remained unchanged in the presence of the transwell (Fig. 6G and Fig. E8B).

To investigate which surface molecules mediate the interaction of ILCs and macrophages, we treated anti-PD-1, anti-ICOS, and anti-CD40L which are co-stimulatory or inhibitory molecule that is expressed by ILCs (37-39). When we co-cultured AM and ILC1/3s with blocking antibodies, there were no changes on M1 and M2 macrophage-related gene expression (Fig. E9A and E9B). Although blocking antibodies treatment seems to reduce the expression of *Arg1* and *Retnla* in co-culture group of alveolar macrophages and ILC2s (*Arg1*, n.s., and *Retnla*, $P < 0.0001$; Fig. E9C and E9D), we failed to find surface molecules that can regulate the polarization of macrophage.

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

Discussion

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

The discovery of ILCs in the lungs has provided a breakthrough in our understanding of the cell types and innate pathways underlying the development of asthma (6, 40). ILCs are an emerging type of immune cells that are lineage-negative but produce large amounts of effector cytokines (7). Fort *et al.* first identified lineage⁻ MHC class II^{high} and CD11c^{dull} ILC- like cells in the lungs of mice and demonstrated that IL-25 induces type 2 cytokine production from these cells (41). Recent studies have intensively examined the role of ILCs following exposure to aero-allergens such as house dust mites, ryegrass or fungal extracts. These aero-allergens were found to cause robust eosinophilic airway inflammation and airway hyperresponsiveness (AHR) and also induce the production of type 2 cytokine from ILC2s in the absence of CD4 T cells in mice (42-44). Moreover, the involvement of ILCs in non-allergic forms of asthma triggered by environmental factors such as air pollutants (45, 46) or obesity (16) has been reported. For example, CCR6⁺ ILC3s that produce IL-17A in the lungs of mice fed with a high-fat diet are vital for the development of obesity-related asthma (16). Moreover, IL-17A in the sputum or peripheral blood of asthmatics is correlated with the severity of the disease

(47, 48). So far, only ILC2s have been implicated in the development of allergic asthma; however, other types of ILCs also appear to play important roles in different forms of asthma. In support of this hypothesis, the current study showed that the numbers of all types of ILCs were significantly increased in the sputum of patients with asthma when compared with those in the healthy controls. This diverse distribution of ILCs might reflect the heterogeneous nature of asthma.

To the best of our knowledge, this is the first study to report the presence of ILC1s and ILC3s in the sputum of asthmatics, unlike previous studies that focused on ILC2s only (26-28, 49). Although the function of ILCs in the development of asthma was originally identified in mice, several human studies have demonstrated their roles in the development of this condition (50, 51). Bartemes *et al.* reported that the prevalence of ILC2s was increased in PBMC from allergic asthmatics when compared with healthy controls (28). Nagakumar *et al.* demonstrated the presence of ILC2s in the airways of children with therapy-resistant asthma (STRA). They first identified ILC2s in induced sputum, where the proportion was found to be higher than that in peripheral blood (49). Although several studies have been reported regarding the role of ILCs in asthma, there are several limitations. First, most studies examined ILCs using PBMC (52-54) rather than BALF or induced sputum. As shown by Gasteiger *et al.*, PBMC does not reflect actual lung environments where ILCs are known to reside (55). In the current study, we demonstrated the presence of ILCs in induced sputum, which might be a more accurate reflection of the profile of ILCs rather than that in blood. Second, there are a limited number of immune cells in the sputum. However, we tried to collect adequate amounts of sputum from the patients to analyze the accurate population of the immune cells and were able to confirm the interactions between macrophages and ILCs in the present study. Third, previously, all experiments were

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

Although all types of ILCs were increased in the sputum from asthmatics, a negative correlation between ILC1s and $\text{NCR}^+\text{ILC3s}$ or $\text{NCR}^-\text{ILC3s}$ was observed. It is well known that $\text{NCR}^+\text{ILC3s}$ and ILC1s exhibit plasticity in the intestine (31). It is also reported that ILC2s and ILC1s demonstrate plasticity in the lungs of COPD mice (56, 57); nonetheless, the conversion of ILC1s and ILC3s in the lung was not clear. Because ILC1 and ILC3 plasticity was not observed in healthy controls in the current study (data not shown), certain inflammatory conditions in asthmatics might be responsible for the induction of ILC plasticity.

The proportion of ILC2s in sputum from eosinophilic asthmatics was higher than that from non-eosinophilic asthmatics. A positive correlation between ILC2s and blood eosinophil counts was observed, whereas no such positive correlations were observed between ILC1s and ILC3s and neutrophils. Alternatively, the number of M1 macrophages in sputum was positively correlated with the number of neutrophils, whereas M2 macrophages proportions were positively correlated with eosinophil counts. These findings suggest that ILCs might directly affect the polarization of macrophages, which could regulate the infiltration of eosinophils or neutrophils into the airways. To confirm this hypothesis, we analyzed other immune cells from sputum and found that increase in the number of macrophages was correlated with the number of ILCs. In other words, ILC2s had a positive correlation with M2 macrophages, whereas both ILC1s and ILC3s demonstrated positive correlations with M1

macrophages. Co-culture experiments using mouse ILCs and AMs showed that activated ILCs could regulate the polarization of macrophages by secreting cytokines as well as cell–cell contact. M1 macrophages can be differentiated by IFN γ , whereas the key cytokines that activate M2 macrophages are IL-4 and IL-13 (22, 58). Therefore, IL-4 and IL-13 from ILC2s could induce the polarization of M2 macrophages, whereas IFN γ or IL-17A from ILC1s and ILC3s could induce the polarization of M1 macrophages. Among markers for M2 macrophages, the gene expression of mannose receptor C-type 1 (*Mrc1*) was down-regulated in alveolar macrophages co-cultured with ILC2s (**Figure 5C**). *Mrc1* is one of typical markers for M2 macrophages, which is up-regulated by IL-4 and IL-13 stimulation (22). However, in tissue-resident macrophages, the expression of *Mrc1* is independent of IL-4 and IL-13 (59). Alveolar macrophages are lung-resident macrophages, so IL-4 and IL-13 released from ILC2s might not affect the expression of *Mrc1*. Also, because alveolar macrophages highly expressed *Mrc1* in steady states (**Figure 2A**), we could know that the expression of *Mrc1* in alveolar macrophages was regulated independent of IL-4 and IL-13. In addition, M2 macrophages do not constitute a uniform population and are further subdivided into M2a, M2b and M2c. MRC1 is expressed in M2a and M2c but not in M2b (60). Therefore, decrease of MRC1 might indicate a change in the M2 subtype although further analysis need to be done. The roles of M1 and M2 macrophages in the development of asthma differ from each other. M1 macrophages are the major effector cells in non-allergic forms of asthma, while M2 macrophages are predominant in the allergic forms of asthma (61). Although the current M1/M2 paradigm with its polarized extremes is oversimplified, our data showed that certain subsets of ILCs could regulate the polarization of macrophages. More interestingly, the polarization of macrophages was only induced when macrophages were co-cultured with ILCs. The combination of recombinant cytokines did not affect the polarization of

macrophages indicating the importance of ILCs during this process. Thus, the ILCs-macrophages axis might be important to induce the development of different types of asthma.

It is worthy to note that polarized M1/M2 macrophages could induce the activation of ILCs. M1 macrophages induce the activation of Th1 cells via TNF α and IL-12 production and by interacting with CD86 and MHCII molecules, thereby affecting the development of non-allergic asthma (62, 63). Similarly, M2 macrophages activate Th2 cells via IL-4 and IL-13 production and by interacting with CCL17 and MRC1 and lead to the development of allergic asthma (22). There is a possibility that ILCs and macrophages interact in a cell contact manner, and the inhibition of this interaction in the transwell system may have reduced cytokine production by the ILCs. Recently, it has been reported that ILCs also express co-stimulatory or inhibitory molecules such as PD-1, ICOS, and CD40L on their surfaces (37-39, 64). Activated alveolar macrophages also express high level of PD-L1 and CD40 (65, 66). Therefore, we hypothesized that ILCs and macrophage could interact through these molecules. When we blocked the ILC1/3s-macrophage interaction using anti-PD-1, anti-ICOS, and anti-CD40L antibodies, M1 and M2 macrophage-related genes were not changed (**Figure E9, A and B**). That means unknown molecules, not PD-1, ICOS, and CD40L, might mediate cell-cell interaction between ILC1/3s and alveolar macrophages. In case of ILC2s-macrophage interaction, the expression of *Arg1* and *Retnla* were down-regulated by these blocking antibodies (**Figure E9, C and D**). However, the gene expression of *Retnla* tended to be increased when we co-cultured ILC2s and macrophage separately using transwell system. This discrepancy between the experiments (i.e, co-culture without transwell or with transwell) might indicate that other unknown molecules could mediate interaction of ILC2 and alveolar macrophages. Although

we could not show the surface molecules that mediate the interaction between ILCs and macrophages in current study, we believe that identifying these interactions will be important for understanding the features of ILCs and macrophages.

Although we have found a close relationship between ILCs and macrophages according to the phenotypes of asthma, we failed to see the correlation between ILCs and macrophages with AHR (**Fig E2**). As far as we know, there was no report about the correlation between ILCs and AHR in human asthmatics. One reason why we couldn't see the positive relationship might due to the sample we obtained. In our study, we recruited newly diagnosed, and treatment-naïve patients to exclude the interference effects of drugs on immune cells. Therefore, most patients we recruited belong to mild asthmatics with the level of FEV1 % was more than 80%. Since AHR was caused by severe inflammation and airway damage, it is hard to see the correlation between AHR and immune cells in our samples which are mostly from mild asthmatics. Also, it is possible that ILCs and macrophages are not direct mediators of AHR. The inflammatory factors that known to induce AHR are histamine and leukotrienes, and these proteins are mostly released from eosinophils and mast cells (67). We could find negative correlation between AHR and eosinophils (data not shown). Perhaps increased ILCs or macrophages could regulate AHR through interaction with other immune cells such as eosinophils and mast cells.

In conclusion, this study shows, for the first time, that in addition to ILC2s various other ILCs are also increased in the airway of subjects with asthma. These ILCs might be essential sources of cytokines that induce the polarization of macrophages and control the development of airway eosinophilia or non-eosinophilic inflammation. Understanding the factors that regulate the activation of ILCs may be important for the treatment of subjects with difficult-to-control asthma because ILCs can crosstalk with several innate immune cells.

ILCs might play key roles in controlling allergic or non-allergic inflammation in asthmatics during the early stages of development of asthma. However, more experiments evaluating the relationships between ILCs, macrophages, and other immune cells in the lungs are warranted.

Conflict of Interest

The authors declare no potential conflicts of interests.

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Tables

TABLE 1. Characteristics of patients

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

The data are presented as the mean $\pm$ SD.

Abbreviations: FEV1, forced expiratory volume in 1 second; FEV1(%), forced expiratory volume in 1 second/forced vital capacity; FVC, forced vital capacity; PC20, provocation concentration of methacholine leading a fall of 20% of FEV1; WBC, white blood cell; N.D, no data

Figure Legends

Figure 1. Innate lymphoid cells are increased in induced sputum of asthmatics. **A.** Representative ILC gating strategy from induced sputum of healthy donor ($n = 18$) and asthmatics ($n = 51$). **B.** Comparison of total ILC frequency (% of CD45⁺ cells) between healthy donors and asthmatic patients. **C.** Comparison of frequency of ILC1s, ILC2s, NCR⁻ILC3s, and NCR⁺ILC3s (% of CD45⁺ cells) between healthy donors and asthmatic patients. **D.** Correlation between ILC1s and NCR⁻ILC3s (% of ILCs) in induced sputum of asthma patients. **E.** Correlation between ILC1s and NCR⁺ILC3s (% of ILCs) in induced sputum of asthma patients. Mann–Whitney or Student's *t* test was used to compare healthy controls and asthma patients, and Pearson's correlation test was used to measure associations between variables. Values represent the mean $\pm$ standard deviation (SD). ** $P < 0.01$, **** $P < 0.0001$.

Figure 2. Macrophages are increased in induced sputum of asthmatics. **A.** Representative macrophages gating strategy from induced sputum of healthy donors ($n = 18$) and asthmatics ($n = 51$). Total macrophages (CD68⁺ cells), M1 macrophages (CD68⁺CD11c⁺CD206⁻ cells), M2 macrophages (CD68⁺CD11c⁻CD206⁺ cells), and alveolar macrophages (AM; CD68⁺CD11c⁺CD206⁺ cells). **B.** Comparison of frequency of total macrophages (% of

CD45⁺ cells) between healthy donors and asthma patients. **C.** Comparison of frequency of M1, M2, and AMs (% of CD45⁺ cells) in induced sputum of healthy donors and asthmatics. **D.** Correlation between M1 and AMs (% of macrophages) in induced sputum of asthma patients. **E.** Correlation between M2 and AMs (% of macrophages) in induced sputum of asthma patients. Mann–Whitney or Student's *t* test was used to compare healthy controls and asthma patients, and Pearson's correlation test was used to measure associations between variables. Values represent the mean $\pm$ SD. **P* < 0.05, *****P* < 0.0001.

Figure 3. Eosinophilic asthmatics show type 2 airway inflammation. **A.** The frequency of each subset of ILCs (% of CD45⁺ cells) in induced sputum of non-eosinophilic and eosinophilic patients. **B.** Frequency of M1, M2, and AMs (% of CD45⁺ cells) in induced sputum of non-eosinophilic and eosinophilic patients. **C.** The gene expression of IFN γ , IL-5, IL-13, and IL-17A in pellets of whole sputum cells. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, using Mann–Whitney test to compare between the non-eosinophilic and eosinophilic asthma patients. Values represent the mean $\pm$ SD.

Figure 4. Different subset of ILCs is correlated with the levels of eosinophils and macrophages. **A.** Correlation between ILC1s, ILC2s, NCR⁻ILC3s or NCR⁺ILC3s and eosinophils in induced sputum analyzed. **B.** Correlation between M1 macrophages and ILCs in induced sputum of asthmatics. **C.** Correlation of M2 macrophages and ILCs in induced sputum of asthmatics. **P* < 0.05, ***P* < 0.01, n.s, not significant, using Pearson's correlation test.

Figure 5. ILCs affect the polarization of alveolar macrophages *in vitro*. **A.** Schematic diagram of the co-culture procedure. **B.** The expression of M1-related genes, *Nos2*, *Cd86*, and *Ptgs2*, in alveolar macrophages co-cultured with ILCs. **C.** The expression of M2-related

genes, *Arg1*, *Retnla*, and *Mrc1* in alveolar macrophages co-cultured with ILCs. The gene expression data were normalized with the expression of *Gapdh*. **D.** IFN γ and IL-17 expression levels in the ILC1s/ILC3s and alveolar macrophages co-cultured group. **E.** Expression levels of type 2 cytokines, IL-4 and IL-13, in the ILC2 and alveolar macrophages co-cultured group. $*P < 0.05$, $***P < 0.001$, $****P < 0.0001$ using Kruskal–Wallis test, followed by Dunn's multiple comparison test. The data are representative of three independent experiments analyzed, and presented as the mean $\pm$ SEM.

Figure 6. ILCs-macrophage interaction affects polarization of macrophages via cell-cell contact **A.** Schematic diagram of the co-culture procedure using the transwell system. **B-C.** Expression of M1-related (*Nos2*, *Cd86*, and *Ptgs2*) (**B**) and M2-related (*Arg1*, *Retnla*, and *Mrc1*) (**C**) genes in alveolar macrophages co-cultured with ILC1s/ILC3s with and without the transwell insert. **D-E.** Expression of M1-related (**D**) and M2-related (**E**) genes in alveolar macrophages co-cultured with ILC2s with and without the transwell insert. Each gene expression was normalized by *Gapdh* expression. **F.** IFN γ and IL-17 production in the ILC1s/ILC3s and alveolar macrophages co-cultured group. **G.** IL-4 and IL-13 production in the ILC2s and alveolar macrophages co-cultured group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$ using Mann–Whitney test. The data are representative of three independent experiments, analyzed, and presented as the mean $\pm$ SEM.

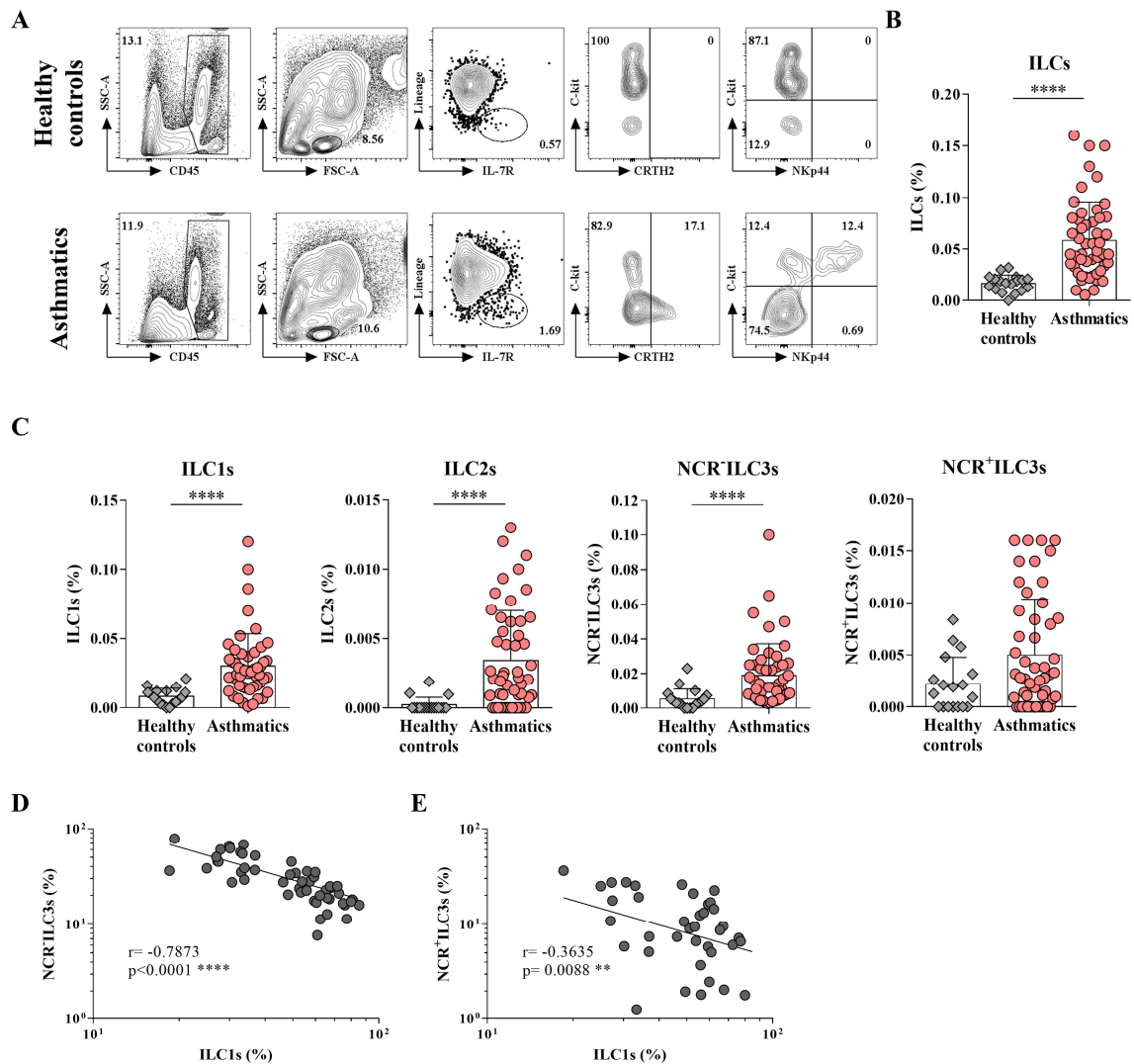
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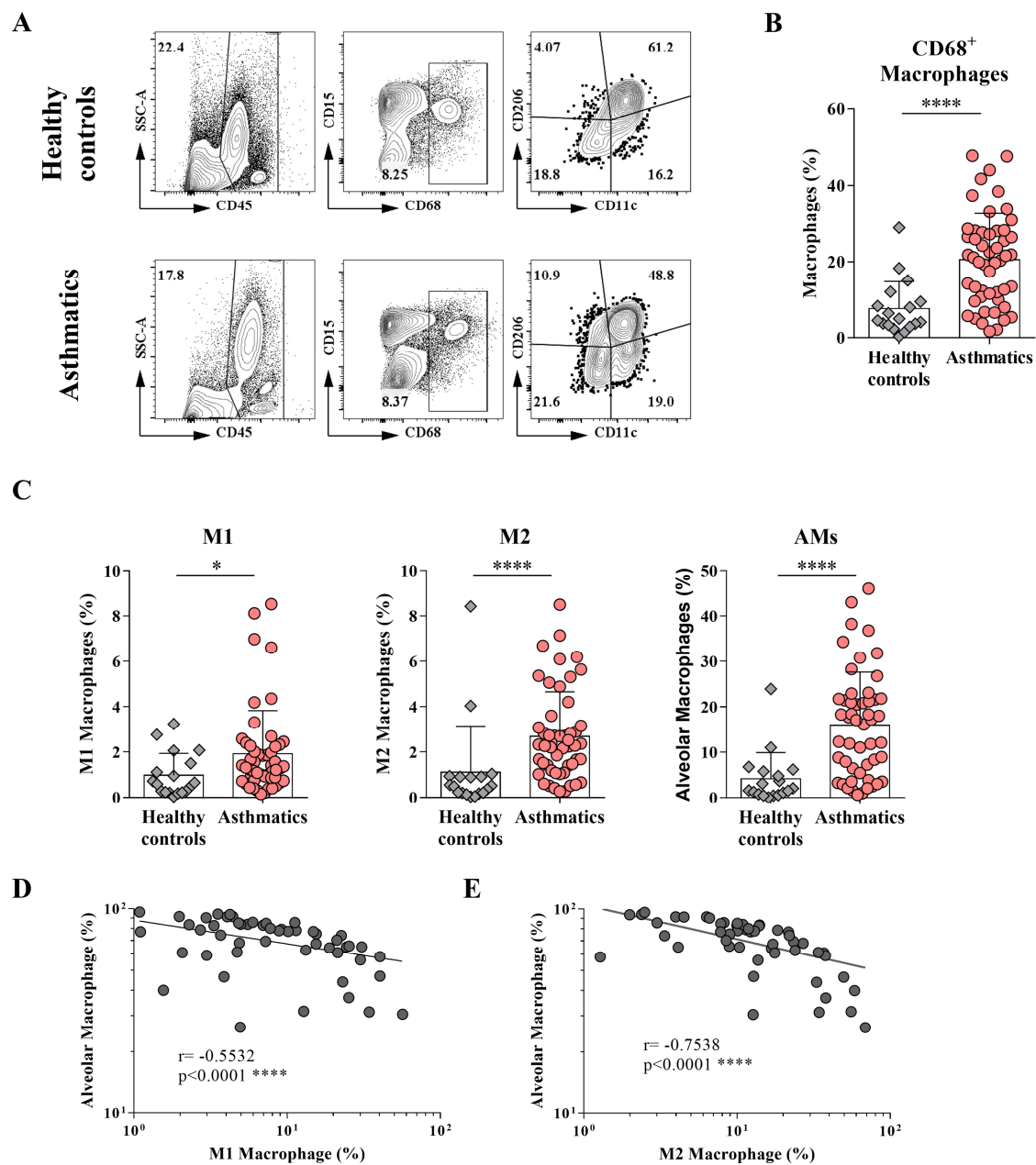
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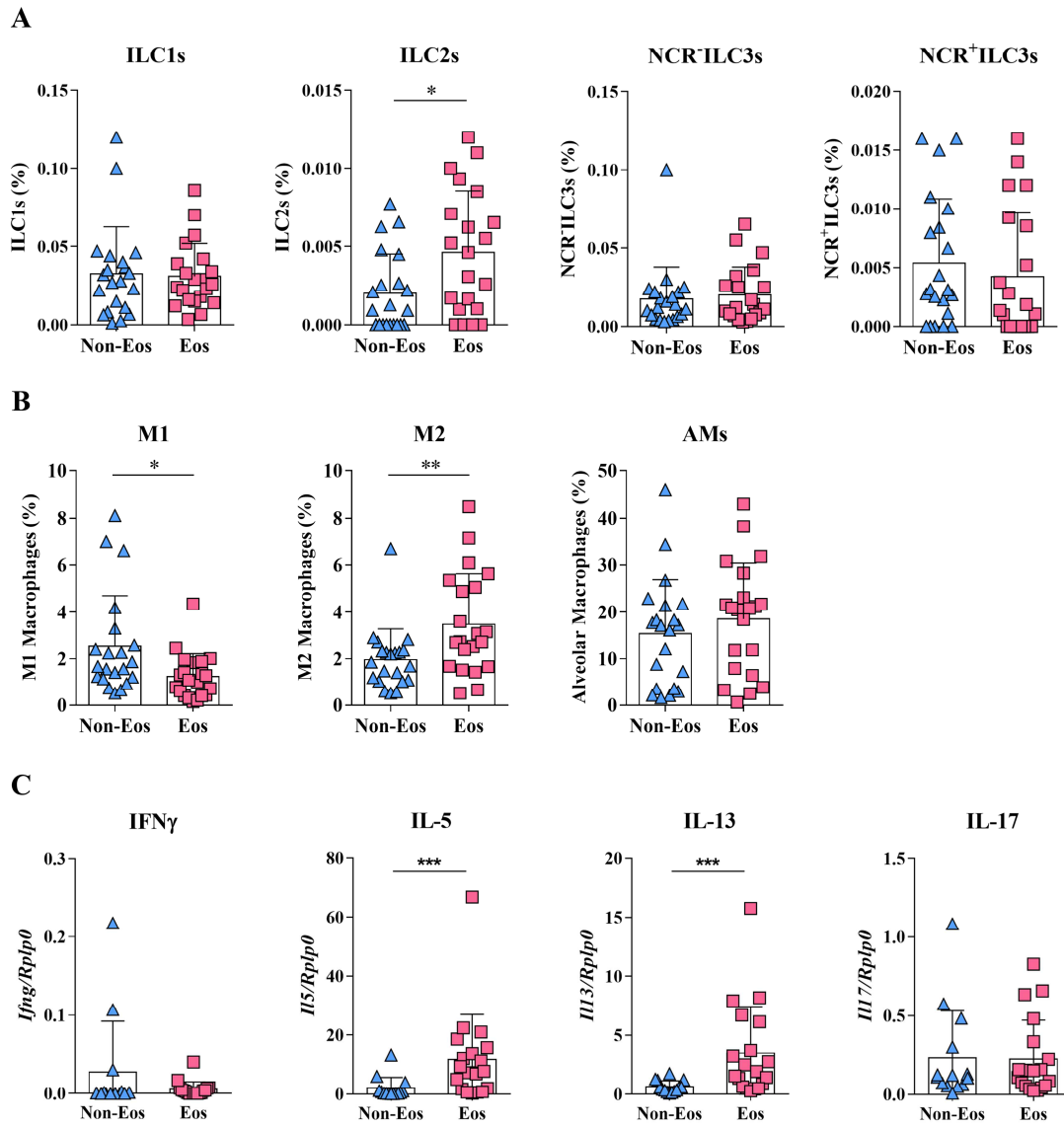
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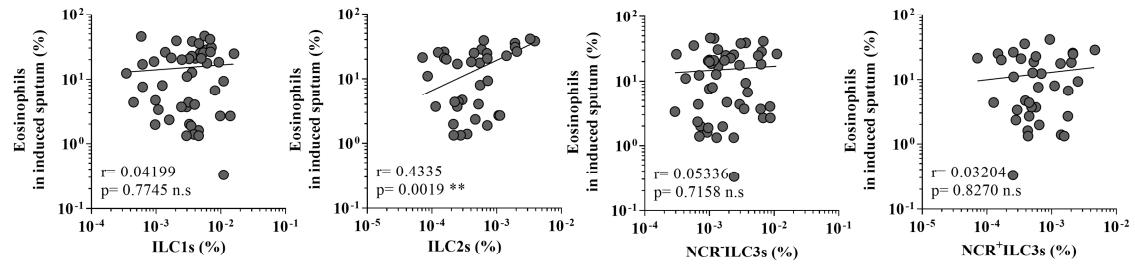
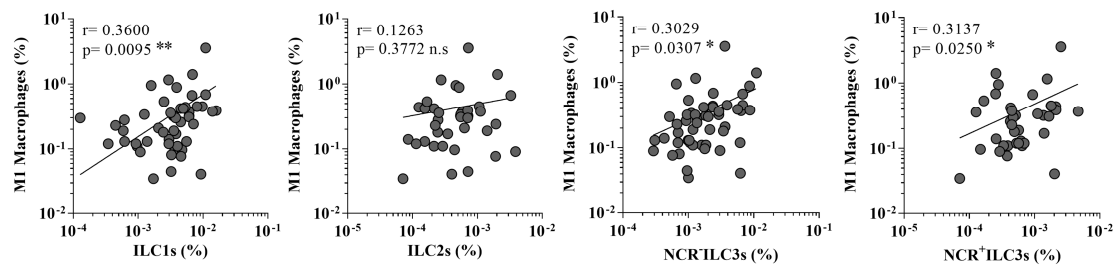
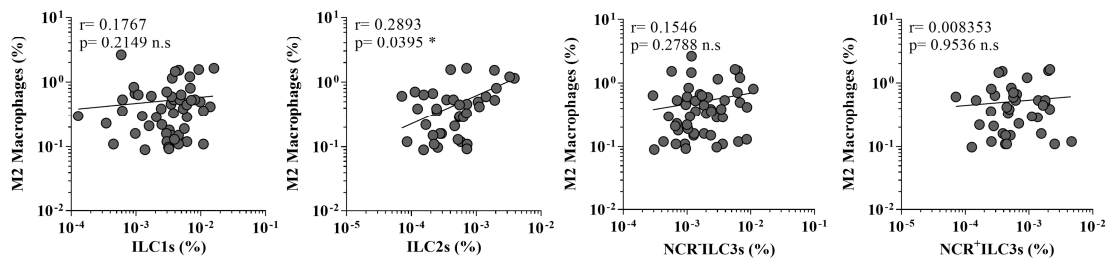
Figure 4**A****B****C**

Figure 5

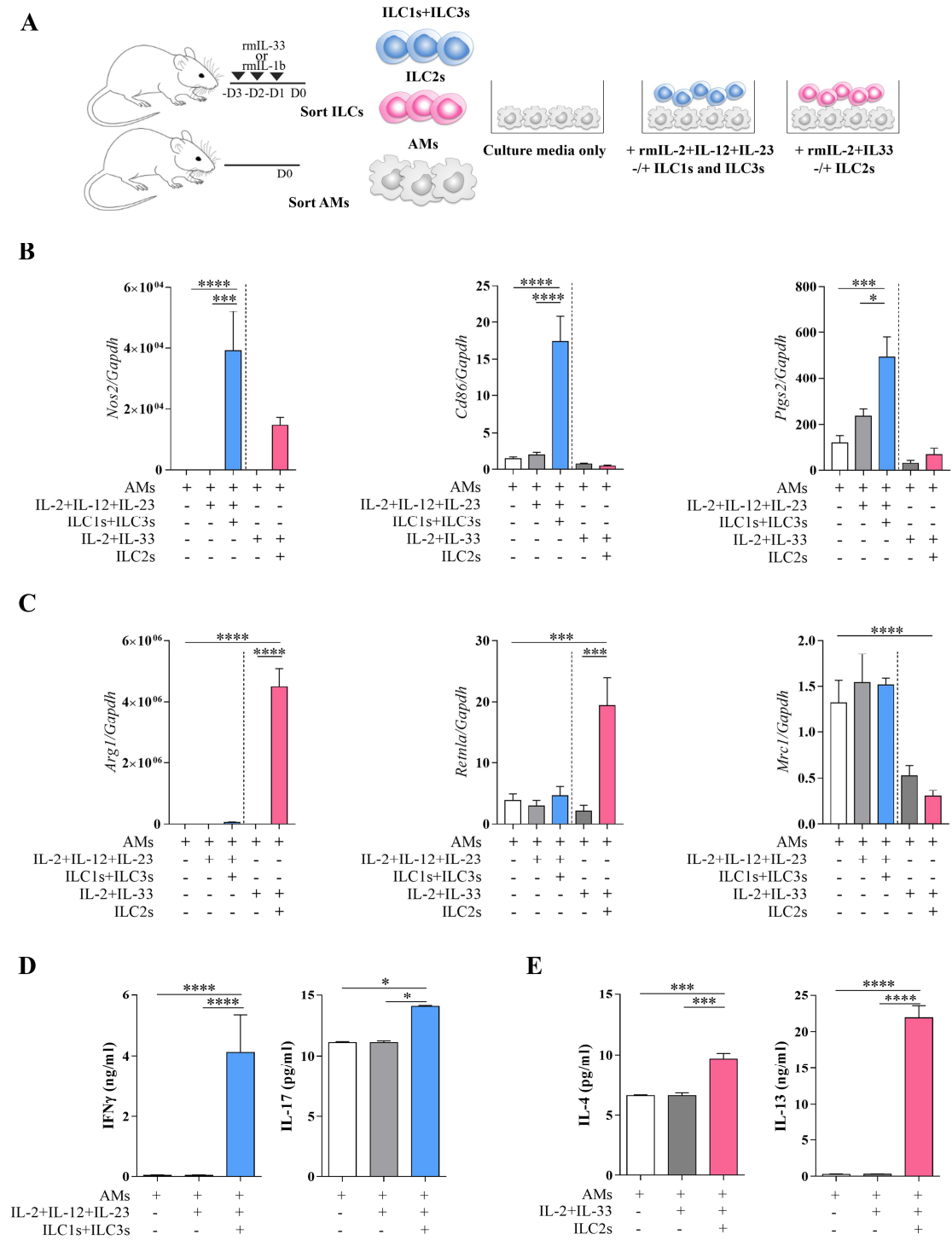


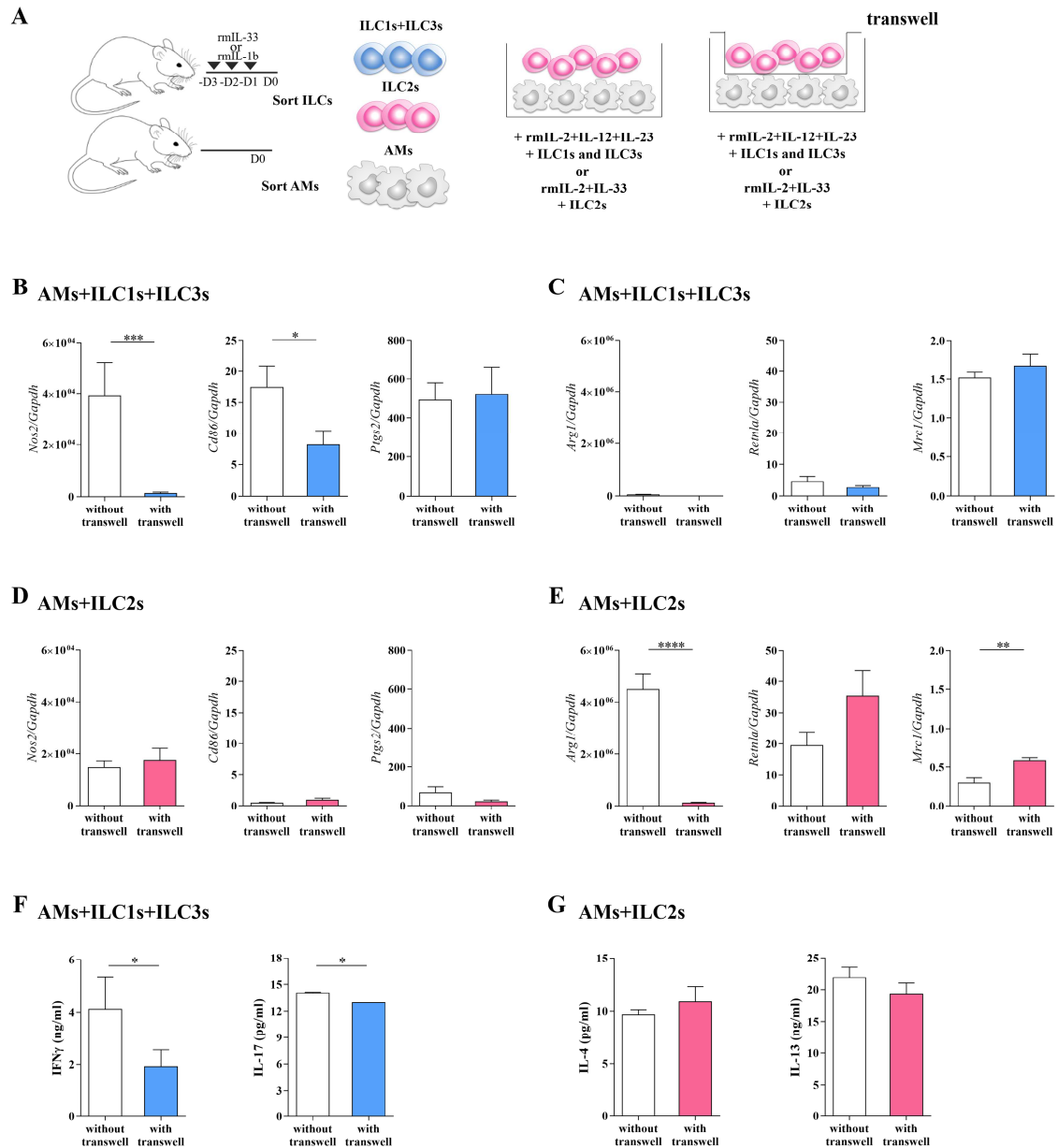
Figure 6

Figure E1. Frequency of CD45⁺ cells and absolute number of ILCs in induced sputum. **A.** The percentage of total CD45⁺ cells in induced sputum. **B.** Absolute number of total ILCs in induced sputum. ** $P < 0.01$, *** $P < 0.001$, using Mann–Whitney test. Values represent the mean $\pm$ SD.

Figure E2. Correlation between clinical data and the percentage of each group of ILCs in induced sputum. The percentage of ILCs based on flow cytometry analysis as described in Figure 1. **A.** Correlation between FEV1/FVC ratio and ILCs. **B.** Correlation between PC20 values and ILCs. PC20 values represent the concentration of provocholine leading to a 20% decrease in FEV1 level. **C.** Correlation between WBC count/uL and ILCs analyzed. WBC counts were based on complete blood cell count results. **D.** Correlation between ILC2s and other ILCs or between NCR⁻ILC3s and NCR⁺ILC3s. n.s, not significant, using Pearson's correlation test.

Figure E3. Comparison of the expression levels of HLA-DR in M1 and M2 macrophages. **A.** Absolute number of total macrophages in induced sputum. **B.** Mean fluorescence intensity (MFI) levels of HLA-DR in M1 and M2 macrophages. **C.** Histogram of HLA-DR expression levels in M1 macrophages and M2 macrophages. **** $P < 0.0001$, using Mann–Whitney test. Values represent the mean $\pm$ SD.

Figure E4. Correlation between clinical data and the percentage of macrophages in induced sputum. The percentage of macrophages was calculated on the basis of flow cytometry analysis as described in Figure 2. **A.** Correlation between FEV1/FVC ratio and macrophages analyzed. **B.** Correlation between PC20 values and macrophages analyzed. **C.** Correlation between WBC count/uL and macrophages analyzed. n.s, not significant, using Pearson's correlation test.

Figure E5. Asthmatics with upper airway diseases have lower NCR⁺ILC3s in induced sputum. **A–D.** Comparison among asthmatic patients based on the presence of allergic rhinitis (AR) and chronic rhinosinusitis (CRS). **A.** Frequency of total ILCs (% of CD45⁺ cells) in induced sputum. **B.** Frequency of each subset of ILCs (% of CD45⁺ cells) in induced sputum. **C.** The frequency of total number of macrophages (% of CD45⁺ cells) in induced sputum. **D.** The frequency of M1, M2, and AM (% of CD45⁺ cells) in induced sputum. ***P* < 0.01, using Kruskal–Wallis test, followed by Dunn's multiple comparison test. Values represent the mean ± SD.

Figure E6. Correlations between ILCs and neutrophil and correlations between macrophages and eosinophils or neutrophils in induced sputum. **A.** Correlation between each subset of ILCs and neutrophils. **B.** Correlation between eosinophils and each subset of macrophages in induced sputum analyzed. **C.** Correlation between neutrophils and each subset of macrophages in induced sputum analyzed. **P* < 0.05, ****P* < 0.001, n.s, not significant, using Pearson's correlation test.

Figure E7. Sorting strategy of ILCs and macrophages. The purity of the cells after FACS sorting with BD FACS Aria™ were as follows: **A.** non-ILC2s (CD45⁺Lineage[−]CD127⁺ST-2[−]CD25[−]), 97.3%; **B.** ILC2s (CD45⁺Lineage[−]CD127⁺ST-2⁺CD25⁺), 96.2%; and **C.** Alveolar macrophages (CD45⁺F4/80⁺CD11c⁺), 97.7%.

Figure E8. The level of IL-5 and IL-9 in co-culture of ILC2s and alveolar macrophages. **A.** The protein level of IL-5 and IL-9 in supernatant of co-culture of ILC2s and alveolar macrophages. **B.** Comparison of protein level of IL-5 and IL-9 in ILC2s and alveolar macrophages co-culture system between with and without transwell. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001 using Mann–Whitney test or one-way ANOVA following by a

Bonferroni's post-test. The data are representative of two independent experiments and presented as the mean $\pm$ SEM.

Figure E9. The changes of gene expression related to M1 and M2 macrophages in alveolar macrophages co-cultured with ILCs using anti-PD-1, anti-ICOS, and anti-CD40L blocking antibodies. **A.** The gene expression of M1-related genes in alveolar macrophages, co-cultured with ILC1/3s using anti-PD-1, anti-ICOS, and anti-CD40L. **B.** The gene expression of M2-related genes in alveolar macrophages, co-cultured with ILC1/3s using anti-PD-1, anti-ICOS, and anti-CD40L. **C.** The gene expression of M1-related genes in alveolar macrophages, co-cultured with ILC2s using anti-PD-1, anti-ICOS, and anti-CD40L. **D.** The gene expression of M2-related genes in alveolar macrophages, co-cultured with ILC2s using anti-PD-1, anti-ICOS, and anti-CD40L. $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$ using one-way ANOVA following by a Bonferroni's post-test. The data are representative of two independent experiments and presented as the mean $\pm$ SEM.

Figure E1

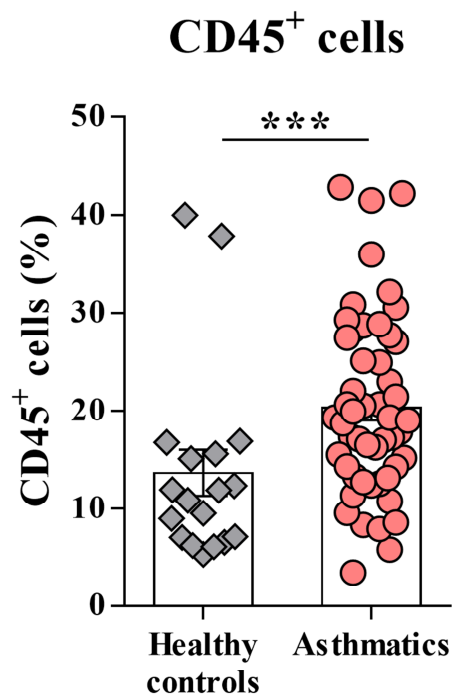
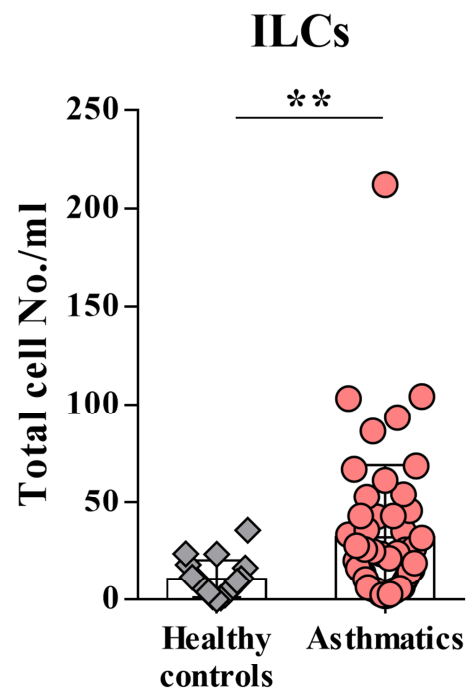
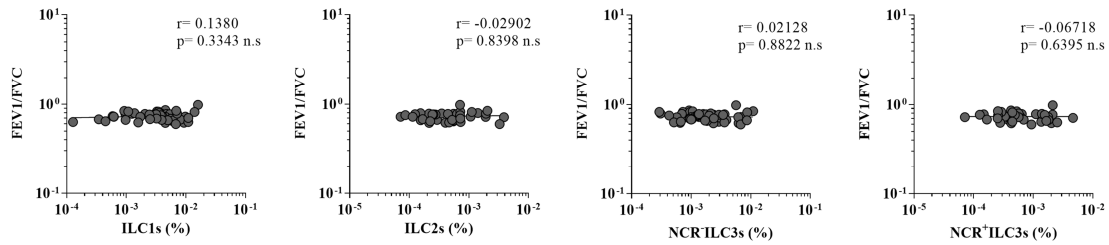
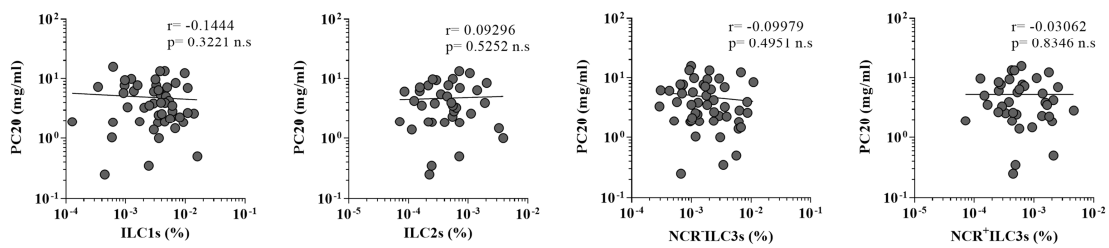
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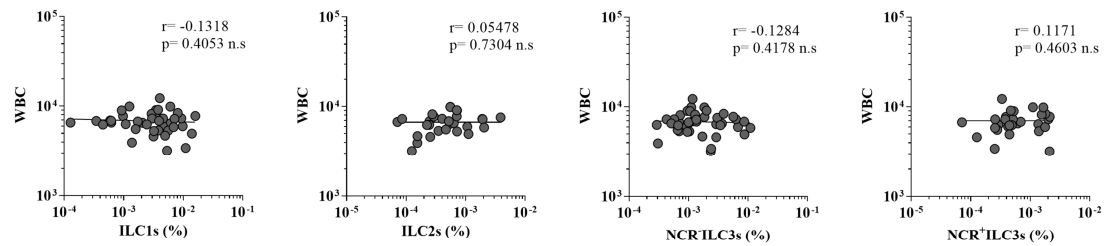
A



B



C



D

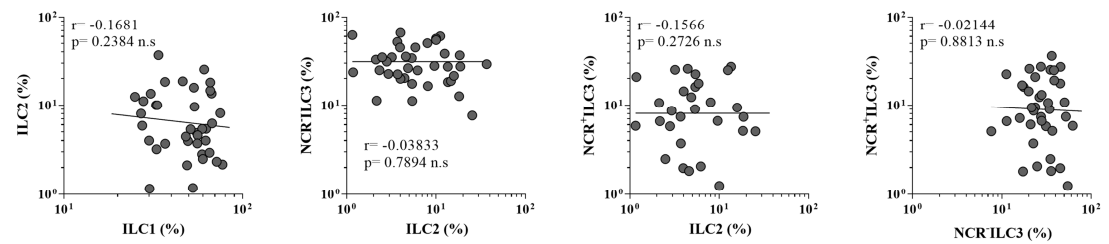


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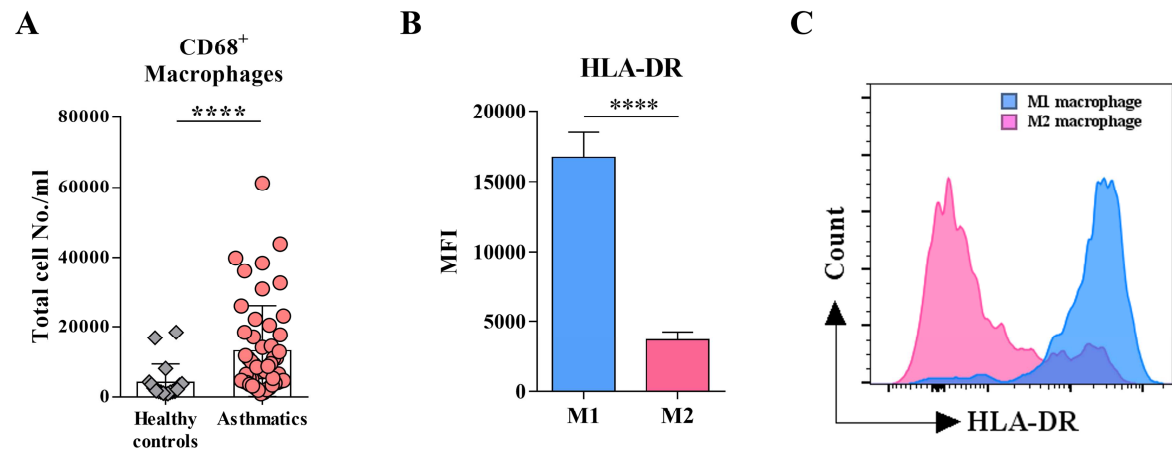


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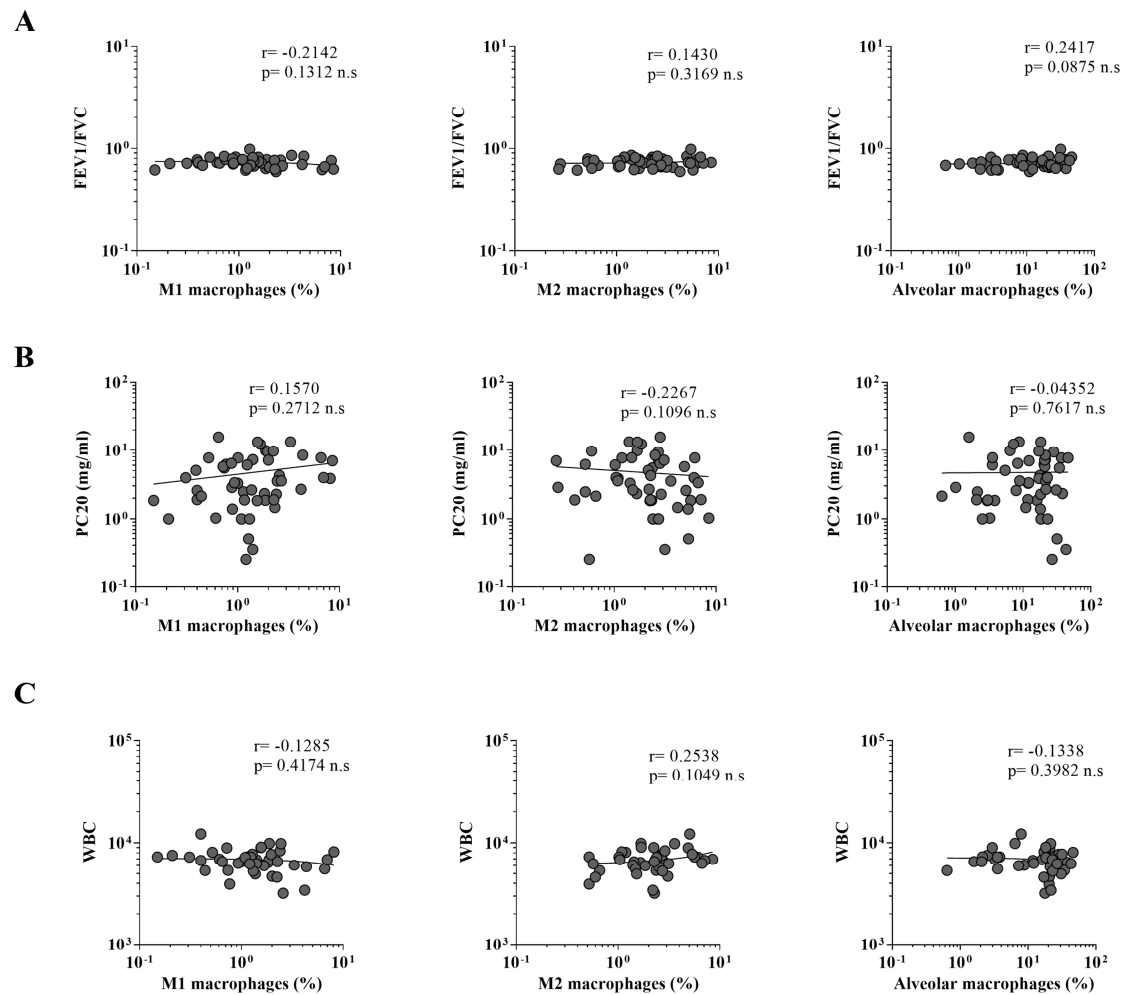


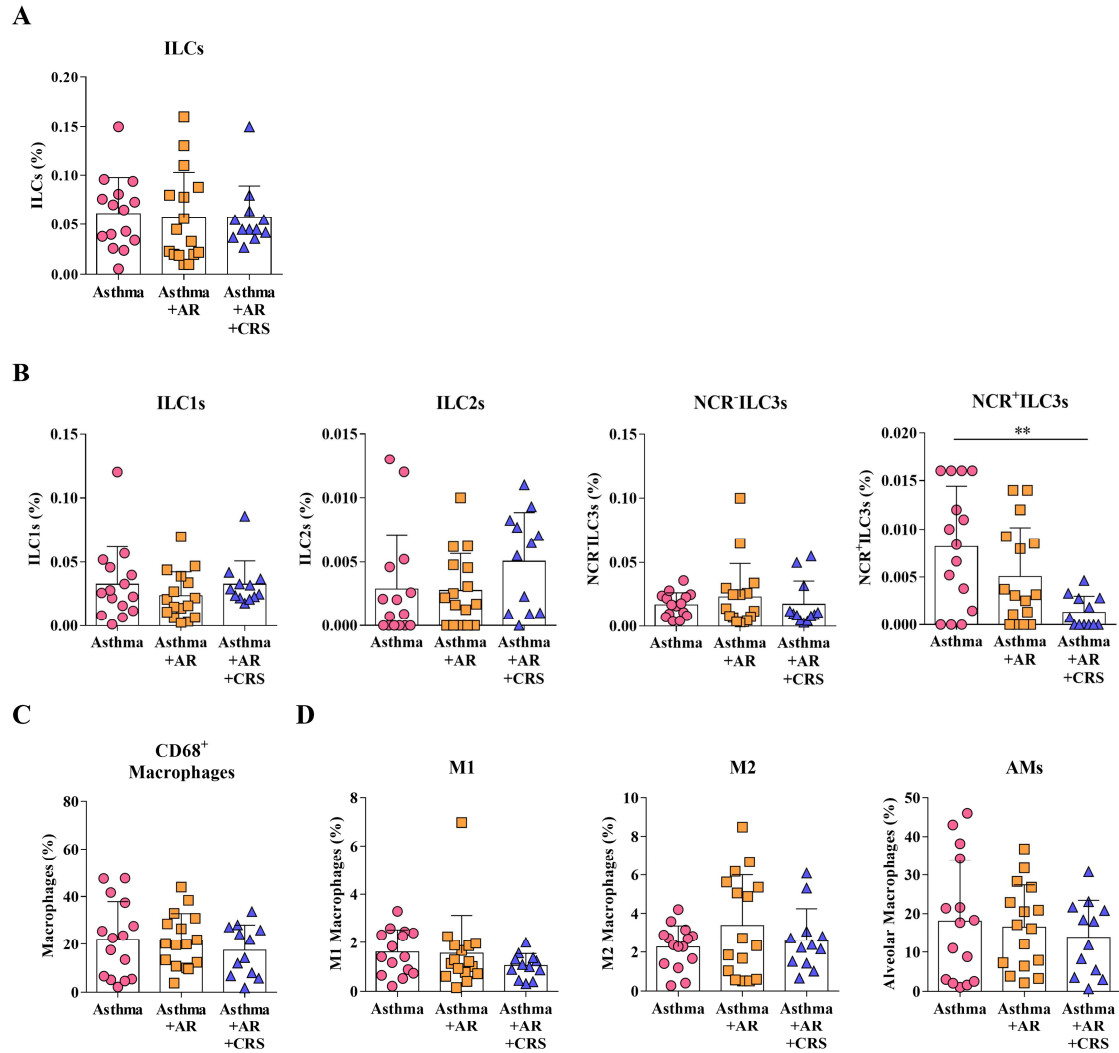
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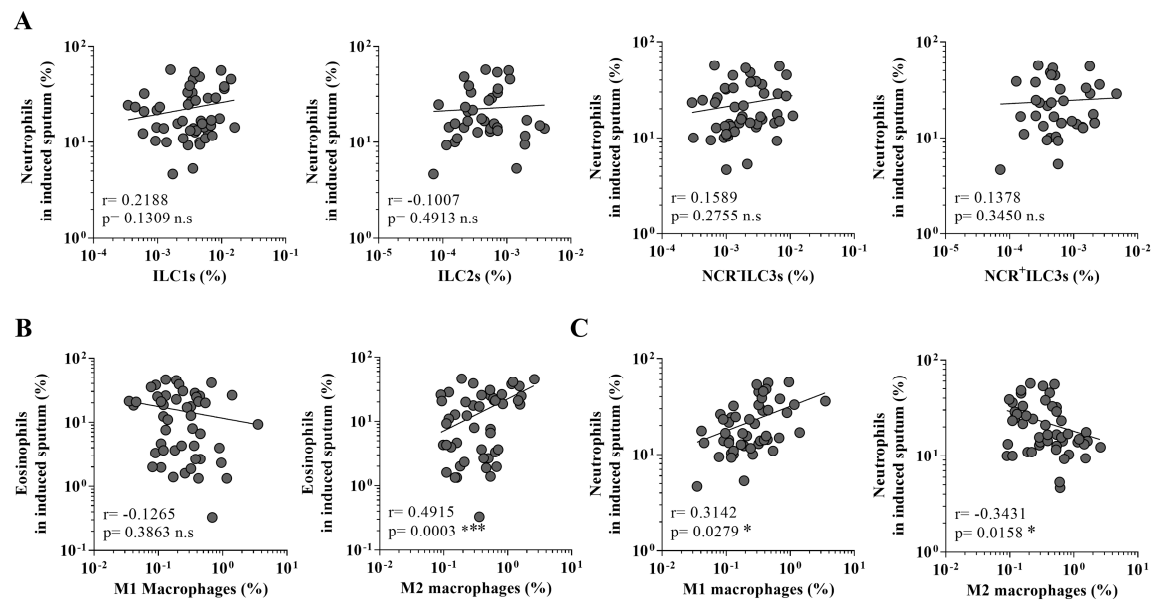
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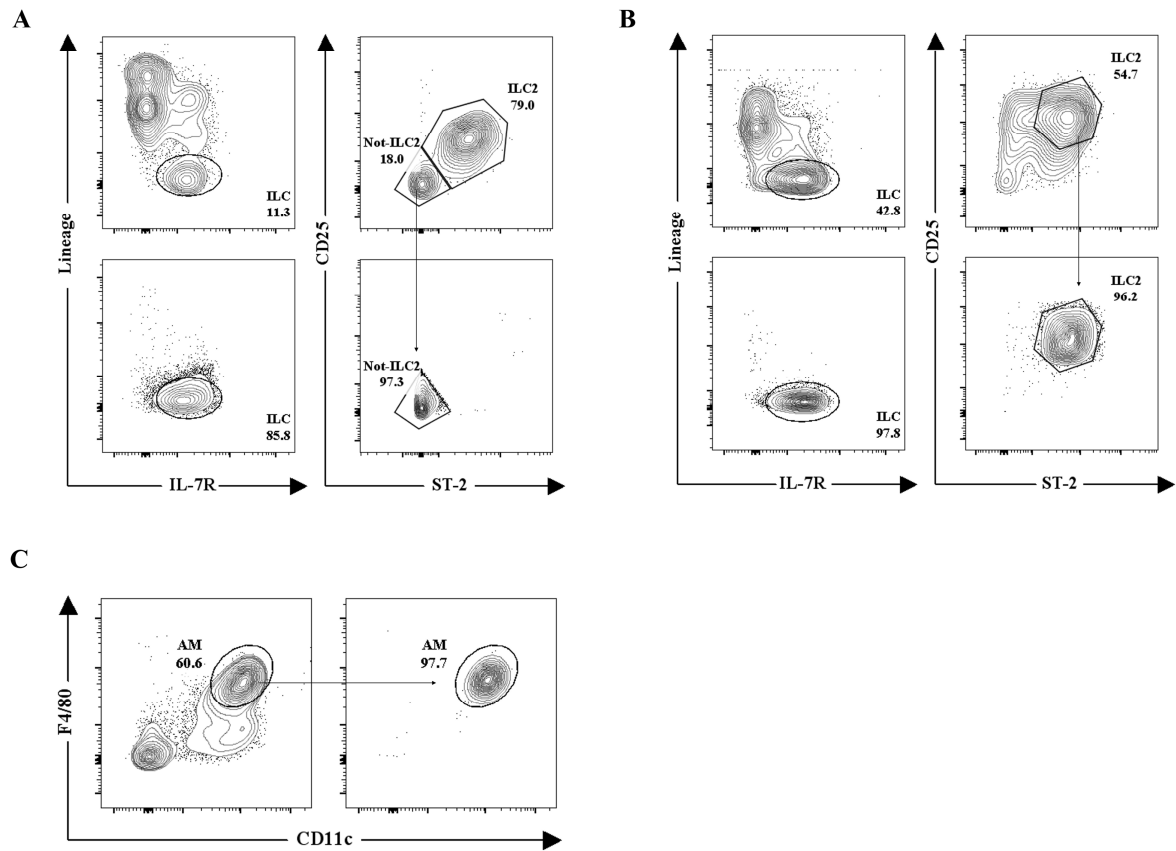
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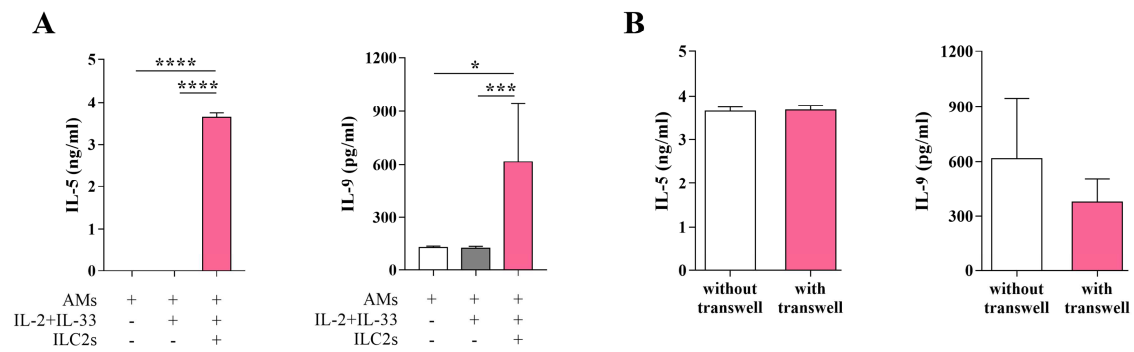
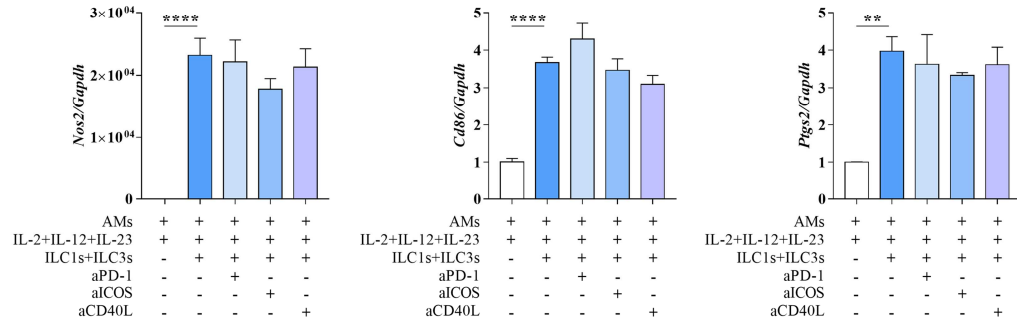
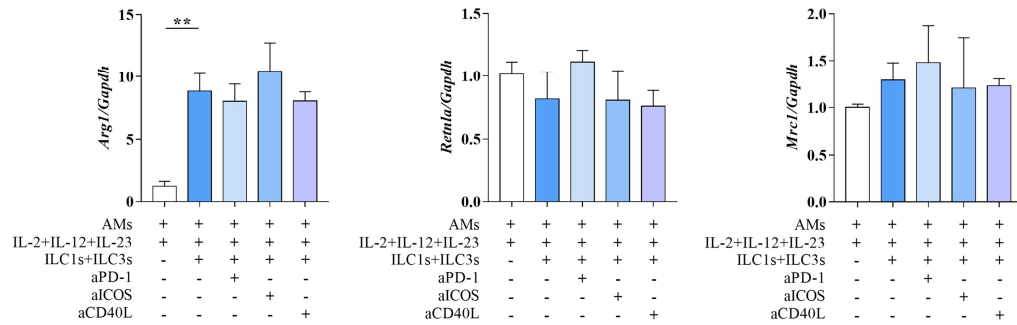


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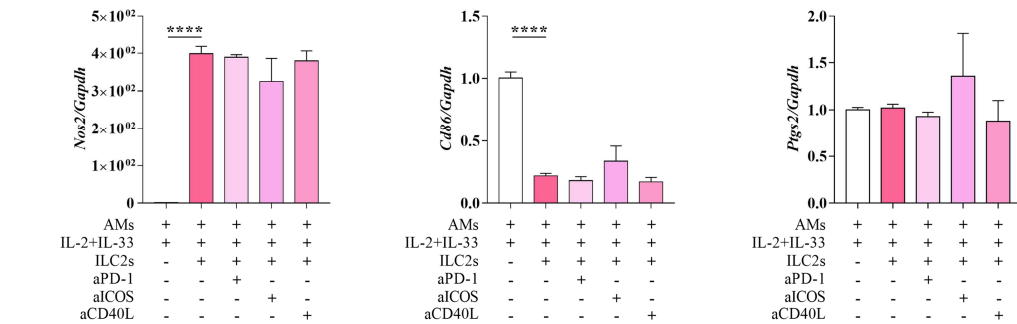
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B AM+ILC1s+ILC3s



C AM+ILC2s



D AM+ILC2s

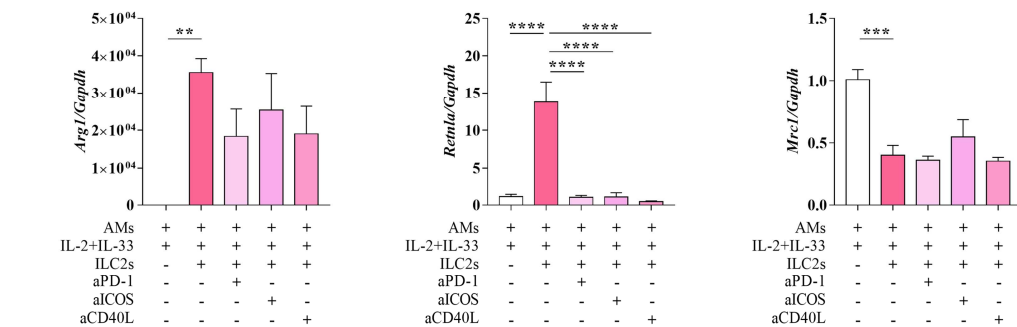


TABLE E1. Comparison of characteristics between non-eosinophilic and eosinophilic asthma patients

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

The data are presented as the mean ± SD.

Abbreviations: FEV1, forced expiratory volume in 1 second; FEV1(%), forced expiratory volume in 1 second/forced vital capacity; FVC, forced vital capacity; PC20, provocation concentration of methacholine leading a fall of 20% of FEV1; WBC, white blood cell

TABLE E2. Comparison of characteristics between asthmatics and asthmatics with other respiratory

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diseases

	Asthma	Asthma+AR	Asthma+AR+CRS	p value
No. of patients	15	16	12	
Age (yr)	60.67±10.15	53.38±20.21	56.75±8.38	0.3813
Sx durations (yr)	4.027±3.61	3.988±4.63	6.633±8.32	0.5891
FEV1	1985±514.2	2133±807.7	2093±641.7	0.8201
FEV1(%)	88.73±14.13	90.81±14.90	84.00±14.21	0.4656
FVC	2806±781.1	2821±990.7	2799±935.8	0.8828
FVC(%)	93.33±17.99	99.63±15.59	94.00±12.01	0.4808
FEV1/FVC	0.7102±0.08	0.7525±0.09	0.7518±0.05	0.2432
PC20(mg/ml)	4.767±4.53	4.944±3.86	3.508±2.22	0.7167
Smoking history (never/ex/current), n(%)	11(73.3)/4(26.7)/0(0)	14(87.5)/0(0)/2(12.5)	10(83.3)/2(16.7)/0(0)	
Induced sputum differential count.				
Macrophages (%)	55.35±15.12	59.81±15.92	57.06±13.35	0.6521
Neutrophils (%)	21.87±10.82	22.63±13.17	17.58±11.44	0.3606
Eosinophils (%)	14.44±14.44	13.57±11.94	21.66±14.71	0.3101
Peripheral blood				
WBC	6775±1654	7272±2022	6413±1435	0.5047
Eosinophils (%)	4.175±3.48	6.227±4.38	6.811±4.46	0.1920
Total IgE	86.13±85.02	228.3±300.4	418.8±482.9	0.1020

The data are presented as the mean ± SD.

Abbreviations: FEV1, forced expiratory volume in 1 second; FEV1(%), forced expiratory volume in 1 second/forced vital capacity; FVC, forced vital capacity; PC20, provocation concentration of methacholine leading a fall of 20% of FEV1; WBC, white blood cell