Investigation of Airway Micro-environmental Cues Modulating Type 2 Innate Lymphoid Cell Activity in Asthma

Xiaotian Ju

McMaster University, jux@mcmaster.ca

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By Xiaotian Ju, BSc

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AUTHOR:	**Xiaotian Ju, BSc (McMaster University)**
SUPERVISOR:	**Roma Sehmi, PhD (McMaster University)**
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LAY ABSTRACT

Asthma is a disease of the airways that makes breathing difficult. About 10% of asthma patients have uncontrolled severe symptoms despite treatment with high doses of corticosteroids which imposes many unwanted side effects. Investigating processes that worsen the disease will help to discover new treatments for asthma. Type 2 innate lymphoid cells (ILC2) are novel cells that produce large quantities of factors which attract and activate effector cells to the lungs which in turn make breathing difficult. This thesis investigated whether controlling ILC2 activity reduces asthma symptoms by studying i) responsiveness of ILC2 to corticosteroids using a controlled allergen exposure through the nose in people with allergic rhinitis and mild asthma, ii) the role of airway nerves and mediators on ILC2 activation, and iii) the ability of signals produced by the lungs to impact factors released by ILC2 and the relationship to effector cells found in the airways of severe asthma. Overall, ILC2 activation can be modulated by corticosteroids, nerve derived factors and lung tissue derived cytokines, and this is associated with changes in the number and type of effector cells in the lungs.

ABSTRACT

Asthma is an inflammatory airways disease affecting over 339 million people of all ages worldwide. More than 10% of asthmatics have uncontrolled severe disease which is insensitive to high doses of oral corticosteroid treatment. Type 2 innate lymphoid cells (ILC2) are pro-inflammatory lymphomononuclear cells proposed as critical drivers of eosinophilic inflammatory disease of the upper and lower airways. Controlling this activity may provide novel therapies for asthma. This thesis aimed to investigate factors that affect the local activation and expansion of ILC2 in the airways including anti-inflammatory medications such as (i) corticosteroids, (ii) neuro-immune regulation of ILC2, and (iii) effect of locally generated cytokines on ILC phenotypes and the relationship to the ongoing airway inflammatory profile.

We firstly investigated the effect of intranasal corticosteroids on activation levels of ILC2 in the upper airway of allergic rhinitics with mild asthma following controlled nasal allergen challenge (Chapter 2). Following pre-treatment with intranasal corticosteroid there was an attenuation in the allergen-induced increase in total ILC2 and IL-5/13^{+} ILC2 in the nasal mucosa. In addition, HLA-DR expression on ILC2 in the nasal mucosa was down-regulated. Overnight culture with IL-2, TSLP or IFN-γ up-regulated HLA-DR expression on ILC2, *in vitro*; an effect that is inhibited in the presence of corticosteroids. Attenuation of HLA-DR expression by ILC2 may be an additional mechanism by which corticosteroids modulate adaptive immune responses in the airways.

We have previously reported that lung ILC2 are activated within 7h following allergen-inhalation challenge. Since airway mucosal tissue is highly innervated, we investigated whether neuroimmune interactions may trigger early and rapid host immune responses (Chapter 3). In a diluent-controlled allergen-inhalation challenge cross-over study, where mild asthmatics

developed early and late bronchoconstrictor responses with sputum eosinophilia (>3%), NMUR1, a receptor for the neuropeptide, neuromedin-U, was up-regulated on sputum ILC2 in 7h post allergen challenge. This was associated with increased expression of IL-5/IL-13 by sputum ILC2 post-allergen and following *in vitro* culture. ILC2 activation was mediated through a MAPK/PI3 kinase dependent-signaling pathway that was attenuated in the presence of dexamethasone. Co-culture with IL-33 and TSLP, *in vitro* up-regulated NMUR1 expression on ILC2 at the protein and transcriptomic level which was attenuated by dexamethasone. The close interplay between neuropeptide signalling and tissue-derived alarmin cytokines may be important interactions for rapid ILC2 activation in airway inflammatory responses in asthma.

We have reported increased ILC2 with the highest level of IL-5/13^{+} ILC2 in the airways of severe asthma with uncontrolled eosinophilia (>3%). The prevalence and phenotypic analyses of innate lymphoid cells subsets in severe asthma with neutrophilic or mixed granulocytic airway inflammatory endotypes remains unclear and was investigated in Chapter 4. Sputum ILC3 were most abundant in severe asthma with neutrophilic airway inflammation where IL-17A^{+} ILC3 correlated with airway neutrophilia. ILC2 were predominant in severe asthma with airway eosinophilia. Importantly, we identified an intermediate ILC2 phenotype displaying ILC3-like markers (c-kit and IL-17A) in severe asthma with neutrophilic and mixed granulocytic airway inflammation. Inflammasome related cytokines, IL-1β and IL-18 were significantly increased in the airways of these patients. At both proteomic and transcriptomic levels, flow sort-purified ILC2 trans-differentiated to the intermediate phenotype when co-cultured with IL-1β+IL-18. Blocking inflammasome-related cytokines may control T2-low severe asthma exacerbations.

Collectively, the findings of this thesis highlight the role of corticosteroids, neuropeptides and airway inflammasome related cytokines as modulators of ILC fate and activity in asthma.

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I am glad to reach the end of my PhD program after spending six brilliant years at McMaster University and in Hamilton, a small town provided me the sense of belongingness. Everything I achieved here would be not possible without the support from my supervisor, committee members, colleagues, friends and family.

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Supplementary Figure E4.8: Gene set enrichment analysis of IL-1β+IL-18 treated ILC2s vs. IL-1β+IL-23+TGF-β treated ILC2s; IL-1β+IL-18 treated ILC2s vs. IL-33+TSLP treated ILC2s and IL-33+TSLP treated ILC2s vs. IL-1β+IL-23+TGF-β treated ILC2s. Genes were pre-ranked based on log2 fold change and adj p value and enrichment score was calculated based on gene set previously reported to be upregulated in **(A)** c-kit$^-$ ILC2s (Bernink *et al. Nat Immunol*. 2019) and **(B)** Th2 cells (Halim L *et al*. *Cell Reports*. 2017). Pathway enrichment analysis using the genes enriched in IL-1β+IL-18 treated ILC2s or IL-33+TSLP treated ILC2s compared with IL-1β+IL-23+TGF-β treated ILC2s. The signalling pathways enriched in IL-1β+IL-18 treated ILC2s or IL-33+TSLP treated ILC2s were shown in **(C).**

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LIST OF ABBREVIATIONS AND SYMBOLS

AA - allergic asthma
AHR - airway hyperresponsiveness
AIC - allergen inhalation challenge
APC - antigen presenting cell
BAFF - B cell activating factor
BAL - bronchoalveolar lavage
CD - cluster of differentiation
CD40L - cluster of differentiation 40 ligand
CGRP - calcitonin gene related peptide
COPD - chronic obstructive pulmonary disease
CRS - chronic rhinosinusitis
DC - dendritic cell
DR3 - death receptor 3
EAR - early asthmatic response
ELISA - enzyme linked immunosorbent assay
FACS - fluorescence activated cell sorting
FcεRI - Fc-epsilon receptor I
FEV_1 - forced expiratory volume in one second
FeNO - fractional exhaled nitric oxide
FMO - fluorescence minus one
FoxP3 - forkhead box p3
FVC - forced vital capacity
FVD - fixable viability dye
GATA3 - GATA binding protein 3
GINA - global initiative for asthma
HC - healthy control
HDM - house dust mite
HLA - human leukocyte antigen
HSC - hematopoietic stem cell
ICOS - inducible T cell co-stimulator
ICS - inhaled corticosteroid
Id2 - inhibitor of DNA binding 2
IF- immunofluorescence
IFN - interferon
IgE - immunoglobulin E
IL - interleukin
ILC- innate lymphoid cell
INCS - intranasal corticosteroid
JAK - janus kinase
KLRG1 - killer cell lectin like receptor subfamily G member 1
LABA - long-acting beta agonist
LAMA- long-acting muscarinic agonist
LAR - late asthmatic response
LPS - lipopolysaccharide

LTRA - leukotriene receptor antagonist
MA - mild asthmatics
MAPK - mitogen activated protein kinase
MCh PC_{20} - provocative concentration of methacholine inducing at least a 20% fall in FEV_1
MHC II - major histocompatibility complex class 2
MyD88 - myeloid differentiation primary response 88
NAC - nasal allergen challenge
NFAT- nuclear factor of activated T cells
NFκB - nuclear factor kappa light chain enhancer of B cells
NK- natural killer
NMU - neuromedin U
NP - nasal polyps
OCS - oral corticosteroid
OVA - ovalbumin
PB - peripheral blood
PC - plasma cell
PNIF - peak nasal inspiratory flow
PRR - pathogen recognition receptor
RNA - ribonucleic acid
ROR - ROR related orphan receptor
ROS - reactive oxygen species
SA - severe asthmatics
SABA - short acting beta agonist
SAC - segmental allergen challenge
STAT - signal transducer and activator of transcription
T-bet - T-box transcription factor
TCR - T cell receptor
TGF - transforming growth factor
TL1A - tumor necrosis factor family member like factor 1A
TLR - toll like receptor
TNF - tumor necrosis factor
TNSS - total nasal symptom score
T_{regs} - regulatory T cells
TSLP - thymic stromal lymphopoietin
VIP - vasoactive intestinal peptide
α - alpha
β - beta
ε - epsilon
γ - gamma
μ - mu

DECLARATION OF ACADEMIC ACHIEVEMENT

The research documented in this thesis is presented as a "sandwich doctoral thesis". The three articles presented in Chapters 2-4 are three independent, but thematically related bodies of research that, as of December 2023, have been or are going to be submitted for peer-review for publication. Although I was the major contributor for the work presented in this thesis, the work required a collaborative effort from several individuals. As such, my contributions, along with those who assisted with the content of each chapter are highlighted and outlined below.

CHAPTER 2

TITLE: Effect of Intranasal corticosteroid treatment on allergen-induced changes in group 2 innate lymphoid cells in allergic rhinitics with mild asthma

AUTHORS: *Yanqing Xie, *Xiaotian Ju, Suzanne Beaudin, Lesley Wiltshire, John Paul Oliveria, Jonathan MacLean, Doron D. Sommer, Ruth Cusack, Olga Li, Prajna Banerjee, Paul K. Keith, Paul M O'Byrne, Rebecca N. Bauer, Tracy Staton, Gail M Gauvreau, Roma Sehmi
* These authors contributed equally to this publication

CORRESPONDING AUTHOR: Drs. Roma Sehmi and Gail Gauvreau, Cardiorespiratory Research, Department of Medicine, McMaster University, Hamilton, Ontario, Canada, Email: sehmir@mcmaster.ca; gauvreau@mcmaster.ca

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AUTHOR CONTRIBUTION: Conception and design: YX, XJ, SB, LW, JPO, JM, DDS, OL, PB, PKK, PMO, RNB, TS, GMG, RS; Patient Recruitment, Sample Collection and Conducting Experiments: YX, XJ, SB, JPO, JM, DDS, RC; Analysis and interpretation: YX, XJ, PMO, RNB, TS, GMG, RS; Drafting the manuscript for important intellectual content: XJ, JPO, PMO, RNB, GMG, RS.

SCIENTIFIC CONTRIBUTION: Following the nasal allergen challenge in allergic rhinitics with mild asthma, ILC2 in nasal mucosa were rapidly activated and the allergen induced ILC2 were well controlled by intranasal corticosteroid treatment. The results from this chapter suggested a steroid sensitive ILC2 in the upper airway at early stage of allergic rhinitis and it is necessary to control the ILC2 to prevent the progression of inflammation into the lower airway and cause more severe symptoms.

CHAPTER 3

TITLE: Neuromedin-U/ NMU-R1 Axis Mediates Rapid Activation of Group 2 Innate Lymphoid Cells in the Airways of Subjects with Mild Asthma following Inhaled Allergen Challenge

AUTHORS: *Xiaotian Ju, *Akimichi Nagashima, Jennifer Wattie, Karen Howie, Guillaume Paré, Reina Ditta, Anna Dvorkin-Gheva, Ruth Cusack, Imran Satia, Paul M O'Byrne, Gail M. Gauvreau and Roma Sehmi
* These authors contributed equally to this manuscript

CORRESPONDING AUTHOR: Dr. Roma Sehmi, McMaster University, HSC 3U31D, 1200 Main St West, Hamilton, ON, Canada L8N 3Z5, Email: sehmir@mcmaster.ca

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AUTHOR CONTRIBUTION: Conception and design: XJ, AN, RC, POB, GMG, RS; Patient Recruitment, Sample Collection and Conducting Experiments: XJ, AN, RC, JW, KH, GP, RD, IS; Analysis and interpretation: XJ, AN, ADG, POB, GMG, RS; Drafting the manuscript for important intellectual content: XJ, AN, GP, POB, GMG, RS.

SCIENTIFIC CONTRIBUTION: This chapter investigated the rapid activation mechanism of ILC2s through NMU-NMUR1 axis within 6 hours following the allergen challenge in mild asthmatics. Also, the NMU induced ILC2 activities were inhibited by corticosteroid or the inhibitors against the downstream signaling mediators of NMUR1 including calcineurin, mitogen activated protein kinase or nuclear factor of activated T cells. Considering the side effect of corticosteroid on asthmatics, the identification of NMU- NMUR1 axis suggested a novel therapeutic target against the early activation of ILC2 in mild allergic asthma as a preventive therapy.

CHAPTER 4

TITLE: Increased Levels of Group 3 Innate Lymphoid Cells and c-kit$^+$ IL-17A$^+$ ILC2 in Severe Asthma with Neutrophilic or Mixed Granulocytic Airway Inflammation: evidence of IL-1β+IL-18 driven ILC plasticity

AUTHORS: Xiaotian Ju, Nahal Emami Fard, Anurag Bhalla, Anna Dvorkin-Gheva, Maria Xiao, Katherine, Radford, Kayla Zhang, Reina Ditta, John Paul Oliveria, Guillaume Paré, Manali Mukherjee, Parameswaran Nair and Roma Sehmi

CORRESPONDING AUTHOR: Dr. Roma Sehmi, McMaster University, HSC 3U31D, 1200 Main St West, Hamilton, ON, Canada L8N 3Z5, Email: sehmir@mcmaster.ca

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SCIENTIFIC CONTRIBUTION: The different frequencies of ILC2 and 3 in severe asthmatics with eosinophilic, neutrophilic or mixed granulocytic airway inflammation suggested that the inflammatory profiles in the airway gave rise to ILC with different phenotypes and functions. Also, it is the first time to identify sputum ILC3 as an important source of IL-17A in severe asthmatics with neutrophilic airway inflammation and IL-17A$^+$ ILC3 were correlated with airway neutrophilia. Of note, an intermediate ILC2 expressing ILC3 markers potentially induced by IL-1β+IL-18 was detected in severe asthma with neutrophilic and mixed granulocytic airway inflammation. Therefore, modulation of ILC3 activation and trans-differentiation of intermediate ILC2s may provide important therapeutic targets for controlling non type 2 severe asthma exacerbations.

CHAPTER 1: Introduction

1.1 Asthma

1.1.1 Epidemiology

Asthma is a chronic airway disease affecting over 300 million people of all ages worldwide, and the increasing number of asthmatic patients is predicted to reach 400 million by 2025 (1). In Canada, more than 8% of the population is reported to have asthma, and the 20-year direct total health and socioeconomic cost of asthma from 2014 to 2034 is predicted to be over 24 billion dollars (2). The symptoms of asthma include recurrent episodes of cough, wheeze, chest tightness, and shortness of breath due to airflow limitation. This is caused by airway constriction, bronchial inflammation, and the excessive narrowing of airways in response to external stimuli termed airway hyperresponsiveness (AHR) (3)(4). Furthermore, the clinical features of asthma consist of goblet cell metaplasia, mucus over-production, airway smooth muscle hyperplasia, and increased vascular permeability (5). There are several comorbidities related to asthma, including rhinitis, sleep apnea and cardiac disease which severely affects patient quality of life and increases the frequency of hospital admission or emergency care visits (5). These asthma-related impacts have imposed a burden on both personal life and social productivity. The prevalence of asthma varies across geographic regions. Compared to Africa and Asia, the incidence of asthma is more prevalent in developed countries, especially America and Europe (6). Of note, Canada is one of the countries in which the asthma incidence rate is significantly higher than average, motivating scientists and clinicians to investigate the etiology of asthma.

The etiological factors of asthma include genetic (high susceptibility genes), environmental (e.g. allergens, pollution), and host (e.g. obesity, bacterial/viral infection, exercise) factors (7). The genetic polymorphism related to asthma were revealed in a systemic genome wide association

study consisting of more than 10,000 subjects with asthma. As a result, the polymorphism of genes encoding interleukin (IL)-18 family cytokines (*IL33*) and their receptors (*IL1RL1* and *IL18R1*) were significantly associated with asthma as compared to unaffected subjects (8). In addition, the polymorphism of type 2 inflammation related genes including *IL13*, human leukocyte antigen-DQ (*HLA-DQ*), IL-2 receptor subunit beta (*IL2RB*) and ROR Related Orphan receptor A (*RORA*) were also highly associated with asthma (8). Furthermore, the seasonal trend of asthma was studied and showed that atopic asthmatics had more severe symptoms during the pollen seasons (mainly spring and summer) (9). Also, the prevalence of influenza and dry air during the winter triggered these symptoms, especially in aged asthmatics. Additionally, along with the influenza virus, rhinovirus also increased the risk of asthma development and triggered the exacerbation of acute asthma attack in asthmatics of all ages (9). Physically, the excess adiposity around the chest wall restricted the total lung capacity and expiratory reversibility, leading to a reduced lung function (10). The association of obesity with asthma was also revealed by reduced lung function in overweight (25 < body mass index (BMI) < 29 kg/m^2) or obese (BMI>30 kg/m^2) asthmatics compared to people of a normal weight (11). Also, weight loss and exercise led to an improvement in lung function (10). In addition, the incidence of asthma might be regulated by sex hormones, due to the fact that a higher asthma incidence was detected in males than females during childhood, and this sex difference is inversed after puberty and then ceased once again after the 5^{th} decade of life (7).

The age difference in asthma incidence was observed when the endotype of asthma was further categorized based on atopy. The highest incidence rate of allergic asthma is detected in children aged from 0-9 years old suggesting an early onset of allergic asthma in a progressive manner due to the encountering of allergens before the pulmonary system is matured (12). The atopic march theory described the progressive development of allergic asthma. Eczema (atopic

dermatitis) is usually the first sign of allergy during the infant stage and is initiated by food allergy. The atopy may proceed into allergic asthma during childhood when airborne allergens stimulate inflammation in the airway (13). Furthermore, there is a high risk for children with allergic asthma to develop allergic rhinitis (14). In contrast, non-allergic asthma has a late-onset and the incidence increases with age, peaking at 50-59 years old and is usually induced by the encounter of occupation-associated reagents and pollution (15). Hospitalization rates are also noted to be higher in asthmatics younger than 5 years old or older than 65 years old (6). Therefore, it is important to diagnose and prevent the development of asthma at an early stage with medical management to prevent the potential risk of developing more severe asthma.

1.1.2 Asthma Diagnosis

Asthma is diagnosed by the medical history of patients, physical examination and objective expiratory airflow limitation test (16). The medical history includes the presence of asthma related symptoms (e.g. allergy, eczema), family history of asthma, and experience of exposure to environmental or occupational triggers (e.g. pollens, smoke, chemicals). Following the medical history, physical examination of the skin (e.g. skin prick test), nose and upper airway for the clinical signs of altered lung function, including wheezing and coughing are helpful for asthma diagnosis (5).

During the clinical examination, reduced lung function is directly assessed by the airflow obstruction test. Spirometry is used to measure the airflow parameters including forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC). Asthma is typically defined by FEV_1< 80% of predicted value or FEV_1/FVC < 75% (5) with the airflow obstruction being reversible in that there is a ≥15% improvement in FEV_1 in response to the administration of a

bronchodilator (4)(16). Apart from the objective airflow limitation test, the airway hyperresponsiveness (AHR) test is also necessary for asthma diagnosis using constrictor agonists such as methacholine or histamine. The hypersensitivity of the asthmatic airway in response to methacholine or histamine is reflected by the concentration (PC_{20}) or dose (PD_{20}) of the inhaled bronchoconstriction stimuli causing a 20% reduction in FEV_1 (17). The commonly accepted threshold of methacholine PC_{20} for asthma diagnosis is less than 16mg/ml (18)(19) and a strong correlation was observed between PC_{20} and asthma severity.

Additionally, there are non-invasive assessments for bronchial inflammation including differential cell counts assessing eosinophil and neutrophil numbers in peripheral blood, bronchioalveolar lavage and induced sputum, or more invasive methods such as bronchial biopsy (3). Collectively, the physical and clinical tests for the diagnosis of asthma assist doctors in determining the severity of asthma and medications to prescribe.

1.1.3 Asthma Severity and Exacerbation

The severity of asthma is categorized based on the frequency of symptoms, lung function test, response to treatment and the intensity of inflammatory features. According to the global initiative for asthma (GINA)-guidelines, asthma severities were divided into three categories: mild asthma, moderate asthma and severe asthma. Patients with mild asthma have an FEV_1>90% of predicted value with asthma associated symptoms occurring more than twice a week (5). Patients with diminished lung function (FEV_1 between 80% to 90%) and symptoms persisting on a daily basis, along with nighttime waking, are diagnosed as having moderate asthma (5). Severe asthmatics have a significantly reduced lung function, registered as FEV_1<80% with continuous symptoms that can be controlled with high dose inhaled corticosteroids and oral steroid therapy;

however more than 10% of severe asthmatics continue to have symptoms despite receiving high dose oral corticosteroid treatment (5)(20). An asthma exacerbation is a progressive worsening of symptoms with loss of lung function from the patients' usual status (under instructed therapy management) which could occur in patients of all severities, but a higher rate of acute exacerbations are observed in patients with severe asthma (21). During the seasonal asthma exacerbations in autumn and winter, human rhinovirus (type A and C) and influenza virus are the two frequent triggers. In contrast, grass pollen and mold spore are active triggers for asthma exacerbation during the allergen season in spring (21). Other potential triggers may include bacterial infections and environmental pollutants such as ozone and cigarette or wild-fire smoke.

1.1.4 Clinical Model of Asthma Study

The baseline immune profile from the blood or airways of asthma sufferers provides only limited insight on the immune response during an asthmatic response or exacerbation. Thus, it is important to apply an *in vivo* stimulatory model to evoke the immune response in the airway of asthmatic patients in a non-invasive manner for research purposes to understand the chronology of the inflammatory response and associated physiological lung changes. The inhaled nasal allergen challenge and whole lung challenge are commonly used to investigate the active type 2 (T2) inflammatory response in the upper and lower airways, respectively. Prior to allergen inhalation challenge, the allergen to which asthmatics are most responsive is determined by a skin prick test. In a wheal and flare response, the allergen causing the biggest response compared to histamine is selected (22). In patients with allergic rhinitis, an intra-nasal allergen challenge induces inflammation in the upper airway. The upper airway inflammatory response is monitored using a total nasal symptom score (TNSS) and peak nasal inspiratory flow (PNIF)(22). On the

other hand, the whole lung challenge to the lower airway is performed using inhalation through a nebulizer and is monitored by the decline of lung function (FEV_1), and the development of airway hyperresponsiveness measured by changes in FEV_1 to a methacholine challenge (PC_{20}).

Following the allergen challenge, two types of allergic responses were normally observed, termed as early and late asthmatic response (EAR and LAR). Mast cell are the primary orchestrators of EAR by releasing histamine, lipid mediators and T2 cytokines in response to surface bound immunoglobulin (Ig)-E cross-linking, which increases vascular permeability, smooth muscle contraction causing bronchoconstriction and some mucus production (3). The EAR is defined by $\geq$ 20% fall in FEV_1 within 10 minutes and resolution within 2 hours after allergen inhalation challenge (23). About 60% of asthmatics proceed to LAR which occurs 3-8 hours after allergen inhalation. This response is associated with an increase in AHR to methacholine challenge measurable at 24h post-challenge and sustained for 2-3 weeks. The LAR is mostly mediated by a cluster of differentiated $(CD)4^+$ Th2 cells and mediators released by pro-inflammatory cells, primarily eosinophils and basophils (23). Eosinophil-derived granule proteins and mediators such as major basic protein, eosinophil cationic protein, leukotrienes and cytokines cause the pathological features of asthma, including tissue remodeling and bronchial hyperresponsiveness (24).

A human model of segmental allergen challenge study was recently conducted by Alladina *et al*. According to the singe cell ribonucleic acid (RNA) analysis on bronchial mucosa from the lower airway of allergic asthmatics, an amplified T2 airway inflammation was observed at the transcriptomic level post allergen challenge. Type 2 helper T cells (Th2) simulated the pro-inflammatory functions of mast cells and T2 dendritic cells; Th2 cells produced IL-4 and 13 to promote goblet cell metaplasia and bronchial epithelium cell remodeling (25). Of note, the IL-4/13

from Th2 cells induced a secretion of matrix metalloproteinase (MMP) from a plastic mononuclear phagocyte to maintain the airway inflammation post allergen challenge (25). Thus, investigating the intricated cellular and molecular makeup underlying the airway inflammation, known as endotype, would help to better understand the pathogenesis of asthma.

1.1.5 Asthma Endotypes

It is important to identify the pro-inflammatory factors mediating the pathogenesis of asthma at molecular and cellular levels for future therapeutic development. The inflammatory profiles including mediators and immune cells underlying the airway hyperresponsiveness are termed as endotype which is mainly subdivided into type 2 (T2) and non-type 2 (non T2) (26).

Around 80% of asthmatic patients have T2 airway inflammation that is induced in an allergen or non-allergen specific manner (27)(28). The mild to moderate T2 asthma is early onset in response to allergens (e.g. house dust mite, dander, pollens), whereas the late onset T2 asthma leads to severe disease mainly induced by viral infection, pollution or exercise (29). The clinical identification of T2 airway inflammation is based on two typical biomarkers (indicators): the eosinophil level (≥300 cells/μL in blood or ≥3% differential cell count in sputum) and fractional exhale nitroxide (FeNO) (≥20 parts per billion) which is the nitroxide released from airway epithelium in response to T2 inflammatory cytokines IL-4 and 13 (30) (**Figure 1.1**). Other biomarkers such as serum IgE and periostin also relate to the diagnosis of T2 asthma (31). The interaction between pro-inflammatory cytokines (IL-33, IL-25, TSLP, IL-4, IL-5 and IL-13) and immune cells (Th2 cells, type 2 innate lymphoid cells (ILC2), dendritic cells, mast cells, basophils and eosinophils) leads to the pathogenesis of T2 inflammation (28). The acute allergic response is mediated by mast cells and basophils through the crosslink of the pre-loaded IgE on the cell surface

by allergens which contribute to a rapid activation of the granulocytes and a release of inflammatory mediators (3). As the major effector granulocyte in the pathogenesis of T2 airway inflammation, the activated eosinophils release cytotoxic proteins (e.g. major binding proteins, eosinophil peroxidase, cationic proteins, eosinophil derived neurotoxins), acute inflammatory proteins (e.g. tumor necrosis factors-α (TNF-α), IL-1β, 6, 8) and cytokines (e.g. IL-4, 5, 13, 9, 25) to enhance the airway inflammation, goblet cell metaplasia and airway smooth muscle thickening (3)(12). Recent studies reported that eosinophils can release extracellular traps (EETs) against pathogens (bacteria, fungi and parasites) and EETs can further bind Charcot Leyden crystals also known as Galetin 10 to alter the composition of mucus making it more cheese-like or elastic gel like (12). Therefore, the eosinophil is considered a critical player as an inflammatory effector cell in T2 airway inflammatory diseases such as asthma and allergic rhinitis.

Non-T2 asthma accounts for about 25% of asthmatic patients and is more commonly observed in asthmatics with moderate to severe disease (about 70%) (32). Non-T2 asthma usually has a late age onset and is induced in a non-allergic manner. Viral/bacterial infection and cigarette smoking are major stimuli leading to lung function loss, attenuated corticosteroid efficacy and increased AHR (12)(32). Also, many overweighted asthmatics are characterized by non-atopic, late-onset asthma symptoms with low eosinophilia in blood and airway, suggesting a high association between obesity and non-T2 asthma (10). Clinically, non-T2 asthma is diagnosed with normal T2 phenotypes including low T2 cytokine level, normal eosinophil (< 2% sputum eosinophilia) and FeNO (< 25 ppb) level in the airways (12). There are two types of clinical features of non-T2 asthma based on differential cellular makeup in the airway: neutrophilic and pauci-granulocytic (31) (**Figure 1.1**). Unlike eosinophils as a signature cell in T2 inflammation, there is no definitive clinical biomarker to diagnose non-T2 inflammation, but a high level of

airway neutrophils is frequently observed (>61% of differential cell count) in neutrophilic non-T2 asthma along with a high MMP9 production (33)(34). As the effector cytokines for neutrophil recruitment, increased levels of IL-17 and IL-22 are observed in asthmatic patients with non-T2 inflammation. Although the clinical symptoms are similar between asthmatics with different pathological endotypes, the different immuno-pathological mechanisms underlying the symptoms may influence the effectiveness of medications on these asthmatic patients. Therefore, understanding the asthma endotypes has the potential to assist scientists in developing therapeutical targets (e.g. pro-inflammatory immune cells or cytokines) for novel asthma therapies.

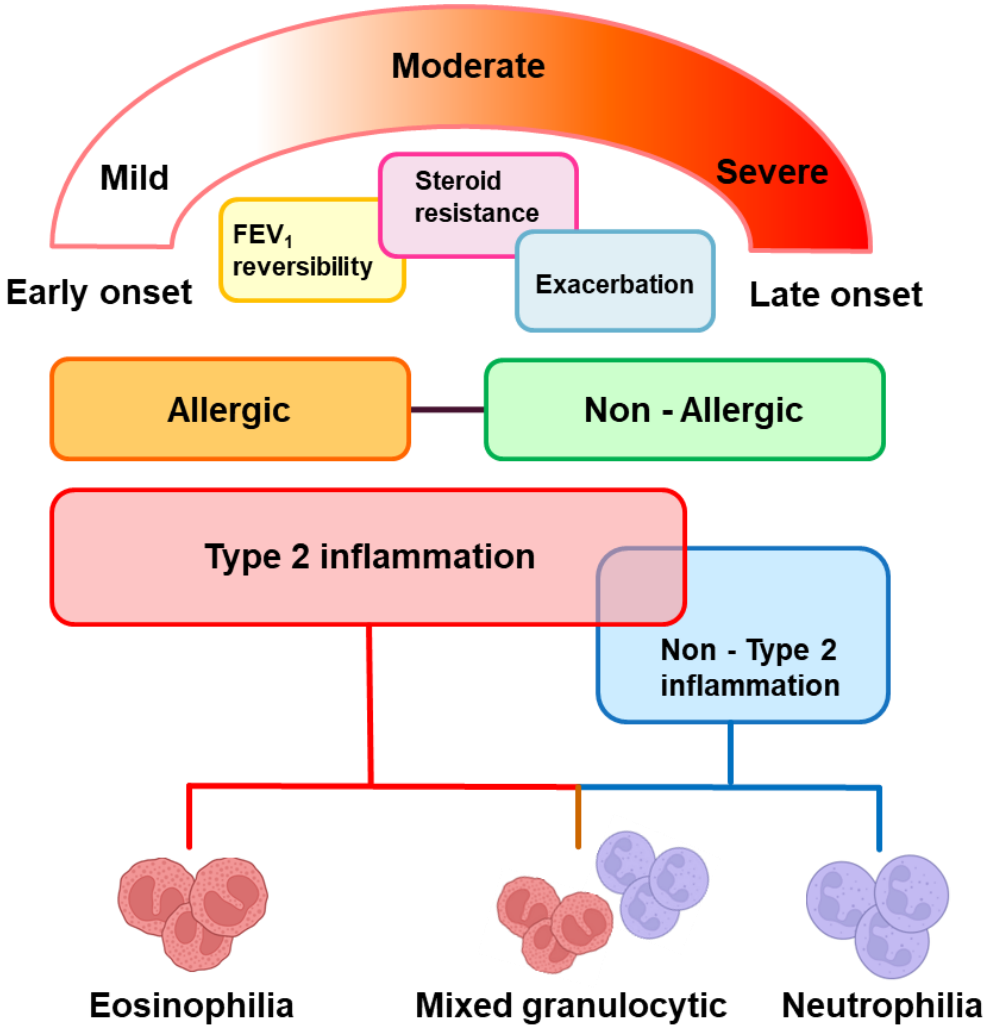

Figure 1.1 Summary of asthma endotypes. (modified based on (35)**)**
About 80% of mild asthmatic patients have type 2 (T2) airway inflammation stimulated in allergen or non-allergen specific manner. The mild to moderate T2 asthma is early onset and the late onset T2 asthma leads to severe disease which is resistant to steroid with more frequent exacerbation rate. The eosinophil is the major effector granulocyte in the pathogenesis of T2 airway inflammation. Furthermore, non-T2 airway inflammation is usually late age onset and accounted for 70% of asthmatics with moderate to severe disease induced in a non-allergen specific manner. The high level of airway neutrophils is a potential biomarker for non-T2 asthma. In severe asthmatics, there are four endotypes of airway inflammation: eosinophilic, neutrophilic, mixed granulocytic and pauci-granulocytic.

1.1.6 Therapeutic Managements for Asthma

Currently, there is no cure for asthma, but long-term medical management can be applied to improve lung function and quality of life for patients with asthma. According to the GINA guideline, an effective therapeutic strategy for asthma should control the symptoms and reduce the risks of asthma related mortality, exacerbation and comorbidity (5). The current pharmacological therapies are divided into two categories: relievers and controllers.

As the most common therapy for asthma, about 80% of asthmatic patients with mild symptoms have their symptoms well controlled by bronchodilators (pharmacologic relievers) including short and long-acting β_2 agonist (SABA and LABA) (16). The intermittent administration of SABA (e.g. salbutamol, salmeterol) rapidly induces the relaxation of smooth muscle in the airways and relieves some symptoms of airway constriction (5). In contrast, LABA (e.g. formoterol) requires daily administration in combination with an inhaled corticosteroid (ICS) because LABA itself contains a high risk of asthma related morbidity (36). During an asthma exacerbation, a combination of LABA and inhaled corticosteroid (ICS) is given along with the additional administration of inhaled long-acting muscarinic antagonist (LAMA) (e.g. tiotropium bromide), which blocks muscarinic receptors (M1-M3) from binding to acetylcholine (37). Other than β_2 agonist, leukotriene receptor antagonist (e.g. montelukast, zafirlukast) is a long-term medication inhibiting the leukotriene signalling pathway which is active in type 2 asthma (38). Furthermore, corticosteroids (inhaled or oral) are a gold standard pharmacologic controller which effectively inhibit the synthesis of pro-inflammatory mediators (cytokines/chemokines) and induce the apoptosis of inflammatory cells (especially eosinophils). Inhaled corticosteroids are prescribed for persistent long-term use, and about 50% of asthmatic patients are well controlled by ICS (e.g. fluticasone) with low side effects (5). For asthmatics with moderate-severe disease or

intermittent exacerbations, oral corticosteroid (OCS) (e.g. prednisone) are prescribed for a short period of time and systemically attenuate the inflammation. However, about 5 to 10% of asthmatic patients with uncontrolled severe asthma are resistant to corticosteroid with less than 15% recovery of FEV_1 after a two-week period of adequate OCS treatment (40 mg) (20) and the high dose OCS may cause significant side effects including weight gain and diabetes (39).

Type 2 biologic medications have been recently developed as a novel therapy which target mediators involved in the pathogenesis of severe asthma with minimized adverse complications as seen with steroids(5). Omalizumab is an anti-IgE immunoglobulin prescribed for T2 (eosinophilic) severe asthmatics with high blood eosinophilia (>150 cells/µl blood) and serum IgE levels. A significant reduction of serum IgE levels and Fc-epsilon receptor I (FcƐRI) on basophils or mast cells was observed in severe asthmatics treated with omalizumab followed by an attenuation of allergic response and asthma exacerbation (40). Furthermore, reslizumab and mepolizumab inhibit the development of eosinophils by targeting free IL-5. A randomized placebo-controlled trial of reslizumab revealed a significant reduction in exacerbation rate along with improved lung function in patients with severe eosinophilic asthma compared to placebo (41). Similar beneficial effects were also observed in a clinical trial using mepolizumab (42). In addition, benralizumab is a monoclonal antibody binding to the IL-5 receptor and leading to a depletion of IL-5R expressing cells through antibody dependent cell mediated cytotoxicity (ADCC) mediated by nature killer cells (NK cells) (43). A significantly reduced oral glucocorticoid dose and asthma exacerbation rate was observed in subjects treated with benralizumab during a randomized placebo controlled trial in severe asthmatics with raised airway eosinophilia (43). Apart from the IL-5/IL5Rα axis, another biologic, dupilumab, is developed to block the IL-4Rα which is a mutual receptor sub-unit for type 2 effector cytokines IL-4 and IL-13. In a randomized placebo controlled clinical trial, a

significantly lower exacerbation risk along with improved lung function was reported in eosinophilic severe asthmatics treated with dupilumab compared with placebo (44). Biologics targeting epithelial derived alarmins are also actively under clinical investigation. More recently, tezepelumab is a monoclonal antibody targeting TSLP which is an epithelial derived cytokine initiating T2 airway inflammation. A placebo controlled clinical trial of AMG157 was conducted on patients with mild allergic asthma. The allergen challenge induced increases in eosinophils (blood and sputum) and FeNO was significantly attenuated by AMG157 (45); and the lung function (FEV_1) of mild asthmatics in response to allergen challenge was significantly improved by the pre-treatment with AMG157 (45). Similar results were also found in two phase 2 studies on moderate-severe asthmatics (regardless of the baseline blood eosinophil level) where a significant reduction of airway hyperresponsiveness to mannitol was observed in the tezepelumab treatment group, along with a significant decrease in eosinophils in airway submucosa or BAL (46)(47). The non-endotype specific benefits of tezepelumab were further revealed by a phase 3 placebo-controlled trial in which tezepelumab imposed a beneficial effect on severe asthmatics with high (>300 cells/μl) or low blood (<300 cells/μl) eosinophil level, including a significantly reduced asthma exacerbation rate, an improvement in lung function (FEV_1 and asthma control questionnaire (ACQ)) and significantly reduced inflammatory biomarkers (blood eosinophil level, IgE and FeNO) (48). Therefore, tezepelumab is the first biological medication which improved lung function and attenuated the AHR in asthmatics of all severities, regardless of endotypes of airway inflammation. However, no significance was detected on the oral corticosteroid sparing effect imposed by tezepelumab, but only a trend of a higher reduction of oral corticosteroid administration was observed in severe asthmatics with baseline blood eosinophil levels higher than 150 cells/μl (49). More recently, a phase 2 trial of itepekimab, a monoclonal antibody against IL-

33, was conducted in moderate-severe asthmatics (50). As a result, the uncontrolled incidence rate was significantly reduced by itepekimab, along with an increase in FEV_1 compared with placebo. However, the blood eosinophil level was not reduced in itepekimab treated subjects possibly due to the presence of other active alarmins (50). Moreover, the macrolide family antibiotic is prescribed to asthmatic patients at high risk of exacerbation caused by bacterial infection. The administration of azithromycin as an add-on therapy had effectively reduced the annual exacerbation rate in moderate-severe asthmatics with eosinophilic or non-eosinophilic airway inflammation (51). However, the high adverse effects of azithromycin are an essential concern, including side effects such as diarrhea. Since an increased level of neutrophils is observed in most asthmatics with non-T2 airway inflammation, the current biological mediations for non-T2 asthma mainly focus on the blockade of the mechanism mediating neutrophil recruitment and activity. Unlike the consistent benefits observed in biological mediations against T2 airway inflammation, the clinical efficacy of biologic mediations targeting non-T2 airway inflammation has encountered several difficulties. Both IL-1β and IL-23 are key cytokines for Th17 activation, and the level of IL-1β is related to the neutrophilic extracellular traps (NETs) in the airway (52). Canakinumab is an anti IL-1β medication prescribed in inflammatory diseases (e.g. arthritis, COPD) but the effect of canakinumab in asthma remains to be investigated clinically in the future. Furthermore, risankizumab is a monoclonal antibody against IL-23p19. During a phase 2a placebo-controlled trial on patients with severe asthma, the length of time until the first exacerbation was shorter in subjects treated by risankizumab, with more rescue medicine required to maintain the lung function following the first administrated dose of the biologic (53). In addition, transcriptomic analyses of sputum extracted cells showed that genes related to IL-23 signalling pathways were significantly attenuated by risankizumab. There was also a decrease in genes related to the anti-

viral immune response which potentially promoted the Th2 response and increased the risk of exacerbation by viral infection (53). Effector cytokines IL-17A/F also play an important role in neutrophil recruitment. A phase 2 randomized clinical trial of anti-IL-17A medication, termed secukinumab, is still under investigation. Of note, a phase 2a placebo-controlled randomized trial of a monoclonal antibody targeting IL-17 receptor (brodalumab) was conducted on severe asthmatics by Busse *et al* (54). No overall improvement of lung function (ACQ score and FEV_1) was detected in subjects treated with brodalumab at any dosage (140mg, 210mg or 280mg) (54). The clinical results from subjects treated with brodalumab were further sub-grouped based on post bronchodilator reversibility (FEV_1 ≥20% improvement), and a significant reduction of ACQ score was observed in high reversibility subjects treated with brodalumab at 210mg compared with placebo, whereas no significant change of ACQ was observed in the low reversibility group (54). However, the baseline demographics of these two subgroups of subjects were similar with a slightly higher atopy in the high reversibility group, suggesting the airway neutrophilia should be a potential criteria for subgrouping in therapies for non T2 asthma in future studies (54).

According to GINA guidelines 2023, bronchodilators, corticosteroids and biologic medications are prescribed based on severity categorizations of patients diagnosed with asthma. Very mild asthmatics with symptoms < 4 times a week are prescribed low dose SABA combined with ICS (5). Furthermore, a combination of relievers and medium dose ICS is prescribed for patients with daily symptoms and reduced lung function. For patients with severe asthma, a high dose of ICS or OCS is prescribed with optional biological medication (5).

The heterogeneity in response to medications in asthmatics poses a continued challenge for the development of asthma therapy. This is due to an incomplete understanding of the underlying drivers and down-stream effects on cellular and molecular mechanisms of asthmatic airway

inflammation. Thus, it is important to further investigate the factors mediating the onset, maintenance and progression of asthma (known as asthma pathogenesis) for a better therapeutic strategy in the future.

1.1.7 Immuno-pathogenesis of asthma

The immunopathogenesis of asthma consists of interactions including pro-inflammatory mediators (e.g. cytokines, chemoattractant), airway structural cells, airway epithelial cells and cells of the adaptive and innate immune pathways. Chronic inflammation in the lung mucosa can lead to a thickening of the airway wall, limiting airflow known as airway remodelling. During the type 2 (T2) airway inflammation, allergens with protease functions (e.g. house dust mite, fungi, pollens) break down the epithelial barrier by cleaving E-cadherin between epithelial cells. In addition, allergens containing ligand functions (e.g. cat dander) can bond to and activate pattern recognition receptors (PRR) on airway epithelial cells (55). As a result, the stimulated airway epithelial cells release a triad of cytokines: IL-25, IL-33 and TSLP, known as alarmins (56)(57). Factors other than allergens including pollution, smoke, chemicals and viral infection also induce the secretion of alarmins in a non-atopic (allergen non-specific) manner (12)(35). Following stimulation by secreted alarmins, conventional dendritic cells (cDC) migrate into mediastinal lymph nodes through C-C Chemokine Receptor (CCR) 7 - CCL (C-C motif Ligand) 19/21 interaction, and promote the differentiation and clonal expansion of type 2 helper T cells (Th2) in an OX40 (tumor necrosis factor receptor superfamily 4)/OX40L dependent manner (35)(58). The mature Th2 cells release IL-4 within the lymph nodes, which stimulates the clonal expansion, and also IgE class-switching of B cells (35). The free IgE secreted by plasma cells can be pre-loaded onto Fc receptors on the surface of mast cells and basophils. Upon subsequent exposure to the specific

allergen, the pre-bound IgE on mast cells and basophils is cross-linked and induces the secretion of pro-inflammatory mediators, including histamine, serotonin, cysteinyl leukotrienes, prostaglandin D2 (PGD_2), promoting vascular permeability, mucus production, and smooth muscle hypertrophy in the airways (35). Moreover, mature Th2 cells enhance airway eosinophilia in epithelial and sub-epithelial mucosa by producing T2 cytokines, including IL-4, IL-5 and IL-13. Both IL-4 and IL-13 induce airway epithelial cells and vascular endothelial cells to release eotaxin which recruit the eosinophil with its progenitors from the bone marrow into the airway (59). In addition, IL-5 enhances the proliferation and inflammatory function of eosinophils in the airway and induces the differentiation of eosinophil progenitor cells in local tissues known as *in situ* eosinophilopoiesis (60). Other than Th2 cell activation by allergens through the adaptive immune pathway, rhinovirus infection is the most frequent trigger in T2 asthma exacerbation in which the type 1 anti-viral response mediated by plasmacytoid DCs is compromised by the T2 inflammation. Schroeder *et al.* proposed that during the T2 response, the binding of IgE to FcƐRI on pDCs blocked its antiviral IFN-α production which expose the airway to a high risk of viral infection (61). Due to the attenuated anti-viral response, the rhinovirus infects the airway cells and reciprocally simulates the epithelial derived IL-33 which further enhances the T2 airway inflammation. In addition, a recent study reported a novel finding of auto-antibodies released by B cells against eosinophilic peroxidase (anti-EPX Abs) in severe asthmatics with chronic ongoing eosinophilic inflammation suggesting a new role played by B cells during the T2 airway inflammation (62) (**Figure 1.2**).

Non-T2 airway inflammation mostly occurred in severe asthmatics, and it includes two immune pathways mediated by type 1 (Th1) or type 17 (Th17) helper T cells, both of which contribute to an elevated neutrophilia in the airway (26). Type 1 inflammation is initiated by the

pathogen associated molecular pattern (PAMP) of intracellular pathogens (mainly virus) or bacteria (at a lesser extent), which are recognized by pattern recognition receptors (e.g. toll like receptor (TLR) 3,4,7,8）on epithelial cells, dendritic cells, and macrophages. Furthermore, the IL-1 family of cytokines is reported to impose an inflammatory effect during the T1 inflammation, especially IL-1β and IL-18. Following the induction of Pathogen Associated Molecular Pattern (PAMP) receptors through TLR ligation, the oligomeric Nod Like Receptor pyrin containing Protein 3 (NLRP3) inflammasome is assembled in an inactive form in the cytosol along with pro-caspase 1, pro-IL-1β and pro-IL-18 (63). Following stimulation from the danger associated molecular pattern (DAMP) such as ATP (adenosine triphosphate), ROS (reactive oxygen species), viral RNA and pore forming toxins, the NLRP3 inflammasome is stimulated and releases the active caspase-1 to catalyze the pro-IL-1β and pro-IL-18 into active forms (63). Besides, PAMPs of virus and bacteria are recognized by TLRs which stimulate the bronchial epithelial cells and effector cells to produce inflammatory mediators (e.g. IL-1β, IL-8, IL-18, TNF-α, granulocyte macrophage colony stimulating factor (GM-CSF)) and promote the antigen presenting cells (APCs) to induce Th1 differentiation (64). The interaction with the antigen presenting cells activates the T cell receptor (TCR) signalling pathway and increases the expression of IL-12 receptors on naïve T cells, leading to the differentiation of Th1 cells in response to IL-12. Additionally, in the presence of IL-12, IL-18 facilitates the maturation of Th1 cells and the mature viral specific Th1 cells produce IFN-γ and TNF-α as an anti-viral response to recruit neutrophils (12)(64).

In response to extracellular pathogens (bacteria, fungi) or pollution, the differentiation of Th17 cells is initiated by the macrophage derived IL-6 and TGF-β, which promote the expression of IL-23R and IL-1R on Th17 cells (12). Upon the stimulation of IL-1β, IL-23, and TGF-β, the Th17 cells produce IL-17A/F to induce the secretion of chemo-attractants (e.g. IL-6, CXCL8,

CCL3) by bronchial epithelial cells promoting neutrophil recruitment (64). In addition, the oxidative stress cell death induced by smoking promotes the secretion of danger associated molecular pattern (DAMP) (ROS, IL-33) from the epithelial layer and enhances neutrophilia in the airway (32). As the most abundant immune cell in non-T2 asthma, neutrophils produce neutrophil elastase, reactive oxygen species, matrix metalloproteinase and cytotoxic neutrophil extracellular traps (NETs), leading to goblet cell metaplasia and damage to both the bronchial epithelial cells and vascular endothelial cells within the airways (12). In addition, the association between obesity and non-T2 severe asthma was recently proposed where the leptin or adipocyte fatty acids release IL-6, TNF-α and monocyte chemoattractant protein 1 (MCP-1) to stimulate M1 macrophages through NLRP3 inflammasome induced caspase 1 (CASP1), which synthesizes IL-1β to promote Th17 cell activity (65)(66) (**Figure 1.3**).

Over the past decade, studies in mice, followed by humans, have identified a novel group of lineage negative cells that lack antigen recognition receptors. These cells are activated in an antigen non-specific manner that can drive innate immune responses and have the capacity to enhance adaptive immune responses. These cells were termed type 2 innate lymphoid cells that produce type 2 cytokines (IL-4, IL-5, IL-13) in a greater amount than $CD4^+$ T cells on a cell per cell basis (24). Investigating the role of these cells in the development of a type 2 inflammatory environment in airway disease and how these cells are modulated by the tissue microenvironment including anti-inflammatory drugs like corticosteroids, neuropeptides and stimulatory cytokines are the main objectives of this thesis.

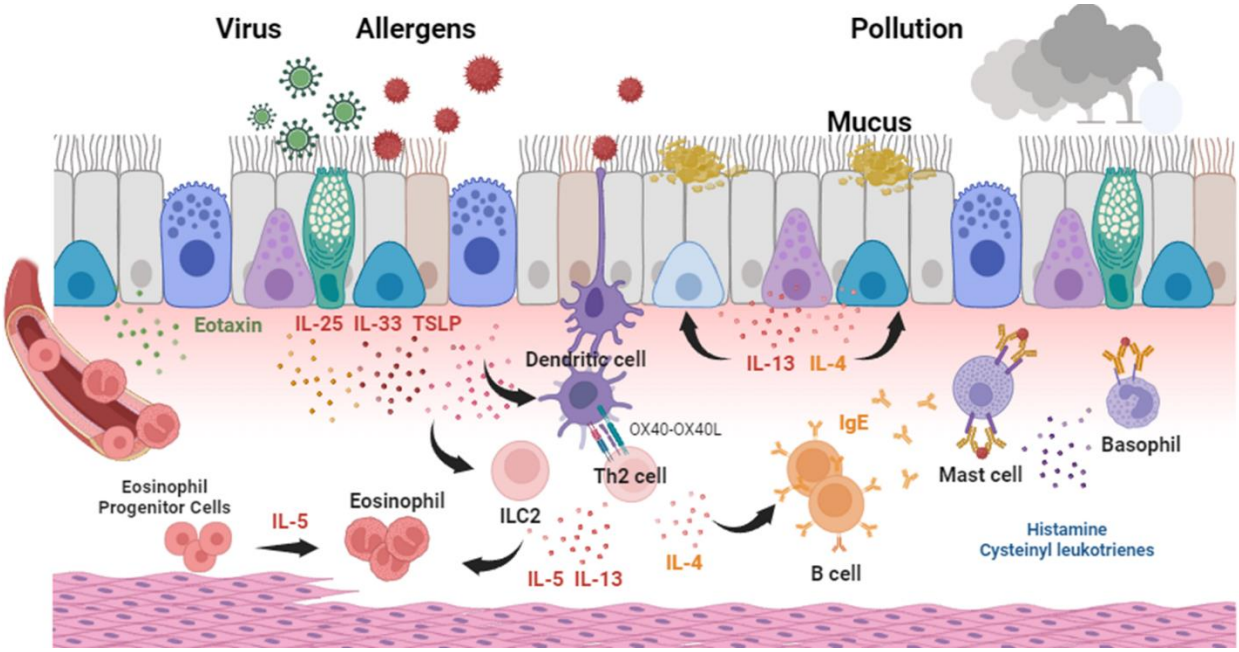

Figure 1.2. The pathogenesis of type 2 airway inflammation.
The type 2 (T2) airway inflammation is initiated by allergens stimulating or breaking the airway epithelial barrier. Apart from allergens, pollution and viral infection also induce the secretion of alarmins in an allergen non-specific manner. Stimulated epithelial cells release interleukin (IL)-25, IL-33 and thymic stromal lymphopoietin (TSLP) to activate conventional dendritic cells (cDC) which can also be activated by allergen binding to pattern recognition receptors. The activated cDCs migrate to mediastinal lymph nodes where through interaction with OX40 naive T cells in an OX40/OX40 ligand dependent manner promote differentiation and clonal expansion of type 2 helper T cells (Th2). Both Th2 cells and type 2 innate lymphoid cells secrete IL-4, IL-5 and IL-13. the IL-4 stimulates B cell antibody class-switch to IgE. The free IgE secreted by plasma cells can be pre-loaded onto Fc receptors on the surface of mast cells and basophils. Upon subsequent exposure to the specific allergen, the pre-bound IgE on mast cells and basophils is cross-linked and induces secretion of pro-inflammatory mediators including histamine, serotonin, cysteinyl leukotrienes, prostaglandin D2. Both IL-4 and IL-13 induce airway epithelial cells and vascular endothelial cells to release eotaxin which recruit the eosinophil with its progenitors from bone marrows into the airway. In addition, IL-5 enhances the proliferation and inflammatory function of eosinophils in the airway and induce differentiation of eosinophil progenitor cells in local tissues known as *in situ* eosinophilopoiesis. Eosinophil- derived granule proteins and mediators such as eosinophil cationic protein and leukotrienes and cytokines cause the pathological features of asthma including tissue remodeling and bronchial hyperresponsiveness.

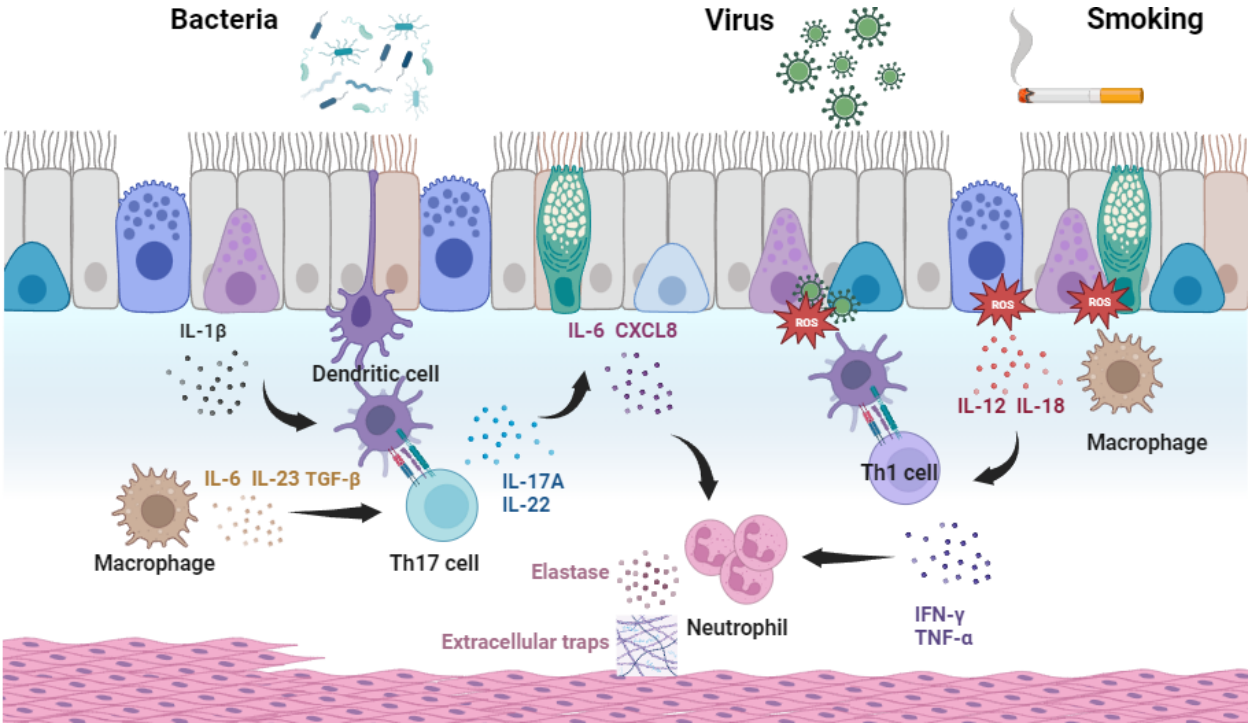

Figure 1.3. The pathogenesis of non-type 2 airway inflammation.
Non-T2 airway inflammation includes two types: type 1 (T1) or type 17 (T17). Type 1 inflammation is initiated by intracellular virus, bacteria and reactive oxygen species which induce the pattern recognition receptors on epithelial cells, dendritic cells and macrophages. Following the stimulations, bronchial epithelial cells and macrophages produce inflammatory mediators mainly IL-12 and IL-18 and promote the antigen presenting cells (APCs) to induce Th1 differentiation. Also, the presence of IL-12 and IL-18 facilitates the maturation of Th1 cells and the mature viral specific Th1 cells produce IFN-γ and TNF-α as an anti-viral response to recruit neutrophils. The type 17 response is induced mainly by extracellular pathogens such as bacteria and fungi. The differentiation of Th17 cells is induced by IL-1β, IL-23, IL-6 and TGF-β which promotes the type 17 helper T cells to produce IL-17A and IL-22 and induce secretion of chemo-attractants IL-6, CXCL8 from bronchial epithelial cells and promote neutrophil recruitment. The activated neutrophils produce neutrophil elastase, reactive oxygen species, matrix metalloproteinase and cytotoxic neutrophil extracellular traps (NETs), leading to goblet cell metaplasia and damage to both the bronchial epithelial cells and vascular endothelial cells within the airways.

1.2 Type 2 Innate Lymphoid Cells

1.2.1 Innate Lymphoid Cells

Innate lymphoid cells (ILC) are lympho-mononuclear cells with similar characteristics to the adaptive immune counterpart T cells (ILC1-Th1, ILC2-Th2, ILC3-Th17, NK cells-CD8 T cells)(67). As a tissue resident cell, ILC are activated rapidly as an early immune response to noxious stimuli because ILC lack the recombination activating genes (RAG)1/2 which are essential for the generation of antigen specific memory in adaptive immune cells (T/B cells) (68). Additionally, the lineage markers of T/B cells (CD3/4/19/20), natural killer (NK) cells (CD2/94), and myeloid cells (CD14/16/FcƐRI) are absent in ILC, which makes ILC a unique category of lymphoid cells (68). The development of ILC starts in the bone marrow, where the common lymphoid progenitors (CLP) differentiate into common innate lymphoid progenitors (CILP). Both NK progenitors (NKP) and common helper innate lymphoid progenitors (CHILP) are differentiated from CILP and the CHILP differentiates into the progenitor cells (ILCP) for each ILC subset (67). During differentiation, DNA binding protein inhibitor 2 (ID2) is essential for the commitment to ILC lineage by inhibiting E-protein transcription factor which is a regulator of T/B cell commitment (69). Circulating naïve ILCs ($CD45RA^+$ $CD62L^-$) have been described as being present in the blood, along with the bone marrow (70). Moreover, c-kit is a cell surface marker of plasticity on ILC committed progenitor cells, and the multipotential c-kit^+ ILC progenitor have been observed by Lim *et al* in human blood. Unlike c-kit^+ ILC3 in local tissue, the peripheral c-kit^+ ILC lack the mature ILC surface markers but express a high level of CD45RA (71). These results suggested the differentiation of ILC can occur in both bone marrow and inflamed tissues in response to local signalling milieu. In general, mature ILC can be subdivided into three groups known as ILC1, 2 and 3 based on surface markers, transcriptional factors, and cytokine expressions.

The lymphoid helper marker IL-7Rα (CD127) is expressed in all human helper ILC subtypes except NK cells (68). Each ILC subtype is stimulated by different cytokines and produces different mediators. Both helper ILC1 ($CD127^+$ $CRTH2^-$ $c\text{-}kit^-$) and NK cells (CD127 $CD56^{+/dim}$ $NKp46^+$) express transcription factor T-bet and produce IFN-γ in response to IL-12, IL-15 and IL-18 during infection with intracellular microbes like viruses in local tissues (e.g. lung, skin, tonsil) (67). However, NK cells express transcription factor Eomesodermin (Eomes) which is absent in ILC1 and NK cells contain a distinctive cytotoxic function similar to CD8 T cells which circulate in the peripheral blood and produce perforins and granzymes to eliminate the viral infected cells or physically stressed cells (e.g. tumor cells)(67). In addition, ILC1 express Runt domain transcription factor 3 (RUNX3) which suppresses the potential of ILC2 and supports the development of ILC1. Furthermore, ILC2 ($CD127^+$ $CRTH2^+$ $c\text{-}kit^{+/-}$) are functionally similar to Th2 cells but ILC2 are directly activated by IL-33, TSLP and IL-25 secreted in response to allergen and helminth exposure to epithelial cells (72). In addition, ILC2 express transcription factors GATA3, B cell lymphoma 11b (Bcl11b) and RORα which mediate the production of type 2 pro-inflammatory mediators (IL-5, IL-13 and amphiregulin) and suppress gene expressions of ILC3 features (69). As a type 2 inflammatory cell, ILC2 are involved in the type 2 immune response including expelling helminth and tissue damage repair to maintain homeostasis (67). Like ILC1, there are two distinctive lineages in ILC3: helper ILC3 ($CD127^+$ $CRTH2^-$ $c\text{-}kit^+$) and lymphoid tissue inducer cells (LTi) which both express transcription factor RORγt. The LTi cells are important for the formation of secondary lymph structures such as lymph node and Peyer's patch during the embryonic development. On the other hand, ILC3 is activated by IL-1β, IL-23 and the aryl hydrocarbon receptor ligand and produce effector cytokines (IL-17 and IL-22) in response to the extracellular microbes (e.g. fungi and bacteria) infection (68) (**Figure 1.4**).

ILC are important innate immune cells required for the homeostasis of local tissues including intestine, lung, skin and tonsils. However, the dysregulation of ILC in the local tissues may lead to inflammatory diseases. The pathogenic role of ILC1 or NK cells has been observed in the airways and gut (e.g. COPD and Crohn's disease, respectively) (67). Also, the overexpression of ILC3 may lead to Crohn's disease, psoriasis (73) and cystic fibrosis with nasal polyps (74). As the most relevant ILC subtype in the pathogenesis of allergy and airway inflammation, the increased number of inflammatory ILC2 is reported in several diseases including atopic dermatitis, chronic rhinosinusitis (CRS) and asthma.

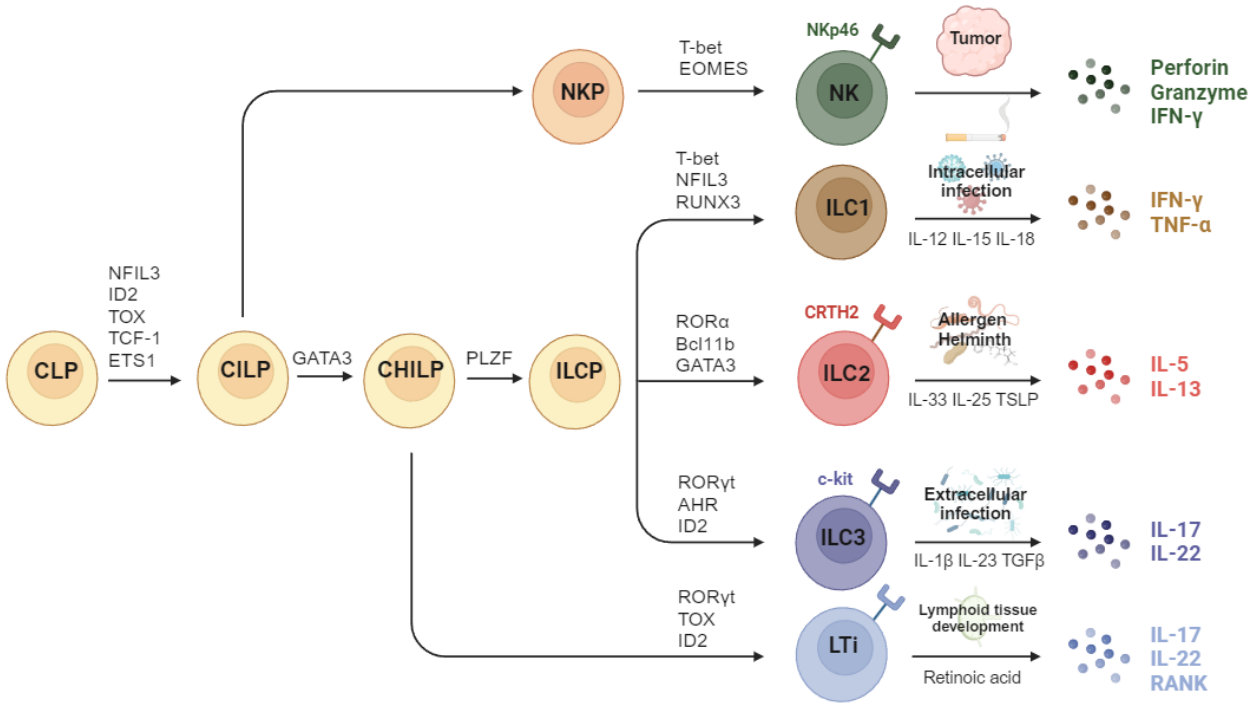

Figure 1.4. Development of innate lymphoid cells. (Modified based on (67)(69)**)**
The development of ILCs starts from common lymphoid progenitors (CLP). The progenitor cells for natural killer cells (NK progenitors) and ILCs (common helper innate lymphoid progenitors) are derived from common innate lymphoid progenitors (CILP). During the differentiation, the transcription factor Id2 is essential for the commitment to ILC lineage and the CHILP further develops into ILC progenitor cells (ILCP). The mature ILCs can be subdivided into three groups: ILC1, 2 and 3. Both helper ILC1s and NK cells express transcription factor T-bet and produce IFN-γ in response to IL-12, IL-15 and IL-18 during the intracellular infection in local tissues. However, NK cells express transcription factor Eomes which is absent in ILC1 and NK cells contain a distinctive cytotoxic function and produce perforins and granzymes to eliminate the viral infected cells or tumor cells. Furthermore, ILC2 are directly activated by IL-33, TSLP and IL-25 in response to allergen and helminth. The transcription factors GATA3, Bcl11b and RORα mediate the production of IL-5, IL-13 and amphiregulin in ILC2. There are two distinctive lineages in ILC3: helper ILC3s and lymphoid tissue inducer cells (LTi) which both express transcription factor RORγt. The LTi cells are important for the formation of secondary lymph structures such as lymph node and Peyer's patch. On the other hand, ILC3 is activated by IL-1β, IL-23 and aryl hydrocarbon receptor ligand and produce IL-17 and IL-22 in response to the extracellular microbes infection.

1.2.2 ILC2 in asthma

The unique role of ILC2 in T2 airway inflammation was first described in a murine model in which chronic airway inflammation and increased methacholine airway resistance were found in allergen challenged mice even though T cells were depleted (*Rag1*$^{-/-}$) (75). Of note, the lung resistance was significantly attenuated when both T cells and ILC2 were genomically knocked out in allergen challenged mice (75). These results suggested that the inflammatory function of ILC2 is not redundant even though ILC2 have similar characteristics with Th2 cells.

The high overlapping symptoms between allergic rhinitis and asthma suggested a common pathogenesis of disease in the upper and lower airways and proposed a "united airway" hypothesis (76). The presence of ILC2 in human respiratory tract was observed in the nostril tissue of upper airways down to the bronchiole tissues of lower airway. As the first line of defense in the airway immune system, the baseline number of ILC2 in the nasal mucosa was found to be significantly higher in moderate-severe asthmatics with allergic rhinitis, compared to healthy subjects (77). Also, in the sinus mucosal tissues, the total number of ILC2 was highest in patients with eosinophilic CRSwNP with asthma as a comorbidity, as compared to healthy controls or non-eosinophilic CRSwNP (78). In response to nasal allergen challenge, the ILC2 number was rapidly increased within 6 hours along with an increased level of eosinophils and IL-5 in the nasal mucosa (77) or nasal lavage fluid of patients with allergic rhinitis (79). According to the atopic march theory, the allergen induced inflammatory response in the upper airway could develop into a more severe disease in the lower airway without proper medical management (13). Thus, this thesis investigated the activation mechanism of ILC2 in the upper airway and the corticosteroid sensitivity of this process in order to develop improved strategies to treat asthma.

In the lower airway, Winkler *et al.* identified a significant increase in total ILC2 with an

elevated level of genes related to chemoattractant receptors (*CCR4, CXCR4*) in the bronchoalveolar lavage (BAL) of mild allergic asthmatics at 24h post allergen challenge (80); in contrast, the decrease of total ILC2s in blood was inversely correlated with the chemoattractant level (PGD_2, CXCL12) in BAL (80). These results suggested a migration of ILC2 from blood into the airway following the T2 airway inflammation in asthmatics. In patients with mild asthma, ILC2 are more activated in BAL than blood by expressing a significantly higher level of effector cytokines (IL-5 and IL-13) and transcripts of activation markers (*CD69, ICOS, TNFRSF9*) (80). Notably, our research group reported an increased number of ILC2 in the airway of mild asthmatics applying the whole lung allergen challenge: Machida *et al* observed an early increase of ILC2 in the airway of mild asthmatics at 7h post allergen challenge compared with the placebo group (81). Furthermore, Chen *et al* found the total number of ILC2 in the sputum of mild asthmatics increased significantly as did the IL-5^+ IL-13^+ ILC2 during the first 24h after inhalation allergen challenge which then declined to baseline levels at 48h post-allergen challenge, whereas a high level of activated $CD4^+$ T cells (IL-5^+ / IL-13^+ or both) was maintained for 48h (82). These studies suggested that ILC2 provide an early, transient source of type 2 immune cytokines and work co-operatively with $CD4^+$ T cells for a maximal type 2 immune response following allergen challenge compared to diluent inhalation challenge (82). However, the mechanism underlying the rapid ILC2 activation (in 7h) in the lower airway is not well understood and it is necessary to identify the mediators contributing to the ILC2 activation as a potential therapeutic target to control the inflammatory ILC2 in the airway.

In addition to the airways, a significantly higher level of ILC2 was also observed within the peripheral blood mononuclear cells (PBMC) of moderate-severe asthmatics compared to patients with allergic rhinitis or healthy controls (83). In addition, similar to the sex difference

observed for asthma incidence, a sex biased difference was also observed in ILC2 activities. In patients with severe asthma (84) or allergic rhinitis with asthma (85), a higher ILC2 level was detected in the blood from female patients compared to male patients. A retrospective study from our group reported a significantly higher level of total airway ILC2 in female patients with mild asthma at baseline (86). However, no significant difference between ILC2 from male or female patients was detected post allergen challenge (86). More inflammatory properties of ILC2 were reported in patients with severe asthma. Golebski *et al.* detected a significant association between the level of peripheral inflammatory ILC2 and the rate of exacerbation or steroid resistance in severe asthmatics (87).

Our group has reported that in patients with prednisone-dependent severe eosinophilic asthma, although the absolute number of $CD4^+$ T cells is more abundant than ILC2 in blood and sputum; ILC2 express a significantly higher proportion of IL-5 and IL-13 in both compartments (24). Furthermore, the greatest number of IL-5^+IL-13^+ ILC2 was found in sputum samples from severe asthmatics with uncontrolled airway eosinophilia (>3% of differential cell count) (24). Therefore, ILC2 are the predominant source of IL-5 and IL-13 in severe eosinophilic asthmatics despite high dose corticosteroid therapy. The functional assay of peripheral blood derived ILC2 from asthmatics with different severities was conducted by Malik *et al.* who reported that the ILC2 from severe asthmatics expressed significantly higher levels of TSLPR and IL-5/13 post-stimulation with IL-33+TSLP *in vitro* (88). Currently, severe asthma is subdivided into four endotypes based on the sputum cytology: eosinophilic (Eos>3%; Neut<63%; TCC<15×10^6cells), neutrophilic (Eos<3%; Neut>63%; TCC>15×10^6 cells), mixed granulocytic (Eos>3%; Neut>63%; TCC>15×10^6cells) and pauci-granulocytic (Eos<3%; Neut<63%)(33). The prevalence and phenotype of ILC (1/2/3) populations in the airways of asthmatics with neutrophilic, mixed

granulocytic or pauci-granulocytic bronchitis has not been described to date.

Thus, the different properties of ILC2 detected in mild and severe asthmatics implied that the activity or activation status of ILC2 might be regulated or altered by micro-environmental cues within the airways. Investigating the interactive mechanism of ILC2 with micro-environmental cues including allergens, corticosteroid therapy, neuroimmune regulation, cytokines and cellular compositions in the airway will be helpful for the development of novel strategies to control the inflammatory ILC2 in the airway.

1.2.3 The Effect of Corticosteroids on ILC2

As the golden standard treatment for asthma, the inhibitory effect imposed by corticosteroids on ILC2 has previously been studied in *in vitro* assays in which the IL-33 or IL-33+TSLP stimulated inflammatory cytokine (IL-4, IL-5, IL-9, IL-13) expressions in blood ILC2s were significantly attenuated after a 6-9 day incubation period with dexamethasone(82)(89). Furthermore, Yu *et al.* proposed a potential inhibitory mechanism of corticosteroids on enriched blood ILC2 *in vitro* through the inhibition of STAT3, 5 and 6 phosphorylation within ILC2 (90). Moreover, chronic rhinosinusitis with nasal polyps (CRSwNP) is a middle age onset sinus disease, which is highly associated with allergic rhinitis and asthma (91). The common triggers of CRSwNP include allergens, smoke, chemicals or anti-inflammatory drugs (91). In patients with CRSwNP, the alarmin stimulated cytokine production (IL-4, IL-5 and IL-13) in the sorted nasal polyps-extracted ILC2 was significantly inhibited by corticosteroids after a 4-day treatment *in vitro* (89).

Other than *in vitro* assays, the clinical administration of corticosteroids to alter the function of ILC2 was conducted in patients with allergic rhinitis and the results from previous studies

suggested a corticosteroid sensitive response of ILC2 in the upper airway. In allergic rhinitics with asthma, a 3-month trial with intra-nasal and inhalation glucocorticoid treatment reported that starting from the second month, the proportion of total ILC2 in PBMCs decreased significantly in a continuous manner, along with a significant improvement of pulmonary function (higher $FEV_{1,}$ FVC; lower FeNO level and ACQ score) (90). However, the IL-13 level in the plasma was not attenuated at the end of treatment (90). Furthermore, Walford *et al*. reported a significant decrease of total ILC2 in the nasal polyp tissue of patients with eosinophilic chronic rhinosinusitis (CRS) after a systemic (IV/oral) steroid treatment for at least 5 days (92). The nasal scraping is a non-invasive sample collection in the upper airway and provided safe access to investigate the direct effect of intranasal corticosteroids on the epithelial layers and immune cells including ILC2. In this thesis, we investigated the corticosteroid sensitivity of ILC2 in the upper airway which is the first line of defense in the respiratory tract; specifically, we investigated the effect of pre-treatment with intranasal corticosteroids on ILC2 in the nasal mucosa of allergic rhinitics with mild asthma in a nasal allergen challenge model.

Compared to mild asthmatics, the effect of corticosteroids on patients with severe asthma is more limited and previous studies from our group have shown that ILC2 are increased in the airways of severe asthmatic patients with airway eosinophilia who are on a high dose of oral corticosteroids (24). Unlike the corticosteroid sensitive property observed in blood ILC2, the expression of IL-5 within ILC2s enriched from the bronchial lavage (BAL) of severe allergic asthmatics was not affected by corticosteroids *in vitro* (93). Of note, the BAL ILC2s enriched from severe asthmatics expressed a higher level of CD127 (receptor for both IL-7 and TSLP) post treatment of dexamethasone *in vitro*. Additionally, a high level of TSLP was detected in BAL of severe asthmatic patients (93) suggesting that dexamethasone induces corticosteroid resistance by

upregulating IL-7Rα surface expression on ILC2 within the airways and the TSLP/IL-7Rα axis may play an important role in corticosteroid resistance of cytokine production by ILC2. Liu *et al.* observed a higher level of downstream mitogen-activated protein kinase (MEK) in bronchial lavage ILC2s stimulated by IL-7 and TSLP (93). The activated MEK translocated into nucleus and expelled the glucocorticoid receptor (GR) co-repressor termed thyroid hormone receptor (SMRT) and the absence of SMRT impaired the anti-inflammatory gene expression mediated by the corticosteroid-GR complex in nucleus (93). In addition, the TSLP/IL-7Rα signalling pathway also induces the phosphorylation of signal transducer and activator of transcription 5 (pSTAT5) which disrupts the translocation of corticosteroid-GR complex into the nucleus for further activity (72). Therefore, TSLP induces corticosteroid resistance of cytokine production by ILC2 in a MEK and STAT5 dependent manner.

Although response of ILC2 to corticosteroid therapy has been clarified in severe asthma, the responsiveness to corticosteroids for ILC2 from milder asthma and allergic rhinitis remains to be clarified and was investigated in the current thesis. The potential for differences in microenvironmental cues to alter the sensitivity of ILC2 to corticosteroid therapy remains to be investigated and was addressed in this thesis.

1.2.4 Regulation of ILC2 Activation by Mediators Acting as Tissue Micro-environmental Cues

Within the human ILC population, ILC2 express the prostaglandin D_2 receptor 2 (CRTH2) which makes ILC2 distinctive from other ILC types by a unique recruitment pathway. The prostaglandin D2 (PGD_2) is a lipid mediator mainly produced by mast cells and is essential for both ILC2 migration and activation (72). Mature ILC2 predominantly reside in mucosal tissue

including lungs, small intestines, skin and adipose tissues (94). The main inflammatory effector cytokines produced by ILC2 are similar to Th2 cells including IL-5 and IL-13 to promote eosinophilia in the local tissues. Additionally, IL-4 and IL-13 produced by ILC2 stimulate the enhanced migration of dendritic cells (95).

Canonically, ILC2 are activated by the epithelial derived cytokines (IL-25, TSLP, IL-33) in response to exposure to allergen, pollution, or viral infections (35). The survival and proliferation of ILC2 is mediated by IL-2 (96) and ILC2 are highly responsive to IL-4 (produced by Th2 and basophils) which functions equivalently to IL-33 for ILC2 activation, acting via the IL-4R (97). Moreover, the IL-9 produced by helper T cells is also important for both survival and cytokine production of ILC2 (98). Of note, ILC2 itself can also produce IL-4 and IL-9 to support its survival in an autocrine manner (98)(99). ILC2 not only produces pro-inflammatory cytokines against noxious stimuli, but it also releases amphiregulin to repair the tissue post-inflammatory response (35). In addition, the expression of co-stimulatory receptor inducible T cell co-stimulator (ICOS) with ICOS ligand on ILC2 is important for the proliferation and IL-13 production in lung ILC2 in a murine allergen challenge model (100). Apart from the epithelial derived alarmins, the activation of ILC2 is also regulated by other mediators including tumor necrotic factor family of cytokines (TNF), lipid mediators (leukotriene, prostaglandin), stimulatory cytokines, sex hormones and neuroimmune mediators.

Leukotriene is an arachidonic acid derived lipid mediator produced by granulocytes (mast cells, basophils, eosinophils) and dendritic cells during the T2 response. The high expression of cysteine leukotriene 1 receptor (CysLT1R) was detected on the lung ILC2 from allergen challenged mice and LTD_4 was reported to induce the expression of T2 effector cytokines (IL-4, IL-5 and IL-13) in murine ILC2 *in vitro* (99). Furthermore, the stimulative function of leukotriene

on human ILC2 was reported by Tojima *et al.* that the IL-5/13 production within the PBMC enriched ILC2 was significantly enhanced by different types of cysteine-leukotrienes (C4, D4, E4) (79). Also, LTE_4 significantly reduced the apoptosis rate of human ILC2 *in vitro* and LTE_4 imposed a synergistic effect with PGD_2 to enhance ILC2 migration and cytokine production (101). Of note, Cai *et al.* showed the combination of LTC_4 or LTD_4 with IL-7+IL-33 induced a significantly higher level of IL-17 expression in murine lung ILC2 (102). Apart from leukotrienes, prostaglandin is another category of lipid mediators which impose both stimulative and inhibitory effects on ILC2. As the ligand for CRTH2, PGD_2 is essential for ILC2 recruitment and activation (72). Upon the stimulation of alarmins, ILC2 can also produce PGD_2 through hematopoietic prostaglandin D synthase (HPGDS) and cyclooxygenase 2 (COX2） metabolism pathways as an endocrine stimulation signal (103). Moreover, the IL-5/13 production in alarmin induced ILC2 was significantly attenuated when CRTH2 or PGD_2 was neutralized, suggesting a crucial stimulatory function of PGD_2 on ILC2 (79). In contrast, PGI_2 significantly attenuated IL-5/13 production in IL-33 or fungi challenged ILC2 in murine airways compared to mice with knocked out IP (PGI_2 receptor). A similar inhibitory effect was also observed when the PGI_2 analog was added to human ILC2 *in vitro* which had significantly attenuated the IL-33 induced IL-5/13 production (104). The regulation of ILC2 by sex hormones (estrogen and androgen) has been studied in murine models. As one of the crucial alarmins for ILC2 expansion and activation, the IL-33 production from human bronchial epithelial cells was promoted by the estrogen treatment *in vitro* (105). Also, the systemic estrogen receptor α (ERα) knock out had significantly reduced the IL-33 level in the airway of mice, suggesting that the estrogen signalling pathway indirectly modulated the ILC2 activities (105). However, the direct effect of estrogen on ILC2 was not evident as IL-5/13 production by ILC2 was not affected when the estrogen receptor was knocked out on lymphocytes

in female mice (105)(106). In contrast, androgens impose an inhibitory effect on ILC2. The increased level of serum testosterone in ERα$^{-/-}$ female mice significantly reduced IL-5 and IL-13 production in ILC2 (106)(107). Furthermore, ILC2 from castrated male mice produced an equivalently high level of IL-5/13 post IL-33 challenge compared to wild type female mice (108) and the inhibition on CD25, IL-5, IL-13 and Rorα expressions in ILC2 from gonadectomized male mice was recovered by *in vivo* androgen treatment (84).

The tumor necrosis factor (TNF) family are important co-stimulatory cytokines regulating immune cell functions and four members of the TNF superfamily of cytokines have been shown to promote the inflammatory response of ILC2 including TNF-α, TL1A (TNFSF15) RANKL (TNFSF11) and GITRL (TNFSF18) (109). Human ILC2 from blood and nasal polyps express a high level of TNFR2 (receptor for TNF-α) and TNF alone or synergizing with IL-33/TSLP significantly promoted IL-5/13 production by ILC2 *in vitro* (110). Furthermore, TNF like factor 1A (TL1A) secreted by antigen presenting cells (macrophages and dendritic cells) also enhanced IL-5/13 production in alarmin induced ILC2. The previous study from our group reported that the expression of dead receptor 3 (DR3; TL1A receptor) on sputum ILC2 was significantly enhanced post allergen challenge in mild asthmatics (81). Also, the TL1A has a synergistic effect with alarmins on promoting IL-5/13 expression in ILC2 and our study showed a corticosteroid insensitivity in ILC2 induced by TL1A+TSLP *in vitro* (81). Moreover, receptor activator of nuclear factor kappa-B ligand (RANKL) is another member of the TNF superfamily which was highly expressed in human nasal polyps and the high expression of its cognate receptor, RANK (TNFRSF11A) on ILC2 in human nasal polyps suggested a potential RANKL-RANK interaction mediating ILC2 activation (111). The synergism of RANK agonist with TSLP promoted IL-5/13 production in ILC2 and this was abrogated by dexamethasone *in vitro* (111). A similar inductive

effect was also observed on GITRL which induced a significant increase of IL-5/13 and also IL-9 production in murine ILC2 when challenged with papain (112). As a regulatory response, the IL-33 promoted CD200 receptor expression on ILC2 in the murine allergen challenge model and induction of CD200R significantly attenuated the type 2 cytokine production along with airway resistance in mice (113). Similar results were also observed on human blood ILC2 in which the IL-33 induced IL-5/13 production was significantly decreased by CD200 induction *in vitro* (113).

To control excessive cell activation in inflammatory responses, there are mediators inhibiting ILC2 activities. The IL-10 is an anti-inflammatory cytokine mainly produced by regulatory T cells to eliminate the excessive inflammation. Ogasawara *et al.* observed an inhibitory effect of IL-10 on type 2 cytokine production in ILC2 derived from blood or nasal polyps *in vitro* (114). Furthermore, IFN-γ and IFN-ß enhanced type 1 inflammation by promoting neutrophilia while inhibiting type 2 immune cells including ILC2. In a murine model, IFN-γ had significantly attenuated the proliferation and cytokine production (IL-5, 13 and eotaxin) in IL-33 or helminth stimulated ILC2 along with an attenuation in airway eosinophilia, basophils, Treg cells, and mucus production (115)(116). Similar results were also observed in IFN-ß treated ILC2s from mouse bone marrow or human cord blood *in vitro* (116). In addition, aryl hydrocarbon receptor (AhR) is an inhibitory transcription factor and the helminth expelling function of ILC2 in the small intestine of mice was significantly enhanced when the *Ahr* was knocked out followed by an increase in IL-5 and IL-13 expression in ILC2 (117).

The interaction between ILC2 with other immune cells is also an important component of airway inflammation. The antigen presenting function of ILC2 is indicated by a high level of major histocompatibility complex (MHC) II expressed on 50% to 70% of the murine naive ILC2 (96). Christopher *et al*. co-cultured the IL-33 induced murine ILC2 with $CD4^+$ T cells in the

presence of ovalbumin (OVA)-derived peptide. As a result, the interaction between ILC2 and $CD4^+$ T cells induced the proliferation of both cell types (96). Additionally, the IL-5/13 expression in nasal ILC2 from OVA challenged mice was significantly enhanced in a co-culture with purified $CD4^+$ T cells (118). The interaction between ILC2 with eosinophils also enhanced the T2 airway inflammation. LeSuer *et al* reported that the enhanced eosinophilia in the airway maintained the local ILC2 proliferation and cytokine production as a positive feedback loop (119). The depletion of eosinophils in an allergen challenged murine model (OVA/HDM) had significantly reduced total ILC2 numbers with IL-5/13 production in the airway followed by an attenuated mucus overproduction (119). Also, the eosinophil stimulated by IL-33 or IL-5 *in vitro* significantly promoted the cytokine production by ILC2 potentially through IL-4 and IL-13, although the exact mediators involved were not described (119). This leads to the idea that not only do ILC2 control eosinophil levels, but that eosinophils themselves may play a role in controlling ILC2 levels. This needs to be further investigated, particularly in the context of T2 biologics that deplete tissue eosinophil numbers.

Additionally, OX40 receptor (TNFRSF4) is a co-stimulatory receptor expressed on T cells. In an allergen challenged murine model, an increased level of OX40L was exclusively induced by IL-33 on ILC2 (not other ILC) (101). Also, the allergen induced T2 airway inflammation features including eosinophilia, high levels of Th2 cells and proinflammatory cytokines (IL-4, IL-5, IL-13) were significantly reduced when ILC or $OX40L^+$ ILC were knocked out in mice (101).

1.2.5 Regulation of ILC2 Activation by Neurotransmitters and Neuropeptides

The studies mentioned above report the stimulative effect of alarmins, sex hormones, lipid mediators, and immune cells on ILC2 activation. However, the mechanism of rapid activation for ILC2 following allergen challenge (especially within 6h) is still unknown. The respiratory tract is highly innervated with afferent sensory neurons with terminal nociceptors which sense noxious stimuli (e.g. smoke, allergen, alarmins) in local tissues. Drake *et al.* detected a significantly higher level of branch points and longer nerve length in the airway of subjects with persistent asthma compared with healthy controls (120). Also, the airway nerve length and branch point were positively correlated with the level of eosinophil peroxidase and airway obstruction (120). Thus, the nerve system in airways is potentially mediating airway inflammation in asthmatics. When the sensory neurons are irritated, the action potential is transmitted to central nerve system (CNS). resulting in an activation of the pre-ganglia neurons connecting to the CNS through cranial nerves (121)(122). Following the activation of pre-ganglia neurons, the post-ganglia neuron of the efferent para-sympathetic (cholinergic) nerve release neuropeptides or neurotransmitters including Neuromedin U, substance P, acetylcholine and vasoactive intestinal peptide (VIP) (121)(122). Of note, the rapid activation of para-sympathetic nerves in trachea and large bronchi was observed during the allergen stimulation in which the synaptic efficacy of the bronchial parasympathetic ganglia reached maximal within minutes leading to the release of pro-inflammatory mediators from immune cells (122).

Furthermore, previous studies had reported the interaction between ILC2 and neurotransmitters or neuropeptides and potentially regulating ILC2 activation in the airway. Epinephrine (adrenaline) is a neurotransmitter of the sympathetic nerve system which binds to G-protein coupled receptor termed beta 2 adrenergic receptor (ARDB2) expressed on epithelial cells

and immune cells including ILC2 (121)(123). The beta 2 adrenergic agonist was widely applied in the bronchodilator to alleviate asthma symptoms due to its inhibitory effects on airway smooth muscle contraction and the inflammatory functions of ILC2 (121)(123). The stimulatory effects of VIP were previously reported that the ILC2 derived IL-5 activating the secretion of VIP from the nociceptors in the airway, and the secreted VIP further enhanced the IL-5 production in ILC2 in a positive feedback loop (124). Of note, calcitonin gene related peptide (CGRP) is a neuropeptide mediating the vasodilation and nerve repair. The pulmonary neuroendocrine cells secreted a significantly higher level of CGRP following the allergen challenge in a murine model (125). Also, a significantly elevated level of CGRP was detected in bronchiole of asthmatics compared with healthy controls (125). The expression of calcitonin receptor like receptor (CALCRL) was detected on ILC2 and the CGRP was reported to facilitate IL-5 production in a synergetic manner with IL-33 and IL-25 in murine ILC2 *in vitro* (125). Although CGRP promoted the IL-33 or IL-25 induced IL-5 production in ILC2 within 3-6h (126)(127)(128), a significantly reduced IL-5 production was detected in murine ILC2 when the timeframe of incubation with CGRP was extended to 3 days (128). In addition, the ILC2 proliferation and IL-13 expression in ILC2 were also significantly suppressed by CGRP through a G-protein coupled receptor complex, leading to a significantly attenuated airway eosinophilia in murine models (126)(128). Thus, CGRP attenuated the ILC2 activation with cytokine productions as a regulatory neuropeptide in the murine airway.

The interaction between ILC2 with NMU was recently detected in a murine model where an increased number of ILC2 was in a closer proximity to sensory neurons expressing NMU in murine parenchymal tissue (129) and cholinergic neuronal termini expressing NMU in murine gut (130) compared to $CD4^+$ T cells. . Additionally, the expression of NMUR1 on lung resident ILC2 was prominently up-regulated post-allergen challenge in mice, and the level of $NMUR1^+$ ILC2

was significantly enhanced in the airways preceding the eosinophilia in response to papain (131). The IL-5 and IL-13 production from gut derived ILC2 was enhanced as rapidly as 4h post stimulation with NMU *in vitro*, whereas similar results were not observed in IL-33 or IL-25 stimulatory conditions (130). These results implied that NMU may be involved in the rapid activation of ILC2 post allergen challenge. Therefore, we investigated the interaction between ILC2 and neuromedin U in the lower airway of mild asthmatics combined with *in vitro* assays in this thesis, which provided a better understanding of the activation mechanism for ILC2 in the lower airway.

1.2.6 ILC2 Phenotypes and Plasticity

The alternation of ILC2 phenotypes occurs when ILC2 are activated during the inflammatory response. Several cell surface markers are reported to be highly correlated with the inflammatory function of ILC2, or its insensitivity to corticosteroids. The conventional ILC2 were identified by typical markers CD127 and CRTH2 within Lin^- cells. Of note, Liu *et al.* had identified the expression of IL-5 within the double negative (DN;$CD127^-$,$CRTH2^-$) ILC from the blood of asthmatics, although it was lower than conventional ILC2 (132). Furthermore, genomic and flow cytometric analysis showed that CD30 and TNFR2 were highly correlated with the expressions of IL-5/13 and GATA3. The high expression of CD30 and TNFR2 was not only detected in conventional ILC2, but also in $CD127^+$ and DN ILCs (132). These results suggested CD30 and TNFR2 as new cell surface markers which are more related to the pro-inflammatory T2 functions of ILC2. However, the involvement of ILC2 expressing CD30 and TNFR2 in airway inflammation remains to be investigated.

The CD45 is a receptor belonging to tyrosine phosphatase family expressing on leukocytes,

with two isoforms of CD45: CD45RA and CD45RO. van der Ploeg and Golebski *et al.* had observed a trans-differentiation of CD45RA into CD45RO on naïve ILC2 in a 7-day *in vitro* stimulation with IL-33+TSLP (87). To compare with $CD45RA^+$ ILC2, the $CD45RO^+$ ILC2 are highly T2 inflammatory prone with a significantly higher level of IL-5 (87). Also, the IL-5/13 expression in $CD45RO^+$ ILC2 was kept high with an enhanced proliferating rate under the incubation with dexamethasone. In terms of its genomic aspect, genes related to glutathione metabolism and detoxification including glutathione s-transferase mu 4 (*GSTM4*) and microsomal glutathione S-transferase 2 (*MGST2*) were elevated in $CD45RO^+$ ILC2, leading to corticosteroid insensitivity by eliminating corticosteroids in cytosol (87). Clinically, a significantly higher level of $CD45RO^+$ ILC2s was detected in the peripheral blood of severe asthmatics who had more than 2 asthma exacerbations during the past 12 months, and the level of $CD45RO^+$ ILC2 was positively correlated with the dose of inhaled corticosteroid (87). Thus, CD45RO is a potential marker on ILC2, which implied the high T2 pro-inflammatory function and corticosteroid insensitivity in ILC2.

Furthermore, KLRG1 is an anti-inflammatory marker expressed on stable blood ILC2. In castrated male mice or androgen receptor knock out mice, the level of $KLRG1^+$ ILC2 was significantly decreased suggesting a potential inductive effect of androgen on KLRG1 expression on ILC2s (106)(108). As an inhibitory sex hormone, androgen inhibits the migration of $KLRG1^-$ ILC2 (inflammatory function) from the bone marrow to the lungs in male mice (106). In addition, the KLRG1 expression on ILC2 is associated with IL-10 production, which is a regulatory cytokine. Under the stimulation of IL-7+IL-33+ retinoic acid, the peripheral blood $KLRG1^+$ ILC produced IL-10 along with a high GATA3 expression *in vitro*. Of note, $IL\text{-}10^+$ $KLRG1^+$ ILC2 had significantly attenuated the proliferation of $CD4^+$ T cells with cytokine expressions in a co-culture

in vitro assay (133). The peripheral blood KLRG1^{+} ILC from atopic subjects had less potential of IL-10 production as compared to healthy controls, and the capability of IL-10 production in ILC2 was neutralized by alarmins, suggesting that the regulatory function of ILC2 is attenuated during the airway inflammation (133).

The identification of T2 inflammation related cell surface markers on ILC2 helped scientists to better distinguish activated ILC2 from its stable state. Once the mature ILC2 are activated in local tissues, the function of the activated ILC2 is not permanently determined. The stimulations from micro-environments lead to a trans-differentiation of ILC2 which makes it acquire the phenotypes and functions of other ILC subsets. In the presence of IL-1β, the effect of non-T2 cytokines (e.g. IL-12, IL-18, IL-23 and TGF-β) on c-kit^{+} ILC progenitors differentiation was discovered by Lim *et al.* that IL-23 and the combination of IL-12+IL-18 stimulated IL-17A and IFN-γ production from ILC committed progenitor cells, respectively (71). In addition to ILC committed progenitor cells, the ILC1 prone plasticity was also found in human peripheral ILC2, in which IL-12 significantly enhanced the IFN-γ production in alarmin induced ILC2 *in vitro* (134). Also, Silver *et al.* using a COPD murine model by treating mice with smoke or influenza virus showed an increased level of T-bet and receptors for IL-12 and IL-18 on lung ILC in mice treated by smoke or virus, implying a potential of ILC2 to ILC1 plasticity mediated by IL-12 and IL-18 (135). In *Tbx21*$^{-/-}$ mice, the initiation of ILC2 to ILC1 differentiation was independent of T-bet but the IFN-γ production was significantly attenuated (135). In patients with COPD, an increasing level of airway ILC1 was detected following the severity classifications (GOLD I-IV) and the ILC2 enriched from blood produced IFN-γ under the stimulation of IL-1β+IL-12, and this procedure was reversed by IL-4, suggesting a direct trans-differentiation from ILC2 into ILC1 (135)(136). Recent studies focusing on the plasticity of ILC2 into ILC3-like cells revealed an

acquisition of IL-17 producing ILC2. Cai *et al* reported that IL-33 alone was identified as a stimulator for IL-17 production in murine lung ILC2s and similar results were not observed in T cells suggesting the ILCs specific mechanism mediated by IL-33 for IL-17 production (102). This increase in IL-17 was significantly attenuated when MyD88 was knocked out as an important signalling moiety for IL-33 mediated effects. In addition, murine $GATA3^+$ ILC2 in the lungs can produce IL-17 independently of the expression of RORγt after stimulation with IL-33 and leukotriene C4/D4 (102). These authors also found in mice intraperitoneally treated with papain, the IL-17 produced by ILC2 was essential for both neutrophil and eosinophil recruitment (102). The IL-17 producing ILC2 was also reported by Huang *et al.* within a group of inflammatory ILC2 (iILC2) in mice phenotyped as $ST2^+$ $KLRG1^+$ and highly responsive to IL-25 (137). Furthermore, the IL-25 induced iILC2 produced a higher level of IL-17 under the stimulation of IL-1β, IL-23, TGF-β and IL-6 compared with Th1 or Th2 conditions (137). In addition, the $IL\text{-}17A^+$ ILC2 have been detected in healthy skin (73) and Golebski *et al.* identified the GATA3 (a transcription factor for ILC2-lineage) expression within the ILC3 like cells ($c\text{-}kit^+$ $CRTH2^-$) from the nasal polyps of patients of cystic fibrosis with nasal polyps (CFwNP) (74). As a cytokine for ILC3 activation, IL-1β itself can also stimulate ILC2s proliferation along with IL-5/13 production (136)(138). Following stimulations with IL-1β, IL-23 and TGF-β *in vitro*, ILC2s can be induced to produce IL-17A in a RORγt dependent manner, with a concurrent decrease in IL-5 production, along with a decrease of GATA3 (73)(74).

Following the worsening of asthma disease, the knowledge related to inflammatory functions of ILC2 in mild asthma is not sufficient to predict the ILC activities in severe asthma because a more intricate inflammatory profile was observed in severe asthma which can be subdivided into four endotypes: eosinophilic, neutrophilic, mixed granulocytic, and pauci-

granulocytic (33). Currently, the phenotype of ILC subsets (including ILC2) with their functions in severe asthma is still unclear. Also, the different endotypes of airway inflammation observed in severe asthmatics suggested a potential trans-differentiation of ILC2 in response to the alternation of the micro-environment in the airway. Therefore, the further investigation of ILC2s in severe asthma with its plasticity may identify novel pro-inflammatory functions of ILC2 which leads to potential therapeutic targets for severe asthmatics who are insensitive to corticosteroid.

1.3 Summary

Collectively, ILC2 are a newly ascribed innate immune cell that are rapidly activated in both upper and lower airways of asthmatics following allergen challenge. This thesis aimed to investigate factors that affect the local activation and expansion of ILC2 in the airways including anti-inflammatory medications, neuropeptides, and cytokines to understand the effect of ILC2s on the inflammatory profile of the respiratory airways in asthma. Specifically, this thesis focused on the corticosteroid sensitivity of ILC2 from the upper airways of mild asthmatics with allergic rhinitis, and mechanisms of rapid activation of ILC2 in the lower airway of mild asthmatics. In severe asthma, the airway inflammatory profiles may vary between eosinophilic, neutrophilic, mixed granulocytic, or pauci-granulocytic. In a baseline cross-sectional study, we investigated the various ILC phenotypes, from the mediators that may regulate ILC plasticity, to subtypes that drive the varying airway inflammation. The capacity of endotype specific cytokines to direct ILC2 trans-differentiation in severe asthma was assessed at the phenotypic and transcriptomic levels to identify potential therapeutic targets for severe asthma (**Figure 1.5**).

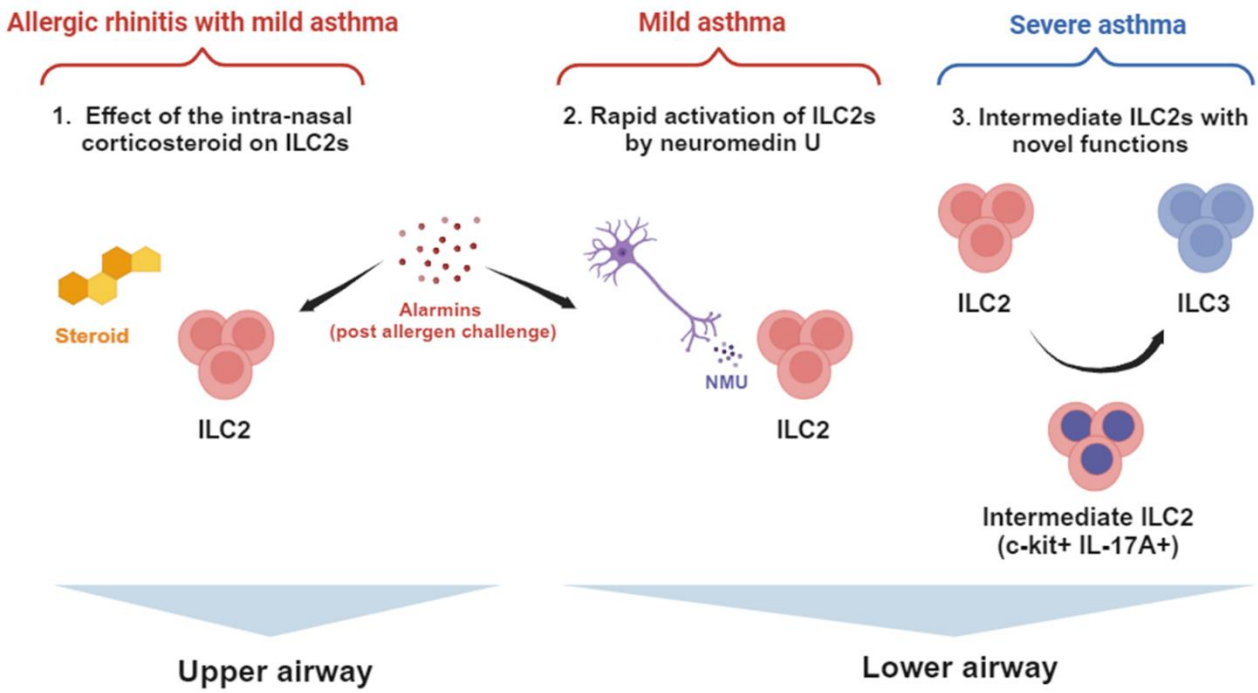

Figure 1.5. Summary
In this thesis, we focused on the corticosteroid sensitivity of ILC2 from the upper airways of mild asthmatics with allergic rhinitis, and mechanisms of rapid activation of ILC2 mediated by NMU-NMUR1 axis in the lower airway of mild asthmatics. In severe asthma, we investigated the phenotypes of various ILC phenotypes mediated by microenvironmental cues and ILC plasticity that drives the varying airway inflammation.

1.4 Hypothesis and Aims

CENTRAL HYPOTHESIS

The type 2 innate lymphoid cell are critical regulators of airway inflammatory responses in the upper and lower airways of subjects with asthma. Airway micro-environmental cues modulate ILC2 activity thereby regulating the inflammatory response.

SPECIFIC HYPOTHESIS AND AIMS

Specific Hypothesis 1: The nasal allergen challenge simulates nasal mucosal ILC2 activation which is inhibited by intranasal corticosteroid treatment in allergic rhinitics with mild asthma (Chapter 2).

Aim 1.1: To evaluate the frequency of ILC2 in nasal mucosa of allergic rhinitics with mild asthma post-nasal allergen challenge.

Aim 1.2: To investigate the effect of inhaled corticosteroid treatment on allergen-induced changes in ILC2 in nasal mucosa of mild allergic rhinitics with mild asthma.

Aim 1.3: To investigate the antigen presenting function of ILC2 during the type 2 inflammation.

Specific Hypothesis 2: Neuromedin U (NMU) triggers the rapid ILC2 activation in the airways of mild asthmatics following whole lung allergen-inhalation challenge (Chapter 3).

Aim 2.1: To identify the responsiveness of ILC2 to NMU in the airway post allergen challenge in mild asthmatics.

Aim 2.2: To identify the endotypical specificity (T2 or T17) of *NMUR1* expression in ILC2.

Aim 2.3: To investigate the stimulatory time course effects of NMU on ILC2 activation.

Aim 2.4: To investigate the effect of corticosteroid and signalling pathway inhibitors on NMU induced ILC2.

Specific Hypothesis 3: The cytokine micro-environment in the airway of severe asthmatics with different pro-inflammatory profiles induces the plasticity of ILC2 to phenotypes that enhance the nature of the airway inflammation (Chapter 4).

Aim 3.1: To characterize and enumerate ILC subsets in the airway of patients with severe asthma.

Aim 3.2: To identify the novel phenotypes of ILC2 (intermediate ILC2) and compare the level of intermediate ILC2s between severe asthmatics with different airway inflammation.

Aim 3.3: To identify the stimulatory cytokines that mediate ILC2 trans-differentiation and novel functional pathways of intermediate ILC2.

CHAPTER 2: Effect of Intranasal corticosteroid treatment on allergen-induced changes in group 2 innate lymphoid cells in allergic rhinitics with mild asthma

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* These authors contributed equally to the development of this manuscript

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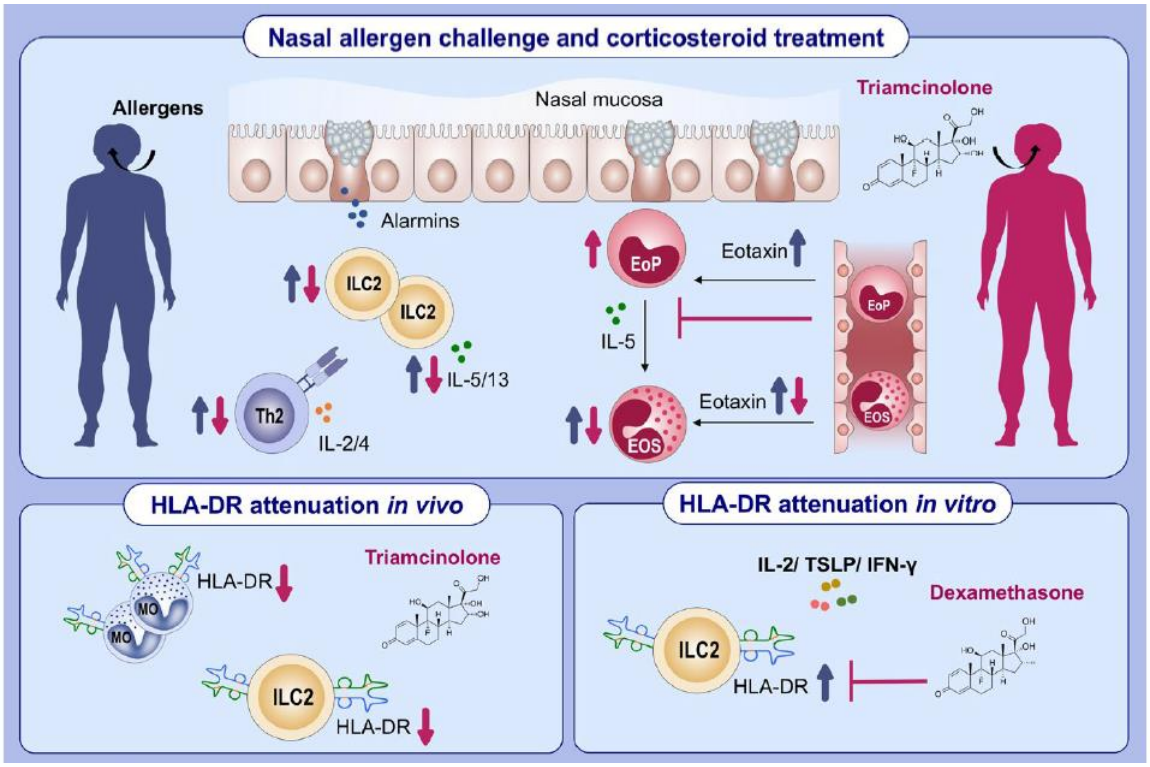
CAPSULE SUMMARY



Group 2 innate lymphoid cells (ILC2s), $CD4^+$ T cells and eosinophils in nasal mucosa of allergic rhinitics with mild asthma were increased significantly after nasal allergen challenge (NAC). The intranasal corticosteroids (INCS) treatment had significantly attenuated the allergen induced ILC2 with IL-5/13 production and human leukocyte antigen (HLA)-DR expression, leading to a significant decrease of eosinophilia in the upper airway.

ABSTRACT

Background: Allergic rhinitis is characterized by rhinorrhea, nasal congestion, sneezing and nasal pruritis. Group 2 innate lymphoid cells (ILC2s), $CD4^+$ T cells and eosinophils in nasal mucosa are increased significantly after nasal allergen challenge (NAC). Effects of intranasal corticosteroids (INCS) on ILC2s remains to be investigated.

Methods: Subjects (n=10) with allergic rhinitis and mild asthma were enrolled in a single-blind, placebo-controlled, sequential treatment study and treated twice daily with intranasal triamcinolone acetonide (220μg) or placebo for 14 days, separated by a 7-day washout period. Following treatment, subjects underwent NAC and upper airway function was assessed. Cells from the nasal mucosa and blood, sampled 24 hours post-NAC, underwent flow cytometric enumeration for ILC2s, $CD4^+$ T and eosinophil progenitor (EoPs) levels. Cell differentials and cytokine levels were assessed in nasal lavage.

Results: Treatment with INCS significantly attenuated ILC2s, IL-5^+/IL-13^+ ILC2s, HLA-DR^+ ILC2s and $CD4^+$ T cells in the nasal mucosa, 24h post-NAC. EoPs in nasal mucosa were significantly increased, while mature eosinophils were significantly decreased, 24h post-NAC in INCS versus placebo treatment arm. Following INCS treatment, IL-2, IL-4, IL-5 and IL-13 were significantly attenuated 24 h post-NAC accompanied by significant improvement in upper airway function.

Conclusion: Pre-treatment with INCS attenuates allergen-induced increases in ILC2s, $CD4^+$ T cells and terminal differentiation of EoPs in the nasal mucosa of allergic rhinitis patients with mild asthma, with little systemic effect. Attenuation of HLA-DR expression by ILC2s may be an additional mechanism by which steroids modulate adaptive immune responses in the upper airways.

INTRODUCTION

Allergic rhinitis and asthma are chronic, heterogeneous airways diseases, with similar underlying inflammatory mechanisms, pathobiology and shared treatment approaches. There is increasing recognition of a link between allergic rhinitis and asthma, with the concept of a "united airway"(1). Epidemiological investigations have shown that 40% of patients with allergic rhinitis have co-morbid allergic asthma, whereas 80 to 90% of allergic asthmatics develop allergic rhinitis. This suggests that inflammatory processes in allergic rhinitis and allergic asthma manifest with similar cellular signaling and activation pathways (2).

Inherent to the pathogenesis of allergic rhinitis and asthma are type 2 immune responses, which contribute to upper and lower airway eosinophilia, tissue remodeling, and bronchial hyperresponsiveness. Group 2 innate lymphoid cells (ILC2s) are lineage negative lympho-mononuclear cells that predominantly reside in mucosal tissues (3)(4). The maintenance and activation of ILC2s is mediated by IL-25, IL-33, and TSLP in both an allergen-specific and non-allergic specific manner (e.g. pollution, smoking, viral infection) (3)(4)(5). ILC2s have been implicated as key players in type 2 immune responses, through the production of copious amounts of IL-5 and IL-13. ILC2s work co-operatively with $CD4^+$ T cells to induce a maximal type 2 immune response during the first 24 hours (h) post-allergen challenge in mild asthmatics (6)(7). The greatest numbers of $IL\text{-}5^+$ $IL\text{-}13^+$ ILC2s have been identified in the airways of prednisone-dependent severe asthmatics with >3% sputum eosinophilia, indicating that these may be steroid insensitive cells that drive persistent airway eosinophilia (5)(8). The production of type 2 cytokines by ILC2s contributing to local eosinophilia by promoting activation, recruitment, and *in situ* differentiation of eosinophil progenitor cells (EoPs) within the airway mucosa (9)(10).

Nasal allergen challenge (NAC) is a validated, reproducible clinical model to investigate

the pathophysiology of allergic rhinitis and assess the kinetics of nasal inflammatory responses including eosinophilia which is sustained to 24h post-NAC(11)(12). Nasal curettage is a minimally invasive method for repeated sampling of the upper airways to assess inflammatory cell changes in mucosal tissue (13) . ILC2s have been identified in the nasal mucosa of allergic rhinitics by immunofluorescence staining (14). In addition, following NAC in moderately severe asthmatics with allergic rhinitis, significant increases in nasal mucosa-extracted ILC2s enumerated by flow cytometry were found which correlated with the level of tissue eosinophilia (15). Although studies have shown that intranasal corticosteroids (INCS) can attenuate the early (0-2h) and late phase (2-7h) response following NAC(16), the changes in ILC2 number, phenotype and activation level within in the nasal mucosa and steroid sensitivity of these cells remains largely unknown. The aim of the current study was to determine whether type 2 inflammation in the nasal mucosa, induced by NAC can be attenuated by intranasal corticosteroid treatment in allergic rhinitis patients with mild asthma, and whether this effect is detected in the systemic circulation.

METHODS

Subjects: Ten non-smokers with allergic rhinitis and mild asthma were enrolled. Subjects (aged 18-65 years) were asymptomatic and not on corticosteroid treatment for 4 weeks prior to study commencement. All subjects had a baseline predicted forced expiratory volume (percent) in one second (FEV_1) of ≥70%; methacholine PC20 (concentration causing a 20% fall in FEV_1) ≤16 mg/ml; positive skin-prick test to common aeroallergens; total nasal symptom score (TNSS) < 3 with each symptom <2 at baseline. Subjects met eligibility if their peak nasal inspiratory flow (PNIF) value was reduced by ≥50% from the baseline and TNSS score increased above 10 following NAC at screening. Baseline subject characteristics are summarized in **(Table 2.1)**. The study (NCT03431961) was reviewed and approved by the Hamilton Integrated Research Ethics Board and all subjects provided informed consent.

Study Design*:* A single-blind, placebo-controlled sequential treatment study detailed in **Figure E2.1** was used to compare 14-day treatment with triamcinolone acetonide (220µg bid) to placebo (saline nasal spray, bid) with a 7-day washout period. A sequential order of treatment, where placebo always preceded the INCS treatment arm to eliminate possible carryover effects of steroids on the post-NAC cellular response. On day 13 of each treatment arm, NAC was performed. TNSS and PNIF were measured at baseline and 5, 15, 30, 60 min and then hourly until 7h post-NAC; blood and nasal lavage were collected at baseline, 7h and 24h post-NAC for cell differentials, cytokine and chemokine levels. Nasal curettage was performed 24h post-NAC (day 14) to enumerate tissue-extracted inflammatory cells by flow cytometry. Negligible cell recovery by nasal curettage at baseline (pre-NAC) precluded the use of this time point. Blood was collected at 1, 4, 7 and 24h post-NAC for cell differentials and 24h post-NAC for enumeration of type 2 cells by flow cytometry. Subjects blinded to the treatment type, recorded drug self-administration in a

diary, and compliance was confirmed by weighing the placebo/steroid dispenser before and after the treatment arms.

Nasal Allergen Challenge: NAC was performed using the Aptar VP7 device (Aptar Pharma) and 100µl of allergen was delivered per nostril. The choice of allergen was based on skin prick test positivity and the challenge cumulative challenge dose was determined by the post-NAC response that caused a reduction in PNIF by ≥50% from the baseline and a total nasal symptom score (TNSS) increase >10 at screening (Table 2.1).

Peak Nasal Inspiratory Flow (PNIF) and Total Nasal Symptom Scores (TNSS): PNIF was performed with peak nasal inspiratory flow meter (GM Instrument) and provided an objective measurement of nasal airflow obstruction. TNSS provided a subjective measurement of symptoms scored by subjects (17).

Nasal Lavage: Pre-warmed physiological saline (0.9%; 10 ml) was instilled using a syringe into each nostril sequentially. A sponge adapter blocking the opposite nostril prevented leakage and up to 7 ml of lavage fluid was recovered. Cytospins were prepared, stained with diff-quik and cell differential cell counts performed to assess percentage of eosinophils, macrophages, neutrophils and lymphocytes. Protease inhibitor was added to the supernatants and stored at -70 °C till assayed for inflammatory mediators using the Meso-Scale Discovery (MSD) and QuickPlex SQ120 Imager.

Nasal Curettage and Peripheral Blood Collection for Flow Cytometry*:* Nasal mucosal samples were collected from both nostrils by scraping the inferior nasal turbinate in each nostril (two-four deep scrapes) using a sterile thermoplastic curette (Rhino-Probe) with zero-degree rigid nasal endoscopy visualization. The tissue sample was resuspended in sterile RPMI+10% FBS with benzonase nuclease on cold beads, washed and then resuspended in RPMI plus 10% FBS.

Heparinized blood (40 ml) was layered by Lymphoprep^{TM} density gradient centrifugation to isolate peripheral blood mononuclear cells (PBMCs).

Immunofluorescence staining and Flow Cytometric Gating Strategy*:* Nasal mucosa-extracted cells and PBMCs were subject to immunofluorescence staining for enumeration of inflammatory cells as detailed in the online supplement.

Mesoplex Assays: Using the Meso-Scale Discovery (MSD) and a QuickPlex SQ120 Imager, nasal lavage samples were assayed for pro-inflammatory cytokines and chemokines as detailed in the online supplement.

Statistical analysis: Data were analyzed using GraphPad Prism 6 software. Statistical analysis of cells, cytokines, and chemokines was performed using two-way ANOVA and *post-hoc* Bonferroni test. Non-parametric paired data from flow cytometric analyses was analyzed using a Wilcoxon test for within group comparisons. A P-value < 0.05 was considered as statistically significant.

RESULTS

Effect of INCS treatment on nasal symptoms and inflammatory response.

Ten subjects (5 males, 5 female) with allergic rhinitis and mild asthma completed the study. Following NAC, mean changes in PNIF (% change from baseline) and TNSS during the early response (0-2h), late airway response (2-7h) and 24h post-NAC are presented in **Figure 2.1A&B**, (individual subject data shown in supplemental **Figure E2.2A&B**). Compared to the placebo treatment, PNIF was significantly improved at 6h post-NAC following 14-day INCS treatment arm (**Figure 2.1A**). Similarly, there was a significant improvement in TNSS at 5 min, 7h and 24h post-NAC following 14-day INCS treatment compared to placebo treatment arm (**Figure 2.1B**). Similar findings were obtained when PNIF and TNSS data were analysed as area under the curve (**Figure E2.2C&D**).

In the nasal lavage, a significant increase in percent eosinophils was detected at 7 and 24h post-NAC compared to baseline in the placebo-and at 24h post NAC in the INCS- treatment arm (**Figure 2.1C**). Between treatment comparison showed a significant reduction in percent eosinophils in the INCS versus placebo treatment arm, 24h post-NAC (**Figure 2.1C**). Absolute eosinophil numbers in blood did not change following NAC in the placebo treatment arm and there was no effect of INCS treatment (**Figure 2.1D**). Although the proportion of macrophages, lymphocytes and neutrophils in nasal lavage did not change at 7h and 24h post-NAC compared to baseline in the placebo group, between treatment comparisons showed a significant increase in the proportion of macrophages and lymphocytes at 7 and 24h post-NAC and a reduction in neutrophils at baseline (supplemental **Figure E2.3**).

Effect INCS pre-treatment on inflammatory cells in nasal mucosa and blood following NAC.

Type 2 innate lymphoid cells were identified by flow cytometry and sequential gating as live, singlet cells with SSA^{low} $Lin^{-}CD45^{+}$ $CD127^{+}$ $CRTH2^{+}$ (**Figure 2.2A and Table E2.1**). Due to low cell recovery pre-NAC, pro-inflammatory cells in the nasal mucosa were only enumerated 24h post-NAC in each treatment arm (supplemental **Figure E2.4**). There was a significant reduction in total ILC2s and IL-5^{+}/IL-13^{+} ILC2s in the nasal mucosa, 24h post- NAC in the INCS versus placebo treatment arm (Figure 2B) but not in blood (**Figure 2.2C**). Of note, we detected expression of HLA-DR on ILC2s extracted from the nasal mucosa (**Figure 2.3A**). Levels of HLA-DR^{+} ILC2s expressed either as a proportion of $CD45^{+}$ cells or as a proportion of total ILC2 cells, showed a significant reduction in the INCS compared to the placebo treatment arm, 24h post NAC (**Figure 2.3B**). Data from *in vitro* cultures show that HLA-DR expression is increased on ILC2s from blood following overnight incubation with optimal concentrations of either IL-2 (10 ng/ml), TSLP (10 ng/ml) or IFN-γ (50 ng/ml) but not IL-4 (10 ng/ml) (**Figure 2.3C & D**). Dexamethasone (10^{-7}M) significantly attenuated IL-2 mediated up-regulation of HLA-DR expression, *in vitro* (**Figure 2.3Ei**). Although dexamethasone showed a trend for attenuating TSLP mediated up-regulation of HLA-DR on ILC2s, this was not significant **(Figure 2.3Eii**).

Recent reports have described unconventional ILC2s (uncILC2s) which compared to conventional ILC2s (cILC2s:Lin^{-} $CD45^{+}$ $CD127^{+}$ $CRTH2^{+}$ cells) are identifiable as Lin^{-} $CD45^{+}$ cells that are non-$CD127^{+}$ ($CD127^{-}$ $CRTH2^{+}$) or non-$CRTH2^{+}$ ($CD127^{+}$ $CRTH2^{-}$) cells (supplemental **Figure E2.5A**) (18). Gating on these population, we found that IL-5/IL-13^{+} and HLA-DR^{+} cell numbers were comparable between all ILC2 sub-populations. However, proportionally INCS treatment had a significant inhibitory effect on IL-5/IL-13 and HLA-DR expression on cILC2s but not on uncILC2s, in particular, non-$CD127^{+}$ cells (supplemental **Figure**

E2.5 B&C).

The proportion of total $CD4^+$ T cells in the nasal mucosa was significantly reduced at 24h post-NAC in the INCS compared to placebo treatment arm (supplemental **Figure E2.6A& B**). In contrast, the proportion of $IL\text{-}5^+/IL\text{-}13^+$ $CD4^+$ T cells, remained unchanged between the two treatment arms (supplemental **Figure E2.6B**). No significant difference was found in total $CD4^+$ T cells or $IL\text{-}5^+/IL\text{-}13^+$ $CD4^+$ T cells in blood, 24h post NAC between the treatment arms (supplemental **Figure E2.6C**). Eosinophil progenitor cells (EoPs; $D34^+CD45^{dim}CD125^+$) (**Figure 2.4A**), expressed as a proportion of total $CD45^+$ cells were significantly increased, 24h post-NAC in the INCS versus placebo treatment arm. However, INCS treatment had no effect on $IL\text{-}5^+/IL\text{-}13^+$ EoPs 24h post-NAC (**Figure 2.4B**). No significant difference between the treatment arms was found in blood EoPs (**Figure 2.4C**). We found no effect of steroid treatment compared to placebo on NAC induced changes in total numbers of hematopoietic progenitor cells or $IL\text{-}5^+/IL\text{-}13^+$ HPCs in either nasal mucosa or blood (supplemental **Figure E2.7**).

Effect of INCS on NAC-induced cytokines and chemokines levels in nasal lavage.

There were significant increases in IL-2, IL-4, IL-5, IL-13 and eotaxin levels in nasal lavage 7h post-NAC compared to baseline in the placebo treatment arm which was significantly attenuated by INCS treatment (**Figure 2.5A-E**). At 24h post-NAC, IL-2 and IL-4 significantly declined relative to 7h post-NAC. IL-13 and eotaxin were sustained up to 24h post- NAC compared to baseline, with trends for an inhibitory effect of INCS although this was not significant. IL-25 levels were increased 7h post-NAC in the placebo treatment arm with a trend for decrease compared to INCS treatment group (P=0.07), although this was not significant; no treatment effect was detected for IL-7 (**Figure 2.5F&G**). IL-33 and TSLP were below the lower limit of detection

in all samples (data not shown). Changes in additional cytokines and chemokines in the nasal lavage are summarized in **Table E2.2**.

DISCUSSION

This study investigated the inflammatory response within the nasal mucosa of allergic rhinitis patients with mild asthma. Our data show the induction of a local type 2 inflammatory response in the nasal mucosa, characterized by increases in ILC2s, $CD4^+$ T cells and associated increases in eosinophils in the nasal lavage in response to intranasal allergen challenge consistent with previous findings (11)(15). Treatment with intranasal steroids attenuated allergen-induced levels ILC2s, $CD4^+$ T cells and type 2 cytokines generation by these cells which may have attenuated terminal differentiation of EoPs in the nasal mucosa and resultant reduction in tissue eosinophilia. Of note, this study detected HLA-DR expression on ILC2s in the nasal mucosa 24h post-NAC which was significantly attenuated in the INCS treatment group. We propose that this may be a potential mechanism by which steroids attenuate local ILC2 mediated adaptive immune responses to allergen challenge in the upper airway.

Our data from subjects with allergic rhinitis and mild asthma shows that compared to placebo, INCS treatment caused a significant inhibition of allergen-induced nasal symptom severity (TNSS) during the early and late response post-NAC, but only had an inhibitory effect on PNIF in the late response. The early response is mediated by the crosslinking of the pre-loaded IgE on the mast cell surface followed by a degranulation of histamine (19). This result is consistent with a study conducted by Liu *et al* who noted that three-day prednisone treatment did not inhibit the increase of mediator levels (histamine, PGD_2 and thromboxane) or the mast cell number in the bronchoalveolar lavage samples five minutes after allergen challenge (20). In addition, Schleimer *et al.* demonstrated that incubation with dexamethasone did not inhibit the release of mediators from human mast cells, *in vitro* (21). Therefore, the significant modulatory effect of INCS on the TNSS but not the PNIF early response may, in part, be explained by 14 days of triamcinolone

acetonide therapy not effectively attenuating mast cell activation in the upper airways. A limitation of the current study was that changes in the proportion of mast cells following treatment with INCS, particularly in the early phase response to NAC were not assessed.

Our findings showed a significant increase in nasal lavage eosinophils, 24h post-NAC in the placebo treatment arm which was significantly attenuated in the INCS treatment group. This is consistent with findings from Benson *et al*., who reported that the eosinophils in nasal lavage was significantly reduced after 1 week of intranasal budesonide treatment during pollen season in allergic rhinitis patients (22). Importantly, we found no effect of NAC in blood eosinophil numbers, in either the placebo or INCS treatment arm indicating the lack of a systemic effect of our NAC in this study. Raised numbers of EoPs have previously been detected in sputum and airway tissue of allergic asthmatics and rhinitics compared to normal healthy controls (9)(10)(23)(24). The capacity of these cells to undergo in-situ differentiation in an IL-5 dependent manner to form mature eosinophils has been demonstrated in *ex-vivo* nasal explant tissue from allergic rhinitics (9). In a clinical trial, where severe eosinophilic asthmatics were treated with mepolizumab, EoPs numbers increased in response to anti-IL-5 treatment with an associated decline in mature eosinophil numbers (10). Similarly, here we suggest that steroid mediated attenuation of tissue IL-5 levels reduces local differentiative processes resulting in a build-up of precursor cells i.e. EoPs, within the nasal mucosa which may in part contribute to the observed attenuation of eosinophilia in the nasal lavage 24h post-NAC in the INCS versus placebo treatment arm. In addition, reduction in eotaxin and IL-13 levels may also reduce migration of mature eosinophils from the peripheral circulation to the nasal mucosa.

ILC2s are central players in type 2 immune response that produce copious amounts of IL-5 and IL-13 (6). We have previously shown significantly increased numbers of ILC2s in the lower

airways of patients with severe steroid-dependent eosinophilic asthma, compared to mild asthmatics, with the highest level of activated ILC2s being found in the sputum of those with uncontrolled eosinophilia (>3% sputum eosinophils) (8). The steroid insensitivity of ILC2s has been further implicated by Liu *et al*., demonstrating that ILC2s from BAL of severe asthmatics, in the presence of IL-7 and TSLP, are resistant to inhibitory effects of dexamethasone (18). In the context of chronic rhinosinusitis (CRS), ILC2s within nasal polyp tissue have been shown to significantly decrease after oral steroid treatment (25). Likewise, blood ILC2s were decreased in asthmatics with allergic rhinitis after a three-month treatment with intranasal and inhaled corticosteroids (26). An attenuation of IL-5 and IL-13 production by alarmin-stimulated ILC2s in the presence of steroids *in vitro* has been shown (26)(27). We have recently showed that in mild asthmatics, total numbers of ILC2s and IL-5^+/IL-13^+ ILC2s in sputum increased significantly during the first 24h after inhalation allergen challenge in mild asthmatics, which then declined to baseline levels by 48h post-allergen challenge (6). Therefore, we selected the 24h time point post-NAC, with a view to capturing maximal change in ILC2 numbers post-NAC and assessing the effects of INCS on these cells in the nasal mucosa. In the INCS treatment arm, there were significantly lower total numbers of ILC2s and IL-5^+/IL-13^+ ILC2s, and significantly lower $CD4^+$ T cells in nasal mucosa, compared to the placebo treatment. In line with this, there was a decrease in type 2 cytokines, including IL-5 and IL-13, within the nasal lavage in the INCS treatment arm. These findings suggest that INCS has the potential to attenuate the ability of ILC2s to drive type 2 inflammation locally within the nasal mucosa.

IL-2 maintains ILC2 survival and cytokine production (28)(29), whereas IL-4 is important for ILC2 proliferation (29)(30). The alarmin cytokines, IL-33, IL-25 and TSLP are important activators of ILC2s, stimulating IL-5 and IL-13 production that drive type 2 inflammatory

responses (29). Furthermore, TSLP and IL-7 produced in high levels within the airways of severe asthmatics are proposed to confer steroid insensitivity to airway ILC2s in this patient population (18). In this study, the NAC stimulated an increase in nasal lavage levels of IL-2, IL-4 and IL-25 at 7h, which were attenuated (not significantly for IL-25) in the INCS treatment arm. As such, a decline in the signals for ILC2 survival and proliferation may have, in part, resulted in lower ILC2 numbers within the INCS treatment arm compared to placebo. In addition low TSLP levels, consistent with findings from other studies in allergic rhinitics challenged with house dust extract (31) may have facilitated the inhibitory effects of steroid on ILC2 numbers and function in the nasal mucosa.

With respect to novel pathways by which ILC2s drive adaptive immune responses, the expression of major histocompatibility complex II (MHC II) has recently been reported on ILC2s, suggesting a possible antigen presenting cell (APC) function of these cells in mice (28)(32). The human MHC II known as Human Leukocyte Antigen (HLA-DR) has also been identified on ILC2s from COPD patients following an acute exacerbation (33). The role of HLA-DR on human ILC2s has not been well studied in the context of allergic airways disease. *In vitro* mouse and human models have shown that the interaction between ILC2s and $CD4^+$ T cells through MHC II results in significant proliferation of both cell types, as well as IL-5 and IL-13 production (28)(32)(33)(34). A novel aspect of this study is the detection of HLA-DR expression on ILC2s. A previous study which reported that ILC2s from non-pathological tissues (lung, skin, tonsil, colon) do not express HLA-DR (35). Here we show that HLA-DR expression is upregulated on ILC2s from chronically inflamed tissue post-allergen challenge and that pre-treatment with intranasal steroids attenuates allergen-induced up-regulation of this receptor. This is supported by our *in vitro* data where overnight culture with either IL-2, TSLP or IFN-γ significantly up-regulated HLA-DR

expression on ILC2s. The IL-2 but not TSLP mediated effect was reversed in the presence of steroids. We therefore propose that steroids attenuate ILC2 mediated activation of T2 inflammatory responses, in part by reducing the antigen presenting capacity of these cells within the nasal mucosa. Further research is needed to determine the relevance of HLA-DR expression in mediating the antigen presenting capacity of ILC2s compared to other antigen presenting cells in human allergic airways disease.

In summary, our data show that nasal allergen challenge induces a local type 2 inflammatory response within the nasal mucosa which is modulated by pre-treatment with intranasal corticosteroids. Increases in ILC2s numbers and activation level in the nasal mucosa was associated with type 2 inflammation, contributing to allergic rhinitis symptoms and inflammation in the nose. The lack of predominance of TSLP or IL-7 within the local upper airways may facilitate steroid sensitivity of ILC2s. Whether the relative proportion of conventional versus non-conventional ILC2s and the steroid sensitivity of these populations varies with severity of allergic rhinitis disease warrants further investigation.

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Table 2.1. Baseline Characteristics of Study Subjects

Sex	Age (yr)	FEV_1 (% Predicted)	FEV/FVC	Allergen	Allergen Dilution	PNIF (L/min)	TNSS
M	37	79.74	0.65	HDMDP	1:128	100	0
F	30	89.59	0.90	Cat	1:8	70	2
M	28	92.51	0.80	Grass	1:2	170	0
F	24	121.35	0.84	HDMDP	1:8	130	1
M	57	85.86	0.62	HDMDP	1:2	150	0
F	22	109.06	0.87	HDMDP	1:8	160	1
M	37	107.82	0.72	Ragweed	1:8	170	2
M	23	98.60	0.85	Horse	1:32	150	0
F	19	91.09	0.75	HDMDP	1:32	130	0
F	21	110.31	0.88	HDMDP	1:8	190	0

Definition of abbreviation: FEV_1= forced expiratory volume in one second; HDMDP= house dust mite protein.

Baseline characteristics of mild allergic rhinitics with asthma (n=10) that were enrolled in the study. Non-smoking subjects aged 21 to 57 were enrolled in this study. Subjects were skin-prick positive to common aeroallergens, FEV_1 greater or equals to 70% of the predicted value. Subjects had no asthma exacerbation and respiratory tract infection during the previous 8 weeks. Subjects had no using of β2-agonist treatment, corticosteroids, aspirin or anti histamine medications prier to the laboratory visit. Choice of allergen was based on largest skin prick test positivity and challenge dilution of the allergen was determined based on a post-nasal allergen challenge that caused a peak nasal inspiratory flow (PNIF) reduction by ≥50% from the baseline and a total nasal symptom score (TNSS) increase above 10.

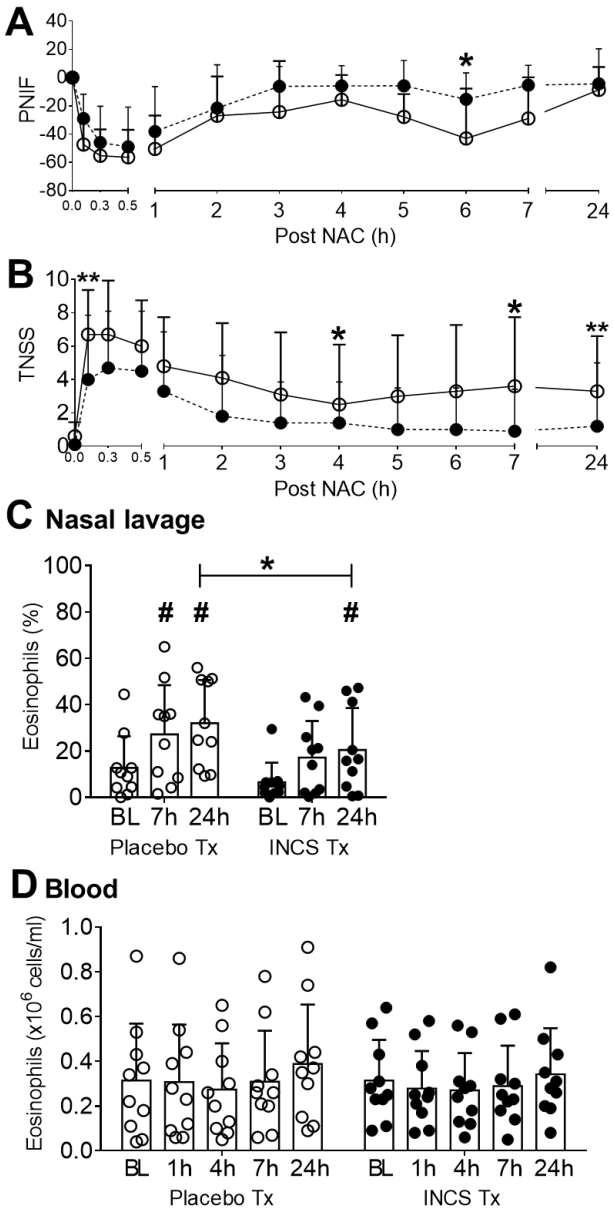


Figure 2.1. Nasal symptoms (PNIF –peak nasal inspiratory flow and TNSS: Total nasal symptom score) and eosinophil levels in the upper and blood detected pre and post-nasal allergen challenge (NAC) in the placebo (open circles) and intranasal corticosteroid (INCS) (closed circles) treatment arms. There was a significant improvement in (A) PNIF in INCS treatment arm 6h post-NAC (n=9) and (B) TNSS at 5 min, 4h, 7h and 24h post NAC in the INCS treatment arm (n=10). (C) NAC-induced increase in the percentage of eosinophils in nasal lavage was attenuated in the INCS treatment arm 24h time post (n=10). (D) Absolute numbers of eosinophils in blood were not affected by NAC in the placebo or INCS treatment arms (n=10). Data are presented as mean ± SD; A Wilcoxon test for non-parametric paired data was used for (A&B) and ANOVA test with Bonferroni post-hoc test was used to detect significance for (C & D). (# $p<0.05$ for within and * $P<0.05$ for between treatment arm comparisons); BL= baseline.

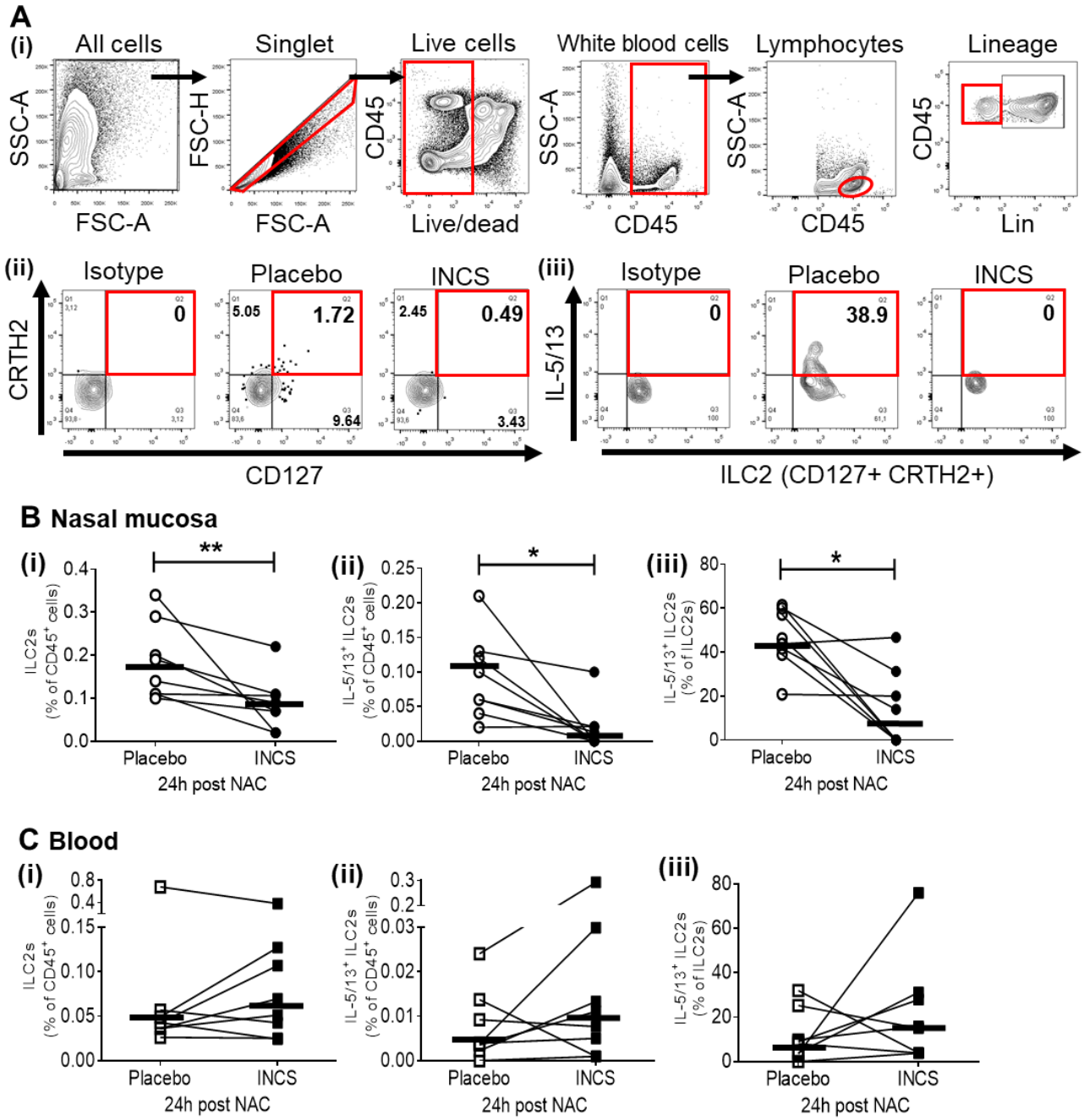


Figure 2.2. (A) Representative FACS plot of ILC2s gated as live, singlet SSC^{low} Lin^{-} $CD45^{+}$ $CD127^{+}$ $CRTH2^{+}$ cells from nasal mucosa. A significant reduction in nasal mucosa levels of (B)(i) total ILC2s and (B)(ii) IL-5^{+}/IL-13^{+} ILC2s expressed as percent of total $CD45^{+}$ cells or (B)(iii) total ILC2s was found 24h post-NAC in the INCS versus placebo treatment arm. (C) No significant change in total ILC2s or IL-5^{+}/IL-13^{+} ILC2s was found in blood, 24h post-NAC when placebo was compared to INCS treatment arm. Data (n=8 of 10 as 2 subjects had insufficient cell recovery by nasal curettage) were analyzed for statistical significance using a Wilcoxon test for non-parametric paired data; horizontal bars represent median of each data set. (*P<0.05 & ** P<0.01).

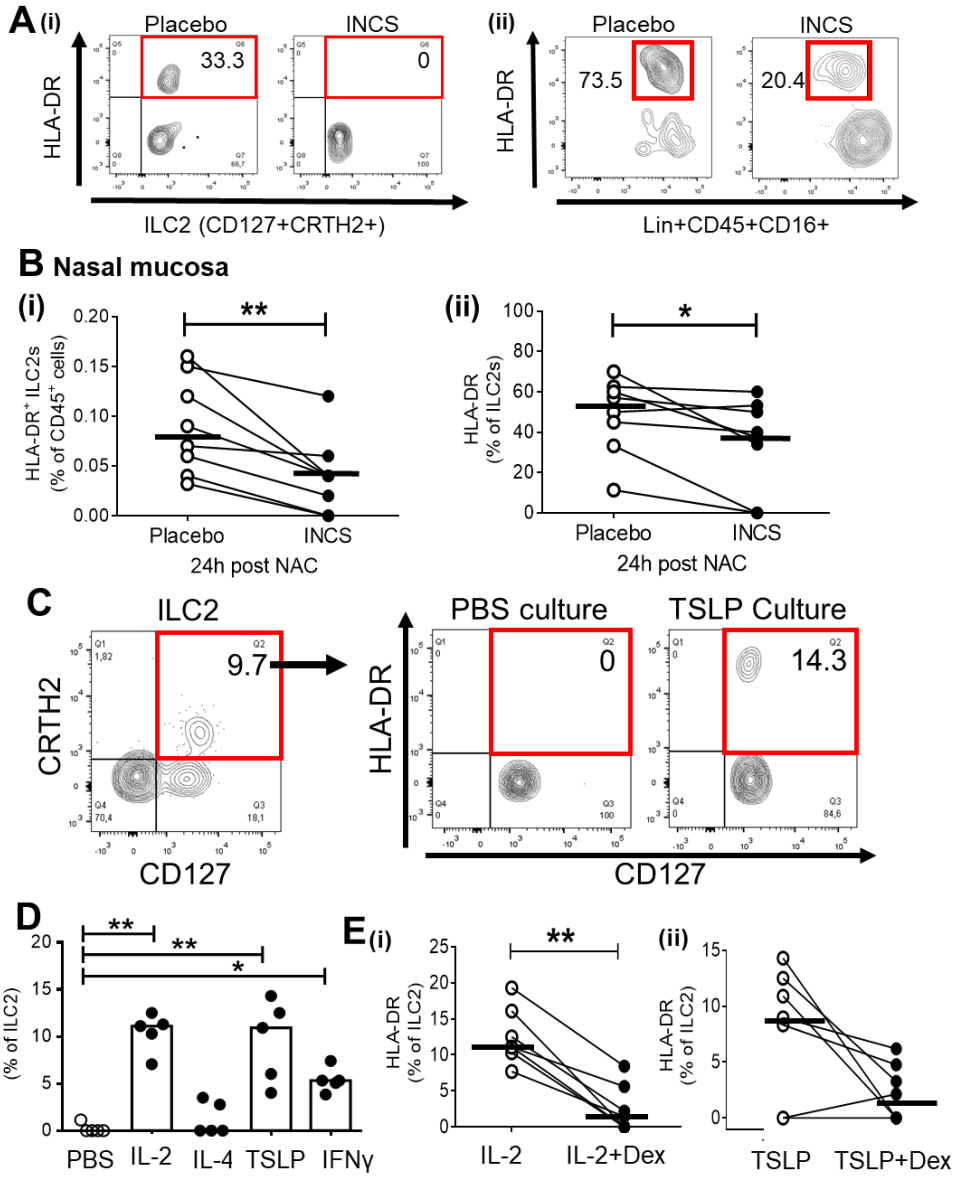


Figure 2.3. Representative FACS plot of HLA-DR expression on (A)(i) ILC2s (Lin^-CD45^+ $CD127^+$ $CRTH2^+$) compared to the positive control (A) (ii) monocytes (Lin^+ $CD45^+$ $CD16^+$ cells) extracted from nasal mucosa. (B) (i) HLA-DR expression was significantly attenuated on ILC2s detected at 24h post NAC in INCS vs placebo treatment arm when data were expressed as a proportion of (B)(ii) $CD45^+$ cells or (B)(iii) as a proportion of total ILC2s. A similar trend was observed with monocytes although this was not significant. (C) FACS gating on PBMCs for HLA-DR expression on ILC2s cultured in PBS vs TSLP (10ng/ml) overnight. (D) $HLA\text{-}DR^+ILC2s$ were increased significantly following overnight culture with optimal concentrations of IL-2, TSLP (10 ng/ml) and INF-γ (50ng/ml) but not IL-4 (10ng/ml). (E) Upregulation of HLA-DR expression by ILC2s was attenuated in the presence of dexamethasone (10^{-7}M). Statistical analyses of data in (B) used a Wilcoxon test for non-parametric paired data; horizontal bars represent median values of each data set, (D) one-way ANOVA with Bonferroni post-hoc and (E) paired T-test.

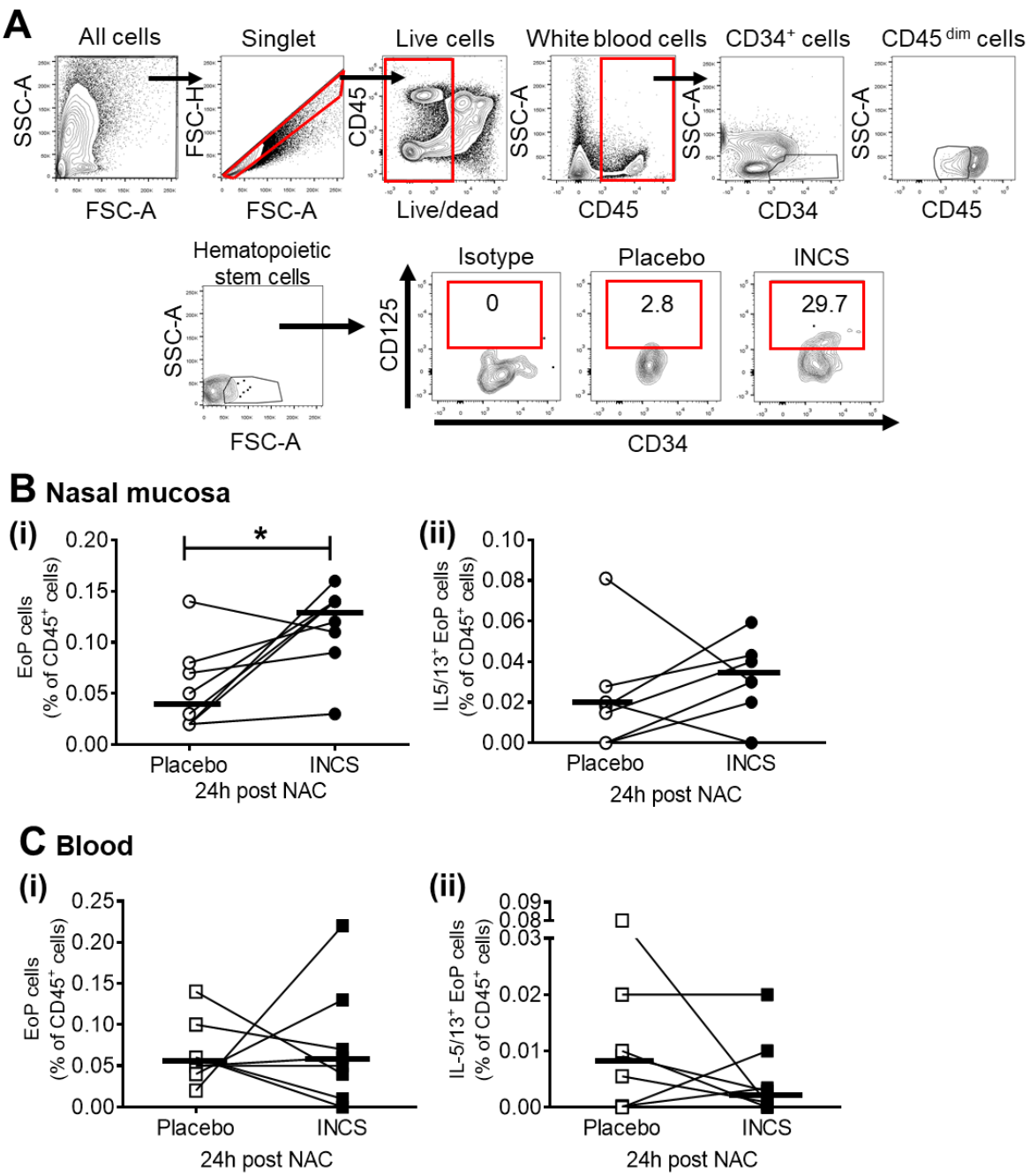


Figure 2.4. Enumeration of Eosinophilic progenitor cells (EoPs) in nasal mucosa and peripheral blood 24 hours after NAC in placebo and INCS treatment arms. (A) EoPs were identified as $CD34^{+}$ $CD45^{dim}$ $CD125^{+}$ cells by FACS. There was a significant increase in (A)(i) total EoPs in nasal mucosa 24h post-NAC in the INCS versus placebo treatment group. No significant change was found for (A)(ii) IL-5^{+}/IL-13^{+} EoPs in nasal mucosa, (B)(i) total blood EoPs or (ii) IL-5^{+}/IL-13^{+} EoPs cells in blood when 24h post-NAC samples were compared in the INCS versus placebo treatment arms **(C).** Data (n=8) were analyzed for statistical significance using a Wilcoxon test for non-parametric paired data; Horizontal bars represent median values of each data set.

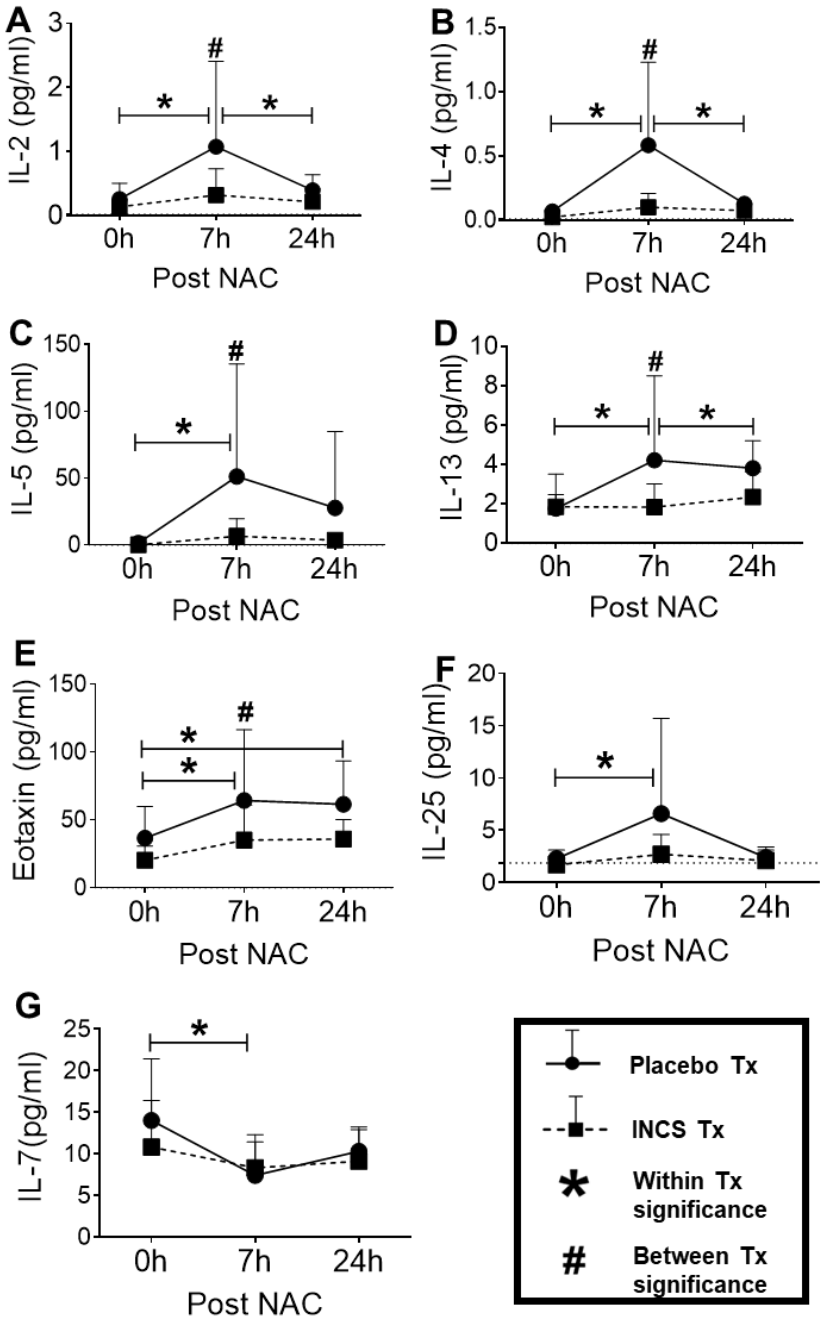


Figure 2.5. Cytokine and chemokines in nasal lavage samples from patients with mild allergic rhinitis and asthma. Subjects were treated with placebo (solid circles) or intranasal steroids (solid squares) for 14 days before NAC. At baseline (BL), 7h and 24h post-nasal Ag challenge, levels of (A) IL-2, (B) IL-4, (C) IL-5, (D) IL-13, (E) eotaxin (F) IL-25 and (G) IL-7 were determined by mesoplex assays on the MSD Platform. A two-way ANOVA test with Bonferroni correction (α/m) was used to determine statistical significance of parametric paired data. Data was presented as mean and SD (n=9). * indicates the significance within the placebo group and # indicates the significance between placebo and INCS groups.

ONLINE SUPPLEMENTAL DATA

Title: Effect of Intranasal corticosteroid treatment on allergen-induced changes in group 2 innate lymphoid cells in allergic rhinitics with mild asthma

Authors: *Yanqing Xie[1,2], *Xiaotian Ju[1], Suzanne Beaudin[1], Lesley Wiltshire[1], John Paul[1,3] Oliveria, Jonathan MacLean[4], Doron D. Sommer[4], Ruth Cusack[1], Olga Li[5], Prajna Banerjee[5], Paul K. Keith[1], Paul M O'Byrne[1], Rebecca N. Bauer[5], Tracy Staton[5], Gail M Gauvreau[1], Roma Sehmi

Immunofluorescence Staining for Flow Cytometric Analyses

Nasal-extracted cells and peripheral blood mononuclear cells were stained with viability Dye (AF700) and incubated in Perm/Fix Buffer (BD Bioscience). Cells were then stained with antibodies for both surface and intracellular markers Lin-FTIC, CD45-APC-H7, IL-5/13-PE, PerCP-Cy5.5-HLA-DR, CRTH2-BV421, CD127-BV605, and CD4-BV510, CD34-BV786 and CD125-APC or relevant isotype controls (BD Bioscience, Mississauga, ON, Canada; eBiosciences, San Diego, CA; R&D Systems, MN, USA). Lin-cocktail antibodies to (CD2, CD3, CD14, CD19, CD20 and CD94) plus FcƐRI excluded T and B lymphocytes, B-cells, monocytes, eosinophils, NK cells and basophils. After incubation, cells were washed and resuspended in fixed in PBS with 1% paraformaldehyde and analyzed by a FACS LSRII flow cytometer (BD Biosciences, CA, US) within 24 h. As cells were treated with Perm/Fix immediately after isolation from the nasal mucosa, a protein transport inhibitor was not required to maintain intracellular cytokine. Gating in the lympho-mononuclear region (low side scatter/low forward scatter) and following acquisition of 300,000 events, data were analyzed using Flow-Jo software (Tree Star, CA, US) where ILC2 were defined as live, singlets with SSA^{low} Lin^{-} $CD45^{+}$ $CD127^{+}$ $CRTH2^{+}$ phenotype expressing intracellular type 2 cytokines, IL-5/IL-13^{+} (Figure 2.2A) or surface HLA-DR (Figure 2.3A).

Gating for $CD4^+$ T cells as live, singlet cells with SSA^{low} $Lineage^+$ [CD2, 3, 14, 16, 19, 20, 94, FcƐRI] $CD45^+$ $CD4^+$) phenotype is described in Figure E6 with a 98% confidence limit. Data are expressed as a proportion of the total $CD45^+$ (white cell population) cells gated unless otherwise stated. Gating for eosinophilic progenitor cells (EoPs; $CD34^+$ $CD45^{dim}$ $CD125^+$) and hematopoietic progenitor cells (HPCs; $CD34^+$ $CD45^{dim}$) is described in **Figure 2.4**.

Mesoplex Assays

Using the Meso-Scale Discovery (MSD) and a QuickPlex SQ120 Imager, nasal lavage samples were assayed using (i) Human V-PLEX 36-cytokine panel to detect chemokines, pro-inflammatory and Th17 cytokines and, (ii) Uplex kit to detect alarmin cytokines (TSLP, IL-17E/IL-25 and IL-33). The lower limit of detection (LLOD) values were IL-2, IL-5 (0.02 pg/ml); IL-4 (0.01 pg/ml); IL-7 (0.06 pg/ml); IL-13 (0.14 pg/ml); IL-25 (1.88 pg/ml); TSLP (1.96 pg/ml); Eotaxin (3.29 pg/ml). Any value below LLOD value was replaced by LLOD/2.

Subjects	$CD45^+$ cells (Events)	ILC2s (Events)	$IL5/13^+ILC2s$ (Events)	ILC2s (%$CD45^+$ cells)	$IL5/13^+ILC2s$ (%$CD45^+$ cells)	$IL5/13^+ILC2s$ (% ILC2s)
01						
Placebo	5465	16	7	0.29	0.13	43.8
INCS	**6740**	**15**	**7**	**0.22**	**0.1**	**46.7**
02						
Placebo	16132	18	7	0.11	0.04	38.9
INCS	**4003**	**1**	**0**	**0.02**	**0**	**0**
03						
Placebo	21728	24	5	0.11	0.02	20.8
INCS	**4704**	**5**	**1**	**0.11**	**0.02**	**20**
04						
Placebo	17293	24	10	0.14	0.06	41.7
INCS	**7854**	**7**	**1**	**0.09**	**0.01**	**14.3**
05						
Placebo	3465	7	4	0.20	0.12	57.1
INCS	**5605**	**4**	**0**	**0.07**	**0**	**0**
06						
Placebo	15704	32	16	0.20	0.10	50
INCS	**4729**	**5**	**0**	**0.11**	**0**	**0**
07						
Placebo	4977	5	3	0.10	0.06	60
INCS	**24425**	**16**	**5**	**0.07**	**0.02**	**31.3**
08						
Placebo	13054	44	27	0.34	0.21	61.4
INCS	**4773**	**1**	**0**	**0.02**	**0**	**0**

Table E2.1. Raw data for FACS gating for total and cytokine positive ILC2 from nasal curettage samples taken from each patient in the current study. These raw data from 24h post-nasal allergen challenge (NAC) samples taken from the placebo and intranasal corticosteroid (INCS) arms are presented as number of gated events and percent of gated $CD45^+$ cells (white blood cells). These data are also presented in figure 2 of the manuscript.

Not significant	**Placebo**						**INCS**					
(pg/ml)	**BL**		**7h**		**24h**		**BL**		**7h**		**24h**	
Cytokines	**Mean**	*SD*	**Mean**	*SD*	**Mean**	*SD*	**Mean**	*SD*	**Mean**	*SD*	**Mean**	*SD*
Eotaxin-3	**359.5**	*811.1*	**649.2**	*1477.1*	**269.2**	*254.3*	**45.9**	*69.5*	**44.1**	*34.6*	**184.2**	*385.8*
IL-1β	**3.2**	*3.3*	**1.4**	*1.1*	**3.2**	*2.2*	**2.2**	*2.0*	**1.5**	*1.6*	**2.7**	*1.9*
IL-6	**1.3**	*0.9*	**2.8**	*2.5*	**3.4**	*3.0*	**1.2**	*0.9*	**4.0**	*5.2*	**3.0**	*3.4*
IL-8	**513.0**	*368.1*	**267.8**	*112.0*	**693.6**	*330.9*	**349.0**	*224.6*	**279.7**	*193.6*	**654.5**	*730.7*
IP-10	**993.3**	*1136.1*	**449.5**	*227.4*	**769.0**	*513.4*	**674.1**	*1046.9*	**435.2**	*279.2*	**421.8**	*234.8*
MCP-1	**24.2**	*8.7*	**41.9**	*28.2*	**35.2**	*15.3*	**29.9**	*9.8*	**42.2**	*27.2*	**32.7**	*12.9*
MCP-4	**5.7**	*8.1*	**3.9**	*3.0*	**5.1**	*4.4*	**1.6**	*1.2*	**2.4**	*1.6*	**2.8**	*1.9*
MDC	**28.7**	*18.6*	**42.2**	*34.8*	**65.4**	*58.0*	**13.1**	*13.1*	**15.4**	*4.9*	**82.1**	*184.1*
MIP-1α	**10.9**	*6.1*	**15.5**	*14.8*	**15.0**	*9.5*	**7.9**	*4.1*	**8.9**	*2.9*	**19.7**	*27.0*
MIP-1β	**12.9**	*17.3*	**32.4**	*42.2*	**46.6**	*66.9*	**5.6**	*3.5*	**13.2**	*15.9*	**28.4**	*42.1*
IL-16	**62.7**	*79.8*	**71.9**	*84.8*	**79.1**	*71.8*	**22.0**	*21.7*	**31.7**	*34.7*	**53.8**	*36.9*
IL-1α	**15.8**	*10.9*	**11.0**	*7.3*	**15.2**	*10.8*	**19.1**	*11.6*	**10.8**	*5.1*	**12.5**	*7.2*
TARC	**15.2**	*11.9*	**57.0**	*66.9*	**92.6**	*115.6*	**8.7**	*4.1*	**14.3**	*7.7*	**47.4**	*59.2*
VEGF	**288.0**	*124.1*	**232.9**	*88.8*	**236.9**	*66.1*	**263.0**	*103.1*	**247.8**	*57.4*	**268.1**	*106.0*

Table E2.2. Additional cytokine and chemokine levels in nasal lavage samples from patients with mild allergic rhinitis and asthma. Subjects were treated with placebo followed by intranasal steroid for 14 days before NAC. At baseline (BL),7h and 24h post-NAC. Cytokines that demonstrated no significant change after INCS treatment are listed above. Data was presented as mean with SD. A two-way ANOVA test with Bonferroni correction (α/m) was used to determine statistical significance of parametric paired data.

*A number of cytokines were below the limit of detection of the assay kit including GM-CSF, IFN-γ, IL-10, IL-12, IL-12p70, IL-15, IL-17, IL-17A/F, IL-33, TSLP, TNFα and TNF-β

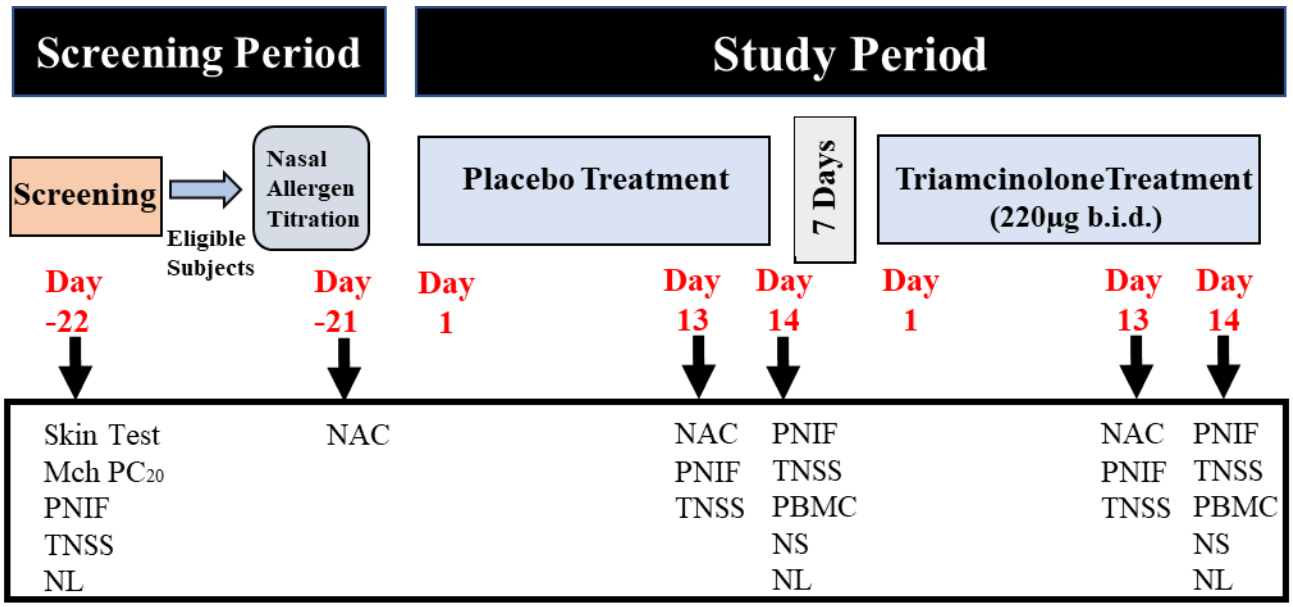


Figure E2.1. Study Design: Eligible subjects (allergic rhinitis patients with asthma) inhaled placebo by aqueous nasal spray twice a day for 14 days before nasal allergen challenge (NAC). Blood and nasal sampling were made 24 hours after NAC. Following a 7 day washout period, subjects inhaled 220 µg triamcinolone acetonide twice daily by aqueous nasal spray for 14 days before the NAC. Blood and nasal sampling were repeated as indicated. NAC= nasal allergen challenge; MCh PC_{20} = concentration of methacholine required to reduce FEV_1 by 20%; INCS= intranasal corticosteroid; PNIF= peak nasal inspiratory flow; TNSS= total nasal symptom score; NL=nasal lavage; PBMC= peripheral blood mononuclear cells; NS= nasal scraping.

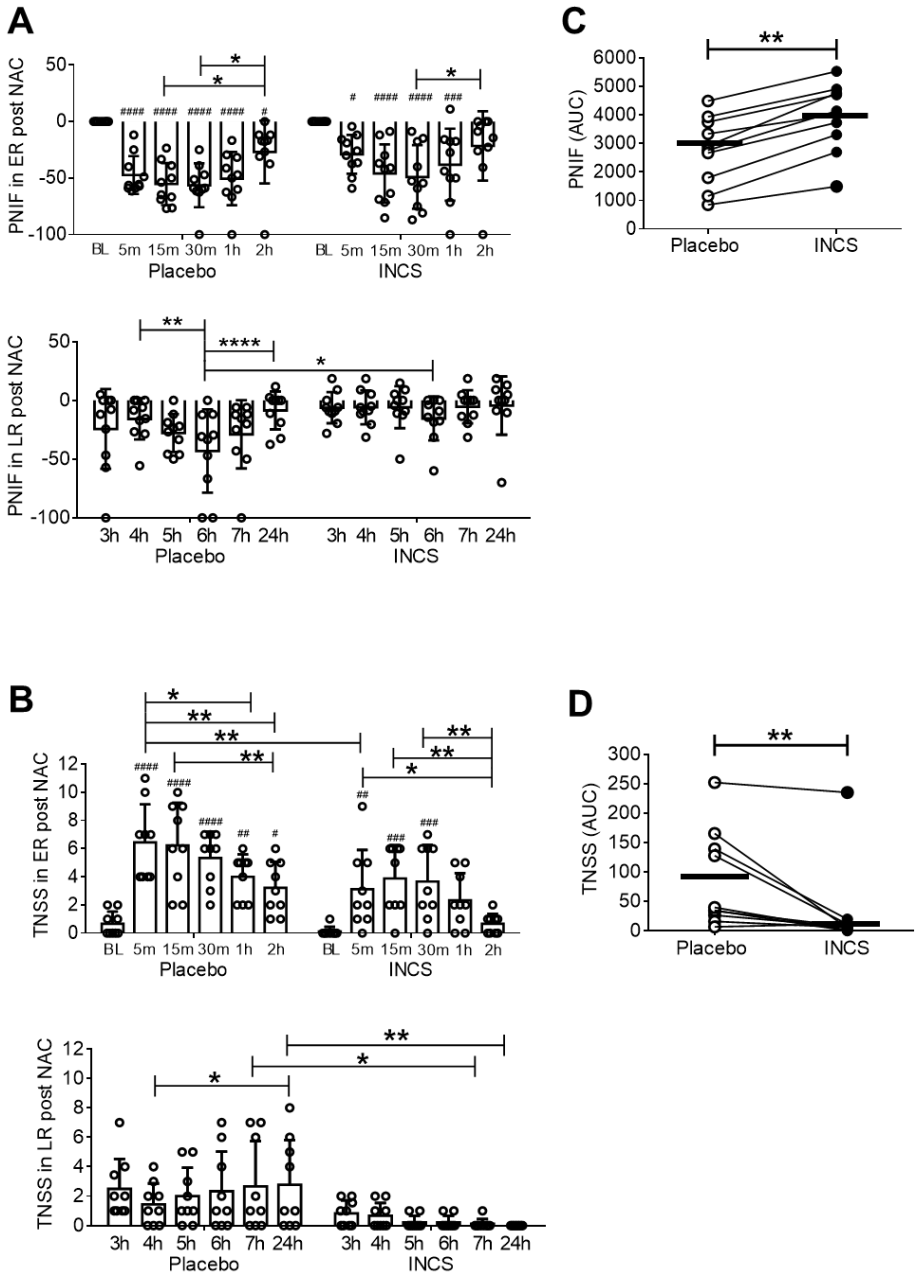


Figure E2.2. Nasal symptoms score (PNIF and TNSS) at baseline and post NAC in placebo and INCS Treatment Arms**.** Data are subdivided based on early and late responses. **(A)** PNIF is presented as % change from baseline (n=10), **(B)** TNSS at baseline and different time points after NAC in placebo and INCS arms (n=10). Nasal symptoms - PNIF **(C)** and TNSS **(D)** were analysed from 0 min to 24 hours post NAC in each treatment arm and data are presented as area under the curve (AUC) for each patient with horizontal bars showing median levels. A two-way ANOVA test with Bonferroni correction (α/m) was used to determine statistical significance of parametric paired data. A Wilcoxon test was used for between placebo and INCS treatment arm comparisons. BL= baseline. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; # indicates comparison to baseline.

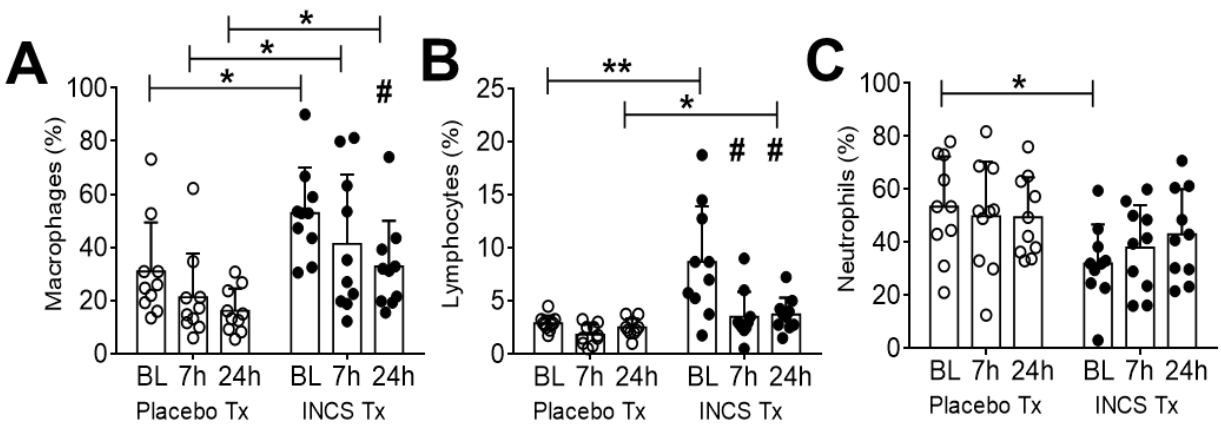


Figure E2.3. Cellular frequencies in nasal lavage fluid taken pre and post allergen challenge in the placebo and INCS treatment arms. INCS pre-treatment resulted in a significant decrease in (A) macrophages and (B) lymphocytes but not (C) neutrophils, 24h post NAC versus pre-NAC in the INCS treatment arm but not in the placebo treatment. Data were presented as mean ± SD; a two-way ANOVA test with Bonferroni correction (α/m) was used to assess significance of parametric paired data. BL= baseline.

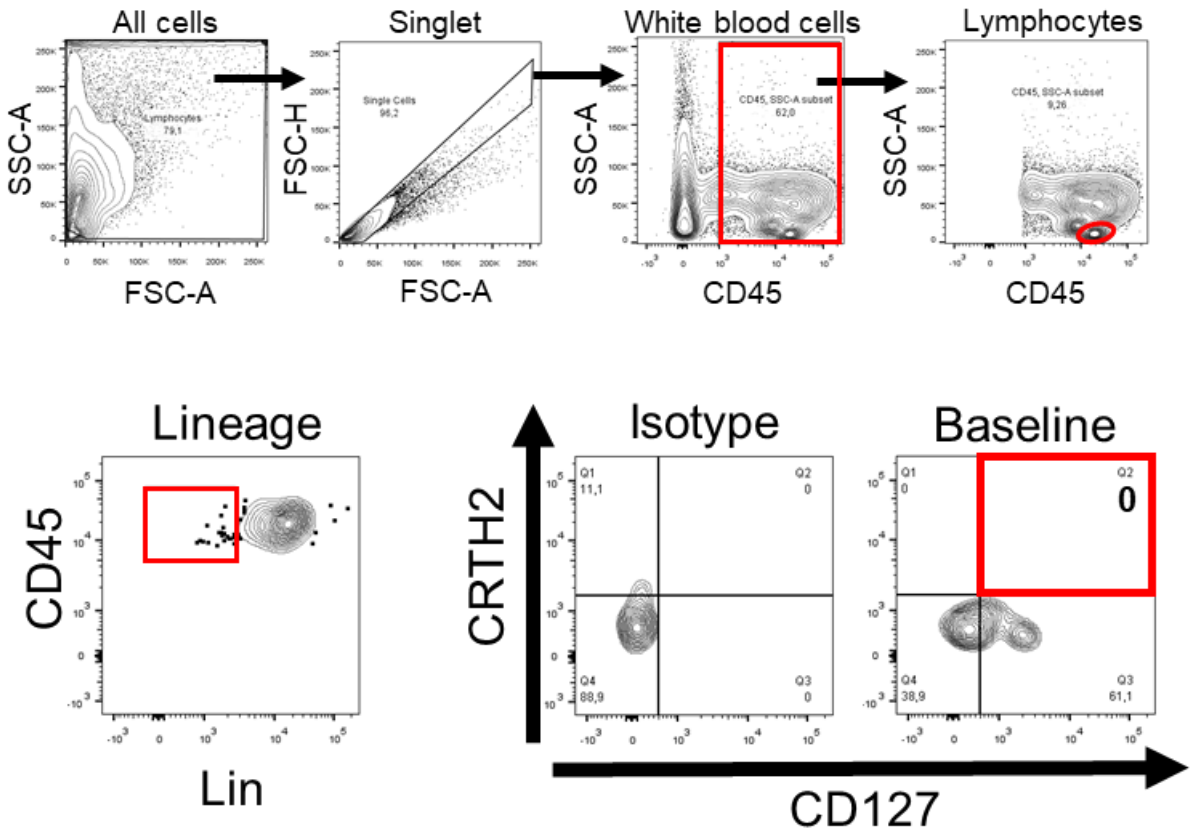


Figure E2.4. FACS gating of a representative nasal curettage sample taken pre-allergen challenge to identify ILC2s as live, singlet cells that are lin-CD45^{+} CRTH2^{+} CD127^{+}. Data show that negligible numbers of ILC2s were detected in pre-allergen challenge samples from mild allergic rhinitics with asthma. This precluded the use of pre-allergen challenge samples and only 24h post-nasal allergen challenge samples were collected in the placebo and steroid treatment arm of this study.

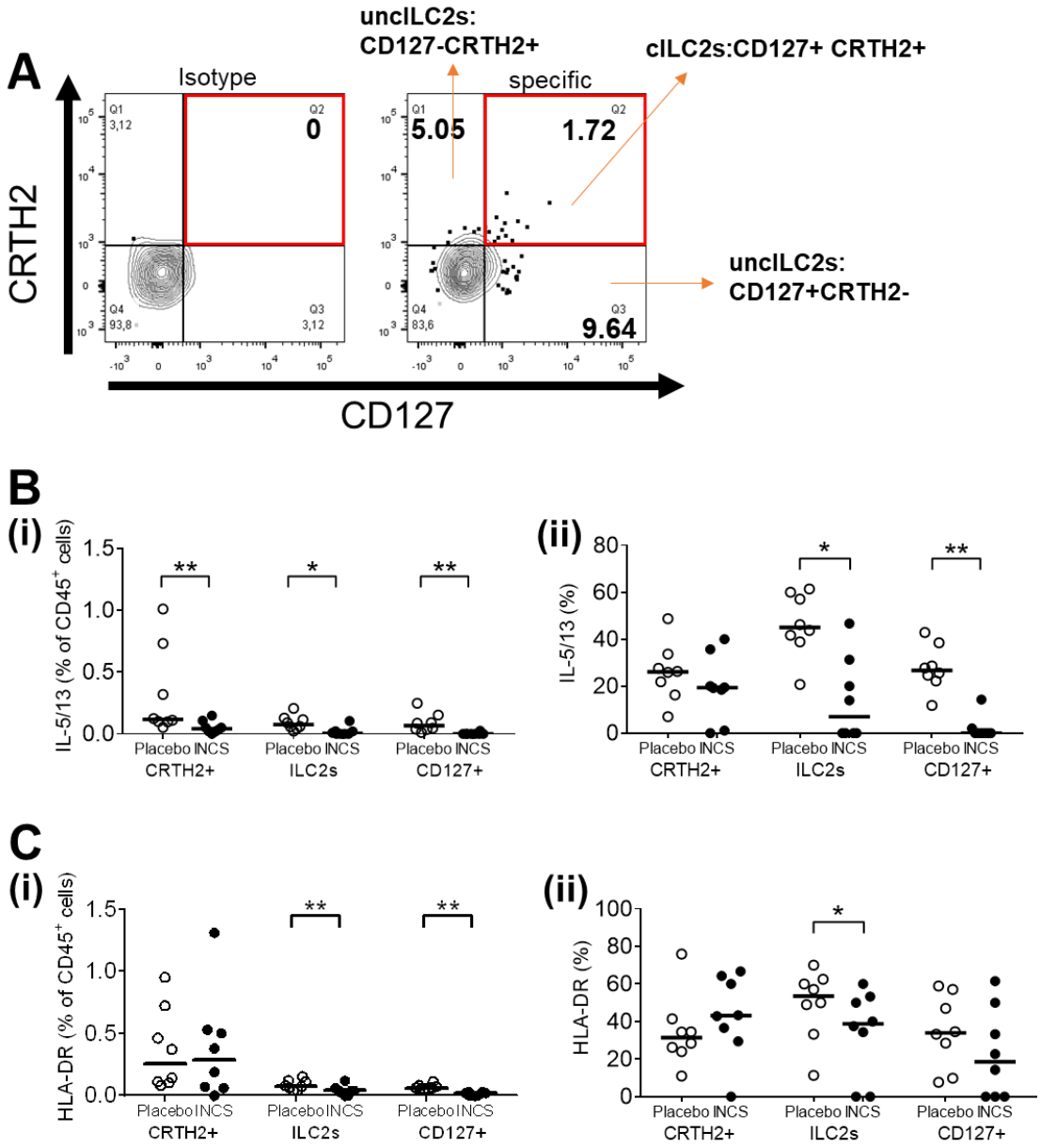


Figure E2.5: (A) Flow cytometric enumeration of nasal mucosa extracted cells identified as conventional ILC2s (cILC2s: Lin$^-$ CD45$^+$ CD127$^+$ CRTH2$^+$) compared to unconventional ILC2s (uncILC2s: Lin$^-$ CD45$^+$ CD127$^-$ CRTH2$^+$ and Lin$^-$ CD45$^+$ CD127$^+$ CRTH2$^-$ cells). Expressed as (B) (i) a percent total CD45$^+$ cells, comparable expression levels of IL-5/13 in all ILC2s sub-groups and all were significantly attenuated by INCS treatment, (ii) a percent of each ILC2 population, INCS treatment had differential inhibitory effects on type 2 cytokine expression. For HLA-DR$^+$ ILC2s expressed as (C)(i) percent CD45 or (C)(ii) percent ILC2 population – although comparable levels of expression were found, INCS treatment only significantly attenuated HLA-DR expression 24h post-NAC in the cILC2 population and not uncILC2s. Data (n=8) were analyzed for statistical significance using a Wilcoxon test for non-parametric paired data; horizontal bars represent median values of each data set. (*P<0.05 and ** P<0.01).

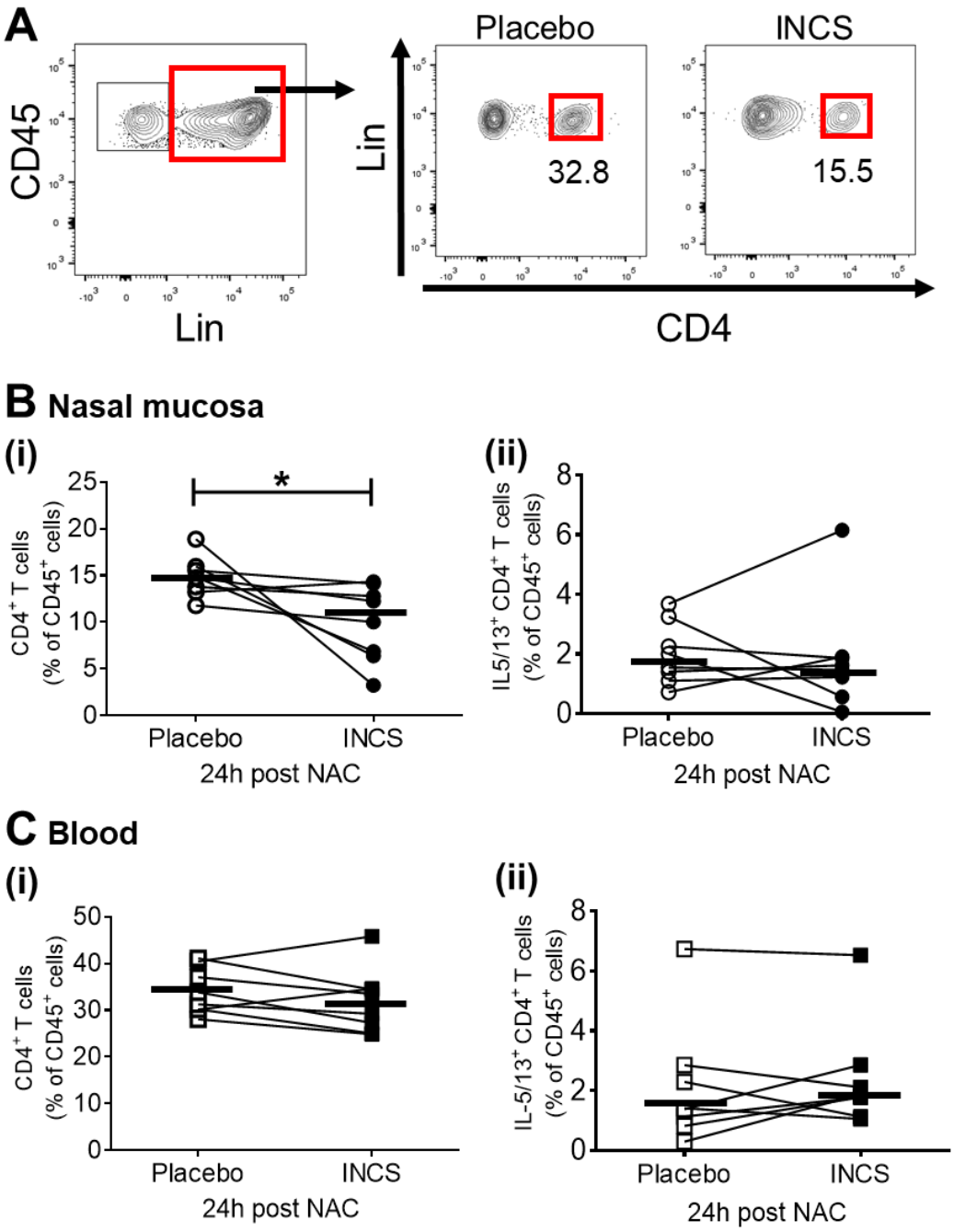


Figure E2.6. $CD4^+$ T cells in nasal mucosa and peripheral blood 24 hours after NAC in placebo and INCS treatment groups. (A) FACS gating of live, singlet $CD45^+$ Lin^+ $CD4^+$ T cells in nasal mucosa extracted cells (B) There was a significant reduction of total $CD4^+$ cells in nasal mucosa 24h post NAC in INCS treatment arm versus placebo (n=8). (C) No significant change was detected in IL-5^+/IL-13^+ $CD4^+$ cells in nasal mucosa, total or IL-5^+/IL-13^+ $CD4^+$ cells in blood (n=8). Data were analyzed for statistical significance using a Wilcoxon test; horizontal bars represent median values of each data set.

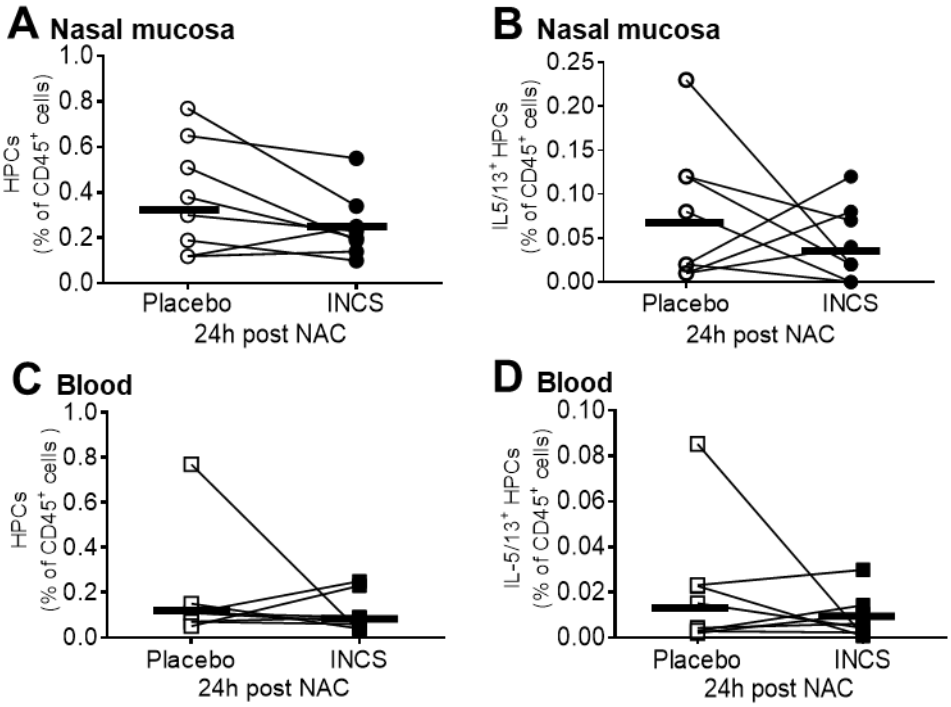


Figure E2.7. Hemopoietic progenitor cell (HPCs) levels in nasal mucosa and peripheral blood 24 hours after NAC in placebo and INCS treatment groups. (A-D) No significant change was detected in HPCs or IL-5^+/IL-13^+ HPCs in nasal mucosa or blood (n=8) after INCS treatment. Data were analyzed for statistical significance using a Wilcoxon test and the horizontal bars represent median values of each data set.

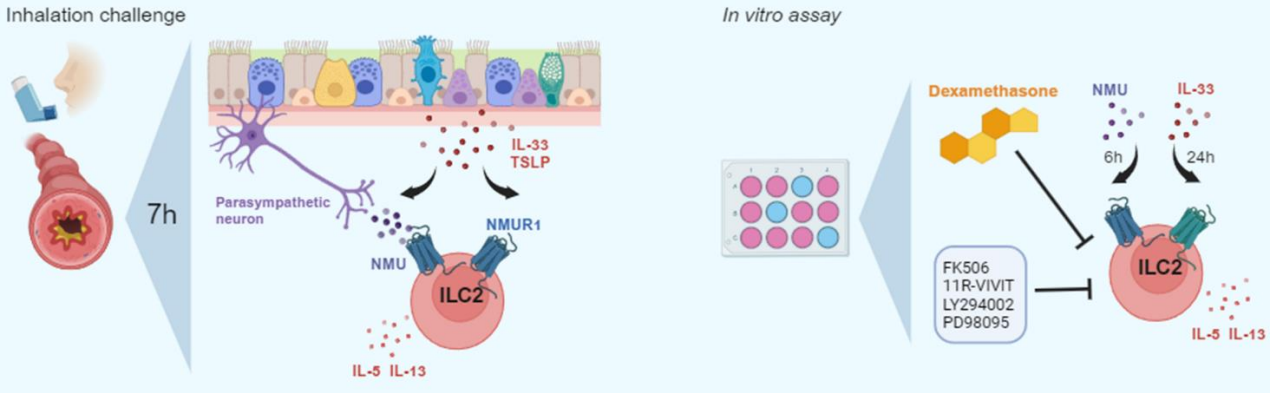
CHAPTER 3: Neuromedin-U/NMU-R1 Axis Mediates Early Activation of Group 2 Innate Lymphoid Cells in the Airways of Subjects with Mild Asthma following Inhaled Allergen Challenge.

The submission of this chapter is pending.

Cite: *Xiaotian Ju, *Akimichi Nagashima, Jennifer Wattie, Karen Howie, Guillaume Paré, Reine Ditta, Anna Dvorkin-Gheva, Ruth Cusack, Imran Satia, Paul M O'Byrne, Gail M. Gauvreau and Roma Sehmi. Neuromedin-U/ NMU-R1 Axis Mediates Rapid Activation of Group 2 Innate Lymphoid Cells in the Airways of Subjects with Mild Asthma following Inhaled Allergen Challenge. (Submitted for publication Nov 2023)

* These authors contributed equally to the development of this manuscript

CAPSULE SUMMARY



ILC2 increased responsiveness to neuromedin U (NMU) by upregulating NMU receptor 1 following allergen challenge in mild asthmatics or alarmins in an *in vitro* culture. NMU directly induced IL-5/13 production in ILC2 within 6h, suggesting that NMU-NMUR1 axis mediate a rapid activation of ILC2. Furthermore, the NMU mediated ILC2 activities were significantly attenuated by corticosteroid or the inhibitors against the downstream signaling mediators of NMUR1 including calcineurin, mitogen activated protein kinase or nuclear factor of activated T cells.

ABSTRACT

Rationale: In asthma, sputum group 2 innate lymphoid cells (ILC2) are activated within 7h after allergen challenge. Neuroimmune interactions mediate rapid host responses at mucosal interfaces. In murine models of asthma, lung ILC2 co-localize to sensory neuronal termini expressing the neuropeptide, neuromedin U (NMU) and NMU stimulates type 2 cytokines secretion by ILC2 with additive effects to alarmins, *in vitro*.

Objectives: Investigate effect of NMU/NMUR1 axis on ILC2 activation in asthma.

Methods: Stable mild asthmatics (n=8) enrolled in a diluent-controlled, allergen-inhalation challenge study. Sputum ILC2 expression of NMU receptor 1 (NMUR1) and T2 cytokines was enumerated by flow cytometry and airway NMU levels in were assessed by ELISA. This was compared to samples from moderate-severe asthmatics (n=9). Flow sort-purified and *ex-vivo* expanded blood ILC2 were used for functional assays and transcriptomic analyses.

Results: A significant increase in sputum ILC2 expressing NMUR1 was found 7h post- allergen versus diluent challenge where the majority of $NMUR1^{+}$ILC2 expressed IL-5/IL-13. Sputum $NMUR1^{+}$ILC2 were significantly greater in mild versus moderate-severe asthmatics and $NMUR1^{+}$ILC2 correlated inversely with the dose of inhaled corticosteroid in the latter group. Co-cultures with alarmins upregulated *NMUR1* in ILC2, attenuated by dexamethasone. NMU stimulated T2 cytokine production by blood ILC2, maximal at 6h, which was abrogated by dexamethasone or specific signaling inhibitors for mitogen-activated protein kinase 1/2, phospho-inositol 3 kinase but not IL-33 signaling moiety MyD88, *in vitro*.

Conclusions: The NMU/NMUR1 axis stimulates rapid effects on ILC2, and maybe an important early activator of these cells in eosinophilic inflammatory responses in asthma.

INTRODUCTION

Asthma is a chronic and heterogeneous airways disease characterized by reversible airflow obstruction, airway hyperresponsiveness and bronchial inflammation affecting over 350 million people of all ages worldwide (1). As the most prevalent asthma phenotype, allergic asthma is mediated by type 2 immune responses involving interleukin (IL)-4, IL-5, IL-9 and IL-13 which predominantly secreted by $CD4^+$ T helper (Th) 2 cells and group 2 innate lymphoid cells (ILC2)(2)(3). ILC2 are lineage-negative lymphomononuclear cells that lack antigen recognition receptors and are copious producers of type 2 cytokines (4) activated by epithelial cell-derived alarmins, lipid mediators, aryl hydrocarbons and cytokines (IL-2, IL-7). ILC2 provide an early, innate source of IL-5 and IL-13 promoting airway eosinophilia in response to common aeroallergens, pollution, viral infections and fungal agents (5-9). Expression of MHC-II by ILC2 (10), with capability for peptide-mediated activation of antigen-specific T cells, indicates that by bridging innate and adaptive immune pathways, ILC2 play a fundamental role as drivers of tissue eosinophilia in atopic and non-atopic asthma (11-13).

ILC2 are primarily tissue resident cells found at mucosal barriers (14) with increased accumulation at sites of eosinophilic inflammation (15)(16). These cells are increased in asthmatics compared to normal controls, with the greatest levels of activated ILC2 detected in the airways of severe asthmatics with uncontrolled eosinophilia (>3%), despite high-dose steroid therapy (17-19). Transcriptomic analyses of sputum cells from the UBIOPRED study support a similar association between ILC2 and severe asthmatics with airway eosinophilia (20). Following allergen-inhalation challenge in mild asthmatics, phenotypic and transcriptomic assessments show temporal- and spatially-constrained activation profiles of ILC2 (21)(22). Specifically, ILC2 are activated *early* in the asthmatic response (within 24h post-Ag) and only within the airways. More

recently we reported that activated airway ILC2 numbers peak at 7h post-allergen challenge in mild asthma (23). Similarly, in allergic rhinitis patients, increases in activated ILC2 in the nasal mucosa have been detected early on in the time course of pro-inflammatory events following allergen or viral exposure (24)(25). Although alarmin cytokines IL-33 and TSLP are key triggers of ILC2 activation, these factors have a delayed onset of action (longer than 12h) (26). Therefore, pathways that mediate the early and rapid activation of airway ILC2 in eosinophilic airway responses in asthma remain to be investigated.

The respiratory tract is innervated by sensory nerves where nerve density correlates with the airway eosinophilia in asthma (27). When noxious stimuli or immune mediators (e.g. IL-33, TSLP, histamine) stimulate nociceptors on the termini of sensory neurons, action potential activates the parasympathetic ganglia to release neuropeptides or neurotransmitters in the airway (28-30). Neuromedin U (NMU) is a neuropeptide generated by enteric cholinergic neurons and airway sensory neurons, modulating smooth muscle contraction and immune cell activation (31)(32). NMU has two G-protein coupled receptors: NMUR1 that is primarily expressed by immune and hematopoietic cells (33); and NMUR2 which is predominantly expressed in the central nervous system (34). NMUR1 is highly expressed by murine ILC2 within the leukocyte populations in the lungs, gastro-intestinal tract and skin (35)(36). Following exposure of mice to papain (35), *Alternaria alternata* or respiratory syncytial virus (37), lung resident ILC2 prominently up-regulate *NMUR1* and significant increases in $NMUR1^{+}$ILC2 numbers are noted which precede development of tissue eosinophilia; an effect that is markedly impaired in $NMUR1^{-/-}$ mice (35). A stimulatory role of NMU on ILC2 in type 2 airway inflammatory responses along an NMU/NMUR1 axis is further supported by visualization of increased numbers of ILC2 in a closer proximity to the dorsal root ganglion of sensory neurons expressing NMU in murine lungs (38).

In *in vitro* studies, a comparable level of IL-5/13 expression was detected in murine ILC2 stimulated with NMU or IL-25 and IL-33 (39, 40) where the neuropeptide synergized with alarmin stimulation (38) suggesting that NMU is an important mediator, other than alarmin cytokines that signals local ILC2 activation in type 2 inflammatory responses. In humans high levels of NMUR1 expression have been detected on ILC2 in blood but only at low levels in the airways of subjects with stable severe asthma (41). Little however is known about the expression of NMUR1 on ILC2 or NMU levels in the airway during an allergic asthmatic response.

Here, we show an early increase in sputum $NMUR1^{+}$ILC2 in the airways of subjects with mild asthma following inhaled allergen challenge and that most of these cells expressed IL-5 and IL-13. This is supported by i*n vitro* findings that NMU stimulates rapid expression of IL-5 and IL-13 by ILC2 within 6h of incubation through a MAPK/PI3 kinase dependent signaling pathway that is steroid sensitive. Co-culture of ILC2 with alarmin cytokines up-regulated NMUR1 expression at the protein and gene level which was attenuated in the presence of dexamethasone. These findings reflect the lower levels of $NMURI^{+}$ILC2 and NMU levels in moderate sever asthmatics compared to steroid naïve mild asthmatics. We propose that the close interplay between neuropeptide signalling and tissue-derived alarmin cytokines maybe be important initiators of ILC2 activation in airway inflammatory responses in asthma.

METHODS

Subjects: Asymptomatic and steroid-naïve non-smokers with stable mild atopic asthma were recruited for inhaled allergen challenges (n=8) and moderate-severe asthmatics for baseline cross-sectional comparison (n=9). Subject demographics are detailed in **Table 3.1** and **Table E3.1**. All experimental procedures were reviewed and approved by the Hamilton Integrated Research Ethics Board and all subjects provided informed consent.

***Study design, methacholine challenge and whole lung allergen challenge*:** Mild asthmatics underwent a randomized, cross-over, diluent-controlled, allergen inhalation challenge with a minimum 2-week washout period (**Fig E3.1**) as previously described (23). Methacholine and allergen/diluent challenges were performed by the tidal breathing method using a Wright nebulizer (42)(43) as previously described (44). And detailed in the online supplement.

Sputum Induction and Cell Isolation: Sputum was induced following inhalation of an aerosol of hypertonic saline (3/5/7%) as previously described (45). Processing details of sputum mucus plugs are provided in the online supplement. Sputum supernatants were collected for proteomic analyses and the cell fraction was used for cytospin preparations which were stained with Diff-Quik (American Scientific Products, McGawPark, IL, US) for differential counts of duplicate slides. Remaining cells were stained for flow cytometry.

Immunofluorescence staining and flow cytometric gating strategy: Sputum-extracted cells were subject to immunofluorescence staining with antibodies to surface markers with relevant isotype controls: Lineage-FITC, FcεRI-FITC, CD94-FITC, CD45-BV510, CD127-BV605, CRTH2-PE-CF594 and NMUR1-PE followed by permeabilization and fixation to stain for intracellular IL-5-BV421 and IL-13-AF700. Gating strategy of flow cytometric enumeration of ILC2 are detailed in the **Fig E3.3A & B** and previously described by us (23).

ILC2 stimulation with NMU and signalling inhibitors, in vitro: The details for *in vitro* time course and functional assays with enriched ILC2 from peripheral blood are summarized in the online supplement.

ELISA Assays: The levels of the neuropeptide NMU and T2 cytokines in sputum supernatants were analysed using a commercially bought NMU ELISA kit (BIOMATIK, ON, CA) or by ELLA^{TM} multiplex automated-ELISA (Bio-Techne, US).

Microarray analysis of ILC2 transcriptome: Enriched blood ILC2 were flow sort-purified and *ex vivo* expanded then cultured with T2 or non-T2 cytokine inducing conditions as described in the online supplement. Following culture, cell-associated RNA was extracted, quantified, amplified, converted to cDNA using biotinylated sense-strand DNA targets and labeled samples were then hybridized to human Clariom S array (Thermofisher Scientific). Raw data were normalized using the RMA algorithm implemented in the limma R-package. Adjusted *p*-values were calculated using the *Benjamini-Hochberg* method. Data were visualized using ggplot22 and p heatmap R-packages.

Statistical Analysis: Data were analyzed using GraphPad Prism 7 software (Graph Pad Software Inc., CA, USA). Data are expressed as mean ± SEM unless stated otherwise. For data not normally distributed, statistical analyses were performed on the log-transformed data. Statistical analysis for cytokine expression and neuronal receptor on ILC2 following inhalation challenges was performed using two-way ANOVA with a Bonferroni post hoc test. All *in vitro* experiment data were analyzed by using one-way ANOVA with a Dunnett post hoc test. P values of < 0.05 were considered significant.

RESULTS

Allergen inhalation challenge induces airway hyperresponsiveness.

All mild allergic asthmatic subjects (n=8) (**Table 3.1**) had a dual bronchoconstrictor response post allergen-inhalation challenge (**Fig. E3.2A&B**), and a significant decrease in methacholine PC_{20} (expressed as Log2) from baseline at 24h post-allergen, but not post- diluent challenge (**Fig. E3.2C, Table 3.2**). Eosinophils in sputum increased significantly at 7h and 24h post-allergen challenge but not post diluent challenge (**Table 3.2**, **Fig. E3.2D**).

Rapid increases in sputum NMUR1$^+$ ILC2 post allergen-inhalation challenge.

Sputum ILC2, enumerated by flow cytometry (multi gating strategy - **Fig. E3.3A&B**), were significantly increased at 7h and 24h post allergen-challenge compared to the baseline level and only at 24h compared to the diluent challenge (**Fig. 3.1A**). The level of NMUR1$^+$ILC2 were significantly increased at 7h post-allergen compared to pre-allergen challenge levels when expressed as absolute cells/ml (**Fig. 3.1B**) and as a percentage of total ILC2, expression of NMUR1 was significantly increased at 7h post-allergen compared to diluent challenge (**Fig. 3.1C**). In addition, the heatmap shows that the frequency of NMUR1 expression was significantly up-regulated only on ILC cells expressing CRTH2 (i.e. ILC2) but not on CRTH2$^-$ ILC (non-ILC2) at 7h post-allergen challenge (**Fig 3.1D, Fig. E3.3C**).

NMUR1$^+$ ILC2 expressed more type 2 cytokines and correlated with airway eosinophilia post-allergen challenge.

Sputum ILC2 expressing intracellular IL-5 and IL-13 were significantly increased at both 7h and 24h post-allergen compared to diluent challenge when data were expressed as cells/ml (**Fig. 3.2A**)

or percentage of total ILC2 **(Fig. E3.3D**). At all time points post-allergen challenge, we found that a significantly greater proportion of IL-5/IL13^{+} expression was found in NMUR1^{+} ILC2 compared to NMUR1^{-}ILC2 cells (**Fig. 3.2B**).

Key pro-inflammatory cytokines were measured in sputum supernatants and IL-5 levels were significantly increased 7h and 24h post-allergen compared to pre-challenge levels (**Fig. E3.4**). Numbers of sputum NMUR1^{+}ILC2 were significantly correlated with sputum eosinophilia, 24h post-allergen challenge (**Fig. 3.2C**) and showed a non-significant positive correlation with airway levels of IL-5 (r=0.71, p=0.06) (**data not shown**). Levels of NMU detected in the sputum supernatants by ELISA did not vary significantly at any time point following allergen or diluent-challenge (**Fig 3.2D**).

Alarmin-mediated upregulation of NMUR1 expression by ILC2 at gene and protein level

To investigate up-regulation of NMUR1 expression on ILC2 at the transcriptomic level, we performed microarray gene analysis on flow sort-purified blood ILC2 that were expanded *ex-vivo* prior to stimulation with IL-2 control, T2-inducing (IL-33+TSLP) or non-T2 (IL-1β+IL-23+TGF-β) inducing cytokines, as previously described (46)(47). A principal components analysis (PCA) was conducted based on 440 genes related to neuro-signalling pathways or T2 inflammation (**Table E3.2**). The resultant PCA plot shows distinctive clusters of ILC2 induced by IL-2 compared to T2- and non-T2 inducing culture conditions with the greatest difference observed between the latter two conditions (**Fig. 3.3A**). The volcano plot shows a significant upregulation of the *NMUR1* expression along with type 2 inflammatory signature genes (*IL1RL1*, *CRLF2*, *CYSLTR2)* in T2 inducing cultures compared to non T2-inducing cultures (**Fig. 3.3B, Table E3.3**). Up-regulation of *NMUR1* was associated with significant up-regulation of type 2 cytokine genes (*IL5* and *IL13)*

(**Fig. 3.3C**). Of note, compared to *NMUR1*, only low levels of *NMUR2* were detected in ILC2 for any stimulatory condition (**Fig. 3.3D**). In contrast, nicotinic acetylcholine receptor related genes (*CHRNA5* and *CHRNA6*) were upregulated in ILC2 stimulated with non-T2 inducing cytokines (**Fig. 3.3E**). In agreement with the transcriptomic data, we found that incubation of enriched ILC2 with IL-33 (1ng/ml) for 6h significantly up-regulated the proportion of ILC2 expressing NMUR1 and that this was attenuated in the presence of dexamethasone (10^{-7}M) (**Fig. 3.3F&G**).

NMU- stimulated rapid T2 cytokine expression by ILC2 was attenuated by Dexamethasone. Data from the allergen-inhalation challenge and microarray assays indicate that NMU/NMUR1 axis mediates rapid type 2 cytokine generation by ILC2. Here we investigated the direct effect NMU on ILC2, *in vitro*. Following stimulation of enriched ILC2 from mild asthmatics with IL-2 plus NMU (1ng/ml) (determined based on the level of NMU in the airway at 7h post allergen challenge in mild asthmatics in a pilot study) over a 0-24h time course, there was an increase in IL-5 and IL-13 expression by ILC2 at 6h but not 24h post-stimulation compared to 0h timepoint (**Fig. 3.4A&B**).

Under optimal stimulatory conditions with NMU (1ng/ml, 6h), dexamethasone (10^{-7}M) significantly attenuated the level of intracellular expression of IL-5 and 13 by ILC2 (**Fig. 3.4C&D, Fig. E3.5A**). By comparison, IL-33 (1ng/ml) showed an only delayed onset of stimulatory effect on IL-5 and IL-13 intracellular expression by ILC2, detectable at 24h but not 6 h of culture, *in vitro* (**Fig. 3.4E&F**). This effect was attenuated in the presence of dexamethasone (10^{-7}M) only at 24h.

NMU/NMUR1 axis of ILC2 activation dependent on MEK, NFAT and PI3 Kinase but not MyD88 signalling.

To investigate the signalling pathway for NMU/NMUR1 axis, enriched ILC2 were incubated with previously optimized concentrations of inhibitors for mitogen-activated protein kinase kinase (MEK), nuclear factor of activated T-cells (NFAT) or myeloid differentiation primary response 88 (MyD88) in the presence of NMU (1ng/ml, 6h, 37°C). There was a significant attenuation in IL-5 and IL-13 expression by ILC2 stimulated with NMU in the presence of inhibitors for MEK while NFAT inhibitors only attenuated IL-5 expression ((**Fig. 3.5A&B,** supplemental **Fig. E3.5B**). Inhibitors for PI3K or calcineurin (CsA) significantly attenuated expression of IL-5 and IL-13 and only IL-5 by NMU stimulated ILC2, respectively (**Fig. 3.5C&D,** supplemental **Fig. E3.5C**). In contrast, blockade of MyD88 signaling had no effect on levels of IL-5 or IL-13 expression by NMU stimulated ILC2, *in vitro* (**Fig. 3.5E&F**).

NMUR1 expression on ILC2 from subjects with moderate severe asthma.

There was a significantly higher proportion of sputum ILC2 expressing NMUR1 from mild asthmatics (40%) compared with moderate-severe asthmatics (16%) currently treated with high dose inhaled corticosteroid therapy (**Fig. 3.6A&B**). Similarly, NMU levels in sputum were significantly higher in mild vs moderate-severe asthmatics (**Fig. 3.6C**). Notably, a significantly inverse correlation was observed between the fluticasone equivalent inhaled corticosteroid dose (μg/day) and the number of $NMUR1^+$ ILC2 in the sputum of moderate-severe asthmatics (**Fig. 3.6D**). On the other hand, the level of calcitonin gene related peptide (CGRP) (**Fig 3.6E**) and vasoactive intestinal peptide (VIP) (**Fig 3.6F**) and in sputum were higher in moderate-severe vs mild asthmatics.

DISCUSSION

Inhaled allergen-challenge model in mild asthmatics induces an early asthmatic response (EAR) followed by a late asthmatic response (LAR)(48). The EAR is defined by ≥20% fall in FEV_1 within 10 minutes and resolving in 2 hours after allergen inhalation challenge; about 60% of asthmatics proceed to LAR which occurs 3-7 hours (48)(49). All mild asthmatic subjects in this study developed LAR with a significant increase of airway eosinophil level at 7h post allergen-challenge and maintained to 24h post-challenge. In agreement with our previous studies using this model, we observed a rapid increase of total ILC2 and IL-5/13$^+$ ILC2 numbers in the airways at 7h post-allergen challenge. The delayed time-course of action on ILC2 activation by alarmins (IL-25, IL-33, and TSLP) was shown in this study and others (26), and our results suggested that NMU-NMUR1 axis may mediate the rapid, early activation of airway ILC2 in mild allergic asthma.

As the only leukocyte expressing a high level of NMUR1 in mice, a close proximity was observed between ILC2 and NMU$^+$ sensory or cholinergic neurons in the murine lung parenchyma (38) and small intestines (39), respectively. Also, Jarick *et al* observed a significant reduction of lung ILC2 and eosinophilia in the house dust mite or papain challenged *Nmur1*$^{-/-}$ mice (35). Here, we show for the first time that a significant increase of NMUR1$^+$ ILC2, with a higher level of IL-5/13 expression in NMUR1$^+$ ILC2 was observed at 7h post allergen challenge in mild asthmatics, suggesting an association between NMUR1 expression and IL-5/13 production by ILC2. In line with our *in vivo* observations, NMU stimulation of enriched ILC2 from blood enhanced IL-5/IL-13 expression within 6h *in vitro*. These results are consistent with the study conducted by Ye *et al* who showed NMU significantly increased IL-5 and IL-13 production by human blood ILC2 *in vitro*, an effect that was synergistically increased in the presence of IL-25, IL-33 and TSLP (41). In addition, Cardoso *et al.* and Klose *et al* administered intraperitoneal (39) or intranasal (40)

injections of NMU into mice which induced and effect equivalent to IL-7/25/33 treatment for enhancing ILC2 expression of IL-5 and IL-13 in the airways associated with increased airway resistance to methacholine and tissue eosinophilia (39). In contrast our rapid effects with NMU, the stimulatory effect of IL-33 on blood ILC2 was not observed until 24h post stimulation *in vitro*. Collectively, our results suggested that the rapid effects of NMU can directly mediate the early time course of ILC2 activation in the airways of asthmatics post-allergen challenge while the prolonged activation seen at 24h post allergen are maintained by alarmin cytokines.

We next investigated the direct effect of alarmins on *NMUR1* expression in ILC2. The upregulation or maintenance of increased NMUR1 gene expression has been reported for both human and murine ILC2 stimulated with IL-25+IL-33 (41) or IL-25 alone (38). In agreement with this, we showed that T2 stimulatory cytokines (IL-33+TSLP) significantly upregulated the *NMUR1* expression in flow-sort purified ILC2 *in vitro,* but not in the non T2 stimulatory conditions. Thus, alarmins up-regulate NMUR1 expression on ILC2 in the airways which in-turn can trigger rapid activation of these cells in response to NMU stimulation. We did not however find significant allergen-induced changes in NMU levels within the airways of mild asthmatics which may be consistent with the short half life of NMU (<5 min) (50) mediated by inactivation of the neuropeptide by peptidase activity within the airways (51). This too may explain why we found lower levels of NMU in the airways of moderate-severe asthmatics compared to mild asthmatics.

Activation of ILC2 by NMU was significantly attenuated in the presence of dexamethasone or signalling pathway inhibitors against mitogen-activated protein kinase kinase (MEK), calcineurin/NFAT and PI3K pathway. In addition, corticosteroid attenuated NMUR1 surface receptor expression on ILC2 from mild asthmatics, *in vitro*. A novel finding here is that treatment of dexamethasone led to a significant reduction of type 2 cytokine expression (IL-5 and IL-13) in

NMU stimulated ILC2 *in vitro*. Furthermore, we observed a significantly lower level of NMUR1 expression on sputum ILC2 in moderate severe asthmatics currently on steroid therapy. At the molecular level, the corticosteroid bind to α-subunit of glucocorticoid receptor (GRα) and form a GCS-GRα homodimer in the cytoplasm, which translocate onto the glucocorticoid responsive element in nucleus and activate the anti-inflammatory genes expression or inhibit the transcription factors (e.g. NFκB, AP-1) mediating the pro-inflammatory genes expression (52). Also, the GCS-GRα homodimer was reported to induce the apoptotic signaling cascade in inflammatory cells (53). Here, we proposed that dexamethasone directly inhibited the IL-5/13 production by ILC2 in response to NMU stimulation, partly by attenuating surface expression of NMUR1 on ILC2.

Through ligation of it's receptor, NMU stimulates three signalling pathways: mitogen-activated protein kinase kinase (MEK), calcineurin/NFAT and PI3K pathway (54). Here we showed that NMU mediated ILC2 activation was significantly attenuated by MEK, NFAT, PI3K or calcineurin inhibitors. These results are consistent with reports of attenuated proliferation of NMU induced murine ILC2 and genomic expressions of cytokines (*Il5*, *Il13*, *Csf2*) in the presence of inhibitors to calcineurin (CsA), MEK or NFAT (39). In contrast, blockade of myeloid differentiation primary response 88 (MyD88), an IL-33 signaling moiety did not affect the IL-5/13 production by NMU stimulated ILC2 indicating independent signaling pathways for NMU versus IL-33 and may explain the difference in time course of action of these mediators on ILC2.

Although the inhibitory effect of corticosteroid or signalling inhibitors on NMU stimulated ILC2 was elucidated by *in vitro* experiment in this study, the clinical effect of corticosteroid on NMU-NMUR1 axis in ILC2 from asthmatic subjects is still unclear. In this study, we observed a significantly higher level of baseline NMUR1 on sputum ILC2 from mild asthmatics compared to high dose corticosteroid dependent moderate-severe asthmatics (median 32% vs 16%) and the

baseline NMU level in sputum supernatant was also significantly higher in mild asthmatics. Ye and colleagues also reported a comparably low level of NMUR1 (median 15%) on ILC2 from the airways of both moderate severe asthmatics and the healthy controls at baseline whereas the baseline expression of NMUR1 on T cells in the lung tissues was around 50% (41). The potential explanation for these observations is that the long-term corticosteroid therapy in severe asthmatics reduced NMUR1 expression on ILC2 which agree with our *in vitro* findings. It is important to note that the national review of asthma deaths in UK found that more than half of reported asthma deaths were mild or moderate asthmatics who were prescribed insufficient amount of inhaled corticosteroid and were on excessive doses of short acting beta agonist therapy (55). It is postulated that the lack of inhaled corticosteroid treatment may have led to life-threatening rapid deterioration (exacerbation) of asthma (55). Our findings suggest that NMU mediated ILC2 activation may be one of triggering pathways that could be adequately controlled by steroid therapy in patients with mild asthma.

NMU positive sensory neurons co-stain positive for other neuropeptides including vasoactive intestinal peptide (VIP) and calcitonin gene-related peptide (CGRP) (39). Cognate receptors for these neuropeptides namely VIP receptor type 2 (VPAC2) and calcitonin receptor-like (CALCRL), respectively, are expressed on ILC2s and shown to potentiate ILC2 activity in type 2 inflammatory responses in mice (56)(57). A limitation of this study was that we did not concurrently explore the relative role of these neuropeptides on ILC2 activation in subjects with mild asthma. We were however constrained by the low recovery of sputum cells numbers in these study subjects.

In conclusion, our study found that the NMU-NMUR1 axis mediates rapid activation of airway ILC2 in mild asthma using an inhaled allergen challenge model. We propose that the close

interplay between neuropeptide signalling and tissue-derived alarmin cytokines maybe be important initiators of ILC2 activation in airway inflammatory responses in asthma.

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Table 3.1. Clinical Characteristics of Subjects with Mild or Moderate Severe Asthma

Characteristics	Subjects with Mild Asthma	Subjects with Moderate Severe Asthma
Patients (n)	8	9
Age (yr)	36 ± 4.2	48 ± 4.5
Sex (n)	M=2; F=6	M=3; F=6
BMI (kg/m^2)		
Median	27	31
Range	23-40	24-42
Prednisone (mg/d		
Median	0	4
Range	0	0-4
ICS dose, (Total daily dose)		
Median	0	500
Range	0	250-1000 *
Long-acting β-agonist (n)	0	5
Leukotriene receptor antagonist (n)	0	2
Sputum eosinophilia (%)		
Median	2.3	10.6
Range	0.5-6.6	3.5-57.2 *
FEV_1% predicted (pre-BD)	91.5 ± 3.1	72.8 ± 6.5 *
FEV_1 absolute, (L)	3.28 ± 0.1	2.04 ± 0.16 *
FVC, (L)	4.2 ± 0.19	2.98 ± 0.25 *
FEV_1/FVC, (%)	79 ± 2.31	69.6 ± 3.71 *

Definition of abbreviations:

BMI=body mass index; ICS= inhaled corticosteroid; pre-BD=prebronchodilator. Mild asthmatics only infrequently used inhaled short-acting bronchodilators as reliever therapy and were asymptomatic for 4 weeks prior to study. For moderate-severe asthmatics, the dose of ICS is equivalent to inhaled fluticasone. Baseline demographic data are presented as either mean ± SEM or median as indicated in the table. Both study subject groups were similar in age, BMI and sex. There were significant differences between the two subject groups for maintenance dose of prednisone and ICSs, sputum eosinophilia, and measures of lung function including FEV_1% predicted, FEV_1(L), FVC(L), and FEV1/FVC (%). *P<0.05 for between group comparisons.

Table 3.2. Lung Function and Sputum Eosinophils before and after Allergen and Diluent challenge

	Allergen Challenge	Diluent Challenge
Methacholine PC_{20}, mg/ml		
Before challenge	5.99 ± 1.52	6.05 ± 2.47
24h after challenge	3.25 ± 1.44	5.3 ± 1.18
Total sputum cells, x10^6 cells/ml		
Before challenge	4.27 ± 0.72	4.04 ± 1.05
7h after challenge	4.57 ± 0.98	6.74 ± 1.38
24h after challenge	11.43 ± 6.24	7.04 ± 2.08
Sputum eosinophils, % (x10^6 cells/ml)		
Before challenge	3.5±0.9 (0.10±0.02)	1.6±0.2 (0.07±0.02)
7h after challenge	10.8±2.5 (0.40±0.11) ***#**	3.1±0.8 (0.18±0.07)
24h after challenge	14.7±2.6 (0.78±0.34) ***#**	2.2±0.3 (0.09±0.01)

Definition of abbreviations: PC_{20}=provocative concentration of methacholine, causing a 20% fall in FEV_1. There were significant allergen-induced changes in methacholine PC_{20} levels and sputum eosinophil numbers compared with diluent inhalation challenge. Data are presented as mean ± SEM. *$p<0.05$ for comparison with baseline; #$p<0.05$ for comparison with diluent.

FIGURES

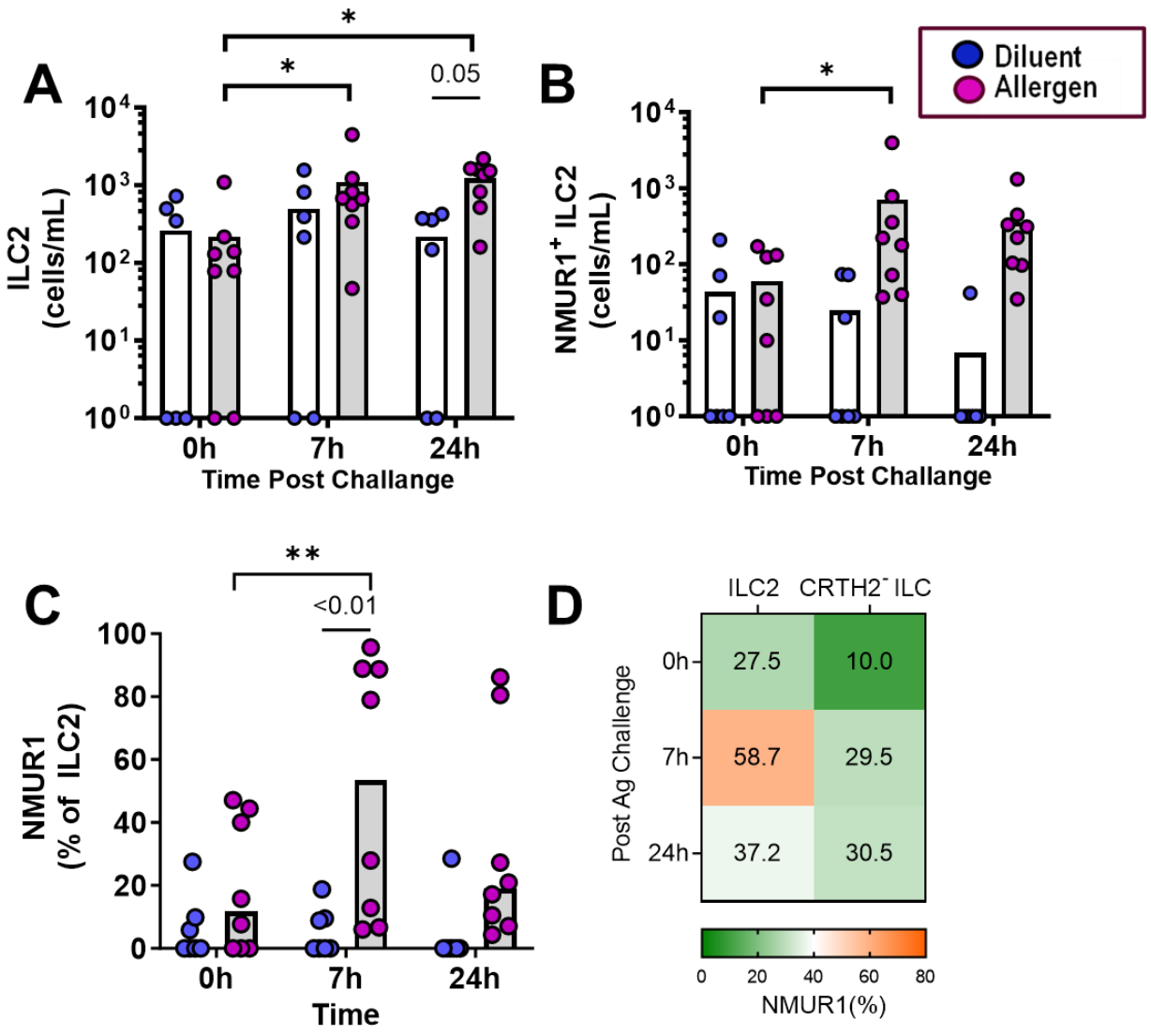

Figure 3.1. Allergen-induced changes in numbers of total and NMUR1 expressing ILC2 in sputum from subjects with mild asthma. Total **(A)** ILC2, **(B)** NMUR1^{+} ILC2 (cells/ml), and **(C)** proportion of ILC2 expressing NMUR1 increased significantly within 7h-post allergen challenge. **(D)** Heatmap shows mean NMUR1 expression was increased on ILC2 (Lin^{-} CD127^{+} CRTH2^{+} cells), but not CRTH2 $^{-}$ ILC cells (Lin^{-} CD127^{+} CRTH2^{-}) at 7h post allergen challenge. Data are presented as a log-scaled y axis and bar plots represent median of each data sets (A-B); (two-way ANOVA with a Bonferroni post-hoc test, *p<0.05).

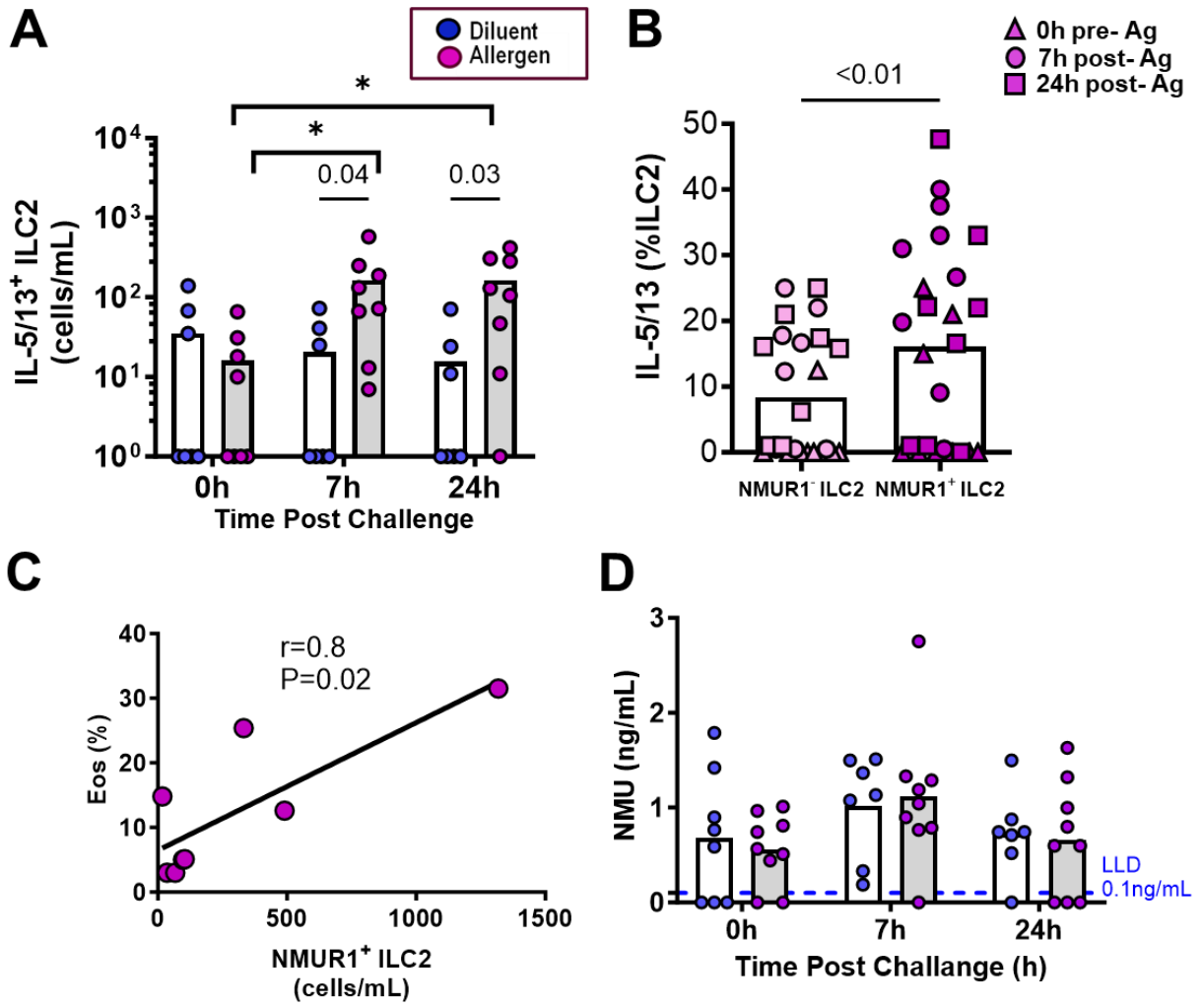

Figure 3.2. Allergen-induced changes in IL-5/13 expression by ILC2 and NMUR1$^+$ ILC2 following inhalation challenge. (A) IL-5/13$^+$ ILC2 were significantly increased at 7h and 24h post- allergen challenge compared to baseline or the corresponding diluent challenge levels. **(B)** A significantly greater proportion of IL-5/13 was detected in NMUR1$^+$ ILC2 compared to NMUR1$^-$ ILC2. **(C)** NMUR1$^+$ ILC2 cell numbers correlated with sputum eosinophilia (%) at 24 post allergen challenge. **(D)** NMU levels in the sputum supernatant of mild asthmatics challenged by diluent or allergen. Bar plots represent median of each data sets (A-B); two-way ANOVA with a Bonferroni post-hoc test for within group and paired t-test for between group comparisons, *p<0.05; Pearsons's correlation coefficient (r) for linear correlations.

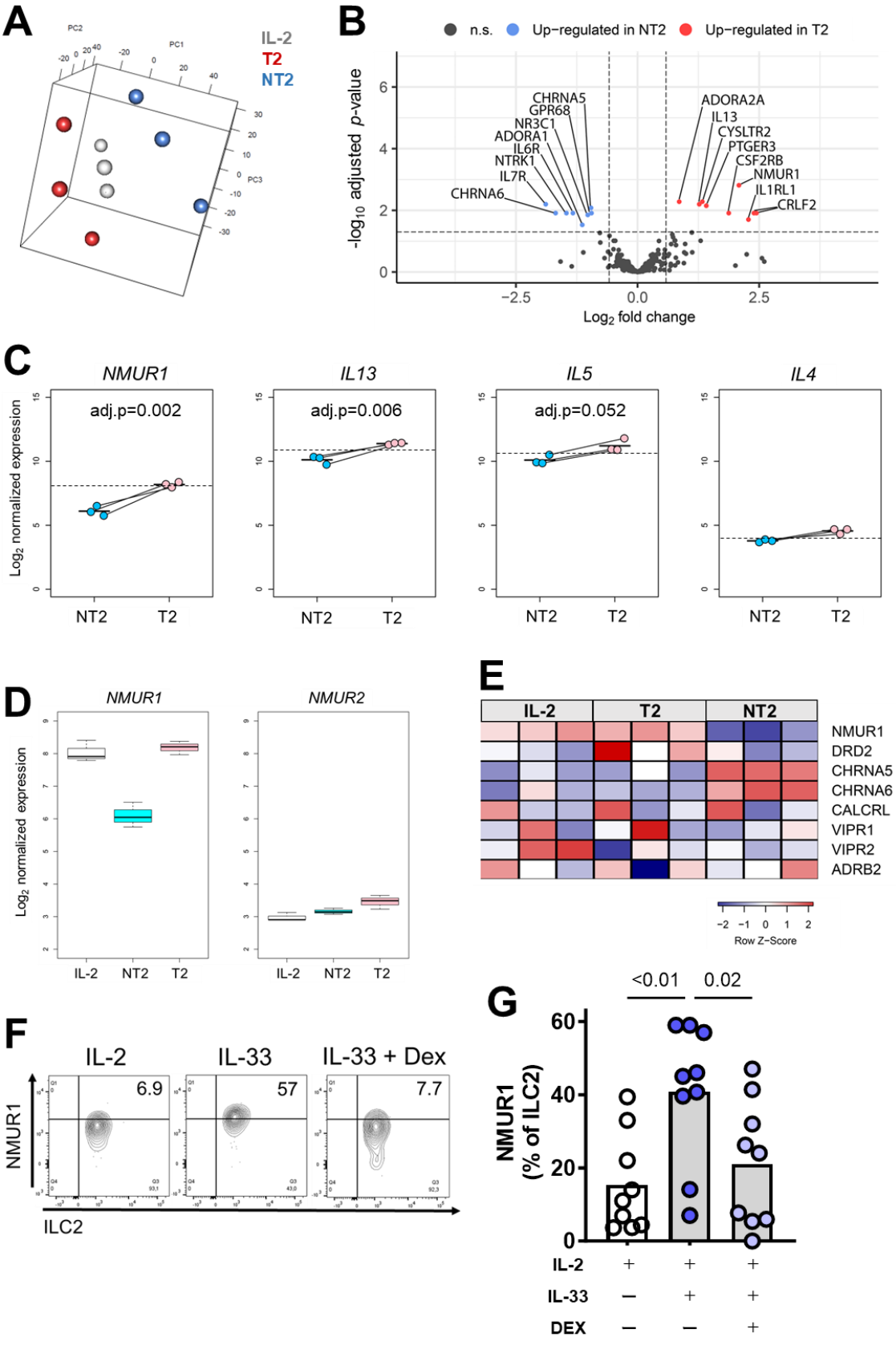
A
IL-2
T2
NT2
B
n.s.
Up−regulated in NT2
Up−regulated in T2
-log10 adjusted p-value
Log2 fold change
CHRNA5
GPR68
NR3C1
ADORA1
IL6R
NTRK1
IL7R
CHRNA6
ADORA2A
IL13
CYSLTR2
PTGER3
CSF2RB
NMUR1
IL1RL1
CRLF2
C
NMUR1
IL13
IL5
IL4
adj.p=0.002
adj.p=0.006
adj.p=0.052
Log2 normalized expression
NT2
T2
D
NMUR1
NMUR2
Log2 normalized expression
IL-2
NT2
T2
E
IL-2
T2
NT2
NMUR1
DRD2
CHRNA5
CHRNA6
CALCRL
VIPR1
VIPR2
ADRB2
Row Z-Score
F
IL-2
IL-33
IL-33 + Dex
6.9
57
7.7
NMUR1
ILC2
G
<0.01
0.02
NMUR1 (% of ILC2)
IL-2
IL-33
DEX

Figure 3.3. Microarray analyses of ILC2 transcriptome. (**A**) Principle component analysis of flow sort-purified and *ex-vivo* expanded ILC2s stimulated with IL-2, T2 (IL-33+TSLP) or non-T2 (IL-1β+IL-23+TGF-β) inducing cultures. (**B**) Volcano plot showing signficantly upregulated genes (fold change > 1.5; adjusted p-value <0.05) from the total 422 genes assessed for neuro-signalling pathway or type 2 inflammation in T2 vs non-T2 inducing cultures of ILC2, *in vitro*. Comparative expression levels of (**C**) *NMUR1, IL13, IL5 and IL4*, (**D**) *NMUR1* and *NMUR2*, and (**E**) neuro-signalling pathway in ILC2 stimulated with IL-2, T2 or Non T2 cytokines. Data are representative of 3 separate experiments. Dash line indicates the average value of genes in IL-2 condition. (**F-G**) NMUR1 expression on ILC2 from mild asthamtics was upregulated following cultures with IL-33 (1ng/ml) and attenuated in the presence of dexamethasone (n=9). Mann-Whitney U test calculated the statistical difference between parametric data.

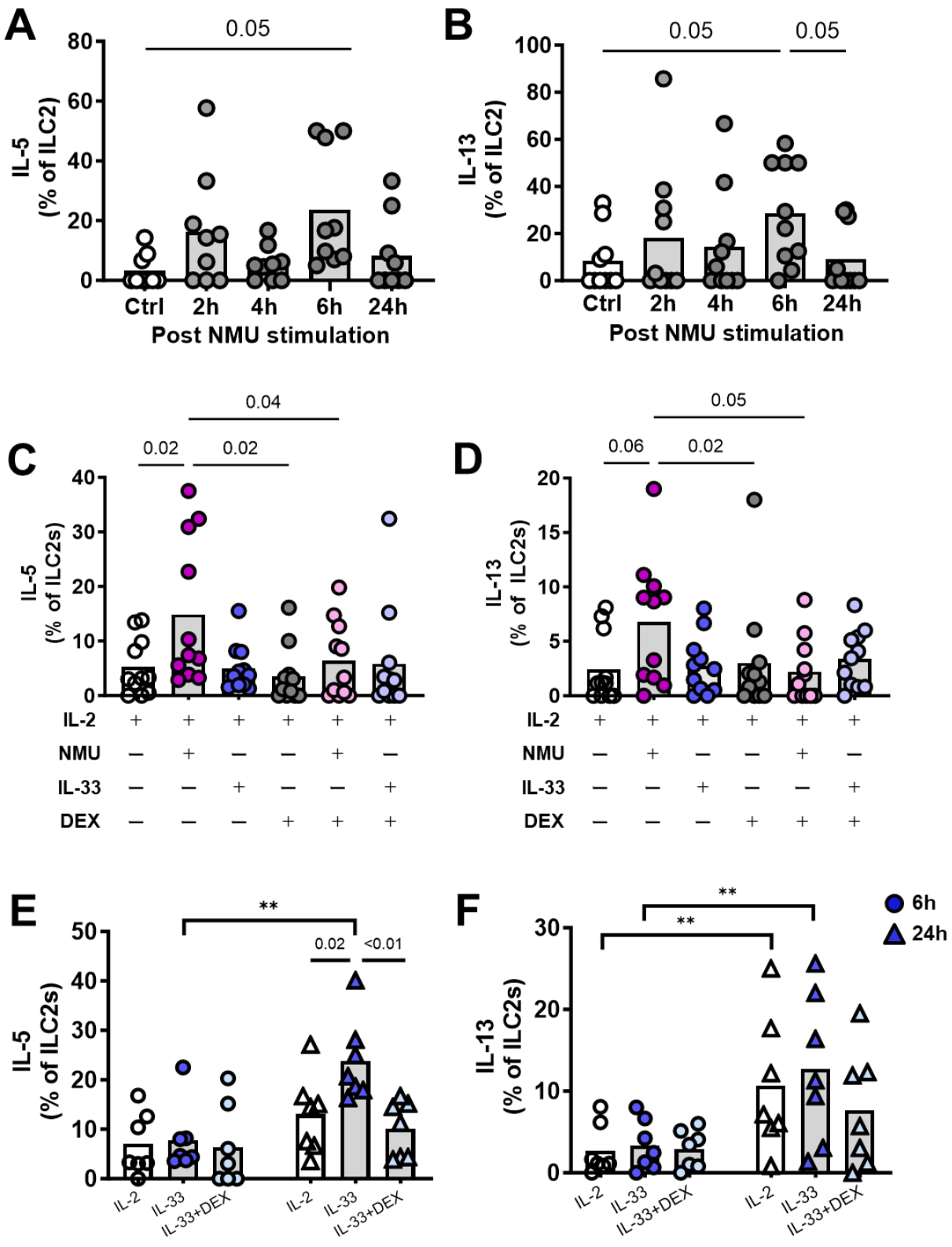
A
0.05
IL-5 (% of ILC2)
Ctrl 2h 4h 6h 24h
Post NMU stimulation
B
0.05 0.05
IL-13 (% of ILC2)
Ctrl 2h 4h 6h 24h
Post NMU stimulation
C
0.04
0.02 0.02
IL-5 (% of ILC2s)
IL-2
NMU
IL-33
DEX
D
0.05
0.06 0.02
IL-13 (% of ILC2s)
IL-2
NMU
IL-33
DEX
E
**
0.02 <0.01
IL-5 (% of ILC2s)
IL-2 IL-33 IL-33+DEX IL-2 IL-33 IL-33+DEX
F
**
**
6h
24h
IL-13 (% of ILC2s)
IL-2 IL-33 IL-33+DEX IL-2 IL-33 IL-33+DEX

Figure 3.4. Rapid time course of ILC2 activation by NMU. There was a signficant increase in intracellular expression of (**A**) IL-5 and **(B)** IL-13 by enriched ILC2 following 6h stimulation with NMU (1ng/ml), *in vitro*. Pre-treatment with dexamethasone (added 30 min prior to stimulation with NMU (1ng/ml, 6h, 37^{o}C)), significantly attenuated expression of **(C)** IL-5 and (**D**) IL-13 by ILC2. The treatment with IL-33 (1ng/ml) signficantly increased expression of **(E)** IL-5 and **(F)** IL-13 in ILC2 at 24h but not 6h *in vitro* and this effect was attenuated by dexmethasone. Bar plots represent mean of each data sets; (one-way or two-way ANOVA with a Bonferroni post-hoc test, n= 6-12, **p<0.01).

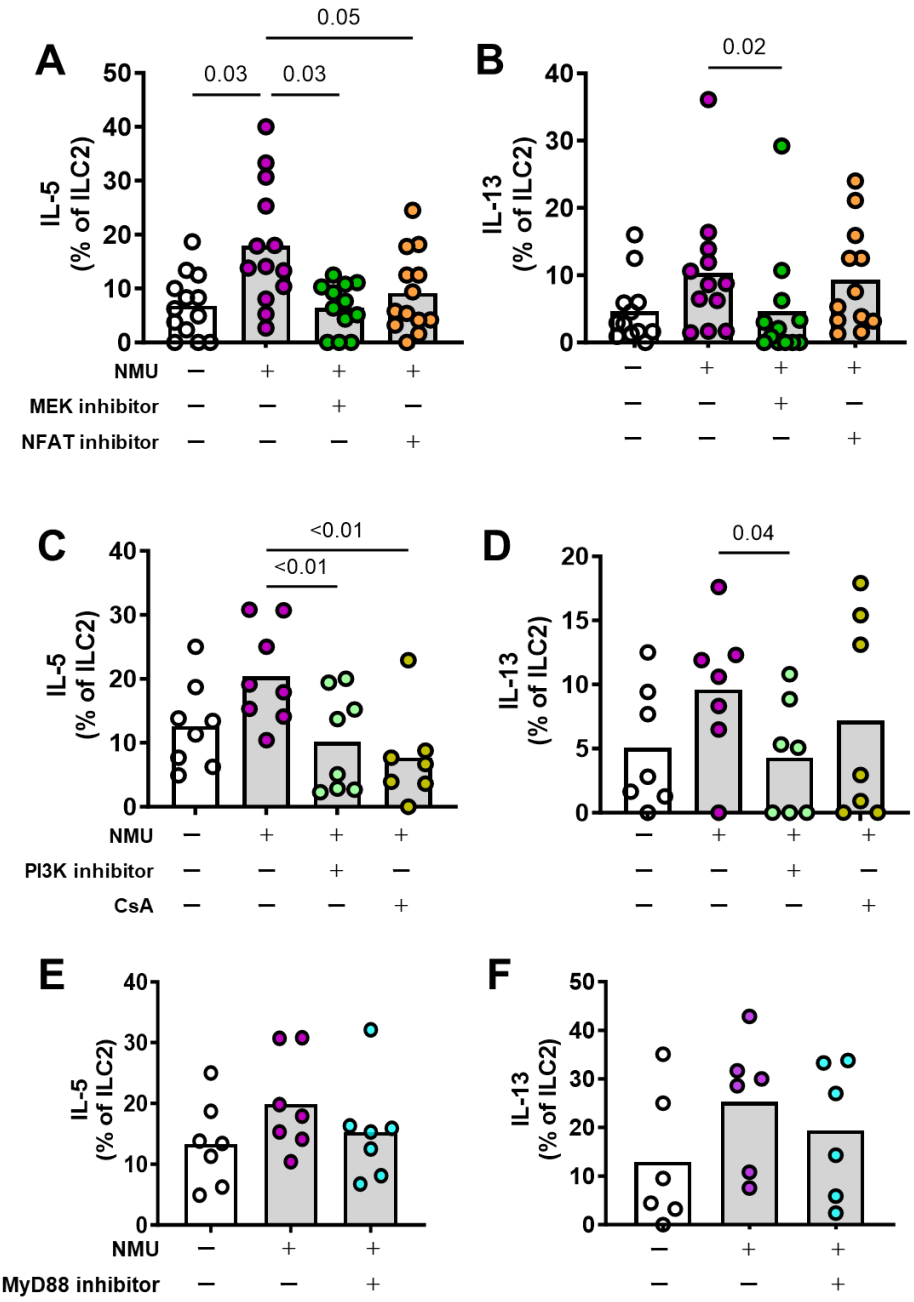

Figure 3.5. NMU mediated signaling pathway in ILC2. In the presence of inhibitors for mitogen-activated protein kinase kinase (MEK, 40µM) or Nuclear factor of activated T-cells (NFAT, 5µM) (pre-treatment for 30 min), there was a significant attenuation of **(A)** IL-5, (**B**) IL-13 expression by enriched ILC2 stimulated with NMU (1 ng/ml, 6h). Blockade of **(C)** phospho-inositol 3 kinase (PI3K, 10µM) significantly attenuated IL-5 and IL-13 while blocking **(D)** calcenuerin (CsA, 50nM) attenuated IL-13 expression by NMU stimulated ILC2. In contrast, (**E-F**) blockade of Myeloid differentiation primary response 88 (MyD88, 1ng/ml) had no effect. Bar plots represent mean of each data sets; (one-way ANOVA with a Bonferroni post-hoc test, n= 6-13).

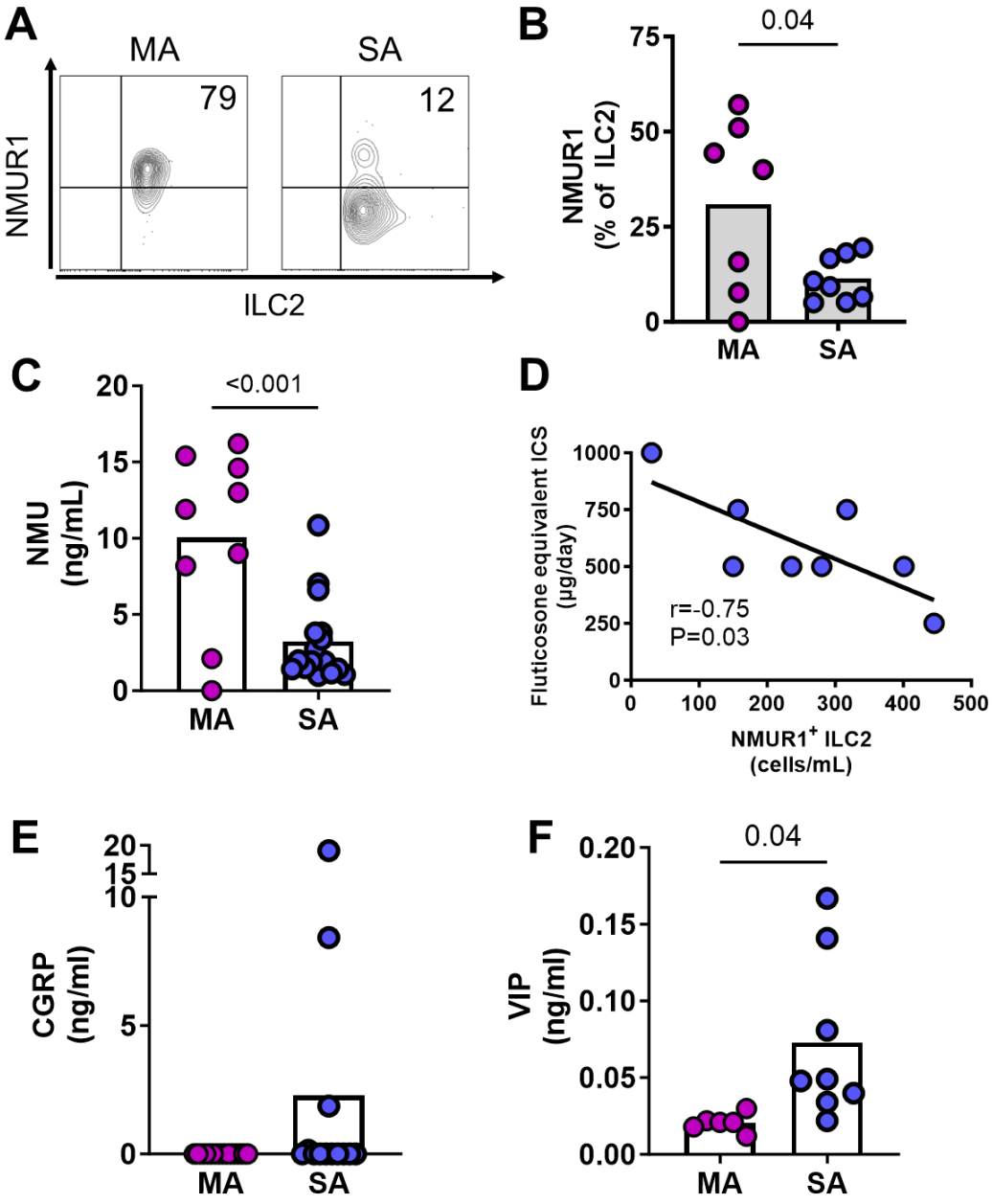

Figure 3.6. NMUR1 expression by sputum ILC2 from mild and moderate-severe asthmatics **(A)** A representative plot showing flow cytometric gating to assess NMUR1 expression by sputum ILC2 from mild and moderate severe asthmatics, **(B)** greater proportional expression of NMUR1 by ILC2 from mild asthmatic (MA, n=7) compared to moderate-severe asthmatic (SA, n=8). **(C)** Sputum levels of NMU detected by ELISA were significantly higher in mild versus moderate severe asthmatics. **(D)** A signficantly inverse correlation was detected between total number of $NMUR1^+$ ILC2 in sputum and fluticosone equvalent inhaled corticosterid (µg/day) in severe asthmatics. Sputum levels of **(E)** CGRP and **(F)** VIP detected by ELISA were higher in moderate severe versus mild asthmatics. Mann-Whitney U test and Unpaired t-test calculated the statistical difference between non-parametric and parametric data, respectively.

SUPPLEMENTARY MATERIAL

Title: Neuromedin-U/ NMU-R1 Axis Mediates Early Activation of Group 2 Innate Lymphoid Cells in the Airways of Subjects with Mild Asthma following Inhaled Allergen Challenge.

Authors: *Xiaotian Ju[1], *Akimichi Nagashima[1], Yoshihisa Tokunaga[1], Jennifer Wattie[1], Karen Howie[1], Guillaume Paré[2], Reine Ditta[2], Anna Dvorkin-Gheva[3], Ruth Cusack[1], Imran Satia[1,3], Paul M O'Byrne[1], Gail M. Gauvreau[1] and Roma Sehmi[1,3]

* These authors contributed equally to the development of this manuscript

Address: [1]Respiratory Research Group, Department of Medicine, McMaster University, Hamilton, Ontario, Canada. [2]Population Health Research Institute, McMaster University, Hamilton, L8N 3Z5, Ontario, Canada. [3]The Research Institute of St. Joe's Hamilton, Firestone Institute for Respiratory Health, St Joseph's Healthcare Hamilton, Ontario

Correspondence: Address correspondence and reprint requests to Dr. Roma Sehmi, McMaster University, HSC 3U31D, 1200 Main St West, Hamilton, ON, Canada L8N 3Z5

Email: sehmir@mcmaster.ca

SUPPLEMENTARY METHODS

Subjects: Ten asymptomatic non-smokers with mild atopic asthma were recruited. All subjects had baseline $FEV_1 \geq 70\%$ predicted, intermittent using β2-agonist, methacholine (MCh) $PC_{20} \leq 16$ mg/ml, no respiratory infection for 4 weeks prior to sampling. Study eligibility was met if subjects exhibited allergen-induced early (0-2h) and late (3-7h) post-challenge bronchoconstrictor responses assessed as a ≥20% and ≥15% fall from baseline FEV_1, respectively (Subject demographics of mild asthmatics and moderate-severe asthmatics (n=9) included as a baseline cross-sectional comparison are detailed in **Table 3.1** and **supplementary Table E3.1**). Demographics of All experimental procedures were reviewed and approved by the Hamilton Integrated Research Ethics Board and all subjects provided informed consent.

Study design: Subjects with mild asthma completed diluent-controlled, allergen-challenge crossover study with a minimum 2-week washout period. Subjects underwent skin prick testing, spirometry, provocative concentration of methacholine causing a 20 % fall in The FEV_1 (PC_{20}), a medical history, and sputum induction on Day 1. On the following day, subjects were challenged with diluent or allergen. Spirometry was performed hourly to 7 hours after challenge, and induced sputum was collected at 7 hours post-challenge. At Day 3 a methacholine challenge was performed, and induced sputum collected for the 24 hours post-challenge. After a washout period, subjects returned for corresponding challenge (allergen or diluent), and matching measurements of sputum induction and spirometry were repeated. Sputum cells were extracted for differential cell count, immunofluorescence staining and flow cytometric analyses; sputum supernatants were collected for T1/T2 cytokine analysis by ELLA™ multiplex automated-ELISA (Bio-Techne, US) or NMU neuropeptide ELISA kit (BIOMATIK, ON, CA).

Methacholine Inhalation and Airway Challenge: Methacholine inhalation challenge was performed by the tidal breathing method using a Wright nebulizer (1). In brief, doubling concentrations of methacholine chloride (Methapharm, ON, Canada), were inhaled orally from a Hans Rudolph valve for 2 min. The FEV_1 was measured following inhalation at 30 second and 90 second or until it stopped falling. The percent fall was calculated from the baseline FEV_1 value. The test terminated when a fall in FEV_1 of at least 20 % of the baseline value occurs.

***Allergen inhalation challenge*:** Subjects with asthma were required to be stable (FEV_1 within 10% of baseline) to proceed with the inhalation challenges. Allergen-inhalation challenge was performed as previously described (2). The extract producing the largest skin wheal was selected from results of a skin prick test for inhalation and the concentration of allergen required to achieve a 20 % decrease in FEV_1 was determined as previously described (3). Spirometry was performed to measure FEV_1 at regular intervals until 7 hours after allergen inhalation. The maximum percentage fail in FEV_1 was recorded for the early (0-2 h post-allergen) and late (3-7 h post-allergen) phase response. Where mentioned physiologic saline was used for diluent-challenge for control.

***Sputum induction and cell isolation*:** Sputum was induced by hypertonic saline inhalation as previously described by Pizzichini et al (4). The sputum samples were processed by selecting the mucus plugs, mixing with 4 times volume of Dulbecco's phosphate buffered saline (DPBS) and 4 times volume of dithiothreitol (DTT), and filtered through a 52-μm nylon mesh. The filtrated cells were pelleted and re-suspended in Dulbecco PBS. The total cells count was determined with a hemocytometer chamber and expressed as the number of cells per gram of sputum. Two cytospins were prepared and subsequently stained with Diff Quick for cell differential cell counts were

averaged. Remaining cells were immune-stained fresh for flow cytometry and sputum supernatants stored at -80℃ for ELISA.

***Peripheral blood collection and ILC2 enrichment*:** Peripheral venous blood was drawn into sodium heparin vacutainers from subjects. Blood was diluted 1:1 with McCoy's 5A (Gibco, CA, US) and layered on Lymphoprep (StemCell, BC, Canada) for density gradient sedimentation (2200 rpm, 20 min). Mononuclear cells were collected from the interface, and subject to ILC2 purification using the EasySep® Human ILC2 Enrichment Kit (StemCell, BC, CA) in enrichment experiments.

ILC2 in vitro stimulation with NMU: Enriched blood ILC2s from mild asthmatics, were suspended in RPMI-complete and incubated with IL-2 (1ng/ml) and NMU (1ng/ml; United States Biological, MA, US) for over different times (2,4,6 and 24 hour) at 37℃, 5% CO2. Optimized culture conditions with for 6h with NMU (10 ng/ml) with dexamethasone (10^{-7}M) or previously optimized doses of signalling inhibitors for mitogen-activated protein kinase 1/2 (MEK1/2; PD98059, 40 µM), nuclear factor of activated T-cells (NFAT; 11R-VIVIT, 5 µM), phospho-inositol 3 kinase (PI3K; LY294002, 10µM), calcineurin A (cyclosporin A, CsA, 50 nM) or a specific inhibitor of MyD88 (NBP2-29328, 1 ng/ml) was also tested. All signalling inhibitors were purchased from Sigma, CA. Transport inhibitor cocktail (Thermo Fisher Scientific Inc., MA, US) was added 4 h prior to terminating the cell incubation and harvested cells for immunostaining to identify total ILC2 and expression of intracellular cytokines and neuropeptide receptors by flow cytometry.

Flow sorting and ex-vivo expansion of ILC2 This procedure is modified from previous studies (5). Briefly, Lin^- cells were isolated from non-adherent peripheral blood lymphomononuclear cells using the EasySep Human ILC2 Isolation Kit (STEMCELL Technologies, CA). Enriched ILC2s

were subject to immunofluorescent cell surface staining as described below and flow-sort purified by gating on Lin^{-} $CD127^{+}$ $CRTH2^{+}$ cells using the BD FACSAria™ III cytometer. The sort-purified cells (2000-3000 cells per well) were cultured in 96-well U-bottom plates with RPMI-complete media plus IL-2 (10ng/ml), IL-33, IL-7 and IL-25 (50 ng/ml) for 7-10 days. Cells were then rested for 3 days in IL-2+IL-7 and then stimulated with various cytokine combinations including (a) IL-2 alone, (b) T2 inducing cultures – IL-33+TSLP or (c) non-T17 inducing cultures: IL-1β, IL-23, TGF-β (all at 50ng/ml) for 7-9 days with media replenishment every 2 days. During the final 4 hours of the cultures, PMA (10ng/ml) and ionomycin (500nM) were added to stimulate cells in the presence of Brefeldin A (1x). Cells were then subject to immunofluorescent staining, resuspended in PBS with 1% paraformaldehyde and analyzed by a BD LSRFortessa™ flow cytometer as described below.

Immunofluorescence staining and flow cytometric gating strategy: Cells from sputum or blood were stained with fluorescently labeled antibodies as previously described (2) to Lineage cocktail-FITC, FcεRI-FITC (Thermo Fisher Scientific Inc., MA, US), CD94-FITC, CD45-BV510, CD127-BV605, CRTH2-PE-CF594, Fixable Viability Stain 780 (Becton-Dickinson Biosciences, ON, CA), NMUR1-PE (Bioss Antibodies Inc., CA, US), or corresponding isotype controls. Lin-cocktail antibodies to (CD2, CD3, CD14, CD16, CD19, CD56, CD235a) plus FcεRI and CD94 excluded T and B lymphocytes, monocytes, eosinophils, basophils, and NK cells. Cells were washed, and then fixed and permeabilize, optimal concentrations of intracellular staining antibodies to IL-5-BV421 (BioLegend Inc., CA, US) and IL-13- Alexa Flour-700 (R&D Systems, MN, USA) or isotype controls were added. After incubation, cells were washed and fixed in PBS with 1% paraformaldehyde and analyzed by a FACS LSR II flow cytometer (Becton-Dickinson Biosciences, ON, CA). Data were analyzed using the FlowJo 10 software (FlowJo LLC., OR, US) to enumerate

neuronal receptor expression and cytokine levels in ILC2s defined as live, singlets with Lin^- $CD45^+$ $CD127^+$ $CRTH2^+$ phenotype (supplementary **Fig. E3.3A and E3.5**). Absolute cell numbers were calculated from the percent positive population from FACS analysis and total white cell count in sputum as described previously (6).

Microarray analysis of ILC2 transcriptome: Flow sort-purified and ex-vivo expanded ILC2 were stimulated in T2 or non-T2 conditions as described in the online supplement followed by resuspension in RNeasy Lysis buffer (RLT) and stored at -80°C. RNA was isolated and purified using the RNeasy kit (Qiagen, US). The concentration of extracted RNA was measured by NanoDrop 8000 Spectrophotometer and Qubit Broad Range assay (Thermo Fisher Scientific) and RNA integrity assessed using the Agilent Bioanalyzer 2100 and RNA Nano kit. Sample preparation was performed using the GeneChip WT Plus Kit (Thermo Fisher Scientific) to amplify the extracted RNA and generate biotinylated sense-strand DNA targets. The labeled samples were then hybridized to human Clariom S array (Thermo Fisher Scientific). Washing and staining was performed by the GeneChip Fluidics Station 450 and the scanning was completed on GeneChip Scanner 3000 (Agilent). Raw data were normalized using the RMA algorithm implemented in the limma R-package. Adjusted *p*-values were calculated using the *Benjamini-Hochberg* method. Data were visualized using ggplot22 and p heatmap R-packages.

Supplementary Tables and Figures

Sex	Age (year)	FEV1 % predicted	FEV1/FVC	Methacholine PC20 (mg/ml)	Allergen	Sputum Eos (%)
F	37	92	0.69	10.56	HDM DF	0.5
F	38	94	0.82	12.2	Grass	0.5
M	59	100	0.72	1.67	CAT	2.4
F	35	104	0.86	1.5	HDM DF	6.6
F	22	92	0.92	5.06	Ragweed	4.8
F	39	101	0.74	9.05	Grass	3.3
M	62	87	0.68	5.45	HDM DF	2.62
F	20	102	0.85	5.25	Horse	2.75
F	21	84	0.83	1.9	Grass	10.25
F	30	77	0.83	2.15	Grass	1.5

Table E3.1.
Baseline characteristics of non-smokers with mild stable asthma (n=10; aged 21-62) enrolled in the study. Subjects had no other lung disease or chronic illness, no lower respiratory tract infection or worsening of asthma for 4 weeks before screening and avoided exposure to sensitizing allergens (Ag) apart from house dust mite. Subjects were not on steroid treatment, infrequently used inhaled ß$_2$-agonist treatment of asthma, and refrained from ß$_2$-agonist and caffeinated beverages prior to laboratory visits.
Definition of abbreviations: FEV_1 - forced expiratory volume in 1 second. Allergen – antigen (at a specified dilution) used for inhalation challenge; MCh PC_{20} - the provocative concentration of methacholine causing a 20% fall in FEV_1. Data are presented as mean ± SD.

Neuroreceptor related genes								ILC related genes
TNFRSF1A	CHRNA1	P2RX7	CRHR1	F2RL2	HTR5A	PTPN3	EBAG9	IL1RL1
ESR1	DRD1	GABRB3	IRAK1	NR4A2	P2RX3	GRIP1	RIC3	CRLF2
RAPSN	GRIA1	GRK2	HTR7	PTPN1	GABRR3	PTPRA	GPR174	KLRG1
ADRB2	IL1R1	TRPV1	ESRRB	GABRG3	MCHR1	NTSR2	GPR12	KLGB1
EGFR	DRD3	GABRB2	VIPR1	SCTR	P2RY4	FZD1	TRPC7	IL7R
MUSK	LRP1	NR1I3	OPRL1	SIGMAR1	TNFRSF18	PRLHR	PAQR8	PTGDR2
CHRNE	F2RL1	GRIA3	GABRA3	LTB4R	GRK6	NR2F1	GPR143	KIT
AR	RXRA	AVPR2	CHRNB2	GABRD	HTR1F	HTR3E	ADGRL3	AREG
LEPR	IRS1	GPER1	CALCR	ACKR2	EPHA4	TRPC4	ADGRL2	GATA3
VDR	ADORA2A	CHRM3	FGFR4	NPR2	TNFRSF4	NR2F2	TRPM2	TBX21
INSR	PPARA	CHRNA4	HRH2	GLRB	CHRNA9	TRPC6	CRCP	RORC
CHRNB1	MET	OXTR	TNFRSF11B	LPAR1	VIPR2	HCAR1	GRINA	IL5
PGR	IL2RB	FPR1	RAMP2	GRIK4	GRM8	FZD7	TRD	IL13
NR3C1	GRIN2B	CCKAR	HTR6	TAAR1	RXRG	PTPRT	GPR153	IL4
RYR2	RYR1	NTRK3	GHRHR	GRM4	TFR2	CHRNA10	PAQR6	CSF2
DRD2	OPRK1	GRM5	CHRNA3	CRHR2	GABRP	TAS2R4	SSTR4	IL10
ERBB2	GRM1	CHRM1	IL1RAPL2	CHRNG	GRM6	IRS4	LGR5	IL17A
TLR4	GRIN1	BMPR2	GRIN2D	IL1R2	GALR2	PTPRN2	GPR68	IL22
GHR	AHR	CCKBR	GABRR2	APLNR	ABL1	NPBWR2	FFAR2	IFNG
IGF1R	FLT1	C5AR1	PTPRC	P2RY6	CHRM5	TRPM8	HTR3D	IL12A
NR3C2	P2RY12	CNR2	TRAF3	NPR3	ITPR2	TAAR6	PTPRU	IL12B
HTR2A	NR1I2	NR4A1	RIPK1	EPHA1	GABRE	NOTCH4	AIP	IL18
ESR2	TNFRSF1B	GRIK1	GRM2	CHRNA5	PTH2R	IL1RAPL1	TRPA1	
PPARG	PTPN11	P2RY1	GABRA4	NPY2R	LGR6	FZD2	NMUR2	
CHRND	GABRG2	SSTR2	HCRTR1	CHRNA2	P2RX4	GPR4	RYR3	
LDLR	IL18R1	BDKRB2	AVPR1A	NPR1	FOLR1	SCARB2	GLP2R	
NR1H2	NTRK2	HTR4	ADORA2B	ACKR1	GABRG1	TRPC5	TAAR5	
OPRM1	OPRD1	ITPR1	FCGR2B	ARNT	GRB10	GPR151	GALR3	
MC1R	GRB2	AGTR2	GRM3	MC3R	LBR	GPR17	FZD5	
GHSR	CHRNA7	PRLR	CSF2RB	TRA	GABARAP	GRID1	GPR19	
HTR1A	EDNRA	GLP1R	GRIK3	SSTR5	OXER1	LRP4	EPS8	
Neuroreceptor related genes								

IL2RA	GRIK2	MC2R	GABRR1	NTSR1	GLRA2	GPR119	GPR26	
CCR5	FSHR	TACR3	RORA	GRIN3A	CHRNA6	P2RX6	PAQR9	
KDR	NCOA1	GABBR2	LRP2	P2RX2	RTN4R	TAAR8	GABARAPL1	
HTR3A	CALCRL	CXCR1	GRIN2C	ITPR3	BRS3	GPR61	GPR21	
AGTR1	GRIN2A	GRIA4	TRHR	NMUR1	SSTR3	FZD8	GPR176	
DRD4	THRA	HRH3	NR0B1	GRM7	NPY4R	TAAR9	GPR88	
SRC	GRIA2	GABRB1	NPY1R	CYSLTR2	P2RY11	HMMR	GPR27	
IL6R	ADORA1	HTR2B	HRH4	NPY5R	RACK1	OGFR	ADGRB1	
MC4R	HTR1B	GABRA2	LPAR3	EPHA3	HTR1E	FZD3	GPR158	
FGFR2	IFNGR2	NCOA3	ESRRA	SSTR1	NPSR1	FZD9	BCAP31	
TGFBR2	GABRA1	DRD5	RAMP3	GRID2	FCER1A	GPR65	TRPM4	
IL1RN	TLR3	NCOR2	ADORA3	INSRR	GPR55	GPR132	NPTXR	
TGFBR1	CHRM2	GLRA1	F2RL3	IL3RA	GALR1	TAAR2	CNIH2	
CNR1	PDGFRA	LIFR	LRP5	P2RX1	TRPC1	RYK	GABARAPL2	
GABBR1	TBXA2R	HTR1D	HCRTR2	GPRC6A	CHRNB3	GRIP2	PLGRKT	
F2R	ERBB4	FPR2	CHRNB4	GRK3	MLNR	PTPRN	SSR1	
FGFR1	TACR2	GABRA6	BDKRB1	NMBR	GLRA3	PAQR7	REEP3	
CASR	EDNRB	RAMP1	ALK	FZD4	HTR3C	ROBO1	CLPTM1	
FAS	NGFR	P2RY2	GRIK5	LRP6	PROKR2	DCC		
HTR2C	HRH1	GABRA5	GRPR	GABRQ	MC5R	ADGRL1		
TACR1	ERBB3	HTR3B	GPBAR1	CHRM4	PTPRF	TRAT1		
NTRK1	GNRHR	PTGER3	ADCYAP1R1	GRIN3B	TRPC3	GPR85		

Table E3.2. The list of 442 genes related to neuroreceptor signalling pathway or ILCs applied for microarray analysis based on Gene Card.

Gene Symbol	Probe ID	nont2 - t2_FC	nont2 - il2_FC	t2 - il2_FC
CRLF2	TC0Y00006873.hg.1	-5.44	-4.33	
	TC0X00008900.hg.1	-5.25	-4.31	
IL1RL1	TC0200016501.hg.1	-4.86		
NMUR1	TC0200016026.hg.1	-4.24	-3.82	
CSF2RB	TC2200007273.hg.1	-3.67		
PTGER3	TC0100014576.hg.1	-2.66		
CYSLTR2	TC1300007148.hg.1	-2.52	-3.09	
IL13	TC0500008642.hg.1	-2.41		
ADORA2A	TC2200009233.hg.1	-1.81	-1.67	
GPR68	TC1400009978.hg.1	1.93		
CHRNA5	TC1500007980.hg.1	1.94	2.00	
NR3C1	TC0500012340.hg.1	2.03		
IL6R	TC0100010078.hg.1	2.51	2.43	
NTRK1	TC0100010196.hg.1	2.76		
CHRNA6	TC0800010297.hg.1	3.21	2.87	
IL7R	TC0500007138.hg.1	3.70		
HCAR1	TC1200012249.hg.1		-3.61	
GRK6	TC0500009622.hg.1		-1.61	
ADORA1	TC0100011245.hg.1		2.48	
TNFRSF18	TC0100012452.hg.1			1.71

Table E3.3. Differential expression of neuro-signalling pathway or type 2 inflammation related genes in group 2 innate lymphoid cells that showed a significant difference between the three culture conditions i.e. Non-T2, T2 and IL-2 control.

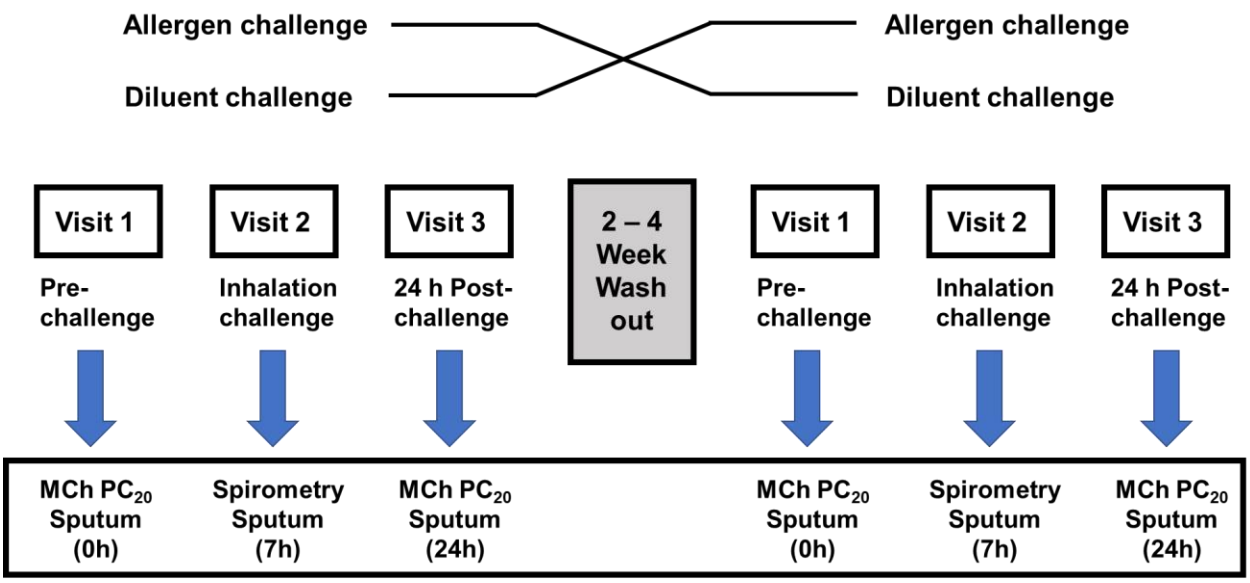

Figure E3.1. In this crossover study, subjects were randomized to allergen or diluent challenge. After 4-week washout recovery, subjects were re-challenged with diluent or allergen. Sputum samples were induced at 0, 7 and 24h post-challenge. Sputum cells were extracted for differential cell count and immunofluorescence staining (FACS) followed by FACS acquisition; sputum supernatant was collected for cytokine analysis by ELISA. MCh PC_{20} = concentration of methacholine required to reduce FEV_1 by 20%.

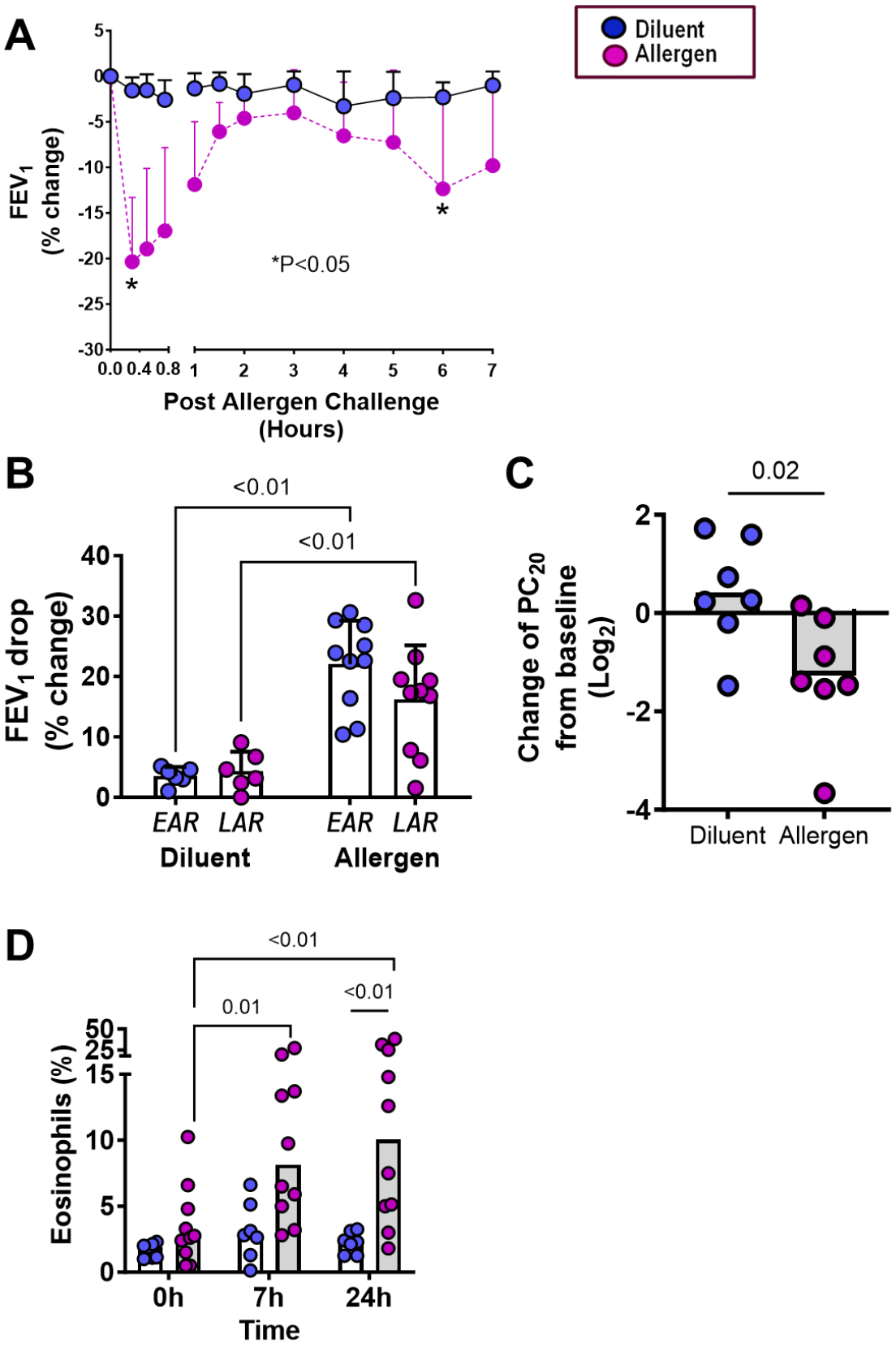
A
Diluent
Allergen
FEV1 (% change)
*P<0.05
Post Allergen Challenge (Hours)
B
<0.01
<0.01
FEV1 drop (% change)
EAR LAR EAR LAR
Diluent
Allergen
C
0.02
Change of PC20 from baseline (Log2)
Diluent Allergen
D
<0.01
<0.01
0.01
Eosinophils (%)
0h 7h 24h
Time

Figure E3.2. (A) Spirometry was performed hourly pre and post allergen- or diluent- challenge for the first 7h. **(B)** Allergen inhalation induced a significantly greater mean maximal fall in FEV_1 from baseline during the early asthmatic response and late asthmatic response compared to diluent control ($p<0.01$). **(C)** A significant decrease of PC_{20} (expressed as Log_2) from baseline was observed in allergen challenge arm compared to diluent challenge arm. **(D)** Sputum eosinophils expressed as % of total cell counts were significantly increased at 7 and 24h post-allergen but not diluent challenge. Data are presented as mean of each data sets; (two-way ANOVA with a Bonferroni post-hoc test).

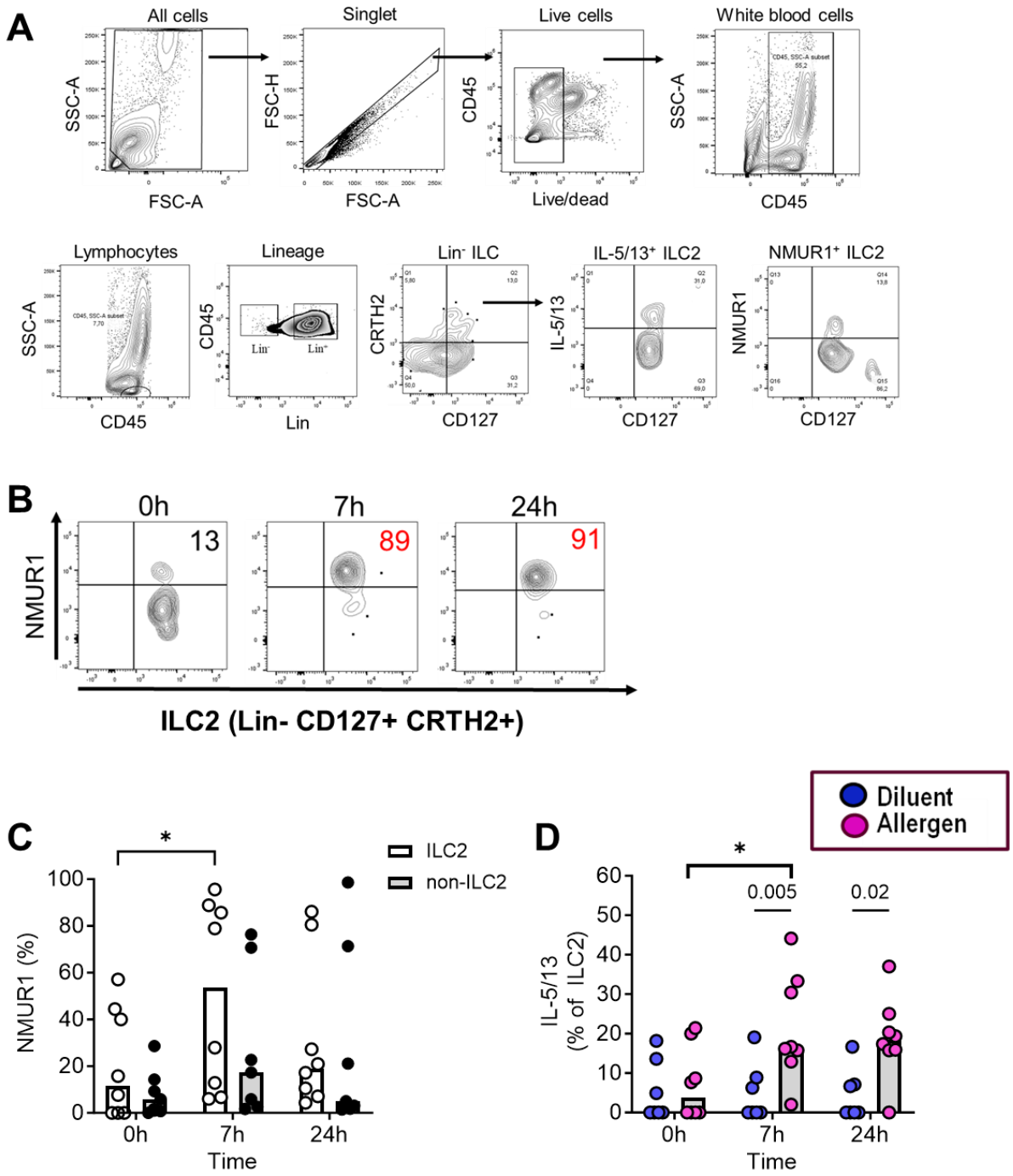

Figure E3.3. (A) Representative flow cytometric gating for ILC2 showing expressions of IL-5/13, NMUR1 in sputum cells. **(B)** Representative flow cytometric gating showing increasing proportion of ILC2 population expressing NMUR1 post-allergen challenge. **(C)** The proportional increase of NMUR1 post allergen challenge was not detected on CRTH2^{-} ILC. **(D)** The proportional expression of IL-5/13 on sputum ILC2 in mild asthmatics were significantly increased at 7h and 24h post allergen challenge compared with 0h and diluent challenge group. Bar plots represent mean of each data sets; (two-way ANOVA with a Bonferroni post-hoc test, * $p<0.05$, ** $p<0.01$).

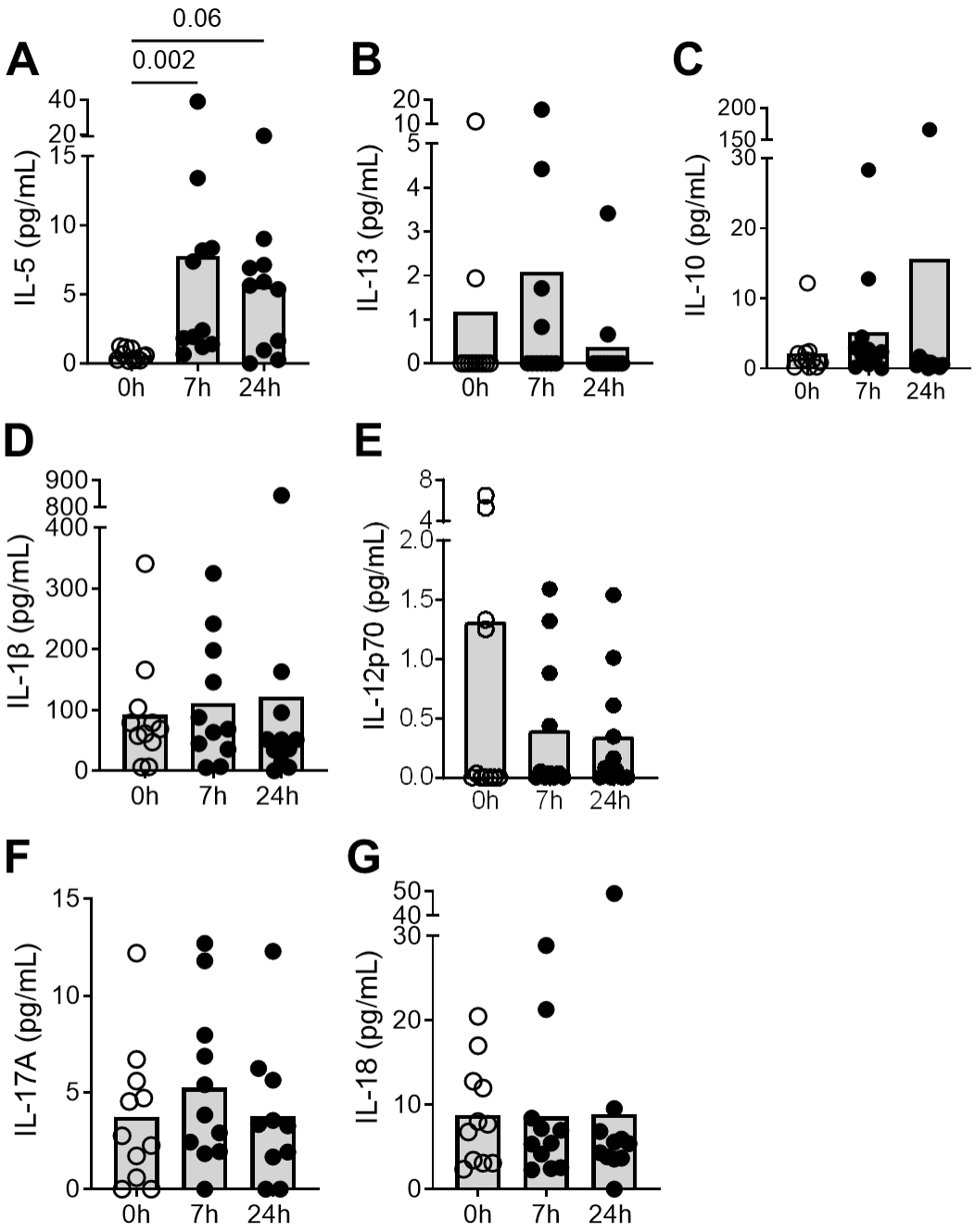

Figure E3.4. The cytokine levels of **(A)** IL-5, **(B)** IL-13, **(C)** IL-10, **(D)** IL-1β, **(E)** IL-12p70, **(F)** IL-17A and **(G)** IL-18 in sputum supernatant were detected by ELLA assay at 0h, 7h and 24h post allergen challenge in mild asthmatics. Bar plots represent mean of each data sets; (one-way ANOVA with a Bonferroni post-hoc test).

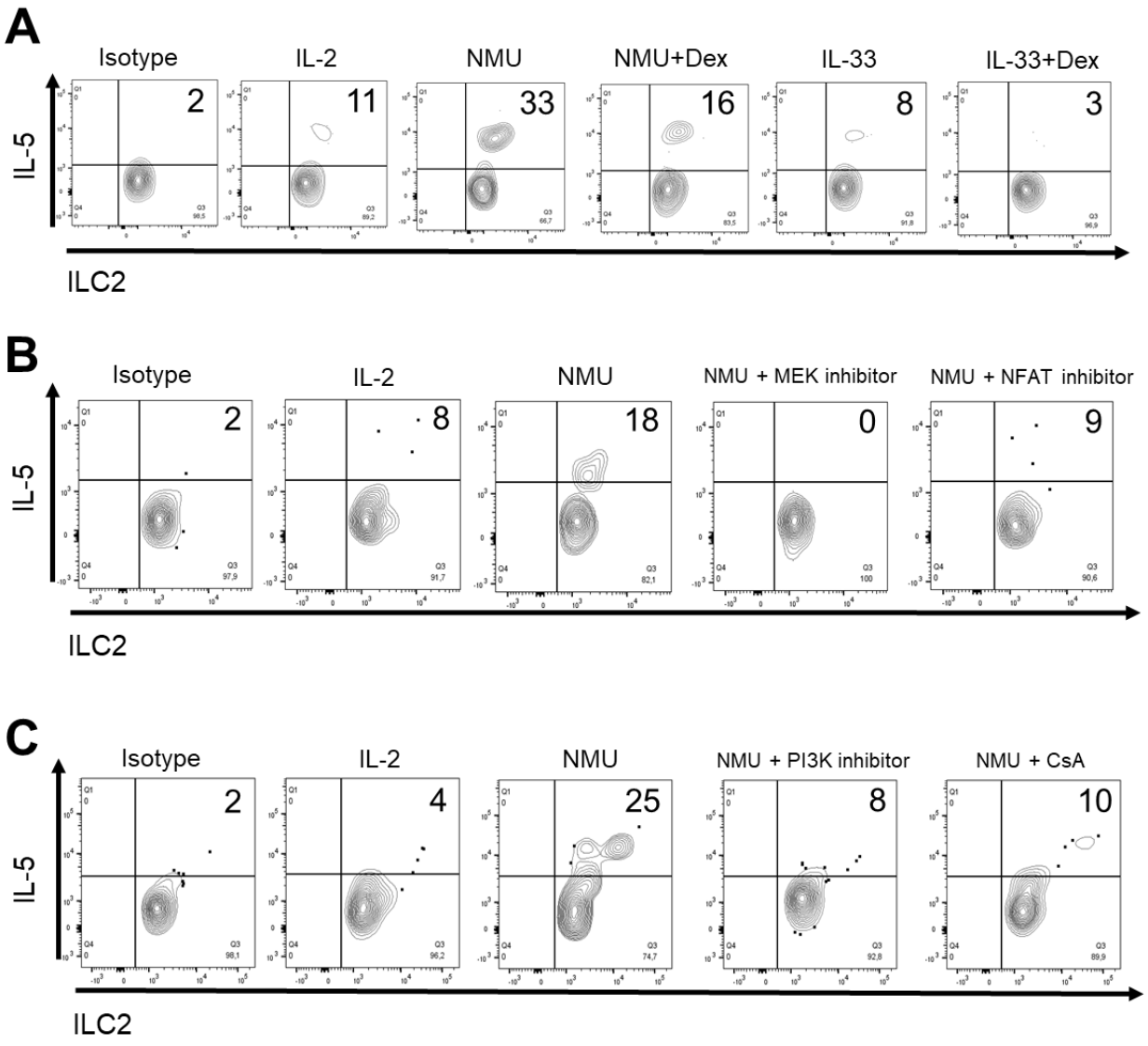

Figure E3.5. Representatives flow cytometric plots of enriched ILC2 in from mild asthmatic blood stimulated with NMU (1 ng/ml) in the presence of **(A)** dexamethasone (10^{-7}M) or **(B, C)** signalling inhibitors at 6h, *in vitro*.

Supplementary References

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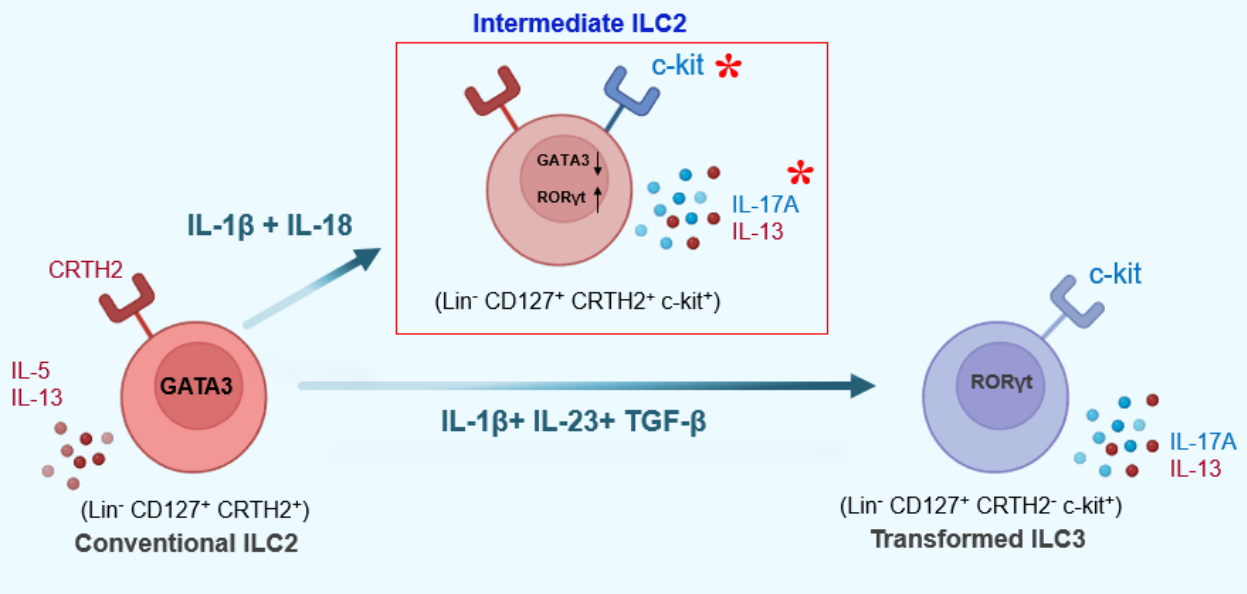
CHAPTER 4: Increased Levels of Group 3 Innate Lymphoid Cells and c-kit$^+$ IL-17A$^+$ ILC2 in Severe Asthma with Neutrophilic or Mixed Granulocytic Airway Inflammation: Evidence of IL-1β+IL-18 driven ILC plasticity

The chapter is full developed for publication and submission for review is pending.

Cite: Xiaotian Ju, Nahal Emami Fard, Anurag Bhalla, Anna Dvorkin-Gheva, Maria Xiao, Katherine, Radford, Kayla Zhang, Reina Ditta, John Paul Oliveria, Guillaume Paré, Manali Mukherjee, Parameswaran Nair and Roma Sehmi. Increased Levels of Group 3 Innate Lymphoid Cells and c-kit$^+$ IL-17A$^+$ ILC2 in Severe Asthma with Neutrophilic or Mixed Granulocytic Airway Inflammation: evidence of IL-1β+IL-18 driven ILC plasticity (Manuscript is in preparation for submission Dec 2023)

CAPSULE SUMMARY



The different airway inflammatory endotypes of severe asthmatics as associated with ILC with different phenotypes and function in the airway. Specifically, ILC2 and ILC3 are associated with the eosinophilic and neutrophilic airway inflammation in severe asthma, respectively. Also, tissue resident ILC2 can trans-differentiate in response to changing microenvironmental cues in the airways which in turn can alter the predominant airway inflammatory profiles in severe asthmatics.

ABSTRACT

RATIONALE: In severe asthma (SA) with airway eosinophilia (>3%) group 2 innate lymphoid cells (ILC2) represent a steroid insensitive source of Interleukin (IL)-5 and IL-13, highlighting these cells as critical drivers of type 2 asthma exacerbations. In contrast, pathobiological pathways driving neutrophilic or mixed granulocytic airway inflammation in severe asthma have not been fully investigated. This study assessed sub-classes of ILC in various endotypes of severe asthma and investigated ILC2 plasticity in response unique airway microenvironmental cytokine cues.

METHODS: Consenting subjects with severe asthma were categorized by differential sputum cell counts into eosinophilic, neutrophilic or mixed granulocytic airway inflammatory endotypes. Induced sputum-extracted cells were subject to immunofluorescence staining; data were acquired using BD LSRFortessa^{TM} and analysed by FlowJo V10 or R studio. Airway cytokines were quantified by ELLA multiplex and plasticity assays were performed with flow sort purified ILC2. Phenotypic and transcriptomic changes were assessed by flow cytometry and gene microarray.

RESULTS: Sputum ILC3 were significantly increased in SA with neutrophilic airway inflammation where IL-17A^{+} ILC3 correlated with airway neutrophilia and were highest in subjects with body mass index >30. Conversely, sputum ILC2 were most prevalent in eosinophilic SA and correlated with airway eosinophilia. Unbiased clustering analyses (*UMAP)* identified an "ILC3 featured ILC2" displaying ILC3 markers (c-kit and IL-17A) in SA with neutrophilic or mixed granulocytic airway inflammation; airway levels of IL-1β and IL-18 were highest these SA endotypes. Co-culture of purified ILC2 with IL-1β+IL-18 significantly increased c-kit and IL-17A expressions *in vitro* and expressed gene profiles related to type 17 inflammation.

CONCLUSIONS: Our data show ILC3, as a source of IL-17A, are most abundant in severe asthmatics with neutrophilic airway inflammation. Detection of "ILC3 featured" intermediate ILC2 in SA with neutrophilic or mixed granulocytic inflammation indicate ILC2 can trans-differentiate in response to changing of airway microenvironmental signals altering the bronchitis. Controlling ILC3 activity and the trans-differentiation of intermediate ILC2 by blocking NLRP3 cytokines may be an important therapeutic control for type 2 low severe asthma exacerbations.

INTRODUCTION

Asthma is a chronic airways disease affecting over 300 million people of all ages worldwide (1). The majority of patients with asthma are well controlled by inhaled corticosteroids (ICS) and bronchodilator therapy (2)(3); however, more than 10% of asthmatics have uncontrolled severe disease that remains inadequately controlled by daily oral corticosteroids (OCS), and accounts for a disproportionate socio-economic and healthcare burden (4). Severe asthma includes four major endotypes based on different inflammatory profiles in the airway: eosinophilic, neutrophilic, mixed granulocytic or pauci-granulocytic (5). Investigating the immunological mechanisms underlying different inflammatory endotypes may provide novel therapeutic targets for optimal control of symptoms and asthma exacerbations and elucidate biological considerations for patient selection strategies for more personalized biologic therapies.

Severe asthma with type 2, eosinophilic airway inflammation is the most studied endotype mediated by type 2 helper T cells (Th2) and group 2 innate lymphoid cells (ILC2) producing type 2 (T2) cytokines interleukin (IL)-5 and IL-13 mediated by transcription factor GATA3 that drive the airway eosinophilia (6). Of note, ILC2 are lineage negative lympho-mononuclear cells which are rapidly activated by bronchial epithelium-derived cytokines IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) following exposure to allergens, infection, smoke or pollutants (7). We and others have previously reported that numbers of total ILC2 and IL-5^+IL-13^+ ILC2 but not $CD4^+$ T cells are increased in sputum from prednisone-dependent severe asthmatics with airway eosinophilia (>3%) highlighting ILC2 as a steroid resistant population (8)(9), and the steroid insensitivity of airway ILC2 is dependent on TSLP or IL-33-driven effects (10)(11). Mechanistic studies with T2 biologics that reported effective reduction of annualized asthma exacerbation rates, steroid sparing effects and attenuation of airway eosinophilia with associated reduction of total

and activated airway ILC2 numbers in severe asthmatics with eosinophil airway inflammation (12)(13)(14)(15).

There is a clinical unmet need of targeted therapy for severe asthmatic patients with a neutrophilic or mixed granulocytic airway inflammation secondary to infection who are prone to recurrent exacerbations. Airway neutrophilia in severe asthmatics is driven by infection (e.g. viral fungal, bacterial) induced non-T2 airway inflammation mediated by type 1 (T1) or type 17 (T17) immune responses (16). Upon viral infection or smoke exposure, airway epithelial derived IL-12 and IL-18 induce T1 helper T cells (Th1) to produce interferon (IFN)-γ, an important cytokine for neutrophil recruitment (17). Bacterial/fungal infections, pollution or fatty acids can stimulate alveolar macrophages to release IL-1β, IL-23 and transforming growth factor (TGF)-β promoting T17 helper T cells (Th17) to produce IL-17A which induces neutrophil chemo-attractant secretion (e.g. IL-6, IL-8) from airway epithelium (17)(18)(19). The role of ILC in the development of severe asthmatics with non T2 airway inflammation has not been fully investigated.

ILC3 are functionally similar to Th17 cells, producing IL-17A and IL-22 in response to IL-1β, IL-23 and TGF-β (20). Transcriptomic profiles in the U-BIOPRED study showed significantly higher levels of ILC3 associated genes in nasal brushings from adult-onset severe asthmatics compared to childhood onset asthma (21). In murine model treated with high fat diet, IL-17A$^+$ ILC3 stimulated by IL-1β were significantly associated with airway hyperresponsiveness (22). Similarly, more IL-17A$^+$ ILC was detected in the bronchoalveolar lavage (BAL) of obese severe asthmatics compared to mild or non-asthmatic subjects. However the specific ILC subsets within the IL-17A$^+$ ILC was not determined (22). Although these studies indicate that IL-17A$^+$ ILC3 may contribute to the pathogenesis of severe asthma associated with obesity and neutrophilic airway inflammation, a clear connection between ILC3 and neutrophilia remains to be clarified.

In this study, we reported that sputum ILC3 are most abundant in severe asthma with neutrophilic airway inflammation and that IL-17A^{+} ILC3 numbers correlated with airway neutrophilia. Importantly, we identified an intermediate ILC2 displaying ILC3 markers (c-kit and IL-17A) in SA with neutrophilic and mixed granulocytic airway inflammation. Inflammasome-derived cytokines, IL-1β and IL-18 were found to be increased in these inflammatory endotypes of severe asthmatics and induced ILC3-like features in canonical ILC2 at the proteomic and transcriptomic levels. We propose that canonical ILC2 can trans-differentiate in response to airway microenvironmental signals and alter the airway bronchitis. Therefore, controlling ILC3 activity or the trans-differentiation of intermediate ILC2 by blocking IL-1β and IL-18 may be an important therapeutic strategy for asthma exacerbations with a neutrophilic or mixed granulocytic bronchitis.

METHODS

Subjects: Subjects with severe asthma (n=36) were current/ex-smokers (<10 pack year) or non-smokers with a pre-bronchodilator forced expiratory volume in 1s (FEV_1) <80% predicted; post-bronchodilator reversibility ≥12% and ≥200 ml in FEV_1, or a historical PC_{20} ≤8 mg/ml; receiving high-dosage ICS or long acting beta 2 agonist (LABA) (>880 μg/day); had ≥6 months history of treatment with OCS (7.5–40 mg daily prednisone or equivalent) in the previous year. Subjects were categorized based on the predominant cellularity of sputum cell differential counts: Eosinophilic (>3% of sputum differential count; n=9), neutrophilic (>61% of sputum cells; $>15\times10^6$ cell total cell count; n=14) and mixed granulocytic (both eosinophilic and neutrophilic characteristics; n=10) airway inflammation (5). Baseline subject baseline demographics are summarized in **table 4.1**. In addition, mild asthmatic patients (n=10) as airway disease controls were recruited at McMaster Health Sciences center.

Sputum Induction and Cell Extraction: Sputum was induced by inhalation of aerosolized hypertonic saline (3/5/7%) and processed by selecting the mucus plugs, mixing with 4 parts 0.1% dithiothreitol and Dulbecco PBS as previously described (23). Isolated cells were filtered through a 52-μm nylon mesh, pelleted and resuspended in Dulbecco PBS. Cytospin preparations were prepared on glass slides and stained with Diff-Quik for differential counts expressed as percentage means of duplicate slides. The remaining cells were used for flow cytometry.

Immunofluorescence staining: Sputum-extracted cells ($1\text{-}2\times10^6$ cells / tube) were stained for extra- and intracellular markers for ILC subsets and $CD4^+$ T cells with antibodies to Lin (FITC), FcƐRI (FITC), CD94 (FITC), CD45 (APC-H7), CD127 (BV605), CRTH2 (PerCP-Cy5.5), CD4 (BV510), c-kit (PE-CF594) and corresponding isotype controls. Lineage cocktail antibodies (CD2, CD3, CD14, CD19, CD20, CD94) and FcƐRI excluded T/B cells, monocytes, mast cells, neutrophils, eosinophils, NK cells and basophils as previously described (24) and the viability of

cells was identified by Fixable Viability Stain 700. For intracellular staining, cells were incubated in 1% Perm/fix Buffer prior to addition of IL-5/13 (PE), IL-17A (BV786), IFN-γ (PE-Cy7) and transcription factors T-bet (BV421), GATA3 (APC) and RORγt (BV650) antibodies. Stained cells were washed and resuspended in PBS with 1% paraformaldehyde and acquired by a FACS LSR II flow cytometer.

Flow cytometric gating strategy: Sequential multi-gating flow cytometric assessments was performed to identify and enumerate ILC2, ILC3 and $CD4^+$ T cell populations in sputum samples using the FlowJo software supplied by BDIS. ILC1 were identified as Lin^- $CD45^+$ $Fc\varepsilon RI^-$ $CD127^+$ $CRTH2^-$ $c\text{-}kit^-$; ILC2 as Lin^- $CD45^+$ $Fc\varepsilon RI^-$ $CD127^+$ $CRTH2^+$ cells, and ILC3 as Lin^- $CD45^+$ $Fc\varepsilon RI^-$ $CD127^+$ $CRTH2^-$ $c\text{-}kit^+$ cells based on previous reports (25)(26) (**Fig. 4.1A**); Although NKp44 staining was used to sub-divide ILC3, the majority of ILC3 in human airways were lacked NKp44 expression (27)(28) in severe asthmatics (**Fig. S4.1A**). $CD4^+$ T cells were identified as $CD45^+$ Lin^+ $CD4^+$ (**Fig. S4.2A**). Since ILC are rare cell type, the cellular level of ILC analyzed by FlowJo was normalized as proportion of total Lin^- cells, Lin^- $CD127^+$ cells and $CD45^+$ cells. Levels of activated cells were determined by calculating the proportion of cells expressing intracellular IL-5/13 or IL-17A as previously described (8)(24). The intracellular cytokine expressions (IL-5/13 and IL-17A) within ILC are also represented by pie charts generated using "Sunburst" plugin on FlowJo based on mean fluorescence intensity of the pre-concatenated FACS data from each subject group.

Visualization of FACS results by Uniform Manifold Approximation and Projection: Scaled values of pre-gated Lin^- regions (Lin^- $CD45^+$ $Fc\varepsilon RI^-$) were exported from FlowJo (29) and data processed using Seurat package in R (30). Specifically, cells from SA with neutrophilic airway inflammation (termed "NEUT") were analyzed separately from those obtained from the mixed granulocytic (MIXED) or eosinophilic (EOS) airway inflammation. The NEUT values were

centered, and 33 clusters were found using a resolution of 0.3. Data of MIXED and EOS were mapped into the NEUT space using FindTransferAnchors and MapQuery functions in Seurat. Based on the pattern of the marker levels, events were merged into 10 distinct clusters and visualized using Seurat package.

ELLA assays: The sputum supernatants were diluted 1:8 during processing and this was factored into the final calculation of cytokines which were quantified using the ELLA multiplex platform (Bio-Techne). The lower limit of quantification (LLOD) values in a Simple Plex Cartridge was IL-4 (0.52 pg/ml), IL-5 (0.13 pg/ml), IL-13 (4.13 pg/ml), IL-10 (0.58 pg/ml), IL-1β (0.16 pg/ml), IL-18 (0.96 pg/ml), IL-12p70 (0.46 pg/ml), IL-17A (1.05 pg/ml), IFN-γ (0.94 pg/ml), IL-15 (0.51 pg/ml), TNF-α (0.3 pg/ml), IL-6 (0.28 pg/ml), BAFF (0.55 pg/ml) and IL-33 (0.75 pg/ml).

ILC2 culture and staining: Lin^- cells were isolated from non-adherent peripheral blood lymphomononuclear cells from healthy subjects by EasySep Human ILC2 Isolation Kit (STEMCELL). The ILC2 (Lin^- $CD127^+$ $CRTH2^+$) were sort-purified from enriched Lin^- cells using BD FACSAria^{TM} III and cultured in 96-well U-bottom plates (2000-3000 cells per well) in the presence of RPMI-complete media with IL-2 (10ng/ml) with IL-33, IL-7 and IL-25 (50 ng/ml) for 7-10 days. Ex-vivo expanded cells were rested for 3 days in IL-2+IL-7 and subject to various stimulatory conditions including IL-2 (10ng/ml), IL-33, IL-1β, IL-23, TGF-β and IL-18 (IL-18 at 10ng/ml and rest of cytokines at 50ng/ml) for 7-9 days with media replenishment every 2 days. During the final 4 hours of the culture, PMA (10ng/ml) and ionomycin (500nM) were added to stimulate the cells in the presence of Brefeldin A (1×). Cells were then subject to flow cytometric analyses as described above.

Microarray analysis: The stimulated ILC2 were lysed using RNeasy Lysis buffer (RLT) and stored at -80°C till RNA was isolated and purified using the RNeasy kit (Qiagen). The

concentration of extracted RNA was measured by NanoDrop 8000 Spectrophotometer and Qubit Broad Range assay. The RNA integrity was assessed using the Agilent Bioanalyzer 2100 and RNA Nano kit. Samples preparation performed using the GeneChip WT Plus Kit (Thermo Scientific) was applied to amplify the extracted RNA and generate biotinylated sense-strand DNA targets. Labeled samples were then hybridized to human Clariom S array (Thermo Scientific). Washing and staining was performed by the GeneChip Fluidics Station 450 then scanned using a GeneChip Scanner 3000. Raw data were normalized using the RMA algorithm implemented in the limma R-package. Adjusted *p*-values were calculated using the *Benjamini-Hochberg* method. Data were visualized using ggplot22 and p heatmap R-packages. The functional interaction networks of differentially expressed genes were generated using STRING (version 12).

RESULTS

Comparison of ILC subgroups in severe asthma with differential airway inflammation

Study Subject demographics are summarized in Table 1. Using sequential multi-gating strategies of flow cytometric data, established sub-groups of ILC in the airways of severe asthmatics (SA) were identified and enumerated and data expressed as a proportion of total ILC ($Lin^-CD45^+CD127^+$) (**Fig 4.1A**). In SA with eosinophilic airway inflammation, the percentage of ILC2 was significantly greater than ILC3 (40% vs 6%), whereas more ILC3 were detected in SA with neutrophilic airway inflammation and a comparable level of ILC2 (21% vs 9%) and ILC3 was detected in SA with mixed granulocytic airway inflammation and mild asthma (MA) cohorts (**Fig 4.1B**). Similar trends were found when ILC were expressed as percentage of total lineage negative (Lin^-) cells (**Fig. S4.1B)**. Although ILC1 accounted for a majority of ILC proportion (**Fig 4.1B**), there was no detectable intracellular IFN-γ or T-bet expression in these cells suggesting that ILC1 were not actively driving the airway inflammation in stable SA subjects (**Fig.S4.1C**). As well, CD34 staining was not performed and so the presence of progenitor cells cannot be excluded from this fraction. Using an unbiased uniform manifold approximation and projection (UMAP) clustering based on expression patterns of CD127, CRTH2 and c-kit within Lin^- gated cells, similar findings for ILC2 and ILC3 prevalence were observed for SA with eosinophilic or neutrophilic airway inflammation (**Fig. 4.1C&D**). No distinct cluster of ILC subsets was identified in the SA with mixed granulocytic airway inflammation or MA cohorts.

In the pooled data from all SA groups, the frequency of total ILC2 correlated significantly with the airway eosinophilia (% of differential cell count in sputum) (**Fig. 4.1E**), while sputum neutrophilia correlated significantly with total ILC3 (**Fig. 4.1E**). Details of ILC correlations for each SA subject group are summarised in **Fig. S4.1D-G.** These data suggest that ILC2 and ILC3

may contribute to driving inflammation in the airways of SA with eosinophilia or neutrophilia respectively. In contrast, there was no significant between-group difference in total $CD4^+$ T cells or cytokine positive $CD4^+$ T cells likely due to suppression of T cell activity in response to steroid therapy (**Fig.S4.2)**.

The association of ILC2 and ILC3 with severe asthma endotypes

In severe asthmatics, significantly greater numbers total ILC2 ($\times 10^6$ cells/mL) and IL-$5/13^+$ ILC2 were detected in SA who received higher dose of corticosteroid (Fluticasone equivalent >500 µg/day) (**Fig. 4.2A&B**). Furthermore, the severe asthmatics with eosinophilic airway inflammation contained the greatest number of total ILC2 ($\times 10^6$ cells/mL or % of CD45 cells) **(Fig. 4.2C, S4.3A)** which was inversely correlated with lung function **(Fig. S4.3B)**. Similarly, ILC2 in SA with eosinophilic airway inflammation expressed the highest proportion of IL-5/13 (41%) compared to other inflammatory endotypes **(Fig. 4.2D)**. Within the IL-$5/13^+$ ILC2 **(Fig. 4.2E)**, the expression of GATA3 was significantly higher than RORγt **(Fig. 4.2F)** and a significantly higher proportion of IL-5/13 was detected in $GATA3^+$ ILC2 or $GATA3^+$ $CD4^+$ T cells compared to $GATA3^-$ counterparts (**Fig. S4.3C-E**) supporting previously published associations between GATA3 and IL-5/13 expression in ILC2.

The total numbers of ILC3 and IL-$17A^+$ ILC3 were positively associated with the high dose of corticosteroid (**Fig. 4.2G&H**). The frequency of total ILC3 number ($\times 10^6$ cells/mL or % of CD45 cells) was highest in SA with neutrophilic airway inflammation **(Fig 4.2I, S4.3F)** with increased intracellular IL-17A expression in these groups **(Fig 4.2J).** Also, the total ILC3 level was significantly correlated with lung function and airway neutrophilia in SA with neutrophilic airway inflammation (**Fig. S4.3G**). As a transcription factor mediating IL-17A production, we

have identified a significant association between RORγt and IL-17A expression within CRTH2$^-$ ILC (non-ILC2) (**Fig. 4.2K&L**); whereas no association was detected between the IL-17A and GATA3 in ILCs or CD4$^+$ T cells (**Fig. S4.3H&I**). Notably, levels of total ILC3 and IL-17A$^+$ ILC3 were significantly higher in SA with neutrophilic airway inflammation who had a body mass index >30 (**Fig. 4.3M&N)**. Similar trend was not observed in ILC2 or IL-5/13$^+$ ILC2 (**Fig. S4.3J&K)**.

C-kit$^+$ IL-17A$^+$ ILC2 phenotype in severe asthmatics with neutrophilic or mixed granulocytic airway inflammation

We detected an intermediate ILC2 population in the airways of SA with neutrophilic or mixed granulocytic airway inflammation which exhibited markers expressed by canonical ILC3 i.e c-kit and IL-17A (**Fig. 4.3A&B and S4.4A&B**). The frequency of IL-17A$^+$ ILC2 and IL-17A$^+$ ILC3 was comparable in SA with airway neutrophilia (**Fig. 4.3C**). Both c-kit$^+$ ILC2 and IL-17A$^+$ ILC2 demonstrated an inverse correlation with lung function (FEV_1) in SA with neutrophilic and mixed granulocytic airway inflammation (**Fig. 4.3D&E**) and the levels of c-kit$^+$ ILC2 and IL-17A$^+$ ILC2 were correlated with airway neutrophilia in these SA sub-groups (**Fig. S4.4C**). Also, there was a significant association between c-kit and IL-17A but not IL-5/13 expression by ILC2 in SA with neutrophilic airway inflammation (**Fig. 4.3F**).

To further explore the presence of an intermediate ILC2, pre-gated Lin$^-$ cells from each SA group were analyzed using an unbiased clustering analysis (**Fig. 4.3G, S4.4D**). The UMAP revealed 6 clusters of ILC (Lin$^-$ CD127$^+$) (cluster 1-6) where clusters 1, 2, 3 were identified as ILC1 (CRTH2$^-$ c-kit$^-$), ILC2 (CRTH2$^+$ c-kit$^-$) and ILC3 (CRTH2$^-$ c-kit$^+$), respectively (**Fig. 4.3H**). Furthermore, cluster 4-6 expressed the phenotypes associated with the intermediate ILC2 including c-kit, IL-17A and RORγt (**Fig. 4.3H; Fig S4.4E-F**). The low expressions in cytokines

(IL-5/13, IL-17A and IFN-γ) and surface markers (CD127, CRTH2 and c-kit) in cluster 7 indicated quiescence or precursor cell stage. Of note, cluster 8-10 were defined as unconventional ILC2/ILC3 as CD127 expression was absent but cells expressed IL-17A and IL-5/13 along with CRTH2 or c-kit (**Fig. 4.3H, S4.4E-F**)(31)(32). We next compared the proportion of each cluster using one proportion z-test with SA with neutrophilic airway inflammation as the reference group. The SA with eosinophilic airway inflammation expressed a significantly higher proportion of c-kit^{-} ILC2 (cluster 4) than SA with neutrophilic and mixed granulocytic airway inflammation (**Fig. 4.3I, table S4.1**). In contrast, the highest proportion of ILC3 (cluster 3), intermediate ILC2 (cluster 5, 6), CD127^{-} c-kit^{+} ILC2 (cluster 9), and CD127^{-} ILC3 (cluster 10) were found in SA with neutrophilic and mixed granulocytic airway inflammation (**Fig. 4.3I, table S4.1**).

Cytokine levels in the airway of severe asthmatics with differential inflammatory profiles

Different frequencies of ILC2, ILC3 and intermediate ILC2 in the various SA inflammatory endotypes suggested a potential effect of airway cytokines on ILC characteristics. Measuring cytokine levels in sputum supernatants, we found the highest level of cytokines related to T2 inflammation (IL-4, IL-5, IL-13 and IL-10) in SA with eosinophilic airway inflammation (**Fig. 4.4A-D**). On the other hand, the levels of IL-1β and IL-18 were significantly higher in SA with mixed granulocytic and neutrophilic airway inflammation (**Fig. 4.4E&F**). A significantly higher level of IL-12p70 was detected in the SA with eosinophilic airway inflammation while IL-17A was comparable between groups (**Fig. 4.4G&H**). Additionally, IFN-γ, IL-15, TNF-α, IL-6, B cell activating factor and IL-33 were also comparable between groups (**Fig. S4.5A-F**).

Acquisition of ILC3 features in ILC2 induced by IL-1β+IL-18, *in vitro*

Detection of increased numbers of intermediate ILC2 (c-kit$^+$ IL17A$^+$ ILC2) co-incident with raised levels of IL-1β and IL-18 in severe asthmatics with either neutrophilic or mixed granulocytic airway inflammation suggested IL-1β and IL-18 as potential mediators for ILC2 trans-differentiation toward "ILC3 featured cells". We therefore cultured blood sort-purified canonical ILC2 with IL-1β+IL-18 compared to IL-33+TSLP or IL-1β+IL-23+TGF-β cytokine combinations which have previously been shown to induce IL-5 or IL-17A/c-kit expression by ILC2, *in vitro* (25)(33), (**Fig. 4.5A, S4.6A&B**). We found that IL-5 and IL-5/13 expression by ILC2 were significantly enhanced by IL-33+TSLP compared to IL-2 control culture (**Fig. 4.5B&C, S4.6F&G**). In contrast, IL-1β+IL-23+TGF-β and IL-1β+IL-18 induced a significantly increased IL-17A and c-kit expression by ILC2 (**Fig. 4.5D&E, S4.6H&I**). To exclude the possibility that IL-1β or IL-18 could alone induce ILC2 trans-differentiation, preliminary experiments using each cytokine alone showed that a combination of both cytokines was required for effective ILC2 trans-differentiation to the intermediate phenotype; optimal cytokine concentrations were also previously optimized (**Fig. S4.6C-E**).

We next systematically explored the phenotypical change of the stimulated ILC2 by RNA microarray assay. The purity of post incubated ILC2 was revealed by a low genomic expression of lineage-markers (Log_2 Signal $\leq$ 5.5) (**Table S4.2**). The principal components analysis (PCA) showed that IL-1β+IL-18 culture condition induced an intermediate ILC2 cluster with closer proximity to IL-33+TSLP induced ILC2 (**Fig. 4.5F**) with few differentially expressed genes (**Fig. 4.5G, S4.7A**). However, the differential expressed genes detected between IL-1β+IL-18 vs. IL-1β+IL-23+TGF-β induced ILC2 and IL-33+TSLP vs. IL-1β+IL-23+TGF-β induced ILC2 were further analysed (**Fig. 4.5G, S4.7A**). We found an upregulation of ILC2 signature genes (34)

encoding effector mediators (*IL5, IL13, IL10, CSF2)* and receptors for TSLP and cysteinyl leukotriene (*CRLF2, CYSLTR2*) in both IL-33+TSLP and IL-1β+IL-18 cultured ILC2 (**Fig. 4.5H**). On the other hand, genes encoding canonical ILC3 biomarkers (34) c-kit and RORγt (*KIT, RORC)* along with ILC3 functional genes including *IL17A, CCL20* were mainly detected in ILC2 cultured with IL-1β+IL-18 or IL-1β+IL-23+TGF-β (**Fig. 4.5I, S4.6D**). Of note, increased expression of *IL4R* in IL-1β+IL-23+TGF-β stimulated cultures of ILC2 was consistent with previous studies (**Fig. 4.5I**)(33)(34). No difference was detected in other ILC signature genes between the different culture conditions (**Fig. S4.7B**). In addition, the low expression of *IFNG* (IFN-γ) in all conditions suggested that differentiation of ILC2 to an ILC1 phenotype was not induced by IL-1β+IL-18, unlike IL-12+IL-18 reported by others (35)(**Fig. S4.7C**). Overall, these results suggested an IL-1β+IL-18 culture can induce "ILC3 featured ILC2" or intermediate ILC2 which express key signature genes of ILC3 while maintaining key ILC2 features.

Intermediate ILC2 induced by IL-1β+IL-18 expressed signature genes of c-kit$^+$ILC2 and Th17 cells

A previous study has identified two distinctive genomic profiles in ILC2 based on c-kit expression. The c-kit$^+$ ILC2 exhibited more ILC3 signature genes whereas c-kit$^-$ILC2 expressed more genes related to conventional ILC2 (25). Based on this, we ranked the top 100 differentially expressed genes (based on fold change) in c-kit$^+$ ILC2 and c-kit$^-$ ILC2 from the database of Berninck and Ohne's study (25) as a reference of signature genes for gene set enrichment analysis (GSEA) on our ILC2 cultured with IL-1β+IL-18, IL-33+TSLP or IL-1β+IL-23+TGF-β. As a result, ILC2 cultured with IL-1β+IL-18 or IL-1β+IL-23+TGF-β showed a significant enrichment of c-kit$^+$ ILC2 signature genes compared with IL-33+TSLP cultured ILC2 (**Fig. 4.6A**). In contrast, c-

kit$^-$ ILC2 related transcripts were significantly enriched in IL-33+TSLP and IL-1β+IL-18 cultured ILC2 conditions compared to IL-1β+IL-23+TGF-β cultured ILC2 (**Fig. S4.8A**). In addition, the gene set of Th17 signatures (36) was also significantly enriched in IL-1β+IL-18 cultured ILC2 compared to IL-33+TSLP or IL-1β+IL-23+TGF-β conditions (**Fig. 4.6B**), while an equivalently high expression of Th2 related genes (36) was detected in ILC2 cultured with IL-1β+IL-18 and IL-33+TSLP (**Fig. S4.8B**).

IL-1β+IL-18 cultures of ILC2 induced signalling pathways and gene interactions related to T17 inflammation

We further explored the gene sets related to inflammatory responses or disease which were uniquely enriched in IL-1β+IL-18 or IL-33+TSLP cultured ILC2. Compared with IL-1β+IL-23+TGF-β cultured ILC2, IL-1β+IL-18 induced an exclusive enrichment of genes involved in pathways of "cytokine cytokine receptor interaction", "neutrophil degranulation" and "inflammatory bowel disease" which included genes related to T17 inflammation (**Fig. 4.6C**; **table S4.3**), whereas IL-33+TSLP related genes involved in "IL-4/13 signalling", "signaling by interleukin" and "GM-CSF pathway" were uniquely enriched in IL-33+TSLP cultured ILC2 (**Fig. 4.6D** ; **table S4.3**). The complete list of signalling pathway was summarized in figure **S4.8C.**

To identify the potential gene interaction induced by IL-1β+IL-18 in ILC2, an interaction network was generated based on the differential expressed genes which were functionally connected in IL-1β+IL-18 compared with IL-1β+IL-23+TGF-β cultures of ILC2. We observed that *CTSH* (cathepsin H) and *PTPRJ* (protein tyrosine phosphatase receptor J) which are T17 inflammation related genes independently connected to human leukocyte antigen family related genes (**Fig. 4.6E&F**). Previous studies had clustered *CTSH* with *IL-17A/F, CCR6* and *RORC* as

Th17 signature genes which mediate neutrophilic inflammation (37)(38). The *PTPRJ* encodes protein tyrosine phosphatase CD148 which is mainly expressed by T/B cells (39). Also, a higher expression of *PTPRJ* was detected in RORγt^{+} ILC3 compared to ILC2 in murine small intestine lamina propria (40). Of note, a connection between heat shock protein related genes including heat shock protein A4 like (*HSPA4L*) and DNAJ heat shock protein family 40 member C5 Beta (*DNAJC5B*) were detected in IL-1β+IL-18 cultured ILC2 (**Fig. 4.6E&F**). Heat shock protein A4 (HspA4) was previously indicated to promote inflammatory cytokine expressions (IL-6, IL-17, TNF-α) in T lymphocytes or myeloid cells and to inhibit immune cell apoptosis (41). Furthermore, *DNAJC5B* is highly expressed in lymphocytes within the BAL of severe asthmatics (42). Thus, the increased level of heat shock protein related genes potentially maintains proliferation and IL-17A expression by IL-1β+IL-18 stimulated ILC2. In contrast, a network connected to IL-13 interaction (T2) pathway was detected in IL-33+TSLP stimulated ILC2: CC motif chemokine receptor 3 (*CCR3*) (43)(44) is essential for the migration and cytokine expression in Th2 cells and ILC2 during the T2 airway inflammation (43)(44) (**Fig. 4.6G&H**). In addition, the kruppel like transcription factor 2 (*KLF2*) and dual specificity phosphatase 1 (*DUSP1*) are related to the migration and tissue residency of ILC2 (34)(45) (**Fig. 4.6G&H**).

DISCUSSION

A comparative analysis of ILC sub-groups and investigation of the role of ILC in the pathogenesis of various airway diseases is being actively pursued including allergic rhinitis (46), cystic fibrosis with nasal polyps (33) and chronic obstructive pulmonary disease (COPD) (47). In the present study, we have characterized the detailed phenotypes of ILC2 and ILC3 in severe asthma with different airway inflammatory profiles. In line with previous studies (8), we found that ILC2 was associated with airway eosinophilia and negatively correlated with lung function in severe asthma.

Notably, this is the first study to report that the highest frequency of ILC3 are detected in the airway of SA with neutrophilic airway inflammation where ILC3 levels corelated with airway neutrophilia (21)(22)(26). Also, we have identified ILC3 as an important source of Interleukin-17A (IL-17A), a pro-inflammatory cytokine that induces the release of chemoattractant (e.g. IL-6, IL-8, leukotriene B_4) from the airway epithelial cells which recruit and activate neutrophils in the airway (48). Neutrophil is a pro-inflammatory granulocyte involved in the non-T2 airway inflammation and exacerbation of severe asthma (49)(50). Unlike eosinophils which can be effectively inhibited by corticosteroids, this therapy promotes neutrophil survival in the airway of severe asthmatics (6)(49). Thus, novel add-on therapies are required to control airway neutrophilia in severe asthma. In this study, the positive correlation between IL-17A$^+$ ILC3 and ICS dosage implied a steroid insensitivity of ILC3 in a non-T2 cytokine environment. Also, the correlation between body mass index and IL-17A$^+$ ILC3 level suggested that obesity maybe a driver of ILC3 activation in line with a previous report (22). Thus, the increased level of ILC3 expressing IL-17A in SA with airway neutrophilia may be an important target to control in non-T2 severe asthma.

Of note, a preliminary study from our group had observed IL-17A$^+$ ILC2 in the sputum of

patients with COPD (51), indicating that ILC2 is an alternative source of IL-17A other than ILC3. Among the mature ILC, c-kit is an ILC3 specific marker used to distinguish from ILC1 within $CRTH2^-$ ILC populations (52). Notably, c-kit^+ ILC2 was recently observed in human blood (25) and c-kit^+ ILC2 expressed higher levels of the RORγt and IL-17A than c-kit^- ILC2 with a maintenance of IL-5/13 expression (53). In this study, we detected a group of intermediate ILC2 expressing ILC3 features (c-kit or IL-17A or both) in SA with neutrophilic or mixed granulocytic airway inflammation and the airway intermediate ILC2 level was significantly correlated with the airway neutrophilia. In addition, the expressions of c-kit and IL-17A were significantly associated in the intermediate ILC2. High levels of c-kit and IL-17A expressions were also identified in unconventional ILC2 ($CD127^-$ $CRTH2^+$) in SA with neutrophilic or mixed granulocytic airway inflammation. The unconventional ILC is absent of CD127 expression (32) and expresses a higher level of CD45RO suggesting them as more differentiated and reactive than conventional ILC (54). Thus, the observation of intermediate ILC2 in severe asthmatics with non T2 airway inflammation indicate that ILC2 may not only drive T2 airway inflammation but may also promote airway neutrophilia potentially by trans-differentiating towards ILC3 like cells.

The trans-differentiation of ILC2 towards ILC3 like cells following bacterial or fungal infection has previously been reported using human skin derived ILC2 which released IL-17A when co-cultured with epidermal cells in the presence of *C.albicans* (25). Also, IL-$17A^+$ ILC2 were observed in a co-culture of human blood ILC2 and nasal polyp-derived epithelial cells incubated with *Staphylococcus aureus* or *Pseudomonas aeruginosa* (55). In both studies (25)(55), the epithelial cells released IL-1β, IL-23 and TGF-β as stimulators for ILC2 trans-differentiation in response to fungal or bacterial exposure. Furthermore, co-culture with these cytokines *in vitro* induced c-kit and IL-17A expression by ILC2 in a RORγt dependent manner with a concurrent

decrease in IL-5 and GATA3 (25)(33). Although IL-1β+IL-23+TGF-β have the potential to induce trans-differentiation of ILC2 into ILC3-like cells in dermal or nasal polyp tissues (25)(33), the drivers for ILC2 plasticity in airway of SA have not been determined.

To identify the stimulators of intermediate ILC2 in the airway of SA, the cytokine level in sputum supernatants was assessed and we found significantly higher levels of T2 cytokines including IL-4, IL-5 and IL-13 in SA with eosinophilic bronchitis. Both IL-12 and IL-23 belong to the IL-12 cytokine family and the high level of IL-12p70 in SA with airway eosinophilia suggested a potential history of exacerbation due to rhinoviral infection (35). The low levels of IFN-γ, IL-15 and B cell activating factor suggested an absence of T1 airway inflammation in our SA subjects. NLRP3 inflammasome-related family of cytokines IL-1β and IL-18 (56)(57) have been detected in fatal/severe asthma, a significantly higher level of IL-18 with its receptor was previously reported in the lung tissues and distal bronchi (58)(59). Here we detected an increased level of IL-1β and IL-18 in SA with neutrophilic or mixed granulocytic inflammation. Since these same patients also had a high frequency of the intermediate-ILC2 population we investigated IL-1β and IL-18 as potential stimulators for ILC2 trans-differentiation.

There are commonly accepted *in vitro* stimulators for ILC2 plasticity: IL-1β+IL-12 (47)(60), IL-1β+IL-23+TGF-β (25)(33) or IL-7+IL-33+retinoic acid (46) which induced ILC2 to acquire functions of ILC1, ILC3 or ILCreg, respectively. Here, we reported that IL-1β+IL-18 induced ILC3 features in ILC2 including an increase expression of c-kit and IL-17A at proteomic level. Also, the RNA microarray identified an elevated level of c-kit^{+} ILC2 and Th17 signature genes in IL-1β+IL-18 induced ILC2, and gene sets related to the signalling pathway of T17 inflammation or non T2 disease were uniquely enriched in IL-1β+IL-18 induced ILC2. We had identified a gene association network including *CTSH* and *PTPRJ* which are signature genes

related to ILC3 and T17 response in IL-1β+IL-18 induced ILC2 and the connection between these two genes with antigen presentation process (*HLA-DQ*) need to be further investigated. In addition, the network of heat shock protein family genes *HSPA4L-DNAJC5B* enriched in IL-1β+IL-18 induced ILC2 was previously reported as important mediators for IL-17 expression of T cells and lymphocytes activity in severe asthma (41)(42). Thus, we identified a network of signatures genes in IL-1β+IL-18 induced ILC2 which might regulate the development of ILC3 and T17 features as a potential therapeutic target.

Type 2 targeted biologics are novel therapies to treat severe eosinophilic asthmatics who are not well controlled by high dose corticosteroids. A positive effect has been reported for T2 biologics targeting IgE (omalizumab)(61), IL-5 (mepolizumab, reslizumab and benralizumab)(13)(15), IL-4 and IL-13 (dupilumab)(62), TSLP (tezepelumab)(63) and to some extent IL-33 (itepekimab)(64). However, the determinant targets for non T2 airway inflammation in SA are not well defined and no clinical efficacy was found for biologics targeting IL-17 or IL-23 (65)(66). Here, we propose that the combination of IL-1β+IL-18 stimulated trans-differentiation of canonical ILC2 to an intermediate ILC2 which may promote neutrophilic (T17) inflammatory responses. These new findings suggested that IL-1β and IL-18, part of the inflammasome family of cytokines, may be a promising a therapeutic target for asthmatics with non T2 airway inflammation.

A few limitations of the current study are that the largest proportion of airway proportion of ILC1 or ILC progenitor like cells identified in the airway of severe asthmatics were not well characterized. Although the relative proportion of ILC1 like-cells to other ILC subgroups were comparable to those reported by Kim *et al* in sputum from mild asthmatics, further characterization is warranted in severe asthma (26). In addition, the phenotypes of airway ILC reported here reflect

a baseline cross-sectional analysis of stable severe asthma. Whether these phenotypes change with the fluctuating airway inflammatory profile in severe asthma patients experiencing an exacerbation remains to be determined and is the subject of an ongoing study. The same may hold true for airway $CD4^+$ and $CD8^+$ T cell populations in the airways.

In conclusion, the novel findings from our study suggested that ILC3 are associated with the pathogenesis of severe asthma with neutrophilic airway inflammation. Also, canonical ILC2 can adapt to changing environmental cues such that when IL-1β+IL-18 cytokines are raised in the airways these cells may differentiate into an intermediate ILC2 phenotype which through the production of IL-17A may contribute to airway neutrophilia in some severe asthmatics. Therefore, ILC3 and intermediate ILC2 might be targets of therapy to control exacerbations of non-type 2 severe asthma.

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Table 4.1: Demographic Information of Study Subjects

	Eosinophilic (n=9)	Neutrophilic (n=14)	Mixed (n=10)	MA (n=10)
Age (y)	61 ± 6	57 ± 12	57 ± 14	39 ± 20
Atopy	1 / 9	7 / 14	7 / 10	7 / 10
Sex (% male)	55.6	57.1	50	50
Smoking Hx (Current / Ex- /Non)	2 / 3 / 4	1 / 6 / 7	3 / 3 / 4	0 / 0 /10
Body Mass Index	27.04 ± 5.1	29.1± 7.1	32.3 ± 5.04	N/A
FEV_1 (% predicted)	65.9 ± 25.5	65.8± 25.1	60.4 ± 18.9	88.7 ± 3.3
Pre-bronchodilator FEV_1 (L)	1.86 ± 0.75	2.11 ± 0.89	1.8 ± 0.82	3.31 ± 0.35
Pre-bronchodilator FVC (L)	2.90 ± 0.82	3.42 ± 1.2	3.05 ± 1.05	4.11 ± 0.44
Pre-bronchodilator FEV_1/FVC ratio (%)	76.5 ± 1.5	77.1 ± 3.4	78.6 ± 3.1	80.1 ± 5
Prednisone mg/d (n)	5 (5, 10) (5/9)	7.5 (5, 20) (9/14)	7.5 (7.5, 50) (7/10)	0
ICS-Fluticasone Equivalent µg/d Median/Mean	1000 / 1056 (0, 2000)	500 / 592 (0, 2000)	1250 / 1325 * (750, 3000)	0
Biologics (N)	3/9	4/14	1/10	0
Sputum total WC $\times 10^6$ cells/ml (g)	11.1 (9.6, 40)	66.7 # (22.2, 108.3)	45.3 (18, 108.4)	4.6 (0.55,8.8)
Sputum Eos %	16.5 # § (3.5, 78.9)	0 (0, 1.8)	7.8 (3.7, 11.3)	2 (1, 4.3)
Sputum Neut %	36.8 (10.7, 67.8)	91.8 # (76, 99)	74 § (65.5, 93.3)	42.6 (29.7, 62.3)
FEGs	3 # § (1, 3)	0 (0, 0)	2 * (1, 3)	N/A
Bacterial infection (n)	0 / 9	13 / 14	5/ 10	0

Subjects with severe asthma had a pre-bronchodilator forced expiratory volume in 1 s (FEV_1) <80% predicted; post-bronchodilator reversibility ≥12% and ≥200 mL in FEV_1, or historical PC_{20} ≤8 mg/mL; receiving high-dose ICS/LABA (>880 μg/day); ≥6 months history of treatment with OCS (7.5–40 mg daily prednisone or equivalent); Sputum cellularity was determined by differential cell counts. Mild asthma subjects were disease control comparison. Data are presented as median(range) or mean ± SD unless otherwise indicated. FEG – free eosinophil granules.
*Significant difference in Mixed Granulocytic vs Neut; # Significant difference in Eos vs Neut; § Significant difference in Eos vs Mixed Granulocytic airway inflammation.

FIGURES

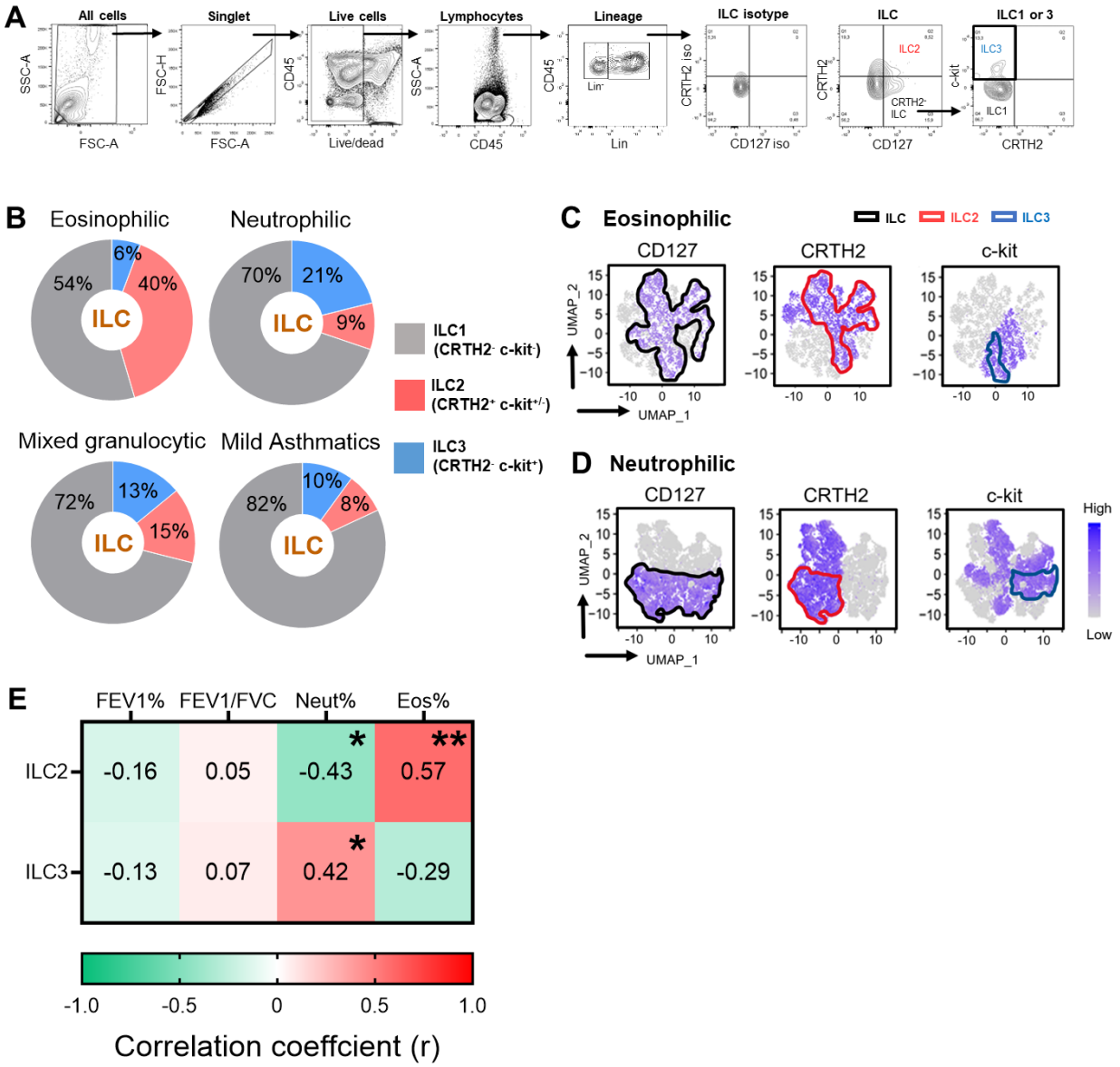


Figure 4.1. Characterization of sputum ILC in severe asthmatics (A) Representative flow cytometric gating for ILC subsets **(B)** frequency of ILC1, ILC2 and ILC3 expressed as % of total ILC. Unbiased clustering analyses (UMAP) of ILC2 and ILC3 in pre-gated Lin$^-$ sputum cells in **(C)** eosinophilic and **(D)** neutrophilic severe asthmatics; expression patterns of CD127, CRTH2 and c-kit are plotted in purple, the intensity of colour representing expression level. **(E)** Heatmap shows correlations between ILC2 (% of Lin$^-$ cells) and eosinophilia, ILC3 (% of Lin$^-$ cells) and neutrophilia using pooled data from all subject groups. Data are assessed for statistical significance using Mann-Whitney U tests and Pearson correlation coefficient (r); horizontal bars represent median values (*P<0.05 and ** P<0.01).

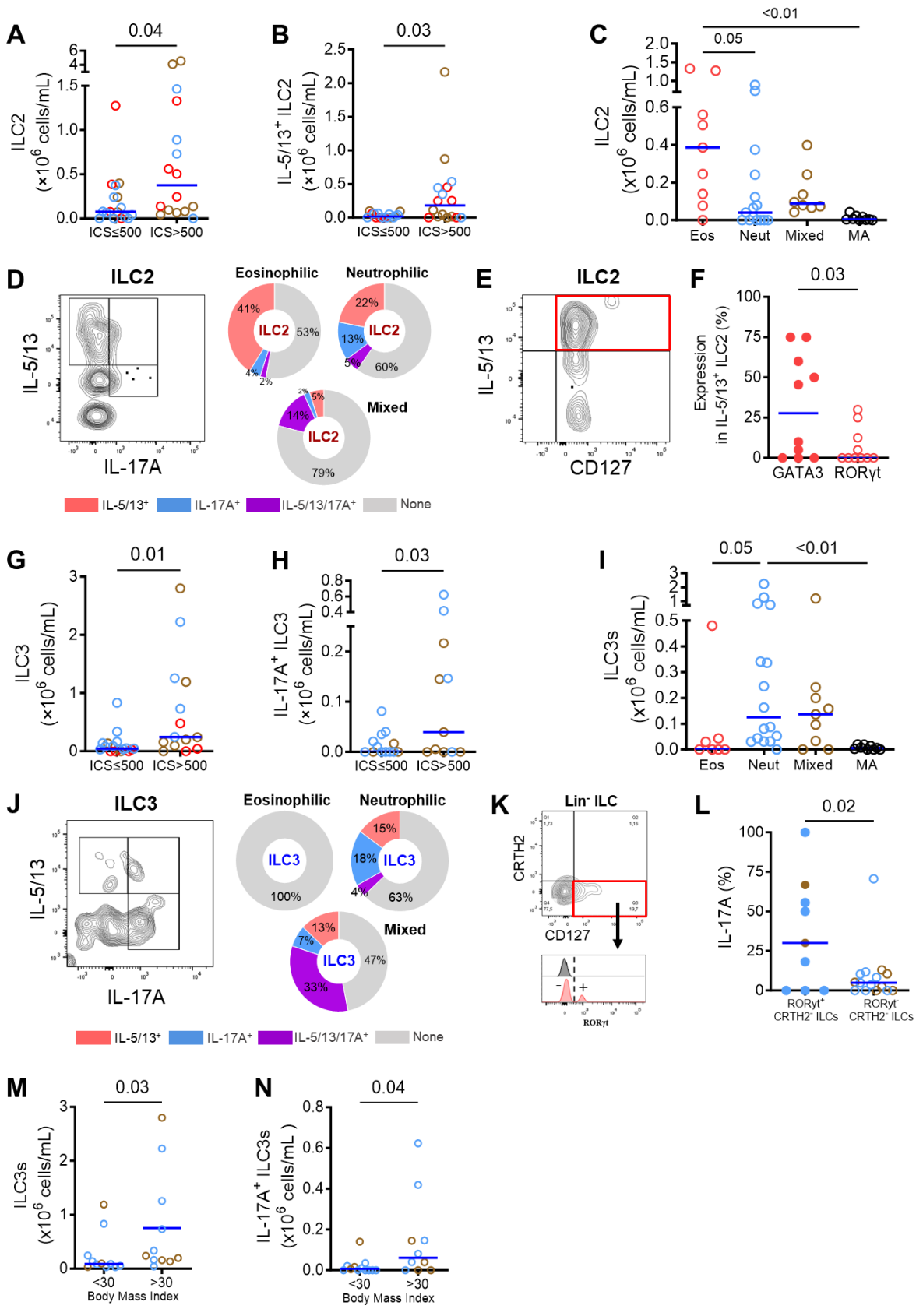

A
0.04
ILC2 (×10^6 cells/mL)
ICS≤500 ICS>500
B
0.03
IL-5/13+ ILC2 (×10^6 cells/mL)
ICS≤500 ICS>500
C
<0.01
0.05
ILC2 (x10^6 cells/mL)
Eos Neut Mixed MA
D
ILC2
IL-5/13
IL-17A
Eosinophilic
Neutrophilic
Mixed
41%
ILC2
53%
4%
2%
22%
13%
ILC2
5%
60%
2%
5%
14%
ILC2
79%
IL-5/13+ IL-17A+ IL-5/13/17A+ None
E
ILC2
IL-5/13
CD127
F
0.03
Expression in IL-5/13+ ILC2 (%)
GATA3 RORγt
G
0.01
ILC3 (×10^6 cells/mL)
ICS≤500 ICS>500
H
0.03
IL-17A+ ILC3 (×10^6 cells/mL)
ICS≤500 ICS>500
I
0.05
<0.01
ILC3s (x10^6 cells/mL)
Eos Neut Mixed MA
J
ILC3
IL-5/13
IL-17A
Eosinophilic
Neutrophilic
Mixed
ILC3
100%
15%
18%
ILC3
4%
63%
13%
7%
ILC3
47%
33%
IL-5/13+ IL-17A+ IL-5/13/17A+ None
K
Lin- ILC
CRTH2
CD127
RORγt
L
0.02
IL-17A (%)
RORγt+ CRTH2- ILCs
RORγt- CRTH2- ILCs
M
0.03
ILC3s (x10^6 cells/mL)
<30 >30
Body Mass Index
N
0.04
IL-17A+ ILC3s (x10^6 cells/mL)
<30 >30
Body Mass Index

Figure 4.2. ILC2, ILC3 with cytokine expression in the sputum from severe asthma subjects. Level of total **(A)** ILC2 and **(B)** IL-5/13^{+} ILC2 in severe asthmatics categorized based on daily treatment with inhaled corticosteroids (500µg fluticasone equivalent). (**C**) Frequency of ILC2 ($\times 10^6$ cells/ml) in sputum of severe asthma subjects with eosinophilic (red), neutrophilic (blue), mixed granulocytic (brown) airway inflammation and mild asthma (black). (**D**) Representative gating shows IL-5/13 and IL-17A expression by ILC2 where data were visualized in pie charts as mean % of ILC2. (**E-F**) Expressions (%) of GATA3 and RORγt in IL-5/13^{+} ILC2. Level of total **(G)** ILC3 and **(H)** IL-17A^{+} ILC3 in severe asthmatics categorized based on daily treatment with inhaled corticosteroids (500µg fluticasone equivalent). **(I)** Frequency of ILC3 ($\times 10^6$ cells/ml) in sputum **(J)** The intracellular expressions of IL-17A and IL-5/13 in ILC3 are shown in a representative flow plot where data are visualized in pie charts as mean % of total ILC3. **(K-L)** Expression (%) of IL-17A in RORγt^{+} CRTH2^{-} ILC (non-ILC2) and RORγt^{-} CRTH2^{-} ILC in severe asthma with neutrophilic (blue) and mixed granulocytic (brown) airway inflammation. Levels of **(M)** total ILC3 and **(N)** IL-17A^{+} ILC3 in severe asthmatics categorized by body mass index (BMI; < 30 or ≥ 30); Statistical differences were assessed using one way ANOVA with Tukey's multiple comparison test for within group and unpaired t-test for between group comparison of parametric data.

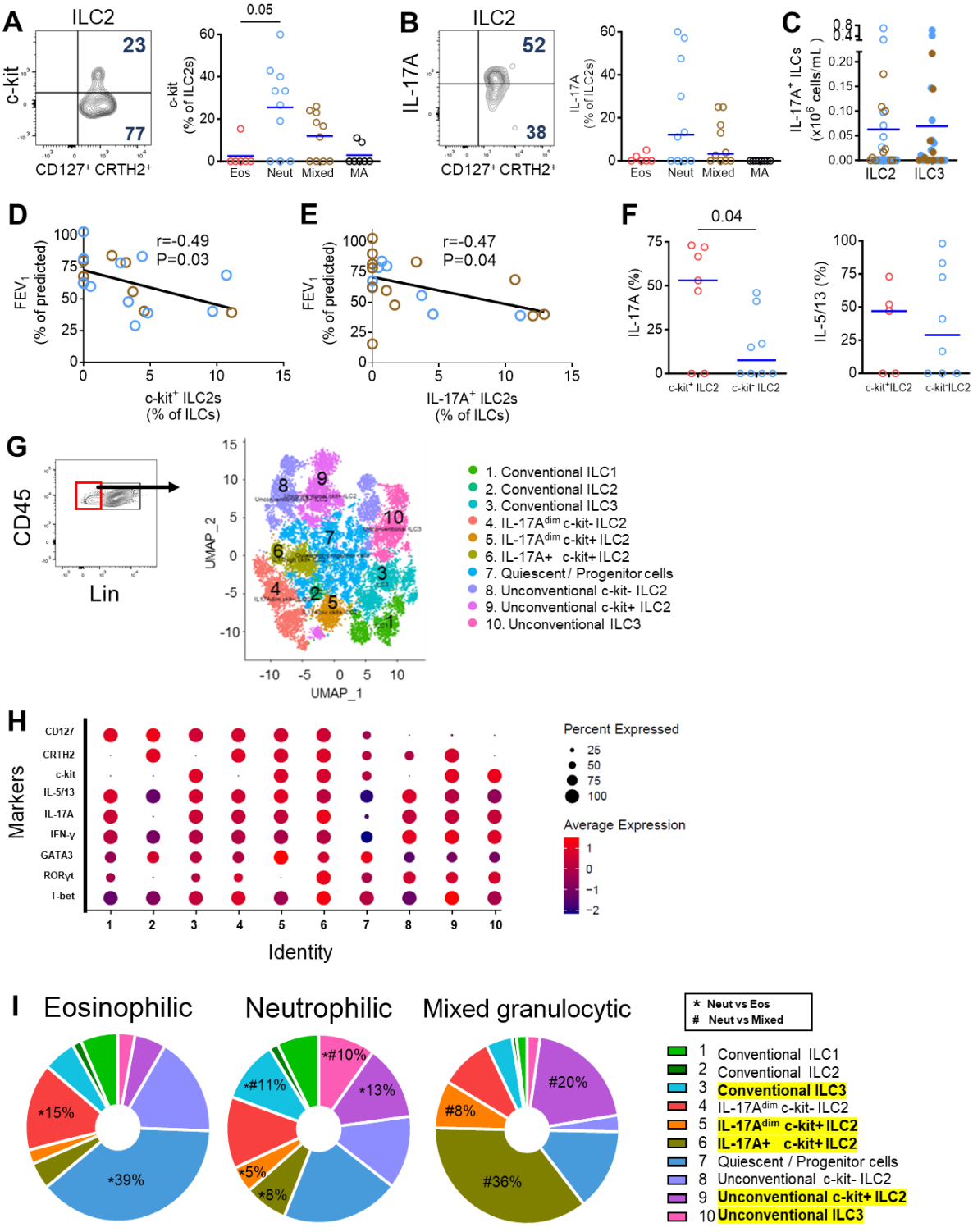

A
ILC2
23
77
c-kit
CD127+ CRTH2+
0.05
c-kit (% of ILC2s)
Eos Neut Mixed MA
B
ILC2
52
38
IL-17A
CD127+ CRTH2+
IL-17A (% of ILC2s)
Eos Neut Mixed MA
C
IL-17A+ ILCs (x10^6 cells/mL)
ILC2 ILC3
D
r=-0.49
P=0.03
FEV1 (% of predicted)
c-kit+ ILC2s (% of ILCs)
E
r=-0.47
P=0.04
FEV1 (% of predicted)
IL-17A+ ILC2s (% of ILCs)
F
0.04
IL-17A (%)
c-kit+ ILC2 c-kit- ILC2
IL-5/13 (%)
c-kit+ILC2 c-kit-ILC2
G
CD45
Lin
UMAP_1
UMAP_2
1. Conventional ILC1
2. Conventional ILC2
3. Conventional ILC3
4. IL-17Adim c-kit- ILC2
5. IL-17Adim c-kit+ ILC2
6. IL-17A+ c-kit+ ILC2
7. Quiescent / Progenitor cells
8. Unconventional c-kit- ILC2
9. Unconventional c-kit+ ILC2
10. Unconventional ILC3
H
Markers
CD127
CRTH2
c-kit
IL-5/13
IL-17A
IFN-γ
GATA3
RORγt
T-bet
Identity
Percent Expressed
25
50
75
100
Average Expression
1
0
−1
−2
I
Eosinophilic
Neutrophilic
Mixed granulocytic
*15%
*39%
*#11%
*5%
*8%
*#10%
*13%
#8%
#36%
#20%
* Neut vs Eos
# Neut vs Mixed
1 Conventional ILC1
2 Conventional ILC2
3 Conventional ILC3
4 IL-17Adim c-kit- ILC2
5 IL-17Adim c-kit+ ILC2
6 IL-17A+ c-kit+ ILC2
7 Quiescent / Progenitor cells
8 Unconventional c-kit- ILC2
9 Unconventional c-kit+ ILC2
10 Unconventional ILC3

Figure 4.3. The intermediate ILC2 in severe asthmatics with neutrophilic and mixed granulocytic airway inflammation.

Expression of **(A)** c-kit (%) and **(B)** IL-17A (%) by ILC2 in severe asthma subjects with eosinophilic (red), neutrophilic (blue), mixed granulocytic (brown) airway inflammation and mild asthma (back). (**C**) Frequency of IL-17A expressing ILC2 and ILC3 in severe asthmatics with neutrophilic (blue) or mixed granulocytic (brown) airway inflammation. The correlation plots between FEV_1 and **(D)** c-kit$^+$ ILC2 or **(E)** IL-17A$^+$ ILC2 in SA with neutrophilic (blue) and mixed granulocytic (brown) airway inflammation. (**F**) The association between c-kit expression and IL-17A or IL-5/13 expression by ILC2 from SA with neutrophilic airway inflammation. (**G**) Unbiased clustering UMAP analyses of live CD45$^+$Lin$^-$ cells in sputum from SA with neutrophilic airway inflammation. (**H**) Dot plot shows expression of surface receptors, cytokines and transcription factors in clusters identified in (G); the size of each dot indicates frequency of ILC that express the receptors, transcription factors or cytokines and the color intensity represents average expression level. **(I)** Pie chart indicates the proportion of each cluster identified in (H) expressed as % of total clusters in SA with eosinophilic, neutrophilic or mixed granulocytic airway inflammation, and the clusters of ILC expressing ILC3 features (c-kit or IL-17A or both) are highlighted in yellow. Pearson's coefficient (r) was calculated for correlational associations. Unpaired t-tests assessed between group statistical difference. One proportion z-test was applied in (I) with SA with neutrophilic airway inflammation as the reference group. (*P<0.05 and *** P<0.001)

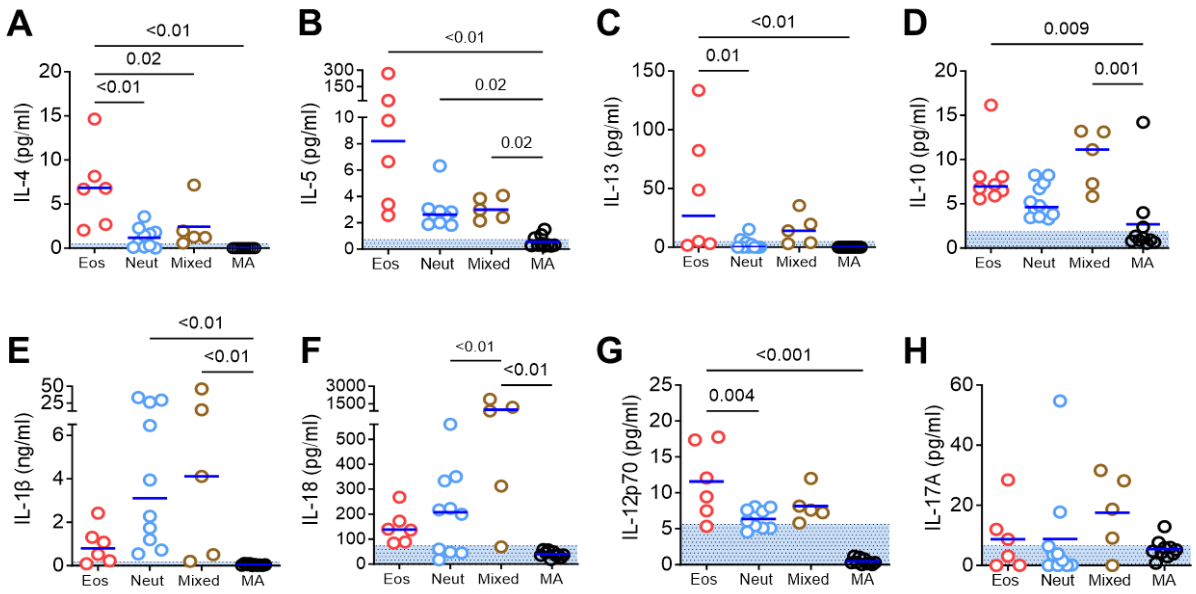
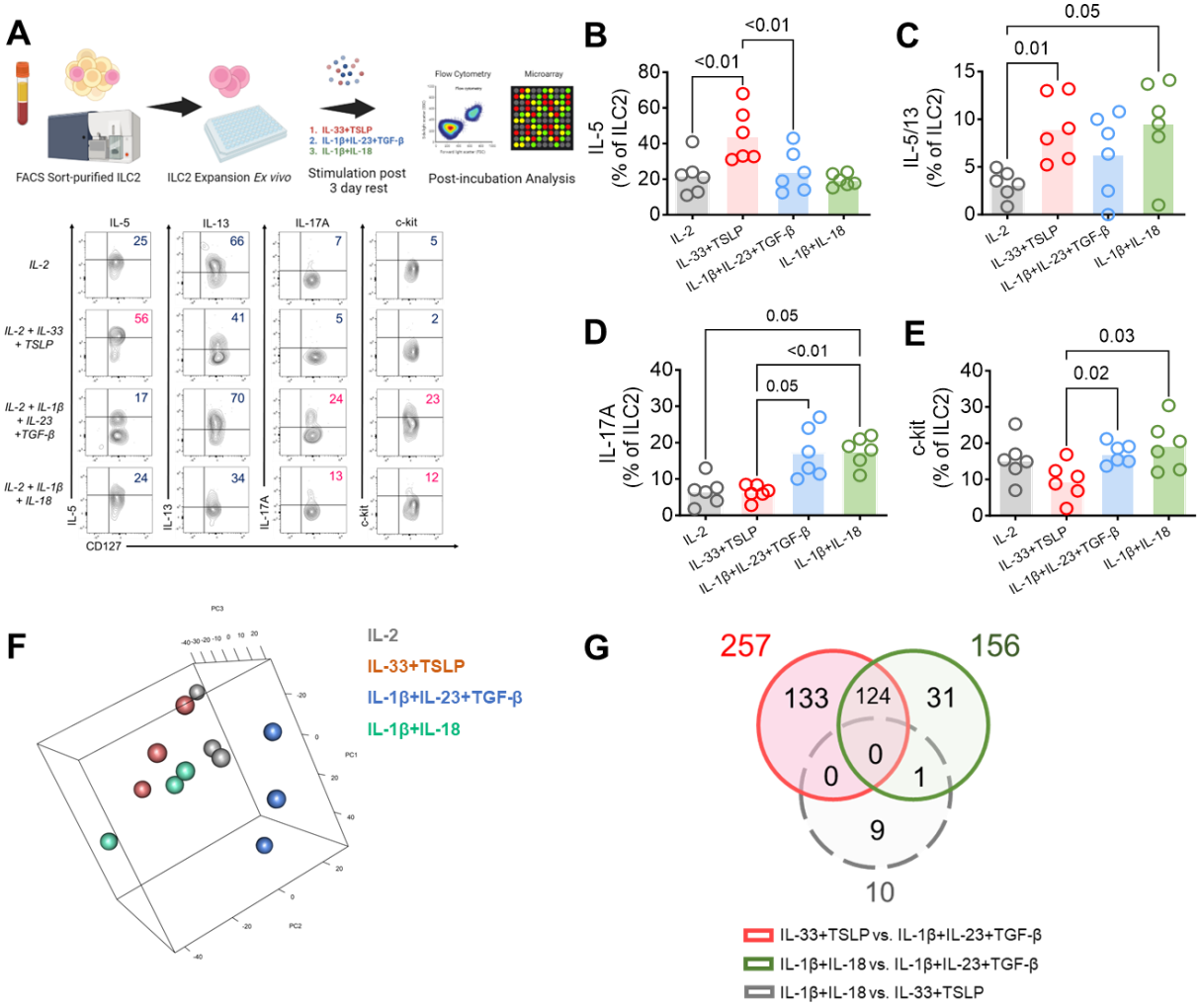
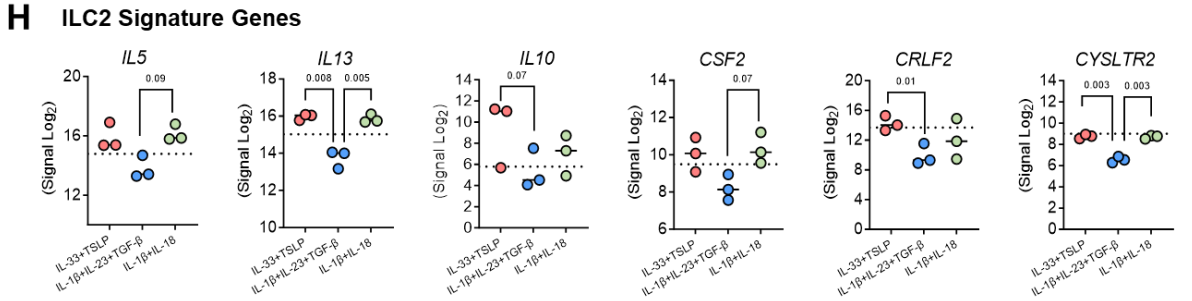


Figure 4.4. Cytokines in sputum supernatants from patients with severe asthma. Sputum supernatants were assessed in severe asthma subjects with eosinophilic (red), neutrophilic (blue), mixed granulocytic (brown) airway inflammation and mild asthma (back) by Ella^{TM} multiplex assay for **(A)** IL-4, **(B)** IL-5, **(C)** IL-13, **(D)** IL-10, **(E)** IL-1β, **(F)** IL-18, **(G)** IL-12p70 and **(H)** IL-17A in severe asthma subjects with eosinophilic, neutrophilic and mixed granulocytic airway inflammation. Kruskal-Wallis test was used to calculate between group statistical difference for non-parametric data; horizontal bars represent median value and the dotted lines represent the 90th percentile levels from sputum supernatant of healthy controls (n=17). **IL-12p70 represents bioactive form of IL-12.


A
FACS Sort-purified ILC2
ILC2 Expansion Ex vivo
1. IL-33+TSLP
2. IL-1β+IL-23+TGF-β
3. IL-1β+IL-18
Stimulation post 3 day rest
Flow Cytometry
Microarray
Post-incubation Analysis
IL-5
IL-13
IL-17A
c-kit
IL-2
IL-2 + IL-33 + TSLP
IL-2 + IL-1β + IL-23 +TGF-β
IL-2 + IL-1β + IL-18
CD127
B
IL-5 (% of ILC2)
<0.01
<0.01
C
IL-5/13 (% of ILC2)
0.05
0.01
D
IL-17A (% of ILC2)
0.05
<0.01
0.05
E
c-kit (% of ILC2)
0.03
0.02
IL-2
IL-33+TSLP
IL-1β+IL-23+TGF-β
IL-1β+IL-18

F
IL-2
IL-33+TSLP
IL-1β+IL-23+TGF-β
IL-1β+IL-18
G
257
156
133
124
31
0
0
1
9
10
IL-33+TSLP vs. IL-1β+IL-23+TGF-β
IL-1β+IL-18 vs. IL-1β+IL-23+TGF-β
IL-1β+IL-18 vs. IL-33+TSLP


H
ILC2 Signature Genes
IL5
IL13
IL10
CSF2
CRLF2
CYSLTR2
(Signal Log2)
IL-33+TSLP
IL-1β+IL-23+TGF-β
IL-1β+IL-18

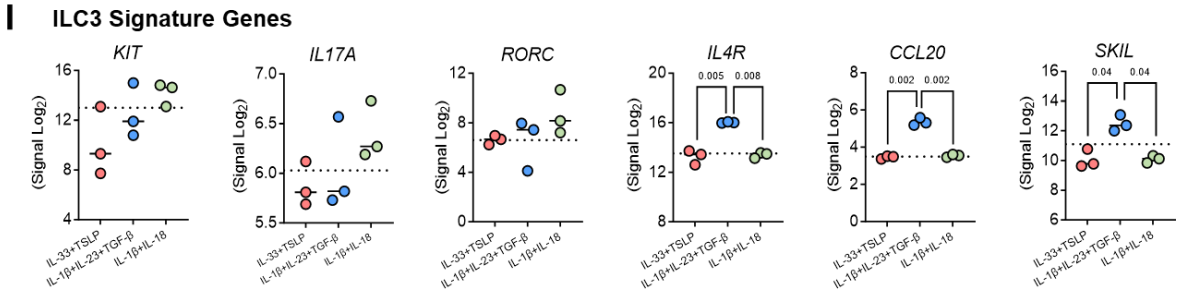

I
ILC3 Signature Genes
KIT
IL17A
RORC
IL4R
CCL20
SKIL
(Signal Log2)
IL-33+TSLP
IL-1β+IL-23+TGF-β
IL-1β+IL-18

Figure 4.5. IL-1β+IL-18 induced intermediate ILC2 with ILC3 features i*n vitro*. (A) Representative gating plots of IL-5, IL-13, IL-17A and c-kit expression by flow-sort purified ILC2 cultured with IL-2 (grey), IL-33+TSLP (red), IL-1β+IL-23+TGF-β (blue) or IL-1β+IL-18 cytokines (green symbols) for 7-9 days then re-stimulated with PMA and ionomycin; data from each experiment presented as proportional expression of **(B)** IL-5, **(C)** IL-5/13, **(D)** IL-17A and **(E)** c-kit. **(F)** Principal components analyses of transcriptome from ILC2 cultured in various conditions, **(G)** Venn diagram summarizing differentially expressed genes (adj $P<0.05$) in ILC2 cultured with IL-33+TSLP vs. IL-1β+IL-23+TGF-β (red circles); IL-1β+IL-18 vs. IL-1β+IL-23+TGF-β (green circles); IL-1β+IL-18 vs. IL-33+TSLP (grey circles). Log_2 mRNA expression of signature genes for **(H)** ILC2 and **(I)** ILC3 in presented for ILC2s from the different culture conditions; the dot line indicates the average value of IL-2 condition. One way ANOVA with Bonferroni's multiple comparison test of parametric data was used to calculate between condition statistical differences.

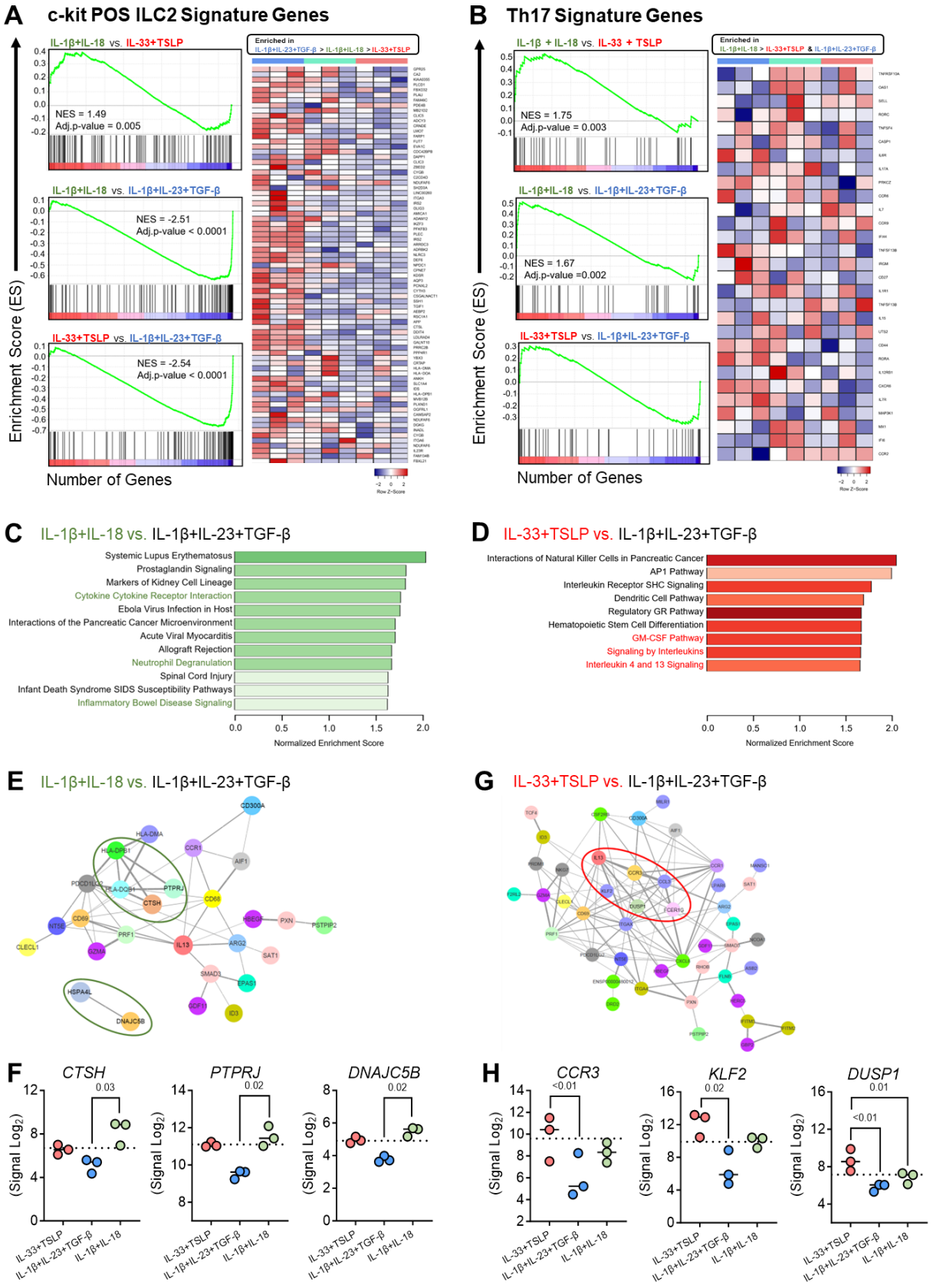

A
c-kit POS ILC2 Signature Genes
IL-1β+IL-18 vs. IL-33+TSLP
NES = 1.49
Adj.p-value = 0.005
IL-1β+IL-18 vs. IL-1β+IL-23+TGF-β
NES = -2.51
Adj.p-value < 0.0001
IL-33+TSLP vs. IL-1β+IL-23+TGF-β
NES = -2.54
Adj.p-value < 0.0001
Enrichment Score (ES)
Number of Genes
Enriched in
IL-1β+IL-23+TGF-β > IL-1β+IL-18 > IL-33+TSLP
B
Th17 Signature Genes
IL-1β + IL-18 vs. IL-33 + TSLP
NES = 1.75
Adj.p-value = 0.003
IL-1β+IL-18 vs. IL-1β+IL-23+TGF-β
NES = 1.67
Adj.p-value =0.002
IL-33+TSLP vs. IL-1β+IL-23+TGF-β
Enrichment Score (ES)
Number of Genes
Enriched in
IL-1β+IL-18 > IL-33+TSLP & IL-1β+IL-23+TGF-β
C
IL-1β+IL-18 vs. IL-1β+IL-23+TGF-β
Systemic Lupus Erythematosus
Prostaglandin Signaling
Markers of Kidney Cell Lineage
Cytokine Cytokine Receptor Interaction
Ebola Virus Infection in Host
Interactions of the Pancreatic Cancer Microenvironment
Acute Viral Myocarditis
Allograft Rejection
Neutrophil Degranulation
Spinal Cord Injury
Infant Death Syndrome SIDS Susceptibility Pathways
Inflammatory Bowel Disease Signaling
Normalized Enrichment Score
D
IL-33+TSLP vs. IL-1β+IL-23+TGF-β
Interactions of Natural Killer Cells in Pancreatic Cancer
AP1 Pathway
Interleukin Receptor SHC Signaling
Dendritic Cell Pathway
Regulatory GR Pathway
Hematopoietic Stem Cell Differentiation
GM-CSF Pathway
Signaling by Interleukins
Interleukin 4 and 13 Signaling
Normalized Enrichment Score
E
IL-1β+IL-18 vs. IL-1β+IL-23+TGF-β
G
IL-33+TSLP vs. IL-1β+IL-23+TGF-β
F
CTSH
PTPRJ
DNAJC5B
(Signal Log2)
IL-33+TSLP
IL-1β+IL-23+TGF-β
IL-1β+IL-18
H
CCR3
KLF2
DUSP1
(Signal Log2)
IL-33+TSLP
IL-1β+IL-23+TGF-β
IL-1β+IL-18

Figure 4.6. Signalling pathway analysis of IL-1β+IL-18 induced intermediate ILC2. Gene set enrichment analysis of ILC2 cultured with IL-1β+IL-18 vs. IL-1β+IL-23+TGF-β, IL-1β+IL-18 vs. IL-33+TSLP and IL-33+TSLP vs.IL-1β+IL-23+TGF-β. Genes were pre-ranked based on log2 fold change and adj p values, enrichment score was calculated based on previously reported gene set upregulated in **(A)** c-kit$^+$ ILC2 (25) and **(B)** Th17 cells (36). Pathway enrichment analysis of genes enriched in IL-1β+IL-18 and IL-33+TSLP treated ILC2 were compared with IL-1β+IL-23+TGF-β treated ILC2. Signalling pathways significantly enriched in IL-1β+IL-18 or IL-33+TSLP treated ILC2 are shown in **(C)** and **(D)**, respectively (adj p-value is indicated by the color intensity). **(E)** The interaction network was generated based on differentially expressed genes enriched in ILC2 cultured with IL-1β+IL-18 compared with IL-1β+IL-23+TGF-β and **(F)** Log_2 mRNA expression of differential expressed genes related to type 17 inflammation. **(G)**. The interaction network was generated based on the differential expressed genes enriched in IL-33+TSLP induced ILC2 compared with IL-1β+IL-23+TGF-β induced ILC2 and **(H)** Log_2 mRNA expression of differential expressed genes connected to IL-13 signalling pathway in IL-33+TSLP induced ILC2.

Supplementary Tables

Table E4.1. Results of one proportion z-test for different cultures groups of ILC in severe asthmatics.

	EOS			NEUT	one proportion z-test p-value
	# of cells	Total # of cells	Proportion out of total # of cells	Proportion out of total # of cells	
1. ILC1	247	3762	0.06565657	0.0737006	5.90E-02
2. ILC2	60		0.01594896	0.0154964	8.22E-01
3. ILC3	215		0.05715045	0.10587974	*2.63E-22
4. IL-17A dim c-kit- ILC2	564		0.14992026	0.12308149	*5.42E-07
5. IL-17A dim c-kit+ ILC2	93		0.02472089	0.04522874	*1.42E-09
6. IL-17A+ c-kit+ ILC2	172		0.04572036	0.07555424	*4.39E-12
7. Quiescent-progenitor cells	1449		0.38516746	0.2072366	*1.12E-159
8. Unconventional c-kit- ILC2	643		0.17091972	0.12508341	*1.92E-17
9. Unconventional c-kit+ ILC2	206		0.05475811	0.12938385	*2.39E-42
10. Unconventional ILC3	113		0.03003721	0.09935493	*7.62E-46

	MIXED			NEUT	one proportion z-test p-value
	# of cells	Total # of cells	Proportion out of total # of cells	Proportion out of total # of cells	
1. ILC1	497	26784	0.018555854	0.0737006	*1.97E-261
2. ILC2	205		0.007653823	0.0154964	*2.71E-25
3. ILC3	1233		0.046034946	0.10587974	*2.36E-222
4. IL-17A dim c-kit- ILC2	2467		0.092107228	0.12308149	*1.03E-53
5. IL-17A low ckit+ ILC2	2220		0.082885305	0.04522874	*2.79E-193
6. IL-17A+ c-kit+ ILC2	9590		0.358049582	0.07555424	*0.00E+00
7. Quiescent-progenitor cells	3799		0.141838411	0.2072366	*1.18E-153
8. Unconventional c-kit- ILC2	761		0.028412485	0.12508341	*0.00E+00
9. Unconventional c-kit+ ILC2	5387		0.201127539	0.12938385	*3.96E-268
10. Unconventional ILC3	625		0.023334827	0.09935493	*0.00E+00

Table E4.2. Expression of lineage-related genes in stimulated ILC2.

Gene Symbol	Description	IL-2 Avg (log2)	IL-33+TSLP Avg (log2)	IL-1β+IL-23+TGF-β Avg (log2)	IL-1β+IL-18 Avg (log2)
CD2	CD2	4.25	4.5	4.67	4.14
CD3G	CD3	4.32	3.97	5.76	4.14
CD4	CD4	4.24	4.83	3.97	4.32
CD8A	CD8a	3.79	3.87	3.5	3.57
CD8B	CD8b	3.27	3.4	3.43	3.37
CD14	CD14	4.21	4.53	4.15	4.2
CD19	CD19	5.93	6.25	6.15	6.55
KLRD1	CD94	3.28	3.04	3.49	3.18
NCAM1	CD56	3.74	3.54	3.5	4
FCER1A	FcER1	3.75	3.74	3.91	4.16
MS4A1	CD20	3.91	4.53	3.95	4.2
FCGR3A	CD16	4.21	4.93	4.78	4.12

Table E4.3. Signalling pathway enrichment analysis on *in vitro* cytokines induced ILC2.

Signalling pathways enriched in IL-1β+IL-18 induced ILC2 vs. IL-1β+IL-23+TGF-β

NAME	SOURCE	NES	FDR q-val	Leading Edge Genes
SYSTEMIC LUPUS ERYTHEMATOSUS	KEGG	2.23	8.32E-05	CD86, IL10, ACTN1, HLA-DQB1, HLA-DPB1, HLA-DMA, HLA-DOA, CD28, HLA-DQA1, HLA-DPA1, HLA-DRB1, HLA-DMB, HLA-DRA, C9, TNF, C1R, FCGR1A, TRIM21, CTSG, FCGR2B, CD80, FCGR2A, HLA-DOB, FCGR2C, GRIN2B, C1QC, ACTN3, C8G
MARKERS OF KIDNEY CELL LINEAGE	WP	2	0.006	NT5E, SMAD1, ALDH1A2, EYA1, SIX1, KIT, AXIN2, KDR, HNF1B, PDGFRB, EPO, BMP4, WNT4, BMP7
PROSTAGLANDIN SIGNALING	WP	2	0.006	CCL3, CXCL8, CXCL10, PTGER2, IL1A, CCR2, CD28, IL6, CASP1, TNF, MMP9, CCL2, IL17A, CXCL1, NLRP3
CYTOKINE CYTOKINE RECEPTOR INTERACTION	KEGG	1.94	0.01	IL22, CCL3, CCR1, CCR8, MET, CXCL8, IL19, CCR3, CXCL10, IL10, ACVR2A, IL1A, IL4, IL13RA1, CSF2, CRLF2, IL3RA, IFNK, CD70, IL7, CCR7, CCR2, IL13, CCR9, IL5, TNFRSF10A, IL26, CCL3L3, TNFRSF17, LTA, KIT, IL6, CCL21, IL3, TNFSF11, CSF2RB, TNFRSF9, TNFRSF10D, CXCL11, KDR, TNF, CLCF1, IL12B, TNFRSF19, IFNA4, CCL14, PDGFRB, IL1R2, OSM, IL23A, TNFRSF1B, TGFBR2, EPO, CXCL14, VEGFC, BMP7, INHBA, CCL16, IFNB1, IL2RA, CCL5, PRLR, ACVR1, CXCR1, CCL2, IL17A, CCL17, LIF, IL1R1, MPL, CCL22, CXCL1, CSF3R, LTB, BMPR1B, CXCL16, IFNA7, INHBC, IFNA8, CXCR2, IL12RB2, PPBP
EBOLA VIRUS INFECTION IN HOST	WP	1.93	0.01	CD300A, TLR4, ACTN1, HLA-DQB1, IL4, ITGB3, BST2, RHOB, HLA-DPB1, HLA-DMA, NPC2, HLA-DOA, HLA-DQA1, HLA-DPA1, HLA-DRB1, HLA-DMB, HLA-DRA, FOLR1, SCIN, ITGA6, RHOC, ITGA4, CAV1, VPS16, C1QBP, ICAM2, IRF3, SOCS3, HAVCR1, FLNB, HLA-DOB, NEDD4
CELL INTERACTIONS OF THE PANCREATIC CANCER MICROENVIRONMENT	WP	1.87	0.02	CXCL10, CD86, CSF2, CCR2, IFITM2, HLA-DRB1, HLA-DRA, NRP1, KDR, CTLA4, CCL5, CD80, CCL2
ACUTE VIRAL MYOCARDITIS	WP	1.87	0.02	AIF1, CCR3, TLR4, IL10, HLA-DMA, CD4, IL6, CASP1, BID, CASP6, ITGB2, TNF, IL12B, TLR3, CAV1, MMP9, LAMA2, CD80, ITGAL, EDN1, ABL2, CASP7, BCL2L1, TICAM1, CCND1, CD40LG, RAC2, SGCA, DAG1, NOD2, CASP9, IL12A, STAT1, PARP1, CYCS
ALLOGRAFT REJECTION	HALLMARK	1.83	0.03	GZMA, CCR1, CD86, IL10, ACVR2A, IL4, PRF1, GBP2, IL7, RIPK2, HLA-DMA, CCR2, HLA-DOA, IL13, CD4, CCND3, CD28, HLA-DQA1, HLA-DMB, HLA-DRA, IL6, PRKCG, HDAC9, BCAT1, ITGB2, TRAT1, ICAM1, TNF, IL12B, STAB1, CDKN2A, PRKCB, TIMP1, CD74, TLR3, THY1, INHBA, MMP9, PTPN6, IL2RA, CCL5, FCGR2B, BCL3, CD80, ITGAL, CCL2, WAS, TAP1, HLA-DOB, IRF4, LIF
NEUTROPHIL DEGRANULATION	REACTOME	1.84	0.03	VNN1, CD300A, CTSH, TMEM63A, FCER1G, CD68, ALDOC, CKAP4, MGST1, VCL, PTPRJ, SELL, BST2, CD33, CEACAM1, NPC2, HPSE, DEFA1, DEFA1B, PRDX4, CEACAM6, GCA, IRAG2, GSTP1, AHSG, ITGAM, PRSS3, ITGAX, ENPP4, CDA, FGL2, STOM, FRMPD3, COTL1, ATP6V0A1, ITGB2, CLEC4D, GM2A, NEU1, DSC1, RAB37, PRCP, CREG1, CPPED1, CD36, JUP, FCAR, MCEMP1, CR1, MME, TNFRSF1B, FTL, IGF2R, S100P, TNFAIP6, TCN1, HP, CRISPLD2, PRTN3, GGH, CTSG, SLC11A1, GPR84, CEACAM8, PNP, MMP9, PTPN6, MS4A3, UNC13D, GUSB, ITGAL, DSP, FCGR2A, PTPRB, ORM1, RAB9B, CXCR1, BPI, PSMB7, LILRB3, GLIPR1, P2RX1, LAMTOR2, SNAP23, CXCL1, SERPINA3, HEXB, CNN2, VAMP8, HRNR, TRPM2, CD14, SERPINB1, CD63, HK3, CXCR2, LTA4H, PTAFR, NCSTN, PRSS2, PPBP, ASAH1, CD59, DOK3, LAMTOR1, DSG1, FOLR3, PKM, PSMA2, HBB, PSMD1, RETN, COMMD9, GNS, PFKL, FUCA1, BST1, RAP2B, QSOX1, SLC27A2, CAMP, RNASE2, TLR2, PSMD3, FGR, PSMB1, TRAPPC1, APEH, CYBB
SPINAL CORD INJURY	WP	1.79	0.04	AIF1, CXCL8, CXCL10, TLR4, GJA1, IL1A, IL4, RHOB, CCR2, IL6, EGR1, SLIT3, ICAM1, TNF, AQP4, RHOC, PTGS2, ACAN, MMP12, GAP43, MMP9, FCGR2A, CCL2, LILRB3, IL1R1, CXCL1, SLIT1, PLA2G5, LTB
SUDDEN INFANT DEATH SYNDROME SIDS SUSCEPTIBILITY PATHWAYS	WP	1.78	0.04	CXCL8, NTRK2, GJA1, IL10, IL1A, TPH1, GATA2, AR, IL13, RYR2, SLC25A4, IL6, IL1RN, EGR1, GABRA1, HDAC9, POU2F2, HES1, MYB, TNF, AQP4, TLX3, CDCA7L
INFLAMMATORY BOWEL DISEASE SIGNALING	WP	1.78	0.04	IL22, TLR4, IL10, RORC, IL1A, IL4, HLA-DMA, IL13, IL5, IL6, TNF, IL23A

Signalling pathways enriched in IL-33+TSLP induced ILC2 vs. IL-1β+IL-23+TGF-β

NAME	SOURCE	NES	FDR q-val	Leading Edge Genes
INTERACTIONS OF NATURAL KILLER CELLS IN PANCREATIC CANCER	WP	2.2	0.0005	FCGR3A, CCL2, FOXO1, NCR3LG1, CSF2, CCL1, IFNG, PRF1, GATA2, CCL3
AP1 PATHWAY	PID	1.86	0.04	CCL2, JUND, EGR1, DMP1, MYB, FOSL2, CDKN1B, CDK1, EP300, BAG1, FOSB, CXCL8, FOS, BCL2L11, DUSP1, IL10, IL6, GATA2, GJA1, IFNG
INTERLEUKIN RECEPTOR SHC SIGNALING	REACTOME	2.17	0.0007	CCL2, MT2A, FOSB, IL5, MYB, EGR1, CSF2, FOS, IFNG, IL4, IL6, DUSP1, IL10, GATA2, CXCL8, GJA1
CYTOKINE PATHWAY	BIOCARTA	1.93	0.01	IL5, IL3, IL13, IL1A, IFNG, IL4, IL6, IL10, CXCL8
INFLAMMATORY RESPONSE	HALLMARK	1.92	0.02	CD48, NOD2, PIK3R5, PDE4B, PTAFR, NFKBIA, CCL22, CD14, SLC31A1, LY6E, TIMP1, LAMP3, PROK2, OSM, LDLR, ICAM1, EMP3, NAMPT, TNFRSF1B, CD55, TNFRSF9, CCL2, GCH1, CMKLR1, RIPK2, CXCL11, KCNA3, ITGB3, BST2, CD69, CD70, MXD1, INHBA, ACVR2A, CCR7, IL1A, IFITM1, ITGB8, CXCL10, SELL, MET, HAS2, HBEGF, IL6, PTGER2, AQP9, NMUR1, IL10, DCBLD2, ABCA1, KCNJ2, CXCL8
DC PATHWAY	BIOCARTA	1.84	0.04	CD33, IL5, TLR7, IL3, IL13, CSF2, IFNG, ITGAX, IL4, IL10
HEMATOPOIETIC STEM CELL DIFFERENTIATION	WP	1.81	0.04	SPI1, PIM1, GYPA, FLI1, CIITA, EPO, NFE2, MX1, FOSB, ITGB3, IL5, MYB, IL3, CSF2, FOS, IL1A, MUC1, IL6, GATA2
GM-CSF PATHWAY	PID	1.81	0.04	STAT5A, PRKACA, MAP2K2, MAPK3, PIM1, LYN, JAK2, KRAS, STAT5B, OSM, CCL2, GAB2, CSF2, SYK, FOS, CSF2RB
SIGNALING BY INTERLEUKINS	REACTOME	1.8	0.05	CXCL1, PSMB5, TALDO1, TCP1, MYD88, PSMA4, PSMB9, JUNB, IL1RAPL1, BOLA2, PSME2, PTPRZ1, VEGFA, CNN2, NOD2, PTAFR, NFKBIA, F13A1, CCL22, JAK2, CCL11, MAPK8, CLCF1, TIMP1, STAT2, IL26, STAT5B, MAP3K8, PTPN13, PSMA6, OSM, PSMB8, IL2RA, MAPK7, IL17C, ICAM1, IL23A, PIK3R2, BATF, IL36B, PTPN20, TNFRSF1B, PSMA1, ITGAM, IL17RE, IRF4, CCL2, RIPK2, CD4, PTGS2, ITGB2, IL1RN, GAB2, IRAK3, PELI1, FOXO1, IL5, IL3RA, IL3, IL13, CSF2, S1PR1, SYK, TEC, IL12RB2, FOS, CCR2, IL1A, IFNG, CXCL10, MUC1, SOCS3, ITGAX, CCL3L3, USP18, CSF2RB, SMAD3, IL7, IL4, IL22, IL1RL1, IL6, FGF2, IL13RA1, STX3, CRLF2, CCR1, CD86, IL10, FCER2, IL19, CXCL8, CCL3
INTERLEUKIN 4 AND INTERLEUKIN 13 SIGNALING	REACTOME	1.8	0.05	F13A1, CCL22, JAK2, CCL11, TIMP1, OSM, ICAM1, IL23A, BATF, TNFRSF1B, ITGAM, IRF4, CCL2, PTGS2, ITGB2, FOXO1, IL13, S1PR1, FOS, IL1A, MUC1, SOCS3, ITGAX, IL4, IL6, FGF2, IL13RA1, IL10, FCER2, CXCL8

Supplementary Figures

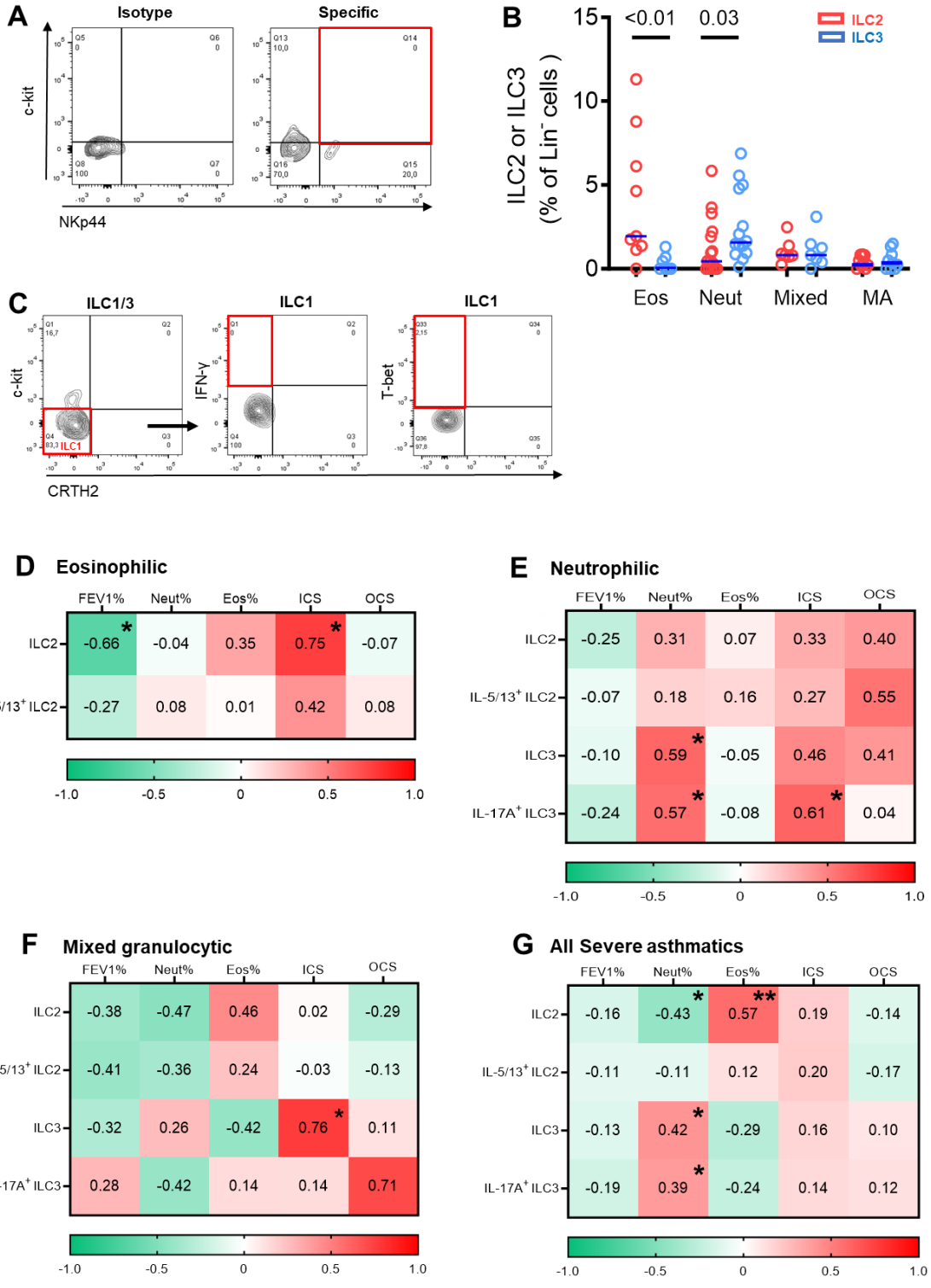


Figure E4.1. (A) Representative flow cytometric analyses of NKp44 expression on ILC3 in the sputum from asthmatic patients. **(B)** Levels of ILC2 and ILC3 cells expressed as % of Lin^- cells. **(C)** Representative flow cytometric analyses of IFN-γ and T-bet in ILC1. Heatmaps show correlation between ILC subsets (% of Lin^- cells) and demographic parameters in SA with **(C)** eosinophilic, **(D)** neutrophilic, **(E)** mixed granulocytic airway inflammation and **(F)** all severe asthmatics. Data were analyzed for statistical significance using a Mann-Whitney test; horizontal bars represent median values of each data set. (*$P<0.05$ and ** $P<0.01$).

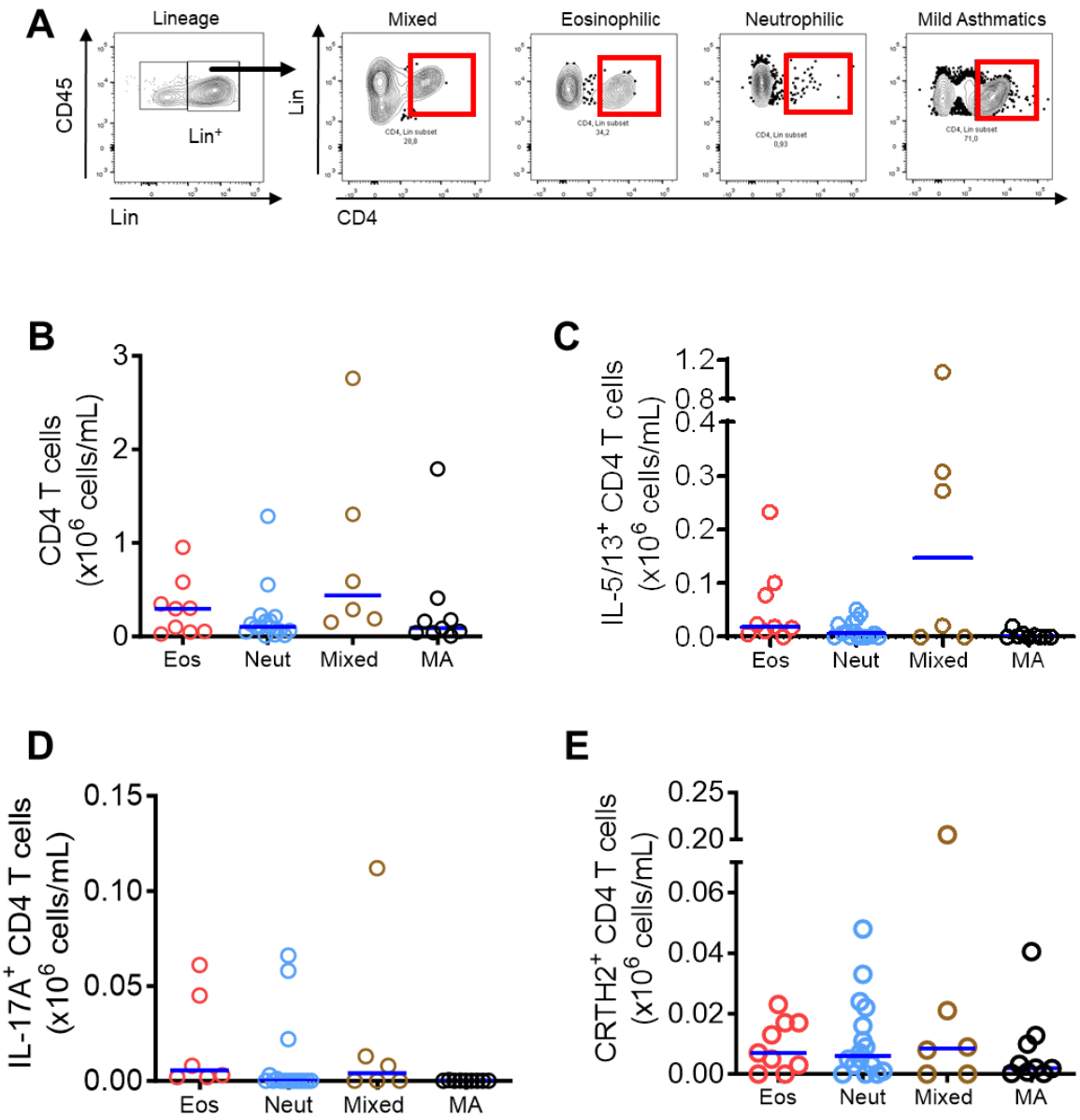


Figure E4.2. (A) Representative flow cytometric gating CD4 T cell in sputum. Frequency of **(B)** total CD4 T cells, and expression of intracellular levels of **(C)** IL-5/13$^+$, **(D)** IL-17A$^+$ and **(E)** CRTH2$^+$ (Th2 cells) expressed as $\times 10^6$ cells/ml. Data were analyzed for statistical significance using a Mann-Whitney U test; horizontal bars represent median values of each data set.

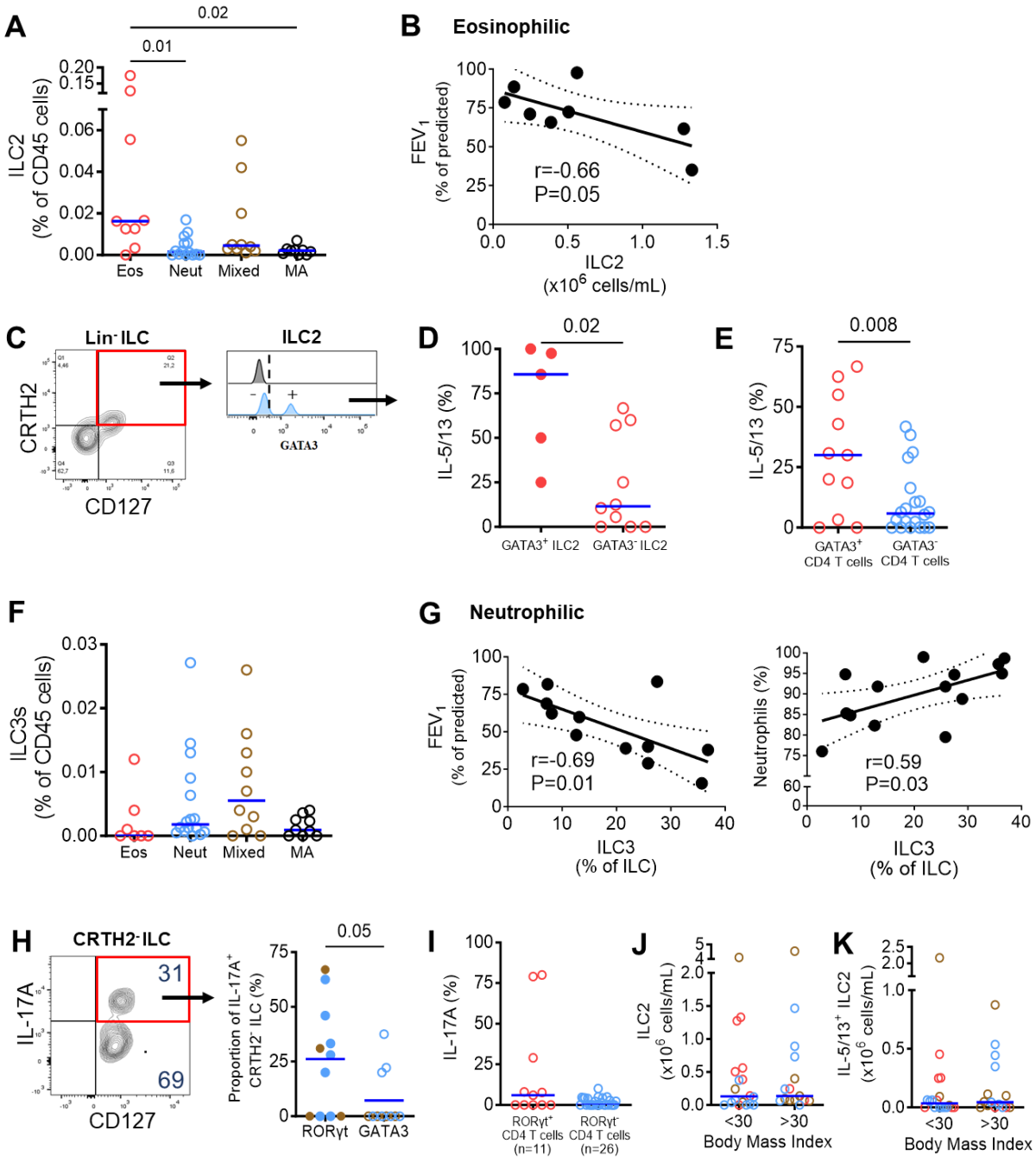

A
0.02
0.01
ILC2 (% of CD45 cells)
Eos
Neut
Mixed
MA
B
Eosinophilic
FEV1 (% of predicted)
r=-0.66
P=0.05
ILC2 (x10^6 cells/mL)
C
Lin- ILC
ILC2
CRTH2
CD127
GATA3
D
0.02
IL-5/13 (%)
GATA3+ ILC2
GATA3- ILC2
E
0.008
IL-5/13 (%)
GATA3+ CD4 T cells
GATA3- CD4 T cells
F
ILC3s (% of CD45 cells)
Eos
Neut
Mixed
MA
G
Neutrophilic
FEV1 (% of predicted)
r=-0.69
P=0.01
ILC3 (% of ILC)
Neutrophils (%)
r=0.59
P=0.03
ILC3 (% of ILC)
H
CRTH2- ILC
IL-17A
31
69
CD127
0.05
Proportion of IL-17A+ CRTH2- ILC (%)
RORγt
GATA3
I
IL-17A (%)
RORγt+ CD4 T cells (n=11)
RORγt- CD4 T cells (n=26)
J
ILC2 (x10^6 cells/mL)
<30
>30
Body Mass Index
K
IL-5/13+ ILC2 (x10^6 cells/mL)
<30
>30
Body Mass Index

Figure E4.3. (**A**) Frequency of ILC2 (% of CD45 cells) in sputum of severe asthma subjects with eosinophilic (red), neutrophilic (blue), mixed granulocytic (brown) airway inflammation and mild asthma (black). **(B)** Correlation between total ILC2 and FEV_1 in severe asthmatics with eosinophilic airway inflammation. **(C)** Representative gating plot of GATA3 expression ILC2 and association with increased in IL-5/13 expression by **(D)** ILC2 and **(E)** $CD4^+$ T cells. (**F**) Frequency of ILC3 (% of CD45 cells) in sputum of severe asthma subjects with eosinophilic (red), neutrophilic (blue), mixed granulocytic (brown) airway inflammation and mild asthma (black). (**G**) Correlations between total ILC3 with FEV_1 and airway neutrophilia in severe asthmatics with neutrophilic airway inflammation. (**H**) Expressions (%) of RORγt and GATA3 in IL-$17A^+$ $CRTH2^-$ ILC (neutrophilic = blue; mixed granulocytic = brown). **(I)** Association between RORγt and IL-17A expression in $CD4^+$ T cells; **(J)** total ILC2 and **(K)** IL-$5/13^+$ ILC2 in severe asthmatics with different airway inflammations subdivided based on obesity (eosinophilic=red; neutrophilic=blue; mixed granulocytic=brown). One way ANOVA with Tukey's multiple comparison test was used to calculate statistical difference for between groups parametric data and Mann-Whitney test for non-parametric intragroup comparisons; Pearson's coefficient (r) was calculated for correlational associations; dash line shows 95% confidence interval. horizontal bars represent median values of each data set.

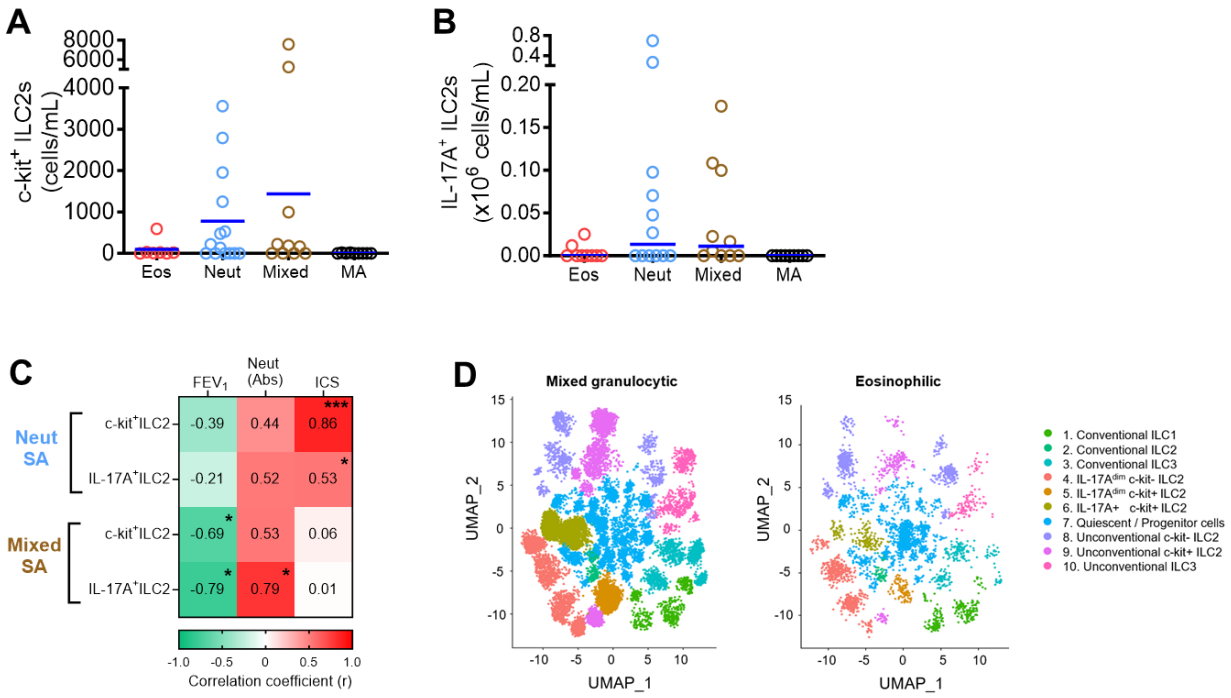

A
c-kit+ ILC2s (cells/mL)
8000
6000
4000
3000
2000
1000
0
Eos
Neut
Mixed
MA
B
IL-17A+ ILC2s (x10^6 cells/mL)
0.8
0.4
0.20
0.15
0.10
0.05
0.00
Eos
Neut
Mixed
MA
C
FEV1
Neut (Abs)
ICS
Neut SA
Mixed SA
c-kit+ILC2
IL-17A+ILC2
-0.39
0.44
0.86
-0.21
0.52
0.53
-0.69
0.53
0.06
-0.79
0.79
0.01
-1.0 -0.5 0 0.5 1.0
Correlation coefficient (r)
D
Mixed granulocytic
Eosinophilic
UMAP_1
UMAP_2
1. Conventional ILC1
2. Conventional ILC2
3. Conventional ILC3
4. IL-17Adim c-kit- ILC2
5. IL-17Adim c-kit+ ILC2
6. IL-17A+ c-kit+ ILC2
7. Quiescent / Progenitor cells
8. Unconventional c-kit- ILC2
9. Unconventional c-kit+ ILC2
10. Unconventional ILC3
E
Mixed granulocytic
F
Eosinophilic
Features
CD127
CRTH2
c-kit
IL-5/13
IL-17A
IFN-γ
GATA3
RORγt
T-bet
1 2 3 4 5 6 7 8 9 10
Identity
Percent Expressed
25
50
75
100
Average Expression
1
0
−1
−2

Figure E4.4. The frequency of **(A)** c-kit$^+$ and **(B)** IL-17A$^+$ ILC2 (cells/mL) in sputum from severe asthmatics (SA). (**C**) Heatmap showing correlation between c-kit$^+$ ILC2 or IL-17A$^+$ ILC2 (cells/mL) with lung function (FEV_1 and FEV_1/FVC), airway neutrophilia, and inhaled corticosteroid level in SA with neutrophilic or mixed granulocytic airway inflammation. **(D)** The UMAP clustering of the pre-gated cells Lin- cells from SA with mixed granulocytic and eosinophilic airway inflammations. Expression of the receptors, transcription factors and cytokines in clusters identified in (D) in SA with **(E)** mixed granulocytic or **(F)** eosinophilic airway inflammation; the size of dot indicated the percentage of cells that express markers and the color intensity represents the average expression level. One way ANOVA with Tukey's multiple comparison test was performed to calculate the statistical difference among groups for parametric data. Pearson's coefficient (r) was calculated for correlational associations.

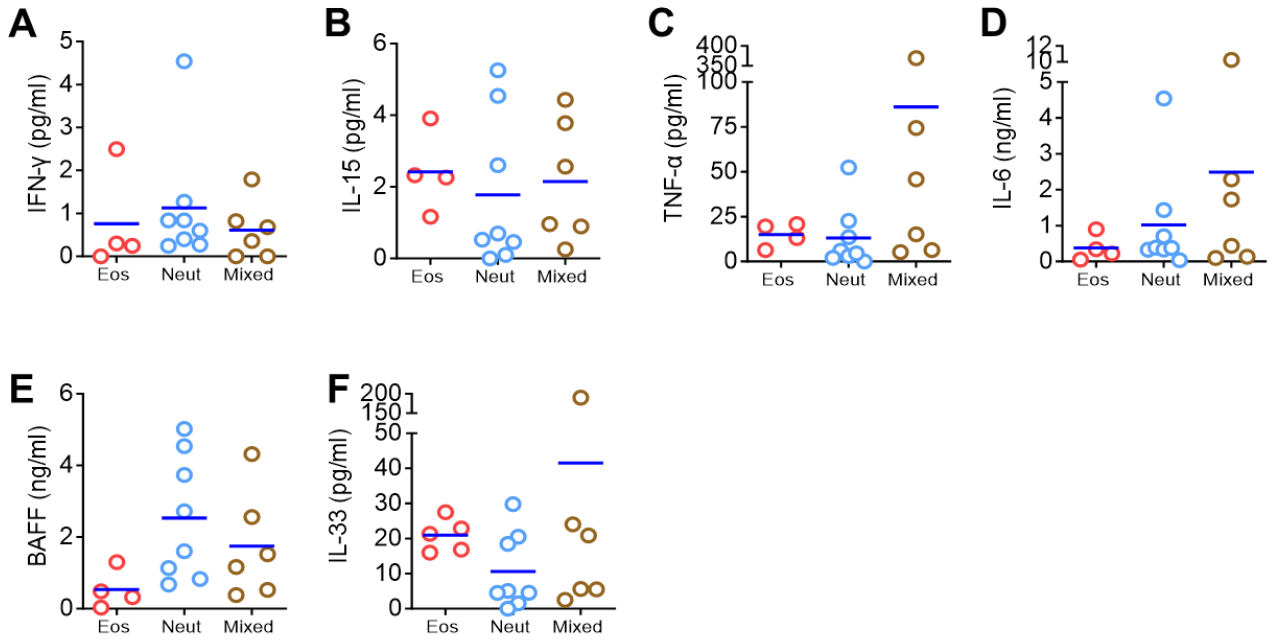


Figure E4.5. Sputum supernatants were assessed by Ella^{TM} multiplex assay for levels of **(A)** IFN-γ, **(B)** IL-15, **(C)** TNF-α, **(D)** IL-6, **(E)** BAFF and **(F)** IL-33 in severe asthmatics with eosinophilic, neutrophilic or mixed granulocytic airway inflammation. Kruskal-Wallis test was performed to calculate between group statistical difference for non-parametric data.

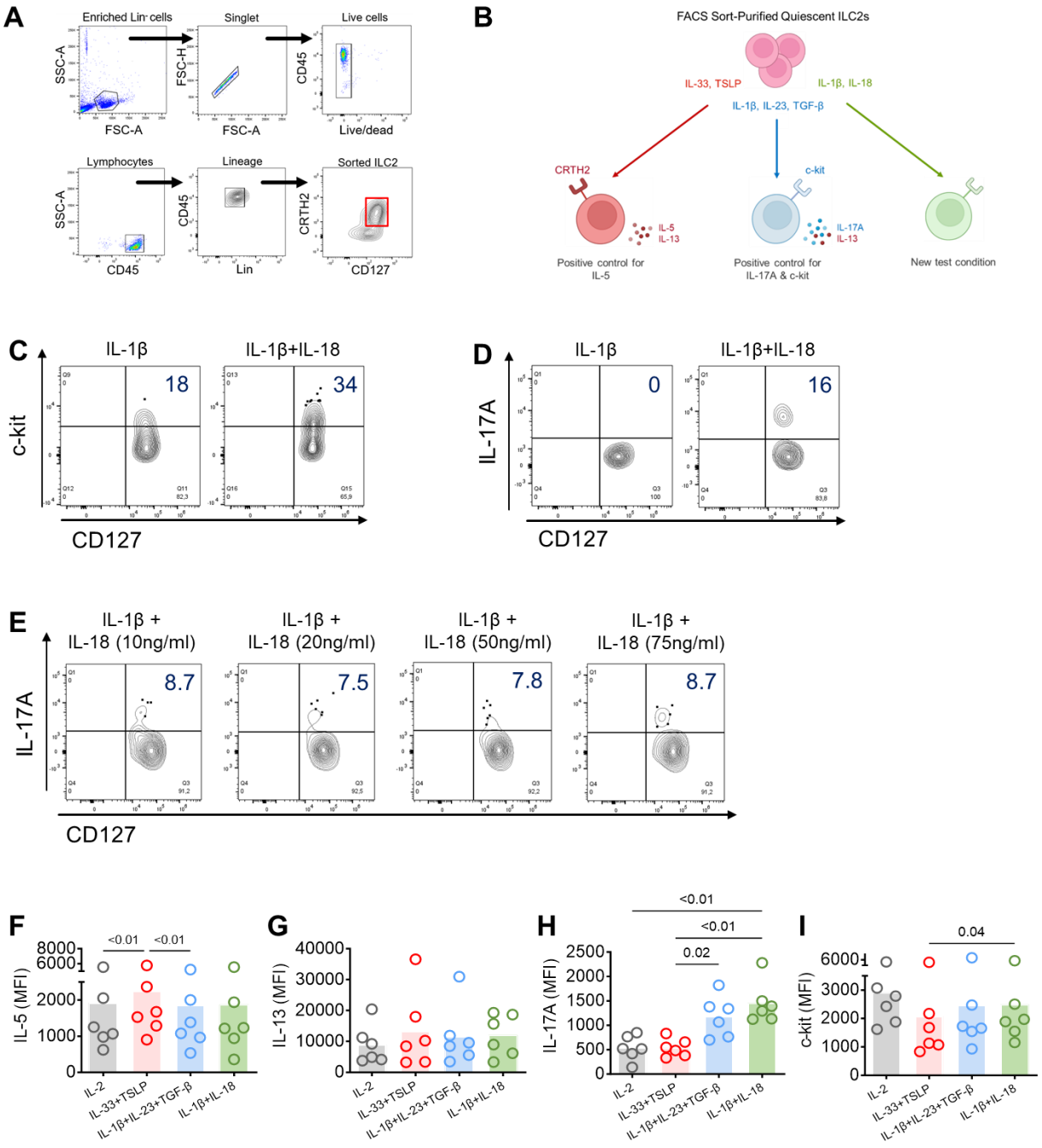


Figure E4.6. (A) Representative plots of flow cytometry-based sorting of ILC2 from blood. **(B)** Schematic summary of the *in vitro* assay. Representative plots of a preliminary trial detecting **(C)** c-kit and **(D)** IL-17A expression by ILC2 cultured with IL-1β or IL-1β+IL-18. **(E)** Representative plots of a titration trial for IL-18 on IL-17A expression in ILC2. The expression of **(F)** IL-5, **(G)** IL-13, **(H)** IL-17A and **(I)** c-kit were quantified as mean florescence intensity by FACS sort-purified ILC2 cultured in various conditions.

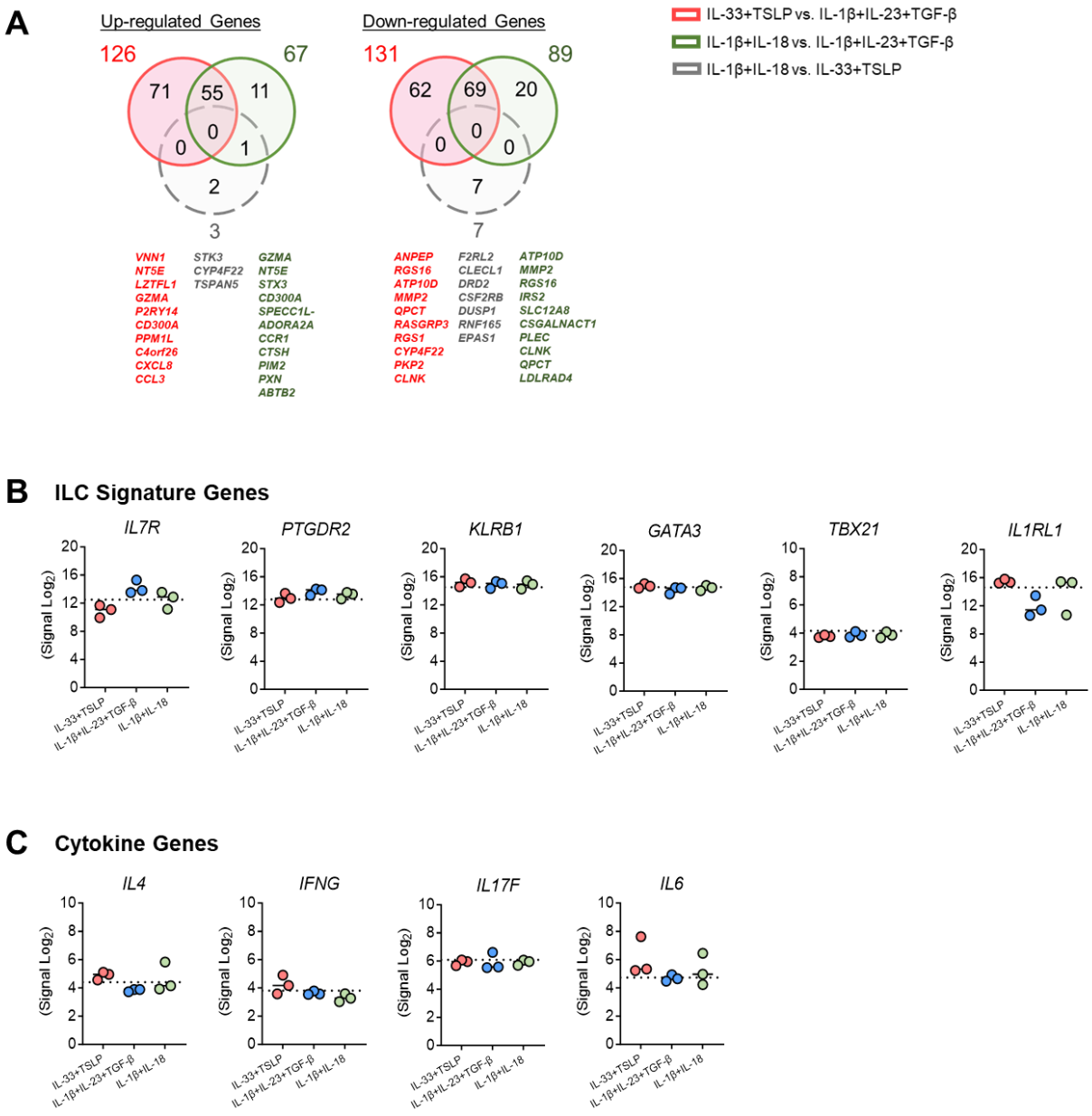
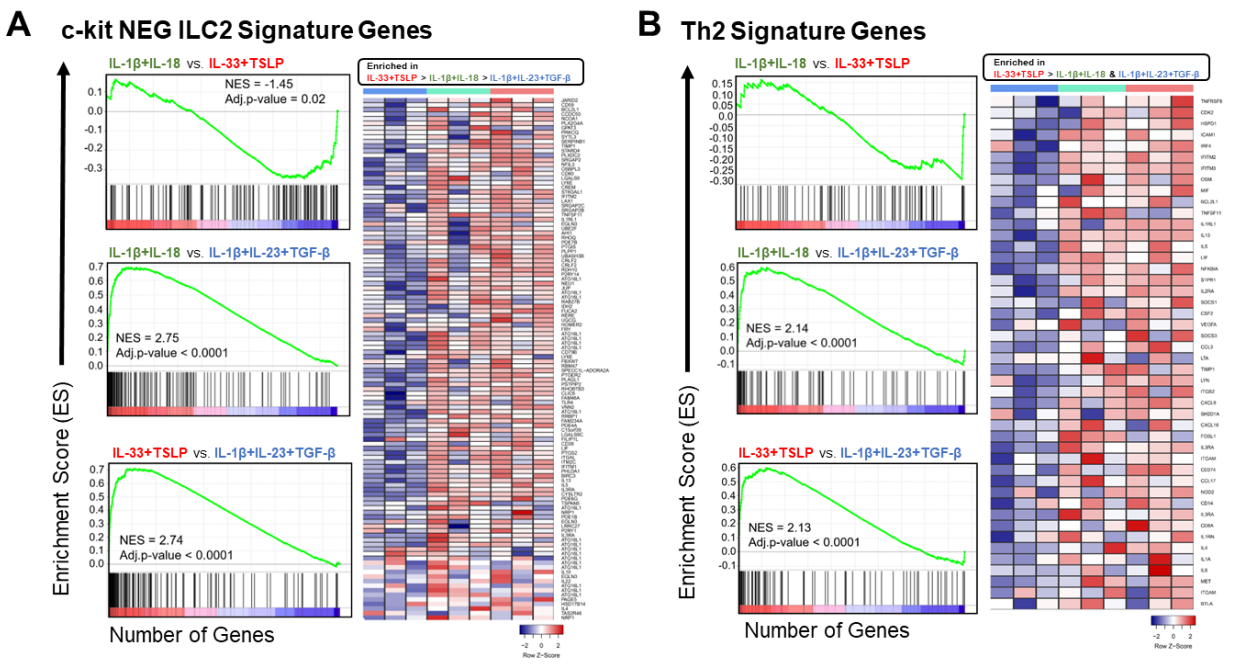


Figure E4.7. (A) Venn diagram summarizes differentially expressed genes (adj P<0.05) with listed top 10 ranked genes based on adj P value. Red circle: IL-33+TSLP treated ILC2 vs. IL-1β+IL-23+TGF-β treated ILC2; green circle: IL-1β+IL-18 treated ILC2 vs. IL-1β+IL-23+TGF-β treated ILC2; grey circle: IL-1β+IL-18 treated LC2s vs. IL-33+TSLP treated ILC2. Log_2 mRNA expression of signature genes of **(B)** ILC and **(C)** cytokines in FACS sorted ILC2 stimulated by IL-33+TSLP, IL-1β+IL-23+TGF-β or IL-1β+IL-18.


A
c-kit NEG ILC2 Signature Genes
IL-1β+IL-18 vs. IL-33+TSLP
NES = -1.45
Adj.p-value = 0.02
IL-1β+IL-18 vs. IL-1β+IL-23+TGF-β
NES = 2.75
Adj.p-value < 0.0001
IL-33+TSLP vs. IL-1β+IL-23+TGF-β
NES = 2.74
Adj.p-value < 0.0001
Enrichment Score (ES)
Number of Genes
Enriched in
IL-33+TSLP > IL-1β+IL-18 > IL-1β+IL-23+TGF-β
Row Z-Score
B
Th2 Signature Genes
IL-1β+IL-18 vs. IL-33+TSLP
IL-1β+IL-18 vs. IL-1β+IL-23+TGF-β
NES = 2.14
Adj.p-value < 0.0001
IL-33+TSLP vs. IL-1β+IL-23+TGF-β
NES = 2.13
Adj.p-value < 0.0001
Enrichment Score (ES)
Number of Genes
Enriched in
IL-33+TSLP > IL-1β+IL-18 & IL-1β+IL-23+TGF-β
Row Z-Score

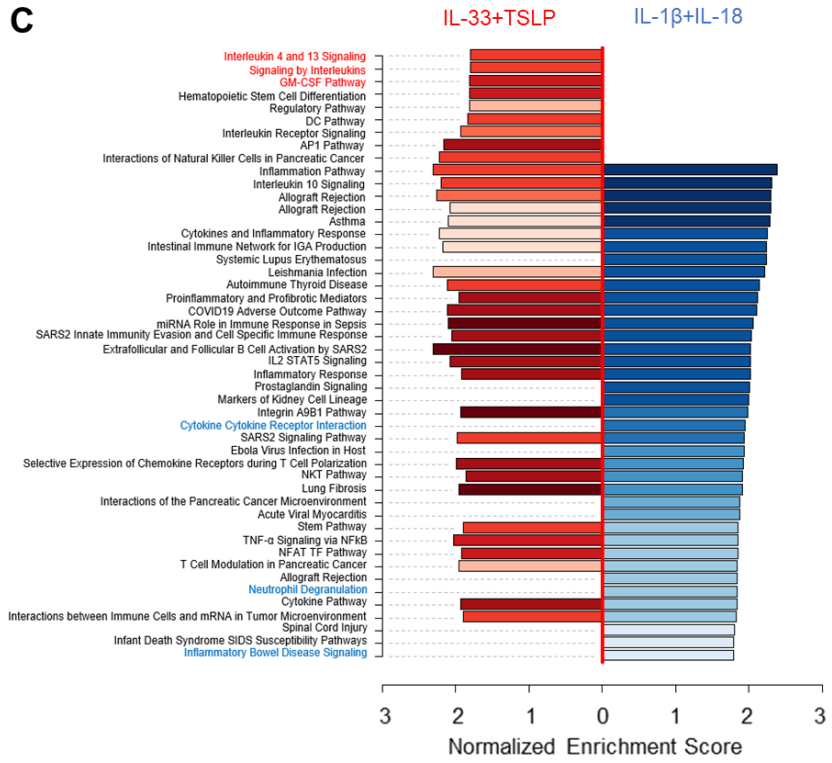

C
IL-33+TSLP
IL-1β+IL-18
Interleukin 4 and 13 Signaling
Signaling by Interleukins
GM-CSF Pathway
Hematopoietic Stem Cell Differentiation
Regulatory Pathway
DC Pathway
Interleukin Receptor Signaling
AP1 Pathway
Interactions of Natural Killer Cells in Pancreatic Cancer
Inflammation Pathway
Interleukin 10 Signaling
Allograft Rejection
Allograft Rejection
Asthma
Cytokines and Inflammatory Response
Intestinal Immune Network for IGA Production
Systemic Lupus Erythematosus
Leishmania Infection
Autoimmune Thyroid Disease
Proinflammatory and Profibrotic Mediators
COVID19 Adverse Outcome Pathway
miRNA Role in Immune Response in Sepsis
SARS2 Innate Immunity Evasion and Cell Specific Immune Response
Extrafollicular and Follicular B Cell Activation by SARS2
IL2 STAT5 Signaling
Inflammatory Response
Prostaglandin Signaling
Markers of Kidney Cell Lineage
Integrin A9B1 Pathway
Cytokine Cytokine Receptor Interaction
SARS2 Signaling Pathway
Ebola Virus Infection in Host
Selective Expression of Chemokine Receptors during T Cell Polarization
NKT Pathway
Lung Fibrosis
Interactions of the Pancreatic Cancer Microenvironment
Acute Viral Myocarditis
Stem Pathway
TNF-α Signaling via NFkB
NFAT TF Pathway
T Cell Modulation in Pancreatic Cancer
Allograft Rejection
Neutrophil Degranulation
Cytokine Pathway
Interactions between Immune Cells and mRNA in Tumor Microenvironment
Spinal Cord Injury
Infant Death Syndrome SIDS Susceptibility Pathways
Inflammatory Bowel Disease Signaling
3 2 1 0 1 2 3
Normalized Enrichment Score

Figure E4.8. Gene set enrichment analysis of IL-1β+IL-18 treated ILC2 vs. IL-1β+IL-23+TGF-β treated ILC2; IL-1β+IL-18 treated ILC2 vs. IL-33+TSLP treated ILC2 and IL-33+TSLP treated ILC2 vs. IL-1β+IL-23+TGF-β treated ILC2. Genes were pre-ranked based on log2 fold change and adj p value and enrichment score was calculated based on gene set previously reported to be upregulated in **(A)** c-kit$^-$ ILC2 (25) and **(B)** Th2 cells (36). Pathway enrichment analysis using the genes enriched in IL-1β+IL-18 treated ILC2 or IL-33+TSLP treated ILC2 compared with IL-1β+IL-23+TGF-β treated ILC2. The signalling pathways enriched in IL-1β+IL-18 treated ILC2 or IL-33+TSLP treated ILC2 were shown in **(C)**.

CHAPTER 5: Thesis Discussion

5.1 Investigating the ILC2 using nasal mucosa and sputum samples in an allergen challenge model gives more clinically relevant results about airway ILC2 in asthma.

ILC2 are rare but active innate immune cells that drive inflammatory responses in local tissues. Here we examined ILC2 activity in the nasal mucosa or sputum from asthmatics. A laboratory-controlled allergen challenge model by intranasal or inhaled mode was used to provide more clinically relevant picture of ILC2 changes in allergic inflammatory responses related to rhinitis and asthma. ILC2 from peripheral blood of asthmatics were previously studied to get an insight of the inflammatory roles of ILC2 in asthma due to the technical limitations on sample collection from airways (83)(85)(87). However, the compartmental difference between ILC2 in blood and airways may potentially affect the accuracy of the conclusion. In this thesis, the nasal mucosa or sputum sample were collected from the airways of asthmatics by using nasal curettage (or deep tissue scraping) or sputum induction which are less invasive methods to sample the airways compared to lung biopsy or bronchial alveolar lavage. We also conducted the nasal or inhalation allergen challenge as a clinical stimulation model to investigate the ILC2 activity during the induced airway inflammation instead of ILC2 at stable state. Detailed flow cytometric analysis was conducted to investigate the frequency of ILC2 in clinical samples and *in vitro* cultures. In addition, we had used transcriptomic microarray to further investigate novel characteristics of ILC2 in this thesis.

5.2 Steroid sensitive ILC2 in nasal mucosa: an important therapeutic target to control the upper airway inflammation.

We firstly identified the corticosteroid sensitive ILC2 in the upper airway of allergic

rhinitis subjects with mild asthma using a nasal allergen challenge model. The nasal mucosa in the upper airway forms the first line of defense against noxious particles in the inhaled air. A previous study observed a significant increase of ILC2 in the nasal mucosa of moderate severe asthmatics with rhinitis at 6h post-nasal allergen challenge (NAC) (77). To our knowledge, it was the first time for our research group to systemically study the ILC2 activity and its responsiveness to inhaled nasal corticosteroid in nasal mucosa post-NAC in allergic rhinitics with mild asthma. Even though the symptoms of mild asthma are well controlled by corticosteroid, the responsiveness of airway ILC2 to corticosteroid had not been studied in mild asthmatics. The corticosteroid insensitive ILC2 were detected in the lower airway of severe asthmatics in previous research (24)(93), but the intrinsic factors mediating the corticosteroid insensitivity of ILC2 remain unclear. In this thesis, we had observed that intranasal corticosteroids directly attenuated the NAC induced ILC2 activities within the nasal mucosa and also reduced the cytokines required for ILC2 survival and proliferation. These results suggested that within the nasal mucosa, ILC2 are rapidly activated in response to allergen exposure in atopic subjects. We found that in our mild asthmatic subjects, ILC2 activation in response to allergen was attenuated by corticosteroid pre-treatment. This is in contrast to findings in more severe asthma patients where high levels of alarmin cytokines within the airways has been proposed to drive ILC2 steroid insensitivity. In these studies, alarmins were shown to change the phenotype of ILC2 and induce the distinct capability for blocking inhibitory effects of steroids in ILC2, including increased expression of CD127 (TSLP receptor)(93) and conversion of CD45RA to CD45RO (87). Investigating targets that mediate ILC2 corticosteroid insensitivity may help to control the symptoms in severe asthmatics.

A novel finding of this thesis was the detection the potential for antigen presenting function of ILC2 from nasal mucosa from mild asthmas subjects with allergic rhinitis (chapter 2). Human

leucocyte antigen-DR (HLA-DR) is an antigen presenting receptor mainly expressed on dendritic cells and macrophages to induce the T cell maturation in lymph nodes. Of note, we detected the HLA-DR expression on ILC2 within the nasal mucosa, which is consistent with previous reports that the interaction between ILC2 and $CD4^+$ T cells through MHC II had significantly enhanced the proliferation and cytokine productions in both cell types in murine models (96)(139). In this thesis, the HLA-DR expression on ILC2 in nasal mucosa was significantly reduced by inhaled corticosteroid treatment. Similarly, we showed that dexamethasone attenuated the up-regulation of HLA-DR expression by ILC2 stimulated with IL-2 or TSLP, *in vitro.* These results suggested a novel function of ILC2 which potentially facilitates type 2 inflammation by inducing the CD4 T cells differentiation in the local tissues, and corticosteroids might attenuate the antigen presenting function of ILC2 to $CD4^+$ T cells, thereby further prevent the disease progression by inhibiting clonal expansion of $CD4^+$ T cells in the upper airways.

As well based on the studies quoted earlier, steroid insensitivity of ILC2 within the airways detected in severe asthma may be driven by higher tissue levels of alarmins (IL-33 and TSLP) compared to moderate severe and mild asthmatics (140). In a previous study from van der Ploeg and Golebski *et al*, stable $CD45RA^+$ ILC2 from human blood were cultured with IL-33+TSLP and this stimulation caused trans-differentiation from $CD45RA^+$ into $CD45RO^+$ ILC2, *in vitro* (87). The genomic profiles between $CD45RA^+$ ILC2 (unstimulated ILC2) and $CD45RO^+$ ILC2 (steroid insensitive ILC2) were compared and an elevated level of genes related to glutathione metabolism and detoxification were detected in $CD45RO^+$ ILC2 which is a mechanism that potentially mediates steroid insensitivity response in $CD45RO^+$ ILC2 (87). To compare with the stimulated blood ILC2 in an *in vitro* assay, ILC2 from airway will contain more pro-inflammatory features related to asthma pathogenesis. Therefore, the corticosteroid responsive ILC2 detected in the upper

airway from this thesis can be applied as a control group to be compared with the steroid insensitive airway ILC2 enriched from severe asthmatics to identify novel molecular factors related to corticosteroid insensitivity in ILC2 at genomic level in a future study, and whether treatment of severe asthmatics with anti-alarmin targeted biologics will reverse steroid sensitivity of ILC2 remains to be investigated.

5.3 NMU-NMUR1 axis: novel mechanism for rapid ILC2 activation in the lower airway of mild asthmatics.

The rapid activation of ILC2 during the early stage of type 2 (T2) airway inflammation was previously observed by our group in which increased levels of total ILC2 and IL-5/13^{+} ILC2 were detected in the airway of mild asthmatics at 7h and 24h post whole lung allergen challenge (81)(82). However, the underlying mechanism of early ILC2 activation within 7h is still unclear. In this thesis, we had described the NMU-NMUR1 axis as a novel mechanism for the rapid activation of ILC2 within 7h post whole lung challenge in mild asthmatics. Specifically, allergen challenge enhanced ILC2 responsiveness to NMU by upregulating its NMUR1 expression within 7h of allergen exposure. The expression of NMUR1 on ILC2 was significantly associated with increased IL-5/13 expression. We further observed an exclusive upregulation of *NMUR1* expression in ILC2 induced by type 2 (T2) stimulatory cytokines (IL-33+TSLP) *ex vivo* at transcriptomic and proteomic levels. In contrast, no increase of *NMUR1* expression was found in non T2 stimulatory cytokine inducing cultures (IL-1β+IL-23+TGF-β) of ILC2. However, an upregulation of nicotine acetylcholine receptor gene *CHRNA6* was observed. These results suggest that different neuropeptide-ILC2 interactions may come into play under the T2 versus non T2 inflammatory conditions and this may lead to different inflammatory profiles within the airways. Also, the

discovery of neuro-immune cell interactions opens up a new frontier of potential therapeutic target development for asthma symptoms driven by both inflammatory and structural cells. As the most widely used anti-neurotransmitter medication for asthma, beta 2 adrenergic agonist (ARDB2) is a bronchodilator that inhibits the binding of epinephrine, a neurotransmitter, to its G-protein coupled receptor on epithelial cells and the inflammatory functions of immune cells including ILC2 have also been shown to be attenuated (121). Also, muscarinic antagonist blocks the binding of acetylcholine to muscarinic receptors to alleviate the bronchoconstriction and cough (16). In addition, dopamine is another neurotransmitter which significantly attenuated the IL-33 induced IL-5/13 expression in ILC2 (141). Clinically, the dopamine level in the serum of asthmatics was significantly lower than health control suggesting dopamine is a potential anti-inflammatory neurotransmitter in response to the type 2 airway inflammation (141). Following the previous studies related to the interaction between neurotransmitters and ILC2, the identification of NMU-ILC2 axis might be helpful to develop new therapy for asthma using neuropeptide as a target.

In line with our *in vivo* findings with sputum ILC2 from the inhaled allergen challenge model, NMU stimulated an early activation and IL-5/13 production by enriched ILC2, *in vitro* which peaked at 6h post-stimulation. On the other hand, a significant increase of IL-5/13 production induced by IL-33 was not observed until 24h. Therefore, we show for the first time using human ILC2 that NMU as a rapid stimulator for ILC2 activation. In addition, the treatment of dexamethasone (Dex) led to a significant reduction of type 2 cytokine expression (IL-5 and IL-13) in NMU stimulated ILC2 *in vitro*. Similarly, the effect of NMU on ILC2 was significantly attenuated when the downstream signalling factor calcineurin (CsA), MEK or NFAT was blocked, whereas the blockade of MyD88 (mainly induced by IL-33) did not interfere the effect of NMU on ILC2. Different signalling pathways underlying the NMU or IL-33 induction potentially

explained the time frame difference between NMU and IL-33 on ILC2 activation, which requires further investigation. These studies would provide targets to modulate the early priming response of ILC2 for enhanced responses to subsequent stimulation by alarmin cytokines thus blocking the onset of allergic symptom in response to allergen exposure. Whether the same neuroimmune stimulation of ILC2 occurs in response to viral or environmental smoke or pollution exposure requires further investigation.

Of note, the attenuative effect imposed by corticosteroid on the expression of NMUR1 on ILC2 was also studied in this thesis. The IL-33 induced NMUR1 expression on ILC2 within 6h was reduced by dexamethasone i*n vitro* which is potentially associated with the observation that the baseline level of NMUR1 on sputum ILC2 from mild asthmatics was higher than ILC2 from corticosteroid dependent severe asthmatics. Thus, we proposed that the long-term corticosteroid administration in severe asthmatics might reduce the NMUR1 expression on ILC2. Furthermore, the mild asthmatic subjects that participated in this allergen challenge study were steroid naïve which means the ILC activity was not previously modulated by corticosteroid. Thus, the NMUR1 might be a potential marker for corticosteroid naïve ILC2. In addition, the different expressions of NMUR1 on ILC2 from mild and severe asthma implied that it is necessary to control the ILC2 before it differentiates into a phenotype which is difficult to be controlled by corticosteroid.

In contrast, we have preliminary data in chapter 3 which show that a higher level of vasoactive intestinal peptide (VIP) was detected in the sputum supernatants from moderate-severe asthmatics compared to mild asthmatics. VIP is a neuropeptide binding to VPAC1/2 and previous studies revealed that the nociceptors in the airway produce VIP in an OVA challenged murine model which enhance ILC2 activities. Furthermore, ILC2 derived IL-5 stimulated the secretion of VIP by nociceptors in the airway in a positive feedforward loop (124). Therefore, our data indicate

that while NMU/NMUR1 axis may play an upstream stimulatory effect that enhances activation of airway ILC2 in steroid naïve or steroid non-dependent atopic asthmatics, ILC2 from severe asthma might be regulated by different neuropeptides. Possible candidates may include VIP on ILC2 although this requires further investigation. An additional neuropeptide that has been reported to stimulate ILC2 function is calcitonin gene related peptide (CGRP) produced by pulmonary neuroendocrine cells found within the bronchial epithelium. The CGRP induced a rapid increase of IL-5 production within 3-6h in a synergetic manner with IL-33 and IL-25 in murine ILC2, and the rapid increase of IL-5 in ILC2 was attenuated by CGRP in 3 days *in vitro* (126)(127)(128). On the other hand, CGRP imposed an inhibitory effect on ILC2 proliferation with its IL-13 expression leading to an attenuated airway eosinophilia in murine models (126)(128). To be consistent with the previous study which detected a higher level of CGRP in the lung of asthmatic patients (125), we identified a trend of higher level of CGRP in the sputum supernatant from moderate severe asthmatics compared to mild asthmatics and the function of CGRP in human asthma requires further investigation.

5.4 ILC2 trans-differentiation: new properties of ILC2 to mediate different airway inflammations in severe asthma.

The increased frequency of ILC2 with IL-5/13 expressions in the airway of mild or severe asthmatics with eosinophilic airway inflammation was observed in this thesis and previous studies (24)(82). However, the pathological role of other ILC subsets in severe asthma with non-eosinophilic airway inflammation is still unclear. Here we show for the first time we systemically investigated ILC subsets in severe asthmatics with different inflammatory profiles. The different endotypes of airway inflammation in severe asthma potentially give rise to ILC with different

phenotypes and functions. Specifically, a higher level of ILC2s with IL-5/13 expression was observed in severe asthmatics with eosinophilic inflammation in which ILC2s correlated inversely with FEV1 implying a role in deteriorating lung functions. On the other hand, ILC3 was exclusively associated with non-T2 airway inflammation in severe asthma with airway neutrophilia as an important source of IL-17A. Thus, the presence of increased numbers of ILC3s expressing IL-17A in severe asthma with neutrophilic airway inflammation suggests a role for ILC3 as a driver of this inflammatory endotype and thus maybe a potential target to modulate in non-T2 severe asthma.

In addition to ILC3, we detected of an intermediate ILC2 phenotype that expresses c-kit and IL-17A (ILC3 like features) while maintaining ILC2-like features (CRTH2, IL-5/13) was described in the airway of severe asthmatics with neutrophilic or mixed granulocytic inflammation. These results suggested that ILC2 was not exclusively involved in T2 airway inflammation but also in non-T2 airway inflammation as an important source of IL-17A. Following the detection of intermediate ILC2 in airway, potential future studies may focus on the intermediate ILC2 in other inflammatory diseases mediated by non T2 inflammation such as chronic obstructive pulmonary disease or nasal polyposis with neutrophilic inflammation and provide a new target to control the development for non T2 inflammation.

We also identified IL-1β+IL-18 as potential inflammatory cytokines mediating non T2 airway inflammation in severe asthma. In severe asthma with eosinophilic inflammation, IL-33, IL-25 and TSLP were well defined as alarmin cytokines that can promote T2 airway inflammation by promoting the generation of T2 cytokines and driving eosinophilia and increased mucus production. Furthermore, the stimulative effects of alarmins on ILC2 for IL-5 and IL-13 production along with corticosteroid insensitivity were also well studied (24)(87). Clinically, the

administration of anti-T2 biologic medications such as mepolizumab, benralizumab and tezepelumab have achieved significant efficacy in severe asthma with attenuating eosinophilic airway inflammation with associated reduction in annualized exacerbation rates (42)(48)(142). In contrast, the pathogenesis of non T2 airway inflammation is more complicated. The canonical cytokines mediating type 17 (T17) response including IL-1β, IL-23 and TGF-β had been discovered in psoriasis and cystic fibrosis with nasal polyps and these T17 inducing cytokines stimulated the trans-differentiation of ILC2 towards an ILC3 featured cell (73)(143). However, the determinant cytokines during the non-T2 airway inflammation in severe asthma is not well defined since no clinical efficacy of anti-IL-17 or anti-IL-23 biologicals were reported and the clinical trial for anti- IL-1β medication is yet to be reported (53).

IL-1β and IL-18 were previously identified as a mediator in T17 and T1 inflammation, respectively (66). Here, it is the first time for us to observe a high level of IL-1β and IL-18 in sputum supernatants from severe asthmatics with neutrophilic or mixed granulocytic airway inflammation and we proposed a new mechanism for ILC2 trans-differentiation induced by IL-1β+IL-18 in the lower airway based on the clinical observations from severe asthmatics which was further confirmed by *in vitro* assays using flow cytometric and genomic analysis. Therefore, the combination of IL-1β+IL-18 stimulated an intermediate ILC2 which expresses signatures for T17 inflammatory response, and these new findings suggested IL-18 may be a novel therapeutic target for asthmatics with non T2 airway inflammation.

5.5 Limitation and future direction.

One of the drawbacks in the nasal allergen challenge (NAC) study is that the rapid activation of ILC2 from baseline to 6h post NAC was not examined. According to the preliminary

trial, mild allergic rhinitics with asthma had few ILC2 in the nasal mucosa prior to NAC (baseline). The 24h post-NAC was chosen based on the report from previous research that the increase in eosinophils was sustained in the nasal mucosa of allergic rhinitics at 24h post NAC (22)(144). However, it is still necessary to detect the level of ILC2 at 6h and 12h post NAC in the upper airway in future study which could provide additional information related to the mechanism of early ILC2 activation. Furthermore, no significant reduction of T2 biomarkers (e.g. eosinophils) was detected at early response (0-6h) post NAC in the INCS treatment arm, possibly due to the lack of intranasal corticosteroid (INCS) effects on the rapidly activated mast cells in the upper airway during the early stage post NAC. The future study will assess the level of mast cells with tryptase as one of the indicators for mast cell activation in the nasal mucosa. Also, the effect of INCS treatment on the allergen induced tryptase in mast cells will be further investigated.

Notably, observations from nasal mucosa and *in vitro* assays in this thesis suggested an attenuative effect imposed by corticosteroid on the HLA-DR expression of ILC2. In future study, the blood ILC2 and Th2 cells will be purified from allergic asthmatics post allergen challenge and co-cultured in the presence of specific antigens or anti-HLA-DR antibodies *in vitro* to further investigate the direct interaction between ILC2 and Th2 cells through HLA-DR signalling pathway *ex vivo*. Furthermore, immunofluorescence (IF) staining is an alternative strategy to quantify ILC2, $CD4^+$ T cells and pro-inflammatory cells (eosinophils and neutrophils) in the nasal biopsies pre- and post-NAC (6h, 12h, 24h) to confirm the induction of inflammatory response in upper airway and the effect of corticosteroid treatment. Also, IF staining will reveal the direct interaction between ILC2 and CD4 T cells such as HLA-DR-T cell receptor, OX40 (tumor necrosis factor super family 4)-OX40L and Inducible T-cell Co-stimulator (ICOS)-ICOSL interactions within the nasal tissues (96)(145). Moreover, the alarmins released by epithelial cells including interleukin

(IL)-25, IL-33 and thymic stromal lymphopoietin (TSLP) will also be IF stained to investigate the type 2 immune response and the effect of steroid treatment on the production of these mediators. Recent studies had applied genomic sequencing to identify novel features of ILC2 or compare the genomic difference between ILC2 in different microenvironments. The identification of corticosteroid sensitive ILC2 in this study led to a future direction in which the genomic features of airway ILC2 from mild asthmatics (steroid sensitive) will be compared with ILC2 from severe asthmatics (steroid resistant) to identify differentially expressed genes which are uniquely associated with the corticosteroid insensitivity in ILC2.

Even though the upregulation of NMUR1 was identified on ILC2 post allergen challenge, a limited level of NMU was detected by ELISA in sputum supernatant in this thesis at any time point. A possible explanation is that NMU was quickly degraded due to its short half life (146) and also the effect of inactivation by peptidases (147). Therefore, immunofluorescence staining using lung biopsies might lead to a more accurate result for NMU level in the airway because the morphology of tissues will be preserved in paraffin. Furthermore, we will monitor the expression of NMUR1 on sputum ILC2 from asthmatics treated with corticosteroid in a clinical study to validate the attenuative effect imposed by corticosteroid on the NMUR1 expression of ILC2s observed in *in vitro* assays. Beyond the NMUR1, a systematic study should be performed to assess the level of other neuropeptide receptors at the genomic level in ILC2 treated with or without corticosteroid to better understand the regulation of neuro-immune interaction on ILC2. In addition, we observed a significantly higher level of vasoactive intestinal peptide (VIP) in the sputum supernatant from severe compared to mild asthma at baseline, suggesting that ILC2 might be regulated by different neuropeptides as the severity of the disease proceeds. In future studies, the VIP receptor (VPAC1/2) on ILC2s should be assessed by flow cytometry using monoclonal

antibodies binding to VPAC1/2. Similarly, change or fluctuation in CGRP receptors should be followed on ILC2 from mild and severe asthmatics to understand the broader role of neuroimmune regulation on ILC2 function. This should also be extended to other agents that activate the airways through an antigen non-specific manner and also ILC2 in various inflammatory endotypes of severe asthma.

The novel mechanism of ILC2 trans-differentiation induced by IL-1β+IL-18 was detected in clinical observation combined with *in vitro* assays in this study. However, the responsiveness of ILC3 to IL-1β+IL-18 is still unclear. The tissue resident ILC3 are identified as CRTH2$^-$ c-kit$^+$ within ILC but the properties of CRTH2$^-$ c-kit$^+$ ILC in blood are dramatically different than the mature ILC3 in the local tissues (71). Also, previous studies showed the blood derived CRTH2$^-$ c-kit$^+$ ILC is not naïve ILC3 (148). Therefore, establishing an *in vitro* model using blood surrogate cells to investigate ILC3s would be a challenge for future study.

We only conducted a baseline cross-sectional study of ILC2 in severe asthma patients whose condition was currently stable. As an analog to allergen challenge, the more clinically relevant results would be observed in comparing severe asthmatics experiencing an asthma exacerbation compared to when stable. The comparison of ILC3 and intermediate ILC2 levels at stable and exacerbation condition in severe asthma would provide better evidence of the potential for ILC2 trans-differentiation *in vivo*. This could also be associated with changes in the airway inflammatory response. For example, the airway levels of intermediate ILC2 in sputum from severe asthma with eosinophilic airway inflammation could be compared to the level of intermediate ILC2 during the asthma exacerbation when the patient developed either a neutrophilic or eosinophilic inflammatory response. Furthermore, with the progress in genomic techniques, less cells are required for single cell RNA sequencing with more simplified protocol for rare cells. It

would be possible to systemically identify and compare each immune cell type based on the unbiased clustering analysis of signature genes in the sputum samples from severe asthmatics when stable and at exacerbation. Potentially, novel ILC subtypes with different features (more than 10,000 markers) will be detected at single cell RNA level compared with flow cytometry which the number of detectable biomarkers is dependent on the fluorochromes capacity of FACS machine (15-20 markers) and the number of cells in a sample. Once the new ILC subtypes are determined, the RNA velocity is a novel analytical tool which can be further applied to determine the transient ILC among different ILC subsets and predict the terminal ILC type of the transient ILC (cell fate trajectories). The initial, transient or terminal state of a ILC subset is determined based on the splicing condition of the ILC signature genes (spliced or un-spliced) and the direction of trans-differentiation in transient ILC2 can be predicted within total ILC population in severe asthma with different inflammatory profiles. This unbiased RNA velocity analysis will help to identify ILC with new pathological relevant phenotypes in severe asthma and also monitor the real time ILC trans-differentiation from its initial to terminal state in airways. In addition, more advanced *in vitro* assay can also be applied in future study. The human airway epithelial cells can be *ex vivo* expanded and differentiated in an air-liquid interface cell culture to establish a human airway model. Macrophages and the bacteria extracts will be added to the air-liquid interface cell culture to mimick the airway during the bacterial infection. Whether macrophages or epithelial cells are a major source of IL-18 could be clarified in this model. The post incubation supernatant will be collected and induce the purified ILC2s with or without anti-IL-18 antibody to further investigate the effect of IL-18 on ILC2s.

In the IL-1β+IL-18 induced ILC2, we have identified an elevated level of genes encoding cathepsin H, protein tyrosine phosphatase receptor J and also heat shock protein family related

genes. However, the specific function of these genes in the respect of the development of non T2 features in ILC2s is still unclear. Thus, the lentiviral vector can be applied to deliver small RNA to silence the genes of interest in ILC2s (149) and investigate the functions of genes elevated in IL-1β+IL-18 induced ILC2.

5.6 Summary

To conclude, we identified the corticosteroid sensitive ILC2 in the upper airway of allergic rhinitics with mild asthma post-nasal allergen challenge with a novel function of HLA-DR expression on ILC2 leading to an enhancement of eosinophilia in the upper airway by potentially inducing $CD4^+$ T cells through antigen presentation. Furthermore, we elucidated the rapid activation of ILC2 via NMU-NMUR1 axis in the lower airway of mild asthmatics within 6h post allergen challenge and the expression of NMUR1 on ILC2 and the NMU mediated ILC2 activities were sensitive to corticosteroid. In severe asthmatics with different endotypes of airway inflammation, ILC2 is insensitive to corticosteroid and can trans-differentiate in response to changing microenvironmental cues in the airways altering the predominant airway inflammatory profiles in severe asthma. Collectively, this thesis investigated the ILC2 activation mediated by different airway micro-environments. The results of thesis suggested that ILC2 is a critical regulator of airway inflammation in asthmatics with different severities, and controlling the inflammatory functions of ILC2 is an important therapeutic strategy for the development of asthma treatment.

CHAPTER 6: Thesis References

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