NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTAMENT OF LIFE SCIENCES

MADALENA CRISTINA BRAVO DE MOREIRA PINTO

BSc in Biology

BRIDGING ATOPIC DERMATITIS AND ASTHMA:

THE ROLE OF MEMORY-LIKE ILC2s IN THE ATOPIC MARCH

MASTER IN MOLECULAR GENETICS AND BIOMEDICINE

NOVA University Lisbon

September, 2023

DEPARTAMENT OF
LIFE SCIENCES

BRIDGING ATOPIC DERMATITIS AND ASTHMA:
THE ROLE OF MEMORY-LIKE ILC2s IN THE ATOPIC MARCH

MADALENA CRISTINA BRAVO DE MOREIRA PINTO

BSc in Biology

Adviser: Itziar Martinez Gonzalez
Assistant Professor, Karolinska Institutet

Co-advisers: Pedro Viana Baptista
Full Professor, NOVA University Lisbon

Examination Committee:

Chair: Doctor Ana Rita Fialho Grosso,
Assistant Professor, FCT-NOVA

Rapporteurs: Doctor Paula Alexandra Quintela Videira,
Associate Professor, FCT-NOVA

Adviser: Doctor Pedro Miguel Ribeiro Viana Baptista,
Full Professor, FCT-NOVA

MASTER IN MOLECULAR GENETICS AND BIOMEDICINE

NOVA University Lisbon
September, 2023

Bridging Atopic Dermatitis and Asthma: The Role of Memory-Like ILC2s in the Atopic March

This document was created with Microsoft Word text processor and the NOVAthesis Word template [1].

Para a minha família, a minha fonte interminável de amor e apoio.

ACKNOWLEDGMENTS

I would like to express my heartfelt gratitude to my supervisor, Professor Doctor Itziar Martinez Gonzalez from Karolinska Institutet, for welcoming me into the 'Immunological Memory of Innate Lymphoid Cells' research group. Her mentorship, constant willingness to help and teach, and her nurturing of team spirit have been invaluable. Her kindness and welcoming nature made me feel comfortable from the very first moment, fostering both team building and a sense of belonging.

I am equally grateful to my co-supervisor, Post-Doctoral Researcher Laura Mathae, for her patience, trust, and immense knowledge. She has been a guiding light, teaching me, helping me become autonomous and ensuring that my thesis was progressing according to plan. Her constructive comments and meticulous attention to detail during the writing process have been indispensable for the completion of this work.

Additionally, I wish to extend my appreciation to my co-supervisor Professor Doctor Pedro Viana Baptista from NOVA School of Science and Technologies. His commitment to the progression of my thesis, assistance with bureaucratic and document-related tasks, and insightful feedback during the writing of the thesis have been immensely beneficial.

A special acknowledgment goes to Professor Doctor Alexandra Fernandes, the Master Coordinator, for her continuous support and guidance, ensuring that the students were well-informed about their responsibilities, deadlines, and ensuring a smooth progression of our thesis.

I am indebted to Karolinska Institutet Biomedicum and the Department of Microbiology, Tumor, and Cell Biology for providing me with the necessary facilities and a conducive environment to conduct my research. I also extend my gratitude to NOVA School of Science and Technology and the Department of Life Sciences for equipping me with the foundational knowledge required for this master’s thesis.

My appreciation also goes to the International Relations Division from NOVA FCT for their unwavering support, patience, and assistance throughout my year abroad, and to the Erasmus+ Scholarship for making my studies abroad possible.

To my lab mates in Stockholm, Claudia, Etienne, and Marta, thank you for making every moment in the lab memorable. Your companionship during coffee breaks, light-hearted gossip in the cell culture, and friendship during bench work have enriched my experience so much. I miss you deeply.

I am also grateful for the friendships forged at residence Fogdevreten, which turned potential moments of stress into memorable moments of laughter and happiness. I will always remember our international dinners, movie nights, hikes, barbecues, and parties with love. My friends from FCT in Portugal, who shared my worries and offered their support, also deserve my heartfelt thanks.

A special mention goes to my dear friend Madalena Branco, whose shared experiences abroad and uplifting phone calls were a source of comfort and guidance.

Most importantly, my deepest thanks go to my family: my parents and my sister, who have been my greatest source of love and support. Without you, I would not be where I am today. I am also thankful to my grandparents for their enduring support and genuine happiness for my accomplishments.

This journey has been made possible by the combined effort and support of each individual mentioned, and for that, I am eternally grateful.

"Strive not to be a success, but rather to be of value." – Albert Einstein.

ABSTRACT

Atopic dermatitis (AD) and asthma are increasingly prevalent atopic diseases characterized by a dysregulated type 2 immune response. It has been observed that children initially diagnosed with AD often develop asthma in subsequent years, a progression termed 'Atopic March' (AM). Central to this type 2 immune dysregulation are the Group 2 Innate Lymphoid Cells (ILC2s). These cells rapidly react to allergens and environmental insults at barrier surfaces and subsequently release type 2 cytokines. This response was associated with enhanced type 2 inflammation in asthma and AD mouse models. Recently, using mouse models of allergic lung inflammation studies revealed that ILC2s can acquire immunological memory. In this research, we aimed to investigate the role of memory ILC2s in connecting different type 2 immune allergic diseases including AD and asthma. We hypothesized that memory ILC2s generated in the skin might play a pivotal role in the onset of asthma later in life. For this, we employed mouse models simulating the conditions of the Atopic March. Specifically, we induced an AD-like phenotype through MC903 skin ILC2 sensitization, followed by lung ILC2 stimulation to model asthma inflammation. Our results underscore the potential influence of ILC2s in the AM, indicating a possible link between MC903 skin treatment and subsequent lung ILC2 activation. Specifically, using a T and B cell-deficient mouse model, and AD-like phenotype potentially pointed to an enhanced ILC2 response. Notably, MC903 treated mice exhibited a significant difference in ST2+ lung ILC2 populations. However, the exact mechanisms for the observed heightened ILC2 response remained unclear. Furthermore, a 'memory' state was not detected with this model. This study provides an initial step towards an atopic march mouse model, potentially leading to novel therapeutic interventions for allergic diseases through continued research.

Keywords: Atopic March, Atopic Dermatitis, Allergic Asthma, Group 2 Innate Lymphoid Cells, Immunological Memory

RESUMO

A dermatite atópica (DA) e a asma são doenças com uma prevalência crescente, caracterizadas por uma desregulação da resposta imunitária do tipo 2. Foi observado que crianças inicialmente diagnosticadas com DA frequentemente desenvolvem asma em anos subsequentes, uma progressão intitulada 'Marcha Atópica' (MA). Centrais nesta desregulação imunitária do tipo 2 são as Células Linfoides Inatas Grupo 2 (ILC2s). Estas células respondem rapidamente a alergénios e insultos ambientais em barreiras epiteliais e, subsequentemente, libertam citocinas do tipo 2. Esta resposta foi associada a uma intensificação de inflamação do tipo 2 em modelos de asma e DA em murganhos. Recentemente, com a utilização de modelos de inflamação alérgica pulmonar em murganhos, estudos revelaram que ILC2s podem adquirir memória imunológica. Neste estudo, procurámos investigar o papel destas ILC2s-memória na conexão entre diferentes doenças alérgicas imunitárias tipo 2, incluindo DA e asma. Hipotetizámos que ILC2s-memória geradas na pele poderiam desempenhar um papel crucial no aparecimento de asma subsequentemente. Com esse objetivo, utilizámos modelos de murganho que simulam as condições da Marcha Atópica. Especificamente, induzimos um fenótipo semelhante à DA através da sensibilização de ILC2s da pele com MC903, seguido de estimulação de ILC2s pulmonares para simular a inflamação da asma. Os nossos resultados destacam a potencial influência das ILC2s na MA, indicando uma possível conexão entre o tratamento de pele com MC903 e a subsequente ativação de ILC2 pulmonar. Usando um modelo de murganho deficiente em células T e B, o fenótipo semelhante ao DA apontou para um potencial aumento da resposta por ILC2s. Notavelmente, murganhos tratados com MC903 exibiram uma diferença significativa nas populações de ILC2 pulmonares ST2+. No entanto, os mecanismos exatos para a resposta observada permaneceram incertos. Ademais, um estado de 'memória' não foi detetado com este modelo. Este estudo fornece um passo inicial na obtenção de um modelo de MA em murganhos, que poderá levar a intervenções terapêuticas inovadoras para doenças alérgicas.

Palavras-Chave: Marcha Atópica, Dermatite Atópica, Asma Alérgica, Células Linfoides Inatas Grupo 2, Memória Imunológica

CONTENTS

LIST OF FIGURES

ACRONYMS

AD	Atopic Dermatitis
AM	Atopic March
APC	Antigen-Presenting Cell
AREG	Growth Factor Amphiregulin
BALF	Bronchoalveolar Lavage Fluid
BCR	B Cell Antigen Receptors
BM	Bone Marrow
CLPs	Common Lymphoid Progenitors
CMPs	Common Myeloid Progenitors
DAMPs	Damage-Associated Molecular Patterns
DCs	Dendritic Cells
dLNs	Draining Lymph Nodes
dsRNA	Double-Stranded RNA
GATA3	GATA Binding Protein 3
HDM	House Dust Mites
HSC	Hematopoietic Stem Cell
HSCT	Hematopoietic Stem Cell Transplantation
i.n.	Intranasal
i.p.	Intraperitoneal
IFNγ	Interferon-Gamma
IgE	Immunoglobulin E
Ig	Immunoglobulin

iILC2s	Induced Inflammatory ILC2s
IL	Interleukin
IL-1	Interleukin 1
IL-12	Interleukin 12
IL-13	Interleukin 13
IL-17	Interleukin 17
IL-17RA	IL-17 Receptor A
IL-17RB	IL-17 Receptor B
IL-18	Interleukin 18
IL-1RAcP	IL-1 Receptor Accessory Protein
IL-22	Interleukin 22
IL-25	Interleukin 25
IL-25R	IL-25 receptor
IL-33	Interleukin 33
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-9	Interleukin 9
ILCP	Common ILC Precursor
ILC	Innate Lymphoid Cell
ILC1	Innate Lymphoid Cell Type 1
ILC2	Innate Lymphoid Cell Type 2
ILC3	Innate Lymphoid Cell Type 3
iTreg	Induced Regulatory T cell
JAK	Janus Kinase
LPS	Lipopolysaccharides
LTA	Lipoteichoic acid
LTi	Lymphoid Tissue Inducer
MAPK	Mitogen-Activated Protein Kinase
MHC	Major Histocompatibility Complex
MHC-I	Major Histocompatibility Complex Class I
MHC-II	Major Histocompatibility Complex Class II

mLN	Mesenteric Lymph Node
NF	Nuclear Factor
NK	Natural Killer
OVA	Ovalbumin
PAMPs	Pathogen-Associated Molecular Patterns
PB	Peripheral Blood
PRRs	Pathogen Recognition Receptors
Rag	Recombination Activating Gene
RORα	Retinoic Acid Receptor-Related Orphan Receptor α
RT	Room Temperature
S1P	Sphingosine-1-Phosphate
SCID	Severe Combined Immunodeficiency
ST2	Interleukin 1 Receptor-Like 1 Receptor
Tc1	Cytotoxic T Cell Type 1
Tc17	Cytotoxic T Cell Type 17
Tc2	Cytotoxic T Cell Type 2
TCR	T Cell Antigen Receptors
Th1	T Helper Type 1
Th17	T Helper Type 17
Th2	T Helper Type 2
TNFα	Tumor Necrosis Factor-Alpha
Tregs	Regulatory T Cells
TSLP	Thymic Stromal Lymphopoietin
WT	Wild-Type

1 | Introduction

1.1. The Immune System

1.1.1. Background

Throughout life humans are constantly exposed to pathogens and toxic or allergic substances that may threat host viability. Thereby, it is essential that the host bears an adequate defense, capable of preventing or eliminating potential threats, while maintaining normal tissue and organ function. In fact, the human body is equipped with diverse specialized immune cells, effector molecules and biochemical processes that work together to provide layers of protection in a complex system known as the immune system.

Immune cells and other blood cellular components are produced during the embryonic development and throughout adulthood through a process termed hematopoiesis **(Fig. 1.1)**.[1] During hematopoiesis in adults, the blood and immune cells are originated from a common precursor that resides in the bone marrow (BM), the hematopoietic stem cell (HSC). HSCs are multipotent stem cells that can self-replicate and differentiate into progenitors that gradually lose certain lineage differentiation potential, including the common myeloid progenitors (CMPs) and the common lymphoid progenitors (CLPs), which separate the myeloid and lymphoid lineages, respectively.[2] The myeloid lineage includes granulocytes (neutrophils, eosinophils, basophils, and mast cells), monocytes, macrophages, and dendritic cells (DCs). Moreover, the lymphoid lineage consists of T and B lymphocytes, natural killer (NK) cells, innate lymphoid cells (ILCs) and lymphoid tissue inducer (LTi) cells.[3,4]

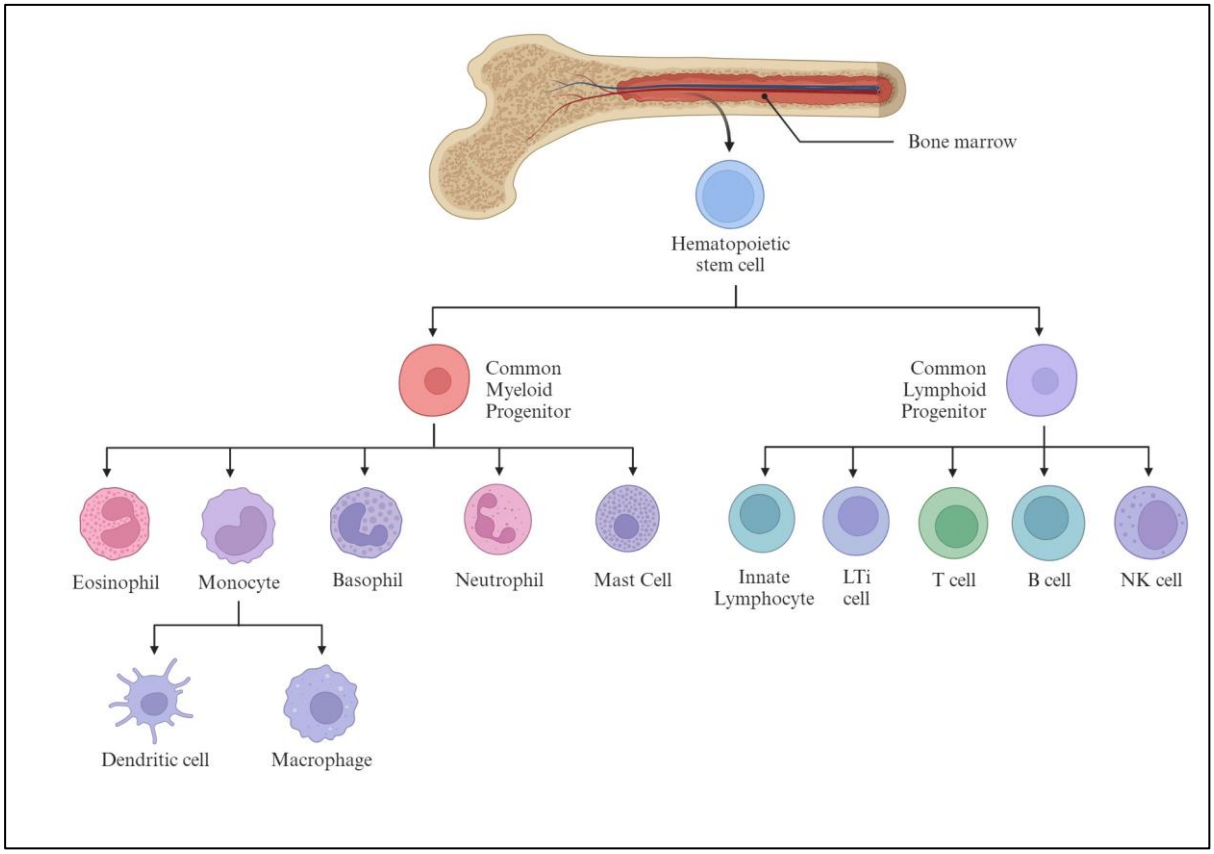

Fig. 1.1 – *Hematopoietic Differentiation Scheme*. Hematopoietic stem cells are multipotent stem cells that differentiate into progenitors with reduced differentiation potential, including the common myeloid progenitors (CMPs) and the common lymphoid progenitors (CLPs), which separate the myeloid and lymphoid lineages, respectively. CMPs give rise to eosinophils, monocytes, basophils, neutrophils, and mast cells. CLPs generate innate lymphocytes, lymphoid tissue inducer (LTi) cells, T cells, B cells, and NK cells. Created with BioRender.com.

Both myeloid cells and lymphoid cells, along with other biochemical processes and molecules, have specialized roles and function together to defend the host against foreign entities like microbes, viruses, cancer cells, and toxins. To function effectively, the immune system must differentiate between the body's own cells and foreign entities, as well as distinguish between non-harmful and harmful invaders. This ability to accurately identify and respond to potential threats is crucial for the body to neutralize harmful substances or organisms without causing harm to its own tissues. To accomplish this, the immune system is structured into lines of defense with complementary functions: the innate immune system offers an immediate, non-specific response, while the adaptive immune system provides a delayed, highly specific, and enduring response.[5,6] However, while the immune response is crucial for defending against invading pathogens, excessive and unregulated inflammatory reactions can themselves become a pathogenic.

1.1.1.1. The Innate Immune System

The innate immune system serves as the body's initial line of defense, and it is accountable for pre-programmed responses dictated by genes in the host's genetic lineage. It uses both mechanisms that prevent an infection (constitutive) and mechanisms that act after an infection (inducible) to maintain homeostasis.[7]

Constitutive innate mechanisms consist of nonhematopoietic components that prevent the entrance or restrict the growth of pathogens.[7] It includes physical barriers that protect outer surfaces, such as the skin; and mucous membranes that protect internal surfaces, such as the respiratory, gastrointestinal, and genitourinary tracts. Additionally, it encompasses molecular processes that regulate the infection or replication of microorganisms such as antimicrobial peptides, antibacterial enzymes, chemical substances that regulate pH, or mucous.[7,8] Constitutive mechanisms provide a level of protection that can combat infections promptly, potentially reducing the need for inducible immune responses and the downstream inflammatory response. Nevertheless, this mechanism does not have the capability to enhance the response and it utilizes energy to stay functional. Therefore, the host's defense mechanisms are primarily inducible when there is an infection, and thus requiring the identification of the pathogen for activation.[9]

Inducible mechanisms consist of hematopoietic components – myeloid and lymphoid cells – that identify shared, conserved molecular patterns found in a broad range of microbial pathogens, as well as the metabolic consequences resulting from inflammation, leading to a fast inflammatory response.[8] Myeloid cells include granulocytes, monocytes, macrophages, and DCs. Moreover, NK cells, ILCs and LTi cells are categorized as innate lymphoid cells.[3,4] Also involved in the inducible response is the complement system, an array of plasma proteins that collaborate as a cascade and promote lysis of infectious organisms, generation of proinflammatory mediators, opsonization of pathogens surface and immune clearance.[5,10]

Cells from the innate immune system do not possess antigen-specific receptors, yet they can be activated during an inflammatory reaction on account of pathogen recognition receptors (PRRs). PRRs are a class of germ-line encoded receptors that recognize highly conserved features shared by multiple pathogens known as pathogen-associated molecular patterns (PAMPs). Examples of PAMPs include bacterial cell wall components such as lipopolysaccharides (LPS) of Gram-negative bacteria and lipoteichoic acid (LTA) of Gram-positive bacteria, β-glucans found in fungal cell walls and double-stranded RNA (dsRNA) produced during viral infection.[5] Furthermore, PPRs recognize common biological consequences of infection and inflammation derived from host cells in the form of damage-associated molecular patterns (DAMPs). DAMPs are endogenous molecules that are upregulated and released upon cell lysis and tissue damage such as endogenous alarmins, heat-shock proteins and uric

acid.[4,8] Once PRRs are activated, they initiate intracellular biochemical cascades that lead to cellular activation.[8] This promotes phagocytosis and the production and secretion of cytokines and chemokines, inducing inflammation and other antiviral responses. NK cells have a cytolytic function and are able to identify and directly kill infected target cells.[11]

The recognition molecules recognized by the innate immune system are typically found across a wide range of pathogens, leading to a fast response after encounter with the pathogen and thus constituting the initial host response. In its role as the initial defense barrier, the innate immune system recognizes, signals, and fights potential invading infectious organisms in an antigen nonspecific way. In response, these signals play a role in triggering the activation of the adaptive immune system which will provide a more robust and specific response in an antigen-specific manner.

1.1.1.2. The Adaptive Immune System

The adaptive immune system is accountable for extremely specific responses to its target antigens. This specificity is due to the presence of antigen-binding proteins expressed on the surface of adaptive immune cells. Antigen-specific receptors are formed through the somatic recombination of gene segments derived from the host's germline, generating a large antigen-binding diversity. Therefore, the adaptive response has the ability to differentiate between 'self' and 'non-self' antigens, leading to the creation of immunologic effector pathways that are specific to the pathogen.[6]

The primary effector cells of adaptive immune responses are the lymphocytes. There are two major lymphocyte lineages: B cells and T cells.[12] These cells develop from HSCs originated in the BM and undergo maturation in the BM or in the thymus, respectively, where assembly of B cell antigen receptors (BCR), including membrane-bound immunoglobulins (Igs), and T cell antigen receptors (TCR) occurs. BCRs directly recognize intact antigens, including proteins, carbohydrates, and lipids.[13] Upon antigen-receptor recognition, B cells undergo a process of activation, proliferation, and differentiation into plasma cells, which are responsible for generating substantial quantities of antibodies to bind and neutralize antigens. TCRs recognize peptide fragments of antigen bound to the major histocompatibility complex (MHC) class I (MHC-I) or class II (MHC-II) molecules present on the surface of other cells. MHC-I is expressed on all mammalian cells and displays peptides derived from intracellular pathogen to T cells that express the co-receptor CD8.[14] MHC-II is expressed on antigen-presenting cells (APCs) including DCs, macrophages, and B cells and displays peptides from extra-cellular sources to T cells that express the co-receptor CD4.[14] Activated T cells undergo clonal expansion generating effector T cells: CD8+ cytotoxic T cells, that directly kill infected or cancerous cells, and CD4+ helper T cells, that stimulate the response of other immune cells.[15] CD4+ T cells can

be further classified into several subsets, including T helper type 1 (Th1), 2 (Th2), 17 (Th17), and regulatory T cells (Tregs), based on the different cytokines they produce and functions they serve within the immune system. Similarly, CD8+ T cells can be classified into cytotoxic T cell type 1 (Tc1), 2 (Tc2) and 17 (Tc17).[16]

A characteristic aspect of the adaptive response is its ability to generate enduring protection by developing immunological memory. Activated immune cells from the adaptive system have the capacity to remember their initial encounter with a particular pathogen and can launch a more robust and faster secondary response, quickly eradicating the threat upon subsequent exposures.[17]

1.1.2. Types of Immunity

The human body is threatened by different pathogens every day. The diversity of threats is faced with a diversity of responses by the immune system, where different types of immune cells and molecules may be activated depending on the nature of the threat to maximize the protective effect against the variety of hazards. Thus, the human immune system is divided into different types of innate and adaptive cell-mediated immunity, comprising type 1, type 2, and type 3 immunity **(Fig. 1.2)**.[16] These classifications are based on the type of immune cells and molecules that are activated in response to a particular pathogen or allergen, and the functions they serve in the immune response.

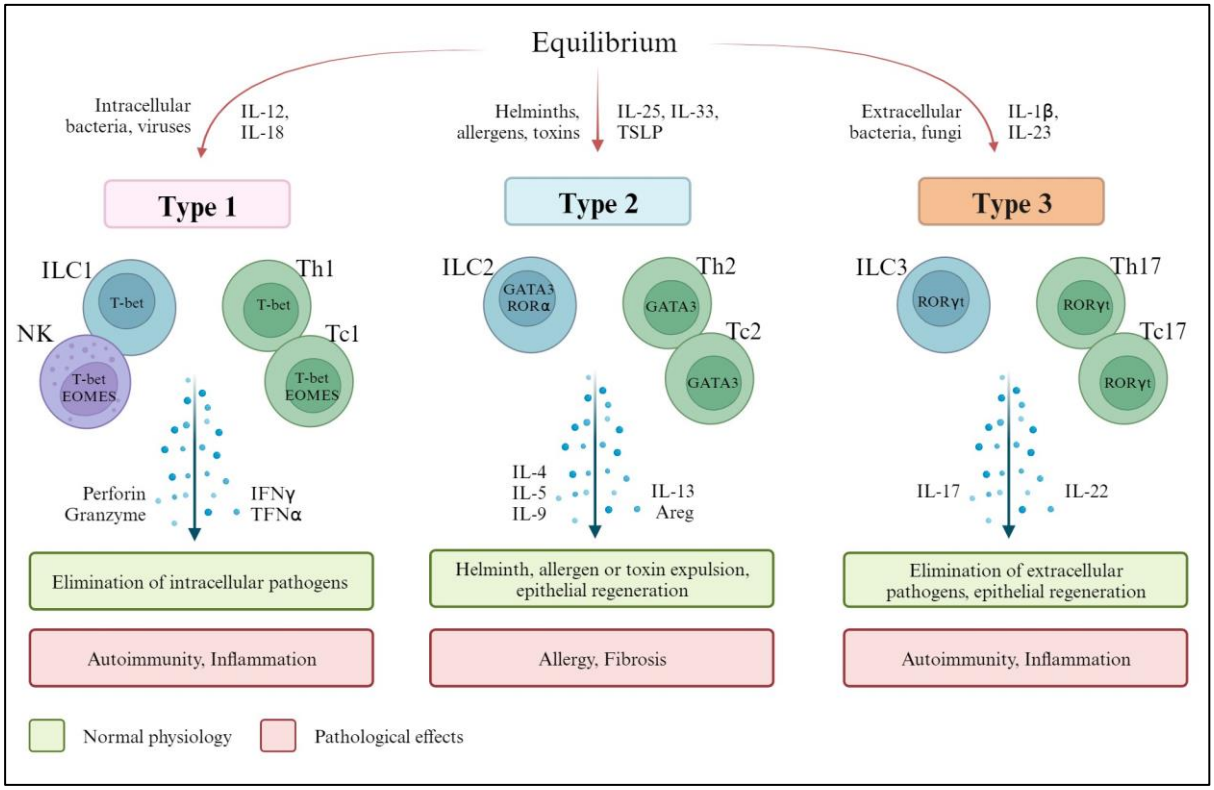

Fig. 1.2 – ***Types of Immunity.*** Type 1 immunity is composed of ILC1s, Th1, Tc1 and NK cells which together produce IFNγ, TNFα, perforin and granzyme. This results in elimination of intracellular pathogens and epithelial regeneration while pathological effects include autoimmunity or inflammation. Type 2 immunity is composed of ILC2s, Th2 cells, and Tc2 cells, which produce IL-4, IL-5, IL-9, IL-13, and Areg. This leads to helminth, allergen, or toxin expulsion, but may also lead to allergy or fibrosis. Type 3 immunity is composed of ILC3s, Th17 cells, and Tc17 cells, producing IL-17 and/or IL-22. This results in elimination of extracellular pathogens and epithelial regeneration but may result in autoimmunity or inflammation. Created with BioRender.com.

1.1.2.1. Type 1 Immunity

Type 1 immunity **(Fig. 1.2)** plays a crucial role in mounting an effective defense against intracellular pathogens such as bacteria, protozoa, and viruses. This type of immunity is characterized by an intense phagocytic activity and the secretion of the inflammatory signaling proteins interferon-gamma (IFNγ) and tumor necrosis factor-alpha (TNFα). Thus, type 1 immunity comprises specialized cells that produce IFNγ and TNFα including T-bet^{+}Eomes^{-} Th1 cells and ILC1s, as well as T-bet^{+}Eomes^{+} cytotoxic lymphocytes, explicitly Tc1 and NK cells.[16,18]

Upon intracellular pathogenic persistence, activated APCs produce and release cytokines, including interleukin 12 (IL-12) and 18 (IL-18). These cytokines induce the initial secretion of IFNγ and TNFα by ILC1s and NK cells before an antigen-specific adaptive immune response occurs.[18,19] IFNγ can bind to about all nucleated cells and promote monocyte differentiation at sites of infection, stimulate phagocytosis, induce MHC expression, enhancing antigen presentation, and control cellular

growth and apoptosis.[20–22] The secretion of IFNγ by innate cells along with the release of IL-12 by activated APC and antigen presentation promotes Tc1 and Th1 differentiation. Tc1 and NK cells have cytotoxic potential and directly eliminate infected cells by releasing perforin and granzymes. Th1 along with other IFNγ- and TNFα-producing cells release these cytokines and further activate the mononuclear phagocytic system thus contributing to the elimination of intracellular pathogens.[16]

This type of immunity can, nonetheless, have a pathogenic role in human diseases. Excessive activation of type 1 immunity has the potential to result in autoimmune diseases like multiple sclerosis and rheumatoid arthritis, along with other chronic inflammatory disorders.[23]

1.1.2.2. Type 2 Immunity

Type 2 immunity **(Fig. 1.2)** responds to helminth infections, allergens, and venom toxins. It is characterized by increased numbers of granulocytes and GATA Binding Protein 3 ($GATA3^+$) effector cells, mainly Th2, ILC2s and Tc2, along with the overproduction of immunoglobulin E (IgE) and type-2 cytokines IL-4, IL-5, IL-9, and IL-13 and the growth factor amphiregulin (AREG).[24]

Helminth infection or allergen/venom exposure on the skin or mucosal surfaces induces the epithelial release of IL-25, IL-33 and thymic stromal lymphopoietin (TSLP).[25] These alarmins directly activate mast cells, granulocytes and ILC2s to produce IL-4, IL-5, IL-9, IL-13, and AREG.[26,27] The epithelium-derived response also activates DCs that, under the influence of IL-4 and IL-13, promote Th2 and Tc2 differentiation and consequent production of IL-4, IL-5, and IL-13.[28] IL-4 and IL-13 are B cell differentiation factors with a role in Ig class switching to IgE, an antibody commonly associated to allergic diseases.[29] IL-13 promotes goblet cell hyperplasia, mucous hypersecretion and smooth muscle hyperreactivity.[30] IL-4 is believed to play a role in Th2 and Tc2 differentiation and induction of GATA3 expression, leading to type-2 cytokine production, although this process seems to be mediated by IL-13.[31] IL-5 is responsible for promoting eosinophilia.[32] The objective of these responses is to expel pathogens or allergens and to encourage the differentiation and growth of epithelial cells, which facilitates the repair of damaged tissue.

Nevertheless, an improper type 2 immune response may arise when this form of response is triggered by continuous exposure to allergens. Consequently, the majority of human diseases associated with type 2 immunity are typically allergic conditions, like atopic dermatitis (AD) or asthma.[16] Dysregulated type 2 inflammation can also lead to tissue fibrosis.[33]

1.1.2.3. Type 3 Immunity

Type 3 immunity **(Fig. 1.2)** is linked to defense against extracellular bacteria and fungi. This immunity is distinguished by the generation of IL-17 and/or IL-22 by RORγt+ lymphocytes, primarily Th17, Tc17, and ILC3s.[16]

Activation of this type of immunity by extracellular bacteria or fungi results in the production and secretion of cytokines IL-1β and IL-23 by APCs triggering ILC3 activation, Th17 and Tc17 differentiation, and consequent type 3 cytokine production. IL-17 and IL-22 have important functions for protection and signal immune and non-immune cells. IL-17 induces recruitment of neutrophils, promotes the expression of anti-microbial peptides, directs tissue remodeling during inflammatory responses and regulates tight junction formation in endothelial cells.[34,35] Just like IL-17, IL-22 stimulates the secretion of anti-microbial peptides. IL-22 also plays a role in safeguarding tissues, promoting cell survival, differentiation, restructuring, and in the production of pro-inflammatory proteins.[35]

Nonetheless, an imbalance in type 3 inflammation can be linked to a variety of autoimmune diseases, including rheumatoid arthritis, psoriasis, inflammatory bowel diseases, uveitis, among others.[16,35]

1.1.3. ILC2s

The traditional perception for type 2 immune responses was primarily accounted for by the activation of adaptive immunity, mainly Th2 and B cells. However, while the activation of the adaptive immune response is important for fighting infections and forming immunological memory, in recent years, it has become clear that ILCs also have a significant function in both immunity and inflammation. In fact, studies have shown that mice that are recombination activating gene (*Rag*) deficient and, thus, T and B cell deficient, can still initiate an innate type 2 response.[36–38] Type 2 innate lymphoid cells (ILC2s) are viewed as the innate equivalents of adaptive Th2 cells, since they exhibit transcription factors and release effector cytokines similar to those of Th2 cells.[3]

ILC2s represent a subgroup in the ILC family, characterized by the elevated expression of the transcription factors retinoic acid receptor-related orphan receptor α (RORα) and GATA3, which are essential for their development.[39,40] These cells do not yet have a single lineage-restricted marker; hence, their identification is based on a combination of markers, which can vary depending on the tissue from which they originate. ILC2s do not possess cell lineage markers that are typically

associated with T cells, B cells, dendritic cells (DCs), macrophages, and granulocytes, including CD3ε, CD4, CD8α, TCRβ, TCRγδ, CD5, CD19, B220, NK1.1, Ter119, Gr-1, CD11b, CD11c and FcεR1α (Lin –).[41] Yet, they do express CD45, Thy1 (CD90), CD127 (IL-7Rα), CD25 (IL-2Rα), Sca-1, CD117 (c-Kit), ST2 (IL-33R), KLRG1, and ICOS.[26,42] Skin ILC2s uniquely express CD103 and IL-18Rα, while the expression of IL-33 receptor (ST2) and IL-25 receptor (IL-25R) differs based on the specific organ.[43]

ILC2s are tissue resident cells that have been located at barrier surfaces, including the lung, intestine, and skin, as well as in the mesenteric lymph node (mLN), BM, spleen, liver, kidney, visceral adipose tissue, and blood.[44,45] These cells do not express antigen specific receptors, instead, their expansion and activation is controlled by cytokines, neurotransmitters and lipid mediators in the local environment of each tissue.[43,46] Upon barrier disruption, epithelial and stromal cells release cytokines, including IL-33, TSLP, and IL-25, which have been demonstrated to foster responses from Th2 cells and also to be potent activators of ILC2s.[26,42,47] Similarly to Th2 cells, activated ILC2s generate type 2 cytokines such as IL-4, IL-5, IL-9, IL-13, and the growth factor AREG, leading to type 2 inflammation. This inflammation is marked by an increased presence of eosinophils and alternative activation of macrophages, Th2 cell differentiation and IgE class switching.[43] Activation of ILC2 occurs within a few hours following exposure to allergens. Thus, ILC2s serve as the initial source of type 2 cytokines and play a crucial role in triggering the adaptive response that is mediated by Th2 cells.

1.1.3.1. Activation of ILC2

Alarmins, also known as DAMPs, are molecules typically found inside cells and are released in response to tissue damage, environmental harm, physiological stress, or cell death (necrosis).[48] ILC2s express receptors for alarmins such as IL-33, IL-25 and TSLP, and are potently activated by them.[43]

IL-33 is a cytokine actively released by necrotic epithelial cells, fibroblasts, and endothelial cells of skin, gastrointestinal tract, and lungs.[42,49] It signals through the heterodimeric receptor formed by interleukin 1 receptor-like 1 receptor (ST2) and the IL-1 receptor accessory protein (IL-1RAcP), which is mainly expressed by immune cells, including ILC2s.[42,49] IL-25 expression can be prompted in an extent of immune cells or is constitutively expressed by epithelial cells in the intestine.[43] IL-25 binds to its receptor IL-25R, composed of IL-17 receptor A (IL-17RA) and IL-17 receptor B (IL-17RB), that is present on various non-immune and immune cells, including ILC2s.[42] TSLP expression is induced in epithelial cells in the lung and skin, whereas it is constitutively expressed in intestinal

epithelial cells.[43,50] It signals through the receptor constituted of IL-7Rα and TSLPR, which is broadly expressed within hematopoietic cell populations, including ILC2s.[42,51]

Binding of IL-33 or IL-25 to its associated receptors leads to the activation of nuclear factor (NF) κB and mitogen-activated protein kinase (MAPK).[43,51] This induces the phosphorylation of GATA3, promoting GATA3 binding to the IL5 and IL13 promoters and ILC2 proliferation.[43,51] Binding of TSLP to its associated receptor activates a signaling pathway involving Janus kinase (JAK) 1/2 and STAT5.[43,51] STAT5 binds to the GATA3 gene and, together, they regulate ST2 expression, enhancing IL-33 induced activation.[52]

The responsiveness of ILC2s to cytokine stimulation differs based on their location within tissues. Within the lung, ILC2s exhibit elevated levels of ST2 expression and TSLPR but low levels of IL-25R, and thus ILC2s are potently activated upon intranasal (i.n.) administration of IL-33 but not with IL-25.[53] *In vitro* experiments showed that IL-33 is essential but not sufficient for ILC2 activation. In fact, *in vitro* activation of ILC2s requires IL-33 plus one more cytokine that acts as enhancer of activation, such as TSLP, IL-25, IL-2 or IL-7.[38] On the contrary, intestine ILC2s display high levels of IL-25R expression and relatively low levels of ST2.[17] Therefore, IL-25 seems to have an important function in provoking ILC2-mediated inflammation in the gastrointestinal tract, in contrast to its role in the lung. Furthermore, ILC2s in mLN, spleen and liver expand upon intraperitoneal (i.p.) administration of IL-25.[54,55] In the skin, ILC2s activation was proven to be independent of IL-33 or IL-25 and dependent on TSLP.[56] Ultimately, IL-33 appears to play a crucial role in the activation of lung ILC2s, while IL-25 and TSLP seem to hold greater significance in the activation of ILC2s within the gut and the skin, respectively.

1.1.3.2. Function of ILC2s

Following activation, ILC2s undergo an expansion phase when they become highly proliferative and secrete high quantities of type 2 cytokines IL-5 and IL-13, but also IL-4, IL-9, and the growth factor AREG **(Fig. 1.3)**.[43] IL-5 plays a role in the differentiation, activation, and survival of eosinophils, resulting in eosinophilia.[57] AREG also contributes to the activation and survival of eosinophils and other immune cells, plus, has an important function in tissue repair and regeneration.[58] IL-13 induces mucous hyperproduction, airway hyperreactivity, and smooth muscle contraction.[29] It also contributes to tissue repair and fibrosis.[29] Furthermore, in conjunction with IL-4, IL-13 provides signals for the differentiation of macrophages into alternatively activated macrophages and fosters antibody class switching to IgE.[29,59] Although IL-4 is thought to play a role in the differentiation and activation of Th2 cells, IL-13 seems to mediate this process.[31] IL-9 contributes to the survival and

proliferation of mast cells and airway remodeling by inducing goblet cell hyperplasia.[60] Furthermore, autocrine IL-9 signaling on ILC2s is important for their expansion and production of type 2 cytokines.[61]

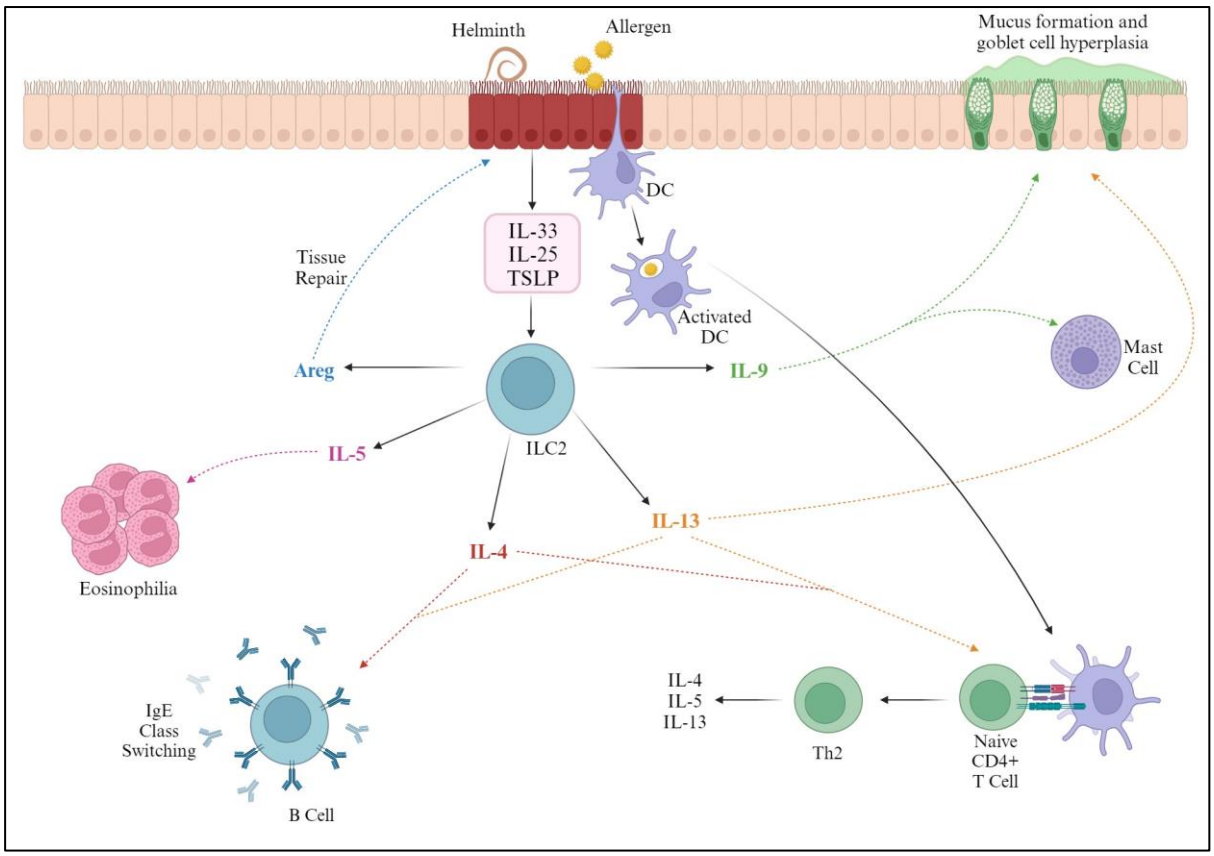

Fig. 1.3 – *Type 2 immune response orchestrated by ILC2s*. Epithelial damage induced by helminth infection or allergen exposure result in epithelial secretion of alarmins, including IL-25, IL-33, and TSLP. These alarmins potently activate ILC2s, resulting in the production of the type 2 cytokines IL-4, IL-5, IL-9, IL-13, and the growth factor Areg. Areg is involved in tissue repair. IL-5 promotes eosinophilia. IL-4 and IL-13 promote IgE class switching and support the differentiation of naïve CD4+ T cells into effector Th2 cells. IL-13 also induces mucous hyperproduction, airway hyperreactivity and smooth muscle contraction. IL-9 contributes to the survival and proliferation of mast cells and airway remodeling by inducing goblet cell hyperplasia. The epithelium-derived response also activates DCs that, in the presence of IL-4 and IL-13, promote Th2 differentiation and consequent production of IL-4, IL-5, and IL-13. Created with BioRender.com.

As mentioned before, ILC2s serve as the primary origin of type 2 cytokines, playing a crucial role in initiating type 2 inflammatory responses. This response is known to be controlled by induced regulatory T cells (iTregs) in the lungs, which also reduce both dendritic and T cell responses in murine asthmatic models. In *Rag2*-deficient mice, which lack mature B and T cells, the adoptive transfer of iTregs reduced both AHR and eosinophilia, thus suppressing ILC2-dependent inflammation *in vivo*.[62] After the expansion phase in the lung, ILC2s either presumably die, remain in the lung as memory-like ILC2s (discussed in more detail later), or become exhausted, indicating their incapacity to incite type 2 immune responses upon recurrent exposure to allergens.[53,63,64] Exhausted ILC2s produce IL-10 and are formed in the mouse lung during persistent inflammation or in response to IL-

2.[63,64] Exhausted ILC2s were induced both in the lung and in the small intestine in *in vitro* experiments.[64]

1.1.3.3. Pathogenicity of ILC2s

Whereas there is a crucial role for Th2 in allergic inflammations, the pathogenicity of ILC2s has been described in allergic diseases, including asthma and AD.[39,56,65,66] Their pathogenic roles in airway inflammation have been shown with the use of asthma mouse models.[38,53,67–70] In these models, ILC2s have been found to accumulate in lungs, bronchoalveolar lavage fluid (BALF), and mediastinal lymph nodes, leading to an exacerbation of type 2 inflammatory responses and increased production of type 2 cytokines, specifically IL-5 and IL-13. This phenomenon has been observed in mice infected with influenza virus or challenged with various allergens such as *Alternaria alternata*, house dust mites (HDM), papain, and ovalbumin (OVA). Additionally, i.n. administration of cytokines such as IL-25 and IL-33 has also resulted in the accumulation of ILC2s in the lungs. Another study showed that, while both these cytokines induced proliferation of and cytokine expression by ILC2s, IL-33 was shown to be a more potent inducer of ILC2 activation than IL-25.[71] These models have yielded valuable insights into the development of asthma-related pathologies. Notably, the increase of ILC2 population in these models was associated with airway eosinophilia, characterized by the infiltration of eosinophils into the airways. Moreover, the activation of ILC2s was also linked to airway hyperreactivity, epithelial cell hyperplasia and enhanced secretion of mucous. Furthermore, activation of ILC2s and consequent enhanced type 2 inflammation was shown to be independent of B and T cells. Other studies observed similar levels of airway inflammation, eosinophil recruitment, and cytokine production upon i.n. administration of papain, IL-25, or IL-33 in *Rag1*/*Rag2*-deficient mice, which lack mature B and T cells, when compared with wild-type (WT) mice.[38,69] The same treatment did not induce asthma-like pathologies in mice lacking B, T cell and ILC2s.[38,69] However, adoptive transfer of ILC2s into these mice reconstituted asthma-like pathologies, including eosinophilia and mucous secretion.[38] Overall, these findings highlight the crucial function of ILC2s in mediating type 2 immune responses and the subsequent development of asthma-related pathologies. Since ILC2s are also present in human lungs and have been found elevated in the peripheral blood (PB) and BALF of asthmatic patients, it implicates that they may play a critical role in human lung diseases. [65,72–76]

ILC2s have also been shown to have a pathogenic role in allergic skin inflammation as the frequency of these cells is also increased in the skin of patients with AD.[56,66] This evidence is supported by experimental data from mouse models of AD. These models were obtained with topical application of the vitamin D3 analog calcipotriol (MC903), which induced an AD-like skin

inflammation phenotype characterized by eczema, elevated serum IgE, skin thickening, dilated blood vessels and dermal inflammatory infiltration of mononuclear leukocytes and granulocytes.[56,77] Additionally, increased frequencies of $CD25^+$ $ST2^+$ ILC2s were observed in the skin and draining lymph nodes (dLNs) of these mice. Moreover, the frequency of IL-5 and IL-13 producing ILCs isolated from dLNs was elevated, implicating their activation in the context of AD-like inflammation. Experiments in which adaptive lymphocyte-deficient *Rag*-deficient mice displayed inflammation, while ILC2 deletion mitigated the dermatitis phenotype, have demonstrated the crucial role of ILC2s in MC903-induced dermatitis.[56] Similarly, subcutaneous HDM challenge results in ILC2 accumulation in human subjects, demonstrating their role in allergic responses. [66]

1.1.3.4. Memory ILC2s

Immunological memory has long been regarded a hallmark of adaptive immunity and is described by the ability of the immune system to recall previous exposure to pathogens and mount a faster more robust response upon re-encountering the same pathogens.[17] This classical definition has focused only on T and B cells of the adaptive immunity. Nonetheless, latest findings revealed the capability of innate immune cells to have immunological-like properties, namely NK cells, monocytes, macrophages and ILC2s.[53,78–83] This type of antigen non-specific immune memory has been termed 'trained immunity'.[84]

Dr. Itziar Martinez-Gonzalez has previously shown that ILC2s are able to acquire immunological memory-like proprieties in the lung.[53] In this study, i.n. administration of allergens, such as papain or *Alternaria oryzae*, or alarmins, such as IL-33, triggered ILC2 expansion and activation in the lung along with the production of cytokines IL-5 and IL-13. The expansion phase continued for a few days and was subsequently succeeded by a contraction phase during which the numbers of ILC2s decreased, the production of cytokines ceased, and the lung eosinophil numbers declined, suggesting the resolution of inflammation. However, lung ILC2 frequency remained higher when compared to naïve lung ILC2s for a long time. Additionally, it was demonstrated that certain previously activated ILC2s persisted for an extended duration, signifying their transition into long-lived cells, a trait commonly associated with memory cells. Upon re-challenge with an unrelated stimulus, these mice showed a heightened airway inflammation with augmented numbers of eosinophils in the lung and higher mucous accumulation in the airways when compared to naïve mice that received the same challenge. This study showed that previously activated ILC2s were able to produce elevated levels of IL-5 and IL-13 in contrast with naïve ILC2s, leading to an exacerbated inflammatory response, which is also distinctive of immunological memory.

The antigen non-specific immune memory of ILC2s might mediate allergic diseases triggered by unrelated allergens, which may describe the mechanism behind reactivity towards multiple allergens seen in allergic patients. However, it is unknown whether ILC2 memory is generated in any other tissues or in humans and whether memory ILC2s can migrate to other tissues to elicit enhanced inflammation in non-resident organs.

1.1.3.5. Migratory ILC2s

ILC2s are derived from HSCs, which initially give rise to CLPs and, through a series of differentiation stages, eventually lead to common ILC precursors (ILCPs). ILCs arise from fetal ILCPs in distinct waves of differentiation throughout embryogenesis.[85] Putative ILCPs are present in the fetal liver and BM, providing a potential source for these cells.[86] ILC precursors have also been detected before birth in peripheral tissues, such as the small intestine, mesentery, and lungs, where they undergo further differentiation and maturation *in situ*. [87–89] These findings suggest that that the initial differentiation of ILC2s takes place in the fetal liver and BM, while their ultimate maturation occurs in peripheral tissues. Once in peripheral tissues, ILC2s receive environmental cues specific to the residing organ and adapt to tissue specific phenotypes.[90] Schneider *et al.* found that the adult tissue ILC2 pool consists of both prenatal and postnatal origins.[85] They observed that the postnatally derived ILC2s were generated *de novo* within the first few weeks of life and persisted into adulthood, with minor contribution from subsequent adult BM hematopoiesis in the majority of the organs. Hence, he fetal and neonatal periods are pivotal moments in the formation of tissue ILC2 pools. Parabiosis studies where mice were surgically joined to share their bloodstreams have unequivocally demonstrated that ILC2 are tissue resident cells, with minimal trafficking through the blood and between tissues. [91,92] Instead, ILC2s establish long-term residence within tissues, both during homeostasis and under inflammatory circumstances. Ultimately, ILC2s predominantly develop during the fetal and neonatal phases and persist as long-lived tissue-resident cells. Thus, the preservation of tissue ILC2s during adulthood and under homeostatic conditions is achieved through their localized expansion, which is not reliant on replenishment by circulating ILC2Ps or ILC2s. It is plausible that each tissue possesses its distinct ILC2P populations that play a role in upholding the ILC2 pool during normal conditions and ILC2 generation during inflammatory responses.

Notwithstanding, various studies have provided evidence supporting the recruitment of ILC2s during inflammatory situations. Gasteiger *et al.* proven the tissue residence of ILC2s under both steady-state and acute inflammatory conditions through parabiotic studies, however, they also observed donor-derived ILC2 infiltration during chronic inflammatory conditions.[92] These findings

indicated the potential mobilization of ILC2s from hematopoietic sources during chronic inflammatory circumstances. Other studies suggested that ILC2s and ILC2Ps migrate from the bone marrow and fill empty niches in disrupted tissues upon recruitment mediated by cytokines.[53,93,94] Human studies also support the idea of recruitment of ILC2Ps from the bone marrow that can generate tissue-resident ILC2s during both steady-state conditions and inflammation.[95–97] In severe combined immunodeficiency (SCID) patients who underwent hematopoietic stem cell transplantation (HSCT), ILC2 reconstitution occurred, indicating the recruitment and repopulation of ILC2s in tissues.[97] Additionally, circulating ILCPs have been observed in human PB, further suggesting their potential to give rise to tissue resident ILCs.[96] In the context of asthma, research has revealed that in humans, ILC2s accumulate in the BALF following allergen exposure, while the numbers of circulating ILC2s decreases. This supports the notion of ILC2 recruitment from the circulation into the lungs during episodes of inflammation.[95] Overall, the available data suggest that ILC2s can be generated *de novo* and recruited from the BM to tissues during inflammation and tissue disruption. It is plausible that the recruitment and local expansion of ILC2s are not mutually exclusive processes but can happen sequentially or concurrently, contingent on the precise inflammatory circumstances.

While ILC2s might be recruited from hematogenous sources to sites of infection in response to inflammatory signals, it is also possible that activated ILC2s migrate between tissues during infection. Huang *et al.* revealed that IL-25 induced inflammatory ILC2s (iILC2s) in the small intestine to proliferate and migrate through the lymphatics and blood circulation into peripheral tissues, including the lung, through a mechanism dependent on sphingosine-1-phosphate (S1P).[98] The same authors proposed that iILC2s are circulating cells that respond to IL-25 during the initial stages of inflammation and, eventually, they transition into ST2+ conventional ILC2s within the tissues.[99] Thus it is conceivable that iILC2s are transient progenitors of ILC2s that migrate, eventually contributing to immunity. In another study, Ricardo-Gonzalez *et al.* found that activation of local ILC2s by tissue-specific alarmins induces ILC2 proliferation, lymph node migration, and blood dissemination, thus systemically distributing type 2 responses.[100] Circulatory ILC2s exhibited 2 distinct phenotypes derived from distinct tissues during the course of infection. These findings suggest that ILC2s, located in different organs, have the capability to enter the bloodstream upon activation and extend local immune responses to systemic type 2 immunity. Recently, Mathä *et al.* reported the increase of ILC2 numbers in the lung, PB, and liver upon i.n. IL-33 treatment in mice.[101] Utilizing a parabiosis model, they demonstrated that the augmentation of ILC2s in the lung resulted from the proliferation of resident ILC2s in the lung, whereas in the liver, it was attributed to the S1P-dependent migration of activated ILC2s from the lung. ILC2s originating from the lung induced fibrotic inflammation in the liver and displayed protective effects in a model of acute hepatitis induced by concanavalin A. This study suggests that ILC2s emigrate from the lung and foster type 2 inflammation in the liver and reduce type 1 inflammation. Thus, ILC2s seem to mediate inflammation in two different organs.

As mentioned above, under both steady-state and acute inflammatory conditions, ILC2s are broadly recognized as cells residing in tissues; however, upon activation these cells appear to possess the ability to move through circulation and migrate between various tissues, and possibly turn local inflammation into systemic type 2 inflammation. This opens the possibility of migratory ILC2s feasibly connecting various type 2 immune diseases. Nonetheless, the mechanisms governing the migration of ILC2s between tissues, the diversity between tissue resident and migratory ILC2s, and their roles in connecting various human diseases are not entirely comprehended.

1.2. Atopy

Atopy is defined as an individual's predisposition to become sensitized and overproduce IgE antibodies in response to substances normally considered harmless in the environment.[102] Usually, IgE is present in the serum in low concentrations, and it may increase as a protective reaction to combat parasitic infections. Nevertheless, individuals with atopic conditions exhibit an unusually heightened capability to generate IgE antibodies in response to common irritants, environmental factors, or allergens which trigger immune system activation upon ingestion, inhalation, or absorption through the skin.[103] Clinically, atopy in patients is termed as an IgE-antibody high-response where IgE sensitization is verified by IgE antibodies in the serum or by a positive skin prick test.[104] This test is a diagnostic procedure where small amounts of potential allergens are applied to the skin using a small needle, leading to localized allergic reactions in sensitive individuals, thereby helping identify specific allergies. Atopic patients have an increased risk to develop one or more atopic disorders that can exist simultaneously or at different points in time within the same individual. The most common manifestations of atopy include allergic asthma, AD, food allergies and allergic rhinitis.[105]

Nowadays, the term 'allergy' is commonly used as a synonym for IgE-mediated atopic diseases. However, it has been noted that serum IgE levels can stay within the normal range even in cases of mild, moderate, and occasionally, severe atopic diseases.[106] Thus, atopic diseases can be categorized into two distinct phenotypes: the extrinsic, allergic (atopic) variant, which arises in the context of sensitization to environmental allergens and is characterized by elevated serum IgE levels and positive skin prick test results; and the intrinsic, nonallergic (nonatopic) variant, characterized by the absence of detectable sensitization, low serum IgE levels, and negative skin prick test results.[103] A variety of risk factors have been identified for atopic diseases, including genetic factors, allergic sensitization, maternal smoke, respiratory virus infection, obesity, and many others.[105] Allergic diseases impose a substantial burden to the healthcare system as their prevalence is increasing in recent decades and now affecting 20% of the population worldwide.[107]

1.2.1. Asthma

Asthma is a non-communicable, chronic inflammatory disease of the airways, characterized by increased mucous production and airway obstruction, airway hyper-reactivity and bronchospasm leading to intermittent outbreaks of breathlessness, wheezing, and coughing.[108] Airway obstruction can occur as a result of mucous production or due to airway remodeling, possibly causing permanent lung function impairment.[109,110] Bronchospasm is an outcome of persistent airway inflammation accompanied by the leakage of plasma and the influx of inflammatory cells that produce cysteinyl leukotrienes, causing smooth muscle contraction.[109,111] Airway hyper-reactivity is a prominent characteristic of asthma, as it characterizes the inclination of the airways in patients to narrow excessively in response to stimuli that would typically have minimal or no effect on healthy individuals.[109] This disorder has a higher incidence in children, but it can affect people of all ages, and it presents an elevated asthma-related healthcare use and mortality rates in adults.[112] In 2019 it was estimated that around 300 million people have asthma worldwide, and that it is likely to increase in the near future.[113] The cause of the disease can be attributed to many risk factors including exposure to tobacco smoke, allergens, air pollution, obesity, viral infections, and many others.[114] However, asthma susceptibility seems to also have genetic and sex hormonal contributions.[115,116]

Presently, asthma is viewed as a heterogeneous condition comprising various clinical presentations (phenotypes) and distinct underlying pathophysiological mechanisms (endotypes). Generally, asthma can be divided into 3 phenotypes: the extrinsic/allergic (atopic) asthma, the intrinsic/nonallergic asthma (non-atopic), and the mixed form.[103,117] Allergic asthma is the most prevalent phenotype of asthma. It develops mostly during childhood, through contact with common allergens such as pollen, animal dander, house dust, certain foods or drugs, and many others.[118] This atopic state is identifiable through elevated total and allergen-specific IgE levels in the serum, as well as positive skin prick test results, which confirm sensitization to common allergens in affected individuals.[103,118] It is also often associated with other allergic disorders and inherited allergic predisposition. Intrinsic asthma occurs in 10-20% of asthmatic individuals and has a later onset, affecting mostly adults, and a more severe clinical course.[103,118] Additionally, this phenotype exhibits a noticeable predominance among females.[103] This type of asthma is prompted by non-allergic stimuli, like environmental pollution, smoke or fumes exposure, exercise, or respiratory infections.[103] Unlike allergic asthma, patients with the intrinsic form synthetize low levels of total IgE, lack IgE specific to seasonal and common allergens and have negative skin prick tests.[103,119]

Asthma classification based on endotypes focuses on cellular and molecular mechanisms behind the development of the disease.[120] Allergic asthma has a Th2-mediated background, marked by the existence of eosinophilic airway inflammation. In contrast, a subset of non-allergic asthma patients

exhibits a non-Th2 inflammation, characterized by neutrophilic or paucigranulocytic airway inflammation.[121] Thus, Th2-mediated asthma is type 2 immunity-driven whereas non-Th2-mediated is non-type 2 immunity-driven.[109,120]

1.2.2. Atopic Dermatitis

Atopic dermatitis is a non-communicable, chronic inflammatory disease of the skin, characterized by a skin disruption known as atopic eczema, which causes persistent pruritus, serous drainage, blister formation, erythema, and scaling, often associated with physiological stress (sleeping disorders or depression).[122,123] However, the clinical manifestation can vary significantly, contingent upon the patient's age and the severity of the disease. The prevalence of AD is estimated to be 15-20% in children and 1-3% in adults, and the incidence is expected to increase in the next decades.[124] Nowadays, AD is one of the most common chronic skin diseases and affects up to a fifth of the population worldwide.[125] Common triggers encompass a range of environmental stimuli, such as heat, perspiration, anxiety, and infections.[123] AD pathogenesis is complex and multifactorial, and its cause can be attributed to many risk factors including filaggrin protein deficiency, climate, air pollution, obesity, among others.[123]

Similarly to asthma, AD can be classified according to two phenotypes – extrinsic, allergic (atopic) AD and intrinsic, non-allergic (non-atopic) AD. Most patients with AD have the extrinsic form, representing 70-80% of the cases.[126] These patients exhibit elevated levels of total serum IgE and allergen specific IgE, along with positive reactions to skin prick tests for common allergens.[126] This disease occurs mainly during childhood in atopic families and is often linked to other atopic diseases. Intrinsic AD represents the minority of AD cases, occurring in 10% of the patients.[126] Clinically, it is observed that this condition is not linked to sensitization to food or airborne allergens. Patients typically have normal total and allergen-specific serum IgE levels and exhibit negative reactions to skin prick tests for common allergens. Nonetheless, these patients may present with similar skin lesions as individuals with elevated serum IgE levels.[103] This phenotype typically manifests at a later stage, but family history and disease duration appear to be comparable to those with extrinsic AD.[127] Furthermore, multiple studies have noted a higher prevalence of female patients among those with nonallergic AD. This observation could be attributed to the influence of sex hormones, which can affect mucosal allergic reactivity.[128,129]

The distinct molecular mechanisms that distinguish disease subtypes of AD, previously defined as endotypes, are poorly described. However, studies hypothesized a dynamic connection between both

forms of AD, suggesting that intrinsic AD could be viewed as a transitional state leading to extrinsic AD.[130]

1.2.3. Atopic March

Atopic diseases are multifactorial disorders as they might be caused by several contributing factors such as genetic susceptibility, perinatal exposure, or environmental factors. Although some patients are affected by only one atopic disorder, others can develop several through life. This progression of atopic manifestations has been termed 'atopic march' (AM).[131] AM usually begins with AD occurrences in patients during childhood which provides the sensitization required for the incidence of other atopic diseases later in life, including food allergies, allergic asthma, or allergic rhinitis.[131] Studies have shown that AM does not always follow this pattern of progression, as some patients may first develop asthma and later develop AD.[132] Also, it has been shown that it may occur at any age, not just during childhood.[133] Nonetheless, there are clinical observations describing AM as the atopic diseases progression starting with an AD diagnosis to subsequent allergies as well as the correlation between these atopic manifestations.[134–139] In fact, over recent years, AD has been considered a threat to public health as 70% of individuals with severe AD progress into asthma later in life compared to approximately 8% of the general population.[107] The presence of AD is also linked to heightened asthma severity and a greater likelihood of asthma persisting into adulthood.[140] The atopic march describes the sequential progression of atopic diseases, which are interconnected through shared genetic and environmental triggers. This progression involves allergen-specific responses mediated by Th2 cells and ILC2s, characterized by a type 2 effector phase. This phase may encompass specific IgE production, activation of granulocytes, and other innate responses such as increased mucus production and tissue swelling. Crucially, the diagnose of one allergic condition amplifies the chance of developing additional ones, contributing to the cumulative nature of the atopic march.

1.3. Thesis objective

The primary objective of this thesis is to comprehensively investigate the potential role of memory-like ILC2s in the context of the Atopic March, a phenomenon that links various forms of atopic diseases. While Th2 cell responses have traditionally been associated to this progression, recent research highlights the significance of ILC2s as initiators of type 2 inflammation during allergic reactions. ILC2s have been shown to contribute to type 2 inflammation in atopic diseases, to have a

role in mouse models of AD and asthma and to have an antigen non-specific immune memory. The antigen non-specific immune memory of ILC2s might mediate allergic diseases triggered by unrelated allergens, which may describe the mechanism behind reactivity towards multiple allergens seen in allergic patients. However, it is unknown whether ILC2 memory is generated in any other tissues or in humans and whether memory ILC2s can migrate to other tissues to elicit enhanced inflammation in non-resident organs. Considering the implication of ILC2s in allergic diseases, the role of ILC2s in mouse models of AD and asthma, and their ability to recall previous activation in an antigen non-specific manner, it is conceivable that memory ILC2s may play a crucial role in AM by linking type 2 inflammation in the skin and lung. Our central hypothesis posits that memory-like ILC2s generated in the skin might contribute to the development of asthma later in life.

To study this hypothesis, we employed mouse models simulating the conditions of the AM. Specifically, we induced an AD-like phenotype by sensitizing skin ILC2s and later stimulated lung ILC2s to model an asthma-like inflammation. This multi-step approach allowed us to track the progression from AD to asthma and investigate the role of memory ILC2s in mediating the AM. Through this comprehensive investigation, we aimed to advance our understanding of the AM underlying mechanisms and open doors for the development of innovative therapeutic strategies for allergic diseases.

2 | Materials & Methods

2.1. Materials

2.1.1. Mice

C57BL/6J (B6) female mice were purchased from Charles Rivers Laboratories (Wilmington, MA, USA). C57BL/6N (B6) and C57BL/6NRj-*Rag2*^{tm1Ciphe}/Rj (*Rag2*$^{-/-}$) female mice were purchased from Janvier Labs (Le Genest, Pays de la Loire, France). Animals were used at 6 to 10 weeks of age. Mice were kept under specific pathogen-free conditions at Comparative Medicine Biomedicum (KM-B), a barrier D animal facility in Karolinska Institutet in Stockholm, Sweden. All animal experimental procedures were performed according to approved guidelines by the Ethical Committee on Animal Experiments (*Djurförsöksetisknämnd*), Sweden.

To reduce the distress and suffering of the mice, they were anesthetized using isoflurane inhalation during intranasal injections and topical applications. They were observed until full recovery from anesthesia in a separate enclosure. The well-being of the mice was evaluated daily throughout the treatments by observing their behavior, appearance, hydration levels, breathing, and any evident signs of pain. Additionally, their weight was monitored daily during topical treatments and one week after the last treatment. At the time of harvest, euthanasia was performed by carbon dioxide asphyxiation followed by cervical dislocation.

2.1.2. Antibodies, Reagents and Flow Cytometry

Fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD3ε (145-2C11), NK1.1 (PK136), CD16/CD32 (2.4G2), TCRβ (H57-597), TCRγδ (GL3), CD8a (53-6.7), CD19 (1D3), CD11c (HL3), Gr1 (RB6-8C5), CD11b (M1/70), Ter119 (TER-119), Alexa Fluor (AF) 700-conjugated anti-mouse CD45 (30-F11), Brilliant Violet (BV) 711-conjugated anti-mouse CD4 (GK1.5), Ly6G (1A8), BV786-conjugated anti-mouse CD3ε (145-2C11), BV605-conjugated anti-mouse IL-33R (ST2, U29-93),

BV510-conjugated anti-mouse CD11b (M1/70), CD103 (M290), BV421-conjugated anti-mouse CD278 (ICOS, 7E.17G9), Allophycocyanin (APC)-conjugated anti-mouse IL-5 (TRFK5), APC-Cy7-conjugated anti-mouse CD90.2 (Thy-1.2, 53-2.1), Phycoerythrin (PE)-conjugated anti-mouse CD11c (HL3), PECy7-conjugated anti-mouse CD127 (SB/199), PECy5-conjugated anti-mouse CD3ε (17A2) and PE-CF594-conjugated anti-mouse CD3ε (145-2C11) were purchased from BD Biosciences (Franklin Lakes, NJ, EUA). BV570-conjugated anti-mouse CD45 (30-F11), FITC-conjugated anti-mouse FcεR1a (MAR-1) and PerCP-conjugated anti-mouse CD90.2 (Thy-1.2, 53-2.1) were purchased from BioLegend (San Diego, CA, EUA). PE-conjugated anti-mouse IL-13 (eBio13A) and PerCP-eFluor 710-conjugated anti-mouse CD170 (SiglecF, 1RNM44N) were purchased from Thermo Fisher Scientific (Waltham, MA, EUA).

eFluor 780 fixable viability dye (eF780) and fixable viability stain 620 (FVS620) were purchased from eBioscience (San Diego, CA, EUA) and BD Biosciences, respectively. Recombinant mouse IL-33, IL-2 and TSLP were purchased from R&D Systems (Minneapolis, MN, EUA). Recombinant mouse IL-25 was purchased from BioLegend. Calcipotriol (MC903) and ionomycin were purchased from R&D Systems. Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma-Aldrich (St. Louis, MO, EUA). Brefeldin A was purchased from eBioscience. GolgiStop (monensin) was purchased from BD Biosciences. Paraformaldehyde (PFA) was purchased from Santa Cruz Biotechnology (Dallas, Texas, EUA).

BD LSR II SORP (BD Biosciences) and Cytek Aurora (Cytek Biosciences, Fremont, CA, USA) were used for flow cytometric analysis and BD FACSAria Fusion (BD Biosciences) was used for cell sorting. Flowjo version 10.0.7r2 was used for data analyses.

2.2. Methods

2.2.1. *In Vivo* Stimulation

Mice were anesthetized by isoflurane inhalation and i.n. injections were given. Mice received i.n. administrations of 0.1 μg IL-33 (R&D Systems) in 30 μL sterile phosphate-buffered saline (PBS) or 0.25 μg IL-33 in 30 μL sterile PBS. For MC903 treatments, mice were topically

treated daily on both ears with 1 nmol of MC903 (calcipotriol, R&D Systems) in 20 μL of ethanol (vehicle) per ear for either 3 or 6 consecutive days, depending on the experiment.[1]

2.2.2. Primary Leukocyte Preparation

Single cell suspensions were prepared from the lung following a modified version of the protocol previously described by Romera-Hernández *et al*.[141] Lungs were harvested from mice, sectioned into small fragments, and subjected to enzymatic digestion in 5 mL of Dulbecco's Modified Eagle's Medium (DMEM, Thermo Fisher Scientific). Two digestion mixtures were used, depending on the experiment. The first consisted of DMEM supplemented with 10% fetal bovine serum (FBS), 142.5 U/mL collagenase type IV (Thermo Fisher Scientific), and 111 U/mL DNase I from bovine pancreas (Sigma-Aldrich). The second consisted of DMEM supplemented with 2% FBS, 71.25 U/mL collagenase type IV, and 55.5 U/mL DNase I from bovine pancreas. The digestion was carried out at 37ºC for 25 minutes, with continuous agitation at 200 rpm. For experiments where the second digestion mixture was used, the enzymatic reaction was terminated with 5 mL DMEM containing 10% FBS. Digested tissues were mechanically dissociated by mashing through 70 μm strainers. The strainers were subsequently washed with 5 mL of DMEM containing 10% FBS and cells were pelleted by centrifugation (4ºC, 5 min, 400 xg). Leukocytes were isolated by Percoll density (36%) centrifugation (20ºC, 10 min, 650 xg, without acceleration or brake). Red blood cells (RBCs) were lysed using 3 mL lysis buffer and washed with 5 mL PBS containing 2% FBS (centrifuged at 4ºC, 5 min, 400 xg).

For single cell suspension of skin cells, both ears were collected from mice. Ear tissues were cut in small pieces and digested in 2 mL PBS containing 2% FBS, 1 μg/mL DNase I and 0.25 mg/mL Liberase TL (Roche, Basel, Switzerland). The digestion was carried out at 37ºC for 45 minutes, with continuous agitation at 200 rpm. Digested tissues were mashed through 70 μm strainers, which were washed with 8 mL DMEM containing 10% FBS. Cells were collected by centrifugation (4 ˚C, 5 min, 400 xg). Leukocytes were isolated by Percoll density (36%) centrifugation (20ºC, 10 min, 650 xg, without acceleration or brake).

For single cell suspension of spleen cells, samples were mashed through 70 μm in 5 mL PBS containing 2% FBS. Strainers were washed with 5 mL of the same buffer and cells were pelleted by centrifugation (4ºC, 5 min, 400 xg). RBCs were lysed using 3 mL lysis buffer and washed with 5 mL PBS containing 2% FBS (centrifuged at 4ºC, 5 min, 400 xg).

[1] It is important to acknowledge that the author of this thesis did not participate in the direct treatment of mice in line with international standards, due to not possessing the FELASA-accredited lab animal certification.

2.2.3. Cell Surface Staining

Isolated leukocytes were incubated in 2.4G2 monoclonal antibody (produced in-house) to block Fc receptors and viable cells were counted using a hemocytometer prior to cell surface staining with fluorochrome-conjugated antibodies for flow cytometry analysis. To identify ILC2s and eosinophils, cells were stained with viability dye FVS620 or eF780. For FVS620 staining, samples were centrifuged (4ºC, 5 min, 400 xg) prior to staining with 100 µl FVS620 stain for 10 minutes at room temperature (RT) in the dark. Afterward, samples were washed twice with plain PBS, stained with 50 µl surface marker master mix (MM) for 30 minutes at 4ºC, and washed with 1 mL PBS containing 2% FBS (4ºC, 5 min, 400 xg). For stains with eF780, this dye was included in the MM. Samples were stained with MM for 30 minutes at 4ºC and washed with 1 mL PBS containing 2% FBS (4ºC, 5 min, 400 xg). To identify lung ILC2s, the MM included FITC-conjugated lineage marker mAbs (CD3ε, FcεR1a, NK1.1, CD16/32, TCRβ, CD8, CD19, TCRγδ, CD11c, Gr1, CD11b, Ter119), BV570- or AF700-conjugated CD45, BV711-conjugated CD4, BV786-, PE-Cy5- or PECF594-conjugated CD3ε-, APC-Cy7- or PerCP-conjugated CD90.2, PE-Cy7-conjugated CD127 and BV605-conjugated ST2. To identify skin ILC2s, the MM included FITC-conjugated lineage marker mAbs (CD3ε, FcεR1a, NK1.1, CD16, TCRβ, CD8, CD19, TCRγδ, CD11c, Gr1, CD11b, Ter119), AF700-conjugated CD45, BV711-conjugated CD4, PECF594-conjugated CD3ε, PerCP-conjugated CD90.2, BV421-conjugated CD278 and BV510-conjugated CD103. To identify eosinophils, the MM included BV570-conjugated CD45, BV711-conjugated Ly6G, BV510-conjugated CD11b, PerCP-eFluor 710-conjugated SiglecF and PE-conjugated CD11c. Stained and washed samples were fixed with 100 µl PFA for 30 minutes at 4°C, washed with 1 ml PBS and resuspended in 100 µl PBS + 2% FBS.

2.2.4. Intracellular Staining

Isolated leukocytes were incubated at 37ºC for 3 hours in 500 µL Roswell Park Memorial Institute (RPMI) 1640 media (Thermo Fisher Scientific) containing 10% FBS, 100 U/mL penicillin/streptomycin (P/S, Thermo Fisher Scientific), 50 µM 2-mercaptoethanol (2-ME, Sigma-Aldrich), Brefeldin A, GolgiStop, 30 ng/mL PMA and 500 nM ionomycin. Samples were blocked, centrifuged (4ºC, 5 min, 400 xg) and surface stained as mentioned before. Intracellular staining for IL-5 and IL-13 was performed after the incubation and surface staining using the Cytofix/Cytoperm Fixation/Permeabilization Solution kit (BD Biosciences). Samples were fixed/permed in 250 µl BD Cytofix/Cytoperm for 12 minutes at 4°C, washed with 2 ml 1x BD wash buffer (centrifuged at 4ºC, 5 min, 400 xg) and stained with 100 µl intracellular antibody cocktail (I/C MM) containing APC-

conjugated IL-5 and PE-conjugated IL-13 for 45 minutes at RT in the dark. Afterwards, samples were washed with 2 ml 1x BD wash buffer (centrifuged at 4ºC, 5 min, 400 xg) and resuspended in 100 µl 1x BD wash buffer.

2.2.5. Immune Cell Identification

Samples were first gated on live cells using fixable viability dye eF780 or FVS620, depending on the experiment. Lung ILC2s were identified as $CD45^+$ Lin (CD3ε, FcεR1a, NK1.1, CD16/32, TCRβ, CD8, CD19, TCRγδ, CD11c, Gr1, CD11b, Ter119)$^-$ $Thy1^+$ $ST2^+$ cells **(Fig. 2.1A)** based on surface markers. Skin ILC2s were identified as $CD45^+$ Lin (FcεR1a, NK1.1, CD16/32, CD8, CD19, TCRγδ, CD11c, Gr1, CD11b, Ter119)$^-$ $Thy1^+$ $CD127^+$ $CD103^+$ $ICOS^+$ cells based on surface markers. Eosinophils were identified as $CD45^+$ $Ly6G^-$ $SiglecF^+$ $CD11c^-$ **(Fig. 2.1B)** based on surface markers. The gating strategies used to identify lung ILC2s, and eosinophils are shown in **Figure 2.1**.

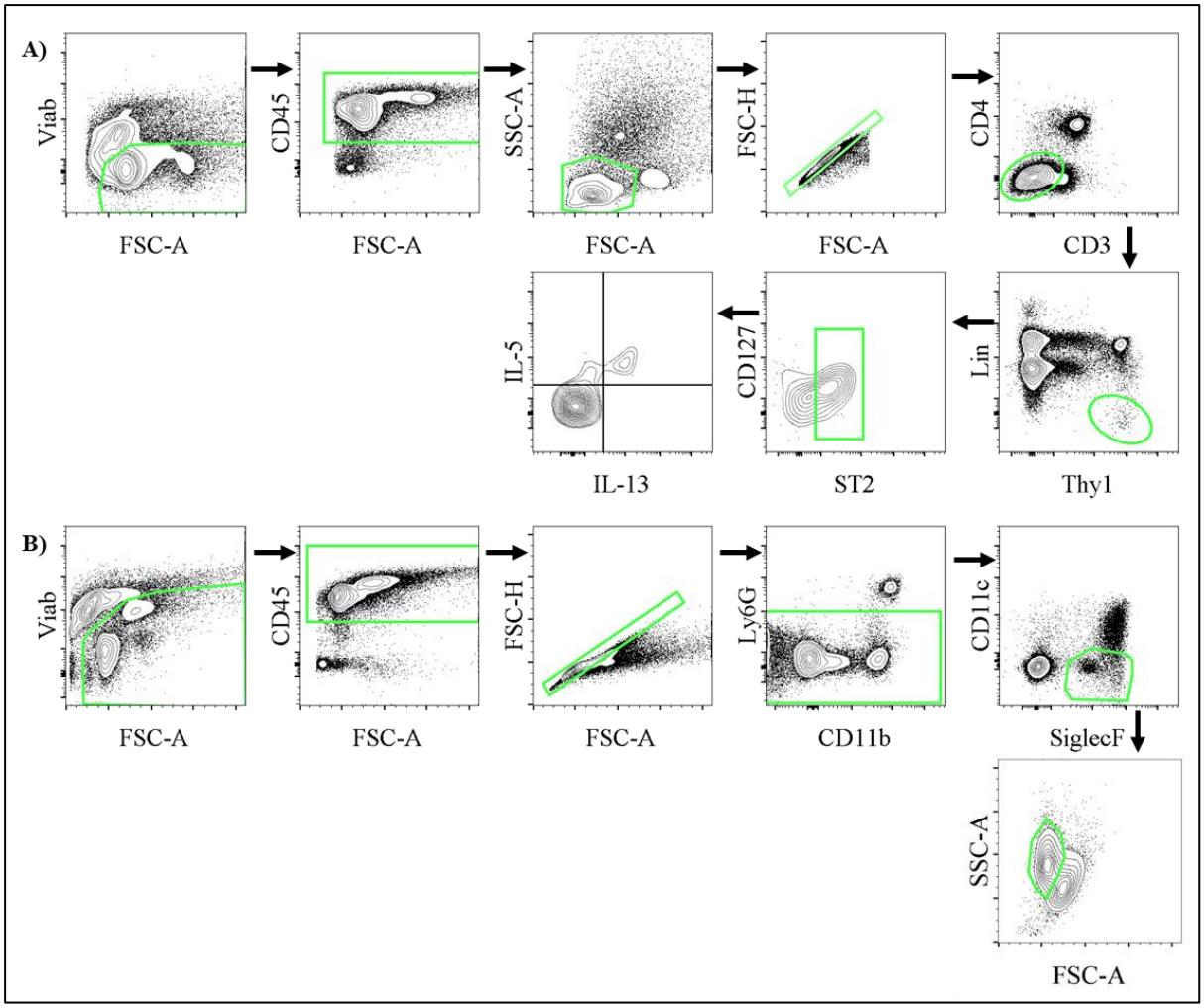

Fig. 2.1 – ***Gating strategies.*** **(A)** Gating strategy used to identify lung activated $ST2^+$ ILC2s. Viable, CD45+, $SSC\text{-}A^{Low}$ $FSC\text{-}A^{Low}$, singlet, $CD3\varepsilon^-$ $CD4^-$, Lin^-, Thy^+, $ST2^+$ cells were gated in adult female lung following

surface and intracellular staining. **(B)** Gating strategy used to identify eosinophils. Viable, CD45+, singlet, $Ly6G^{-}$, $CD11c^{-}$ $SiglecF^{+}$ cells were gated in adult female lung following surface staining. Samples were first gated on live cells using eFluor 780 fixable viability dye or fixable viability dye 620. Lin cocktail contains CD3ε, FcεR1a, NK1.1, CD16, TCRβ, CD8, CD19, TCRγδ, CD11c, Gr1, CD11b and Ter119. Cell debris and cell doublets were excluded from the analysis based on scatter signals.

2.2.6. *In Vitro* Stimulation

ILC2s were FACS-sorted from lung and skin after cell surface staining. Cells were cultured in 200 μL RPMI-1640 media supplemented with 10% FBS, 100 U/mL P/S, and 50 μM 2-ME. The culture medium was further supplemented with cytokines, either IL-33 (5 ng/mL), IL-25 (5 ng/mL) and TSLP (5 ng/mL), or IL-33 (5 ng/mL) and IL-2 (5 ng/mL). The culture was maintained at 37ºC with 5% CO_2. Culture supernatants were collected at 48, 96, and 144 hours post-culture.

2.2.7. Quantification of Cytokines

In vitro culture supernatants were analyzed for IL-5 and IL-13 using R&D Systems DuoSet enzyme-linked immunosorbent assay (ELISA) kit according to manufacturer's protocol.

2.2.8. Statistics

GraphPad Prism version 8.0.1 was used for data analysis. Unpaired two-tailed t test, one-way ANOVA (analysis of variance) with Bonferroni correction, or two-way ANOVA with Bonferroni correction was used to determine statistical significance, with a P value <0.05 being significant, as indicated in each figure. Data in graphs represent the mean ± SEM (standard error of the mean). *P⩽0.05, **P⩽0.01, ***P⩽0.001, ****P⩽0.0001, n.s, not significantly different (P>0.05).

3 | Results & Discussion

3.1. Reducing Enzyme Concentration During Digestion Step Maintained Efficiency in Primary Leukocyte Preparation

In order to obtain a single cell suspension from lung with highest viability, minimal cellular debris or aggregates, and preserved cell surface antigens, a modified enzymatic digestion procedure was developed. Specifically, adjustments to the amounts of collagenase IV and DNase I in the enzymatic digestion step were made, based on the original protocol for primary leukocyte preparation described by Romera-Hernández *et al* [141]. To evaluate the effects of these modifications, we conducted a comparative analysis on the total numbers of ILC2s and eosinophils, as well as on cell viability in the lungs of B6 WT mice treated with topical MC903 for 5 consecutive days. B6 WT mice treated with EtOH for 5 consecutive days were used as controls. Two conditions were examined: one where the usual concentration of enzymes (142.5 U/mL collagenase type IV and 111 U/mL DNase I) was used and the other where half the usual concentration of enzymes (71.25 U/mL collagenase type IV and 55.5 U/mL DNase I) was used. The results of these analyses are presented in **Figure 3.1**.

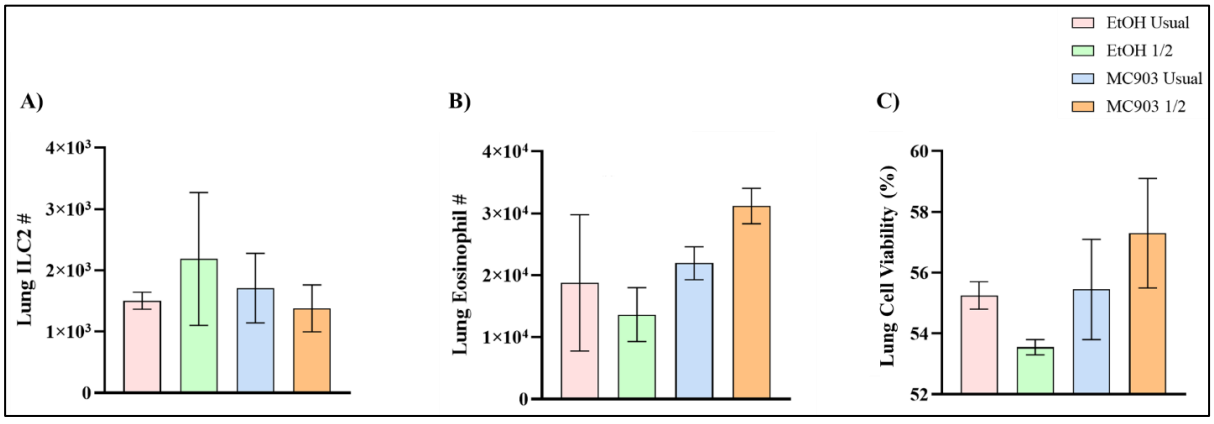

Fig. 3.1 – ***Reducing the enzyme quantity by half in the digestion step does not impact cell numbers or viability.*** WT mice were topically treated with MC903 for 5 consecutive days. After the last treatment, lung ILC2 numbers (gated as $CD45^+$ Lin^- $Thy1^+$ $ST2^+$ cells) **(A)**, lung eosinophil numbers (gated as $CD45^+$ $Ly6G^-$ $SiglecF^+$ $CD11c^-$ cells) **(B)** and lung cell viability **(C)** were analyzed by flow cytometry. Bars in the graphs are color coded for each condition as shown. Data represented are mean ± SEM. n = 2.

The results of this study demonstrate that there is not a statistically significant difference in the numbers of $ST2^+$ ILC2s (**Fig. 3.1A**) and eosinophils (**Fig. 3.1B**) in the lungs of MC903-treated mice or EtOH-treated mice in both conditions tested. This suggests that the lower enzyme concentration in the digestion step was still effective in dissociating the tissue and releasing the cells, not compromising the efficiency of isolating ILC2s and eosinophils from the tissue. Furthermore, when examining cell viability, the data show no statistically significant difference in the total viable cell percentage in both EtOH and MC903 treated mice (**Fig. 3.1C**). The lack of significant difference in cell viability between the two conditions suggests that the lower enzyme concentration used in the digestion step was still effective in maintaining cell viability, similar to the condition with usual enzyme concentration. This implies that the lower concentration is sufficient for dissociating the tissue without compromising the viability of the cells.

Overall, the outcomes of this study reveal that utilizing half the standard enzyme concentration for tissue digestion led to comparable outcomes in terms of $ST2^+$ ILC2 and eosinophil numbers, along with cell viability in the lungs of MC903-treated mice and EtOH-treated mice. Considering these findings, it is prudent to consider the practical implications. Adopting a reduced enzyme concentration for the digestion procedure not only has no negative effects on efficiency but also is cost-effective, as it saves enzyme costs still obtaining valid results. Thus, this modified enzymatic digestion procedure with reduced enzyme concentration in tissue processing protocol was adopted for the following studies.

3.2. MC903 3-Day Treatment did not Fully Activated Lung ILC2s in the Presence of T And B Cells

ILC2s have been shown to have a pathogenic role in both allergic airway and skin inflammation.[39,56,65,66] However, it is still unknown whether ILC2s have a role in the AM – the progression of an initial allergic disorder, like AD, to a secondary allergic disorder, such as asthma. To study the potential involvement of ILC2s in AM connecting these two diseases, we investigated whether WT mice with an AD-like phenotype were more prone to developing type 2 inflammation in the lungs when compared to control mice without the phenotype. To replicate the progression of the AM, this approach involved a two-step process. Initially, an AD-like phenotype was generated in mice by the topical application of the vitamin D3 analog calcipotriol (MC903) which was previously reported to induce an AD-like skin inflammatory response associated with type 2 cytokine production

which mimics human AD.[142] Subsequently, a lung inflammation was induced in these mice with i.n. administration of IL-33, allowing to simulate the transition to secondary allergic disorders like asthma in the context of the AM. IL-33 was previously reported to directly stimulate ILC2s *in vivo* which, when activated, lead to the production of type 2 cytokines and later promoting IgE class switch, a common pathway of allergic lung inflammation in response to a broad range of allergens.[31] Preliminary data from our laboratory suggested that skin ILC2 frequency reaches a peak on the third day of MC903 treatment, followed by a subsequent decline thereafter (data not published). Consequently, a three-day treatment regimen was adopted based on the hypothesis that it would effectively enhance the activation and function of ILC2s without interference from Treg cells, which are known to suppress immune responses in a later stage of inflammation. Furthermore, mice undergoing a short-term MC903 treatment are unlikely to experience significant weight loss, ensuring that the overall well-being of the animals is maintained. Thus, B6 WT mice were topically treated with MC903 for 3 consecutive days (**Fig. 3.2A**), resulting in increased ear thickness by day 3 (**Fig. 3.2B**) without overall health impairment or weight loss with ethical implications (**Fig. 3.2C**). B6 WT mice topically treated with EtOH for 3 consecutive days were used as controls. However, after a subsequent challenge with i.n. IL-33 administration, it was not observed elevated ILC2 or eosinophil numbers in the lungs of MC903-treated WT mice in comparison to the EtOH-vehicle-treated mice at the time point investigated (**Fig. 3.3A, B**). The percentage of lung ILC2s double positive for intracellular IL-5 and IL-13 was also measured (**Fig. 3.3C**). Nonetheless, the results did not indicate a higher ILC2 activation in the lungs of MC903-treated WT mice when compared to the control group.

Based on the hypothesis that ILC2s might have a role in the AM, we additionally wanted to understand if AM can occur independently of T cells. To investigate this, *Rag2*$^{-/-}$ mice were used, which are characterized by lacking the *Rag2* gene, crucial for the development of functional B and T cells. Thus, these mice produce no mature T cells or B cells. The same treatment regimen was employed, where *Rag2*$^{-/-}$ mice were topically treated with MC903 for 3 consecutive days (**Fig. 3.4A**), resulting in increased ear thickness by day 3 (**Fig. 3.4B**) without overall health impairment or weight loss with ethical implications (**Fig. 3.4C**). *Rag2*$^{-/-}$ mice topically treated with EtOH for 3 consecutive days were used as controls. Interestingly, after a subsequent challenge with intranasal IL-33 administration a trend for a higher ILC2 number in MC903-treated *Rag2*$^{-/-}$ mice lungs was observed in comparison to vehicle-treated mice at the time-point investigated (**Fig. 3.3A**). Similarly, a greater tendency for lung eosinophilia in MC903 treated *Rag2*$^{-/-}$ mice was observed (**Fig. 3.3B**). The percentage of lung ILC2s double positive for IL-5 and IL-13 was also measured (**Fig. 3.3C**). The results did not indicate significant differences in ILC2 activation between the two treatment groups in *Rag2*$^{-/-}$ mice. However, we did see a trend for greater activation in MC903-treated mice in comparison to vehicle-treated.

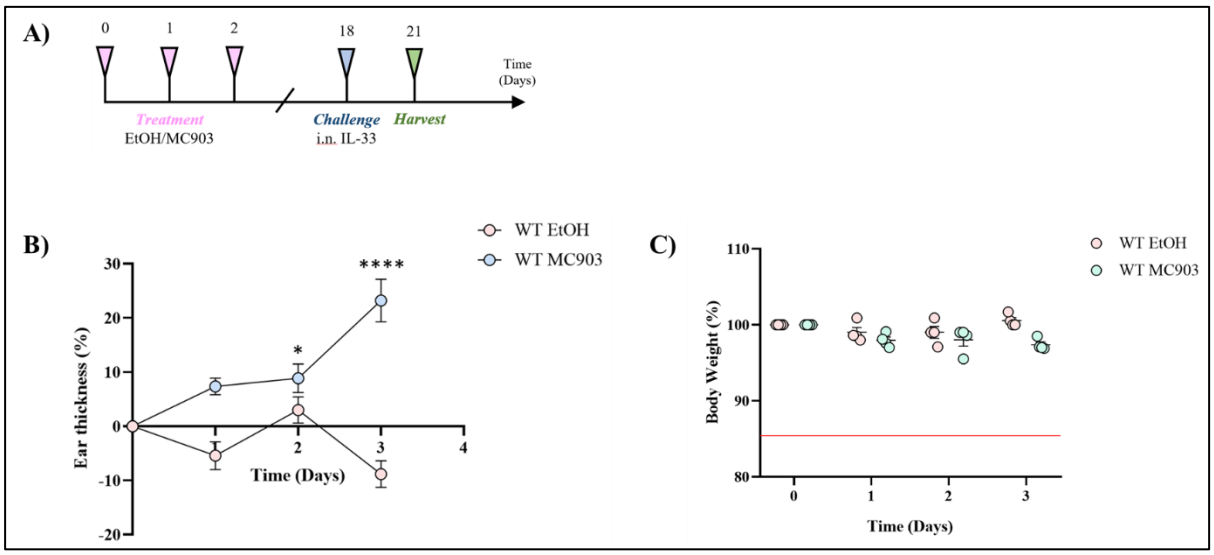

Fig. 3.2 – ***Treatment scheme and mice monitoring.*** WT mice were topically treated with EtOH or MC903 for 3 consecutive days and intranasally challenged with 0.25 µg IL-33 2 weeks after the last treatment. Samples were analyzed 72H after the challenge. **(A)** *In vivo* treatment scheme. Ear thickness **(B)** and weight **(C)** were measured during the course of the treatment. Dots in the graphs are color coded for each condition as shown. Data represented are mean ± SEM. n = 4. Two-Way ANOVA was used to assess statistical significance in comparison to day 0, with a P value <0.05 being significant. *P ⩽ 0.05, ****P ⩽ 0.0001.

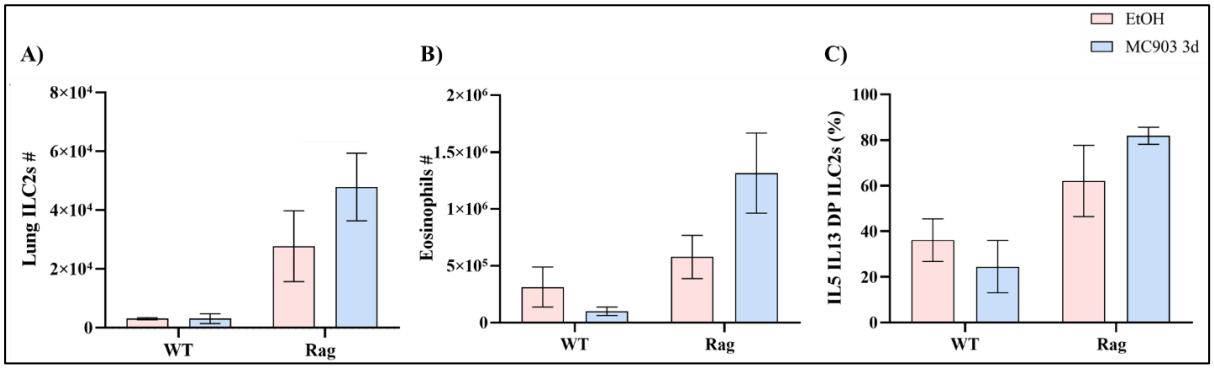

Fig. 3.3 – ***3-day MC903 treatment is not optimal for ILC2 activation in WT mice.*** WT and *Rag2*$^{-/-}$ mice were topically treated with EtOH or MC903 for 3 consecutive days and challenged intranasally with 0.25 µg IL-33 2 weeks after the last treatment. Samples were analyzed 72H after the challenge. Lung ILC2 numbers (gated as CD45^{+} Lin^{-} Thy1^{+} ST2^{+} cells) **(A)**, lung eosinophil numbers (gated as CD45^{+} Ly6G^{-} SiglecF^{+} CD11c^{-} cells) **(B)** and percentages of intracellular IL-5^{+} and IL-13^{+} lung ILC2s **(C)** were analyzed by flow cytometry. Bars in the graphs are color coded for each condition as shown. Data represented are mean ± SEM. n = 4.

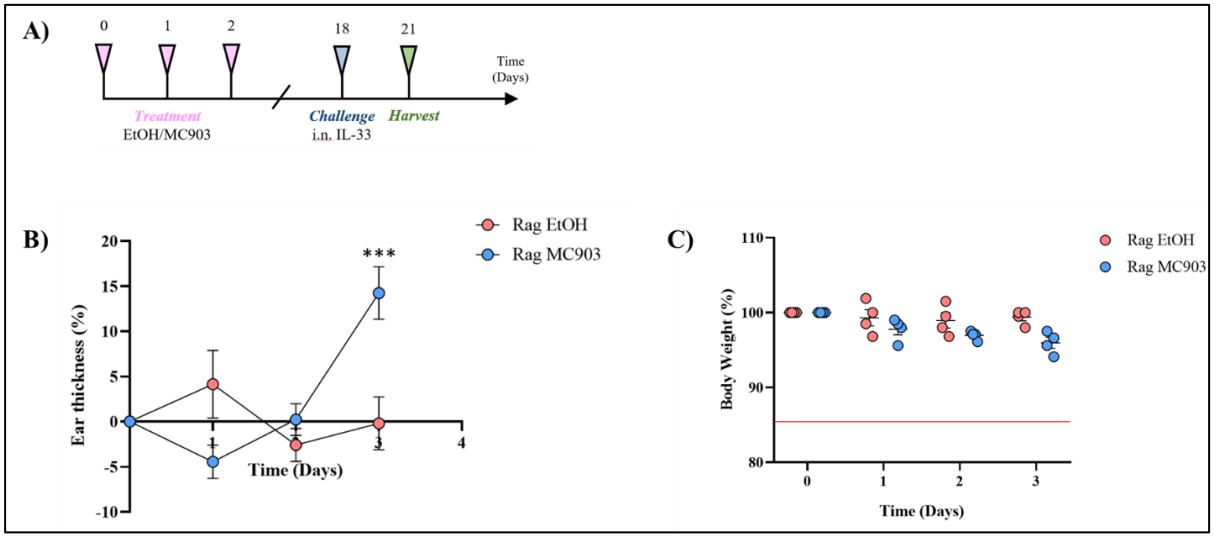

Fig. 3.4 – ***Treatment scheme and mice monitoring.*** *Rag2*$^{-/-}$ mice were topically treated with EtOH or MC903 for 3 consecutive days and intranasally challenged with 0.25 µg IL-33 2 weeks after the last treatment. Samples were analyzed 72H after the challenge. **(A)** *In vivo* treatment scheme. Ear thickness **(B)** and weight **(C)** were measured during the course of the treatment. Dots in the graphs are color coded for each condition as shown. Data represented are mean ± SEM. n = 4. Two-Way ANOVA was used to assess statistical significance to day 0, with a P value <0.05 being significant. ***P≤0.001.

Martinez Gonzalez *et al.* characterized memory-like ILC2s in the lung of mice, where ILC2s that have previously been activated responded more strongly to a secondary challenge compared to naïve ILC2s.[53] However, it is unknown whether memory ILC2s can be generated in other tissues. Our results suggest that, in the absence of T or B cells, mice with an AD-like phenotype prior to the lung inflammation had a trend for a greater ILC2 response in the lung. Thus, the AD-like phenotype might be prompting the generation of memory-like ILC2s leading, upon challenge, to an exacerbated ILC2 activation and enhanced type 2 inflammation in the lung. This observation could be corroborated by the increased eosinophilia, which is an indication for type 2 airway inflammation. The development of lung eosinophilia can be attributed to IL-33-induced ILC2 activation and consequent IL-5 production. In fact, we did see a trend for higher lung ILC2 activation in Rag KO mice with an AD-like phenotype. However, although it was observed a trend for a greater ILC2 response in mice with an AD-like phenotype in the absence of adaptive immunity, it was not reported an ILC2 response in WT mice with an AD-like phenotype. The absence of significant differences or trends in ILC2s and eosinophil numbers in the lung of WT mice following the IL-33 challenge suggests that the sensitization process may not have been sufficiently established, meaning that the initial activation required to generate memory-like ILC2s did not occur. Thus, it was necessary to improve the stimulation to fully activate ILC2s in the presence of T and B cells. To investigate this, we next tested a longer MC903 treatment duration in order to achieve more pronounced effects and generation of memory ILC2s in WT mice. Additionally, in order to understand if the trend for enhanced ILC2 response in the absence of adaptive immunity was attributed only to previously sensitized ILC2s, it

was also tested the dosage of IL-33. Research suggests that ILC2s, once sensitized, can exhibit heightened responses to minimal stimuli, a characteristic of immunological memory. [17] By adjusting the IL-33 dosage, the goal was to distinguish the nuances of ILC2 reactions and comprehensively assess the implications of prior sensitizations, especially the potential memory-like responses to diminished stimuli.

3.3. Increased ILC2 Response to IL-33 Appears to Originate from Naïve ILC2s

To determine the optimal duration of the topical MC903 treatment we compared the effects of a 3-day treatment with a longer 5-day treatment. Further, we assessed the optimal dose of IL-33 that only activated sensitized ILC2s, without eliciting a response from naïve ILC2s. Thus, B6 WT mice were topically treated with MC903 for 3 or 5 consecutive days **(Fig. 3.5A)**, resulting in the development of an AD-like phenotype without overall health impairment or weight loss with ethical implications **(Fig. 3.5B)**. B6 WT mice topically treated with EtOH for 3 or 5 consecutive days were used as controls. After, mice were challenged with 0.25 μg or 0.1 μg IL-33 on day 18 **(Fig. 3.5A)**. We previously observed that lung ILC2s in WT mice treated with MC903 for 3 days did not exhibit a response to 0.25 μg of IL-33 **(Fig. 3.3A, C)**. As a result, the effects of a lower dose, 0.1 μg, were not assessed under the same conditions. Concordantly, we observed that mice treated with MC903 for 3 consecutive days and then challenged with 0.25 μg of IL-33 did not have an increase in ILC2 or eosinophil numbers **(Fig 3.6A, B)**. Additionally, the percentage of activated ILC2s was not significantly elevated in these mice, similar to previous results **(Fig 3.3C, 3.6C)**. Mice subjected to a 5-day MC903 treatment showed elevated ILC2 and eosinophil numbers upon challenge when compared to those treated for 3 days **(Fig. 3.6A, B)**. Moreover, the percentage of activated ILC2s in the lungs was increased in the 5-day treatment group in comparison to the 3-day treatment group **(Fig. 3.6C)**. However, a similar increase was observed in the control group, indicating that the observed augmentation was not exclusively attributable to the MC903 treatment. When mice with a 5-day MC903 treatment were subsequently exposed to a challenge with 0.25 μg of IL-33, they exhibited a higher ILC2 response in comparison to mice challenged with the lower dose of 0.1 μg IL-33. This enhanced response was evident through higher counts of ILC2s and eosinophils in the lungs **(Fig. 3,6A, B)**, alongside a heightened level of ILC2 activation **(Fig. 3.6C)**.

Overall, a higher tendency for ILC2 expansion or activation in MC903-treated WT mice was not observed when contrasted to vehicle-treated controls. This assessment reflects that extending the MC903 treatment to a 5-day regimen did not yield more pronounced effects on the generation of

memory-like ILC2s in these mice. Further, it is noteworthy that a 0.25 µg dose of IL-33 induced a greater lung ILC2 activation when compared to the 0.1 µg dose of IL-33. Since we did not observe any difference in ILC2 response between controls and MC903-treated mice, nor a heightened responses to minimal IL-33 stimuli, it is possible that the responsive population to the IL-33 challenge was constitute of naïve ILC2s instead of previously sensitized ILC2s. Memory cells, distinct from naïve cells, are known for two characteristics: they react more intensely to lower doses of stimuli and they respond faster upon re-exposure to a familiar antigen.[17] Given that we might be allowing naïve ILC2s ample time to respond to IL-33, the next logical step would be to adjust the timing of lung tissue collection. By identifying an optimal time point where only the sensitized ILC2s have had the chance to respond, we can better distinguish between the responses of naïve and memory-like ILC2 populations. For this, we tested different time points for the lung tissue harvesting after the IL-33 challenge.

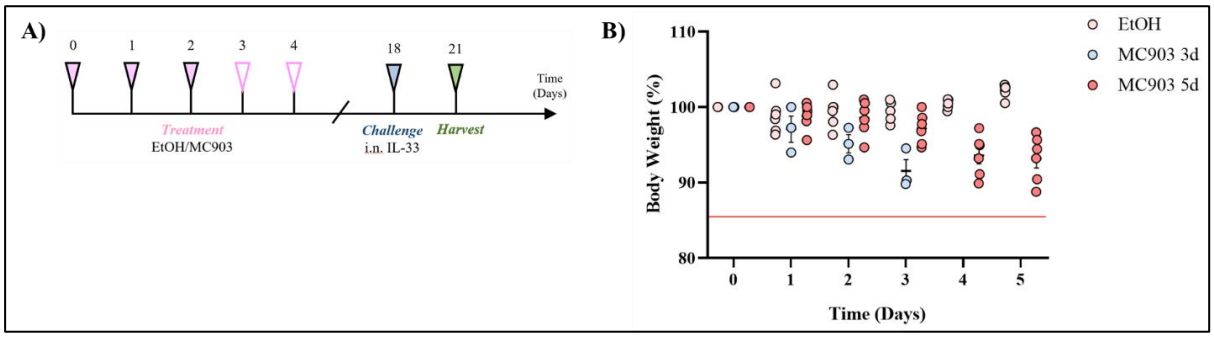

Fig. 3.5 – ***Treatment scheme and mice monitoring.*** WT mice were topically treated with MC903 for 3 or 5 consecutive days and intranasally challenged with 0.25 µg or 0.1 µg IL-33 2 weeks after the last treatment. Samples were analyzed 72H after the challenge. **(A)** *In vivo* treatment scheme. Weights **(B)** were measured during the course of the treatment. Dots in the graphs are color coded for each condition as shown. Data represented are mean ± SEM. n = 3 – 6.

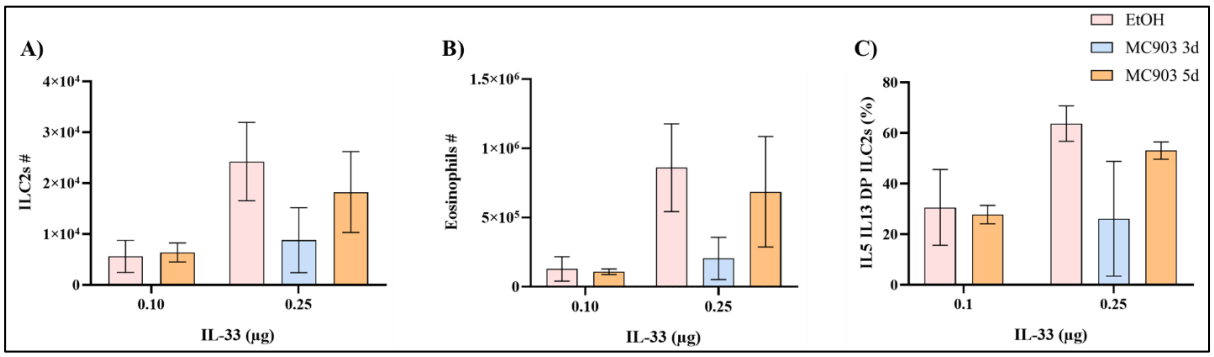

Fig. 3.6 – *Higher IL-33 dose induces greater lung ILC2 activation in WT mice.* WT mice were topically treated with MC903 for 3 or 5 consecutive days and challenged intranasally with 0.1 or 0.25 µg IL-33 2 weeks after the last treatment. Samples were analyzed 72H after challenge. Lung ILC2 numbers (gated as $CD45^+$ Lin^- $Thy1^+$ $ST2^+$ cells) **(A)**, lung eosinophil numbers (gated as $CD45^+$ $Ly6G^-$ $SiglecF^+$ $CD11c^-$ cells) **(B)** and percentages of intracellular $IL\text{-}5^+$ and $IL\text{-}13^+$ lung ILC2s **(C)** were analyzed by flow cytometry. Bars in the graphs are color coded for each condition as shown. Data represented are mean ± SEM. n = 3.

3.4. Harvesting at 48-Hour Time Point Appears Beneficial to Prevent Naïve ILC2 Response

Immunological memory is characterized by a cell's ability to generate a stronger and faster secondary response that quickly eliminates a threat upon subsequent exposures.[17] In line with this principle, we hypothesized that naïve cells might not exhibit a discernible response to a single dose of IL-33 within a short timeframe. We wanted to understand whether the ILC2 response observed following a single dose of IL-33 originated from naïve ILC2s or previously sensitized ILC2s. For this, we tested different time points for lung tissue collection following intranasal administration of IL-33, specifically comparing the outcomes at 48-hour and 72-hour. Thus, B6 WT mice were treated with a single dose of i.n. IL-33 injection or PBS, for controls, and subsequent analyses were performed at 48H and 72H time points **(Fig. 3.7A)**. We did not observe statistically significant differences in ILC2 numbers in the lungs of mice examined at 48 hours and 72 hours when compared to the control group **(Fig. 3.7B)**. While there was a noticeable increase in the number of ILC2s at the 72-hour time point in contrast to 48 hours, the difference was not statistically significant either. It is important to note the presence of an outlier in the 72-hour time point data as well, which likely contributed to the observed divergence. Further, we understand that ILC2 numbers in both time points are similar to the control group. Additionally, we assessed the percentage of ILC2s expressing both IL-5 and IL-13 **(Fig. 3.7C)**. We found that in both time points, there was a statistically significant activation of ILC2s when compared to the control group, with no discernible distinction between the two time points.

IL-33 is known to induce the activation and proliferation of ILC2s, leading to the release of various inflammatory mediators.[56,72] By examining mice at 48 hours and 72 hours after an IL-33 challenge, it was understood that there was a slight expansion and mild activation of naïve ILC2s devoid of prior sensitization. Nonetheless, naïve ILC2s did not significantly respond to the challenge in both conditions. At 72-hour time point, there was a slight inclination toward greater expansion compared to the 48-hour point, although this differential lacked statistical significance and was likely due to the outlier. Overall, these results showed us that at a 48-hour time point, naïve ILC2s are consistently not responding, with similar numbers as the control group, and without outliers. Thus, this time point for harvesting seems beneficial to avoid naïve ILC2 response in further experiments. To attenuate the influence of naïve ILC2 response, we made the decision to proceed with experiments targeting the 48-hour time point for harvesting. This time frame elicited a comparatively subdued reaction among this naïve ILC2s.

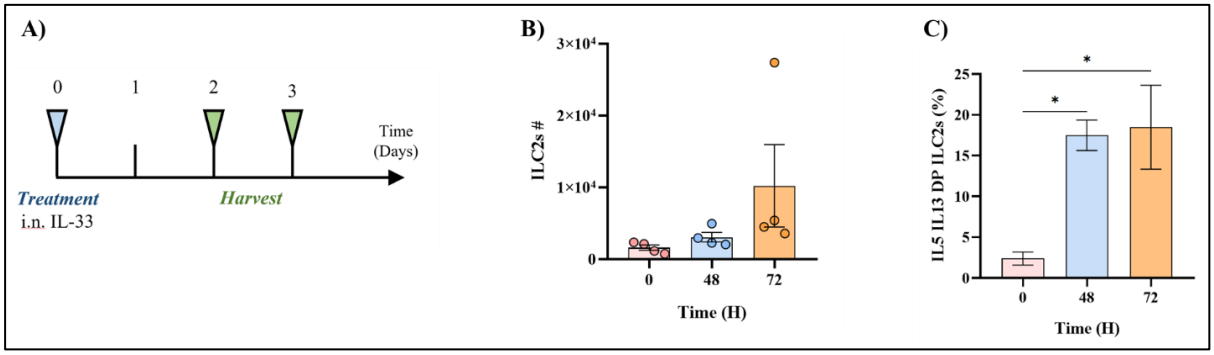

Fig. 3.7 – *Naïve ILC2s do not expand but mildly activate with a single dose of IL-33*. WT mice were challenged intranasally once with 0.25 µg IL-33. Samples were analyzed 48H or 72H after challenge. **(A)** *In vivo* treatment scheme. Lung ILC2 numbers (gated as $CD45^+$ Lin^- $Thy1^+$ $ST2^+$ cells) **(B)** and percentages of intracellular IL-5^+ and IL-13^+ lung ILC2s **(C)** were analyzed by flow cytometry. Bars in the graphs are color coded for each condition as shown. Data represented are mean ± SEM. n = 4. One-Way ANOVA was used to assess statistical significance, with a P value <0.05 being significant. *P<0.05.

3.5. MC903 Treatment Seems to Modulate Lung Immune Environment, Potentially Attributed to Extrinsic Factors

The mechanism of ILC2 activation has been investigated in an atopic dermatitis model with topical application of MC903 to mouse skin.[56] In line with our findings, we aimed to discern potential changes in the ILC2 population in the lung upon MC903 skin treatment. For this, B6 WT mice were topically treated with MC903 for 5 consecutive days **(Fig. 3.8A)**, without overall health impairment or weight loss with ethical implications **(Fig. 3.8B)**. B6 WT mice topically treated with EtOH were used as controls. On the fifth day of treatment, mice were euthanized one hour after the treatment, and the lungs were harvested and analyzed. Interestingly, we observed a statistically significant difference in $ST2^+$ ILC2s numbers in the lungs of M903 treated mice when compared to EtOH treated mice **(Fig. 3.8C)**. Modulating the immune response in the skin through MC903 treatment could be indirectly impacting the lung's immune environment, potentially influencing the development or progression of allergic diseases. In fact, this increase might indicate a potential link between the skin treatment with MC903 and the development of lung-associated allergic reactions, supporting the concept of atopic march. Overall, these findings indicate that there is an increased activation and responsiveness of ILC2s in the lung of topically MC903 treated mice. However, the underlying mechanisms responsible for this enhanced responsiveness remained unclear. We next aimed to understand whether the increased responsiveness observed was due to a cell intrinsic memory-like phenotype or if it is driven by environmental factors.

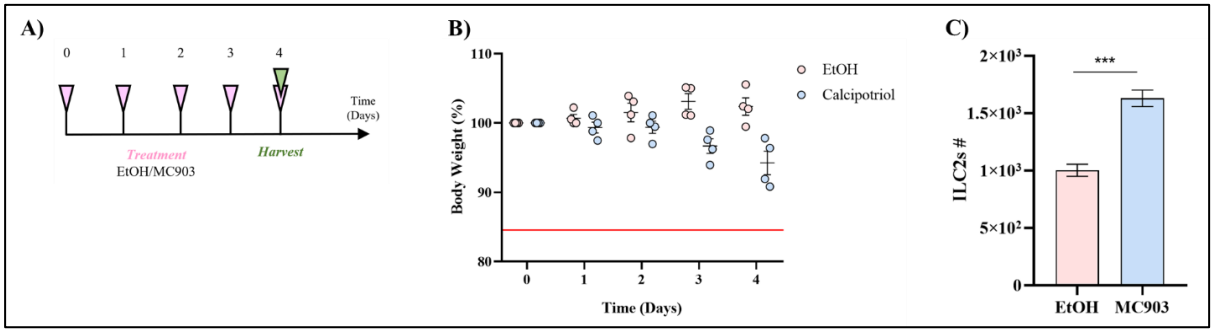

Fig. 3.8 – *MC903 Skin Treatment in WT Mice Increased ILC2 Numbers in The Lung*. WT mice were topically treated with MC903 for 5 consecutive days. Samples were analyzed 1H after the last treatment. **(A)** *In vivo* treatment scheme. Weights **(B)** were measured during the course of the treatment. Lung ILC2 numbers (gated as $CD45^+$ Lin^- $Thy1^+$ $ST2^+$ cells) **(C)** were analyzed by flow cytometry. Dots and bars in the graphs are color coded for each condition as shown. Data represented are mean ± SEM. n = 4. Unpaired two-tailed t-test was used to assess statistical significance, with a P value <0.05 being significant. ***P⩽0.001.

We aimed to investigate whether the increased responsiveness of previously sensitized ILC2s is primarily driven by environmental factors or intrinsic changes within the ILC2 population. By removing ILC2s from the lung microenvironment and comparing their responsiveness to stimuli with those isolated from EtOH treated mice, we intended to dissect the contributions of the microenvironment and ILC2 intrinsic properties. Furthermore, we aimed to explore the distribution of memory ILC2s between the skin and lung to gain insights into the location of heightened memory responses. For this, B6 WT mice were topically treated with MC903 for 5 consecutive days **(Fig. 3.9A)**, resulting in increased ear thickness by day 3 without overall health impairment or weight loss with ethical implications **(Fig. 3.9B)**. Two weeks after the last treatment, lung and skin ILC2s were sorted and cultured under a 5 ng/mL dose of IL-33, IL-25 and TSLP, or a combination of IL-2 together with IL-33, combinations known to stimulate ILC2s, for 48, 96 and 144 hours **(Fig. 3.9A)**.[38] Supernatant was harvested at these time points and the media was refreshed with the same dose of cytokines. ILC2s from the ears of MC903-treated mice exhibited an enhanced response to the cytokine stimulation, as evidenced by up to a fivefold increase in IL-5 and IL-13 production compared to ILC2s from EtOH treated ears **(Figure 3.10A, 3.10B)**. Thus, MC903-experienced skin ILC2s are inherently more responsive than naïve ILC2s to the same concentrations of cytokines. In contrast, naïve lung ILC2s demonstrated a superior response to the same concentration of stimulating cytokines compared to lung ILC2s from MC903-treated mice **(Fig. 3.10C, 3.10D)**. This observation suggests that the heightened responsiveness of lung ILC2s observed in prior studies could be attributed to extrinsic factors in the lung microenvironment, including cytokines that act as alarmins, inflammatory signals or cellular interactions. Alternatively, it is conceivable that the diminished response of MC903-treated lung ILC2s might be a manifestation of immune cell exhaustion due to persistent stimulation with cytokines. Immune cell exhaustion is characterized by the progressive loss of a cell's ability to function optimally, which in this context might mean a lower ability to produce cytokines upon stimulation.[63,64] Importantly, our data also suggest that these lung ILC2s may not be in a 'memory'

state two weeks into our investigation. To corroborate this hypothesis and better understand the dynamics of memory state formation and potential exhaustion in ILC2s, it would be beneficial to extend our investigation over a longer 1-month period between skin treatment and the challenge. Furthermore, our findings also highlight the importance of carefully modulating the level of ILC2 cytokine stimulation in our experiments. Over-stimulation of these cells could contribute to an exhaustion state. Therefore, finding the optimal balance of stimulation is crucial in accurately understanding the functional dynamics of ILC2s and to avoid artificially inducing a state of cellular exhaustion. For this, it would be interesting to analyze ILC2s in the same conditions but only stimulating with cytokines once, when cells are cultured.

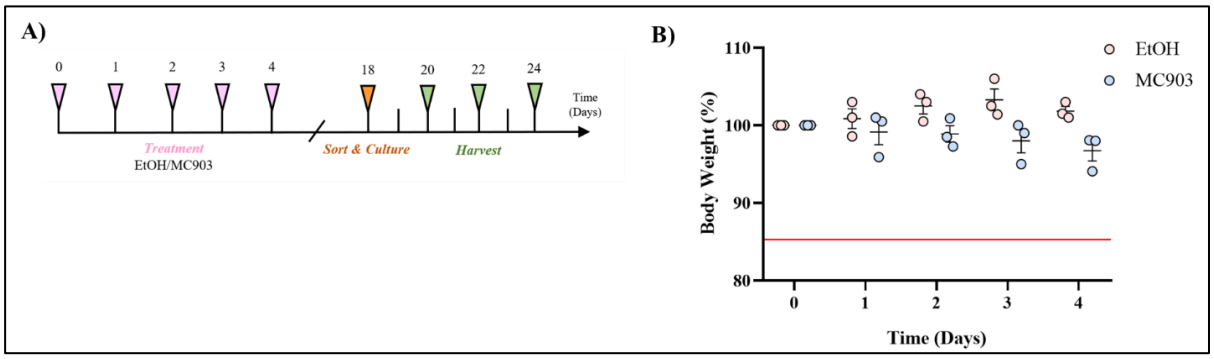

Fig. 3.9 – ***Treatment scheme and mice monitoring.*** WT mice were topically treated with MC903 for 5 consecutive days. Skin and lung ILC2s were sorted and put into culture and supernatant was harvested 48, 96 and 144 hours later. **(A)** *In vivo* treatment scheme. Weights **(B)** were measured during the course of the treatment. Dots in the graphs are color coded for each condition as shown. Data represented are mean ± SEM. n = 3.

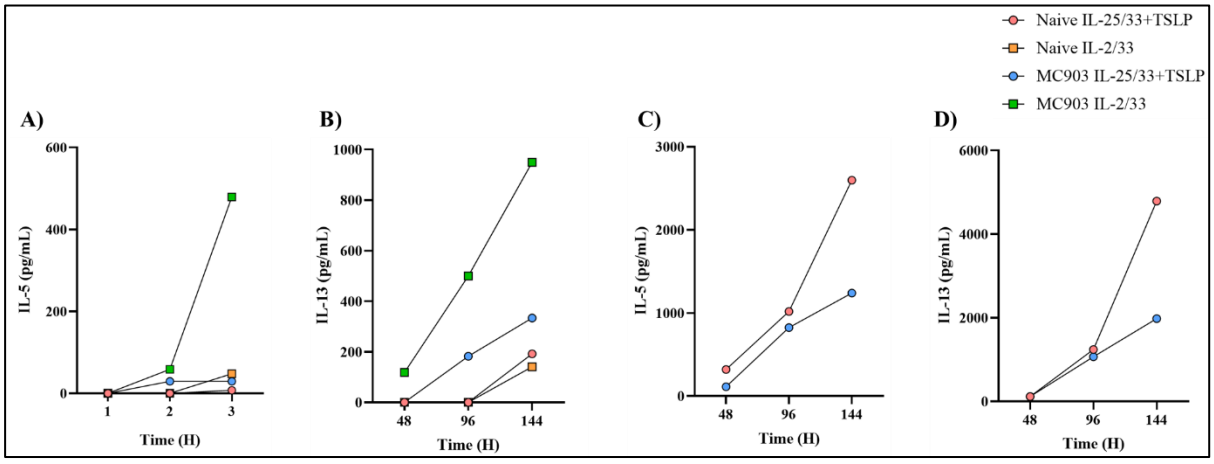

Fig. 3.10 - ***Differential Responsiveness of skin and lung ILC2s.*** WT mice were topically treated with MC930 for 5 consecutive days. Skin **(A, B)** and lung **(C, D)** ILC2s (gated as $CD45^+$ Lin^- $Thy1^+$ $ST2^+$ cells) were sorted 2 weeks later. Sorted ILC2s were cultured for 48, 96 and 144 hours with IL-25 (5 ng/mL), IL-33 (5 ng/mL) and TSLP (5 ng/mL) (810 cells) or IL-2 (5 ng/mL) and IL-33 (5 ng/mL) (500 cells), and the amounts of IL-5 and IL-13 in the culture supernatants were determined by ELISA. Dots in the graphs are color coded for each condition as shown.

4 | Conclusion & Future Perspectives

The global rise of atopic disorders, especially AD and asthma, is adding a significant burden to our healthcare systems. A notable percentage of individuals with atopic diseases experiences a progression known as the Atopic March, where one atopic condition evolves into another over time. This progression of atopic disorders has been studied over the last years. Imbalanced type 2 immune responses are correlated with the diagnose of atopic disorders characterized by epithelial inflammation, such as AD and asthma; or fibrosis. Type 2 immunity is the body's adaptive response, triggered by innate immunity, to helminths, toxic venoms, or allergens. At the forefront of this type 2 allergic inflammatory responses are Group 2 Innate Lymphoid Cells. ILC2s are tissue resident cells that have been found at barrier surfaces, including the lung, intestine, and skin. They are considered the innate counterparts of adaptive Th2 cells as they express transcription factors and effector cytokines that mirror Th2 cells. Upon barrier disruption, epithelial and stromal cells release cytokines, including IL-33, TSLP, and IL-25, which potently activate ILC2s. Activated ILC2s produce type 2 cytokines, including IL-4, IL-5, IL-9, IL-13, and the growth factor AREG, resulting in type 2 inflammation characterized by eosinophilia, alternative activation of macrophages, Th2 cell differentiation and IgE class switching. The pathogenicity of ILC2s has been described in allergic diseases, including asthma and AD. In asthma and AD mouse models, ILC2s have been found to accumulate in lungs or skin, leading to an exacerbation of type 2 inflammatory responses and increased secretion of type 2 cytokines, specifically IL-5 and IL-13. Recently, studies discovered that ILC2s can acquire immunological memory in a mouse model of allergic lung inflammation. Thus, this work aimed to investigate the potential role of memory ILC2s in connecting different type 2 immune allergic diseases including AD and asthma. We hypothesized that memory ILC2s generated in the skin during AD might play a pivotal role in the onset of asthma later in life. In this study we employed mouse models simulating the conditions of the Atopic March. Specifically, we induced an AD-like phenotype in mice by the topical application of the vitamin D3 analog calcipotriol (MC903). Subsequently, we induced lung inflammation in these mice with intranasal administration of IL-33, allowing us to simulate the transition to secondary allergic disorders, like asthma, in the context of the AM.

We were able to quantify and identify ILC2s, eosinophils and assess cell viability in our samples through methodologies such as tissue processing, single-cell isolation, and flow cytometry. All these techniques were optimized and tested during the work in order to obtain the most accurate

results. We were able to identify ILC2 populations in both skin and lung samples. Our study suggested the potential link between MC903 skin treatment and lung ILC2 immune responses, lending support on the AM concept. Using a mouse model that lacked T or B cells, we observed an AD-like phenotype that seemed to increase ILC2 numbers and activation in the lung. This raised the possibility that the AD-like phenotype could prime the lung for exacerbated type 2 inflammation. However, our findings from the WT model showed that the sensitization process might not have been sufficiently established, potentially indicating the absence of the initial activation essential for the generation of memory-like ILC2. Extending our study by increasing the MC903 treatment duration, we aimed to observe heightened memory responses in the lung. However, our expectations were met with results that did not exhibit a clear MC903-related ILC2 response. To specifically target sensitized ILC2 responses, we analyzed and adjusted the IL-33 treatment dosage and harvest time-point based on the knowledge that memory cells tend to react more rapidly and to lower stimuli than naïve cells. Interestingly, after an MC903 skin treatment and analyzing the lung, we found a significant difference in $ST2^+$ ILC2s activation in the lungs of MC903-treated mice compared to their EtOH treated counterparts. This underlined that skin immune responses, when modulated via MC903 treatment, could indirectly influence the lung's immune environment, possibly impacting allergic diseases progression and reinforcing the hypothesis of the atopic march. Although there was an observable trend of heightened activation and responsiveness of ILC2s in MC903-treated mice's lungs, the mechanisms underlaying this responsiveness remained elusive. Our subsequent investigation into whether this increased activity was a result of an intrinsic memory-like phenotype or environmentally driven showed a leaning towards the latter. Extrinsic factors within the lung microenvironment, including cytokines, inflammatory signals, or cellular interactions, might play a significant role. Conversely, the subdued response observed in MC903-treated lung ILC2s could be symptomatic of immune cell exhaustion, especially considering the continuous cytokine stimulation.

In conclusion, our study presents preliminary findings suggesting that skin treatment with MC903 could be modulating lung ILC2 responses. Still, a definitive 'memory' state remained undetected two weeks post-treatment. The role of memory-like ILC2s in the AM necessitates further exploration, underscoring the need for more extended investigations. Subsequent studies should consider extending the period between skin treatment and i.n. challenge to a longer one-month period, potentially fostering memory cell generation. Further, inducing a more chronic skin inflammation with extended MC903 treatment could lead to an increased susceptibility to atopic reactions in order to see further allergen responses in the lung. It would also be interesting to try the i.n. challenge with a different allergen such as papain or IL-25 and compare the different results. Moreover, reassessing this optimized atopic march mouse model in *Rag* KO mice and comparing with previous results from this study could offer insightful results. Examining the model with the use of ILC2 KO mice will be pivotal to discerning the ILC2 dependence in this AM mouse model. Additionally, isolating the factors

influencing ILC2 responsiveness, whether they are intrinsic or extrinsic, will require analyzing ILC2s under conditions where cytokine stimulation is limited during cell culture, thereby averting potential exhaustion states. Finally, if the migration of ILC2s was inhibited it could be helpful to understand if ILC2s resident in tissue are sufficient to drive AM inflammation. This comprehensive understanding will aid in more effective therapeutic strategies and interventions for atopic disorders in the future.

5 | Bibliography

1. Jagannathan-Bogdan, M., & Zon, L. I. (2013). Hematopoiesis. *Development* (*Cambridge, England*), *140*(12), 2463–2467. https://doi.org/10.1242/dev.083147

2. Kondo, M. (2010). Lymphoid and myeloid lineage commitment in multipotent hematopoietic progenitors. *Immunological reviews*, *238*(1), 37–46. https://doi.org/10.1111/j.1600-065X.2010.00963.x

3. Vivier, E., Artis, D., Colonna, M., Diefenbach, A., Di Santo, J. P., Eberl, G., Koyasu, S., Locksley, R. M., McKenzie, A. N. J., Mebius, R. E., Powrie, F., & Spits, H. (2018). Innate Lymphoid Cells: 10 Years On. *Cell*, *174*(5), 1054–1066. https://doi.org/10.1016/j.cell.2018.07.017

4. Kaur, B. P., & Secord, E. (2019). Innate Immunity. *Pediatric clinics of North America*, *66*(5), 905–911. https://doi.org/10.1016/j.pcl.2019.06.011

5. Marshall, J. S., Warrington, R., Watson, W., & Kim, H. L. (2018). An introduction to immunology and immunopathology. *Allergy, asthma, and clinical immunology: official journal of the Canadian Society of Allergy and Clinical Immunology*, *14*(Suppl 2), 49. https://doi.org/10.1186/s13223-018-0278-1

6. Chaplin D. D. (2010). Overview of the immune response. *The Journal of allergy and clinical immunology*, *125*(2 Suppl 2), S3–S23. https://doi.org/10.1016/j.jaci.2009.12.980

7. Paludan, S. R., Pradeu, T., Masters, S. L., & Mogensen, T. H. (2021). Constitutive immune mechanisms: mediators of host defence and immune regulation. *Nature reviews. Immunology*, *21*(3), 137–150. https://doi.org/10.1038/s41577-020-0391-5

8. Turvey, S. E., & Broide, D. H. (2010). Innate immunity. *The Journal of allergy and clinical immunology*, *125*(2 Suppl 2), S24–S32. https://doi.org/10.1016/j.jaci.2009.07.016

9. Medzhitov, R., & Janeway, C., Jr (2000). Innate immune recognition: mechanisms and pathways. *Immunological reviews*, *173*, 89–97. https://doi.org/10.1034/j.1600-065x.2000.917309.x

10. Dunkelberger, J. R., & Song, W. C. (2010). Complement and its role in innate and adaptive immune responses. *Cell research*, *20*(1), 34–50. https://doi.org/10.1038/cr.2009.139

11. Paul, S., & Lal, G. (2017). The Molecular Mechanism of Natural Killer Cells Function and Its Importance in Cancer Immunotherapy. *Frontiers in immunology*, *8*, 1124. https://doi.org/10.3389/fimmu.2017.01124

12. Sanchez-Trincado, J. L., Gomez-Perosanz, M., & Reche, P. A. (2017). Fundamentals and Methods for T- and B-Cell Epitope Prediction. *Journal of immunology research*, *2017*, 2680160. https://doi.org/10.1155/2017/2680160

13. Cooper, M. D., & Alder, M. N. (2006). The evolution of adaptive immune systems. *Cell*, *124*(4), 815–822. https://doi.org/10.1016/j.cell.2006.02.001

14. Mørch, A. M., Bálint, Š., Santos, A. M., Davis, S. J., & Dustin, M. L. (2020). Coreceptors and TCR Signaling - the Strong and the Weak of It. *Frontiers in cell and developmental biology*, *8*, 597627. https://doi.org/10.3389/fcell.2020.597627

15. Germain R. N. (2002). T-cell development and the CD4-CD8 lineage decision. *Nature reviews. Immunology*, *2*(5), 309–322. https://doi.org/10.1038/nri798

16. Annunziato, F., Romagnani, C., & Romagnani, S. (2015). The 3 major types of innate and adaptive cell-mediated effector immunity. *The Journal of allergy and clinical immunology*, *135*(3), 626–635. https://doi.org/10.1016/j.jaci.2014.11.001

17. Martinez-Gonzalez, I., Ghaedi, M., Steer, C. A., Mathä, L., Vivier, E., & Takei, F. (2018). ILC2 memory: Recollection of previous activation. *Immunological reviews*, *283*(1), 41–53. https://doi.org/10.1111/imr.12643

18. Ivashkiv L. B. (2018). IFNγ: signalling, epigenetics and roles in immunity, metabolism, disease and cancer immunotherapy. *Nature reviews. Immunology*, *18*(9), 545–558. https://doi.org/10.1038/s41577-018-0029-z

19. Zook, E. C., & Kee, B. L. (2016). Development of innate lymphoid cells. *Nature immunology*, *17*(7), 775–782. https://doi.org/10.1038/ni.3481

20. Livingston, D. H., Appel, S. H., Sonnenfeld, G., & Malangoni, M. A. (1989). The effect of tumor necrosis factor-alpha and interferon-gamma on neutrophil function. *The Journal of surgical research*, *46*(4), 322–326. https://doi.org/10.1016/0022-4804(89)90195-9

21. Tau, G., & Rothman, P. (1999). Biologic functions of the IFN-gamma receptors. *Allergy*, *54*(12), 1233–1251. https://doi.org/10.1034/j.1398-9995.1999.00099.x

22. Gocher, A. M., Workman, C. J., & Vignali, D. A. A. (2022). Interferon-γ: teammate or opponent in the tumour microenvironment?. *Nature reviews. Immunology*, *22*(3), 158–172. https://doi.org/10.1038/s41577-021-00566-3

23. Romagnani S. (1997). The Th1/Th2 paradigm. *Immunology today*, *18*(6), 263–266. https://doi.org/10.1016/s0167-5699(97)80019-9

24. Haase, P., & Voehringer, D. (2021). Regulation of the humoral type 2 immune response against allergens and helminths. *European journal of immunology*, *51*(2), 273–279. https://doi.org/10.1002/eji.202048864

25. Hammad, H., & Lambrecht, B. N. (2015). Barrier Epithelial Cells and the Control of Type 2 Immunity. *Immunity*, *43*(1), 29–40. https://doi.org/10.1016/j.immuni.2015.07.007

26. Martinez-Gonzalez, I., Steer, C. A., & Takei, F. (2015). Lung ILC2s link innate and adaptive responses in allergic inflammation. *Trends in immunology*, *36*(3), 189–195. https://doi.org/10.1016/j.it.2015.01.005

27. Salter, B. M., Oliveria, J. P., Nusca, G., Smith, S. G., Tworek, D., Mitchell, P. D., Watson, R. M., Sehmi, R., & Gauvreau, G. M. (2016). IL-25 and IL-33 induce Type 2 inflammation in basophils from subjects with allergic asthma. *Respiratory research*, *17*, 5. https://doi.org/10.1186/s12931-016-0321-z

28. Hammad, H., & Lambrecht, B. N. (2015). Barrier Epithelial Cells and the Control of Type 2 Immunity. *Immunity*, *43*(1), 29–40. https://doi.org/10.1016/j.immuni.2015.07.007

29. de Vries J. E. (1998). The role of IL-13 and its receptor in allergy and inflammatory responses. *The Journal of allergy and clinical immunology*, *102*(2), 165–169. https://doi.org/10.1016/s0091-6749(98)70080-6

30. Gurram, R. K., & Zhu, J. (2019). Orchestration between ILC2s and Th2 cells in shaping type 2 immune responses. *Cellular & molecular immunology*, *16*(3), 225–235. https://doi.org/10.1038/s41423-019-0210-8

31. Halim, T. Y., Steer, C. A., Mathä, L., Gold, M. J., Martinez-Gonzalez, I., McNagny, K. M., McKenzie, A. N., & Takei, F. (2014). Group 2 innate lymphoid cells are critical for the initiation of adaptive T helper 2 cell-mediated allergic lung inflammation. *Immunity*, *40*(3), 425–435. https://doi.org/10.1016/j.immuni.2014.01.011

32. Pelaia, C., Paoletti, G., Puggioni, F., Racca, F., Pelaia, G., Canonica, G. W., & Heffler, E. (2019). Interleukin-5 in the Pathophysiology of Severe Asthma. *Frontiers in physiology*, *10*, 1514. https://doi.org/10.3389/fphys.2019.01514

33. Gieseck, R. L., 3rd, Wilson, M. S., & Wynn, T. A. (2018). Type 2 immunity in tissue repair and fibrosis. *Nature reviews. Immunology*, *18*(1), 62–76. https://doi.org/10.1038/nri.2017.90

34. Valle-Noguera, A., Ochoa-Ramos, A., Gomez-Sánchez, M. J., & Cruz-Adalia, A. (2021). Type 3 Innate Lymphoid Cells as Regulators of the Host-Pathogen Interaction. *Frontiers in immunology*, *12*, 748851. https://doi.org/10.3389/fimmu.2021.748851

35. Eyerich, K., Dimartino, V., & Cavani, A. (2017). IL-17 and IL-22 in immunity: Driving protection and pathology. European journal of immunology, 47(4), 607–614. https://doi.org/10.1002/eji.201646723

36. Fort, M. M., Cheung, J., Yen, D., Li, J., Zurawski, S. M., Lo, S., Menon, S., Clifford, T., Hunte, B., Lesley, R., Muchamuel, T., Hurst, S. D., Zurawski, G., Leach, M. W., Gorman, D. M., & Rennick, D. M. (2001). IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. *Immunity*, *15*(6), 985–995. https://doi.org/10.1016/s1074-7613(01)00243-6

37. Hurst, S. D., Muchamuel, T., Gorman, D. M., Gilbert, J. M., Clifford, T., Kwan, S., Menon, S., Seymour, B., Jackson, C., Kung, T. T., Brieland, J. K., Zurawski, S. M., Chapman, R. W., Zurawski, G., & Coffman, R. L. (2002). New IL-17 family members promote Th1 or Th2 responses in the lung: in vivo function of the novel cytokine IL-25. *Journal of immunology (Baltimore, Md. : 1950)*, *169*(1), 443–453. https://doi.org/10.4049/jimmunol.169.1.443

38. Halim, T. Y., Krauss, R. H., Sun, A. C., & Takei, F. (2012). Lung natural helper cells are a critical source of Th2 cell-type cytokines in protease allergen-induced airway inflammation. *Immunity*, *36*(3), 451–463. https://doi.org/10.1016/j.immuni.2011.12.020

39. Halim, T. Y., MacLaren, A., Romanish, M. T., Gold, M. J., McNagny, K. M., & Takei, F. (2012). Retinoic-acid-receptor-related orphan nuclear receptor alpha is required for natural helper cell development and allergic inflammation. *Immunity*, *37*(3), 463–474. https://doi.org/10.1016/j.immuni.2012.06.012

40. Ghaedi, M., & Takei, F. (2021). Innate lymphoid cell development. *The Journal of allergy and clinical immunology*, *147*(5), 1549–1560. https://doi.org/10.1016/j.jaci.2021.03.009

41. Licona-Limón, P., Kim, L. K., Palm, N. W., & Flavell, R. A. (2013). TH2, allergy and group 2 innate lymphoid cells. *Nature immunology*, *14*(6), 536–542. https://doi.org/10.1038/ni.2617

42. Halim, T. Y. F., & McKenzie, A. N. J. (2013). New kids on the block: group 2 innate lymphoid cells and type 2 inflammation in the lung. *Chest*, *144*(5), 1681–1686. https://doi.org/10.1378/chest.13-0911

43. Mathä, L., Martinez-Gonzalez, I., Steer, C. A., & Takei, F. (2021). The Fate of Activated Group 2 Innate Lymphoid Cells. *Frontiers in immunology*, *12*, 671966. https://doi.org/10.3389/fimmu.2021.671966

44. Herbert, D. R., Douglas, B., & Zullo, K. (2019). Group 2 Innate Lymphoid Cells (ILC2): Type 2 Immunity and Helminth Immunity. *International journal of molecular sciences*, *20*(9), 2276. https://doi.org/10.3390/ijms20092276

45. Lei, A. H., Xiao, Q., Liu, G. Y., Shi, K., Yang, Q., Li, X., Liu, Y. F., Wang, H. K., Cai, W. P., Guan, Y. J., Gabrilovich, D. I., & Zhou, J. (2018). ICAM-1 controls development and function of ILC2. *The Journal of experimental medicine*, *215*(8), 2157–2174. https://doi.org/10.1084/jem.20172359

46. Halim T. Y. (2016). Group 2 innate lymphoid cells in disease. *International immunology*, *28*(1), 13–22. https://doi.org/10.1093/intimm/dxv050

47. Islam, S. A., & Luster, A. D. (2012). T cell homing to epithelial barriers in allergic disease. *Nature medicine*, *18*(5), 705–715. https://doi.org/10.1038/nm.2760

48. Rider, P., Voronov, E., Dinarello, C. A., Apte, R. N., & Cohen, I. (2017). Alarmins: Feel the Stress. *Journal of immunology (Baltimore, Md. : 1950)*, *198*(4), 1395–1402. https://doi.org/10.4049/jimmunol.1601342

49. Drake, L. Y., & Kita, H. (2017). IL-33: biological properties, functions, and roles in airway disease. *Immunological reviews*, *278*(1), 173–184. https://doi.org/10.1111/imr.12552

50. Soumelis, V., Reche, P. A., Kanzler, H., Yuan, W., Edward, G., Homey, B., Gilliet, M., Ho, S., Antonenko, S., Lauerma, A., Smith, K., Gorman, D., Zurawski, S., Abrams, J., Menon, S., McClanahan, T., de Waal-Malefyt Rd, R., Bazan, F., Kastelein, R. A., & Liu, Y. J. (2002). Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nature immunology*, *3*(7), 673–680. https://doi.org/10.1038/ni805

51. Kabata, H., Moro, K., & Koyasu, S. (2018). The group 2 innate lymphoid cell (ILC2) regulatory network and its underlying mechanisms. *Immunological reviews*, *286*(1), 37–52. https://doi.org/10.1111/imr.12706

52. Guo, L., Wei, G., Zhu, J., Liao, W., Leonard, W. J., Zhao, K., & Paul, W. (2009). IL-1 family members and STAT activators induce cytokine production by Th2, Th17, and Th1 cells. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(32), 13463–13468. https://doi.org/10.1073/pnas.0906988106

53. Martinez-Gonzalez, I., Mathä, L., Steer, C. A., Ghaedi, M., Poon, G. F., & Takei, F. (2016). Allergen-Experienced Group 2 Innate Lymphoid Cells Acquire Memory-like Properties and Enhance Allergic Lung Inflammation. Immunity, 45(1), 198–208. https://doi.org/10.1016/j.immuni.2016.06.017

54. Neill, D. R., Wong, S. H., Bellosi, A., Flynn, R. J., Daly, M., Langford, T. K., Bucks, C., Kane, C. M., Fallon, P. G., Pannell, R., Jolin, H. E., & McKenzie, A. N. (2010). Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature*, *464*(7293), 1367–1370. https://doi.org/10.1038/nature08900

55. Price, A. E., Liang, H. E., Sullivan, B. M., Reinhardt, R. L., Eisley, C. J., Erle, D. J., & Locksley, R. M. (2010). Systemically dispersed innate IL-13-expressing cells in type 2 immunity. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(25), 11489–11494. https://doi.org/10.1073/pnas.1003988107

56. Kim, B. S., Siracusa, M. C., Saenz, S. A., Noti, M., Monticelli, L. A., Sonnenberg, G. F., Hepworth, M. R., Van Voorhees, A. S., Comeau, M. R., & Artis, D. (2013). TSLP elicits IL-33-independent innate lymphoid cell responses to promote skin inflammation. *Science translational medicine*, *5*(170), 170ra16. https://doi.org/10.1126/scitranslmed.3005374

57. Kouro, T., & Takatsu, K. (2009). IL-5- and eosinophil-mediated inflammation: from discovery to therapy. International immunology, 21(12), 1303–1309. https://doi.org/10.1093/intimm/dxp102

58. Zaiss, D. M. W., Gause, W. C., Osborne, L. C., & Artis, D. (2015). Emerging functions of amphiregulin in orchestrating immunity, inflammation, and tissue repair. *Immunity*, *42*(2), 216–226. https://doi.org/10.1016/j.immuni.2015.01.020

59. Gour, N., & Wills-Karp, M. (2015). IL-4 and IL-13 signaling in allergic airway disease. *Cytokine*, *75*(1), 68–78. https://doi.org/10.1016/j.cyto.2015.05.014

60. Townsend, J. M., Fallon, G. P., Matthews, J. D., Smith, P., Jolin, E. H., & McKenzie, N. A. (2000). IL-9-deficient mice establish fundamental roles for IL-9 in pulmonary mastocytosis and goblet cell hyperplasia but not T cell development. *Immunity*, *13*(4), 573–583. https://doi.org/10.1016/s1074-7613(00)00056-x

61. Turner, J. E., Morrison, P. J., Wilhelm, C., Wilson, M., Ahlfors, H., Renauld, J. C., Panzer, U., Helmby, H., & Stockinger, B. (2013). IL-9-mediated survival of type 2 innate lymphoid cells promotes damage control in helminth-induced lung inflammation. *The Journal of experimental medicine*, *210*(13), 2951–2965. https://doi.org/10.1084/jem.20130071

62. Rigas, D., Lewis, G., Aron, J. L., Wang, B., Banie, H., Sankaranarayanan, I., Galle-Treger, L., Maazi, H., Lo, R., Freeman, G. J., Sharpe, A. H., Soroosh, P., & Akbari, O. (2017). Type 2 innate lymphoid cell suppression by regulatory T cells attenuates airway hyperreactivity and requires inducible T-cell costimulator-inducible T-cell costimulator ligand interaction. *The Journal of allergy and clinical immunology*, *139*(5), 1468–1477.e2. https://doi.org/10.1016/j.jaci.2016.08.034

63. Miyamoto, C., Kojo, S., Yamashita, M., Moro, K., Lacaud, G., Shiroguchi, K., Taniuchi, I., & Ebihara, T. (2019). Runx/Cbfβ complexes protect group 2 innate lymphoid cells from exhausted-like hyporesponsiveness during allergic airway inflammation. *Nature communications*, *10*(1), 447. https://doi.org/10.1038/s41467-019-08365-0

64. Seehus, C. R., Kadavallore, A., Torre, B., Yeckes, A. R., Wang, Y., Tang, J., & Kaye, J. (2017). Alternative activation generates IL-10 producing type 2 innate lymphoid cells. *Nature communications*, *8*(1), 1900. https://doi.org/10.1038/s41467-017-02023-z

65. Monticelli, L. A., Sonnenberg, G. F., Abt, M. C., Alenghat, T., Ziegler, C. G., Doering, T. A., Angelosanto, J. M., Laidlaw, B. J., Yang, C. Y., Sathaliyawala, T., Kubota, M., Turner, D., Diamond, J. M., Goldrath, A. W., Farber, D. L., Collman, R. G., Wherry, E. J., & Artis, D. (2011). Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nature immunology*, *12*(11), 1045–1054. https://doi.org/10.1031/ni.2131

66. Salimi, M., Barlow, J. L., Saunders, S. P., Xue, L., Gutowska-Owsiak, D., Wang, X., Huang, L. C., Johnson, D., Scanlon, S. T., McKenzie, A. N., Fallon, P. G., & Ogg, G. S. (2013). A role for IL-25 and IL-33-driven type-2 innate lymphoid cells in atopic dermatitis. *The Journal of experimental medicine*, *210*(13), 2939–2950. https://doi.org/10.1084/jem.20130351

67. Kim, H. K., Lund, S., Baum, R., Rosenthal, P., Khorram, N., & Doherty, T. A. (2014). Innate type 2 response to Alternaria extract enhances ryegrass-induced lung inflammation. *International archives of allergy and immunology*, *163*(2), 92–105. https://doi.org/10.1159/000356341

68. Chang, Y. J., Kim, H. Y., Albacker, L. A., Baumgarth, N., McKenzie, A. N., Smith, D. E., Dekruyff, R. H., & Umetsu, D. T. (2011). Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity. *Nature immunology*, *12*(7), 631–638. https://doi.org/10.1038/ni.2045

69. Klein Wolterink, R. G., Kleinjan, A., van Nimwegen, M., Bergen, I., de Bruijn, M., Levani, Y., & Hendriks, R. W. (2012). Pulmonary innate lymphoid cells are major producers of IL-5 and IL-13 in murine models of allergic asthma. *European journal of immunology*, *42*(5), 1106–1116. https://doi.org/10.1002/eji.201142018

70. Wilhelm, C., Hirota, K., Stieglitz, B., Van Snick, J., Tolaini, M., Lahl, K., Sparwasser, T., Helmby, H., & Stockinger, B. (2011). An IL-9 fate reporter demonstrates the induction of an innate IL-9 response in lung inflammation. *Nature immunology*, *12*(11), 1071–1077. https://doi.org/10.1038/ni.2133

71. Barlow, J. L., Peel, S., Fox, J., Panova, V., Hardman, C. S., Camelo, A., Bucks, C., Wu, X., Kane, C. M., Neill, D. R., Flynn, R. J., Sayers, I., Hall, I. P., & McKenzie, A. N. (2013). IL-33 is more potent than IL-25 in provoking IL-13-producing nuocytes (type 2 innate lymphoid cells) and airway contraction. *The Journal of allergy and clinical immunology*, *132*(4), 933–941. https://doi.org/10.1016/j.jaci.2013.05.012

72. Christianson, C. A., Goplen, N. P., Zafar, I., Irvin, C., Good, J. T., Jr, Rollins, D. R., Gorentla, B., Liu, W., Gorska, M. M., Chu, H., Martin, R. J., & Alam, R. (2015). Persistence of asthma requires multiple feedback circuits involving type 2 innate lymphoid cells and IL-33. *The Journal of allergy and clinical immunology*, *136*(1), 59–68.e14. https://doi.org/10.1016/j.jaci.2014.11.037

73. Smith, S. G., Chen, R., Kjarsgaard, M., Huang, C., Oliveria, J. P., O'Byrne, P. M., Gauvreau, G. M., Boulet, L. P., Lemiere, C., Martin, J., Nair, P., & Sehmi, R. (2016). Increased numbers of activated group 2 innate lymphoid cells in the airways of patients with severe asthma and persistent airway eosinophilia. *The Journal of allergy and clinical immunology*, 137(1), 75–86.e8. https://doi.org/10.1016/j.jaci.2015.05.037

74. Mjösberg, J. M., Trifari, S., Crellin, N. K., Peters, C. P., van Drunen, C. M., Piet, B., Fokkens, W. J., Cupedo, T., & Spits, H. (2011). Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161. *Nature immunology*, *12*(11), 1055–1062. https://doi.org/10.1038/ni.2104

75. Barnig, C., Cernadas, M., Dutile, S., Liu, X., Perrella, M. A., Kazani, S., Wechsler, M. E., Israel, E., & Levy, B. D. (2013). Lipoxin A4 regulates natural killer cell and type 2 innate lymphoid cell activation in asthma. *Science translational medicine*, *5*(174), 174ra26. https://doi.org/10.1126/scitranslmed.3004812

76. Bartemes, K. R., Kephart, G. M., Fox, S. J., & Kita, H. (2014). Enhanced innate type 2 immune response in peripheral blood from patients with asthma. *The Journal of allergy and clinical immunology*, *134*(3), 671–678.e4. https://doi.org/10.1016/j.jaci.2014.06.024

77. Li, M., Hener, P., Zhang, Z., Ganti, K. P., Metzger, D., & Chambon, P. (2009). Induction of thymic stromal lymphopoietin expression in keratinocytes is necessary for generating an atopic dermatitis upon application of the active vitamin D3 analogue MC903 on mouse skin. *The Journal of investigative dermatology*, *129*(2), 498–502. https://doi.org/10.1038/jid.2008.232

78. Nabekura, T., & Lanier, L. L. (2016). Tracking the fate of antigen-specific versus cytokine-activated natural killer cells after cytomegalovirus infection. *The Journal of experimental medicine*, 213(12), 2745–2758. https://doi.org/10.1084/jem.20160726

79. Sun, J. C., Beilke, J. N., & Lanier, L. L. (2009). Adaptive immune features of natural killer cells. *Nature*, *457*(7229), 557–561. https://doi.org/10.1038/nature07665

80. Paust, S., Gill, H. S., Wang, B. Z., Flynn, M. P., Moseman, E. A., Senman, B., Szczepanik, M., Telenti, A., Askenase, P. W., Compans, R. W., & von Andrian, U. H. (2010). Critical role for the chemokine receptor CXCR6 in NK cell-mediated antigen-specific memory of haptens and viruses. *Nature immunology*, *11*(12), 1127–1135. https://doi.org/10.1038/ni.1953

81. Cooper, M. A., Elliott, J. M., Keyel, P. A., Yang, L., Carrero, J. A., & Yokoyama, W. M. (2009). Cytokine-induced memory-like natural killer cells. Proceedings of the National Academy of Sciences of the United States of America, 106(6), 1915–1919. https://doi.org/10.1073/pnas.0813192106

82. Saeed, S., Quintin, J., Kerstens, H. H., Rao, N. A., Aghajanirefah, A., Matarese, F., Cheng, S. C., Ratter, J., Berentsen, K., van der Ent, M. A., Sharifi, N., Janssen-Megens, E. M., Ter Huurne, M., Mandoli, A., van Schaik, T., Ng, A., Burden, F., Downes, K., Frontini, M., Kumar, V., … Stunnenberg, H. G. (2014). Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. *Science (New York, N.Y.)*, *345*(6204), 1251086. https://doi.org/10.1126/science.1251086

83. Cheng, S. C., Quintin, J., Cramer, R. A., Shepardson, K. M., Saeed, S., Kumar, V., Giamarellos-Bourboulis, E. J., Martens, J. H., Rao, N. A., Aghajanirefah, A., Manjeri, G. R., Li, Y., Ifrim, D. C., Arts, R. J., van der Veer, B. M., Deen, P. M., Logie, C., O'Neill, L. A., Willems, P., van de Veerdonk, F. L., … Netea, M. G. (2014). mTOR- and HIF-1α-mediated aerobic glycolysis as metabolic basis for trained immunity. *Science (New York, N.Y.)*, *345*(6204), 1250684. https://doi.org/10.1126/science.1250684

84. Netea, M. G., & van der Meer, J. W. (2017). Trained Immunity: An Ancient Way of Remembering. *Cell host & microbe*, *21*(3), 297–300. https://doi.org/10.1016/j.chom.2017.02.003

85. Schneider, C., Lee, J., Koga, S., Ricardo-Gonzalez, R. R., Nussbaum, J. C., Smith, L. K., Villeda, S. A., Liang, H. E., & Locksley, R. M. (2019). Tissue-Resident Group 2 Innate Lymphoid Cells Differentiate by Layered Ontogeny and In Situ Perinatal Priming. *Immunity*, *50*(6), 1425–1438.e5. https://doi.org/10.1016/j.immuni.2019.04.019

86. Constantinides, M. G., McDonald, B. D., Verhoef, P. A., & Bendelac, A. (2014). A committed precursor to innate lymphoid cells. *Nature*, *508*(7496), 397–401. https://doi.org/10.1038/nature13047

87. Bando, J. K., Liang, H. E., & Locksley, R. M. (2015). Identification and distribution of developing innate lymphoid cells in the fetal mouse intestine. *Nature immunology*, *16*(2), 153–160. https://doi.org/10.1038/ni.3057

88. Koga, S., Hozumi, K., Hirano, K. I., Yazawa, M., Terooatea, T., Minoda, A., Nagasawa, T., Koyasu, S., & Moro, K. (2018). Peripheral PDGFRα+gp38+ mesenchymal cells support the differentiation of fetal liver-derived ILC2. *The Journal of experimental medicine*, *215*(6), 1609–1626. https://doi.org/10.1084/jem.20172310

89. Ghaedi, M., Shen, Z. Y., Orangi, M., Martinez-Gonzalez, I., Wei, L., Lu, X., Das, A., Heravi-Moussavi, A., Marra, M. A., Bhandoola, A., & Takei, F. (2020). Single-cell analysis of RORα tracer mouse lung reveals ILC progenitors and effector ILC2 subsets. *The Journal of experimental medicine*, *217*(3), jem.20182293. https://doi.org/10.1084/jem.20182293

90. Ricardo-Gonzalez, R. R., Van Dyken, S. J., Schneider, C., Lee, J., Nussbaum, J. C., Liang, H. E., Vaka, D., Eckalbar, W. L., Molofsky, A. B., Erle, D. J., & Locksley, R. M. (2018). Tissue signals imprint ILC2 identity with anticipatory function. *Nature immunology*, *19*(10), 1093–1099. https://doi.org/10.1038/s41590-018-0201-4

91. Moro, K., Kabata, H., Tanabe, M., Koga, S., Takeno, N., Mochizuki, M., Fukunaga, K., Asano, K., Betsuyaku, T., & Koyasu, S. (2016). Interferon and IL-27 antagonize the function of group 2 innate lymphoid cells and type 2 innate immune responses. *Nature immunology*, *17*(1), 76–86. https://doi.org/10.1038/ni.3309

92. Gasteiger, G., Fan, X., Dikiy, S., Lee, S. Y., & Rudensky, A. Y. (2015). Tissue residency of innate lymphoid cells in lymphoid and nonlymphoid organs. *Science (New York, N.Y.)*, *350*(6263), 981–985. https://doi.org/10.1126/science.aac9593

93. Karta, M. R., Rosenthal, P. S., Beppu, A., Vuong, C. Y., Miller, M., Das, S., Kurten, R. C., Doherty, T. A., & Broide, D. H. (2018). β2 integrins rather than β1 integrins mediate Alternaria-induced group 2 innate lymphoid cell trafficking to the lung. *The Journal of allergy and clinical immunology*, *141*(1), 329–338.e12. https://doi.org/10.1016/j.jaci.2017.03.010

94. Stier, M. T., Zhang, J., Goleniewska, K., Cephus, J. Y., Rusznak, M., Wu, L., Van Kaer, L., Zhou, B., Newcomb, D. C., & Peebles, R. S., Jr (2018). IL-33 promotes the egress of group 2 innate lymphoid cells from the bone marrow. *The Journal of experimental medicine*, *215*(1), 263–281. https://doi.org/10.1084/jem.20170449

95. Winkler, C., Hochdörfer, T., Israelsson, E., Hasselberg, A., Cavallin, A., Thörn, K., Muthas, D., Shojaee, S., Lüer, K., Müller, M., Mjösberg, J., Vaarala, O., Hohlfeld, J., & Pardali, K. (2019). Activation of group 2 innate lymphoid cells after allergen challenge in asthmatic patients. *The Journal of allergy and clinical immunology*, *144*(1), 61–69.e7. https://doi.org/10.1016/j.jaci.2019.01.027

96. Lim, A. I., Li, Y., Lopez-Lastra, S., Stadhouders, R., Paul, F., Casrouge, A., Serafini, N., Puel, A., Bustamante, J., Surace, L., Masse-Ranson, G., David, E., Strick-Marchand, H., Le Bourhis, L., Cocchi, R., Topazio, D., Graziano, P., Muscarella, L. A., Rogge, L., Norel, X., … Di Santo, J. P. (2017). Systemic Human ILC Precursors Provide a Substrate for Tissue ILC Differentiation. *Cell*, *168*(6), 1086–1100.e10. https://doi.org/10.1016/j.cell.2017.02.021

97. Vély, F., Barlogis, V., Vallentin, B., Neven, B., Piperoglou, C., Ebbo, M., Perchet, T., Petit, M., Yessaad, N., Touzot, F., Bruneau, J., Mahlaoui, N., Zucchini, N., Farnarier, C., Michel, G., Moshous, D., Blanche, S., Dujardin, A., Spits, H., Distler, J. H., … Vivier, E. (2016). Evidence of innate lymphoid cell redundancy in humans. *Nature immunology*, *17*(11), 1291–1299. https://doi.org/10.1038/ni.3553

98. Huang, Y., Mao, K., Chen, X., Sun, M. A., Kawabe, T., Li, W., Usher, N., Zhu, J., Urban, J. F., Jr, Paul, W. E., & Germain, R. N. (2018). S1P-dependent interorgan trafficking of group 2 innate lymphoid cells supports host defense. *Science (New York, N.Y.)*, *359*(6371), 114–119. https://doi.org/10.1126/science.aam5809

99. Huang, Y., Guo, L., Qiu, J., Chen, X., Hu-Li, J., Siebenlist, U., Williamson, P. R., Urban, J. F., Jr, & Paul, W. E. (2015). IL-25-responsive, lineage-negative KLRG1(hi) cells are multipotential 'inflammatory' type 2 innate lymphoid cells. *Nature immunology*, *16*(2), 161–169. https://doi.org/10.1038/ni.3078

100. Ricardo-Gonzalez, R. R., Schneider, C., Liao, C., Lee, J., Liang, H. E., & Locksley, R. M. (2020). Tissue-specific pathways extrude activated ILC2s to disseminate type 2 immunity. *The Journal of experimental medicine*, *217*(4), e20191172. https://doi.org/10.1084/jem.20191172

101. Mathä, L., Romera-Hernández, M., Steer, C. A., Yin, Y. H., Orangi, M., Shim, H., Chang, C., Rossi, F. M., & Takei, F. (2021). Migration of Lung Resident Group 2 Innate Lymphoid Cells Link Allergic Lung Inflammation and Liver Immunity. *Frontiers in immunology*, *12*, 679509. https://doi.org/10.3389/fimmu.2021.679509

102. Johansson, S. G., Bieber, T., Dahl, R., Friedmann, P. S., Lanier, B. Q., Lockey, R. F., Motala, C., Ortega Martell, J. A., Platts-Mills, T. A., Ring, J., Thien, F., Van Cauwenberge, P., & Williams, H. C. (2004). Revised nomenclature for allergy for global use: Report of the Nomenclature Review Committee of the World Allergy Organization, October 2003. *The Journal of allergy and clinical immunology*, *113*(5), 832–836. https://doi.org/10.1016/j.jaci.2003.12.591

103. Novak, N., & Bieber, T. (2003). Allergic and nonallergic forms of atopic diseases. *The Journal of allergy and clinical immunology*, *112*(2), 252–262. https://doi.org/10.1067/mai.2003.1595

104. Johansson, S. G., Bieber, T., Dahl, R., Friedmann, P. S., Lanier, B. Q., Lockey, R. F., Motala, C., Ortega Martell, J. A., Platts-Mills, T. A., Ring, J., Thien, F., Van Cauwenberge, P., & Williams, H. C. (2004). Revised nomenclature for allergy for global use: Report of the Nomenclature Review Committee of the World Allergy Organization, October 2003. *The Journal of allergy and clinical immunology*, *113*(5), 832–836. https://doi.org/10.1016/j.jaci.2003.12.591

105. Thomsen S. F. (2015). Epidemiology and natural history of atopic diseases. *European clinical respiratory journal*, *2*, 10.3402/ecrj.v2.24642. https://doi.org/10.3402/ecrj.v2.24642

106. Rackemann F. M. (1969). Nervous factors in asthma. *Transactions of the American Clinical and Climatological Association*, 80, 50–53.

107. Bantz, S. K., Zhu, Z., & Zheng, T. (2014). The Atopic March: Progression from Atopic Dermatitis to Allergic Rhinitis and Asthma. *Journal of clinical & cellular immunology*, *5*(2), 202. https://doi.org/10.4172/2155-9899.1000202

108. Locksley R. M. (2010). Asthma and allergic inflammation. *Cell*, *140*(6), 777–783. https://doi.org/10.1016/j.cell.2010.03.004

109. Boonpiyathad, T., Sözener, Z. C., Satitsuksanoa, P., & Akdis, C. A. (2019). Immunologic mechanisms in asthma. *Seminars in immunology*, *46*, 101333. https://doi.org/10.1016/j.smim.2019.101333

110. Dunn, R. M., Busse, P. J., & Wechsler, M. E. (2018). Asthma in the elderly and late-onset adult asthma. *Allergy*, *73*(2), 284–294. https://doi.org/10.1111/all.13258

111. Gregson, K. S. & Bennett, J. D. (2017). Drugs Acting on the Respiratory System. *Pharmacology and Therapeutics for Dentistry: Seventh Edition*. 392–403. https://doi.org/10.1016/B978-0-323-39307-2.00027-8

112. Dharmage, S. C., Perret, J. L., & Custovic, A. (2019). Epidemiology of Asthma in Children and Adults. *Frontiers in pediatrics*, *7*, 246. https://doi.org/10.3389/fped.2019.00246

113. Song, P., Adeloye, D., Salim, H., Dos Santos, J. P., Campbell, H., Sheikh, A., & Rudan, I. (2022). Global, regional, and national prevalence of asthma in 2019: a systematic analysis and modelling study. *Journal of global health*, *12*, 04052. https://doi.org/10.7189/jogh.12.04052

114. Eder, W., Ege, M. J., & von Mutius, E. (2006). The asthma epidemic. *The New England journal of medicine*, *355*(21), 2226–2235. https://doi.org/10.1056/NEJMra054308

115. Ober, C., & Yao, T. C. (2011). The genetics of asthma and allergic disease: a 21st century perspective. *Immunological reviews*, *242*(1), 10–30. https://doi.org/10.1111/j.1600-065X.2011.01029.x

116. Fuseini, H., & Newcomb, D. C. (2017). Mechanisms Driving Gender Differences in Asthma. *Current allergy and asthma reports*, *17*(3), 19. https://doi.org/10.1007/s11882-017-0686-1

117. Nieves, A., Magnan, A., Boniface, S., Proudhon, H., Lanteaume, A., Romanet, S., Vervloet, D., Godard, P., & ARIA (2005). Phenotypes of asthma revisited upon the presence of atopy. *Respiratory medicine*, *99*(3), 347–354. https://doi.org/10.1016/j.rmed.2004.08.004

118. Bochner, B. S., Undem, B. J., & Lichtenstein, L. M. (1994). Immunological aspects of allergic asthma. *Annual review of immunology*, *12*, 295–335. https://doi.org/10.1146/annurev.iy.12.040194.001455

119. Peters S. P. (2014). Asthma phenotypes: nonallergic (intrinsic) asthma. *The journal of allergy and clinical immunology*. *2*(6), 650–652. https://doi.org/10.1016/j.jaip.2014.09.006

120. Kuruvilla, M. E., Lee, F. E., & Lee, G. B. (2019). Understanding Asthma Phenotypes, Endotypes, and Mechanisms of Disease. *Clinical reviews in allergy & immunology*, *56*(2), 219–233. https://doi.org/10.1007/s12016-018-8712-1

121. Svenningsen, S., & Nair, P. (2017). Asthma Endotypes and an Overview of Targeted Therapy for Asthma. *Frontiers in medicine*, *4*, 158. https://doi.org/10.3389/fmed.2017.00158

122. Sroka-Tomaszewska, J., & Trzeciak, M. (2021). Molecular Mechanisms of Atopic Dermatitis Pathogenesis. *International journal of molecular sciences*, *22*(8), 4130. https://doi.org/10.3390/ijms22084130

123. Hadi, H. A., Tarmizi, A. I., Khalid, K. A., Gajdács, M., Aslam, A., & Jamshed, S. (2021). The Epidemiology and Global Burden of Atopic Dermatitis: A Narrative Review. *Life (Basel, Switzerland)*, *11*(9), 936. https://doi.org/10.3390/life11090936

124. Asher, M. I., Montefort, S., Björkstén, B., Lai, C. K., Strachan, D. P., Weiland, S. K., Williams, H., & ISAAC Phase Three Study Group (2006). Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. *Lancet (London, England)*, *368*(9537), 733–743. https://doi.org/10.1016/S0140-6736(06)69283-0

125. Weidinger, S., & Novak, N. (2016). Atopic dermatitis. *Lancet (London, England)*, *387*(10023), 1109–1122. https://doi.org/10.1016/S0140-6736(15)00149-X

126. Chen, L., Overbergh, L., Mathieu, C., & Chan, L. S. (2008). The development of atopic dermatitis is independent of Immunoglobulin E up-regulation in the K14-IL-4 SKH1 transgenic mouse model. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology*, *38*(8), 1367–1380. https://doi.org/10.1111/j.1365-2222.2008.02987.x

127. Schmid-Grendelmeier, P., Simon, D., Simon, H. U., Akdis, C. A., & Wüthrich, B. (2001). Epidemiology, clinical features, and immunology of the "intrinsic" (non-IgE-mediated) type of atopic dermatitis (constitutional dermatitis). *Allergy*, *56*(9), 841–849. https://doi.org/10.1034/j.1398-9995.2001.00144.x

128. Stübner, U. P., Gruber, D., Berger, U. E., Toth, J., Marks, B., Huber, J., & Horak, F. (1999). The influence of female sex hormones on nasal reactivity in seasonal allergic rhinitis. *Allergy*, *54*(8), 865–871. https://doi.org/10.1034/j.1398-9995.1999.00961.x

129. Yamatomo, T., Okano, M., Ono, T., Nakayama, E., Yoshino, T., Satoskar, A. R., Harn, D. A., Jr, & Nishizaki, K. (2001). Sex-related differences in the initiation of allergic rhinitis in mice. *Allergy*, *56*(6), 525–531. https://doi.org/10.1034/j.1398-9995.2001.056006525.x

130. Novembre, E., Cianferoni, A., Lombardi, E., Bernardini, R., Pucci, N., & Vierucci, A. (2001). Natural history of "intrinsic" atopic dermatitis. *Allergy*, *56*(5), 452–453. https://doi.org/10.1034/j.1398-9995.2001.056005452.x

131. Han, H., Roan, F., & Ziegler, S. F. (2017). The atopic march: current insights into skin barrier dysfunction and epithelial cell-derived cytokines. *Immunological reviews*, *278*(1), 116–130. https://doi.org/10.1111/imr.12546

132. Barberio, G., Pajno, G. B., Vita, D., Caminiti, L., Canonica, G. W., & Passalacqua, G. (2008). Does a 'reverse' atopic march exist?. *Allergy*, *63*(12), 1630–1632. https://doi.org/10.1111/j.1398-9995.2008.01710.x

133. Burgess, J. A., Dharmage, S. C., Byrnes, G. B., Matheson, M. C., Gurrin, L. C., Wharton, C. L., Johns, D. P., Abramson, M. J., Hopper, J. L., & Walters, E. H. (2008). Childhood eczema and asthma incidence and persistence: a cohort study from childhood to middle age. *The Journal of allergy and clinical immunology*, *122*(2), 280–285. https://doi.org/10.1016/j.jaci.2008.05.018

134. Shaaban, R., Zureik, M., Soussan, D., Neukirch, C., Heinrich, J., Sunyer, J., Wjst, M., Cerveri, I., Pin, I., Bousquet, J., Jarvis, D., Burney, P. G., Neukirch, F., & Leynaert, B. (2008). Rhinitis and onset of asthma: a longitudinal population-based study. *Lancet (London, England)*, *372*(9643), 1049–1057. https://doi.org/10.1016/S0140-6736(08)61446-4

135. Burgess, J. A., Walters, E. H., Byrnes, G. B., Matheson, M. C., Jenkins, M. A., Wharton, C. L., Johns, D. P., Abramson, M. J., Hopper, J. L., & Dharmage, S. C. (2007). Childhood allergic

rhinitis predicts asthma incidence and persistence to middle age: a longitudinal study. *The Journal of allergy and clinical immunology*, *120*(4), 863–869. https://doi.org/10.1016/j.jaci.2007.07.020

136. Leynaert, B., Neukirch, C., Kony, S., Guénégou, A., Bousquet, J., Aubier, M., & Neukirch, F. (2004). Association between asthma and rhinitis according to atopic sensitization in a population-based study. *The Journal of allergy and clinical immunology*, *113*(1), 86–93. https://doi.org/10.1016/j.jaci.2003.10.010

137. von Kobyletzki, L. B., Bornehag, C. G., Hasselgren, M., Larsson, M., Lindström, C. B., & Svensson, Å. (2012). Eczema in early childhood is strongly associated with the development of asthma and rhinitis in a prospective cohort. *BMC dermatology*, *12*, 11. https://doi.org/10.1186/1471-5945-12-11

138. Carlsten, C., Dimich-Ward, H., Ferguson, A., Watson, W., Rousseau, R., Dybuncio, A., Becker, A., & Chan-Yeung, M. (2013). Atopic dermatitis in a high-risk cohort: natural history, associated allergic outcomes, and risk factors. *Annals of allergy, asthma & immunology: official publication of the American College of Allergy, Asthma, & Immunology*, *110*(1), 24–28. https://doi.org/10.1016/j.anai.2012.10.005

139. Saunes, M., Øien, T., Dotterud, C. K., Romundstad, P. R., Storrø, O., Holmen, T. L., & Johnsen, R. (2012). Early eczema and the risk of childhood asthma: a prospective, population-based study. *BMC pediatrics*, *12*, 168. https://doi.org/10.1186/1471-2431-12-168

140. Strachan, D. P., Butland, B. K., & Anderson, H. R. (1996). Incidence and prognosis of asthma and wheezing illness from early childhood to age 33 in a national British cohort. *BMJ (Clinical research ed.)*, *312*(7040), 1195–1199. https://doi.org/10.1136/bmj.312.7040.1195

141. Romera-Hernández, M., Mathä, L., Steer, C. A., Ghaedi, M., & Takei, F. (2019). Identification of Group 2 Innate Lymphoid Cells in Mouse Lung, Liver, Small Intestine, Bone Marrow, and Mediastinal and Mesenteric Lymph Nodes. *Current protocols in immunology*, *125*(1), e73. https://doi.org/10.1002/cpim.73

142. Li, M., Hener, P., Zhang, Z., Kato, S., Metzger, D., & Chambon, P. (2006). Topical vitamin D3 and low-calcemic analogs induce thymic stromal lymphopoietin in mouse keratinocytes and trigger an atopic dermatitis. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(31), 11736–11741. https://doi.org/10.1073/pnas.0604575103