The role of Interleukin-4 induced gene 1 (IL-4i1) in allergic asthma and atopic dermatitis

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Declaration

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Abstract

Allergies are described as an unnecessary immune response to non-harmful substances known as allergens. Both allergic asthma and atopic dermatitis (AD) are said to be induced by elevated levels of immunoglobulin E (IgE) and T helper 2 (Th2) immune cells and inflammatory associated cells such as eosinophils, mast, and basophils. Globally, asthma is affecting more than 300 million people and is characterized by chronic airway inflammation, reversible airflow limitation, and airway hyperreactivity. AD is affecting approximately 15%-20% of the pediatric population and 7%-10% of adults in the world and is characterized by dysregulation of skin barrier and immunity, eczematous lesions, dry and itchy skin. Dysfunctional tolerogenic immune response to these innocuous allergens has been described as a leading cause of allergic disease pathogenesis. Interleukin-4 induced gene 1 (IL-4i1) is a secreted L-amino acid oxidase enzyme mainly expressed by antigen-presenting cells (APCs) and upon activation by IL-4 and CD40, can be induced in B lymphocytes. IL-4i1 converts phenylalanine into phenylpyruvate, ammonia, and hydrogen peroxide which can induce effector T cells suppression by inhibiting their activation, proliferation, and cytokine production while promoting a regulatory T cell (Tregs) arm. The contribution of IL-4i1 and its immunoregulatory potential has not yet been explored in allergic asthma and atopic dermatitis. Thus, we proposed to investigate the role of IL-4i1 during allergic asthma and atopic dermatitis using acute mouse models. Female mice of 8-12 weeks old either sufficient (IL-4i1$^{+/+}$) or deficient of IL-4i1 (IL-4i1$^{-/-}$) backcrossed to BALB/c genetic background were used in this study. For induction of allergic asthma, a high dose (100µg/per mouse) of house dust mite (HDM) was used in sensitizing mice intratracheally at day 0 and challenged at day 7 to 11 intranasally under anaesthesia. To assess the development of asthma features, we measured lung function on day 14 and collected blood for ELISAs, mediastinal lymph nodes, and lung tissues for FACS and RNA. For induction of AD, a skin irritant vitamin D3 analog (MC903) was used to topically sensitize shaved mice (IL-4i1$^{+/+}$ or IL-4i1$^{-/-}$) for 9 consecutive days. We assessed disease score and skin inflammation at day 10 and collected blood, inguinal lymph nodes, and skin for ELISAs, FACS, RNA, and histology analysis. In both disease models, we saw a significant reduction in total IgE in IL-4i1- deficient mice compared to IL-4i1$^{+/+}$ littermate controls. A significant upregulation of Th2 cytokines and increased eosinophilia was seen in IL-4i1 deficient mice in the allergic asthma model with no changes in airway hyperresponsiveness. In AD model, we observed a protective effect in the absence of IL-4i1, which was demonstrated by no changes in body weight a reduced skin epidermal thickness, and reduced systemic type 2 cytokines, TSLP, IL-5, and IL-13 producing CD8 T cells. Furthermore, type 2 alarmin, TSLP was reduced at disease site. These results suggest a dichotomy of IL-4i1 in regulation of type 2 immune responses depending on disease site. This data further suggests that IL-4i1 may be a potential target for therapy against these diseases. Studies are currently underway to

understand how IL-4i1 is induced and how it regulates downstream effector molecules and how these target molecules can be inhibited.

Abbreviations

AD – Atopic Dermatitis

AHR – Airway hyperresponsiveness

AhR – Aryl hydrocarbon receptor

AMP – Antimicrobial peptides

AP – Alkaline phosphatase

APC – Antigen presenting cells

BAL – Bronchoalveolar lavage

BALF – Bronchoalveolar lavage fluid

BCR – B cell receptor

CD – Cluster designation

cDNA – complementary deoxyribonucleic acid

CNS – Central nervous system

COPD – Chronic obstructive pulmonary disease

cRPMI – complete Roswell Park Memorial Institute

DCs – Dendritic cells

EDTA – Ethylenediaminetetraacetic acid

ELISA – Enzyme-linked immunosorbent assay

FACS – Fluorescence-activated cell sorting

FBS – Fetal bovine serum

FITC – Fluorescein Isothiocyanate

FLG – Filaggrin

GA – Granuloma annulare

gAMPs – granular anti-microbial peptides

GINA – Global Initiative for Asthma

H&E – Haematoxylin and Eosin stain

HDM – House dust mite

HRP – Horseradish peroxide

ICS – Inhaled corticosteroids

IFN-γ – Interferon gamma

Ig – immunoglobulin

IL – Interleukin

ILC – Innate lymphoid cells

iLNs – Inguinal lymph nodes

KO – Knock out

MAIT – mucosal-associated invariant T Cells

MDSCs – Myeloid-derived suppressor cells

MLNs – Mediastinal lymph nodes

MMPs – Matrix metalloproteinases

NETS – Neutrophil extracellular traps

NK – Natural killer cells

NKT – Natural killer T cell

NO – Nitric Oxide

PAMPs – Pathogen-associated molecular patterns

PAS – Periodic acid-Schiff stain

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PMA – Phorbol 12-myristate 13-acetate

PRRs – Pattern-recognition receptors

qRT-PCR – Quantitative reverse transcription polymerase chain reaction

RNA – Ribonucleic acid

ROIs – Regions of interest

ROR – Retinoic acid orphan receptor

ROS – Reactive oxygen species

RT – Room temperature

RT-PCR – Reverse transcription polymerase chain reaction

SB – Stratum basale

SC – Stratum corneum

SD – Standard deviation

SG – Stratum granulosum

SLE – Systemic lupus erythematosus

SS – Stratum spinosum

STAT – Signal transducer and activator of transcription

TCRs – T cell receptors

TEWL – Trans-epidermal water loss

Th – T helper cells

TLRs – Toll-like receptors

TMB – 3.3',5.5' – Tetramethylbenzidine

TNF – Tumour Necrosis Factor

Tregs – Regulatory T cells

TSLP – Thymic stromal lymphopoietin

WT – Wild-type

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Chapter 1: Literature review

1. Immune system

The immune system is described as an organization of molecules and cells with functional roles in protecting against disease[1]. As a defence system, our immune system consists of physiological and first-line anatomical barriers (skin, respiratory tract, and gastrointestinal mucosa), innate immunity, and adaptive immunity[2,3]. Non-specific recognition of pathogens is employed by cells of the innate immunity and includes eosinophils, dendritic cells, neutrophils, natural killer cells (NK cells), macrophages, innate lymphoid cells, and mast cells[4,3,5,6]. The innate immune system which utilizes a set of invariant pattern-recognition receptors (PRRs) for recognising pathogen-associated molecular patterns (PAMPs), acts as a second line of defence that is fast and strikingly effective in the clearance of most invading pathogens[7].

In contrast, the adaptive immune system relies on specific recognition of antigens via B cell or T cell receptors and is slower compared to innate immunity[4]. Characteristics of adaptive immunity include clonal expansion to produce an adequate number of cells to attack the pathogen, differentiation to effector cells in order to destroy and terminate the pathogen, and lastly the maintenance of small fragments of antigen-specific memory cells to mount a faster and stronger response upon pathogen re-encounter[3].

In 1908, a Nobel prize in Medicine was given to Elie Metchnikoff for discovering phagocytic cells which were capable of engulfing and destroy invading pathogens, and this laid foundation for innate immunity[8,9,2]. Another Nobel prize in Medicine was given to Paul Ehrlich and Emil Behring for identifying antibodies that neutralize microbial toxins, and this formed the basis for adaptive immunity[8,9]. Infectious non-self-theory of immunity was then introduced in 1989 by Charles Janeway. This theory stated that the innate immune system acts as a sensor of pathogenic invaders and that antigen-presenting cells (APCs) employed PRRs in discriminating between infectious non-self and non-infectious self[7,10]. Recently, a joint Nobel prize in Medicine was given to Jim Allison and Tasuku Honjo for discovering cancer therapy through inhibition of negative immune regulation[8].

1.1 Innate cells

1.1.1 Macrophages

Macrophages are said to be the mature form of monocytes. Monocytes are produced in the bone marrow by hematopoietic stem cells and circulate in the blood in low amounts, they then undergo differentiation to become macrophages. Macrophages are also thought to develop from fetal liver and yolk sac to become residents in tissues like the lung (alveolar macrophages), brain (microglia), and liver (Kupffer cells) and are long-lived[11]. These tissue-resident macrophages can be

replenished from bone marrow-derived monocytes, particularly during infection[12]. Macrophages detect and directly destruct pathogens through phagocytosis, initiate inflammatory responses, and eliminate malignant cells[3]. Macrophages also function as APCs as they can internalize pathogens and present processed peptides on their surfaces to T cells, thus activating adaptive immune response[4,3]. Through secretion and production of interleukin-1 (IL-1) and Tumour Necrosis Factor (TNF), which subsequently results in activation and recruitment of more immune cells, macrophages are able to directly or indirectly eliminate target pathogens[3,4].

1.1.2 Dendritic cells (DCs)

Dendritic cells originate from hematopoietic myeloid progenitor cells. They first transform into immature DCs which then travel from the blood and reside in tissues. At this stage, they possess macropinocytic and phagocytic abilities and also ingest huge amounts of surrounding extracellular fluid[4,9]. Pattern recognition receptors such as Toll-like receptors (TLRs) are used by the immature DCs when scouting for pathogens in the surrounding environment[13]. When they encounter a pathogen, they mature rapidly and migrate to secondary lymph tissues where they facilitate activation of antigen-specific T lymphocytes[9]. DCs are also defined as professional antigen-presenting cells because of their efficient ability to recognize PAMPs, taking up and processing pathogens to present MHC-bound antigens to T lymphocytes[3].

1.1.3 Basophils

Basophils arise from $CD34^+$ progenitor cells, after differentiation and maturation in the bone marrow they circulate in the periphery with a half-life of a few days[14]. Basophils express cytokine receptors (IL-5R, IL-3R, GM-CSFR), prostaglandin D_2 receptor 2 (CRTH2), chemokine receptors (CCR3, CCR2), complement receptors (CD35, CD11b, CD88), TLRs and Fc receptors ($Fc\varepsilon RI$, $Fc\gamma RIIb$). Together, these are important for basophil activation, degranulation, mediator release, and granule exocytosis[14]. Physiologically, the role of basophils remains unknown however they are said to play a role in host defence against parasites through secretion of early IL-4 and acting as APCs[15,16]. Basophils can infiltrate the skin of patients with atopic dermatitis and airways of patients with respiratory inflammation[14,17].

1.1.4 Mast cells

They derive from hematopoietic stem cells in the bone marrow, terminally differentiate as immature progenitors in tissues where they eventually become residents[17,9]. Interleukins IL-3, -4, and -9, nerve growth factor and stem cell factor are involved in mast cells growth

and differentiation in both human and rodent immune system[17]. Mast cells contribute to wound healing and homeostasis, once activated they release mediators such as tryptase, histamine, heparin, and newly synthesized eicosanoids[14,18]. These mediators are known to promote vascular permeability[9,18]. Mast cells activation through the IgE high-affinity receptor FcεRI is critical to allergic diseases pathogenesis as this leads to the release of immunomodulatory cytokines such as TNF-α, IL-4, and IL-5[14,18,19].

1.1.5 Eosinophils

Eosinophils originate from the bone marrow and are released into circulation post-stimulation with IL-5[14]. The development of eosinophils from $CD34^+$ hematopoietic progenitor cells is promoted by IL-5, GM-CSF, and IL-3, but eosinophil development and differentiation, maturation, and recruitment is specific to IL-5 only[14,18,17]. Expression of IL-5 receptor, CD34, and CCR3 is used in the identification of progenitors committed to eosinophil lineage[14]. Eosinophils are said to be important mainly in defence against parasitic infections[9]. However, in allergic diseases, an increase in tissue eosinophils numbers, eosinophil numbers in bronchoalveolar lavage fluid and sputum suggest a role of eosinophils in airway hyperreactivity, airway remodelling, and mucus secretion[14].

1.1.6 Neutrophils

Neutrophils develop from the bone barrow and circulate briefly in peripheral blood as the most abundant leukocytes before they subsequently migrate to tissues[20,21]. Granulocyte-macrophage colony-stimulating factor receptor and the subset of transcription factors (PU1, STAT3, HoxB7) are responsible for regulating the development of neutrophils from default myeloid progenitor[22]. Neutrophils act as the first line of defence against a wide range of microbial insults (fungi, bacteria, protozoa). Their host defence mechanisms include expulsion of granular anti-microbial peptides (gAMPs), matrix metalloproteinases (MMPs), microorganism phagocytosis followed by their degradation via reactive oxygen species (ROS) within phagolysosomes and trapping and applying microbicidal effects through neutrophil extracellular traps (NETs)[21,23].

1.2 Adaptive cells

1.2.1 T helper cells (Th1, Th17, T regs)

Production of T-lymphocytes (T cells) occurs in the bone marrow from the pluripotent hematopoietic stem cell, and they migrate to the thymus for completion of their development[24,1].

Antigen recognition by T cells is achieved by their T cell receptors (TCRs), and TCRs of some T cells develop as they mature, and these possess a binding affinity to MHC class II antigens, these cells will then become T helper (Th) cells and are distinguished by the expression of CD4 molecule ($CD4^+$ T cells)[24], while other T cells during their maturation develop TCRs which possess an affinity to bind to MHC class I antigens and become cytotoxic T cells expressing CD8 molecule ($CD8^+$ T cells)[24]. $CD4^+$ T helper cells play a significant role in initiating immune response by supplying assistance to other cells (B cells, macrophages). T helper cell activation occurs after antigen presentation by MHC II molecules expressed on the surface of APCs[24]. T helper cells subsets are categorised into Th1, Th2, and Th17 characterized by their prominent cytokine production and functions[24,25] (Figure 1.1). Recently, identification of T helper 22 (Th22) and T follicular helper (Tfh) T cells has broadened the spectrum of CD4+ T cell subsets[26].

1.2.1.1 Th1 cells

Th1 cell phenotype arises from naïve $CD4^+$ T cells activation in the presence of IL-12[5]. Production of Th1 cells is essential in response to eliminate intracellular pathogens and contributes to cell-mediated immunity and delayed-type hypersensitivity reactions[4]. Type II interferons and IL-12 activate STAT4 in naïve T helper cells, while STAT1 becomes activated by a robust TCR signalling. STAT1 then facilitates T-bet expression, resulting in the production of IFN- γ thus protecting the host from microbial and viral threats[27,4,25]. The signature cytokine that defines Th1 cells is the expression of transcriptional factor T bet.

1.2.1.2 Th22 cells

Differentiation of Th22 cells occurs upon expression of aryl hydrocarbon receptor, and act on epithelial cells via secretion of the cytokine IL-22. These cells are also known to promote wound healing and tissue protection against damage[26]. This IL-22 produced by Th22 cells is said to be responsible for skin barrier impairment and epidermal hyperplasia[28].

1.2.1.3 Tfh cells

Some naïve CD4+ T cells upon activation upregulate the transcription factor Bcl6 and migrate into B cell follicles where they become resident Tfh cells contributing to germinal center formation[26]. These Tfh cells are also known to play a role in isotype switching and affinity maturation[29].

1.2.1.4 Th17 cells

Th17 cells are essential in host defence against fungal and bacterial infections and autoimmune diseases development and secrete IL-17A cytokine[25,27,4,24], however, an implication of these cells in moderate to severe asthma has been reported[25]. Th17 cell differentiation from naïve cells is programmed and regulated by a combination of cytokines (IL-6, TGF-β, IL-1β) and TCR stimulation[25]. These cytokines induce the expression of RORγt transcription factor, which is

crucial in Th17 development[4,25]. IL-23 cytokine is thought to be essential in the maintenance of these cells.

1.2.1.5 Regulatory T (T reg) cells

They are defined as a specific $CD4^+$ T cell population that is essential for maintaining immunological tolerance to self to prevent autoimmune diseases[30,24]. T regs secrete IL-10 that induces an immunosuppressive effect on Th1/Th2 effector cells, and reports have suggested that TGF-β secreted by T regs has essential regulatory effects[31]. Transcription factor Foxp3 is a crucial regulator of T reg cell development and function[32,30].

1.2.2 T helper 2 (Th2) cells

Signal transducer and activator of transcription 6 (STAT6) activation downstream of IL-4 receptor alpha signalling pathway are needed in the development of Th2 cells from naïve T cell[33,34]. STAT6 phosphorylation leads to the induction of transcription factor GATA3, which plays a crucial part in the differentiation of Th2 cells, and also serves as a transcriptional activator of Th2 cytokine genes[35,36]. This differentiation of Th2 cells is induced upon TCR stimulation in combination with Th2 cell-favouring B7 co-stimulatory molecules and cytokines expressed by DCs[25]. Differentiation from naïve $CD4^+$ T cells occurs only when allergens are presented on MHC class II complex molecules by DCs[37]. Through secretion of cytokines IL-4, -5, and -13, Th2 cells drive B cell isotype class switching to IgE and IgG1, polarization of macrophages into M2-like phenotype, promote eosinophil recruitment in airways, and induce mucus secretion through goblet cell metaplasia[38,39,33,40,41]. Th2 cells are essential for host immunity against extracellular parasites such as helminths, however, they are also implicated in asthma development and other allergic inflammatory diseases[41,42].

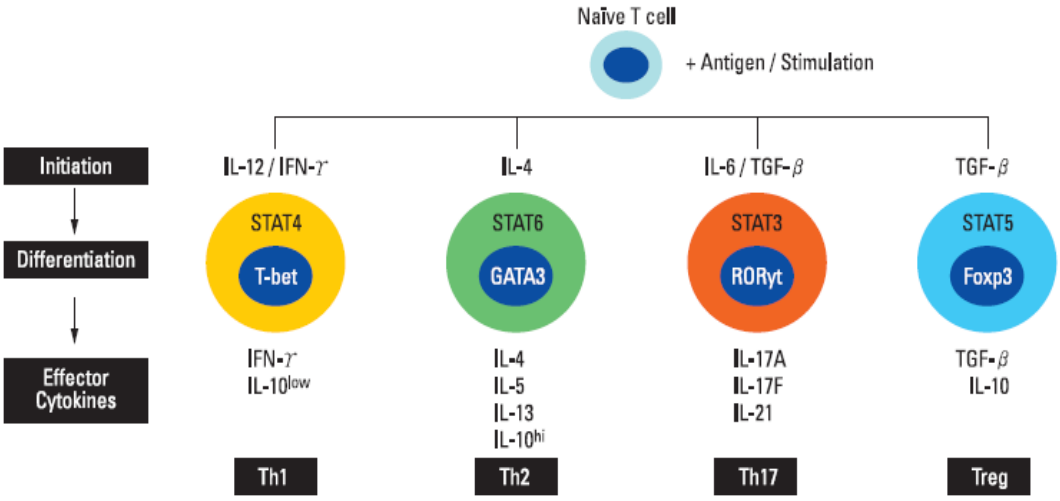

Figure 1.1: Naïve CD4+ T helper cell diversification into several effector T helper cell lineages. Adapted from Lee *et al*., 2009. Upon antigen stimulation, naïve $CD4^+$ T cells can differentiate into diverse effector subsets[43]. IFN-γ and IL-12 maintain and express transcription factor T-bet and are key for the development of Th1 cells[43,44]. Th2 cells are dependent on IL-4 and STAT6 for the increased GATA3 expression, while TGF-β and IL-6 are polarizing cytokines for Th17 cell development and RORγt regulates their differentiation[44]. TGF-β and transcription factor Foxp3 expression regulate Tregs[44].

1.2.3 B cells

B cells develop in the bone marrow from pluripotent hematopoietic stem cells, and migrate to secondary lymphoid organs for completion of their maturation and express B cell receptor (BCR) on their membrane for antigen binding[24,45]. Upon activation by foreign antigens, B cells undertake activation, clonal activation, and differentiation to become antibody-secreting cells known as plasma cells[5]. IL-4 production promotes antigen-stimulated B cells to class switch their constant region isotype from immunoglobulin M (IgM) and IgD expressed in naïve cells to IgG or IgE isotypes[46]. Two signals which are provided by Th2 cells induce B cells isotype switching to IgE production.

The first signal is supplied by IL-4/IL-13 interaction with receptors on the B cell surface. These then transduce their signal by activating the Janus family tyrosine kinases (JAK1 and JAK3), which then results in phosphorylation of the transcriptional regulator STAT6[9]. The second signal is a costimulatory interaction between CD40 ligand on the T cell surface with CD40 on the B cell surface. This interactivity is significant for all antibody class switching[9]. Through allergen cross-linking, synthesized specific IgE can bind to high-affinity IgE receptors (FcεRI) on mast cells and basophils[47]. This then results in the degranulation of mast cell and the release of vasoactive peptides such as histamine[48].

IgE participates in defence against parasitic infections, however, is also associated with allergic reactions and is said to be functioning by upregulating the expression of an Fc receptor on some myeloid cells, while IgG is involved in the direct neutralization of toxins and assists in complement activation[49,5].

1.2.4 Natural Killer, Natural Killer T cells, and Mucosal associated invariant T cells

Natural Killer (NK) cells develop from hematopoietic stem cells and are involved in the killing of malignant and tumour cells[50]. Temporal orchestration of transcription factors Id2 and PU.1 is required to regulate NK cells development[50]. NK cells secrete cytotoxic granules (perforins and granzyme) in their immune synapse resulting in the killing of infected target cells by apoptosis[5,6,45]. Natural killer T cells (NKT) possess cell surface receptors that belong to NK and T cells and are believed to be implicated in suppressing cell-mediated autoimmune responses and have been implicated in *Aspergillus fumigatus* induced allergic asthma[51,5]. Mucosal-associated invariant T (MAIT) cells develop from the thymus and are said to be the most abundant subset of T cells which are involved in tissue repair and homeostasis and antimicrobial host defence[52,53]. MAIT cells are defined by their expression of semi-invariant αβ TCRs which are capable of recognizing microbial-derived derivatives of riboflavin synthesis presented on the MHC-related protein-1 (MR1)[54,55].

2. Hypersensitivity

2.1 Gell and Coomb's classification of four types of hypersensitivity

Hypersensitivity reactions are described as undesirable and extreme immune responses against an antigen or an allergen[45]. These reactions are classified by Gell and Coomb into four types based on the type of immune response and effector mechanism responsible for cell and tissue damage: type I, immediate/IgE-mediated; type II, cytotoxic or IgG/IgM-mediated; type III, immune complex-mediated; and type IV, delayed-type hypersensitivity[56,57,58].

Atopic persons may produce allergen-specific IgE antibodies in response to allergens present in foods, drugs, and the environment[56]. The formed IgE antibodies bind to high-affinity IgE receptors which are found on the surface of mast cells and basophils causing them to be sensitized, upon re-exposure to the allergen crosslinks the bound IgE on sensitized cells and cause degranulation and secretion of mediators such as histamine[56,59]. This is known as the immediate hypersensitivity reaction. And examples of this type of reaction include allergic asthma, angioedema, anaphylaxis, atopic dermatitis[56,59,45].

Type II hypersensitivity reactions occur when IgG and IgM antibodies bind to the patient's cell surface molecules and form complexes that activate the complement system. This is then followed by cell opsonization, agglutination of red blood cells, cell lysis, and death by phagocytes expressing Fc receptors for antibodies and complement proteins[45,56]. Antibody-dependent cell-mediated cytotoxicity is another subtype of type II hypersensitivity reaction, whereby cells displaying the foreign antigen are tagged with IgG or IgM, these cells are then recognized by macrophages and NK cells, which subsequently kill these cells[59,60]. Examples of this reaction include Goodpasture's syndrome, immune thrombocytopenia[59].

Type III hypersensitivity reactions develop when IgG and IgM antibodies bind to soluble proteins and thus form immune complexes in the blood that then deposit in tissues and activate the classical complement pathway, with a consequent reduction in serum complement levels[57,56]. Activation of the complement components promotes neutrophil influx and mast cell degranulation and results in tissue injury and inflammation[56]. Examples of type III hypersensitivity reactions include systemic lupus erythematosus (SLE), celiac disease, serum sickness, and Arthur's reaction[59].

Type IV delayed hypersensitivity reactions are antibody-independent and cell-mediated and are induced by overstimulation of T cells, monocytes/macrophages by an antigen in a complex, which promotes the release of pro-inflammatory cytokines, tissue damage, and cell death[45]. Examples of this reaction include contact dermatitis, tuberculosis, and chronic transplant rejection[45,59].

2.2 Allergy and allergens

Allergy is described as an exaggerated immune response to a harmless environmental substance (allergen)[61]. An allergen can only be defined operationally as substances that elicit an allergic response, examples include pollens, animal dander, house dust mites, and grass, these can trigger the immune system to respond in a harmful way[61]. House dust mite (HDM) is known to be a common trigger of asthma in humans, and in mice, chronic exposure to HDM induces asthma-like pathology[62]. *D. pteronisunnus* and *D. farinae* are the two most common HDM species that are widely spread around the world[63]. In allergic diseases such as allergic rhinitis, anaphylaxis, and allergic asthma, allergic responses are identified by the involvement of allergen-specific IgE and Th2 cells that are involved in the recognition of allergen-derived antigens[64]. The allergen's ability to induce a Th2 cell reaction, in which IL-4 and IL-13 drive IgE production by promoting immunoglobulin class-switch recombination by B cells is known as sensitization[64].

2.3 Hygiene hypothesis versus non-hygiene hypothesis

An increased frequency of persons with allergies worldwide has been proposed to be explained by the hygiene hypothesis[61]. This hypothesis suggested that a cleaner environment may lead to allergic disease development, noting that in children early exposure to parasites and microbes led to a reduction in diseases[61,31,65]. Changes in diet, living environment, weight, and lifestyle greatly influence the diversity and composition of gut and skin microbiome[65]. Thus, alternatively to the hygiene hypothesis, an 'old friend' hypothesis by Rook and Haahtelah- 'the biodiversity hypothesis of allergy were proposed stating that the observed increase in allergies is due to symbiotic relation loss with bacteria and parasites that were once beneficial to our evolution[65].

3. Allergic asthma

3.1 Epidemiology, symptoms, phenotypes, and treatment of asthma

The Global Initiative for Asthma (GINA) describes asthma as a common heterogenous disease, generally categorized by chronic inflammation of the airways[66]. It is described by the history of respirational symptoms such as shortness of breath, cough, wheezing and chest tightness that may differ in intensity over time[67]. The heterogeneity of this disease is characterized by the age of onset, differential levels of severity, type of inflammation, triggers, and response to treatments[68]. More than 300 million people worldwide are affected by asthma, and this disease is causing approximately 250 000 deaths annually[68]. Epidemiological and clinical studies have reported a gender disparity in asthma which switches at puberty, where prevalence is highest in boys when they are children, whereas women have the highest prevalence as adults[69].

Allergic asthma is described by the occurrence of chronic Th2 inflammatory response upon exposure to an inhaled allergen, which in predisposed individuals activates and triggers airway epithelium and dendritic cells[70]. Inhaled allergens are captured by DCs and presented via MHC II molecules to TCR of $CD4^+$ T cells, simultaneously, cytokines produced by damaged epithelial cells boost DCs to polarize CD4 T cells and promote GATA3-expressing Th2 cells[71]. Th2 cells then produce cytokines implicated in inducing hallmark characteristics of asthma including goblet cell metaplasia, tissue eosinophilia, and airway hyperresponsivess[71]. This Th2 inflammatory response is said to be resulting from the activation of molecular pathways of adaptive and innate immune responses.

Asthma phenotypes

Asthma has been classified into various phenotypes to materialize treatment strategies that correspond to individual cases[72]. The phenotypes are described using unbiased approaches based on physiological, hereditary characteristics, clinical and molecular features[73]. Some of the most common clinical phenotypes include non-allergic asthma, whereby certain patients have asthma that is non-linked with allergy[74]. Patients with this phenotype often show less short-term response to inhaled corticosteroids (ICS)[74,29]. The second phenotype is asthma with obesity, whereby certain obese patients with asthma have little eosinophilic inflammation and prominent respiratory symptoms[75]. The third one is asthma with persistent airflow limitation, whereby the patients develop airflow limitation that is reversible as a result of airway wall remodelling[72,76]. Adult-onset (late-onset) asthma has also been classified as another common phenotype, whereby some adults, mostly women, present with asthma for the first time in adult life[77]. These patients have a tendency to be non-allergic and often need higher dosages of ICS or are relatively resistant to corticosteroid treatment[76].

Another classified phenotype is allergic asthma that often begins in childhood[78,79]. This allergic asthma phenotype is linked with a past and/ or family history of allergic diseases such as eczema, food or drug allergy, and allergic rhinitis[76]. Eosinophilic airway inflammation is often revealed when examining induced sputum of patients with allergic asthma before treatment, and these patients generally have a good response to ICS treatment[76]. Severe asthma has been classified as another phenotype of asthma and is linked with increased Th17 cytokines (IL-17A, IL-17F), and neutrophils in the bronchoalveolar lavage fluid of patients[80]. To achieve asthma control and reduce exacerbations, a combination of high-dose corticosteroids and long-acting β_2-adrenergic receptor agonist is required in severe asthmatics[81].

Biological treatments such as the humanized monoclonal antibodies (Omalizumab, Mepolizumab, and Dupilumab) are used in treating patients with severe type 2 asthma[82]. Omalizumab binds to free IgE and inhibits it from binding to the high-affinity FcεRI on mast cells and basophils, and is reported to be effective in treating allergen-exacerbated asthma[82,83,84]. Mepolizumab acts by recognizing and blocking IL-5 signalling and inhibit its binding to IL-5 receptor alpha (IL-5Rα) subunit on the eosinophil surface, thus reducing sputum and blood eosinophil counts on patients with severe asthma[84]. Dupilumab binds interleukin 4 receptor alpha (IL-4Rα) and inhibits the alpha subunit of IL-4/IL-13 receptor signalling, it has been shown to reduce asthma exacerbations, suppresses Th2 inflammation, and boosts lung function in patients with elevated eosinophils numbers and in patients with moderate-to-severe asthma[82,85,84].

3.2 Development of allergic asthma

The airway epithelium acts as a first-line defence against inhaled harmful material and pathogens[86]. However, allergens such as HDMs, and cockroach possess protease activity, and proteases such as Der P 1 and papain act on epithelial cells to disrupt barrier functions via cleavage of tight junction proteins (claudins, occluding, ZO1,2 and 3) and thus inducing an innate cytokine response through stimulation of protease-activated receptors (PARs)[87,88,89]. Upon primary exposure to an allergen, the underlying dendritic cells (DCs) and airway epithelium are activated. Activation of epithelial cells induces secretion of cytokines TSLP, IL-25, and IL-33 which stimulate subepithelial DCs maturation, ILCs and mast cells to recruit innate and adaptive cells, and initiate Th2 cytokine release[90]. Recruitment of immature DCs to the lungs occurs through the secretion of CCL2 and CCL20 by activated airway epithelial cells[25,91] (Figure 1.2).

DCs phagocytose an inhaled allergen, upregulate expression of chemokine receptor CCR7, undergo maturation and subsequently home to the draining lymphoid organs[92]. In the mediastinal lymph nodes,

fully matured DCs express adhesion and co-stimulatory molecules, process and present the antigen on the surface of a cell via MHCII molecule to naïve $CD4^+$ T helper cells which recognize allergens through selective T cell receptor[93]. The antigen is also presented via MHC class II by B lymphocytes, which in turn receive IL-4 signal and class switch to produce IgE that binds to high-affinity FcεRI on mast cells and basophils[94].

The recognition of an antigen by the TCR, in turn, activates naïve T cell proliferation and differentiation into effector T helper cells[95]. It is unknown how DCs induce this differentiation, however, the possibility is that they activate a subset of CD4 T cells, the $NK1.1^+$ subset that produces abundant IL-4 that can induce CD4 T cells to differentiate into Th2 cells after stimulation with an antigen[96]. Basophils, ILC2s and B cells have also been implicated as potential sources of IL-4[16,97–99]. Cytokines such as TSLP, IL-33 and IL-25 which are derived from airway epithelial cells, stimulate allergic inflammation through the recruitment of immune cells to inflamed areas[100]. These epithelial-derived cytokines are also crucial in activating DCs, basophils, type-2 innate lymphoid cells and Th2 effector cells. This activation leads to the production of cytokine-producing cells and thus contributing towards the initiation of a Th2 immune response[92].

T helper cells activation by DCs leads to the production of cytokines that are involved in regulation of isotype switching of B cells in their IgE production[101]. $CD4^+$ Th2 cell activation and differentiation occurs via downstream phosphorylation of GATA-3 and activation of STAT6[102]. Crucial cytokines implicated in Th2 inflammatory response are said to be those encoded in the IL-4 cluster of genes on chromosome 5q31, containing the genes encoding IL-4, IL-5, IL-9, IL-10, IL-13, and GM-CSF[102]. Th2 cell-associated cytokines, IL-4 and IL-13 signals transmit through the IL-4Rα/IL-13Rα1 subunit[103]. IL-4 induces IgE isotype switching in B cells and upregulates FcεRI on target cell surface, IL-5 recruits, activates and promotes eosinophils migration to airways thus triggering bronchial inflammation while IL-13 induces mucus hypersecretion and promotes airway hyperresponsiveness[104,101,94,105].

Re-exposure to an allergen results in an exacerbated allergic inflammation of the airways initiated by crosslinking of IgE bound to the FcεR1 on mast cells, thus consequently leads to mediator release. This causes what is known as the early phase immune response with allergic symptoms such as mucus overproduction and bronchoconstriction[106]. The late immune response is characterized by further recruitment of mast cells and eosinophils within hours. The allergen is taken up by the DCs, leading to activation of Th2 memory cells for cytokine production and release of other mediators, resulting in tissue remodelling and chronic allergic inflammation[106].

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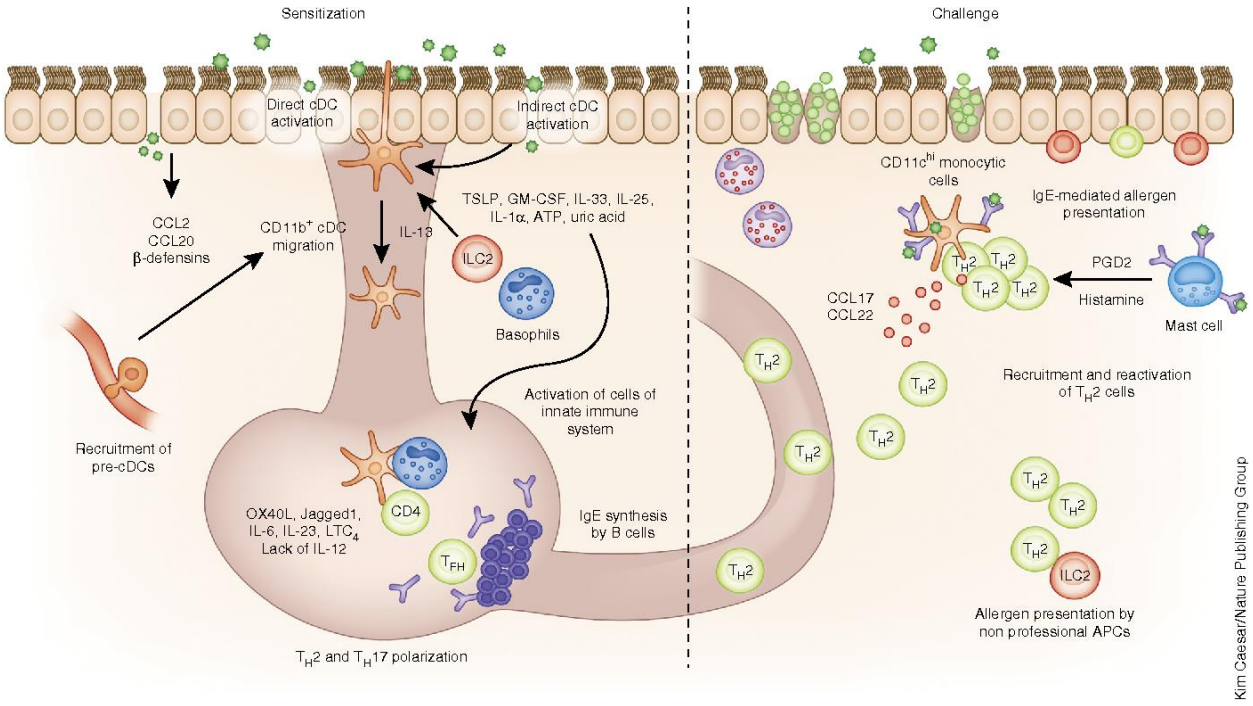

Figure 1.2: Sensitization and effector/ challenge phase immune response during allergic asthma. Adapted from Hammad and Lambrecht, 2015. During sensitization, lung conventional DCs (cDCs) and epithelial cells are activated by allergens with protease activity. Epithelial cells induce the production of alarmins (IL-25, -33 and TSLP) which favour $CD11b^+$ cDCs maturation. Activated lung $CD11b^+$ cDCs migrate to mediastinal lymph nodes and induce Th2 and Th17 polarization. This migration is stimulated by ILC2-derived IL-13. Some T helper cells produce IL-21 and assume a follicular helper T (Tfh) cell phenotype for inducing class switching to IgE in B cells. Th2 responses are sustained by DCs via assistance from basophils. Through CCL17 and CCL22 production, poorly migratory $CD11c^{hi}$ monocytic DCs can locally restimulate effector functions in lung-resident lymphocytes or recruit effector Th2 cells during allergen challenge[87].

4. Atopic dermatitis (AD)

4.1 Epidemiology, clinical symptoms, and treatment of AD

Skin biology

The skin is the largest mammalian organ and constitutes about 15% of total adult body weight[107]. Skin serves as a first-line protective barrier between the host and its external environment, it also serves as an immunological barrier, prevents excessive water loss and facilitates thermoregulation of the body[108,107,109]. The skin consists of three distinct layers which are the epidermis, dermis and subcutaneous tissue[26,107,109]. The epidermis which is the regenerating outermost skin layer is described as the squamous stratified epithelium composed mainly of keratinocytes, through continuous differentiation these keratinocytes induce synthesis of keratin, which is a thread-like long protein that provides protection[107,110]. Based on keratinocyte morphology and the position of their differentiation, the epidermis is made up of stratum basale (SB), stratum spinosum (SS), stratum corneum (SC), and stratum granulosum (SG)[110,109].

Proliferative action of the keratinocytes with the SB confers continual tissue renewal while in the SS, cell differentiation provides tissue stability as a result of robust cell-cell adhesion via a huge number of desmosomes[110]. Tight junction structures are found within the SG and these structures are known for restricting the movement of molecules within the intercellular space by sealing the paracellular pathway[110]. The SC forms a continuous sheet of cells called corneocytes which are enclosed in a lipid intercellular matrix and protect the body by denying entry of microbes and their products into the body[109]. The epidermis layer continually renews and form derivative structures such as nails, sweat glands and pilosebaceous apparatuses. The renewal of the outer epidermis takes place when the basal cells of the epidermis undergo proliferation[107].

The dermis which is the middle layer is made up of connective tissue that contains collagenous, elastic, reticular fibres and fibroblasts which provide elasticity and stability to the skin[111]. Additionally, several immune cells such as dendritic cells, mast cells, some T cells, lymphatic and blood vessels, sebaceous gland, hair follicles are located in the dermis compartment[26,107,109,110]. The sweat glands express antimicrobial peptides, while the sebaceous glands lubricate the hair and skin[112]. The subcutaneous tissue is the lowermost layer of the skin where subcutaneous fat is stored in adipocytes, this layer supplies nerves and blood to the skin, it also acts as a cushion, energy reservoir and provides thermoregulation[110,109].

Background and heterogeneity of AD

Atopic dermatitis (AD) is defined as an extremely heterogenous skin disorder and is characterised by a variety of phenotypes/subtypes based on age, ethnicity, disease chronicity, IgE and filaggrin (FLG) status, as well as fundamental underlying molecular mechanisms[113]. FLG plays an important role in maintaining the physical strength of stratum corneum and also restricts entry of foreign antigens[114]. Loss of function mutations of FLG-encoding gene located on the epidermal differentiation complex on 1q21.3 is the most crucial genetic risk factors for AD[115,108]. Recently, studies have reported a three to fivefold increase in risk for an individual to develop AD if one or both parents possess a history of AD[115].

AD characteristics include epidermal barrier dysfunction with intense itch as the main symptom, dry skin, chronic cutaneous inflammation[108]. Manifestation of AD depends on age and stage: in infants, the skin lesions occur on the cheeks and scalp, in toddlers it mostly affects the extensor surfaces of joins while in adults it affects flexural surfaces of the joints[26,108]. Recent studies suggest that AD is the initial step in atopic march which leads to asthma and/or allergic rhinitis in most susceptible individuals[116].

The onset of AD occurs predominantly in childhood and is said to precede allergic diseases induced by IgE sensitization to environmental allergens[117]. In children, there is about 15%-20% of AD prevalence, while in adults there is about 7%-10% prevalence in developed countries[26,118,119,120]. However, westernized way of living and globalization process have prompted an increasing AD prevalence in low-income countries of East Asia and Africa[121,122,122,121]. During late childhood about 70% of patients outgrow symptoms, however, some remain affected, and some may suffer a new disease onset in adulthood[117,123]. It is a challenge to accurately measure AD frequency because of its clinical heterogeneity, nonetheless, evidence shows that it is amongst the most prevalent chronic diseases globally[124].

Another AD hallmark is skin dysbiosis, with a move toward a pathogenic microbiome, in which favourable commensals, such as *Staphylococcus epidermidis* are replaced by different species, such as *Staphylococcus aureus,* and thus the whole skin microbiota of patients decreases in diversity[125]. This *Staphylococcus aureus* colonizes about 80%-100% of AD patients, in comparison to only about 5%-30% of healthy persons[126]. So far it is poorly understood which mechanisms are controlling the development of acute and chronic AD. This disorder is said to develop from the complex interchange between defects in immune abnormalities, the function of skin barrier, environmental and transmissible agents[116]. Thus there is a need to deeply understand the cellular and molecular mechanisms which underlie this disorder, as that may lead to identifying novel target

molecules, and consequently, to developing advanced therapeutic approaches [26].

Treatment of AD

The most effective and common treatment for AD is topical corticosteroid therapy, but, it can amplify recurrence risk and adverse consequences, this may include dermal atrophy and thinness of the skin[127]. Other therapies being used for the treatment of AD include emollients and moisturizers. It has been said that they work by enhancing the skin barrier, and distribute a lipid layer on the skin surface, and thereby reducing water loss and increasing the skin moisture[128]. The significance of IL-4 and IL-13 in AD pathogenesis has prompted studies to examine how to inhibit their function. A humanized monoclonal antibody (Dupilumab) against IL-4 receptor alpha (IL-4Rα) has shown efficacy in treating patients with moderate to severe AD[129,117,130]. IL-4 and IL-13 share the IL-4Rα subunit, and thus blockage of IL-4Rα inhibits signalling of these cytokines[117].

4.2 Development of atopic dermatitis

Epidermal barrier dysfunction results in the development of AD and also enhanced sensitization to allergens[123]. Dry skin affecting lesional and non-lesional areas of skin is one of the features of AD. The dry skin in AD parallels an increase in trans-epidermal water loss (TEWL), which indicates a skin barrier disruption in AD[131]. Exposure to lessened environmental humidity over an extended period accelerates TEWL, this amplifies barrier dysfunction by promoting more cytokine signalling of inflammatory molecules[132]. A majority of AD patients exhibit an accelerated IgE production in the serum[133]. This IgE is well known for its mediation in mast cell activation, which can induce the expression of proinflammatory cytokines by keratinocytes as well as migration of DCs[134].

Epidermal barrier defects lead to the penetration of the skin by epicutaneous antigens, which then encounter Langerhan and dermal DCs that activate Th2 cells, IL-13 and IL-4 production[135] (Figure 1.3). This in turn leads to the production of allergen-specific IgE, which is induced in a Th2 dependent manner as well as the development of dermatitis characterized by infiltration of $CD3^+$ T cells, eosinophils, neutrophils and local expression of IL-4, IL-5, and interestingly IFNγ[131]. Chronic exposure to protein allergens, especially those with protease activity induces TSLP expression in the epidermis. Under certain conditions when FLG is mutated, the skin barrier dysfunction is the primary cause of the development of AD[131].

Inflammatory mediators of Th2 cells and DCs induce an increase of Th22 cells in AD skin, resulting in the production of IL-22 which is most significantly increased in chronic AD. This IL-22 also induces epidermal hyperplasia during the chronic stage[136]. Th17 cells are downregulated in patients

with AD possibly due to the inhibitory effect of the cytokines produced by Th2 cells. IL-17 plays a role in the regulation of antimicrobial peptides (AMPs), and thus the suppressive action of Th2 cytokines on Th17 cells leads to reduced AMPs in patients with AD[137]. IL-4 and IL-13 levels are upregulated in lesional skin, these cytokines are key regulators of several hallmark features of AD which include T cell chemokines and eosinophil production, dysfunctional skin barrier and epidermal thickening[129,138,139].

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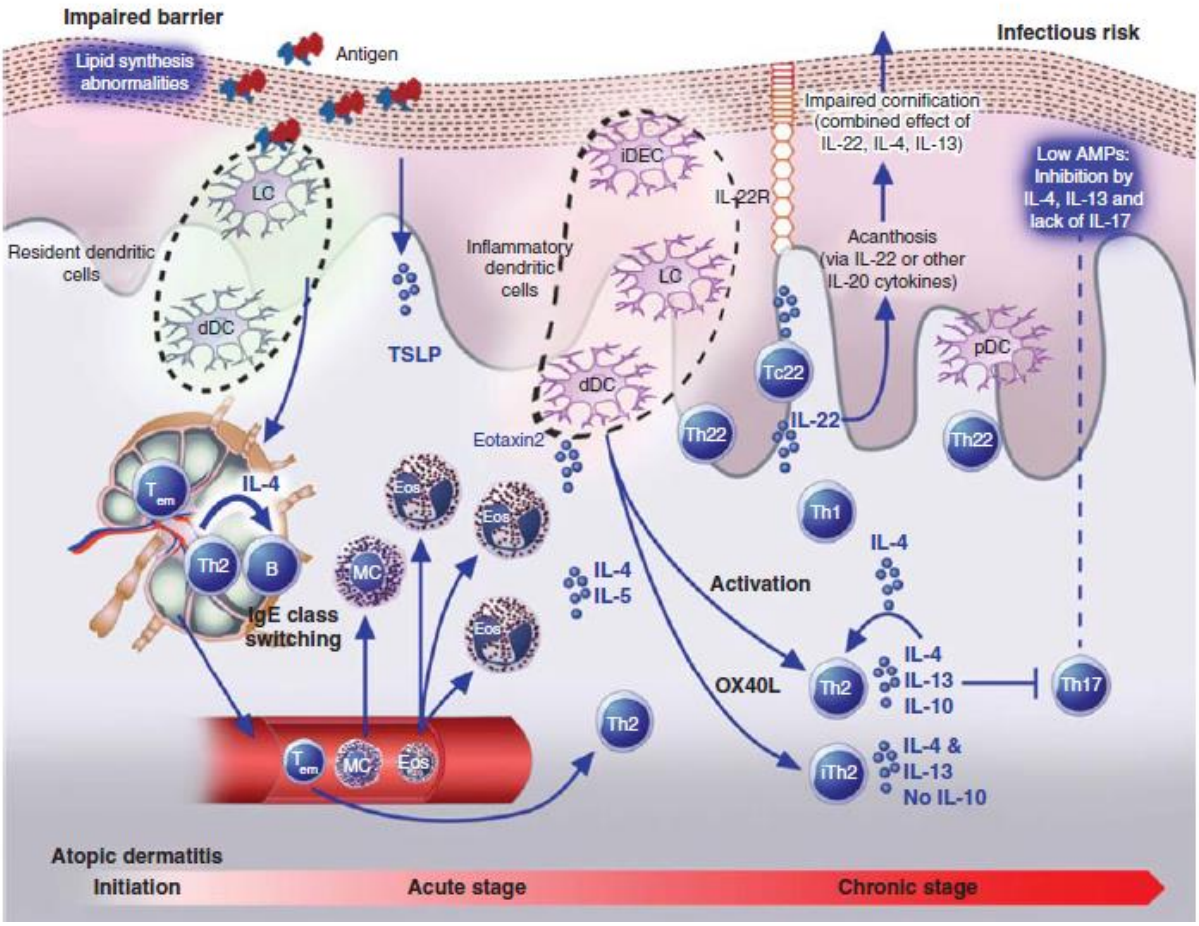

Figure 1.3: Atopic dermatitis disease pathogenesis from non-lesional stage and progressing to acute lesional and chronic stage. Adapted from Guttman-Yassky et al., 2013[140]. The development of the disease has three phases. Epithelial barrier defects result in penetration of epicutaneous antigens which then encounter Langerhan cells and dermal dendritic cells (dDC) resulting in activation of Th2 cells and production of IL-4 and IL-13[140,141,142]. These cytokines increase the survival of Th2 cells and induce IgE class switching by B cells. Th2 cytokines increase gradually from non-lesional to chronic stage of disease and this directly poses effects on the epidermis[140]. The effects include the induction of keratinocytes to

produce TSLP, inhibition of antimicrobial peptides (AMPs) production and impairment of epidermal differentiation. Th2 T cells and inflammatory DCs (iDC) induce peripheral mast cells and eosinophils[142]. Additionally, there is an increase in Th22 cells which promote IL-22 production. IL-22 suppresses terminal differentiation, promotes epidermal hyperplasia and acanthosis, which are major features of chronic AD. Low levels of IL-17 in the chronic stage result in reduced AMPs which further increases AD-associated infections[142,140]. IL-31 primarily expressed by Th2 cells is reported to be a pruritogen and proinflammatory cytokine that is upregulated in AD skin[143]. This cytokine is suspected to probably orchestrate reactive epidermal hyperplasia development seen in chronic AD skin lesions[144].

5. L-amino acid oxidase-interleukin-4 induced gene 1 (IL-4i1)

Interleukin-4 induced gene 1 (IL-4i1) is a secreted mammalian L-amino acid oxidase mainly expressed by antigen-presenting cells and mononuclear phagocytes such as dendritic cells, monocytes and macrophages[145,146]. During infection, IL-4i1 mRNA expression is limited to lymphoid tissues, with the most significant quantities found in the spleen and lymph nodes[147]. The leukocyte-receptor compound is where the human gene is located on chromosome 19, and the location for the mouse gene is chromosome 7 in a region that is in association with susceptibility to systemic lupus erythematous[148].

Upon activation by IL-4 and CD40, this enzyme has also been detected in B lymphocytes, but in lower amounts, and it has been reported that IL-4i1 is expressed by IL-4 activated murine B cells in just under 2 hours of stimulation [149,150]. IL-4i1 converts phenylalanine into phenylpyruvate, ammonia and hydrogen peroxide (Figure 1.4)[151]. This enzyme is described to be localized to lysosomes, which are a subcellular compartment associated with antigen processing, and para-aminobenzoic and benzoic acid are said to be the aromatic inhibitors of IL-4i1[152,153]. In human Th17 cells, a microarray genes analysis post initial events of TCR signalling revealed that the expression of IL-4i1 was solely reliant on retinoic acid orphan receptor (ROR)C[154]. At the transcriptional level, IL-4i1 expression is controlled by RORγT in $CD4^+$ T cells and hence it is also found in Th17 cells and T cells which are under differentiation from regulatory or naïve T cells[148].

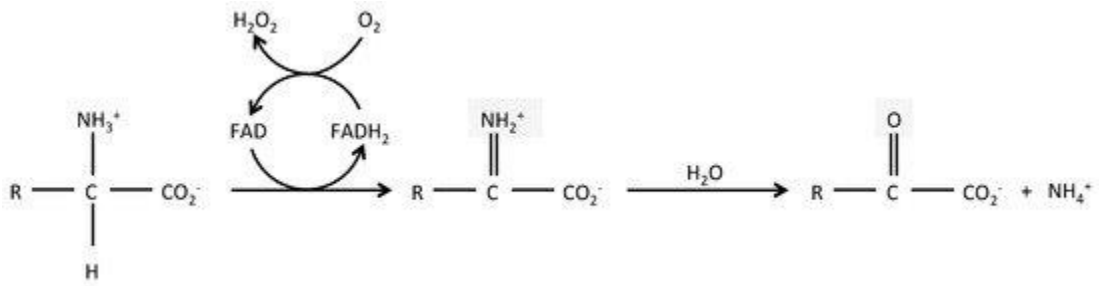

Figure 1.4: Chemical reaction that is catalysed by the L- amino acid oxidase (LAO); adapted from Castellano and Molinier-Frenkel[155]. This is a two-step reaction. Firstly, a formation of an imino intermediate occurs via a proton transfer from the amino group of the substrate to flavin adenine dinucleotide (FAD) ring; the amino acid is then hydrolysed non-enzymatically in its α-keto acid and ammonia (NH_3^+). Secondly, the reaction is completed when molecular oxygen enters the catalytic site to induce re-oxidization of the reduced FAD, resulting in the production of hydrogen peroxide (H_2O_2)[155]. R denotes amino acid group.

More recent findings in humans have suggested that IL-4i1 may be directly involved in tumour progression through catabolizing tryptophan into two aryl hydrocarbon receptor (AHR) ligands namely indole-3-pyruvic acid (I3P) and kynurenine and kynurenine acid (Figure 1.5)[156]. Mechanistically, IL-4i1 enhances systemic Tryptophan-catabolism, which contributes to a systemic tumour-promoting environment that permits tumour cells to migrate and provides their protection from being detected and destroyed.

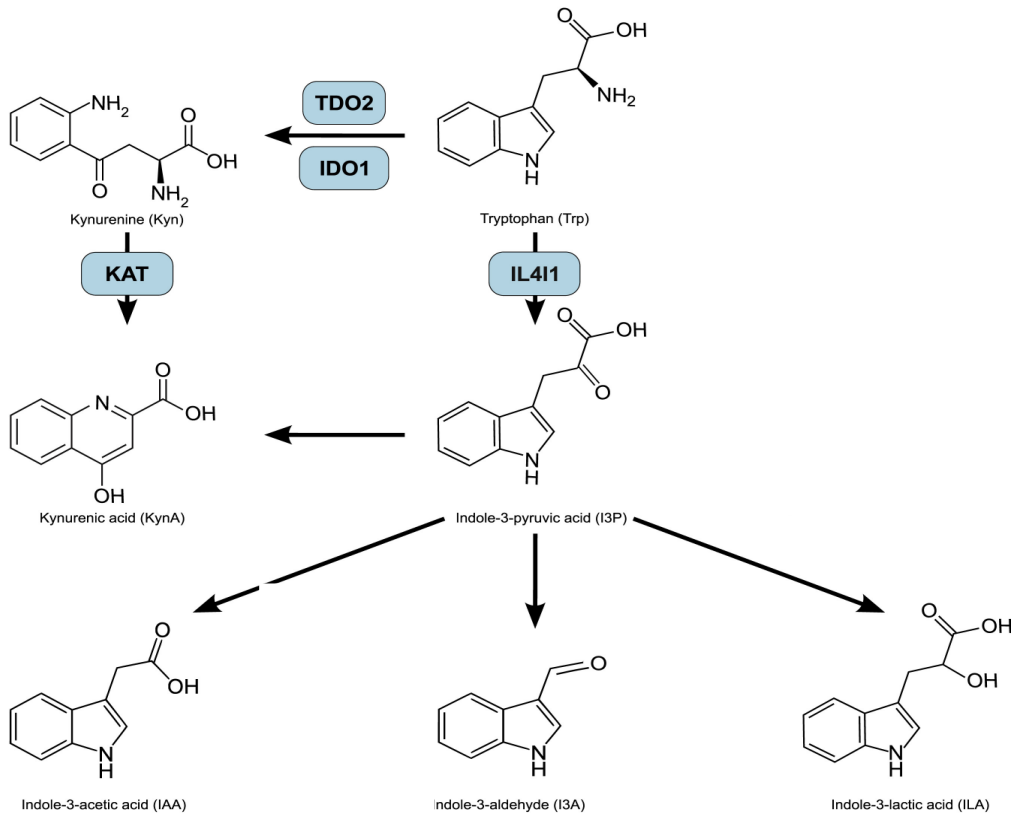

Figure 1.5: Tryptophan catabolism via Indole-3-Pyruvic Acid gives rise to the AHR agonists Kynurenic Acid and Indole-3-Aldehyde. Tryptophan can also be catabolised through IL-4i1 independent pathways which involve Kynurenine which leads to Kynurenic acid. Adapted from Sadik *et al*., 2020 Cell.

5.1 IL-4i1 in immune regulation

The ability of IL-4i1 to produce hydrogen peroxide results in inhibition of T cell activation, proliferation and cytokine production, but favours naïve $CD4^+$ T cell proliferation into Tregs *in vitro*[157]. IL-4i1 is also expressed in circulating induced Treg ($Ailos^+$ + Treg) population in a mouse model of melanoma, thus further implicating IL-4i1 in mediating immune escape of tumours through inhibition of effector T cell responses[145]. This enzyme is also shown to inhibit mammalian rapamycin complex (mTORC)1 but not mTORC2 signalling through phenylalanine depravation[158]. The mechanism may include the direct downregulation of the expression of the CD3ƶ chain through hydrogen peroxide release or macrophage polarization toward an M2 phenotype[159,147]. The M2 macrophages possess anti-inflammatory qualities that can hinder T cell activation, most probably in a STAT-6 and STAT-3-dependent manner[146]. These findings depict IL-4i1 as a crucial immunoregulatory molecule of different T cell functions[145].

In another study by Bod *et al*., they demonstrated that in a mouse model of spontaneous melanoma, IL-4i1 reduces the tumour infiltration by B cells and they also observed that IL-4i1 produced by B cells themselves, controls plasma differentiation and germinal center reaction[160]. This provides evidence that IL-4i1 is also a key regulator in B cell biology. Santarlasci *et al*., also showed that upregulation of IL-4i1 in human Th17 cells restricts their TCR-mediated expansion via maintaining high Tob1 levels and blockage of the molecular pathway implicated in IL-2 promoter activation, which impairs entry to the cell cycle[161].

Aubatin *et al* evaluated the impact of IL-4i1 on the formation of APC-T cell synapse, TCR signalling and T cell phenotype modulation. They observed that the enzyme influences the phosphorylation of immediate proteins associated with TCR signalling at early time points post activation[162]. They also reported that the decrease in signalling was independent of phenylalanine depletion nor products of enzyme activity. After analysing immune synapse between T cells and APCs, they saw a secretion of IL-4i1 in a polarized manner, this reduced proximal and distal phosphorylation of ZAP-70 and ERK. They concluded that IL-4i1 presence during T cell activation decreases early signalling events downstream of TCR signalling especially after CD28 co-stimulation[162]. In a mouse tumour model crucially expressing IL-4i1 developed by Lasoudris *et al*., they were able to show that IL-4i1 expression promotes tumour growth through inhibition of the $CD8^+$ antitumour T cell response *in vivo*[163]. Overall, this suggests that IL-4i1 possesses qualities as an immunosuppressive enzyme.

5.2 IL-4i1 in immunopathology and infection

The enzyme IL-4i1 is a bactericidal enzyme due to its production of toxic NH_3 and H_2O_2, and its antibacterial effects have been seen in $Gram^+$ and $Gram^-$ bacteria[149]. A study by Puiffe *et al*., revealed that

mice that were injected with *Eschericia coli* (*E.coli*) and *Staphylococcus aureus* (*S.aureus*) bacterial strains had a decreased bacterial burden in the spleen after IL-4i1 was added, and this concurrently reduced the inflammatory response[149]. Myeloid cell-derived IL-4i1 has been detected in high levels in tuberculosis granulomas, which led to downregulation of the Th1 response via limitation of T cell proliferation and inhibiting pro-inflammatory chemokines and IFN-γ secretion[164]. This shows that IL-4i1 production is triggered by infection with different pathogens and may promote containment of the infectious agent and suppression of immunopathology mediated by effector Th1 cells.

Secretion of IL-4i1 by mucosal-associated invariant T cells (MAITs) after contact with target cell was recently shown as a possible mechanism for bactericidal effects against *E. coli* BL21-fed THP-1 cells. This bactericidal activity is associated with a distinct granzyme effector composition which likely involves IL-4i1, thus suggesting IL-4i1 as a novel constituent of MAIT cell lytic granules[53]. IL-4i1 secreted by alternatively activated macrophages is increased at the onset of inflammation resolution and remyelination in lesions of mouse central nervous system, and interestingly intravenous injection of IL-4i1 into mouse models of autoimmune encephalomyelitis at the onset of disease remarkably reversed disease severity and thus leading to recovery from paralysis of hindlimb[165]. The contribution of IL-4i1 with immunoregulatory mechanisms has not yet been explored in allergic diseases such as asthma and atopic dermatitis.

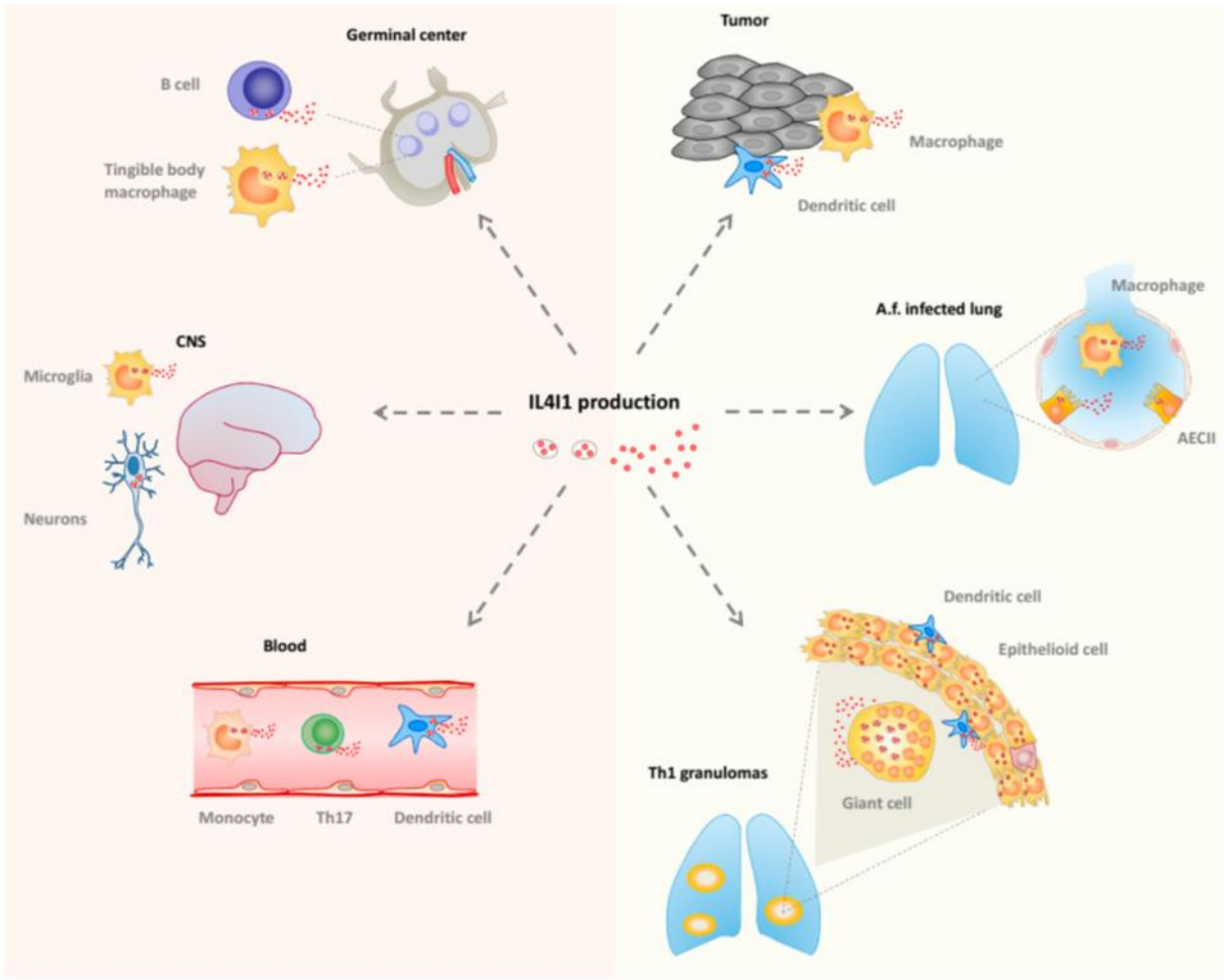

Figure 1.6: *In vivo* production of IL-4i1. Left: Homeostatic and acute and chronic inflammatory. IL-4i1 is detected in germinal center B cells and tangible body macrophages during T cell-dependent development of antibody responses conditions. An isoform 2 of IL-4i1 is detected in central nervous system (CNS) rare cells. In blood, IL-4i1 is produced by dendritic cells, monocytes and Th17 cells. On the right: Under inflammatory conditions, IL-4i1 detection is observed in Th1 inflammatory lesions inclusive of tuberculosis granuloma and sarcoidosis. High-density granules of IL-4i1 are found in dendritic cells (DCs), giant multinucleated cells and macrophage-derived epithelioid. In the lung of *Aspergillus fumigatus* (Af)-infected persons, type II alveolar epithelial cells (AECII) induce the production of IL-4i1. Appearance of allergy to fungi occurs as Type I immediate, IgE hypersensitivity. Furthermore, allergy to *A. fumigatus* is common in atopic asthma and cystic fibrosis[166]. In tumours, IL-4i1 is secreted by macrophages and infiltrating DCs, regardless of the tumour type. Adapted from Castellano and Molinier-Frenkel[155].

6. Rationale for targeting IL-4i1 in allergic asthma

Prevalence of all allergic diseases has increased dramatically in recent decades. This creates a burden financially on health care systems and affects the quality of life for those who have the disease[167]. The pathogenesis of allergic diseases entails a dysfunctional tolerogenic immune response to allergens[168].

Analysis of Genome-wide transcription of M1 macrophages activated with IFN-γ and M2 macrophages activated with IL-4 or IL-4/IL-13 and infected with *Mycobacterium tuberculosis* (Mtb) strain HN878 showed IL-4i1 as part of the upregulated candidate genes in M2 macrophages, indicating a contribution of IL-4i1 during infection with Mtb[169]. Marquet *et al*., demonstrated that DCs and macrophages are the major producers of IL-4i1 *in vitro* and *in vivo* under inflammatory conditions[170]. They also observed a high expression of IL-4i1 in granulomas *in vivo* from chronic tuberculosis Th1 lesions[170]. This was in agreement with another study where microarray data showed induction of IL-4i1 transcription in mouse lung by aerogenic Mtb infection [171,172].

IL-4i1 has also been reported to influence macrophage polarization towards an M2 phenotype which induces anti-inflammatory properties, this occurs via STAT3 and STAT6 phosphorylation, and partly via arginine and L-tryptophan depletion[146]. The functional role of IL-4i1 in allergic disease setting has not yet been investigated, thus we propose to investigate the role of IL-4i1 during allergic asthma and atopic dermatitis using acute mouse models.

7. Rationale for targeting IL-4i1 in atopic dermatitis

Psoriasis is characterised by Th17 cells and a severe form of scarring. A recent study elucidated transcriptional signatures that separate psoriasis from other skin disease conditions such as acne, alopecia areata, granuloma annulare (GA) and leprosy in lesional and non-lesional areas of the skin. Using single-cell RNA sequencing, IL-4i1 was significantly expressed in skin lesional areas of psoriasis patients when compared to other disease conditions[173].

The main cells that expressed IL-4i1 in lesional areas were macrophages, consistent with previous studies that this enzyme is expressed mainly on APCs[173]. IL-4i1 was observed to be highly upregulated in lesional skin of AD patients and was amongst the 50 top genes that were dysregulated in lesions[174]. Furthermore, OX40L which is mainly expressed by APC was found to be the main target for blockade which could inhibit IL-4i1[174].

In a study of human cutaneous melanoma, IL-4i1 secretion was found to modify anti-tumour properties of melanoma immune infiltrate and thus facilitated tumour escape from the immune response[151]. IL-4i1

expression by $CD11b^+$ myeloid was also observed in a mouse model of spontaneous melanoma, and its activity correlated with disease aggressiveness[175]. These studies validate the rationale for therapeutic targeting of IL-4i1 as one of the key immune regulators in diseases.

Aim of the study

This study aimed to investigate the role of IL-4i1 in allergic asthma and atopic dermatitis.

Objectives

1. To understand the role of IL-4i1 in the immune regulation of allergic asthma response by using in *vivo* mouse models
2. To understand the role of IL-4i1 in the immune regulation of atopic dermatitis response by using in *vivo* mouse models
3. To investigate the mechanism by which IL-4i1 regulates the Th2 immune responses during allergic asthma and atopic dermatitis

Hypothesis

We hypothesised that IL-4i1 will be important in the regulation of type 2 inflammation as it is induced by IL-4 and is downstream of IL4Rα.

Chapter 2: Materials and Methods

2.1 Statement of ethics

All experiments in this MSc study were carried out in accordance with the University of Cape Town (UCT) Faculty of Health Sciences Animal Ethics Committee. (FHS- AEC). The experimental protocols (018/013 and 017/004) were reviewed and approved by the FHS-AEC.

2.1.1 Mouse strains

IL-4i1 deficient mice (IL-4i1$^{-/-}$) were generated using gene targeting in 129/SvEvBrd mice-derived embryonic stem cells (ES) [(B6; 129S5-Il4i1tm1Lex)] by Lexicon. IL-41$^{-/-}$, heterozygous (IL-4i1$^{-/+}$) and wild-type control littermates (IL4$^{+/+}$) were purchased from *Mutant Mouse Resource and Research Centr*e (MMRRC, USA). Chimeric mice were bred to C57BL/6J albino mice to generate F1 heterozygous mice which were then inter-crossed to produce wild-type, heterozygous and homozygous mutant progeny. Mice from this progeny were backcrossed 9 generations to wild-type BALB/c and C57BL/6 mice. Mice in the C57BL/6 genetic background are not optimal in studying airway hyperresponsiveness after allergen challenge as they tend to yield a more resistant phenotype[176], whereas in atopic dermatitis the C57BL/6 strain yields a Th1-biased immune response. BALB/c strain is optimal for Th2 responses and has been used extensively to study allergic asthma and atopic dermatitis, because of ease in inducing AHR and translucence of ear and easily accessible draining lymph nodes [177]. Thus, only the BALB/c strain was used in studying the role of IL-4i1 in allergic asthma and atopic dermatitis. Female mice of about 6-12 weeks old with a deficiency in the IL-4i1 gene were used and were maintained under specific pathogen-free conditions in the research animal facility of UCT.

2.1.2 Genotyping and confirmation of IL-4i1 deletion

Mice were genotyped by using the polymerase chain reaction (PCR). IL-4i1 deletion was confirmed by isolating DNA from tail biopsies and splenocytes of naïve IL-4i1$^{-/-}$ mice and control littermates. The primer sequences that were used are: Neo3a forward 5'- GCAGCGATCGCCTTCTATC-3' and reverse 5'-GTGCTCACTTCCTCTTTGCGACT-3' and wild-type primers: forward 5'-TTGAGACCTTTCTTTCCGAGCAG-3' and reverse 5'-AGGCTAAACCTTG-3. The PCR was then separated by electrophoresis on ethidium bromide-stained agarose gel, followed by visualization under UV light. The amplicon size for the wild-type was 325 base pairs (bp) and 187 bp for IL-4i1$^{-/-}$. Confirmation of IL-4i1 deletion from splenic tissues harvested from IL-4i1$^{-/-}$ and wild-type littermate control was performed by quantitative PCR amplicon 200bp normalised to hprt1.

2.2 Induction of allergic asthma

In this study, we used acute House dust mite (HDM, Greer Laboratories, USA) (*D. pteronyssinus*, 37.40 mg protein/vial, 181.90 mg dry wt/vial) allergic asthma model. IL-4il$^{-/-}$ and IL-4i1$^{+/+}$ littermate control mice under anaesthesia with xylazine (Rompun; 16 mg/kg, Bayer, Isando, South Africa) and ketamine (ANaket-V; 80 mg/kg, Centaur Labs, South Africa) and were sensitized on day 0 intratracheally with 100µg (high dose) and 1µg (low dose) of HDM or phosphate-buffered saline (PBS) in control mice. On days 7-11, mice were challenged intranasally while under anaesthesia with 10µg of HDM and PBS for control mice (Figure 1).

Invasive lung function measurement

On the last day of the experiment (day 14), mice were anaesthetized with xylazine/ketamine intraperitoneally. Their tracheas were then cannulated with an 18G size metal tracheal cannula and ventilated at 150 breaths/min at 3.0cm water positive end-expiratory pressure. Two total lung capacity perturbations were performed before baseline measurement and subsequent methacholine challenges. Methacholine was aerosolized for 10 s followed by 10 s of ventilation with an ultrasonic nebulizer. 15 Resistance measurements were made using a 1.25-s, 2.5-Hz volume-driven oscillation applied to the airways by a computer-controlled piston (FlexiVent SnapShot perturbation). The procedure was repeated for 0, 5, 10, 20 and 40 mg/ml concentrations of β- acetyl-methylcholine (Sigma-Aldrich, South Africa). Dynamic resistance (R) and Elastance (E) was determined by the flexiVent software. The maximum R and E values with a coefficient of determination of 0.9 or greater (as determined by the flexiVent software) were used to determine the dose-response curve. After measurement of lung function, mice were euthanized by inhaled halothane and tissues collected for processing.

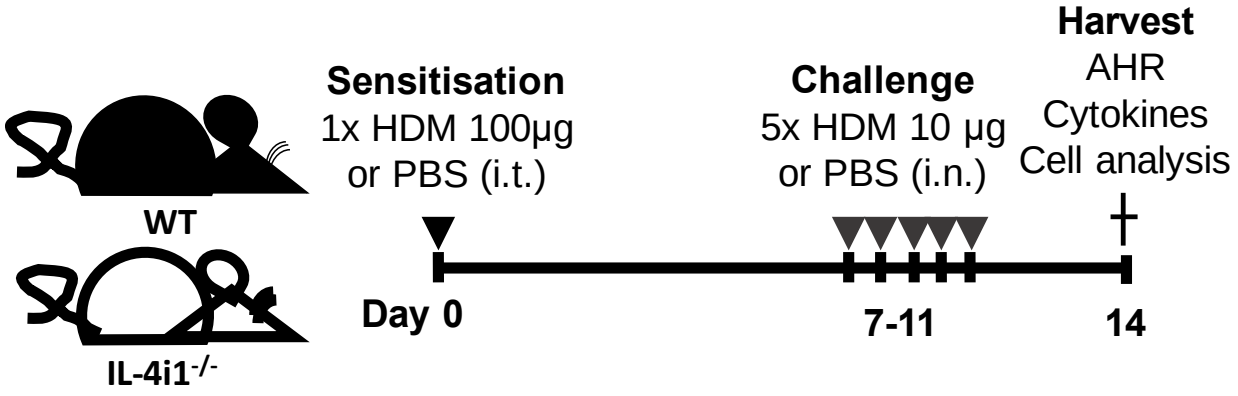

Figure 2.1: Timeline for sensitization and challenge of IL-4i1 knockout (IL-4i1$^{-/-}$), IL-4i1 littermate control (IL-4i1$^{+/+}$) and wildtype BALB/c mice. Mice were sensitized intratracheally with high (100µg) or low (1µg) dose HDM at day 0 and challenged intranasally with 10µg HDM on days 7-11. Lung function and tissues processing was done on day 14.

Following euthanization, bronchoalveolar lavage fluid (BAL) was collected by flushing the lungs with 1ml of cold PBS with 5mM EDTA. Lobes of the lungs were collected for RNA, histology and for fluorescent activated cell sorting (FACS). Blood was collected to perform serum Enzyme-linked immunosorbent assay (ELISA) for the secreted level of total IgE, antigen-specific antibodies (IgG1, IgG2a, IgG2b, IgE and IgM) and Th1 and Th2 cytokines (IL-4, IL-5, IL-13, IFN-γ and IL-17). Mediastinal lymph nodes were collected as well for FACS.

2.2.1 Cardiac puncture

The cardiac puncture method was used to collect blood samples following euthanization. A 25G needle was inserted just below the rib cage with a graded side and flat side of the needle facing up. Approximately 1mL of blood was collected in serum separator tubes (Microtainer^{TM}, Dickinson, USA), when serum was to be used for ELISA or blood collected in EDTA/heparin-coated tubes (Microtainer^{TM}, Dickinson, USA) when it was to be used for FACS. To separate serum from red blood cells, blood was centrifuged at 5500rpm for 7 minutes at room temperature (RT). Separated serum was stored at -80°C until use.

2.2.2 Measurement of antibody levels from serum by ELISA assay

For the measurement of total IgE, 96-well flat-bottom ELISA plates were coated overnight with 50 µl rat anti-mouse IgE antibody (clone 84.1C, 2µg/ml) in 1X PBS at 4°C. The following day, plates were washed three times with a 1X wash buffer (144g $Na_2HPO_4.2H_2O$, 20g KH_2PO_4, 800g NaCl, 50 ml Tween-20 dissolved in 5 litres of ddH_2O). Plates were then incubated at 37°C in an enclosed chamber for three hours with 200 µl of blocking buffer (2% BSA powder, 1X PBS). After incubation, the plates were washed three times with wash buffer. Recombinant IgE standard (Clone C48-2) was diluted in dilution buffer (1% BSA powder, 1X PBS) from 500µg/ml stock (BD Pharmingen), and 1/3 dilution steps were made starting from 1µg/ml. Serum samples were then added in a 1/3 dilution series starting with a 1/10 serum dilution in 1% dilution buffer. The plates were then incubated overnight at 4°C. The following day, plates were again washed three times with a wash buffer, and alkaline phosphatase (AP)-labelled biotinylated secondary antibody (Rat Anti-mouse IgE ε chain specific, Southern Biotech) was diluted 1/1000 in dilution buffer and then added to the plates. Plates were then incubated for three hours at 37°C followed by washing three times, and then the addition of 4 Nitrophenyl phosphate disodium salt hexahydrate (PNP, Merck, Germany) (1mg/ml in substrate buffer) (0.2g NaN_3, 97 ml diethanolamine, 0.8g $MgCl_2.6H_2O$ dissolved in 1 litre of

ddH_2O) at 50µl/ well. Plates were incubated in the dark until a colour change developed. The reaction was stopped with 50 µl 1M sodium hydroxide (NaOH), plates were then read with Versamax plate reader (Molecular Devices, USA) at 405 nm with a reference wavelength at 492 nm.

2.2.3 Antigen-specific antibodies ELISA assay

Blood samples were collected using the same method as described in 2.2.1. For measuring the expression levels of HDM-specific IgE, IgG1, IgG2a, IgG2b and IgM, 96-well flat-bottom plates were coated overnight at 4°C with 5µg/ml HDM in 50µl PBS. The following day, plates were washed 5 times thoroughly with 300 µl of 1X wash buffer, followed by blocking with 200µl of 2% BSA in PBS, and incubation for 3 hours at 37°C. After incubation, plates were thoroughly washed with 1X wash buffer. Serum samples were then added in a 1/3 dilution series starting with a 1/10 serum dilution for HDM-specific IgE, and 1/100 for HDM-specific IgG1, IgG2a, IgG2b and IgM. Plates were incubated overnight at 4°C, which was followed by washing 5 times with 300µl of wash buffer. Isotype specific AP-conjugated secondary antibody (50µl /well) was added in a 1/1000 dilution. Plates were then be incubated at 37°C for 3 hours. After incubation, plates were washed 5 times with wash buffer. The colour change from a clear liquid into yellow in the plates was then developed with 50 µl PNP substrate. The reaction was stopped with 50 µl 1M NaOH, and the plates were read with a Versamax plate reader at 405 nm with a reference wavelength of 492 nm.

2.2.4 Lymph node processing and cell stimulation

Mediastinal lymph nodes (MLNs) were collected in 1ml of complete Roswell Park Memorial Institute (cRPMI) media (Gibco, Paisley, United Kingdom) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 5 ml L-glutamine and 5 ml of penicillin/streptomycin. The MLNs were crushed through a 40 µm strainer using a 2 ml syringe. About 2 ml of media was added with a pipette over the strainer to wash it and then transferred into tubes labelled for cell suspensions. The cell suspensions were centrifuged (1500 rpm, 4°C, 5 minutes). The supernatant was aspirated, and the pellet was then resuspended in 500 µl of red cell lysis buffer (155 mM NH_4Cl, 12 mM $NaHCO_3$, 0.1 mM EDTA) and left for about 1 minute. The lysis reaction was stopped with 2 ml of media. The cell suspension was centrifuged again (1500 rpm, 4°C, 5 minutes). The supernatant was aspirated, and the pellet was suspended in 1 ml of media. The number of cells was counted in a 1/10 dilution in trypan blue dye using single-use chamber slides (Biosigma, Italy) under a microscope. Following counting, cells were reconstituted in cRPMI media to make up 2×10^6 cells/ml. A 96-well flat bottom plate was coated with 100 µl of anti-CD3 (10 µg/ml, clone 145-2c-11) diluted in sterile PBS. The plate was incubated for 2 hours at 37°C. After 2 hours, anti-CD3 was aspirated and 100 µl of reconstituted cell suspensions were then added for anti-CD3 stimulation. For HDM stimulation, 100 µl of HDM (30 µg/ml, media) was added to the appropriate wells. For unstimulated wells,

100 µl of cell suspensions were added to wells. The plate was then incubated for 5 days at 37°C, after which the supernatant was either used immediately for cytokine ELISA assay or stored at -80°C for future use.

2.2.5 Cytokine ELISA assay

IL-4 (Clone 11b11), IL-5 (Clone BD/554393), IL-13 (Clone R&D MAB413), IL-17 (R&D MAB721) and IFN-γ (Clone AN18KL6) capture antibodies were diluted in 1X PBS in a 1/500 dilution for IL-4, IL-5, IL-17 and IFN-γ, and 1/250 for IL-13. After that, 96-well flat-bottom plates were coated with 50µl/well of the capture antibodies and incubated overnight at 4°C. On the next day, the plates were washed four times with 1X wash buffer. The plates were then blocked with 200µl of blocking buffer (2% BSA powder, 1X PBS) and incubated for 3 hours at 37°C. After incubation, plates were again washed four times with wash buffer. Standards appropriately diluted in dilution buffer (1% BSA powder, 1X PBS) were then added to the plates in a 1/3 dilution series starting from 100ng/ml for IL-4, 5, 17, IFN- γ and 50ng/ml for IL-13. Anti-CD3/HDM restimulated and unstimulated samples were diluted in dilution buffer (1% BSA, 1X PBS) in a ½ dilution, and 50 µl was added to the plates. The plates were then incubated overnight at 4°C. On the following day, plates were washed 4 times with wash buffer, and 50 µl of biotinylated secondary antibodies in dilution buffer (1/1000 IL-4, 5 and IFN- γ, 1/500 IL-17, 1/125 IL-13) were added and the plates were incubated for 3 hours at 37°C. Thereafter, the plates were washed 4 times with wash buffer, and plates for IL-4, IL-5, IFN-γ were incubated with streptavidin-AP conjugate (1/1000 dilution) for 1 hour at 37°C. Plates for IL-13 and IL-17 were incubated with streptavidin-horse radish peroxidase (HRP) conjugate (1/5000 dilution) for 1 hour at 37°C. Thereafter, the plates were washed 4 times with wash buffer. The plates incubated with streptavidin-AP conjugate were developed with a PNP substrate (1mg/ml in substrate buffer) at 50 µl per well. A yellow colour was developed, the reaction was stopped with 1M NaOH. The plates were then read using the Versamax plate reader at 450 nm and the reference filter at 540 nm. The plates incubated with streptavidin-HRP were developed with TMB peroxidase substrate (equal volumes of solution A and B, ThermoFisher Scientific, Rockford, USA). A blue colour was developed, and the reaction was stopped with 1M H_2SO_4. The plates were read at 450 nm with a reference filter of 540 nm on the Versamax plate reader.

2.2.6 Lung processing for the acquisition of immune cell populations by flow cytometry

The largest lobe of the lung was collected and chopped into very small pieces in 5 ml digestion media containing RPMI media + 50 U /ml collagenase I (Gibco, Massachusetts) + 13 µg/ml DNase I (Roche, South Africa). Lungs were then incubated for 45 minutes at 37 °C. The top part of the plunger of a 5 ml syringe was used to mash the lung tissue through a 70 µm cell strainer with 3 ml media. The cells were then centrifuged (1500 rpm, 4°C, 6 minutes). The supernatant was aspirated, and the cells were resuspended in

2 ml red cell lysis buffer. The lysis reaction was stopped after a minute with 5 ml of cRPMI media. The cells were centrifuged again (1500 rpm, 4°C, 6 minutes). After spinning, the cells were then suspended in 3 ml media. The cells were counted in a 1/5 dilution in trypan blue dye using single-use chamber slides (Biosigma, Italy) under a microscope. Cells were reconstituted in media to make 2×10^6 cells/ml.

2.2.7 BALF processing for the acquisition of immune cell populations by flow cytometry

BAL tubes were weighed to record the volume of BALF collected. The tubes were then centrifuged at 5500 rpm for 5 minutes at 4°C. After spinning, supernatants were transferred to fresh Eppendorf tubes and stored at -80°C. The remaining pellet was resuspended in lysis buffer (250 µl/sample), after a minute the lysis reaction was stopped with 1 ml of cRPMI media. The tubes were centrifuged again at 5500 rpm for 5 minutes at 4°C. After spinning the supernatants were discarded, and the pellet was suspended in 0.250 ml of RPMI media. Thereafter, cells were counted in a ½ dilution in trypan blue dye using single-use chamber slides (Biosigma, Italy) under a microscope.

2.2.8 Antibodies that were used in flow cytometry panels for analysis of different immune cell populations

Cells were stained for acquisition on LSR II Fortessa flow cytometer (BD Biosciences, Belgium). BAL, Lung and MLN cells were transferred into V-bottom plates. The cells were then centrifuged at 1500 rpm for 3 minutes at 4 °C. Thereafter the supernatants were discarded. The remaining pellets were stained for analysis of B cells and their subpopulations with a cocktail mix (50 µl/sample) containing the following antibodies: FITC anti-CD24 (1/320 dilution), FITC anti-IgD (1/80 dilution), PE anti-GL7 (1/320 dilution), PE anti-IgE / BV786 anti-IgE (1/80 dilution), Biotin FAS (1/200 dilution), Biotin anti-IgM (1/200 dilution), PE-Cy7 anti-CD23 (1/320 dilution), V450/BV421 anti-CD80 (1/320 dilution), V450/BV421 anti-IgG (1/320 dilution), V500/BV510 anti-B220 (1/320 dilution), Qdot605 live/dead (1/2000 dilution), APC anti-CD21/35 (1/80 dilution), APC anti-CD138 (1/80 dilution), APC/Cy-7 anti-CD19 (1/320 dilution), AlexaFluor700(AF700) anti-CD86 (1/320 dilution), AF700 anti-MHCII (1/640 dilution) (Appendix 2), for analysis of T cells, the cells were stained with a cocktail mix (50 µl/sample) containing the following antibodies: PE anti-CD44 (1/320 dilution), PerCP-Cy5.5 anti-CD4 (1/320 dilution), V450 anti-CD62L (1/1280 dilution), AF700 anti-CD3 (1/320 dilution),V500 anti-CD8 (1/320 dilution) (Appendix, Table 4) in FACS buffer (1x PBS in 0.1% BSA) with 1% inactivated rat serum (iRS) and 1% anti-Fc RII/III (2.4G2, 2mg/ml) and incubated for 20 minutes on ice in the dark. After incubation, the cells were washed with 300 µl of FACS buffer and centrifuged at 1500 rpm for 3 minutes. Thereafter the remaining pellets were stained with 50 µl/sample of secondary detection antibody mix containing Streptavidin Texas Red (1/1000 dilution) in FACS buffer and incubated for 20 minutes on ice in the dark. After incubation, the cells were washed

with 200 µl of FACS buffer and centrifuged at 1500 rpm for 3 minutes at 4°C. Thereafter the supernatants were discarded, and the pellets were suspended in 150 µl of FACS buffer for acquisition.

2.2.9 Antibodies that were used for the analysis of myeloid cell populations from lung and BAL cell suspensions

Cells were stained for acquisition on LSR II Fortessa flow cytometer (BD Biosciences) as described above, but without the addition of a secondary detection antibody (streptavidin) since there were no biotin antibodies in this cocktail mix. The cells were stained with the following antibodies: FITC anti-Ly6G (1/320 dilution), PE anti-Siglec-F (1/320 dilution), PerCP-Cy5.5 anti-Ly6C (1/320 dilution), PE-Cy7 anti-F4/80 (1/640 dilution), V450 anti-CD11b (1/320 dilution), Qdot605 live/dead (1/2000 dilution), APC anti-CD11c (1/640 dilution)/ AF 700 anti-CD11c (1/320 dilution) (Appendix, Table 4) in FACS buffer with 1% iRS and 1% anti-Fc RII/III (2.4G2, 2mg/ml).

2.3 Induction of acute atopic dermatitis

A low calcemic analog of vitamin D3 (MC903) was used to induce atopic dermatitis-like symptoms. IL-4i1$^{-/-}$ and IL-4i1$^{+/+}$ littermate control mice, while under anaesthesia, were shaved on the ventral side between the pectoral and pelvic region 3 days before the start of the treatment with MC903. On day 04, 100 µl of 45 µM MC903 dissolved in absolute ethanol, were administered on the shaved area, absolute ethanol was administered to the control mice. IL-4i1$^{-/-}$ and IL-4i1$^{+/+}$ littermate control mice were weighed to record baseline weight prior to the start of treatment and were subsequently weighed daily throughout the course of treatment. IL-4i1$^{-/-}$ and IL-4i1$^{+/+}$ littermate control mice were treated with MC903 and absolute ethanol for nine consecutive days and on day 10 they were euthanized with halothane. Blood samples were collected as described in 2.2.1 for ELISA assay, thereafter the mice were shaved again, skin (2cm × 2cm) was collected and divided into two for qPCR and for histological analysis. Inguinal lymph nodes were also collected for analysis by FACS.

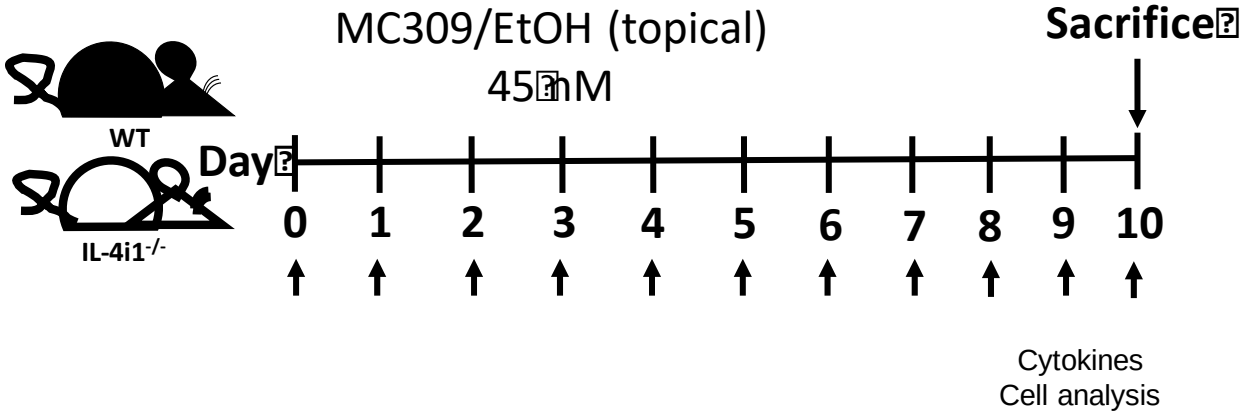

Figure 2.2: Timeline of skin sensitization protocol: IL-4i1$^{-/-}$ knockout mice or their littermate controls (IL-4i1$^{+/+}$) which were 6-12 weeks old were shaved 3 days before commencement of treatment with MC903 (45μM) dissolved in 100% ethanol or 100% ethanol as control. After 3 days the mice were treated with either MC903 or ethanol as a vehicle for 10 days.

2.3.1 Measuring of serum cytokine levels

Blood was collected by the cardiac puncture method as described in 2.2.1 above. The serum was processed as described in 2.2.4. The secreted levels of the following cytokines: IL-4, IL-5, IL-17, TSLP, IL-33, IL-25, and IL-13 were measured in serum by cytokine ELISA assay as in 2.2.5.

2.3.2 Lymph node processing and stimulation of cells for intracellular cytokine staining

Inguinal lymph nodes (iLNs) were collected in 1ml of cRPMI. The cells were then counted after processing in a 1/10 dilution using trypan blue dye using single-use chamber slides (Biosigma, Italy) under a microscope. After counting, cells were reconstituted in media to make up 2×10^6 cells/ml. The cells were then transferred into U-bottom plates with a lid. A mix of 1/200 Phorbol 12-myristate 13 acetate (PMA) (50ng/ml, Sigma-Aldrich), 1/200 ionomycin (250ng/ml, Sigma-Aldrich) and 1/250 monensin (200μM, Sigma-Aldrich) in sterile cRPMI medium was made. Thereafter, about 10 μl of this mix was added to each well containing 200 μl of cells. The plate was then incubated for 4-5 hours at 37°C. After incubation, the plate was centrifuged at 1500 rpm for 3 minutes, thereafter the supernatants were discarded and washed the plate with FACS buffer.

The cells were then stained for surface molecules with a primary antibody mix (50 μl/sample) containing the same antibodies in the B cell analysis panel as in 2.2.8. In addition, cells were stained for follicular helper T cells analysis with 50 μl/sample of antibody cocktail mix containing FITC anti-PD-1 (1/320 dilution), PE anti-CD44 (1/320 dilution), PerCP-Cy5.5 anti-CD69 (1/320 dilution), PE-Cy7 anti-CXCR5 (1/320 dilution), V450 anti-CD62L (1/1280 dilution), BV510 anti-CD4 (1/320), AF700 anti-CD3 (1/320 dilution) (Appendix, Table 5) in FACS buffer (1x PBS in 0.1% BSA) with 1% inactivated rat serum (iRS) and 1% anti-Fc RII/III (2.4G2, 2mg/ml). This was followed by incubation in the dark on ice for 20 minutes. After incubation, the cells were washed with 300 μl FACS buffer and centrifuged for 3 minutes at 1500 rpm. Thereafter the supernatants were discarded, and cells were resuspended in 300 μl permeabilization wash buffer (BD Pharmingen Transcription Buffer set, BD Biosciences) followed by centrifuging for 3 minutes at 1500 rpm. For fixation and permeabilization of cells, 50 μl/sample of fixation-permeabilization buffer (1 part concentrate + 3 parts of perm diluent) was added and incubated at 4°C for 30 minutes. After incubation, cells were washed with 300 μl permeabilization buffer, centrifuged for 3 minutes at 1500 rpm. For intracellular cytokine and intranuclear transcription factor analysis, 50 μl/sample of cytokine antibody

cocktail containing Alexafluor 488/FITC anti-IL-4 (1/100 dilution), PE anti-IL-5 (1/100 dilution), PE-Cy7 anti-IL-13 (1/100), APC anti-FoxP3 (1/50 dilution) and AF 700 anti-IFN-γ (1/100) (Appendix, Table 5) in permeabilization buffer with 2% iRS and 1% FcγRII/III blocker was added and incubated at 4°C for 45 minutes. The cells were then washed with permeabilization buffer and centrifuged at 1500 rpm for 3 minutes. The supernatants were discarded, and the cells were resuspended in 150 µl FACS buffer for acquisition.

2.4 Analysis of gene expression by reverse-transcription polymerase chain reaction (RT-PCR) assay

2.4.1 RNA extraction

Skin samples were firstly homogenized in 1ml Qiazol lysis reagent, thereafter RNA was extracted using Qiagen kit according to manufacturer's instructions (Qiagen, Germany). The supernatant was transferred into a fresh tube. About 140 µl of chloroform was added and the tube was then capped. The tubes were then shaken vigorously with a vortex for 15 seconds. The tubes were then placed at room temperature for 2-3 minutes, thereafter they were centrifuged at 12 000 rpm for 15 minutes at 4°C. The centrifuge was then heated to room temperature. The upper aqueous layer (RNA) was transferred to a fresh collection tube. About 375 µl of 100% ethanol was added and then mixed thoroughly by pipetting. A volume of up to 700 µl of sample including any precipitate was pipetted into RNeasy mini-column in a 2 ml collection tube. The tubes were then centrifuged at 8000 rpm for 15 seconds at room temperature. The flow-through was discarded. The above step was repeated using the remainder of the sample.

2.4.2 DNase digestion

On-column DNase digestion was carried out using Qiagen Mini Kit according to the manufacturer's instructions. Briefly, 350 µl of buffer RW1 was added into the RNeasy mini spin column and centrifuged for 1 minute at 8000 rpm to wash. The flow-through was discarded, and the collection tube was reused. A volume of 10 µl of DNase I stock solution was added to 70 µl buffer RDD per sample and mixed by inverting the tube. A volume of 80 µl of DNase I mix was added directly onto the RNeasy mini spin column membrane on the benchtop at 20-30°C for 15 minutes. Thereafter, 350 µl buffer RW1 was added into the RNeasy mini spin column and centrifuged for 15 seconds at 8000 rpm. The flow-through was discarded.

2.4.3 Elution of RNA

500 µl buffer RPE was added onto RNeasy mini-column and centrifuged for 15 seconds at 8000 rpm. The flow-through was discarded. The column was then carefully removed to prevent contact with the flow-through. The RNeasy mini-column was placed in a new 2 ml collection tube. The tube was then centrifuged at full speed for 1 minute. The RNeasy mini-column was then transferred to a new 1.5 ml collection tube

supplied by Qiagen. 40 µl of RNase-free water was added directly onto the RNeasy mini column membrane and centrifuged for 1 minute at 8000 rpm to elute RNA.

2.4.4 RNA quantification

The amount of RNA in each sample was quantified using Nanodrop One (Thermo scientific). RNase-free water was used to clean the pedestal prior to measuring and after measuring each sample. RNase-free water was also used to blank the instrument, then 1 µl of the sample was used in measuring the amount of RNA present. The purity of RNA was determined by an approximate ratio of 1.9 – 2 between 260 nm and 280 nm.

2.4.5 cDNA synthesis

Transcriptor First Strand cDNA Synthesis Kit (Roche) was used following the manufacturer's guidelines. RNase-free water was used in normalizing all RNA samples to the lowest RNA concentration. 10 µl of RNA and water was added to a primer mix of anchored-oligo (dT) primer and random hexamer primer as in Table 1. Thereafter, the template-primer mix was denatured by heating the tube for 10 minutes at 65°C in a thermal block cycler (Bio-Rad, PTC 100) with a heated lid. The samples were then immediately cooled on ice and 7 µl of First-Strand Synthesis cocktail (see Table 1 below) that contains Transcriptor reverse transcriptase reaction buffer, Protector RNase inhibitor, Deoxynucleotide mix, and Transcriptor reverse transcriptase were added to the template-primer mix with a pipette. The tubes were then centrifuged briefly to collect the sample on the bottom of the tube. The samples were incubated in a thermal block cycler for 10 minutes at 25°C, 60 minutes at 50°C, 5 minutes at 85°C, and 2 hours at 4°C. The samples were then diluted 1/10 in RNAse-free water and were stored at -20°C until qPCR was carried out.

Table 1: Reagents used in transcriptor first-strand cDNA synthesis

Component	Volume	Final concentration
Anchored oligo (dT) 18 primer, 50 pmol/µl	1 µl	2.5 µM
Random hexamer primer, 600 pmol/ µl	2 µl	60 µM
Transcriptor reverse transcriptase reaction buffer, 5× concentration	4 µl	1× (8 mM) $MgCl_2$)
Protector RNase inhibitor, 40 U/µl	0.5 µl	20 U
Deoxynucleotide mix, 10 mM each	2 µl	1 mM each

Transcriptor reverse transcriptase, 20 U/µl	0.5 µl	10 U

2.4.6.1 RT-qPCR assay

The LightCycler® 480 SYBR Green I Master Kit (Roche) was used for this assay. Decontamination of tools was carried out by exposing them to UV light for 5 minutes beforehand. Thereafter, 18 µl of the PCR mix containing 4.8 µl of LightCycler® 480 SYBR Green I Master PCR-grade water, 10 µl of 2× Master Mix, and 1.6 µl of 6.25µM Forward and Reverse Primer was added to the LightCycler® 480 multi-well plate. After that, 2 µl of cDNA was added to the PCR mix to make a total volume of 20 µl reaction mixture, and water only was added for negative controls. The multiwell plate was then covered with star seal advanced polyolefin film (Star Lab International GmbH) and centrifuged at 1200 rpm for 2 minutes at 4°C.

2.4.6.2 Thermocycler conditions and gene expression quantification

Amplification of PCR products was carried out using this programme: 1 cycle of activation at 95 °C for 10 minutes; 55 cycles of amplification consisting of denaturation for 5 seconds at 95 °C, primer annealing for 15 seconds at 55 °C, primer extension for 15 seconds at 72 °C, and data acquisition for 1 second at 80 °C; 1 cycle of melting curve analysis consisting of denaturation for 10 seconds at 95 °C, re-annealing for 30 seconds at 65 °C, and melting for 0.11 °C/second at 95 °C; and 1 cycle of cooling at 40 °C for 30 seconds. The concentration of mRNA transcripts was normalized to the mRNA level of the beta-actin house-keeping gene. Relative fold changes in gene expression were calculated using the formula $2^{-\Delta\Delta Ct}$, a method previously descibed[178]. Expression levels of each gene were plotted using GraphPad Prism 8 software.

Table 2: Primer sequences (Integrated DNA Technologies, USA) used for qRT-PCR

Gene name	**Forward primer sequence (5’→ 3’)**	**Reverse primer sequence 5’→ 3’)**
IL-5	TCA CCG AGC TCT GTT GAC AA	CCA CAC TTC TCT TTT TGG CG
IL-13 GEX	CTC CCT CTG ACC CTT AAG GAG	GAA GGG GCC GTG GCG AAA CAG
IL-33	AAC CAG CTG GCT CTA GTG GA	ACT GTG GTG CCT GCT CTT CT
IL-25	CAT TCT TGG CAA TGA TCG TG	GAA GAC CGT CGT GTT GTG GT
TSLP GEX	TTC ACC ACC ATG GAG AAG GC	GGC ATG GAC TGT GGT CAT GA

IL-4	TCG GCA TTT TGA ACGA GGTC	GAA AAG CCC GAA AGAG TCTC
IFN-γ	GCT CTG AGA CAA TGAA CGCT	AAA GAG ATA ATC TGGC TCTGC
IL-17	CTC CAG AAG GCC CTCA GACT AC	AGC TTT CCC TCC GCAT TGAC ACAG
Beta-actin	TGG AAT CCT GTG GCA TCC AGA AAC	TAA AAC GCA GCT CAG TAA CAG TCC G

2.5 Histology

2.5.1 Lung histology

Lung sections were fixed in 4% formaldehyde in PBS and embedded in paraffin. Sections were cut in 5-7 µm and stained with haematoxylin and eosin (H&E) for tissue inflammation and periodic acid-Schiff reagent (PAS) for mucus production. The processing and staining procedures were performed by Ms Lizette Fick from Groote Schuur Hospital (Department of Surgery).

Slides were scanned at 20x magnification on the Virtual slide VS120 microscope (Olympus, Japan). Quantification of mucus was carried out using the automated NIS elements software by defining regions of interest (ROIs) which are the individual bronchioles on cut lung sections and using threshold quantification of mucus stain in specific ROIs (NIS elements; Nikon Instruments, Japan).

2.5.2 Skin histology

Skin samples were fixed in 4% formaldehyde in PBS and embedded in paraffin, as described in 2.5.1. The sections were stained with H&E for inflammation, Toluidine blue for mast cell recruitment, and Sirius red for eosinophil recruitment. The epidermal thickness of the skin was then quantified using the QuPath v2.0.2-m2 software by opening a VSI file in QuPath software and used the line tool to make annotations in the region of interest (epidermis) to measure the length (µm) of thickness. The average length was calculated on excel and a graph of epidermal thickness was plotted using GraphPad Prism.

2.6 Quantification of nitric oxide production by cells

Cell supernatants were thawed at room temperature for about 30 minutes. Griess reagent 1 (solution A) and Griess reagent 2 (solution B) were put at room temperature to equilibrate as previously described[179]. A dilution was then prepared by putting 50 µl of RPMI media in each well standard in a 95-well flat-bottom plate. Other wells were used as blanks and had only 50 µl of media. A standard of 1mM $NaNO_2$ was serially

diluted in the wells designated for standard. About 50 µl of the sample was added to the other well. A volume of 25 µl/well of solution A was added and incubated in the dark for 5 minutes at room temperature. After 5 minutes, 25 µl of/well of solution B was added and incubated for 5 minutes in the dark until a purple colour was visible. The plate was then read at 540nm and reference at 690nm.

2.7 Statistical analysis

Statistical analysis was performed by one-way anova and t-tests for comparison of differences between the groups of interest. GraphPad Prism software (version 8.0.2, San Diego, CA) was used for one-way anova and unpaired student t-tests analysis. Two-way anova with Bonferroni post tests was used in airway hyperresponsiveness analysis. All data were presented as mean ± SEM or mean ±SD, where significance is shown by $*p< 0.05$, $**p<0.01$, $***p<0.001$.

Chapter 3: Role of IL-4i1 in HDM-induced allergic asthma

Abstract

Background

Allergic asthma is driven by abnormal Th2 and Th17 immune responses against innocuous allergens. Cytokines IL-4, IL-15 and IL-13 are produced by allergen-specific effector Th2 cells and promote the production of IgE by B cells, eosinophil recruitment and activation, AHR and mucus secretion, respectively. Defects in immunosuppressive action by regulatory T cells (Tregs) may provide an explanation for the development of allergic reactions. In humans and mice, IL-4i1 is expressed by antigen-presenting cells (APCs) and portrays an immunomodulatory factor via inhibition of T cells immune activation through secretion of toxic metabolites.

Objective

The immunoregulatory activity of IL-4i1 in allergic asthma has not yet been elucidated, thus in this study, we wanted to investigate the role of this enzyme in a house dust mite (HDM)-induced allergic asthma.

Method

IL-4i1 knockout (IL-4i1$^{-/-}$), IL-4i1 wildtype littermate control (IL-4i1$^{+/+}$), and BALB/c mice were sensitized intratracheally (I.T) on day 0 with a high (100 μg) dose of HDM or phosphate-buffered saline (PBS) in control mice. The mice were then challenged intranasally (I.N) with 10 μg HDM or PBS on day 7-11. On day 14, lung function was assessed, and tissues were collected for processing and analysis.

Results

High dose HDM I.N sensitization and I.T challenge resulted in a significant reduction of total IgE, reduced mucus secretion, increased Th2 cytokines and eosinophils in IL-4i1 deficient mice compared to the littermate controls. These data suggest an immunomodulatory role of IL-4i1 in allergic inflammation.

3.1 Introduction

T helper 2 (Th2) and Th17 immune responses against innocuous allergens drive the chronic inflammatory disease known as allergic asthma[180]. In early life, it has been suggested that reduced exposure to microbes results in polarization of allergen-specific T cell memory towards Th2 instead of protective Th1 immune response[40]. Type 2 immunity to House dust mite (HDM), a major allergen source in humans, occurs via epithelial cell sensing of LPS and HDM compounds and the release of pro-allergic alarmins that direct lung dendritic cells (DCs) to sample allergens, transport them to the draining lymph nodes leading to the induction of allergen-specific $CD4^+$ Th2 cells[181].

Activated Th2 cells induce the production of various cytokines such as IL-4, IL-5 and IL-13, where IL-4 promotes IgE production by B cells, IL-5 participates in recruitment and activation of eosinophils, IL-13 promotes mucus hypersecretion and AHR[182,40,183,94]. Studies of animal models have shown that depletion of $CD4^+$ T cells prevents the development of asthma, thus suggesting Th2 cells as fundamental lymphocytes in allergic asthma immune response[184,103]. Th1 cells develop from naïve $CD4^+$ cells in response to microbial activation of APCs in the presence of IL-12. As differentiated cells, they secrete IFN-γ, a cytokine playing an essential role in the intracellular destruction of phagocytosed microbes[40].

Regulatory T cells (Tregs) play an important role in suppressing dysregulated immune response in asthma pathogenesis, maintain tolerance and homeostatic balance of immune responses[185,186]. However, individuals with allergic asthma are reported to possess fewer and defective Tregs and this leads to uncontrolled effector cell responses and thus promoting pro-asthmatic responses of Th2 and Th17 cells[185]. Th17 cells with their signature cytokine IL-17 are associated with severe asthma, mediate neutrophilic inflammation and exacerbate Th2-mediated allergic inflammation[186].

Interleukin-4 induced gene 1 (IL-4i1) is expressed by APC in both humans and mice. Microarray data have identified significantly increased IL-4i1 mRNA levels in tumour-induced murine myeloid-derived suppressor cells (MDSCs), implicating IL-4i1 in negative feedback regulation of T cell activation[187]. Under physiological temperature and pH, IL-4i1 deaminates phenylalanine into pyruvate, ammonia and H_2O_2[149]. This demonstrates an immunomodulatory factor of IL-4i1 via the inhibition of T cell proliferation associated with a downregulation of CD3zeta chain expression through its enzymatic activity[150,187], furthermore, IL-4i1 has been shown to promote Treg cell expansion[188]. The immunoregulatory property of IL-4i1 has not yet been explored in allergic asthma, thus we induced allergic asthma in IL-4i1 deficient mice, and their wild-type littermate controls to investigate *in vivo* role of this enzyme in this disease. We hypothesized that in the absence of IL-4i1 there would be a lack of Tregs and expansion of T cell activation leading to exacerbated allergic asthma. In the absence of IL-4i1, we found reduced mucus production, total

IgE production which correlated with reduced GCs and increased Type 2 associated cytokines which correlated with increased eosinophils.

3.2 Results

Effect of IL-4i1 in the humoral immune response during HDM-induced allergic asthma

IL-4il is highly expressed in Mycobacterium tuberculosis-induced lung granulomas[189]. However, very little is known about the role of this enzyme in other lung diseases, especially those that involved effector T cells and T regulatory cells. To understand the role of IL-4il in allergic asthma, type 2 mediated lung disease, we sensitized IL-4i1$^{-/-}$, and IL-4i1$^{+/+}$ (wildtype littermate control) mice intratracheally with HDM (100 µg) and a week later challenged with 10 µg HDM as shown in chapter 2 (figure 2.1). We assessed the production of allergen-specific antibodies as well as total IgE in the serum taken on day 14. We observed a significant reduction in total IgE levels (Fig **3.1 A**) in IL-4i1$^{-/-}$ mice when compared to wildtype-littermate control mice. No notable differences were observed in the levels of HDM-specific IgE (Fig **3.1 B**), IgG1 (Fig **3.1 C**), IgG2a (Fig **3.1 D**), IgG2b (Fig **3.1 E**) and IgM (Fig **3.1 F**) between IL-4i1$^{-/-}$ mice and wildtype-littermate control mice. This suggests that IL-4il promotes certain features of type 2 associated antibodies, but not allergen-specific antibody production. Low dose HDM (1 µg) sensitization and challenge yielded no significant difference in AHR, humoral immune response and cytokine production between IL-4i1 knockout mice and their wildtype littermate controls (Appendix, Figure A1).

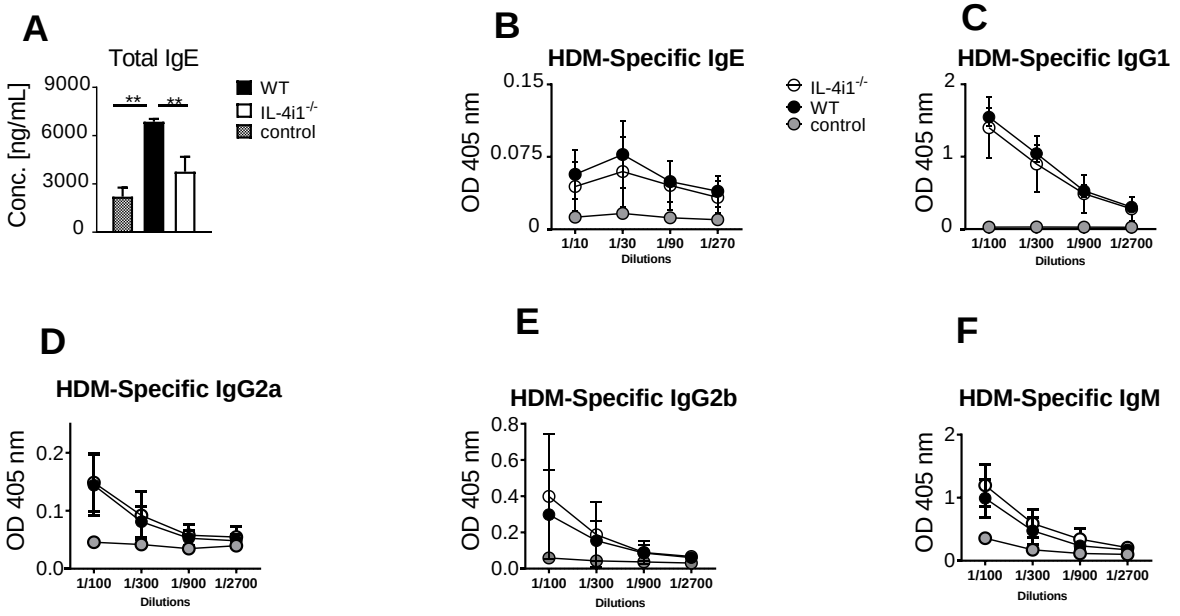

Figure 3.1: Antibody production in the serum measured by ELISA 14 days post sensitization and challenge with HDM. A) Total IgE levels in the serum of IL-4i1$^{-/-}$ mice treated with HDM (white square, Wild-type littermate control mice (black square) and BALB/c wildtype mice as control treated with PBS (Grey square). Data shown as Mean ±SD of 1 experiment from 3 independent experiments (n= 4-6 mice/group). Statistical significance was evaluated with Mann-Whitney student t-test ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$). **B-F)** Serum levels of HDM-specific antibodies (IgE, IgG1, IgG2a, IgG2b and IgM) measured by ELISA in IL-4i1$^{-/-}$ mice treated with HDM (white circle), Wild-type littermate control mice treated with HDM (black circle), and Control mice treated with PBS (grey circle). Data shown as Mean±SDs from 1 representative experiment of 3 independent experiments (n=4-6 mice/group).

IL-4i1 deletion induces an increase in the production of Th2 immune response during HDM induced allergic asthma

To assess the role of IL-4i1 in the production of cytokines, mediastinal lymph nodes (MLNs) from IL-4i1$^{-/-}$ HDM, wild-type littermate control (IL-4i1$^{+/+}$ HDM) and control mice (BALB/c PBS) were stimulated *in vitro* with anti-CD3 and HDM for 5 days. In the absence of IL-4i1, we saw a significant increase in levels of IL-5 and IL-13, but not IL-4 in the anti-CD3 stimulated MLNs (Fig **3.2 A**). Also, there were no significant differences in levels of IFN-γ and IL-17 between IL-4i1$^{-/-}$ and wild type littermate control mice (Fig **3.2A**). When we stimulated MLNs with the allergen (HDM) we did not detect any levels of IL-13, IL-17 and IFN-γ, however, we saw a significant increase in levels of IL-5 (Fig **3.2 B**) but not IL-4 (Fig **3.2 B**) in IL-4i1$^{-/-}$ mice compared to IL-4i1 wild-type littermate control mice.

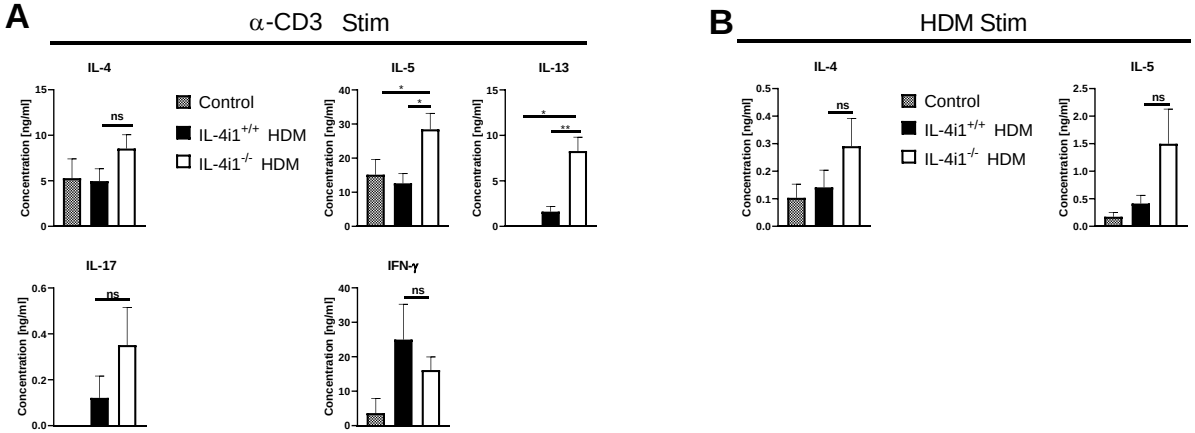

Figure 3.2: IL-4i1 deletion promotes a significant increase in the secretion of Th2 cytokines (IL-5, IL-13) but not IL-4. Cytokine production was measured in ex vivo stimulated MLNs by ELISA. **A)** IL-4, IL-5, IL-13, IL-17 and IFN-γ levels in anti-CD3 (10 µg) stimulated MLNs. **B)** IL-4 and IL-5 levels in HDM (30 µg) stimulated MLNs 14 days post sensitization and challenge with HDM. Data is shown as Mean±SEM of pooled data from 3 independent experiments (n=8-12). Statistical significance was evaluated with Mann-Whitney student t-test ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$).

Since we have observed an increase in type 2 immune response-related cytokines at protein level, we assessed expression levels of cytokines in HDM-challenged mice at mRNA level using quantitative PCR. Total RNA was extracted from lung tissue using the Qiagen kit and was reverse transcribed into cDNA. We did not detect any transcripts of the cytokines (IL-4, IL-5, IL-17 and IFN-γ) in all the groups of mice. We observed a slight increase in the expression levels of IL-13 and IL-33 in the IL-4il knockout mice compared to littermate mice, however, the difference was not statistically significant. Overall, these results demonstrate that IL-4il dysregulates type 2 immune response cytokines and detection of IL-4il exacerbates Th2 cytokine secretion.

Figure 3.3: IL-4il deletion in mice did not affect mRNA level cytokine expression in HDM-induced allergic airway disease. Quantitative analysis of **A)** IL-13 and **B)** IL-33 expression in the lung by RT-qPCR. Data are shown as Mean±SEM of pooled data from 2 individual experiments (n= 8-12)

Deletion of IL-4i1 in mice has no impact on airway hyperresponsiveness and total cells in the lung but significantly increases eosinophils

Airway hyperresponsiveness (AHR) and airway inflammation form part of hallmarks implicated in asthma pathophysiology. Inflammation is characterized by infiltration of inflammatory cells (T cells and eosinophils)[190]. Since we observed a significant increase in IL-5 and IL-13 secretion in the IL-4i1 knockout mice, we then sought to assess whether IL-4i1 has any impact on lung function. On day 14, IL-4i1$^{-/-}$ and IL-4i1 wild type littermate control mice challenged with HDM, or BALB/c control mice challenged with PBS were cannulated and given increasing doses of methacholine while they were under anaesthesia to measure lung function. We did not observe any differences in lung resistance between IL-4i1 knockout mice and the wild-type littermate control mice (Fig **3.4 A**). Lung elastance (Fig **3.4 B**) was also similar between IL-4i1 knockout mice and the wild-type littermate control group. This suggests that IL-4i1 has no overall impact on AHR. Furthermore, we assessed immune cell profiles in the lung by flow cytometry and we found no differences in the total number of cells infiltrating the lungs (Fig **3.4 C**) between the IL-4i1$^{-/-}$ and IL-4i1 wild-type littermate control mice. We also observed similar numbers of $CD3^{+}CD4^{+}$ T cells (Fig **3.4 D**) in the lungs of IL-4i1$^{-/-}$ and IL-4i1 wild-type littermate control mice.

We then assessed myeloid cell subsets infiltrating the lungs, and we observed a significant increase in the number of infiltrating eosinophils, alveolar macrophages, dendritic cells (DCs) and monocytes in the HDM-challenged IL-4i1$^{-/-}$ compared to littermate control mice (Fig **3.4 E**). Percentages of myeloid immune cells infiltrating the lungs were also similar to the cell numbers in Fig **3.4 E** except for the percentage of alveolar macrophages which was not significantly different between IL-4i1$^{-/-}$ mice and their littermate control mice (Fig **3.4 F**). We then assessed mucus production in the lungs by Periodic Acid Schiff (PAS) staining and we observed a slight reduction in mucus produced by` IL-4i1$^{-/-}$ mice compared to IL-4i1 wild-type littermate control mice (Fig **3.4 G**). Taken together, these results suggest that IL-4i1 is required to regulate some aspects of allergic asthma such as eosinophilia, but not AHR and mucus production.

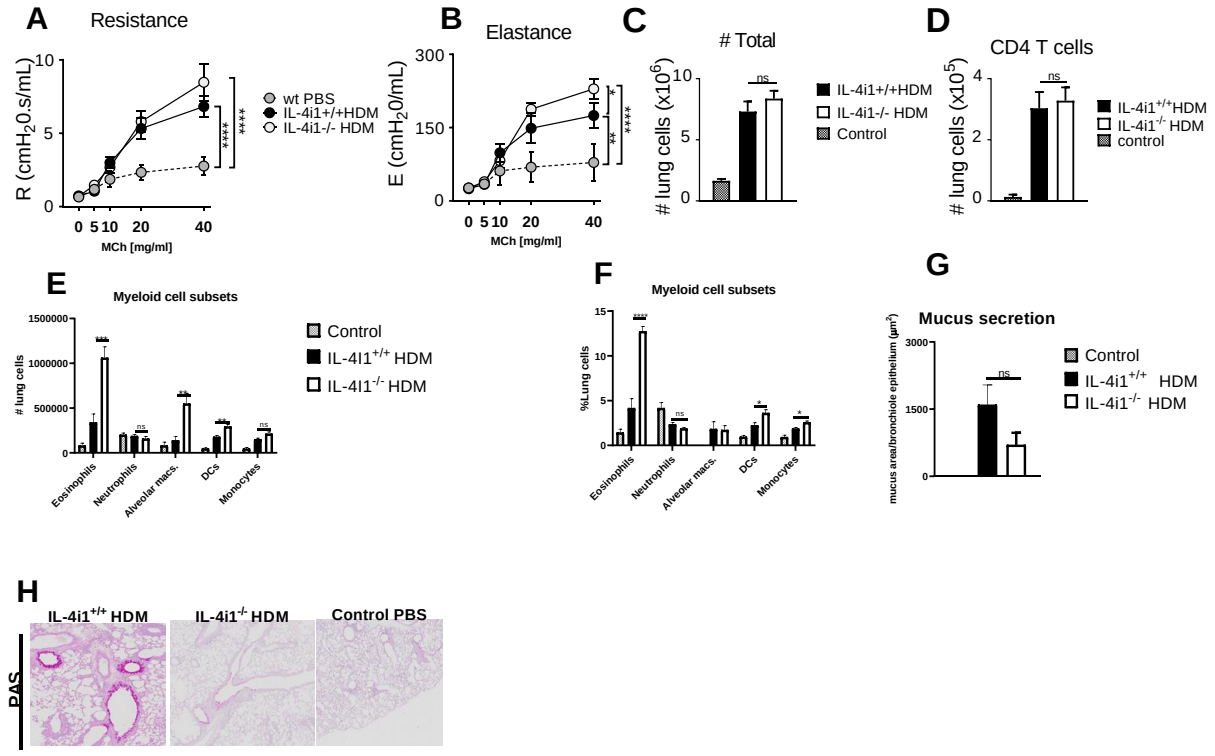

Figure 3.4: Absence of IL-4i1 induces an increase in the number of eosinophils, DCs and alveolar macrophages in the lungs. A) Airway resistance and **B)** Lung elastance were measured on day 14 by exposing mice to increasing doses of methacholine. Shown is 1 representative of 3 independent experiments (n=4-6 mice/group), data shown as Mean±SD where significance was analysed with ANOVA repeated measures with Bonferroni post-test. **C)** Total number of cells infiltrating the lung, **D)** Frequency of $CD3^+CD4^+$ T cells, data shown as Mean±SD of 1 representative of 3 independent experiments. **E)** Number of myeloid cell subsets: Eosinophils ($CD11c^-CD11b^+Ly6G^-SiglecF^+$), Neutrophils ($CD11c^-CD11b^+Ly6G^+$), Alveolar macrophages ($CD11c^+SiglecF^+$), Dendritic cells ($CD11b^+CD11c^+$) and Monocytes ($Ly6G^-Ly6C^+$) infiltrating the lung, **F)** Percentages of myeloid cell subsets infiltrating the lung. Data are shown as Mean±SD of 1 representative of 3 independent experiments (n=4-6 mice/group). Differences were analysed with One Way ANOVA with Tukey's multiple comparison tests. **G)** Quantification of the area of mucus staining per bronchiole epithelial lining was analysed with NIS imaging software, **H)** Histology of the lung with Periodic Acid Schiff (PAS) staining. Data are shown as Mean±SEM of pooled data from 3 individual experiments (n=8-14). ($^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$), ns: Not significant.

When we assessed the infiltration of B cells and their subsets by flow cytometry in the lung and MLNs, there were no differences in the total cells infiltrating MLNs between the IL-4i1$^{-/-}$ HDM-challenged mice

and their HDM-challenged littermate control mice (Fig **3.5 A**). There were no differences in the frequencies of $CD19^{+}B220^{+}$ B cells and follicular B cells (FO B cells) between the IL-4i1 knockout mice and their littermate control mice in the MLNs (Fig **3.5 B**). However, we observed a significant increase in the number and percentages of marginal zone B cells (MZ B cells) and a significant reduction in the cell numbers and percentages of germinal center B cells (GC B cells) in the MLNs of IL-4i1$^{-/-}$ mice compared to IL-4i1$^{+/+}$ littermate control mice challenged with HDM (Fig **3.5 B**). Also, in the lung, there were no differences in the numbers and percentages of $CD19^{+}B220^{+}$ B cells between the IL-4i1 knockout mice and their littermate control mice (Fig **3.5 C**). Similarly, to the MLNs, there was a significant increase in cell numbers and percentages of marginal zone B cells in the IL-4i1$^{-/-}$ mice compared to littermate control mice (Fig **3.5 C**). In contrast to what was observed in the MLNs, the percentage of follicular B cells was significantly reduced, and the cell numbers of germinal centre B cells were significantly upregulated in the IL-4i1$^{-/-}$ mice compared to their littermate control mice (Fig **3.5 C**). Nitric oxide production was significantly increased in anti-CD3 restimulated MLNs supernatants of IL-4i1$^{-/-}$ mice compared to the IL-4i1 littermate control mice. (Fig **3.5 D**). In the bronchoalveolar lavage fluid (BALF), nitric oxide production was increased but none significantly in the IL-4i1$^{-/-}$ mice compared to littermate control mice (Fig **3.5 E**). Taken together, these results demonstrate that IL-4i1 regulates B cell plasma producing cells in the MLNs and nitric oxide production in the lung tissue during HDM-induced allergic airway disease.

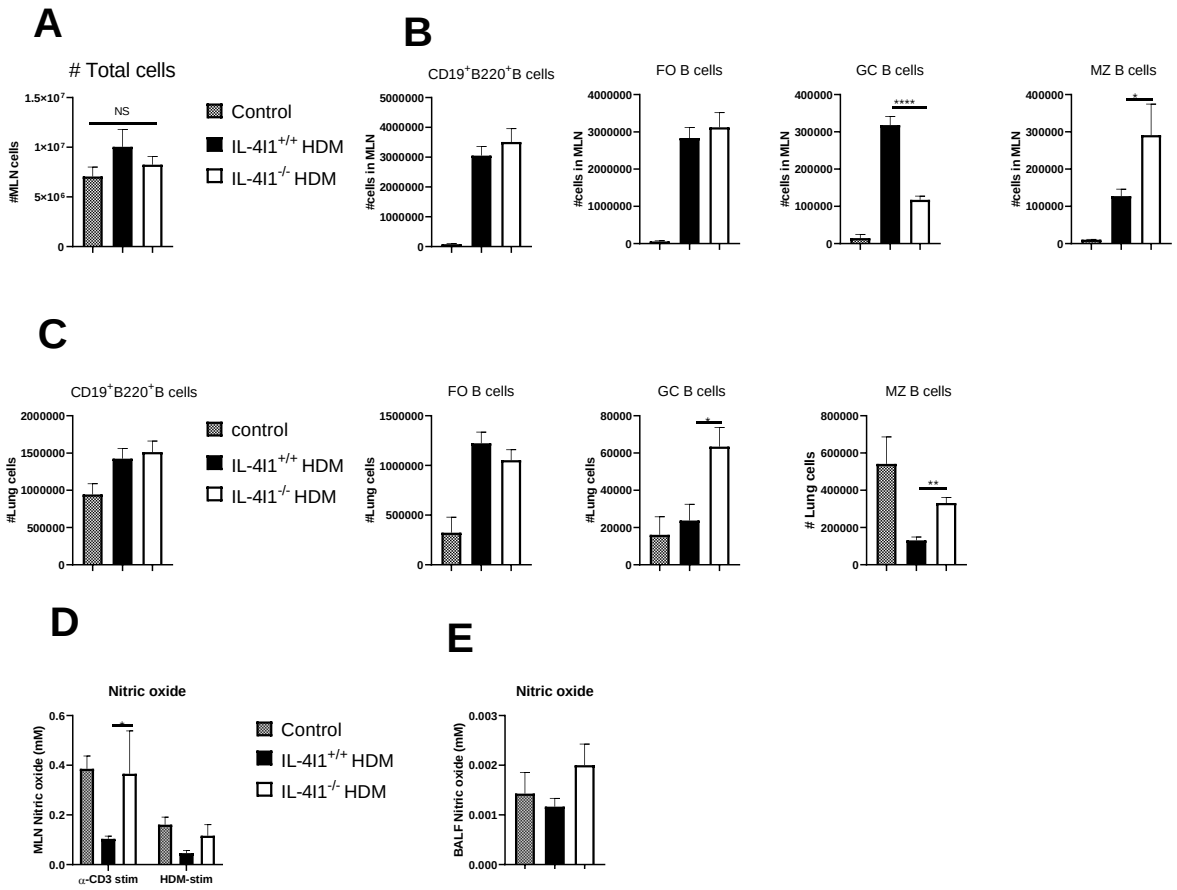

Figure 3.5: IL-4i1 deletion significantly reduces the number of GC B cells in MLNs, but not in the lung during HDM-induced allergic asthma. Mediastinal lymph nodes (MLNs) were collected and processed into cell suspensions on day 14 post sensitization and challenge with HDM. Cell suspensions from groups of mice (IL-4i1$^{-/-}$ HDM, IL-4i1$^{+/+}$ HDM, and Control PBS) were stained for flow cytometry analysis of **A)** Total cell numbers in MLNs, **B)** Cell numbers of B cells subsets; CD19^{+}B220^{+}, Follicular (CD21/35^{+}CD23^{+}), Germinal center (GL7^{+}CD19^{+}) and Marginal zone (CD21/35^{+}CD23^{-}) B cells in MLNs. **C)** Cell numbers of B cells subsets; CD19^{+}B220^{+}, Follicular, Germinal center and Marginal zone B cells in the lung. Data are shown as Mean±SD of 1 representative experiment of 3 independent experiments (n=4-6 mice/group). Statistical significance was evaluated with Mann-Whitney student t-test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. **D)** Nitric oxide from anti-CD3/ HDM- restimulated MLN supernatants. **E)** Nitric oxide from Bronchoalveolar lavage fluid (BALF) supernatants. Data shown as Mean±SEM of pooled data from 2 individual experiments (n=8-12), statistical significance was evaluated with Mann-Whitney student t-test (*p<0.05).

3.3 Discussion

IL-4i1 exerts an immunoregulatory role *in vitro*, particularly on T lymphocytes[154]. *In vitro*, IL-4i1 suppresses effector/memory T cell proliferation and downregulates inflammatory chemokines and Th1 cytokines (IL-2, IFN-γ) production[159,153,150,164]. Suggested mechanisms imply that IL-4i1 directly downregulates CD3ζ chain expression via production of H_2O_2[150] or through polarization of macrophages toward an M2 phenotype[146] and/or indirect inhibition through stimulation of naïve $CD4^+$ T cell differentiation into Tregs[158]. *In vivo* role of IL-4i1 on asthma and mechanisms involved have not yet been explored. In this study, we aimed to investigate the role of IL-4i1 in HDM-induced allergic asthma in mice. We used IL-4i1 knockout (KO) mouse model and found reduced total IgE production, upregulation of type 2 cytokines, increased lung eosinophilia and increased marginal zone B cells in both lung and mediastinal lymph nodes in IL-4i1-deficient mice.

Systemically, we observed that the absence of IL-4i1 significantly impaired total IgE production. In contrast, allergen-specific antibody responses were unchanged as there were no major differences in the production of HDM-specific IgE, IgG1, IgG2a, IgG2b and IgM between IL-4i1 knockout mice and their wildtype littermate controls. Surprisingly, type 2 cytokines IL-4, IL-5 and IL-13 were increased in *ex vivo* anti-CD3 and HDM-restimulated lymph nodes of IL-4i1$^{-/-}$ mice in comparison to their wild-type littermate control counterparts.

Furthermore, we observed a slight increase of Th17 cells related cytokine (IL-17) in IL-4i1$^{-/-}$ mice, while the secretion of Th1 cells related cytokine (IFN-γ) was slightly reduced in IL-4i1$^{-/-}$ mice compared to their wildtype littermate controls. IFN-γ secreted by Th1 cells is reported to possess repressive effects on Th2 cells, and it suppresses IgE isotype switching and can further stimulate cell-mediated cytotoxic effects during allergic inflammation[186]. The increase in type 2 cytokines, specifically IL-4 and IL-13 in IL-4i1$^{-/-}$ mice was unexpected and remains unclear. Since total IgE production was significantly reduced in the knockout mice, we had expected a reduction as well especially in IL-4 secretion in the knockout mice since IL-4 secreted by Th2 cells is said to be the main driver of IgE production by allergen-specific B cells[191,192,193,194].

When we assessed lung function, we observed a significant increase of airway resistance in the IL-4i1$^{-/-}$ mice sensitized and challenged with HDM compared to the control mice treated with PBS, however, this difference was not significant when comparing IL-4i1$^{-/-}$ mice with their wildtype littermate control counterparts. We saw a significantly higher airway elastance in IL-4i1$^{-/-}$ mice compared to IL-4i1 wildtype littermate control mice and the control PBS group. This slight increase in AHR although not significant in IL-4i1$^{-/-}$ mice was consistent with a significantly increased recruitment of eosinophils to the lung. In a study

of multiple sclerosis, Psachoulia *et al*., reported that deficiency of IL-4i1 in mice resulted in an intensified and unresolved inflammation in lesions, which led to an aggravated axonal injury and remyelination impairment[195]. In contrast, IL-4i1 deficient mice showed a slight but statistically insignificant decrease in mucus secretion around the bronchioles. This demonstrates a dichotomous effect of IL-4i1 in the regulation of allergic pathology. IL-4i1 has been found to inhibit human $CD8^+$ and $CD4^+$ T lymphocyte proliferation *in vitro* via production of H_2O_2, with a preference towards memory T lymphocytes, suggesting an essential role IL-4i1 may play in regulating adaptive immune responses[147]. In our case, the number of total cells in the lung were comparable between the IL-4i1$^{-/-}$ mice and wildtype littermate control, this trend was also the same in the frequency of $CD4^+$ T cells in the lung.

We also observed a significant increase in the number of DCs recruited to the lung in the IL-4i1$^{-/-}$ mice compared to wildtype littermate counterparts. The $CD11b^+$ cDC subset is said to be an abundant population in the lung, they are said to be important for allergen-induced Th2 response, and in addition, they promote differentiation of Th2 and Th17 via a dectin-2-dependent mechanism in an HDM-mediated asthma model[180]. Overall, this suggests that IL-4i1 does not affect T cell recruitment in the lung tissue and that in the absence of IL-4i1 DCs function of allergen uptake and presentation is not impaired. This requires further investigation as we do not know if these DCs are the ones promoting these Th2 cytokines since we did not do intracellular staining.

Total cell recruitment in the MLNs was slightly reduced in the IL-4i1-deficient mice, although without statistical significance. We observed comparable numbers and percentages of $CD19^+B220^+$ and FO B cells between IL-4i1-deficient mice and the wild-type littermates in MLNs. Furthermore, GC reaction was impaired in IL-4i1-deficient mice as denoted by the decreased expression of $GL7^+$ $CD19^+$ cells. In contrast, MZ B cells were significantly increased both in numbers and percentages in MLNs of IL-4i1$^{-/-}$ mice compared to wild-type littermates. This is in contrast with a previous study by Bod et al, where IL-4i1 deficient mice had an increase in FO and GC B cell and concluded that IL-4i1 deficiency enhanced T cell-dependent (TD) B cell immune response[160]. In the lung, the frequency of $CD19^+B220^+$ B cells was similar; however, the percentage of FO B cells was significantly reduced in IL-4i1$^{-/-}$ mice while GC B cells number was significantly upregulated in IL-4i1 deficient mice. As seen with MLNs, IL-4i1-deficient mice displayed a significant in MZ B cells in IL-4i1$^{-/-}$ compared to the wildtype littermates. Overall, these data suggest an immunoregulatory role of IL-4i1 in B cell differentiation and B cell immune responses during allergic asthma.

To a lower extent, IL-4i1 is reported to catabolize semi-essential amino acid arginine resulting in the production of toxic metabolites such as H_2O_2 and NO[160,196]. We assessed IL-4i1 enzymatic activity by

measuring nitric oxide (NO) production in anti-CD3 stimulated lymphoid cells and in BALF. In IL-4i1 deficient mice, NO production was significantly increased in anti-CD3 stimulated lymphocytes and this increasing trend was also observed in BALF, although this was not statistically significant. NO is a highly reactive, free radical molecule that is synthesized from L-arginine by nitric oxide synthase[197,198]. It has been reported that NO contributes to eosinophil migration from the circulation into the lung tissue and that high quantities of NO indirectly promote Th2 cell activation due to eosinophil recruitment[199,197]. By speculation, we think the increased NO production explains the upregulation of eosinophils and increased type 2 cytokines that we saw in the IL-4i1 deficient mice. Analysis of cytokine mRNA expression in the lung tissue revealed similar but slightly increased expression of IL-13 and IL-33 by the IL-4i1 deficient mice, thus indicating an important regulatory effect of IL-4i1 in type 2 allergic inflammatory conditions.

Conclusion

To our knowledge, we have shown for the first time that IL-4i1 regulates T and B cell response responses during allergic asthma. Deletion of IL-4i1 promotes lung inflammation, production of type 2 cytokines and reduced total IgE secretion. However, the mechanism by which IL-4i1 exerts these immunoregulatory properties still needs to be established.

Chapter 4: Role of IL-4i1 in MC903-induced atopic dermatitis (AD)

Abstract

Background

Atopic dermatitis (AD) is an inflammatory, relapsing, chronic disease with a prevalence of 15-30% in children and 2-10% in adults. AD is characterized by scaling, redness, epidermal hyperplasia, and itchy skin. T helper 2 (Th2) and Th1 responses dominate skin inflammation, and it is noted that both immune dysregulation and skin barrier dysfunction contribute to the development of disease. IL-4i1 is a secreted L-amino acid oxidase expressed by tumour-associated myeloid cells of solid tumours, including melanoma. IL-4i1 has been reported to facilitate tumour growth in a mouse model of melanoma via suppression of $CD8^+$ T cell-mediated immune response.

Objective

IL-4i1 has been shown to immunoregulatory properties in conditions like various skin cancers, however, its role in AD has not been elucidated. We wanted to examine the role of IL-4i1 in the development of MC903-induced AD.

Method

IL-4i1-deficient ($IL\text{-}4i1^{-/-}$) mice and their wildtype littermate control ($IL\text{-}4i1^{+/+}$) mice were topically administered with 45μM MC903 or ethanol (ETOH) as a control for 10 consecutive days with daily weight recorded. After 10 days, blood, skin, and inguinal lymph nodes (iLNs) were harvested for analysis.

Results

The absence of IL-4i1 significantly ameliorated MC903-induced AD pathology, most remarkably demonstrated by the decrease in serum total IgE, reduced epidermal thickness and reduced serum type 2 cytokines. Thus, this chapter has shown IL-4i1 to be crucial in the development of MC903-induced AD and perhaps may be a potential therapeutic target for AD treatment.

4.1 Introduction

Atopic dermatitis (AD) is defined as a chronic inflammatory skin disease that is most common in children (15-30%), and least common in adults (2-10%)[200]. Complaints of discomfort on patients with AD usually consist of pruritus, scaling or active dryness, and red or inflamed skin[201]. AD brings immense social and financial problems to the patients, their families and the community at large[128]. Currently, AD is considered as a biphasic T cell-mediated disease, whereby the acute phase is predominantly Th2 biased, and a switch from Th2 to Th1 promotes disease chronicity[202].

AD pathophysiology is considered to be complex, multifactorial and involves elements of dysregulation in cell-mediated immune responses, barrier dysfunction, environmental factors, loss of function mutations in filaggrin and IgE-mediated hypersensitivity[132,203,127]. When activated, Th2 cells secrete cytokines such as IL-4, IL-5, IL-13 and IL-10 to enhance humoral immune response[201]. IL-4 has been implicated in inhibiting the Th1 cell function, and thus exerting a Th2 dominance and Th2/Th1 imbalance[201].

Th17 and Th22 cells producing cytokines; IL-17, IL-19 and IL-22 have also been implicated in the initiation and maintenance of AD[200]. Keratinocytes release cytokines crucial for inflammation including thymic stromal lymphopoietin (TSLP), IL-25 and IL-33 under the influence of factors such as exposure to allergens, scratching and microbial action[200].

MC903 is a vitamin D3 analog which when topically applied to mouse skin induces an increase in serum IgE, infiltration of inflammatory cells in the skin, TSLP expression and epidermal thickening[204,134,205]. IL-4i1 is a secreted L-amino acid oxidase expressed by mononuclear phagocytes, dendritic cells and tumour associated myeloid cells[149]. Mouse tumour models have shown that IL-4i1 inhibits $CD8^+$ T cell antitumor response, thus promoting tumour and immune evasion[163]. IL-4il has been shown to be increased in skin cancers and leukemic lymphomas and associates with poor prognosis and early death[156]. The enzyme is also upregulated in lesion areas of psoriasis and atopic dermatitis patients[173,174]. In this study, we show that IL-4i1 promotes type 2 skin inflammation in an MC903-induced AD.

4.2 Results

To study the role of IL-4i1 in induced atopic dermatitis, IL-4i1$^{-/-}$ and littermate control IL-4i1$^{+/+}$ mice were shaved three days prior to topical application of MC903 (10nM to 45 µM), a vitamin D analogue that causes acute skin inflammation and skin irritation when applied to the shaved skin area. The three days are necessary for skin recovery post hair removal which can induce skin trauma. Mice were treated for 10 consecutive days with MC903 (100 µl/skin area) or ethanol as a control. The mice were weighed daily throughout the course of treatment.

We observed weight loss in both IL-4i1-deficient and IL-4i1 littermate control mice starting at day 7 post treatment with MC903. However, on days 9-10 post treatment, IL-4i1 knockout mice regained body weight while the littermate control mice lost more weight until the last day of treatment (Fig **4.1 A**). We analysed skin histopathology in haematoxylin and eosin (H&E)-stained sections using QuPath (Fig **4.1 B**). We observed a significant reduction in epidermal thickness in IL-4i1$^{-/-}$ mice compared to littermate control mice. (Fig **4.1 B**, **C**). Epidermal thickness was marginally increased in mice treated with ethanol but was not changed between IL-4il$^{-/-}$ and littermate controls. We then measured levels of total IgE in serum by ELISA and found significantly reduced levels of IgE in IL-4i1$^{-/-}$ mice compared to IL-4i1$^{+/+}$ littermate mice (Fig **4.1 D**). We also observed significant changes in total IgE levels between IL-4il$^{-/-}$ mice and IL-4il$^{+/+}$ littermate control mice treated with ethanol. IL-4il also has essential roles in metabolism through depletion of amino acids and formation of H_2O_2 and NO. We restimulated inguinal lymph nodes (iLNs) with PMA (50 ng/mL)/Ionomycin (250 ng/mL) and collected supernatants. We measured nitric oxide; a metabolite released by IL-4il dependent catabolism of L-tryptophan. We found an increase in nitric oxide in mice treated with MC903 compared to ethanol-treated mice, however, there was no difference between IL-4il$^{-/-}$ and littermate controls (Fig **4.1 E**). Collectively, these results indicate that IL-4i1 promotes skin inflammation in MC903-induced atopic dermatitis. Lower concentrations of MC903 (4.5nMol, 9nMol) were not sufficient to induce significant phenotypic differences in skin inflammation and cytokine responses in both mice (Appendix, Figure A2, A3).

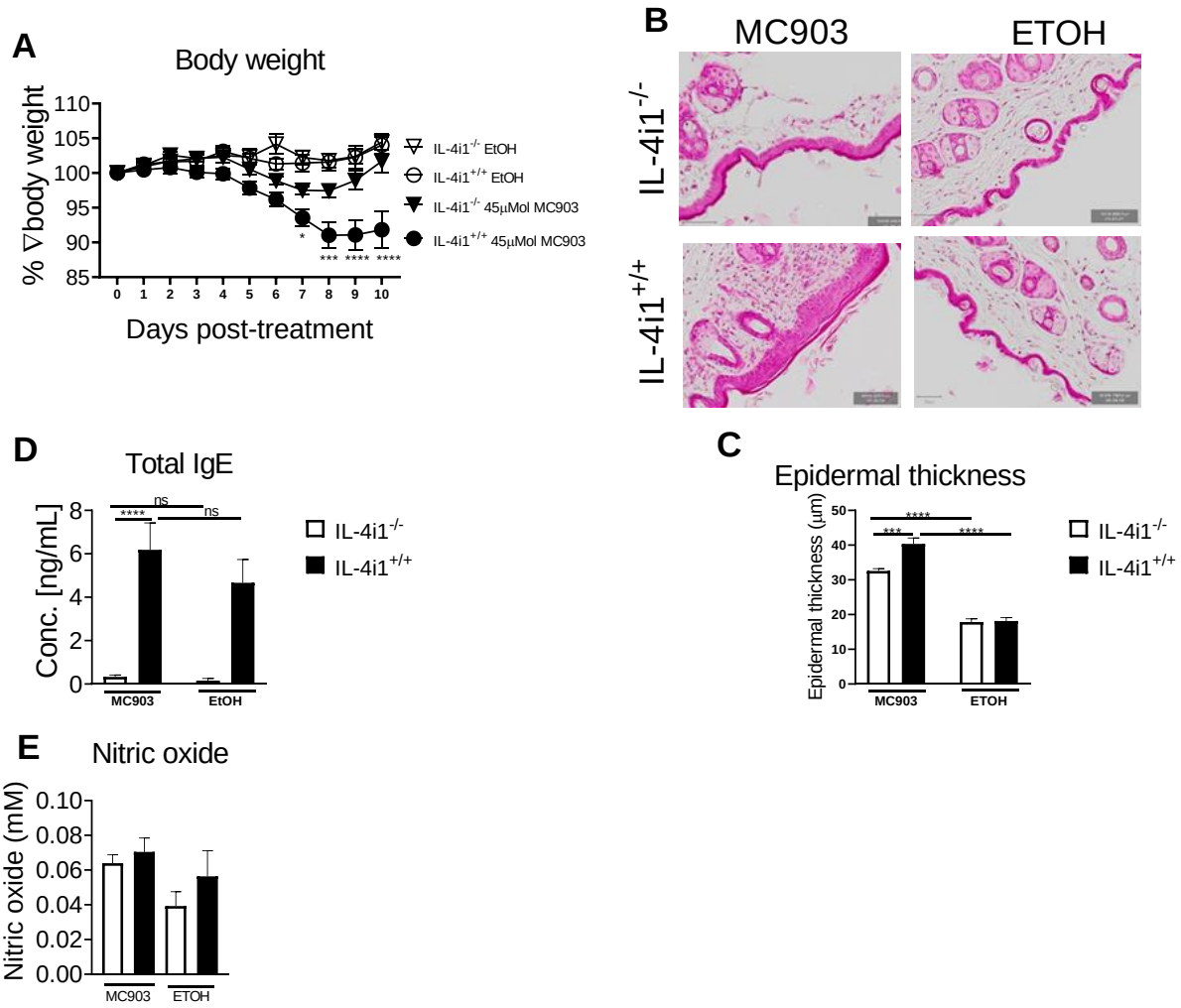

Figure 4.1: MC903 induces skin inflammation in mice. IL-4i1$^{-/-}$ and IL-4i1$^{+/+}$ mice were treated topically with MC903 and ethanol for 10 consecutive days (n=5-6 mice/group). **A)** Percentage changes in body weights of mice during treatment, **B)** Skin histology; representative pictures of haematoxylin and eosin (H&E) staining, 40X magnification (scale bar = 50µm), **C)** Quantification of epidermal thickness using QuPath, **D)** Serum total IgE levels, **E)** Nitric oxide production by iLNs. Data are shown as Mean±SEM of pooled data from 2 individual experiments. Statistical significance was analysed with Ordinary Two-way ANOVA and Mann-Whitney student t-test ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$). n.s., Not significant.

IL-4i1 promotes systemic inflammation during acute AD through increased secretion of T helper 2 cytokines and TSLP

IL-4il has mainly been implicated in immune regulatory functions including induction of T regs which

suppresses effector CD8 T cells[148]. We assessed the role of IL-4i1 in systemic immune responses. Serum levels of Th2 cytokines; IL-4, IL-5, IL-13; Th1 associated cell cytokine; IFN-γ; Th17 associated cell cytokine; IL-17 and epithelial-derived cytokines; IL-33, IL-25 and TSLP were measured by ELISA. IL-4il-deficient mice had significantly reduced levels of TSLP, IL-5 (Fig **4.2 A** and **C**), and a non-significant reduction in IL-4 (Fig **4.2 B**) levels. In contrast, IL-4i1-deficient mice treated with MC903 had increased levels of IFN-γ (Fig **4.2 D**) and IL-17 (Fig **4.2 E**) compared to littermate control mice. However, the differences were not statistically significant between the groups (Fig **4.2 D** and **E**). We did not detect any levels of IL-13, IL-25, and IL-33 in all our mice. Overall, this data demonstrates that IL-4i1 regulates systemic type 2 immune responses in MC903-induced atopic dermatitis.

Role of IL-4il in cytokine expression in the skin after topical treatment with MC903

We verified systemic cytokine ELISA data by analysing cytokine mRNA expression at the site of inflammation using quantitative PCR. The IL-4il$^{-/-}$ mice had a small reduction in Tslp expression (Fig **4.3 A**) and a slight increase in IL-33 expression (Fig **4.3 B**) compared to the littermate control mice. We had also measured the expression of IL-4, IL-5, IL-13, IL-17, IL-25 and IFN-γ but these were not detected.

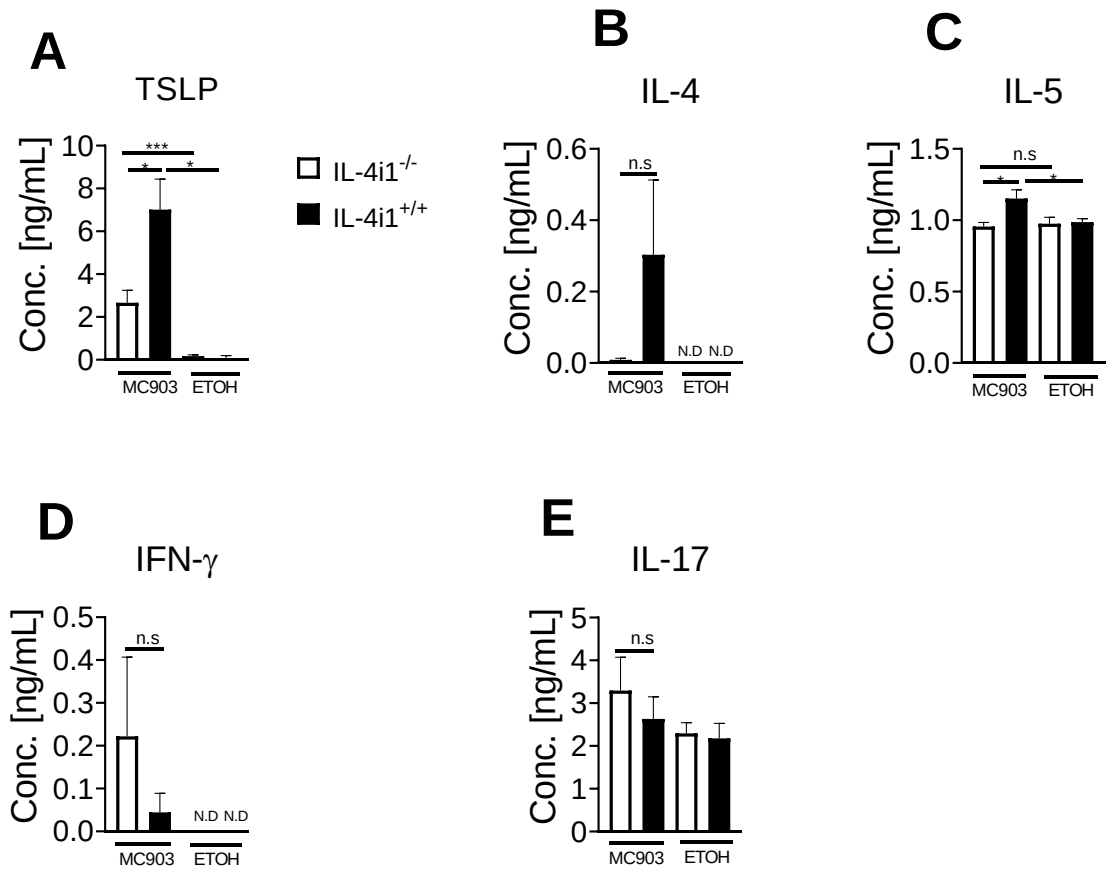

Figure 4.2: Deletion of IL-4i1 reduces systemic secretion of TSLP, IL-5 and IL-4, but not IFN-γ and IL-17 in the development of AD. Serum levels of **A)** Thymic stromal lymphopoietin (TSLP), **B)** IL-4, **C)** IL-5, **D)** IFN-γ and **E)** IL-17 were measured by ELISA. Data is shown as Mean±SEM of pooled data from 2 independent experiments (n=10-12). Statistical significance was analysed with Mann-Whitney student t-test ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$). n.s., Not significant, N.D., Not detected.

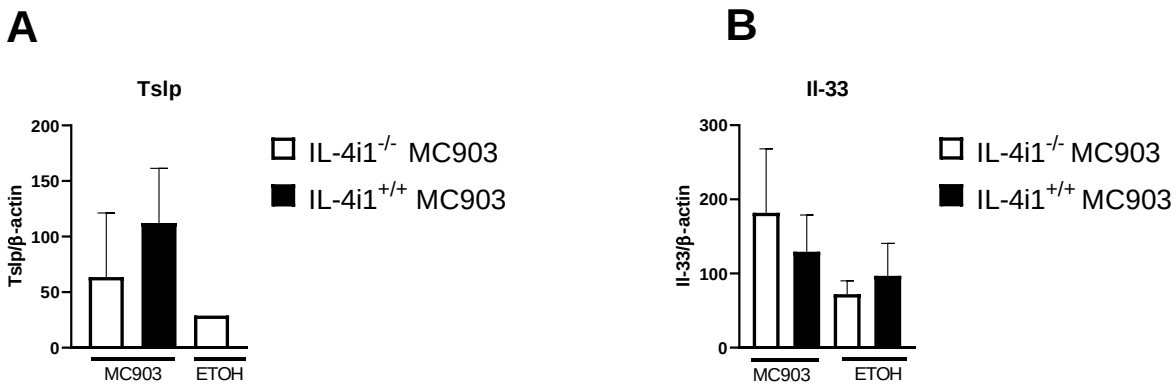

Figure 4.3: IL-4i1 promotes skin inflammation in AD through upregulation of Tslp expression. Quantitative analysis of **A)** Tslp and **B)** Il-33 expression in skin by RT-qPCR. Data are shown as Mean±SEM of pooled data from 2 individual experiments (n=10-12).

IL-4i1 is essential for CD8 T cell IL-13 production, but not CD4 T lymphocytes and their cytokine production

Inguinal lymph nodes (iLNs) were re-stimulated with PMA/ionomycin in the presence of monensin and frequency of CD4+ T cells and intracellular cytokine (IL-4, -5, -13 and IFN-γ) production was analysed. Total iLN cells (Fig **4.4 A**) were comparable between the IL-4i1$^{-/-}$ mice and littermate control mice post MC903 treatment. The trend was also similar in the frequency of CD4^{+} T cells (Fig **4.4 B**) and in the numbers of CD4^{+} T cells producing IL-4,5 and IFN-γ (Fig **4.4 C**, **D**, and **F**). We only observed a slight reduction in the number of CD4^{+} T cells producing IL-13 in the IL-4i1-deficient mice compared to littermate control mice (Fig **4.4 E**). The frequency of CD8^{+} T cells was slightly lower in the IL-4i1$^{-/-}$ mice compared to littermate control mice (Fig **4.4 G)**. The number of CD8^{+} T cells producing IL-4,5, and IFN-γ (Fig **4.4 H**, **I**, and **K**) were comparable between the groups, and in contrast, we observed a significant reduction in the number of CD8^{+} T cells producing IL-13 (Fig **4.4 J)**. These results demonstrate that the absence of IL-4i1 impairs CD8^{+} T cells in IL-13 production and that IL-4i1 has no overall impact on the recruitment and activation of T cells during MC903 AD.

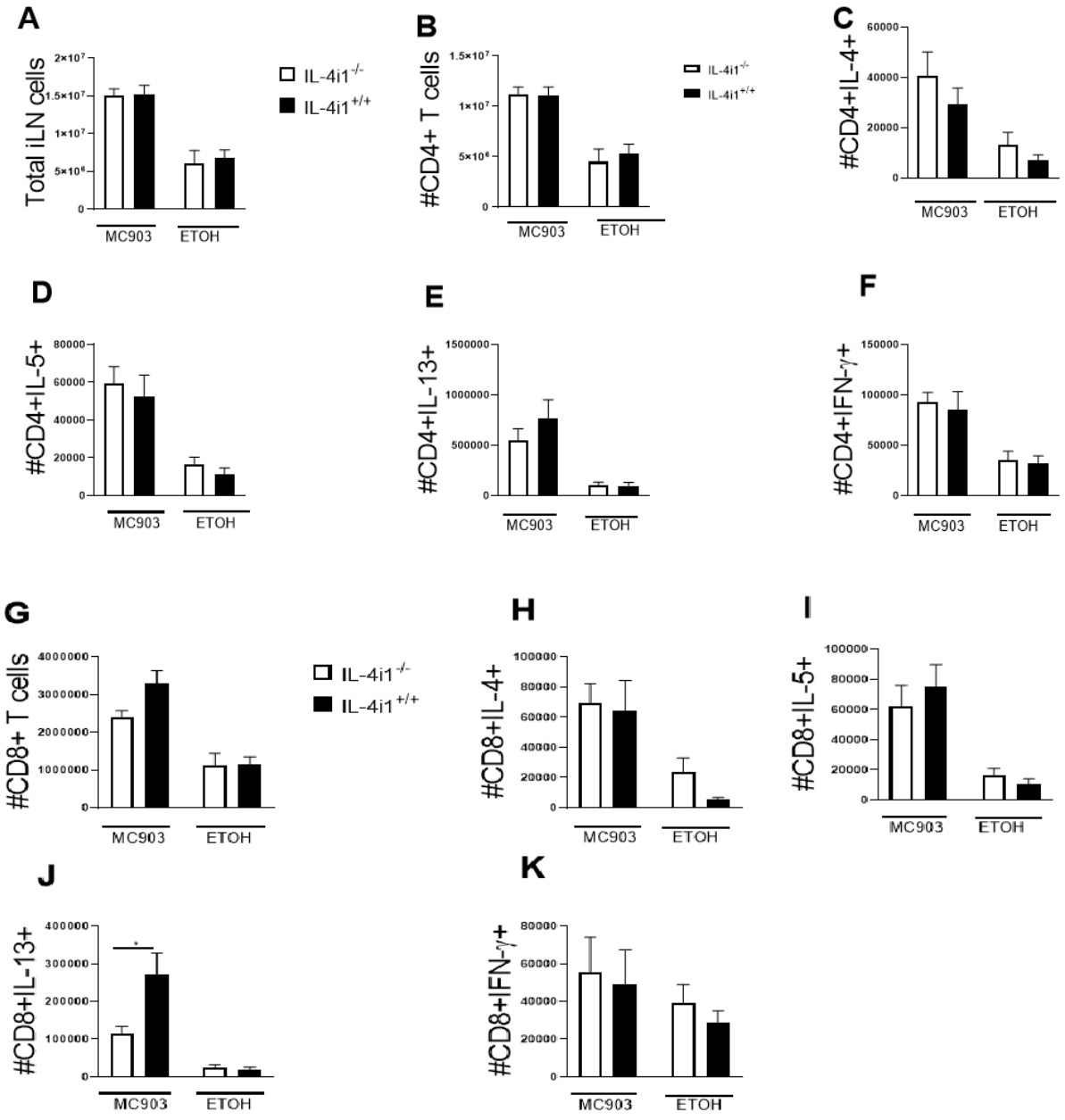

Figure 4.4: Absence of IL-4i1 reduces the number of CD8$^+$ T cells producing IL-13 in an MC903-induced atopic dermatitis disease model. A) Total number of cells in iLNs, **B)** Frequency of CD4+ T cells analysed by flow cytometry and **C)- F)** Flow cytometry analysis of single-cell suspensions of iLNs that were stimulated with PMA/Ionomycin and monensin to induce intracellular cytokine secretion by CD4+ T cells. **G)** Frequency of CD8$^+$ T cells. **H)-K)** Flow cytometry analysis of single-cell suspensions of iLNs that were stimulated with PMA/Ionomycin and monensin for 5 hours to induce intracellular cytokine secretion by CD8$^+$ T cells. Data is shown as Mean±SD of 1 representative experiment of 2 individual

experiments (n=5-6 mice/group). Statistical significance was analysed with Mann-Whitney student t-test ($^{*}p<0.05$).

We also measured by flow cytometry the frequency of $CD19^{+}B220^{+}$ B cells, $GL7^{+}CD19^{+}$ germinal center B cells (GC B cells), $CD4^{+}Foxp3^{+}$ regulatory T cells, $CD62L^{-}CD44^{+}$ T effector cells and $PD\text{-}1^{+}CXCR5^{+}$ Follicular T helper cells in iLNs. We observed comparable numbers of $CD19^{+}B220^{+}$ B cells (Fig **4.5 A**) and Germinal centre B cells (Fig **4.5 B**) in the iLNs of both groups of mice that were treated with MC903. We observed a slight statistically insignificant increase in the number of T effector cells (Fig **4.5 C**), $CD4^{+}Foxp3^{+}$ regulatory T cells (Fig **4.5 E**) and Follicular helper T cells (Fig **4.5 D**) in the IL-4i1-deficient mice compared to littermate control mice. Taken together, this suggests that IL-4i1 deletion has no overall impact on $Foxp3^{+}$ Treg and effector T cell regulation during acute AD.

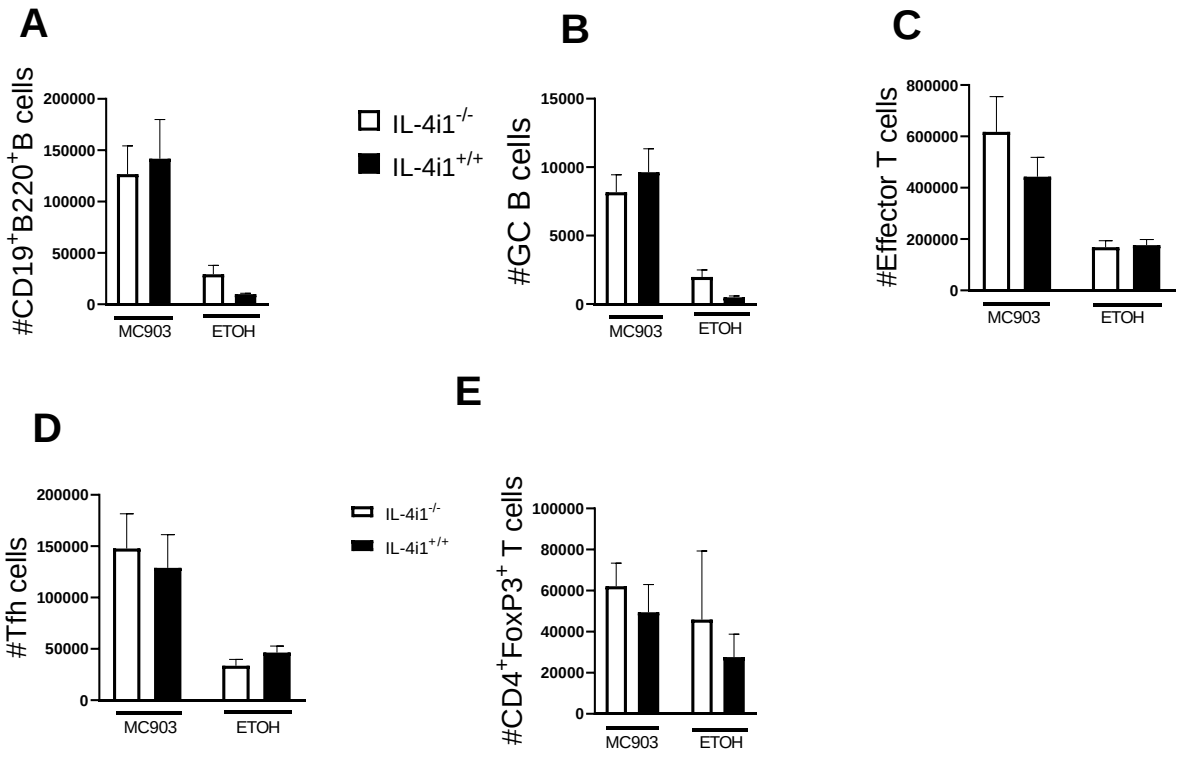

Figure 4.5: Deletion of IL-4i1 slightly increases the accumulation of effector T cells and regulatory T cells in the inguinal lymph nodes. **A)** Frequencies of B cells ($CD19^{+}B220^{+}$), **B)** Germinal center B cells ($GL7^{+}CD19^{+}$), **C)** Effector T cells ($CD62L^{-}CD44^{+}$), **D)** Follicular helper T cells ($PD\text{-}1^{+}CXCR5^{+}$) and **E)**

Regulatory T cells ($CD4^+FoxP3^+$) in inguinal lymph nodes measured by flow cytometry. Data is shown as Mean±SD of 1 representative experiment of 2 individual experiments (n=5-6 mice/group).

4.3 Discussion

IL-4i1 is an L-phenylalanine oxidase primarily produced by dendritic cells as well as macrophages in the setting of pro-T helper type 1 (Th1) inflammatory stimuli[170]. The IL-4i1 enzyme has been shown as an inhibitor of T cell activation *in vitro* and *in vivo*, partly via H_2O_2 production[147]. In this study, we investigated the role of IL-4i1 in the development of MC903-induced AD, and we used mice deficient in IL-4i1. Genetic deletion of IL-4i1 in mice significantly reduced serum total IgE, skin inflammation, and prevented drastic change in body weight of mice during MC903 treatment.

Topical application of MC903 attenuated skin inflammation in IL-4i1-deficient mice in comparison to IL-4i1 wildtype littermate counterparts. This was shown histologically by the significant reduction in epidermal thickness. IL-4i1$^{-/-}$ mice did not experience loss in body weight during treatment with MC903. This shows that IL-4i1 plays a significant inflammatory role in the pathogenesis of MC903-induced AD.

A compromised epidermal barrier due to filaggrin deficiency in AD patients allows penetration of chemical pollutants, bacteria and allergens[108,206]. An increase in these xenobiotics in combination with barrier access has been demonstrated to act on keratinocytes to induce the aryl hydrocarbon receptor (AhR) which has been shown to cause AD worsening, and even a downstream activation of the Th2 pathway and TSLP release[206]. IL-4i1 has been reported to activate the AhR via generation of kynenuric acid and indole metabolites[156]. IL-4i1 reduces survival in glioma patients, facilitates cancer motility and is associated with suppression of adaptive immunity, thereby enhancing chronic lymphocytic leukaemia progression in mice[156].

In the acute phase of the disease, Th2 cytokines (IL-4, IL-13) predominantly mediate skin inflammation in AD. Furthermore, this type 2 cytokine milieu is a known suppressor of antimicrobial peptides (AMPs) production[117]. In this study, we saw a significant reduction in systemic cytokines (IL-5, TSLP) production and IL-4, although with no statistical significance in MC903-treated IL-4i1$^{-/-}$ mice compared to wild-type littermate controls. Th1 associated cytokine (IFN-γ) and Th17 associated cytokine (IL-17) were increased in MC903-treated IL-4i1$^{-/-}$ compared to the wild-type littermate counterparts, although this increase was not statistically significant. We did not detect any levels of IL-13 in the serum in both groups of mice. This

reduction in Th2 cytokine production was consistent with a significant reduction in serum total IgE in MC903-treated IL-4i1$^{-/-}$ mice compared to the wild-type littermates.

IL-4 and IL-13 are known to promote IgE production by B cells, whereas IL-5 is essential for eosinophil generation[207]. Taken together, these data suggest that IL-4i1 regulates type 2 cytokine production by Th2 cells and is involved in maintaining low levels of IFN-γ and IL-17. In addition, IL-4i1 may be involved in increasing serum IgE via stimulation of IL-4 production.

The absence of IL-4i1 did not affect lymphocyte recruitment in the iLNs as we observed comparable total cells and comparable frequencies of CD4$^+$ T cells. CD4$^+$ T cells producing cytokines IL-4, IL-5 and IFN-γ were slightly higher in MC903-treated IL-4i1-deficient mice compared to wildtype littermate mice, although this was not statistically significant. Furthermore, CD4$^+$ T cells producing IL-13 were slightly reduced in MC903-treated IL-4i1$^{-/-}$ mice in comparison to IL-4i1 wildtype littermate counterparts.

In a mouse model of melanoma, Lasoudris *et al*., reported that IL-4i1 expression by tumour-associated macrophages enables tumour growth via inhibition of CD8$^+$ T cell antitumor response[163]. Using Ret mice (mice expressing proto-oncogene c-ret) in a spontaneous melanoma model, IL-4i1 expression was shown to contribute toward tumour progression and aggression via promoting myeloid cell subsets recruitment, and by interfering with T lymphocytes antitumor properties within the primary tumor[159]. Furthermore, genetic inactivation of IL-4i1 delayed the occurrence of primary tumour and tumour-cell dissemination to skin and distant areas, thus arguing for a detrimental role of IL-4i1 in tumour progression[159,208].

Intriguingly, in this study, we found a slightly lower frequency of CD8$^+$ T cells in iLNs of MC903-treated IL-4i1$^{-/-}$ mice, which corresponded with significantly reduced CD8$^+$ T cells producing IL-13. IL-13 is said to be one of the primary cytokines implicated in AD inflammation[209]. CD8$^+$ T cells producing cytokines IL-4 and IFN-γ were similar in their frequency. This suggests that IL-4i1 regulates intracellular cytokine production by CD8$^+$ T cells in MC903-induced AD. Topical administration of MC903 induced slightly increased infiltration of effector and FoxP3 Treg cells in IL-4i1$^{-/-}$ mice in comparison to wildtype littermate control mice. IL-4i1 is known to inhibit proliferation of T cells via H_2O_2 toxicity on memory/effector T cells, and also facilitates the generation of FoxP3 Tregs *in vitro* in mouse and human T cells[158].

We could not see any significant differences in the frequencies of CD19$^+$B220$^+$ and GC B cells infiltrating the iLNs. Therefore, the role of IL-4i1 in B cell development during AD remains unclear. Only TSLP and IL-33 mRNA expression were detected in the skin after the 10-day treatment. TSLP expression was reduced slightly in MC903-treated IL-4i1$^{-/-}$ mice compared to wild-type littermate counterparts. This was consistent with the reduced serum TSLP secretion. The slight increase in IL-33 mRNA expression in IL-4i1$^{-/-}$ mice

was unexpected, but we think that could be explained by the fact that IL-33 has been implicated in pro-and anti-inflammatory processes in various cells and tissues[210].

Conclusion

In this study, we have shown that MC903-induced AD and associated inflammation characterized by increased serum IgE levels, systemic upregulation of Th2 cytokines, and skin epidermal thickness is alleviated in IL-4i1$^{-/-}$ mice. These data suggest IL-4i1 as a potential therapeutic target for AD.

Chapter 5: General discussion, future perspectives, and conclusion

5.1 General discussion

Allergic asthma is a complicated heterogeneous disease and is expected to affect approximately 400 million people by 2025 worldwide[211]. Allergic asthma is mainly characterized by structural thickening of the epithelial layer, inflammatory cell recruitment to the airways, excessive mucus production and elevated IgE levels[211]. Asthma results in healthcare expenditure and considerable morbidity and is deemed as one of the most common chronic diseases[212], furthermore, there is currently no cure for asthma[211]. AD is a chronic inflammatory skin disorder and its features include intense itching and recurring eczematous lesions, these conditions are long lasting[213]. In low-income countries of Africa and East Asia, the prevalence of AD is reported to have increased[120]. AD is deemed a costly disease that leads to impaired quality of life[214]. We targeted IL-4i1 since it was shown to be upregulated candidate genes in M2 macrophages during Mtb infection and, was found to be highly upregulated in lesional skin of AD patients in a transcriptome study of AD.

We aimed at investigating the role of IL-4i1 in allergic asthma and atopic dermatitis. We used gene-deficient mouse models. In our first objective, we sensitized mice with low dose (1 µg) HDM to induce allergic asthma, where we did not observe any significant differences in cytokine production, antibody secretion, AHR, lymphocytes and eosinophil recruitment in the lung between IL-4i1$^{-/-}$ and wild-type littermate control mice. We then increased the dose and sensitized mice with a high dose (100 µg) HDM. Indeed, the high dose was sufficient to induce type 2 inflammation. IL-4i1 deletion resulted in an increased type 2 pathway-driven pro-inflammatory phenotype in mice during HDM-induced allergic asthma.

In our second objective, we used an acute model of skin irritant and showed that lower concentrations of MC903 (4,5nmol and 9nmol) were not sufficient to induce AD skin inflammation with distinct differences in our groups of mice. Higher concentration (45µM) of MC903 induced skin inflammation mostly with a Th2 bias phenotype. IL-4i1 in this setting promoted AD pathophysiology in mice topically treated with MC903, as mice lacking this enzyme were protected from developing severe disease symptoms. Topical application of MC903 has been demonstrated by preclinical studies to sufficiently induce allergic skin inflammation similar to AD, and the resulting phenotype incorporates dermal hyperplasia, epidermal thickening and upregulation of inflammatory cells in the skin[204].

We found IL-4i1 to be central in regulating epithelial and epidermal primary defence organs that protect the lung and skin from external allergenic irritants. In the AD mouse model, skin epidermal thickness was reduced significantly in IL-4i1-deficient mice which was corroborated by a similar reduction in mucus

secretion by epithelial cells in the allergic asthma model. This immunoregulatory role was further supported by a reduction in total serum IgE levels in IL-4i1-deficient mice in both disease models. In B cells, IL-4 is the only cytokine known to induce IL-4i1 via stimulation of the IL-4/IL-13 receptor and consequent phosphorylation of the transcription factor STAT6[215]. IL-4i1 through regulating B cell class switching could likely be key in its function to regulate IgE, although the mechanisms are currently speculative. We observed a reduction in GC formation particularly in secondary lymphoid organs which may suggest a reduced class switch recombination and possibly somatic hypermutation.

In previous studies, IL-4i has additionally been identified in B lymphoma cells deriving from germinal center B cells such as follicular B cell lymphoma[148]. In follicular B cell lymphoma, high levels of IL-4i1 were associated with a better outcome[215]. Additionally, since IL-4i1 is expressed by GC B cells, it is suggested IL-4i1 may play a role in the maturation of T-dependent humoral immune response, furthermore, IL-4i1 is a target of mutations promoted by the activation-induced cytidine deaminase, which promotes somatic hypermutations and class switch recombination in GC cells[145].

Dendritic cells and macrophage populations from chronic Th1 granulomas of tuberculosis, but not M2 biased granulomas from schistosomiasis are strong IL-4i1 producers[148]. In murine macrophages, IL-4i1 expression is said to be regulated by different mechanisms, since it was reported to be stimulated by type 2 stimuli such as IL-4[146]. Here, it is likely that regulation of IL-4i1 was context and site-specific as we observed increased type 2 in the lung, but reduced type 2 inflammation in the skin, suggesting a dichotomy in its regulation. Future studies should delve into how the same enzyme could promote Th2 responses in one organ and yet curb the same type 2 responses in a different organ. Cell-specific deletion of IL-4i1 could further explain this dichotomy as it is known to be expressed by multiple cell types.

Amino acid deficiency is shown to dampen the effectiveness of the immune system and also induces susceptibility of individuals to disease[216]. Arginine is reported to be an enhancer of T cell function and upregulates antibody production, whereas tryptophan metabolites are influencers of innate and adaptive immune systems and exert a local immunosuppressive activity that can regulate T cells[216].

IL-4i1 is part of immunosuppressive enzymes controlling intracellular and extracellular amino acid content while concurrently producing toxic metabolites[217]. It has been shown that these enzymes contribute to immune regulation by influencing proliferative and differentiation abilities of T cells[218], and modifying the balance of effector/Treg cell differentiation[219]. However, there is a paucity of information about the functions and enzymology of IL-4i1/ L-amino acid oxidases (LAAOs), their kinetics, substrate ranges, and the role of downstream metabolites resulting from amino acids[220]. IL-4i1 is known to deplete phenylalanine,

to a lesser extent, this enzyme also catabolizes tryptophan and arginine degradation[219]. Ketoacid indole-3-pyruvate is produced as a consequence of IL-4i1 activity towards tryptophan and may serve as a precursor that can enter the kynurenine pathway[219]. Kynurenine is one of the endogenous ligands of aryl hydrocarbon receptor (AhR)[221].

IL-4i1 is also reported as an activator of AhR via the generation of indole metabolites and kynurenic acid[156]. In a chronic bronchitis setting, studies have shown that AhR activation increases mucin 5AC expression in airway epithelial cell line, and this AhR-induced upregulation of mucus was reported to be partially mediated by reactive oxygen species (ROS) generation[222]. In asthma, chronic obstructive pulmonary disease (COPD) and AD, AhR plays a significant role as a regulator of Treg and Th17 cell differentiation[223]. Furthermore, AhR is implicated in the development of lung cancer[224].

5.2 Limitations and setbacks of the study

This study was conducted in inbred mice, so this may have a limited impact on human disease. In addition, translation of this data from animal experiments to human disease may sometimes fail. We were unable to perform H_2O_2 assay, this would have given us confirmation of IL-4i1 enzymatic activity whether it is the one involved in immune response regulation. An IL-4i1 antibody is a polyclonal antibody and due to that, we were unable to reliably detect the protein expression via flow cytometry. Lastly, the AD model is only limited to acute skin irritant which may not represent other allergens such as HDM.

5.3 Future perspectives

Since IL-4i1 has been reported to activate AhR via the production of indole metabolites and Kynurenic acid in cancer settings, we think it will be beneficial to look and analyse metabolites downstream of the kynurenic pathway following tryptophan catabolism by IL-4i1 in allergic disease settings. We will also assess the role of IL-4i1 in macrophage polarization, this would be beneficial in understanding whether IL-4i1 has a tissue-specific function during induced allergic asthma and AD. It would be interesting to use other allergens such as HDM or ovalbumin in the AD model that are more chronic. It would be beneficial to generate a cell-specific IL-4i1 knockout using Floxed mice and cre system, mainly to identify which cell type plays a key role, specifically where we see differences in type 2 regulation between asthma and AD.

Bone marrow transfers or sorting of specific cells from WT and transferring to KO mice would enable us to see if inflammation in AD would be restored or if type 2 inflammation in asthma would be reduced. Translation of this study into human disease could be done by doing metabolic profile via liquid chromatography-mass spectrometry (LC-MS/MS) either for targeted indole metabolites or broad metabolites from skin biopsies and blood of AD patients. Possible therapy for skin inflammation in AD

could be developed by blocking the enzyme itself directly or its metabolites.

5.4 Conclusion

To the best of our knowledge, we have unravelled a new role of IL-4i1 in diseases such as allergic asthma and atopic dermatitis. In HDM-induced allergic asthma settings, we have shown that IL-4i1 regulates IgE production by B cells and dysregulated Th2 airway inflammation due to the increased lung eosinophilia, upregulated Th2 cytokines in secondary lymphoid tissue and increased NO production by restimulated T lymphocytes. The exact mechanism of how IL-4i1 regulated these events remains to be further investigated. We have also shown a novel role of IL-4i1 in a setting of MC903-induced AD. In this setting, we have demonstrated that IL-4i1 promotes type 2 skin inflammation in mice topically treated with MC903. IL-4i1 deletion in this regard attenuated type 2-mediated skin inflammation depicted by the significant reduction in serum total IgE, epidermal thickness, and systemic Th2 cytokine production. These results suggest IL-4i1 as a target molecule for AD therapy.

Appendix

Table 3: List of cytokines used for ELISA

Cytokine/antibody		Clone	Dilution	Company
IL-4	Primary/Capture	11B11	1/500	BD Biosciences
	Secondary/Detection	BD/554390	1/1000	BD Biosciences
	Standard	BD/550067	Conc. 100ng/ml	BD Biosciences
IL-5	Primary/ Capture	BD/554393	1/500	BD Biosciences
	Secondary/ Detection	BD/554397	1/1000	BD Biosciences
	Standard	BD/554581	Conc. 100ng/ml	BD Biosciences
IL-13	Primary/ Capture	R&D MAB413	1/250	R&D Systems, Minneapolis
	Secondary/ Detection	R&D BAF413	1/125	R&D Systems, Minneapolis
	Standard	BD/554599	Conc. 50ng/ml	BD Biosciences
IFN-γ	Primary/ Capture	AN18.KL6	1/500	BD Biosciences
	Secondary/ Detection	BD/55410	1/1000	BD Biosciences
	Standard	BD/554587	Conc. 100ng/ml	BD Biosciences
IL-17	Primary/ Capture	R&D MAB721	1/500	Biolegend
	Secondary/ Detection	R&D BAF421	1/500	Biolegend
	Standard	R&D 421ML	Conc. 100ng/ml	WhiteSci
TSLP	Primary/ Capture	28F12	1/500	Biolegend
	Secondary/ Detection	65B12	1/1000	Biolegend
	Standard	1259414	Conc. 100ng/ml	R&D Systems, Minneapolis
IL-33	Primary/ Capture	R&D DY3626	1/500	R&D Systems, Minneapolis
	Secondary/ Detection	R&DY3623	1/500	R&D Systems, Minneapolis
	Standard	1188524	Conc. 10ng/ml	R&D Systems, Minneapolis

Table 4: List of antibodies used for flow cytometry analysis of B cells, T cells and granulocytes in an HDM-induced allergic asthma model

Target	Conjugate	Clone	Dilution	Vendor
CD24	FITC	M1/69	1/320	BD Pharmingen
IgD	FITC	11-26c.2a	1/80	Biolegend
Ly6G	FITC	1A8	1/320	BD Biosciences
GL7	PE	GL7	1/320	BD Biosciences
IgE	PE	23G3	1/80	
Siglec-F	PE	E50-2440	1/320	BD Biosciences
FAS	Biotin	15402D	1/200	BD Pharmingen
IgM	Biotin	2gMα	1/200	BD Pharmingen
CD23	PE-Cy7	B3B4	1/320	
F4/80	PE-Cy7	BM8	1/640	eBiosciences
CD80	V450/BV421	16-10A1	1/320	BD Biosciences
IgG	V450/BV421	A85-1	1/320	BD Biosciences
CD11b	V450/BV421	M1/70	1/320	Biolegend
B220	V500/BV510	RA3-6B2	1/320	BD Biosciences
Yellow stain	Qdot605	Dead exclusion	1/2000	eBiosciences
CD21/CD35	APC	7G6	1/80	BD Biosciences
CD138	APC	281-2	1/80	BD Biosciences
CD19	APC-Cy7	1D3	1/320	BD Biosciences
CD86	AF700	GL1	1/320	BD Biosciences
MHCII	AF700	M5/114	1/640	eBiosciences
CD11c	AF700	HL3	1/640	BD Biosciences
Ly6C	PerCP-Cy5.5	AL-21	1/320	BD Biosciences
CD44	PE	KM114	1/320	BD Biosciences

Table 5: List of antibodies used for flow cytometry analysis of B cells and intracellular cytokines in a MC903-induced atopic dermatitis model

Target	Conjugate	Clone	Dilution	Vendor
IgM	FITC	2gH	1/320	BD Biosciences
IL-4	FITC	11B11	1/100	BD Biosciences
PD-1	FITC	29F.1A12	1/320	BD Biosciences
GL7	PE	GL7	1/320	BD Biosciences
IgE	BV786	R35-72	1/80	BD Horizon
IL-5	PE	TRFK5	1/100	BD Biosciences
CD44	PE	KM114	1/320	BD Biosciences
FAS	Biotin	15402D	1/200	BD Pharmingen
CD19	PerCP-Cy5.5	1D3	1/640	BD Biosciences
CD69	PerCP-Cy5.5	H1.2F3	1/320	BD Biosciences
CD23	PE-Cy7	B3B4	1/320	BD Biosciences
IL-13	PE-Cy7	eBio13A	1/100	eBiosciences
CXCR5	PE-Cy7	2G8	1/320	BD Biosciences
CD80	V450/BV421	16-10A1	1/320	BD Biosciences
CD4	V450/BV421		1/640	
CD62L	V450/BV421	MEL-14	1/1280	BD Biosciences
B220	V500/BV510	RA3-6B2	1/320	BD Biosciences
CD4	V500/BV510	RM4-5	1/320	BD Biosciences
Yellow stain	Qdot605	Dead exclusion	1/2000	eBiosciences
MHCII	AF700	M5/114	1/640	eBiosciences
IFN-γ	AF700	XMG1.2	1/100	BD Biosciences
CD3	AF700	145-2C11	1/320	BD Biosciences

CD8	APC	53-6.7	1/320	BD Biosciences
FoxP3	APC	MF23	1/50	BD Biosciences

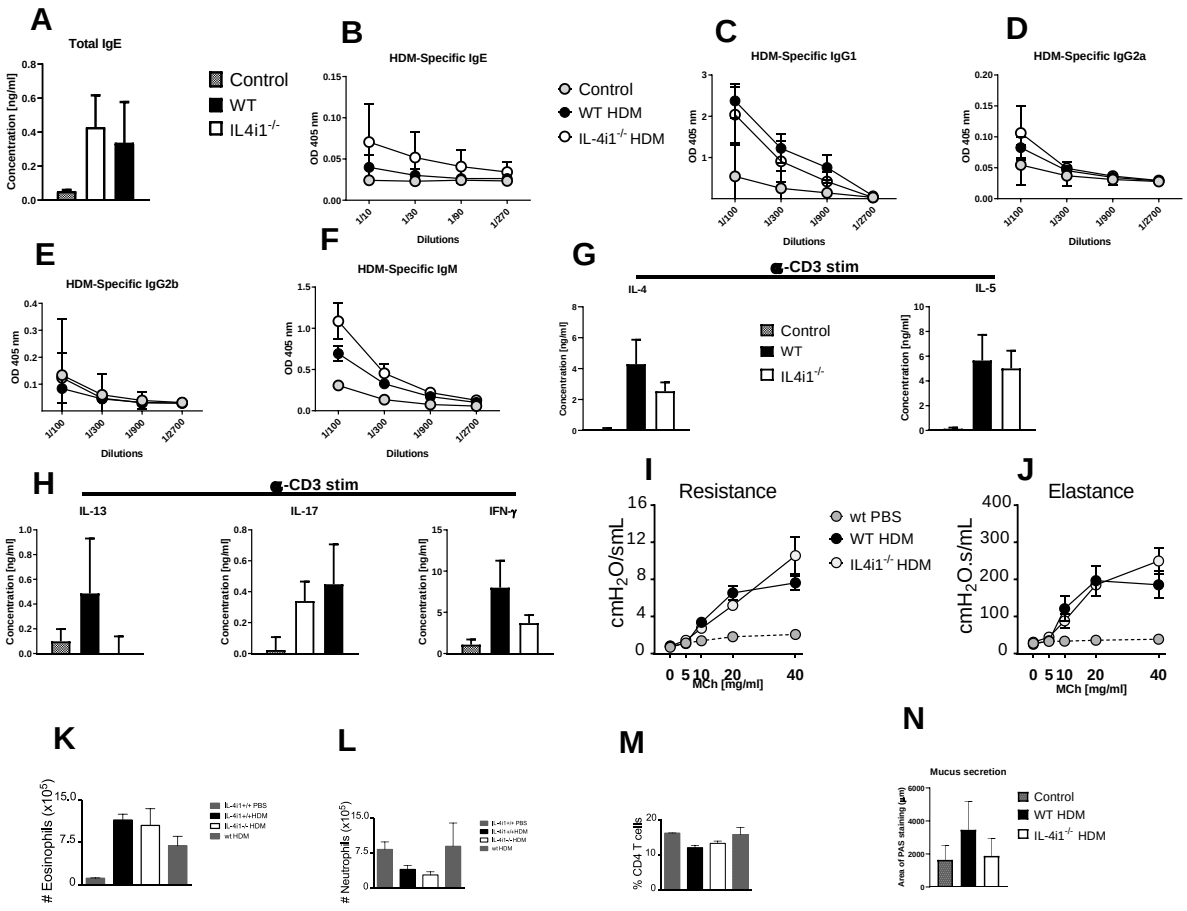

Figure A1: Results of low dose (1 µg)-induced allergic asthma. A) Serum total IgE levels measured by ELISA. **B) – F)** Allergen-specific IgE, IgG1, IgG2a, IgG2b, and IgM production measured by ELISA 14 days post sensitization with HDM. **G) – H)** Mediastinal lymph node cell suspensions were restimulated with anti-CD3 and measured cytokine production by ELISA. **I)** Measurement of airway resistance and **J)** elastance in response to methacholine. **K)** Number of lung eosinophils, **L)** Neutrophils and **M)** $CD4^+$ T cells were measured in the lung by flow cytometry. **N)** Lung tissues were stained with PAS stain and mucus production was quantified using NIS software.

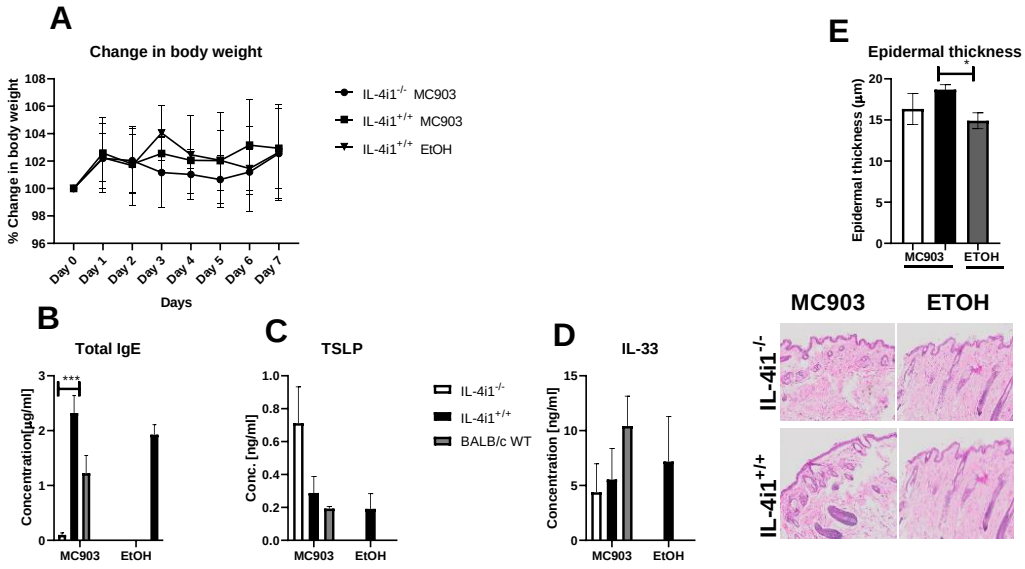

Figure A2: Induction of AD with 4.5nMol MC903. A) Change in bodyweight was calculated daily till the last day of treatment. **B)** Serum total IgE levels were measured by ELISA after day 7. **C)** Serum TSLP and **D)** IL-33 production was measured by ELISA after day 7. **E)** Skin sections were stained with H&E and quantified epidermal thickness using QuPath software.

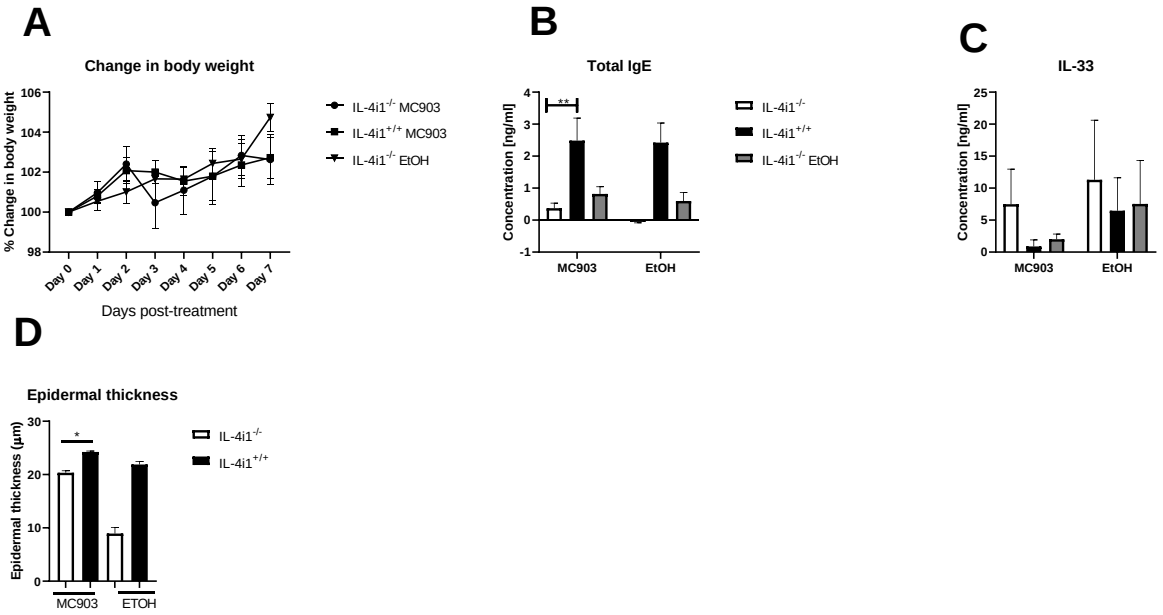

Figure A3: Induction of AD in male mice with 9nMol MC903. A) Change in bodyweight was calculated daily till the last day of treatment. **B)** Serum total IgE levels were measured by ELISA after day 7. **C)** Serum IL-33 secretion was measured by ELISA after day 7. **D)** Skin sections were stained with H&E and quantified epidermal thickness using QuPath software.

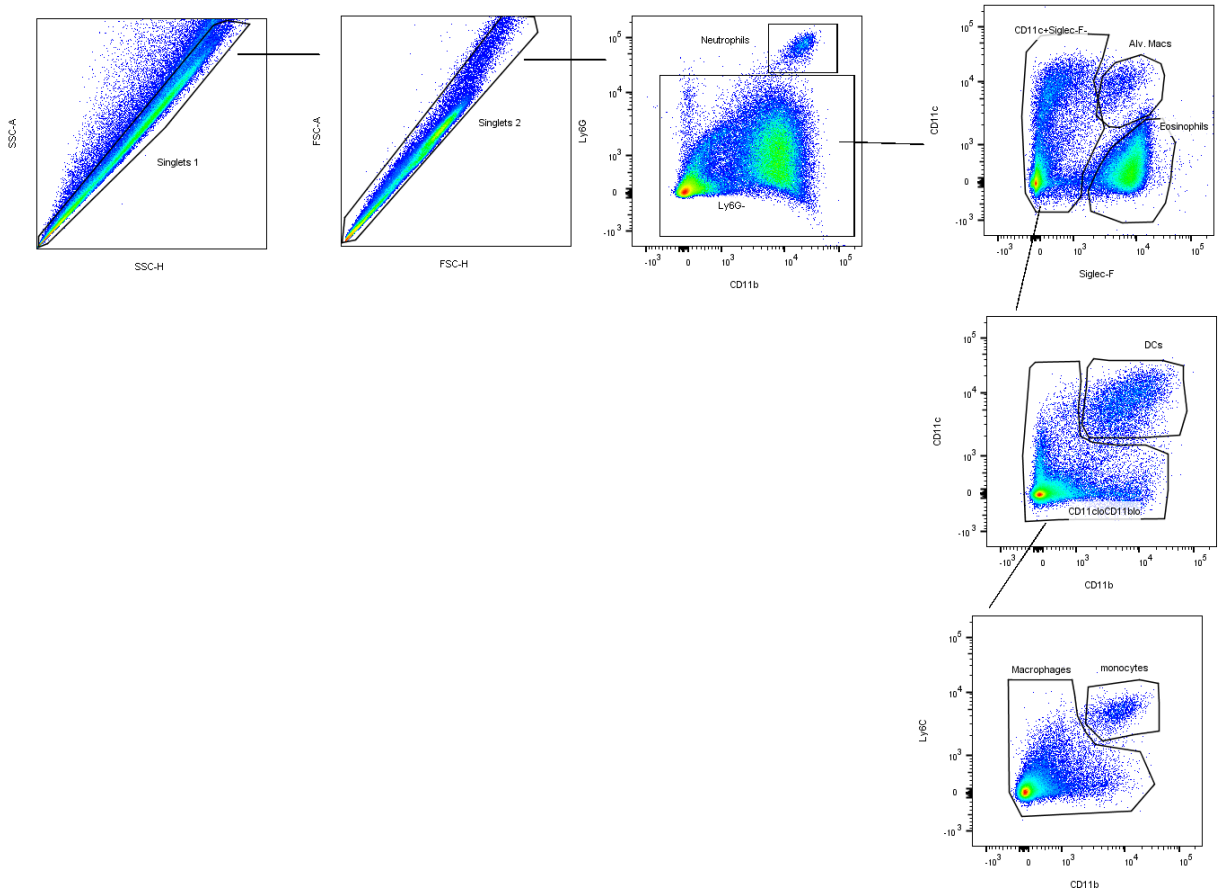

Figure A4: Gating strategy for myeloid cell populations in the lung. We gated on single cells using the SSC and FSC plots. To exclude doubles, we then gated on singlets 2. From singlets 2 we then gated for neutrophils ($Ly6G^+CD11b^+$) and $Ly6G^-$ cells. From the $Ly6G^-$ cells we gated for alveolar macrophages ($CD11c^+Siglec\text{-}F^+$), Eosinophils ($Siglec\text{-}F^+CD11c^-$) and $CD11c^+Siglec\text{-}F^-$ cells. From $CD11c^+Siglec\text{-}F^-$ gate, we then gated for dendritic cells ($CD11b^+CD11c^+$) and $CD11c^{lo}CD11b^{lo}$ cells. From the $CD11c^{lo}CD11b^{lo}$ gate, we then gated for monocytes ($Ly6C^+CD11b^+$). Analysis was done using FlowJo V10.6.

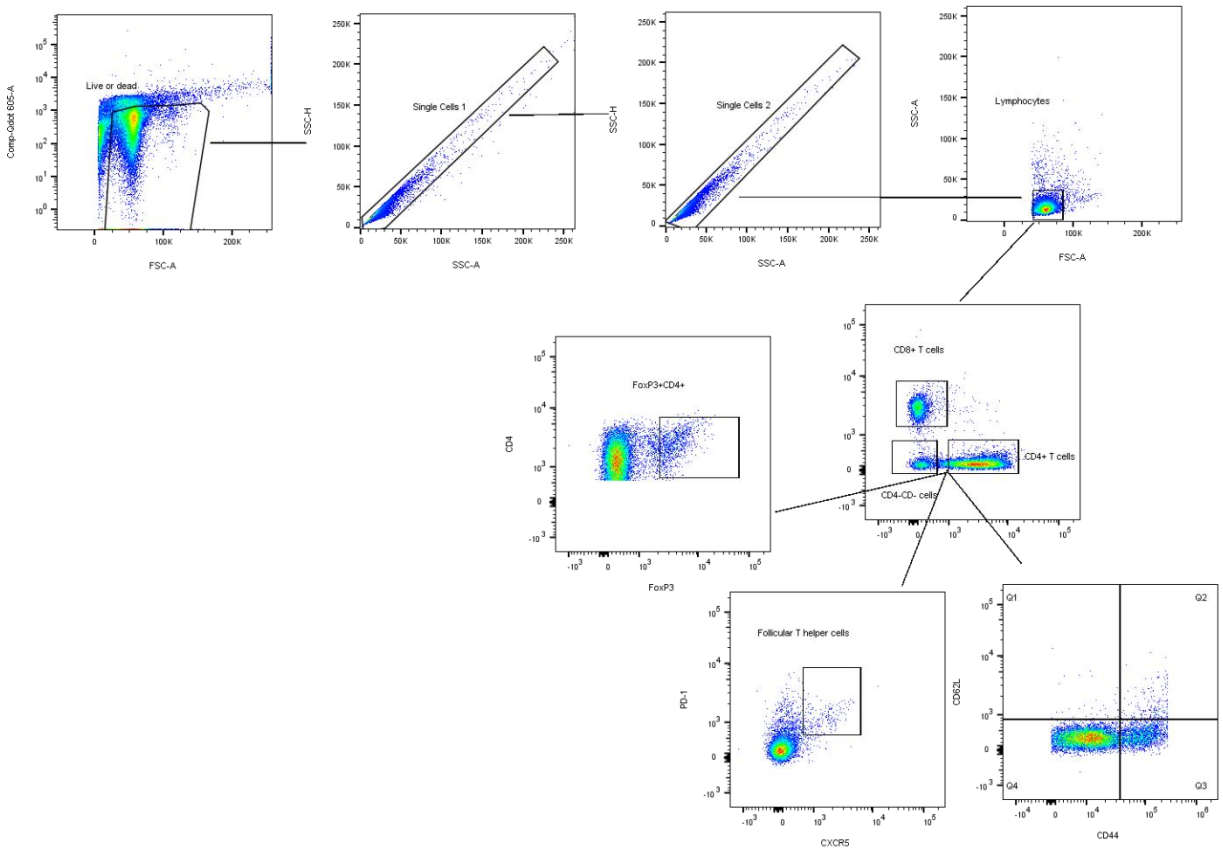

Figure A5: Gating strategy for T cell populations in lung, mediastinal and inguinal lymph nodes. We gated for live cells from Qdot and FSC-A. From the live cells we gated for singlets 1. To remove doublets, we gated for singlets 2 from the singlets 1 gate. From singlets 2 we gated for lymphocytes. From the lymphocytes gate, we gated for $CD4^+$, $CD8^+$ T cells. From $CD4^+$ T cells we gated for $FoxP3^+$ ($CD4^+FoxP3^+$), Follicular helper T cells ($CXCR5^+PD\text{-}1^+$) and effector T cells ($CD62L^-CD44^+$). Analysis was done using FlowJo V10.6.

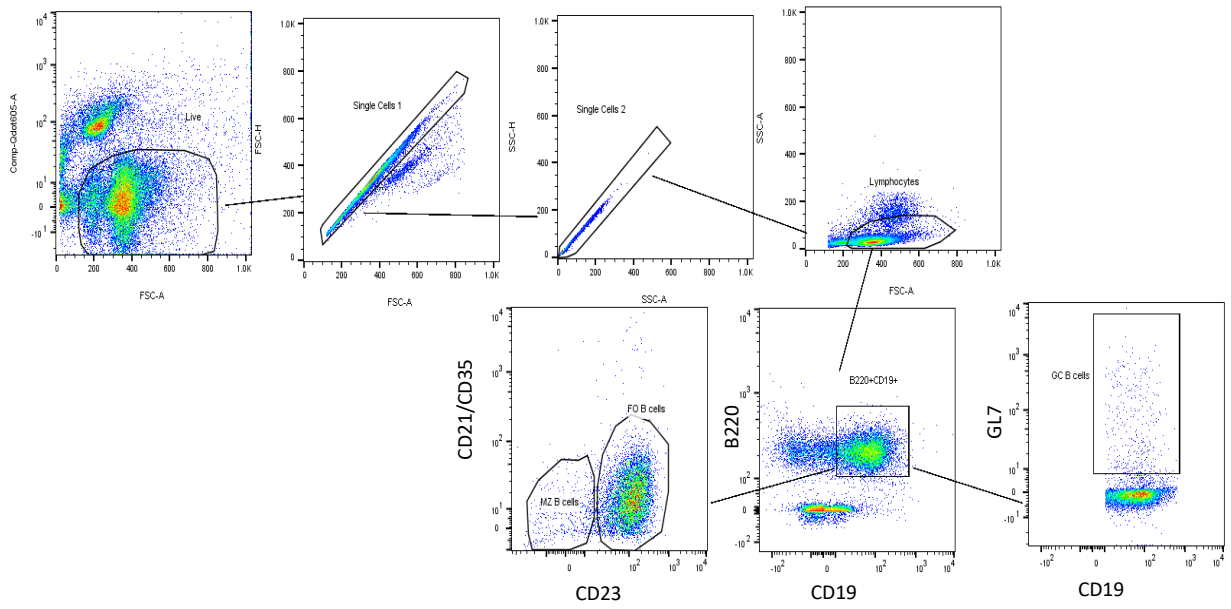

Figure A6: Gating strategy for B cell populations in lung, mediastinal and inguinal lymph nodes. We gated for live cells from Qdot and FSC-A. From the live cells we gated for singlets 1. To remove doublets, we gated for singlets 2 from the singlets 1 gate. From singlets 2 we gated for lymphocytes. From the lymphocytes gate, we gated for $CD19^{+}B220^{+}$ B cells. From $CD19^{+}B220^{+}$ B cells we gated for germinal centre B cells ($GL\text{-}7^{+}CD19^{+}$), Follicular B cells ($CD23^{+}CD21/CD35^{+}$) and marginal zone B cells ($CD21/CD35^{+}CD23^{-}$). Analysis was done using FlowJo V10.6.

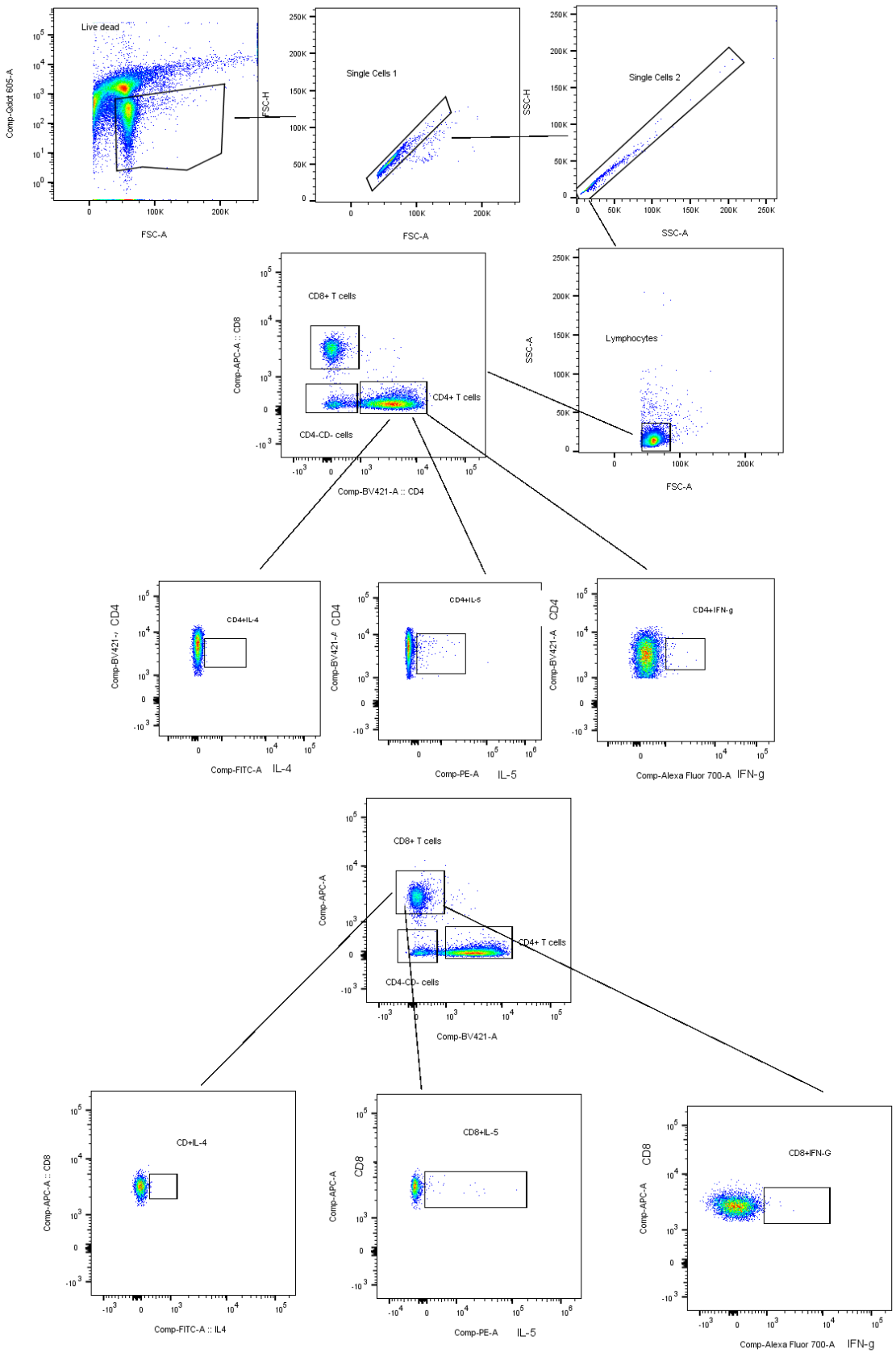
Live dead
Comp-Qdot 605-A
FSC-A
Single Cells 1
FSC-H
FSC-A
Single Cells 2
SSC-H
SSC-A
CD8+ T cells
CD4+ T cells
CD4-CD- cells
Comp-APC-A :: CD8
Comp-BV421-A :: CD4
Lymphocytes
SSC-A
FSC-A
CD4+IL-4
Comp-BV421-A :: CD4
Comp-FITC-A IL-4
CD4+IL-5
Comp-BV421-A :: CD4
Comp-PE-A IL-5
CD4+IFN-g
Comp-BV421-A :: CD4
Comp-Alexa Fluor 700-A IFN-g
CD8+ T cells
CD4+ T cells
CD4-CD- cells
Comp-APC-A
Comp-BV421-A
CD+IL-4
Comp-APC-A :: CD8
Comp-FITC-A :: IL4
CD8+IL-5
Comp-APC-A CD8
Comp-PE-A IL-5
CD8+IFN-G
Comp-APC-A CD8
Comp-Alexa Fluor 700-A IFN-g

Figure A7: Gating strategy for cytokine secretion in mediastinal and inguinal lymph nodes. We gated for live cells from Qdot and FSC-A. From the live cells we gated for singlets 1. To remove doublets, we gated for singlets 2 from the singlets 1 gate. From singlets 2 we gated for lymphocytes. From the lymphocytes gate, we gated for $CD4^+$ and $CD8^+$ T cells. From $CD4^+$ and $CD8^+$ T cells we gated for expression of cytokines IL-4, IL-5, IL-13 and IFN-γ. Analysis was done using FlowJo V10.6.

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