CHARACTERIZING T CELL AND PLASMACYTOID DENDRITIC CELL SIGNATURES IN EARLY LIFE OF ANTIBIOTIC EXPOSED NEONATES

by

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submitted by Sia Cecilia Jan-Abu in partial fulfilment of the requirements for

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in Biomedical Engineering

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Abstract

The prevalence of allergic diseases including food allergies and asthma are increasing worldwide and substantial evidence suggests that this is driven, at least in part, by changes in Western lifestyle and early life environmental factors. The complex interaction between genetics and environment from gestation to the first year of life are believed to drive perturbations in immune development and education which significantly affect allergic diseases susceptibility and development. In mice vancomycin exposure from gestation up to the first 3 weeks after birth induces enhanced systemic allergy later in life. This systemic allergy mouse model was used to study allergy development in the perinatal period by interrogating T cell and plasmacytoid dendritic cell (pDC) signatures in 7-, 10- and 14-day old mice. By flow cytometry we found that the frequency of $CD4^+$ T cells and type 2 $CD8^+$ T cells (Tc2 cells) were temporally altered in the bone marrow of naive vancomycin exposed mice which has implications for allergy development since normal hematopoiesis can be impacted by this T cell activity. pDC activation state in vancomycin primed mice was also significantly different. Furthermore, serum IgE analysis suggests that by day 14 after birth, allergy prone immune development is ensuing. Overall, this study provides insight into specific cell subsets that may drive pro-allergic immune development in early life, and it provides a specific timeline in which such critical changes are occurring.

Lay Summary

The incidence of allergy and asthma are increasing world-wide. The immune system which develops in the first few years of life is drastically affected by allergy, hence perturbations in immune development in early life may affect one's susceptibility to developing allergy. Studies in humans and mice have linked perinatal environmental exposures to increased risk of allergy development in progeny. To investigate how immune changes in early life may influence allergy development we characterized the cell signature and localization of T cells and plasmacytoid dendritic cells (pDCs) in 7 to14-day old mice primed or not primed in-utero to develop allergy through antibiotic exposure. We found that T cell subset composition and their localization and pDC activation in some lymphoid organs was different in primed versus unprimed mice. This suggests that changes in the development of these cells may support allergy development in allergy primed mice.

Preface

All the work presented here was conducted in the Biomedical Research Center, McNagny Laboratory at the University of British Columbia, Point Grey campus. All projects and associated methods were approved by the University of British Columbia's Research Ethics Board [certificate #20190510-01ABC].

I was the lead investigator, responsible for all major areas of concept formation, data collection and analysis, as well as manuscript composition. Dr. Kelly McNagny was the supervisory author on this project and was involved throughout the project in concept formation and manuscript composition.

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List of Abbreviations

AAI	Allergic Airway Inflammation
AHR	Airway Hyperresponsiveness
APC	Antigen Presenting Cell
Areg	Amphiregulin
BALF	Bronchial Alveolar Lavage Fluid
BAP	Butyrate, Acetate, And Propionate
CBMCs	Cord Blood Mononuclear Cells
cDC	Conventional Dendritic Cell
CHILD	Canadian Health Infant Longitudinal Study
CSR	Class Switching Recombination
cys-LTs	Cysteine Leukotriene
DAMPS	Damage Associated Molecular Patterns
DC	Dendritic Cell
FcεR1	High Affinity Immunoglobulin E Receptor
GATA3	GATA Binding Protein 3
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HDM	House Dust Mite
HLA	Human Leukocyte Antigen
HSCs	Hematopoietic Stem Cells
ICOSL	Inducible T Cell Costimulatory Ligand
IFNγ	Interferon Gamma
IgE	Immunoglobulin E

IgG	Immunoglobulin G
ILC	Innate Lymphoid Cells
LTi	Lymphoid Tissue Inducer
MHC II	Major Histocompatibility Receptor II
NK cell	Natural Killer Cell
PAF	Platelet Activating Factor
PBMC	Peripheral Mononuclear Blood Cells
PD-L1	Programmed Death Ligand 1
pDC	Plasmacytoid Dendritic Cell
PGD_2	Prostaglandin D2
RORγt	Retinoic Acid-Receptor-Related Orphan Nuclear Receptor Gamma 2
RORα	Retinoic Acid-Receptor-Related Orphan Nuclear Receptor Alpha
SCFAs	Short Chain Fatty Acids
STAT6	Signal Transducer and Activator of Transcription 6
T-bet	Transcription Box Factor Tbx21
T1	Type 1 Immune Phenotype
T2	Type 2 Immune Phenotype
T3/17	Type 3 Or 17 Immune Phenotype
Tc	$CD8^+$ Cytotoxic T Cells
Tc2	Type 2 Cytotoxic $CD8^+$ T cell
TCR	T Cell Receptor
TGFβ	Transforming Growth Factor Beta
Th1	$CD4^+$ T Helper 1 Cell

Th2	$CD4^+$ T Helper 2 Cell
Th3/17	$CD4^+$ T Helper 3/17 Cell
TLR	Toll-Like Receptor
TLR	Toll-Like Receptor
TNFα	Tumour Necrosis Factor Alpha
Tregs	Regulatory T Cells
TSLP	Thymic Stromal Lymphopoietin
VANC	Vancomycin
wt	Wild Type

Acknowledgements

I extend my gratitude to Dr. Kelly McNagny, for your caring guidance and counsel throughout my research. I also thank my colleagues and friends; Michael Hughes, Julyanne Brassard, Melina Messing and Ahmed Kabil who have mentored and supported me along this challenging yet rewarding journey.

Dedication

To my unwavering support system – my family, whose love and encouragement fueled my academic journey. Your efforts helped put me in a position to receive the highest levels of education and your trust in me to succeed has motivated me each step of the way. A very special thank you to Mama Jan-Abu, you made this possible.

Chapter 1: Introduction

1.1 Overview of allergic disease

1.1.1 Defining allergic disease and origin

Allergic diseases are a group of immune mediated disorders caused mainly by immunoglobulin E (IgE) dependent or independent immunological reaction to innocuous environmental antigens[1]. Allergens are typically of two forms, aeroallergens (e.g., dust mites, pollen, animal dander etc.) and food allergens (soy, nuts, dairy etc.)[1,2]. Long term persistent exposure to allergens induces chronic inflammation which remodels affected tissue and organs leading to functional abnormalities. Many cell types are involved in allergic disease initiation and progression. Depending on the site of antigen contact, different clinical manifestations may develop typically on mucosal surfaces; the airways, GI-tract, and the skin[3]. Disease symptoms could include airway, skin and GI-inflammation, coughing, wheezing, excessive mucus production, rashes, hives, pruritus etc. and in severe cases anaphylactic shock and death[3–5].

1.1.2 Prevalence of allergic disease

In recent decades, as the western lifestyle has become more prevalent around the world, there has been a corresponding increased incidence of atopic disorders including atopic dermatitis (eczema), allergic rhinitis (hay fever), atopic (allergic) asthma and food allergies of which asthma and food allergies are the most well-documented[6]. In 2007 the costs in lost productivity and hospitalizations associated with allergic asthma in the United States, alone, were estimated at $56 billion; a 6% increase[7]. As of 2016, 1 in 4 Canadians were diagnosed with an allergic disease and 1 in 13 Canadians have had an allergic reaction related to food over their lifetime[8]. Additionally, between 2001 and 2012 the proportion of Canadians living with diagnosed asthma

increased by 67%[9]. Even though asthma affects people of all ages the greatest burden of disease is in children 18 years and younger[9,10]. In childhood boys are disproportionately affected compared to girls and the opposite is observed during adulthood. Some have suggested that this may be due to testosterone driving a decrease in some key allergy immunomodulators and effector cells[11]. Overall, affected individuals experience loss in productivity, due to missing school and work, poor quality of life due to disease restrictions and having to carry on the economic burdens of the disease.

1.1.3 Causal Factors

In recent decades the hygiene hypothesis has been the most popular theory used to explain the increased atopy in developed populations. It argues that the excessive use of antibiotics, detergents, vaccination and limited exposure to microbes and infection early in life skews immune development towards a more atopic, type 2(T2) immune phenotype[12]. This theory is supported by evidence from children who grow up on farms, have siblings, pets or infection in early life experiencing a lower incidence of allergy[13–15]. A variety of factors have been linked to the susceptibility and severity of allergy and asthma. These include genetic predisposition, environmental exposures (pre/perinatal, pollutants, microbial and viral exposure etc.), lifestyle (nutrition and sanitation) and interactions among these variables[5,16,17]. However, it is important to note the difference between atopy and allergy when discussing these factors.

Atopy is the genetic predisposition to develop allergic type disorders and is linked to an IgE mediated immune reaction[18]. It is typically diagnosed by the skin prick test or by detecting allergen-specific serum IgE in affected individuals[18]. Whereas allergy is an abnormal reaction to a harmless substance, an allergen[1]. Atopy and allergy can occur together and independently, and

atopic individuals may present with one or more allergic disease over time and this phenomenon is describes as an atopic march[18–20]. Causal factors discussed will be in the context atopic IgE mediated allergy (figure 1.1).

1.1.3.1 Genetic factors

Genetic factors undisputedly have a role in the high incidence of atopy since they dictate the rate of IgE synthesis and turnover. Investigation into different atopy associated genes revealed polymorphisms associated with higher atopic susceptibility which can be inherited via autosomal transmission[21,22]. For example, polymorphisms in a gene cluster associated with cytokines that regulate immunoglobulin class switching, effector cell activation and migration and other allergic mechanisms have been implicated in allergy[22,23]. Polymorphisms in the promoter region, beta, and alpha subunit of the high affinity IgE receptor (FcεR1) have been associated with a higher incidence of atopic asthma[24–26]. In addition, the presence of specific human leukocyte antigen (HLA) alleles has been linked to higher atopic susceptibility[27]. However, population-wide genetic changes usually occur over extremely long periods of time so it can be argued that environmental changes may be more culpable for the recent rise in atopic disease.

1.1.3.2 Environmental factors

A wide range of environmental factors have been implicated in allergic disease including pollutants (like smoke and noxious gases) and in-utero exposure to immune modulatory molecules in the maternal environment. Air pollutants like tobacco smoke and particulate matter have been related to acute respiratory tract illness as they can induce decreasing lung function and can trigger exacerbations in asthma symptoms[28,29]. In the maternal environment,

transplacental exposure to maternal immune mediators like immunoglobulin, short chain fatty acids (SCFAs), hormones, cytokines etc. are believed to impact foetal immune education prior to birth[30,31]. During birth, travel through the birth canal exposes infants to microbiota which may have a protective or inflammatory role [31]. The extensive impact of the maternal environment on infant health will be discussed further in a later section. All this taken together propound a very complex interaction between genetics and the environment converging to create a health emergency.

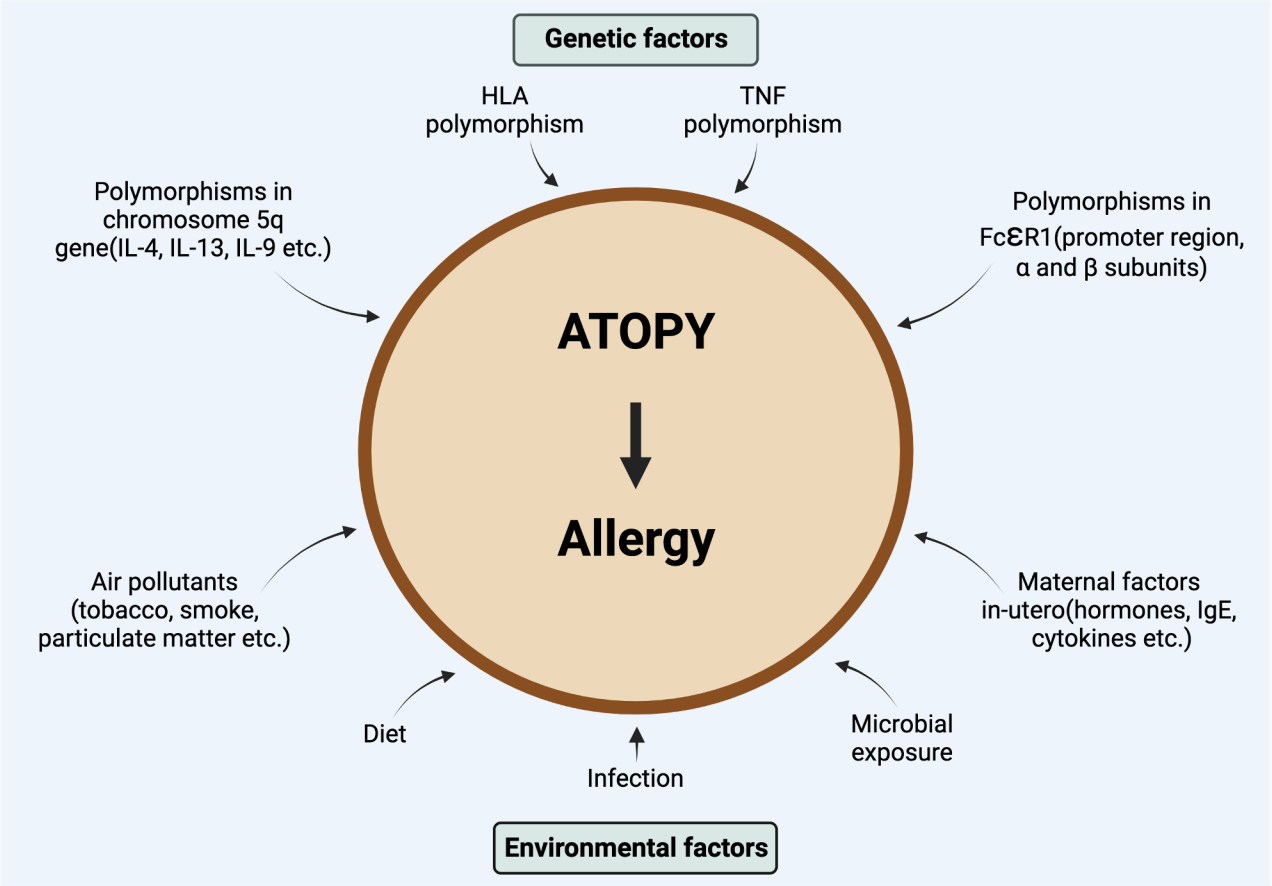

Figure 1.1 Genetic and environmental factors driving atopy and allergy

Specific genetic polymorphisms have been linked to increased incidence of atopy. Most of these polymorphisms are found in genes whose products are involved in the development and progression of allergic disease. In individuals this genetic bias coupled with environmental factors promote sensitization to allergens and the subsequent development of allergy. HLA = human leukocyte antigen, TNF= tumour necrosis factor, IgE = immunoglobulin E

1.2 Immune mechanisms in immunoglobulin E mediated allergy

1.2.1 Overview of cell mediated effector immunity

To better understand the nature of the dominant immune environment during an allergic reaction it is important to clarify the three major types of cell-mediated effector arms of immunity summarized in table 1.1.

Type 1(T1) immunity is driven by transcription box factor TBX21 (T-bet) expressing cells that protect against viruses and other intracellular pathogen. They also have a role in inflammation and autoimmunity through the secretion of T1-associated cytokines like interferon gamma (IFNγ) and tumour necrosis factor alpha (TNFα)[32,33]. Type 1 effector cells include $CD4^+$ T helper 1 (Th1) cells, $CD8^+$ cytotoxic T cells (T_C) and type 1 innate lymphoid cells (ILC1). Th1 and $CD8^+$ T cells polarize upon stimulation with IL-12, IL-15, and IL-18[32].

T2 immunity is driven by the GATA binding protein 3 (GATA3) expressing cells involved in protection against helminths, allergic inflammation, tissue repair and tissue remodelling through the production of T2-associated cytokines IL-4, IL-5, IL-9, IL-13 and amphiregulin (Areg). T2 effector cells; CD4+ T helper 2 (Th2) cells and Type 2 innate lymphoid cells (ILC2) undergo polarization upon stimulation with IL-4 and IL-33, IL-25 and thymic stromal lymphopoietin (TSLP) respectively[32,34].

Finally type 3/17 (T3) immunity is driven by retinoic acid related orphan receptor gamma t (RORγt) expressing cells that produce anti-bacterial responses and promote lymphoid organogenesis. T3-associted cytokines include IL-17 and IL-22 which are secreted by Th17 cells, Tc17 cells and ILC3s[32].

Table 1.1 The three types of cell-mediated effector immunity
Immune effector cells, transcription factors, cytokine and functions are summarized

	Effector cells	Transcription factors	Cytokines	Function
Type 1	Th1, Tc1, ILC1	Tbet, eomes	IFNγ, TNFα	Viral immunity, intracellular pathogens, chronic inflammation
Type 2	Th2, Tc2, ILC2	GATA3, RORα	IL-4, IL-5, IL-13, IL-9 Areg	Helminths defence, allergic inflammation, tissue repair and tissue remodelling
Type3	Th3/17, Tc17, ILC3	RORγt, Tbet	IL22, IL-17	Defence against extracellular bacteria and fungi and chronic inflammation

1.2.2 Allergen sensitization

We are exposed daily to aeroallergens like pollen, animal dander, dust mite and other types of allergens. To such exposure, non-atopic individuals typically produce small amounts of immunoglobulin G (IgG) and may experience mild proliferation of Th cells which preferentially produce IFNγ over IL-4[35,36]. In contrast atopic individuals develop allergies from such exposure through allergen sensitization. Allergen sensitization is the process by which antigen specific Th2 cells polarize and eventually antigen specific IgE is produced to an inhaled, absorbed, or ingested allergen.

Sensitization to an allergen is suggested to occur through innate mechanisms given that the adaptive immune response takes time. In the airway, mechanical damage to the epithelial barrier and entry into the mucosa by an allergen triggers the release of factors that can stimulate tissue resident ILC2s, Dendritic cells (DC), mast cells and basophils to secrete T2 cytokines and activate immature DCs (figure 1.2)[37–39]. These T2 Cytokines and other immunomodulatory factors then facilitate antigen presentation and polarization of antigen specific Th2 cells and immunoglobulin isotype class switching recombination (CSR) in B cells to produce allergen specific IgE (figure 1.3). The presence of antigen specific Th2 cells and IgE enhances the T2

immune reaction upon allergen re-exposure leading to disease. However, the timing and mechanism of this initial reaction has been difficult to resolve as allergy symptoms do not develop from allergen sensitization[17].

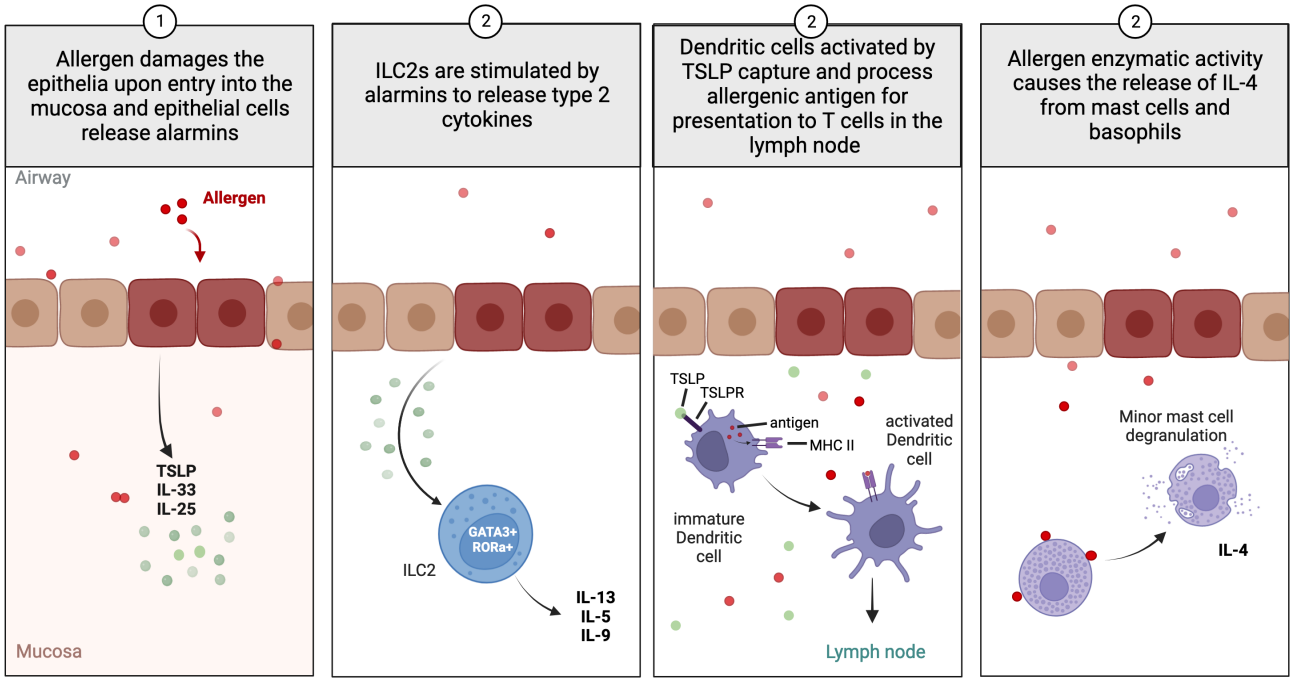

Figure 1.2 Innate mechanisms in allergic airway sensitization

Identifying the specific mechanisms in allergen sensitization has been a challenge. In the case of airway sensitization, the activation of cells in mucosa is driven by the release of alarmins TSLP, IL-33 and IL-25. ILC2s stimulated by these alarmins produce cytokines IL-13, IL-5 and IL-9 which all have downstream effects on both innate and adaptive cells that promote airway allergy. TSLP stimulation of dendritic cells facilitates their activation, and these cells can efficiently present allergens to promote Th2 polarization of naïve T cells in the lymph node. Enzymatic activity by allergens drives mast cells and basophils to secret IL-4 a key cytokine in allergy development. All or some of these mechanisms may drive sensitization at the same time.

One important factor that may influence this sensitization event is the nature of the allergen. Antigen presenting cells (APCs) are constantly surveying epithelial barrier organs for foreign molecules and they may have easier access to antigens when barrier cells are compromised i.e., become permeable or are damaged. To address the non-immunological variables that may influence allergic sensitization significant work has been done to discern the characteristics of

allergens that facilitate their crossing epithelial barriers. Even though complete structural information on the most common allergens is not available most of them seem to have common enzymatic activities that can cause epithelial damage[40,41]. The best characterized allergen with enzymatic activity is the group of mite derived allergens. For example, Der p1 is a house dust mite (HDM) cysteine protease that can disrupt epithelial cells tight junctions and may disrupt the protease-antiprotease balance in different tissue enhancing the activity of endogenous serine proteases facilitating sensitization in allergy. In murine models, papain which is a plant derived cysteine protease is also capable of inducing airway inflammation like HDM[42,43]. In addition to disrupting mucosal barriers some allergens may be involved in inducing mast cell and basophil degranulation, IL-4 and IgE secretion[37]. They may also be involved in cleavage of lymphocyte surface molecules which may affect lymphocyte activation and function[44].

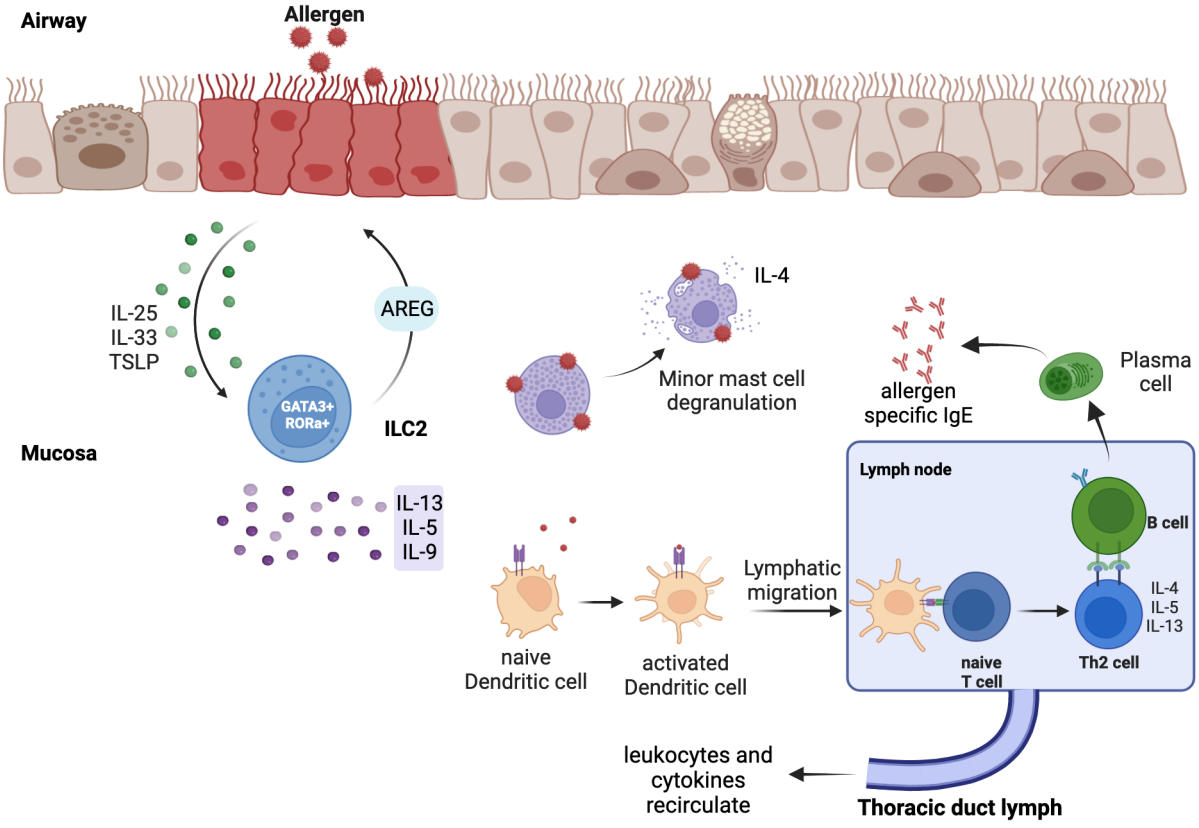

Figure 1.3 Allergen sensitization in the airway

Following epithelial insult, the release of alarmins TSLP, IL-33, and IL-25 facilitate IL-13, IL-5, and IL-9 secretion by ILC2s. These cytokines support the migration of TSLP activated DC to the lymph node where they present allergen epitopes to naïve T cells which polarize into Th2 cells in the presence of IL-4. These cells then secrete more T2 cytokines and stimulate IgE production by B-cells. These leukocytes recirculate are now primed to respond to their cognate antigen upon allergen re-exposure.

1.2.3 Early phase reaction

The mechanism in an acute allergic reaction is very similar to that of the initial allergen sensitization. This reaction occurs within minutes of allergen encounter and is mediated by an allergen epitope crosslinking to IgE bound to FcεR1 on mast cells and basophils[45]. Antigen crosslinking causes aggregation of FcεR1 on mast cell surface (may also include basophils) which leads to a kinase activation cascade that drives the degranulation of preformed mast cells granules[46]. These granules release immune mediators like histamine, leukotrienes, proteases and increase the production of cytokines, chemokines, and growth factors[47]. These locally secreted factors rapidly generate a range of responses and symptoms depending on the reaction site[46]. These include vasodilatation, smooth muscle contraction, mucus hypersecretion, increased vascular permeability, erythema etc.[17,46]. Anaphylaxis occurs in severe cases where large amounts of histamine and then leukotrienes are realeased[48].

1.2.4 Late phase reaction

A late phase reaction results from the long-lasting effects of some early phase mediators and tissue resident and infiltrating leukocyte activity. This reaction occurs 2-9 hours after allergen exposure. In the airway epithelium, allergic airway inflammation (AAI) which occurs in asthma and allergic rhinitis is characterized by a T2 immune response. Disease hallmarks include airway

hyperresponsiveness (AHR), eosinophilic inflammation, mucus hypersecretion, and the secretion of the canonical Th2-associated cytokines IL-4, IL-5, IL-9, and IL-13[49]. Although Th2 cells are the best-known source of these cytokines, various innate cells; mast cells, eosinophils, basophils and ILC2s have also been implicated in their production[50].

In the late phase (figure 1.4) stimulated tissue resident ILC2s continue to produce IL-5 and IL-13. In addition to IL-5 and IL-13, granulocyte macrophage colony stimulating factor (GM-CSF) and TSLP secreted by epithelial cells attract and activate DCs and other APCs which uptake allergic antigens and migrate to nearby lymph nodes where they present these antigens in a major histocompatibility receptor II (MHCII)-dependent manner to naïve T cells[49,51]. In the lymph node, IL-4, acting through its receptor on naïve T cells upregulates GATA3 a key Th2 transcription factor[52]. GATA3, in turn promotes Th2 cell differentiation. The resulting antigen specific Th2 cells secrete high level of IL-4, IL-5 and IL-13 enhancing the T2 immune reaction. Some Th2 cells then exit the draining lymph nodes, enter circulation through the thoracic duct lymph and are recruited to the sites of ongoing inflammation. Additionally, within the lymph nodes some Th2 cells via IL-4, IL-13, and other costimulatory molecules, stimulate B cells to class switch and produce IgE. Secreted IgE coats mast cells and basophils by binding FcεR1 [53]. Antigen-IgE crosslinking causes FcεR1 aggregation on mast cell and basophils leading to their degranulation and the release of more immunomodulatory molecules promoting allergy symptoms. IL-5 produced by Th2 cells and ILC2s facilitates eosinophil recruitment and proliferation at the site of inflammation. Eosinophils are one of the most important responder cells driving allergic inflammation as they produce a wide range of immune mediators that promote allergic pathology[54].

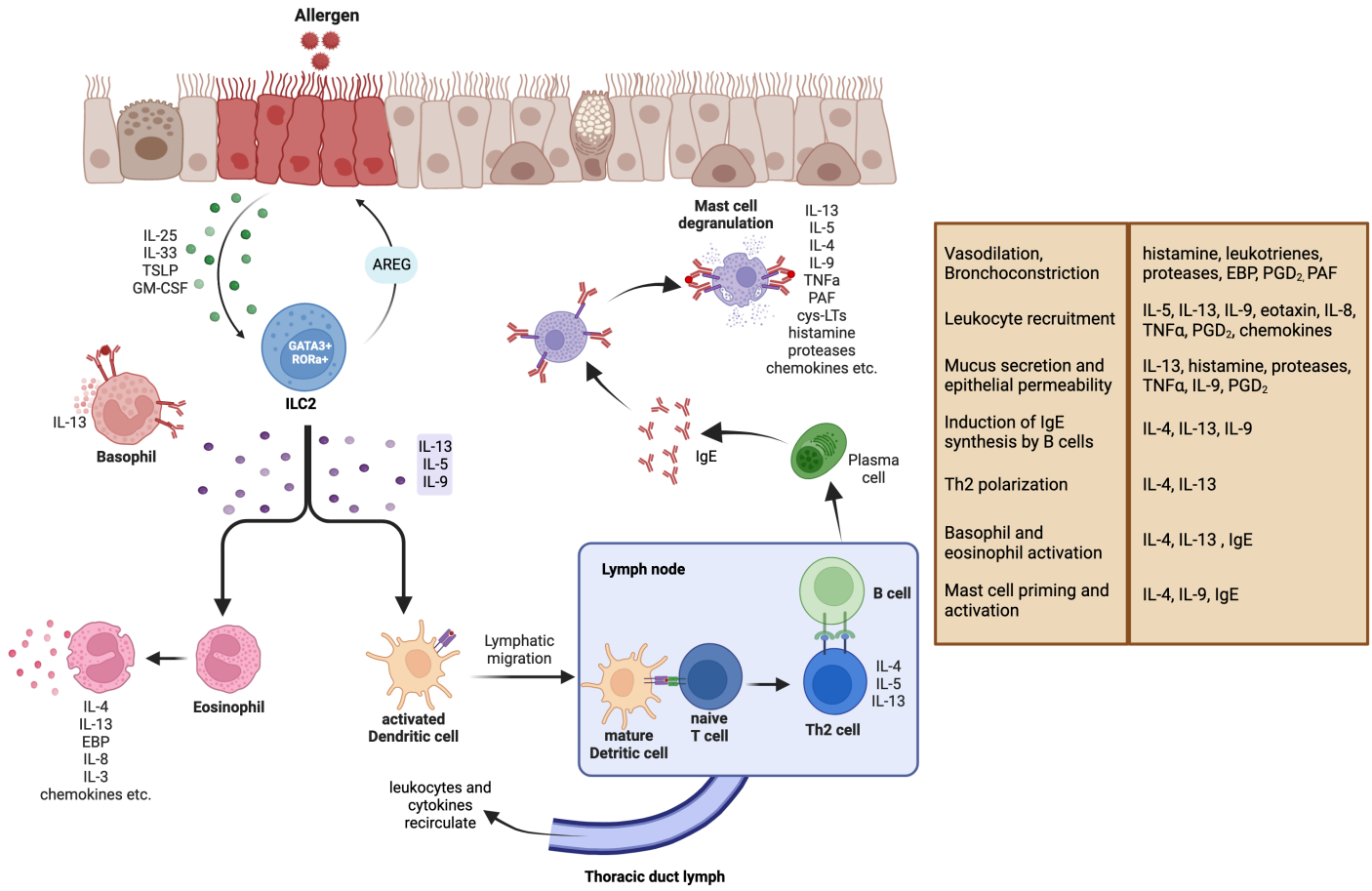

Figure 1.4 Late phase allergic reaction

Alarmins produced by epithelial cells and T2 cytokine secreting ILC2 are still active in the late phase. These cytokines facilitate DC antigen presentation to naïve T cells in the lymph nodes expanding the population of antigen specific Th2 cells. They also support leukocyte recruitment (eosinophils, mast cells, basophils etc.) to the inflamed mucosa. Th2 cells facilitate IgE production by B cells. IgE in the mucosa coats mast cells and basophils which degranulate upon antigen IgE crosslinking. Degranulation of eosinophils also occurs in the late phase. All these cells release cytokines (TNFα, IL-5, IL-4, etc.), chemokines, lipid-derived mediators (PDG_2, cys-LTs etc.), PAF, proteases, histamine etc. that increase blood flow, smooth muscle contraction, mucous hypersecretion, leukocyte recruitment etc. resulting in allergic disease exacerbation.

It is important to note that in some sensitized individuals there is not a clear demarcation between an early and late phase reaction. In fact, some may not experience a late phase reaction at all[55]. Factors that influence this difference and the mechanism driving the resolution of early phase or late phase reactions are still not well understood but it is believed that regulatory T cells

(Tregs) secreting factors like IL-10, transforming growth factor beta (TGF-β) and increased production of cytokines like IFNγ, IL-18 and IL-12 can facilitate the shift from a T2 towards a more T1 environment and may, at least in part, be responsible[56].

1.2.5 Chronic allergic inflammation

Chronic inflammation occurs from consistent allergen exposure causing persistent inflammation[57]. The mechanism behind the transition from repeated early and late phase reactions to chronic inflammation is not clear. Whereas early and late phase reactions can be studied quite easily in humans, studying of chronic allergic inflammation is more challenging and typically done in animal models (usually mice) where the genetic and physiological differences impact the type of conclusions that can be made. Chronic inflammation is characterized by an altered morphology of the structural cells at the site of inflammation which usually translates to altered functioning of the affected tissue or organ[58,59]. For example, in chronic asthma, extensive epithelial damage and repair leads to production of more cytokines that can drive T2 immunity that, in turn, leads to an increase in the number of mucus producing goblet cells, thickening of airway muscles and increase vascularity[58,60]. In atopic dermatitis, skin remodelling results in excessive fibrosis causing barrier dysfunction and dry skin lesions with fibrotic papules[61]. Overall, cells and factors like those in the early and late phase are involved in the progression of clinical chronic inflammation. The main difference here is that there is less effective resolution or dampening of the T2 immune environment.

1.3 Cells involved in allergic reactions

Allergic disease progression is dependent on a complex interplay between cell-mediated activity and soluble immunomodulators in different microenvironments. Hence it is important to get a better understanding of how different cells (tissue resident and infiltrating) exert their effects. Both innate and adaptive immune cells have a role in allergy and in some instances these roles may blur the distinction between innate and adaptive cell function. Some cells may have innate and adaptive like activity bridging the gap between the initial non-specific innate response and an antigen specific adaptive response during an allergic reaction.

1.3.1 T cells

Several T cell subsets have been implicated in allergic disease initiation and potentiation, most notably $CD4^+$ T cells and T regulatory cells[62,63]. Following T cell development in the thymus, naïve T cells exit into the periphery where they may encounter antigens and costimulatory ligands presented by APCs to become activated effector, memory, or regulatory T cells[64].

1.3.1.1 Th2 cells

$CD4^+$ Th2 cells have a pivotal role in allergic pathology through their contribution of T2 cytokines and costimulatory activity[65]. Allergic disease development and progression is supported by a T2 immune environment and an elevation in the number of Th2 cells in affected tissue is a hallmark of allergic disease[66]. T2 cytokine upregulation is correlated with antigen exposure, eosinophilic infiltration, and airway hyperresponsiveness in murine models[67,68]. Furthermore, asthmatic patients have elevated levels of T2 cytokines in their blood and in

bronchial alveolar lavage fluid (BALF) following antigen challenge further emphasizing the importance of Th2 cells in allergy development[68].

Th2 polarization is favoured in the presence of IL-4, an effector cytokine for Th2 cell development[52]. An active area of investigation is the source of IL-4 during the initial allergen sensitization event. Even though mast cell, basophils, eosinophils, and other T lymphocytes are potential sources there is no consensus on the mechanism and conditions that trigger IL-4 secretion during sensitization, probably because there may be multiple mechanisms[52,69–71]. Within the lymph nodes, T cell receptor (TCR)-MHC II dependent antigen presentation with CD28 co-stimulation activates naïve T cells. IL-4 stimulation of these cells facilitates the phosphorylation of signal transducer and activator of transcription 6 (STAT6) which sets off a cascade of events that lead to the upregulation of GATA3 and the subsequent production of large amounts of IL4, IL-5, IL-9, and IL-13 from these now, antigen-experienced Th2 cells[52,72]. Once these cells leave the lymph nodes and enter the inflamed organs, these cells and their cytokines then reinforce the T2 immune environment through various effects. IL-5 is involved in eosinophil activation and recruitment to the affected tissue, IL-13 drives smooth muscle contraction and mucus hypersecretion by goblet cells while IL-9 supports mast cell degranulation[72]. The secretion of IL-4 creates a positive feedback loop for more IL-4 secretion and the polarizing more naïve T cells towards a Th2 phenotype. Multiple naïve T cell populations can be sensitized to different allergenic epitopes (one epitope per T cell population) generating a wide array of different epitope specific Th2 cell clones which might provide a partial explanation for increased disease severity over time. IL-4 and IL-13 are also important co-factors in B cell class switching to produce antigen specific IgE[72]. Non-cognate activation of

Th2 cells with innate cytokines like IL-33 has also been shown to stimulate cytokine production by memory Th2 cells and may help drive pathogenesis[73].

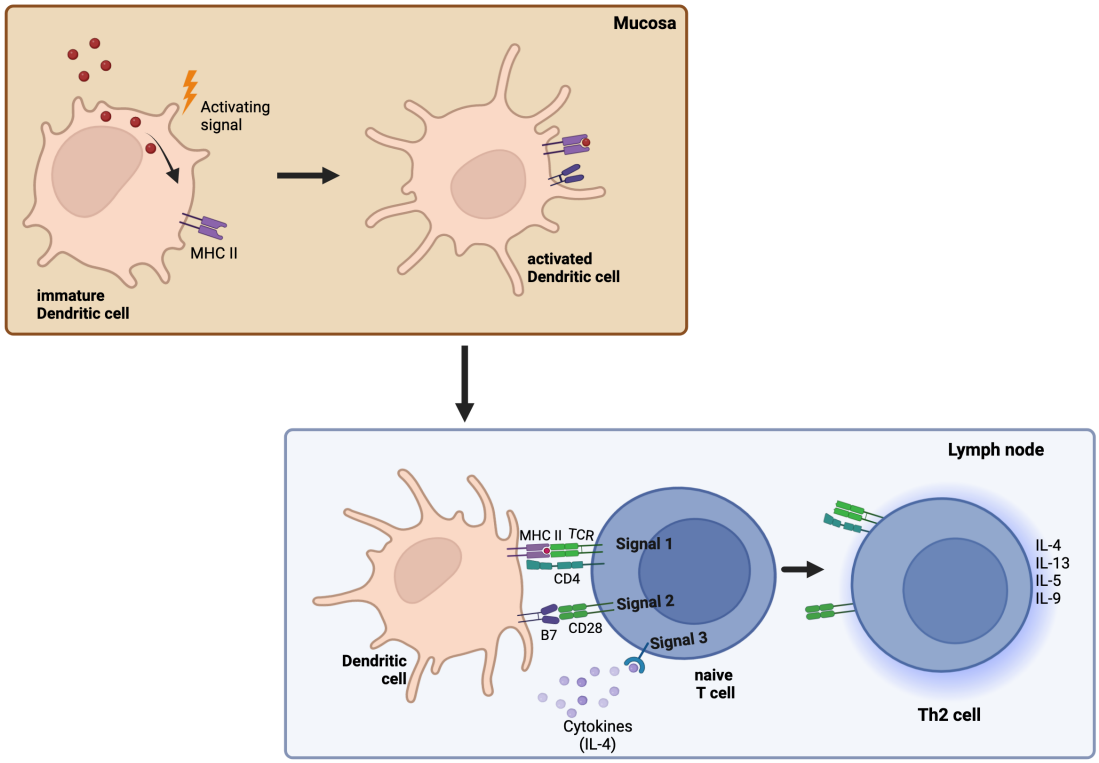

Figure 1.5 Antigen specific Th2 polarization

Upon exposure to allergen immature DCs are uptake antigen for processing. Processed antigen are then displayed by MHC II molecules on activated DCs. These DCs migrate to the lymph node where they encounter naïve T cells. Naïve T cells are primed by these DCs via MHC II-TCR interaction (signal 1), co-stimulation (signal 2) and cytokine stimulation (signal 3) to give rise to Th2 cells capable of producing T2 cytokines.

1.3.1.2 Th1/ Th2 balance

Unlike Th2 cells, Th1 and Treg cells have been shown to antagonize allergic pathology and it has been postulated that specific host factors may drive an imbalance in immune homeostasis that favours skewing towards a type of immune environment that facilitates disease resolution or progression[57,63,74]. The hygiene hypothesis is supported by this idea given the rapid increase in

atopic disease in western countries that is difficult to explain through changing genetics[75]. Some believe that, in early life, our extremely sanitized environment and vaccinations leads individuals to encounter fewer microbes and hence they are unable to develop efficient immunoregulatory checkpoints and therefore become more susceptible to T2 skewed immune responses when exposed to innocuous antigens like pollen. One supporting bit of evidence for this theory is the markedly lower level of atopy in children who grow up on farms and in more rural areas[13]. Hence, finding and restoring a balance between a T1 and T2 immune environments and naturally upregulating Treg activity has been an active area of study for potential treatments of allergic inflammation.

1.3.1.3 Th1 cells

Th1 cell expansion usually occurs as a response to viral infection or intracellular pathogens. IL-12 and IL-18 are the main effector cytokines for Th1 polarization just like IL-4 for Th2 polarization[52]. In IL-12 rich environments Th2 cell polarization is blocked by Th1 cells through the secretion of IFNγ which inhibits Th2 differentiation and downstream IL-4 production[52]. In murine mouse models IFNγ was shown to directly inhibit eosinophilia and mucus production in antigen-sensitized mice[76]. Hence, interventions that drive creation of a T1 immune environment may be effective in tempering acute allergic pathology.

1.3.1.4 Regulatory T cells

Tregs are typically defined as $CD4^+CD25^+$FOXP3 expressing cells that produce soluble factors like IL-10 and TGF-β that have anti-inflammatory properties and can also exert immunomodulatory effects by direct cell to cell interaction[56]. Studies in murine model have

revealed that the depletion of Tregs prior to antigen challenge increases Th2-mediated allergic inflammation and, *in vitro,* Tregs inhibit Th2 differentiation[77]. Hence, it has been suggested that Tregs function may be attenuated in atopic individuals. In addition to acting on Th2 cells, IL-10 produced by these cells has wide ranging suppressive function on other cells involved in allergic disease. For example, it blocks cytokine production by eosinophils and mast cells, blocks IgE induced mast cell activation, reduces APC function, and modulates B cell IgE generation[78].

1.3.1.5 Th17 cells

The Th1/Th2 paradigm of immune homeostasis dominated discussions since the discovery of these two subsets of T cells. However, the revelation in murine models that some previously Th1-associated autoimmune diseases were not resolved by IFNγ deficiency challenged the aetiologic role of Th1 cells in the purported "Th1-driven" pathology[79,80]. IL-17 producing Th cells (Th17) in these diseases provided insight into a different molecular mechanism driving autoimmune pathology[81]. Furthermore, these IL-17 producing cells were also found to contribute to allergic disease pathology by shifting the paradigm of immune homeostasis and maintenance from just Th1/Th2 to Th1/Th2/Th17[82]. Late in allergic asthma, IL-17 mRNA, and protein are increased in serum, BALF and sputum of individuals with asthma and the levels correlate with disease severity[83,84]. IL-17 was found to stimulate epithelial cells, airway smooth muscles and bronchial fibroblasts to produce IL-6, IL-8 and several other immune modulators. These molecules are potent chemoattractant and activators of neutrophils and mast cells but did promote eosinophil driven Th2 inflammation hence contributed to non-Th2 (eosinophilic) asthma (figure 1.6)[82,84]. Similar results have been found in murine models but with much variation depending on the route of priming, type of allergen and mouse background[82].

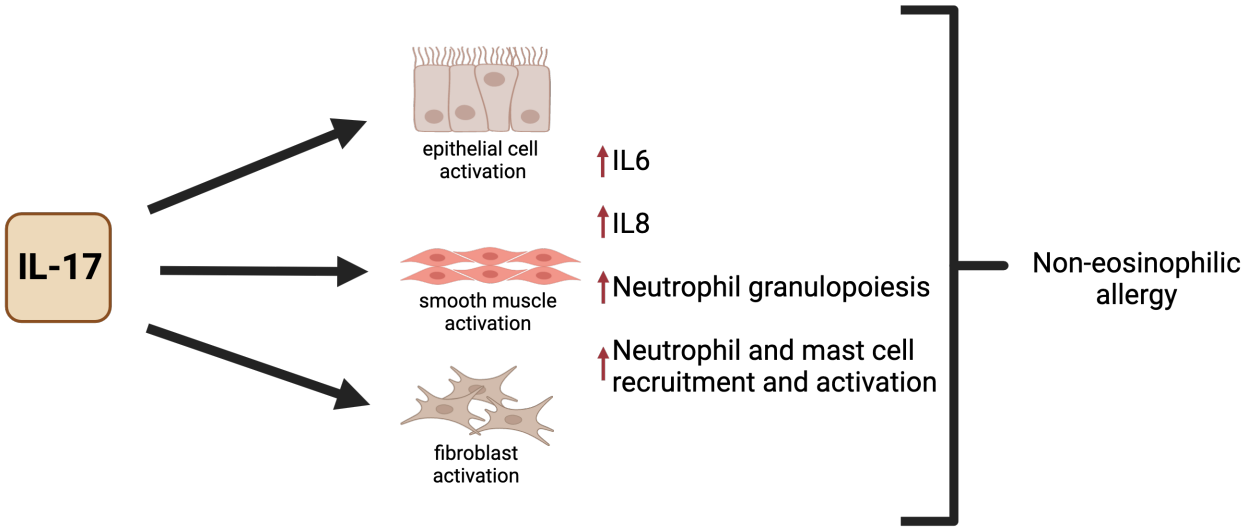

Figure 1.6 IL-17 and allergy

IL-17 produced by Th17, Tc17 and ILC3s has been shown to be involved in allergy pathogenesis. However, IL-17 seems to drive a different allergy endotype. IL-17 activation of epithelial cells, smooth muscle and fibroblasts causes the secretion of IL-6, IL-8, and other factors by these cells. These molecules support neutrophil recruitment activation and granulopoiesis exacerbating non-eosinophilic asthma. In the chronic allergic inflammation these factors also drive fibrosis.

1.3.1.6 $CD8^+$ T cells

Cytotoxic $CD8^+$ T cells (Tc) have established roles in viral immunity however a subtype of $CD8^+$ T cells are known to produce T2 cytokines (Tc2 cells)[85]. Like Th2 cells, Tc2 cells are enriched in the serum of patients with severe eosinophilic asthma suggesting they may play a role in allergy pathogenesis[85]. These cells are activated and recruited in a TCR independent manner by lipid-derived mediators; prostaglandin D2 (PGD_2) and cysteine leukotriene (cys-LTs)[86]. T2 cytokine production by these cells is also enhanced by PGD_2 and cys-LTs.

Thus, different T cell subsets have either a protective or an inflammatory role in allergic disease and could be leverage for their therapeutic potential.

1.3.2 B cells and B regulatory cells

B cells under different stimulation conditions can differentiate into long-lived and short-lived plasma cells that can secrete a wide array of antibody isotypes to different antigens[87,88]. Their principal contribution to allergic pathology is the secretion of IgE isotype antibodies. B cell receptor stimulation in the presence of IL-4 and co-stimulation with CD40 by Th2 cells facilitates B cells class switching recombination to produce antigen specific IgG1 and IgE[89]. This secreted IgE binds FcεR1 on mast cells and basophils and antigen crosslinking can trigger their degranulation[58]. In addition to their role as antibody-producing cells, some B cells have been shown to have a regulatory role analogous to T cells through their production of IL-10 and IgG4 which are important for allergen tolerance[90,91].

1.3.3 Dendritic cells

Dendritic cells (DC) are the principal sentinel cells of the immune system. They have a role in innate and adaptive immunity, antigen presentation and regulation of tolerance. Two subsets; conventional DCs (cDC) and plasmacytoid DCs (pDC), have been identified in both humans and mice[92].

1.3.3.1 Conventional dendritic cells (cDCs)

In allergy cDCs are crucial for stimulating T cells through antigen presentation and the secretion of cytokines that drive disease or allergen tolerance. Immature cDCs survey the lymphoid and non-lymphoid tissues for invading pathogens and uptake antigens they encounter for processing and MHC restricted presentation to naïve T cells (figure 1,5)[93]. DC morphology upon activation

and maturation is characterized by long cytoplasmic processes, a large nucleus, and scattered mitochondria[94].

During allergic reactions epithelial cells secrete factors like GM-CSF which facilitate DC recruitment to affected sites. In this environment different signals support DC maturation; antigen encounter in addition to CD40 ligation, cytokines (TNFα, Type 1 IFNs), DNA and RNA molecules interacting with DC toll-like receptors (TLRs) etc. Another cytokine secreted by epithelial cells that affects DC function is TSLP. In humans, TSLP was shown to trigger DC migration and activation and TSLP activated DC were potent polarizers of naïve T cells to Th2 cells which drive allergic disease[95].

Following antigen uptake, mature DCs upregulate the expression of MHC II and other T cell costimulatory molecules like B7-1(CD80), B7-2(CD86) and CD40. They also modify their chemokine receptor repertoire to express receptors for chemokines that promote their migration to afferent lymphatics[96]. As DCs migrate to the draining lymph nodes they process the acquired allergenic antigen and present specific epitopes to naïve T cells in the lymph via MHC II - TCR interaction providing the first signal for T cell activation. Costimulatory signals like CD28-CD80/86 interactions act as the second activation signal[97,98]. Depending on the cytokine environment DCs can prime naïve T cells towards a Th2 or non-Th2 phenotype. DCs secreting IL-12 and/or in a TNFα rich microenvironment have been suggested to drive Th1 polarization, while Th2 polarization is facilitated by IL-4, prostaglandin E2 (PGE_2) and IL-10 (possibly) which inhibit IL-12 action[97]. A single DC can activate hundreds on naïve T cells and DC Th2 polarization serves to amplify the effects of T2 cytokines that then worsening allergic disease.

1.3.3.2 Plasmacytoid dendritic cells(pDCs)

Plasmacytoid DCs (pDCs) and cDCs can be distinguished by their well-defined surface marker expression in both humans and mice. Even though they share similar morphology pDCs are much poorer at antigen presentation and activation of effector T cells than cDCs[99]. However, pDCs counts in BALF and skin of both humans and mice correlate with disease severity suggesting that pDCs may play a role beyond antigen presentation and T cell activation[100,101]. pDCs act as innate defenders against viral infection via endosomal toll like receptor 7 and 9 (TLR7 & TLR9) which sense foreign RNA and DNA respectively. They are unique among hematopoietic cells in their ability to produce large amounts of type 1 interferon. In allergy pDCs have been shown to ameliorate disease severity or interfere with allergy initiation all together. In humans pDCs counts inversely correlate with childhood atopic asthma. Lower levels of pDCs during infancy correlated with respiratory tracts infections, wheezing and asthma diagnosis up to 5-years of age and this phenomenon has been recapitulated in several murine models[102–108]. pDC depletion by inhibitory antibodies or pDC knockout / transgenic mice exhibit increased sensitization, worsened airway, and intestinal inflammation and increased T2 cytokine secretion by Th2 cells. Intriguingly, subsequent pDC reconstitution ameliorated most allergic symptoms[103,109,110]. Several processes are believed to be involved in the pDC response to allergic inflammation. Through the secretion of IFNα and IL-12, pDCs may interfere with Th2 cells polarization and T2 cytokine production since both cytokines inhibit IL-4 action (a major T2 effector cytokine) [103,111]. IFNα has also been shown to interfere with GM-CSF and IL-33 production by epithelial cells impairing the recruitment of cDC, eosinophils and ILC2s to sites of inflammation[107]. Activated and resting pDCs also express T cell inhibitory ligands like programmed death ligand 1 (PD-L1) and inducible T cell costimulatory (ICOSL) [101]. Ligation to

their corresponding receptors on T cells promotes development of tolerance, cell anergy and even death, effectively decreasing the pool of effector T cells[101,112]. Furthermore, pDC have been shown to stimulate naïve T cell polarization to FOXP3^{+} regulatory T cells, some of which are antigen specific[112]. These cells then produce tolerogenic cytokines like IL-10 and TGF-β which dampen the allergic response and disease severity.

1.3.4 Innate Lymphoid cells (ILCs)

Innate lymphoid cells (ILCs) are a rare family of recently discovered tissue resident lymphocytes involved in early host defences. They are important for immune modulation at steady state and during inflammation, tissue homoeostasis and repair[113]. They are found in both lymphoid and non-lymphoid tissues but mostly accumulate at mucosal barrier surfaces like the airway and gut and respond to a sentinel stimulus to produce the appropriate non-antigen specific response[33]. There are 5 major subtypes of ILCs: natural killer (NK) cells, ILC1, ILC2, lymphoid tissue inducer (LTi), and ILC3, each classified based on their transcription factor profiles, functional characteristics, and cytokine expression profile[33]. ILC1, ILC2 and ILC3 are the non-antigen-specific cell counterparts to Th1, Th2 and Th17 cells, respectively, due to similarities in their developmental repertoire and cytokine expression profiles. As result of these similarities some in the field have deemed these cells functionally redundant[114,115]. However, evidence from ILC studies have implicated ILC2s and ILC3s specifically in allergic disease pathology independent of Th cell function and action[116].

1.3.4.1 Type 2 innate lymphoid cells (ILC2)

In studies of mice lacking T and B cells, ILC2s were identified in spleen, adipose tissue, lymph nodes, intestine, lungs, and nasal mucosa and, upon in vivo stimulation with IL-25 and IL-33 and in vitro with IL-25, were shown to be potent producers of IL-5 and IL-13 but minuscule amounts of IL-4. As previously discussed, these three cytokines are crucial effector cytokines in allergic pathology[43,117]. ILC2s have been revealed as essential in the development of AAI in HDM and papain mouse models[43]. The main mouse model used for ILC2 deficiency studies in our lab are mice with the "staggerer" mutation due to retinoic acid-receptor-related orphan nuclear receptor alpha (*RORα)* deletion[118]. These mice have a staggering gait and neurological defects which give them a very shortened life span but, importantly, also selectively lack ILC2s. To obviate the issue linked to neurological defects in RORα mutants, we routinely generate bone marrow chimeric mice in which wild type (wt) mice are engrafted with hematopoietic stem cells (HSCs) from RORα mutants[118]. This permits a focused analysis of the role of ILC2s in inflammatory disease models. ILC2 deficiency in $RORα^{sg/sg}$ chimeric mice challenged intranasally with HDM resulted in a significant decrease in leukocyte infiltration, especially eosinophils, and a decrease in serum IgE and lung transcripts of IL-4 and IL-5 suggesting attenuation of the downstream adaptive Th2 immune response[117]. Likewise, ILC2 depletion via anti-CD25 antibody prior to a papain challenge was linked to a significant decrease in BAL eosinophil number, mucus production and lung histopathology in $Rag1^{-/-}$mice, which are T cell and B cell deficient. Allergic disease pathology was recapitulated upon ILC2 transplant in these mice[43]. Thus, ILC2s were shown to be important for local T2 priming and eosinophilic inflammation in the lungs without the need for adaptive cells and ILC2 deficiency ameliorates much of the disease immunopathology associated with allergic asthma. ILC2 have also been suggested to play an

essential role in Th2 cell polarization due to their activity in the early phase of the immune response (figure 1.4). ILC2s support Th2 cell migration and proliferation in the lungs and in the draining lymph nodes in response to antigen via cytokine secretion[119].

1.3.4.2 Type 2 and 3 innate lymphoid cells (ILC2 and ILC3)

The development of fibrosis is one of the main outcomes of chronic allergic inflammation. Fibrosis occurs due to excessive extracellular matrix deposition leading to scar tissue that replaces normal functioning tissues when the tissue repair processes are dysregulated[120]. The presence of the elevated ILC2 activating alarmin, IL-25, and IL-13 were linked to worsened fibrosis in mouse models and in the BALF of patients suffering from idiopathic pulmonary fibrosis (IPF). In mouse model, of pulmonary fibrosis, ILC2-deficient mice exhibited a significant decrease in lung collagen deposition. This suggests that targeting ILC2s may be beneficial in attenuating lung fibrosis [121]. However, ILC2s are involved in tissue repair through their secretion of Areg so depending on the microenvironment they may have pathogenic or beneficial roles. Similarly, ILC3 have been implicated in pathogenic and protective roles in the liver and gut fibrosis [122,123]. In a chronic *Salmonella* intestinal fibrosis model ILC3 derived IL-17 was found to drive severe intestinal fibrosis through a RORα dependent mechanism[124]. Furthermore, pulmonary fibrosis can be driven by an IL-13 independent mechanism involving IL-17A and IFNγ which are both Th17 cytokines, suggesting a role for ILC3s in lung fibrosis development[125]. In a hepatic fibrosis model, $IL5^+$ ILC2s colocalize with $IL\text{-}17^+$ lymphocytes in a $IL33^+$ adventitial fibroblast niche. $IL5^+$ ILC2 were maintained by IL-33 in this niche and were found to regulate IL-17 driven fibrosis, again, suggesting type 2 and type 3 lymphocyte crosstalk may be beneficial in fibrosis attenuation.

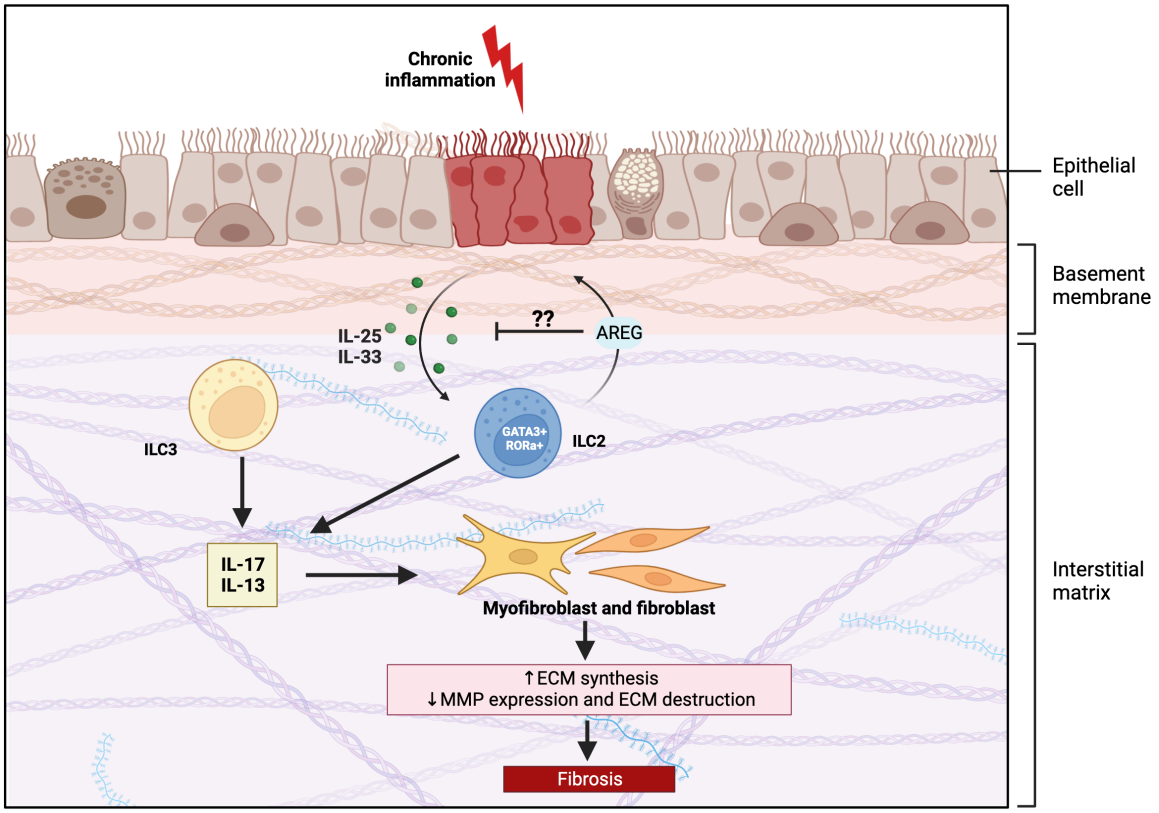

Figure 1.7 ILC2 and ILC3 cells drive fibrosis in chronic allergy

IL-13 produced by IL-25 and IL-33 stimulated ILC2s and IL-17 produced by ILC3s facilitate fibroblast differentiation excessive deposition of extracellular matrix. Over time a decrease in matrix metalloproteinase (MMP) causes and increase in collagen deposition and thickening of tissue wall leading to fibrosis.

1.3.5 Mast cells, basophils, and eosinophils

1.3.5.1 Mast cells

Mast cells are a heterogenous population of granular cells that develop in the bone marrow and then move into the periphery where they mature into distinct phenotypes based on the tissue microenvironment[126]. The localization of long-lived mast cells in vascularized tissue and on mucosal surfaces allows them (and dendritic cells) to be one of the first points of contact with pathogens and foreign antigens. These cells are involved in immunomodulation, homeostasis,

angiogenesis, wound healing, and tissue remodelling through the secretion of a broad range of mediators like histamine, proteases, cytokines, growth factors, lipid mediators etc[46].

In allergic disease mast cells are established as the main effector cells in the early phase hypersensitivity reaction and they also have a significant role in late phase reaction, chronic inflammation, and anaphylaxis[46,127–129]. An increase in mast cell counts in the airway smooth muscle and in the bronchial and nasal epithelium are associated with pulmonary inflammation in both human asthma patients and murine models. In the early phase of an allergic reaction, antigen specific IgE (produced during sensitization) is bound to FcεR1 on tissue resident mast cells, and crosslinking with antigen leads to cellular degranulation and the release of preformed immune mediators from cytoplasmic granules, as well as the subsequent production and secretion of newly formed cytokines and chemokines[47]. The extent of mast cell degranulation is dependent on the type of the signal received. For example, low levels of antigen stimulation may not drive full degranulation and instead result in release of only a subset of mediators from specialized granules through a process known as "piece-meal degranulation"[130]. In contrast, systemic degranulation in mast cell driven anaphylaxis is an example of how overstimulation of these effector cells can have fatal consequences[48].

1.3.5.2 Basophil

Basophils are granular cells like mast cells that express FcεR1 and during allergy are decorated with B cell derived IgE. Antigen crosslinking of the receptor bound IgE results in basophil degranulation to release immune mediators like histamine. Basophils were once considered mast cell precursors due to their functionally and phenotypic similarities. However, they were later shown to be a unique cell population which, unlike mast cells, develop and mature in the bone

marrow and have a short lifespan of 2-3 days with IL-3 and TSLP being important factors for their expansion [131,132]. In asthma, basophil numbers are increased in the lungs of asthmatic patients compared to healthy individuals suggesting a role for basophils in asthma pathology[133]. Nevertheless, others have shown that basophils may be dispensable for Th2 polarization and that IL-33, IL-25 and ILC2s are the real drivers of Th2 polarization in the lungs[42,43,134]. Furthermore, it is suggested that basophils may play a role in the initial allergen sensitization event since they are a great source of IL-4. In food allergy basophils and mast cells were shown to drive peanut-induced anaphylaxis [134]. In summary, there are data to support basophils as important effector cells in allergic disease however they may have some redundant roles that can be performed by other cells like mast cells and ILC2s.

1.3.5.3 Eosinophils

Eosinophils are granular cells with a bi-lobed nucleus that develop in the bone marrow and following their differentiation traffic to different tissues through the blood. In severe disease, eosinophil precursors have been observed in the blood and bronchial biopsies of atopic asthmatics and in the lung of AAI in murine models[135,136]. IL-3, IL-5 and GM-CSF synergistically support the development of mature eosinophils and they are involved in a myriad of cellular processes including tissue damage, remodelling and repair, pathogenic clearance, and disease persistence[137]. Eosinophils carry out these functions via the secretion of several mediator molecules from cytoplasmic granules (like mast cells and basophils) and through different types of receptors expressed on their cell surface.

Allergic disease pathology is very complex and involves many cell types with some having a bigger role than others. It can be argued that eosinophils are one of the most important effector

cells in allergic disease to the extent that atopic asthma phenotypes are now characterized based on the extent of eosinophilic infiltration as "eosinophilic" or "non-eosinophilic" asthma[138]. Eosinophils are believed to be crucial for maintaining long term inflammation in asthma. High concentrations of circulating eosinophils are usually observed in asthmatics and eosinophil counts in sputum, blood and tissue correlate with disease pathology[138]. During allergic reactions IL-5 secreted by Th2 cells, ILC2, basophils and mast cells facilitate eosinophil migration from the bone marrow to sites of inflammation. This migration is synergistically supported by IL-4, IL-13 and eotaxin (ab eosinophil-specific chemokine) secreted by activated eosinophils, epithelial cells, and other leukocytes[139]. Eosinophils express Fc receptors for all immunoglobulin isotypes however unlike mast cells and basophils, eosinophil express few FcεR1 and antigen crosslinking of FcεR1 bound IgE is not sufficient to trigger eosinophil activation and degranulation[140]. Instead, GM-CSF and PAF from mast cells and other sources trigger eosinophil degranulation and release of immunomodulatory molecules. Overall, eosinophils are crucial to the allergic disease mechanisms and, as a result, there are several drugs on the market that target the different cytokines and receptors important for eosinophil function as general corticosteroids have not been very successful at treating eosinophilic asthma[141].

1.3.6 Epithelial cells

The discussion of the various cell types involved in allergic disease initiation and progression cannot be concluded without a brief note on the role of epithelial cells which act as a physical barrier to invading pathogen. In addition to serving as key barrier cells, mucosal epithelial cells can detect different allergens and pathogens (parasites, virus, bacteria etc.) responding to stimuli and providing instructive signals to immune cells which mount the appropriate immune

response[142]. In the context of allergy upon exposure to an allergen, damage to the epithelial cell wall causes the release of damage associated molecular patterns (DAMPS) like DNA, ATP, uric acid etc[65,142]. These DAMPs facilitate the secretion of a variety of cytokines and chemokines including IL-25, IL-33, TSLP, GM-CSF, CCL17, CCL22, eotaxin etc. by epithelial cells. IL-25 and IL-33 are important for the maintenance and activation of ILC2s in lungs and gut and together with TSLP these alarmins facilitate ILC2 expansion and the secretion on T2 cytokines[49]. GM-CSF, TSLP, and eotaxin are involved in DC and eosinophil recruitment (in the lungs) and activation[49,51,143].

1.4 Treatments

The complexity of allergic disease phenotypes has resulted in the creation of a plethora of drugs targeting different aspects of the disease. Typically, mild cases of asthma and other atopic diseases are treated with over-the-counter antihistamines, corticosteroids, β_2-adrenergic agonists and, over time, bronchodilators and leukotriene inhibitors have been added to the mix[144,145]. However, in more severe cases these drugs are not enough to address the recurrence of disease exacerbations and respiratory distress resulting in hospital visits. As a result, adjunctive treatments are continuously being developed. One of the first such adjunctive was omalizumab[145], an anti-immunoglobulin humanized antibody that sequesters IgE and blocks IgE-mediated pathology. Patients on this drug have less severe exacerbations and better airflow. Mepolizumab, Reslizumab and Benralizumab target IL-5 and IL-5Rα and are typically used to treat eosinophilic asthma and Dupliumab targets IL-4Rα which is a shared receptor for IL-4 and IL-13[146]. This is not an exhaustive list of all the drug available now or in development, however it is important to note that even though most of these biologics help with disease management

they are not a cure and may not be an effective solution for treatment for some patients. Hence a better understanding of immune mechanism leading to the development of atopy and allergy is important for better disease management and possibly cure.

1.5 Early life immune development and the microbiome

The mechanisms that facilitate the development and progression of allergic disease are much more clearly understood than the origins of the underlying immune dysregulation that drive allergy susceptibility. However, one thing that is clear from both murine and human studies is that hematopoietic skewing that favours allergy development occurs very early in life. Genetic and environmental factors drive epigenetic changes leading to allergy. An indubitable characteristic of epigenetically mediated processes is that they exert their strongest effect in a specific time frame ("window of opportunity/vulnerability") and the period between conception and the first year of life seem to be crucial for such processes[147,148]. The immune system which develops in the first few years of life is very sensitive to perturbations in the external environment that may affect the balance required to develop appropriate immune responses later in life. Wide ranging evidence exists on the role of maternal influences on the development of atopic diseases in children[149]. Also, the impact from internal and external microbiota on immune development is well established[150].

1.5.1 Maternal influences on immune development

Environmental factors during early life including prenatal exposure to infection can affect immune development and one's ability later in life to generate appropriate responses to immune challenges. Evidence from the study of different diseases has shown that the foetal environment

and foeto-maternal crosstalk can influence disease development in progeny[151]. In both human and murine studies maternal phenotypic traits, genetic traits, infection during pregnancy and immunomodulatory molecules have been linked to allergy development (figure 1.8)[149].

In one study, maternal T1 and T2 cytokine ratios (specifically IFNγ/IL-4 and IFNγ/IL-13) from mitogen stimulated maternal peripheral mononuclear blood cells (PBMCs) collected during and after pregnancy were inversely related to asthma development in offspring from moms without asthma[152]. No significant relationship was found to moms with asthma or paternal cytokines. Others found a relationship between IL-5 upregulation during pregnancy and in-utero sensitization to urban indoor allergens in offspring suggesting a link between maternal cytokine production and allergy development in progeny[153].

In addition to T2 cytokines maternal IgE and sensitization may correlate with progeny IgE production postnatally[154]. However, the specificity of IgE produced by offspring at 12 months after birth suggest that sensitization to the same antigen as mom is rare, rather it seems the prenatal environment may set up conditions for atopy development and environmental exposure and timeline of exposure determines allergen specificity in progeny[155].

Furthermore, maternal T2 immune responses and asthma attacks during pregnancy have been associated with differential methylation profiles in infants' PBMC DNA at 12 months hinting at a possible mechanism for maternal influence on allergy susceptibilty[156]. In support of the idea that maternal disease or infection may skew immune development one murine study showed that in-utero exposure to chronic maternal schistosomiasis infection skewed helper T cell responses in progeny[157]. Naïve T cells from these pups had a stronger capacity to differentiate into Th1 cells whereas Th2 differentiation was impaired due to epigenetic changes impacting the promoter region for some critical Th2 genes resulting in lower asthma susceptibility[157].

Additionally, prenatal asthma protection was conferred through maternal exposure to a common farm bacterium, Acinetobacter Iwoffii F78[158]. This protection was facilitated via maternal TLR signalling highlighting that maternal exertion on immune development may be initiated by different factors (including infection and microbial exposure) and may occur through various mechanisms. This data emphasizes the critical role of maternal influences in immune development during perinatal period.

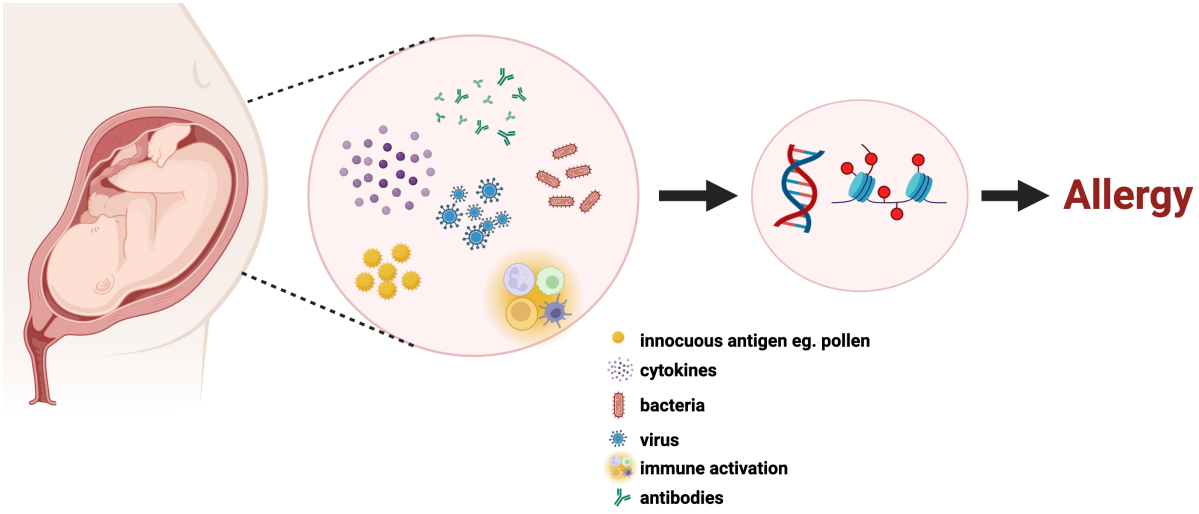

Figure 1.8 Maternal factors affecting allergy development in the perinatal period

Foeto-maternal crosstalk is established to influence development of disease in progeny. Perturbations in fetal immune development due to maternal dysbiosis, infection (viral and bacterial), immune activation due to allergy exacerbations etc. can result in epigenetic changes that may skew immune development and increase allergy susceptibility in progeny. After birthing these immunomodulatory molecules may still be transferred through breast feeding extending maternal influences beyond gestation.

1.5.2 Antibiotics and microbiome

The human gut is colonized by a multitude of microorganisms of which bacteria are the most abundant[159,160]. Alterations in the composition of commensal bacteria populations have been associated with the development of allergic disease. Studies have shown that children treated

with broad spectrum antibiotics and children that develop allergies have altered commensal bacteria communities and, in both cases, antibiotic treatment and changes in microbiome diversity preceded allergy development[161,162]. So, in addition to other types of maternal influences changes that drive dysbiosis in early life have been implicated in allergy development. As stated earlier immune education in early life through microbial exposure and infection is pivotal to developing the correct protective and tolerogenic responses in later life. However, our use of antibiotics and ever decreasing exposure to the external environment in early life is believed to result in the acquisition of a less diverse microflora hence missing out on the protective benefits offered by some commensal bacteria against atopic disease[150]. As infants we acquire most of our microbiome from maternal sources as we pass through the birth canal and after birth the microbiome is modified as we are exposed to the external environment. To get a better understanding of how the microbiome facilitates protections against atopy and the time frame within which the protective effects are most critical several mouse models and birth study cohorts were developed.

Antibiotic use during pregnancy and in early life drives dysbiosis and may facilitate the development of some atopic diseases[161,163]. Oral broad spectrum antibiotic treatment in wildtype mice resulted in reduction of gut bacteria from specific phyla, a significant increase in serum IgE and in the frequency of basophils[164]. Using mouse models for AAI our lab and others were able to show that exposure to antibiotics in the perinatal period increased allergy susceptibility in adult animals[163]. Furthermore, we were able to show that a Th2 bias was antibiotic specific. Vancomycin (VANC) a broad-spectrum antibiotic used in the treatment of gram-positive bacterial infections was shown to preferentially deplete short chain fatty acids (SCFAs) producing gut bacteria enhancing susceptibility to AAI[163,165]. Contrarily, treatment with

streptomycin had more subtle effects on the microbiome and Th2 polarization and rather exacerbated Th17 driven hypersensitivity pneumonitis[117,163,166]. Naïve T cells from VANC-treated mice were preferentially prone to Th2 polarization and DCs from these mice were inherently better at priming Th2 polarization[167]. VANC-induced dysbiosis was completely reversed by adding SCFAs; butyrate, acetate, and propionate (BAP) in the drinking water of VANC-treated mice. BAP could reverse VANC-induced DC Th2 polarization and the T cell maturation bias however it had no significant effect on the microbiome[167]. It is noteworthy that butyrate is a by-product of dietary fibre fermentation by gut microbiome and is a potent histone deacetylase hence may be an example of one of many microbiome metabolites capable of influencing hematopoiesis[168].

1.5.3 CHILD study

To investigate the role of dysbiosis on inflammatory bias in humans we participated in a multi lab study examining stool samples from the Canadian Health Infant Longitudinal (CHILD) study[169]. CHILD collected data on environmental exposures (traffic, stress, pets, cleaning products etc.), parent allergic status and archived biological samples from 3800 Canadian children over their first 8 years of life[170]. A significant correlation was found between the development of wheeze and atopy (predictors of future allergy) and a reduction in 4 genera of bacteria in 3month old stool. Interestingly by 6 months dysbiosis normalized and no differences were observed in the stool of these children regardless of future allergy status. This data emphasized the importance of the window opportunity in early life (gestation + 100 days postnatal in humans) where immune perturbation impacting hematopoietic development may have their strongest effect.

In addition to the stool analysis, we recently carried out RNA-sequencing and CyTOF analysis on cord blood mononuclear cells (CBMCs) from the CHILD cohort[171]. The aim of this ongoing study was to get a better understand of the how immune cell transcriptional changes and surface protein expression are different at the time of birth in kids that go on to develop allergy and those that do not. From CyTOF we observed the most striking differences between the healthy (control group) and the wheeze and atopy (WA) group. The most significant difference was observed in the proportion of naïve $CD8^+$ T cells which were increased in the WA group. Some differences were also observed in pDC proportions even though not significant (figure 1.9).

Knowing that in-utero VANC exposure increases susceptibility to allergy development in mice and given the preliminary data from the CHILD cohort study this project aims to explore differences in the phenotypes and localization of naïve T cells and pDCs in antibiotic exposed neonatal mice to explore insights not perceived from the human CBMC study.

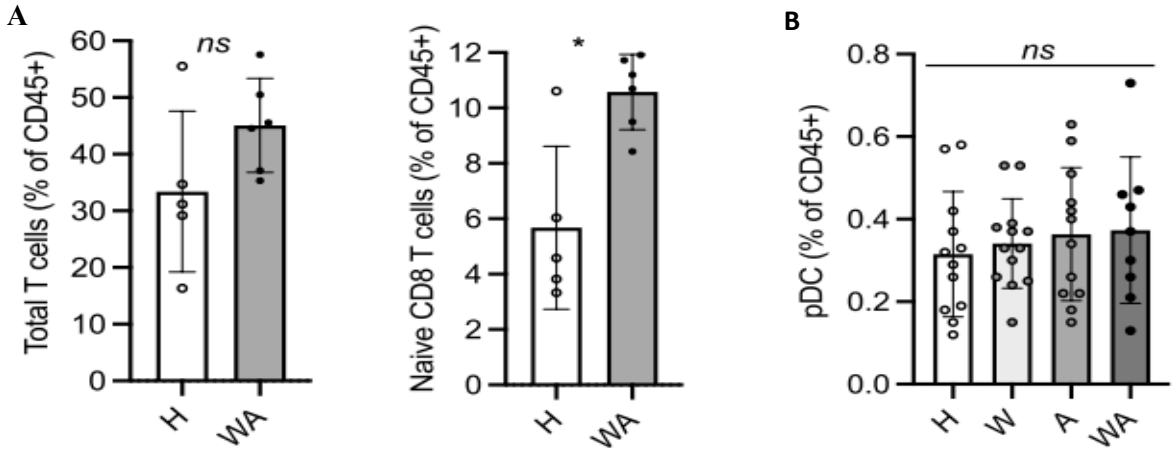

Figure 1.9 T cell and pDC frequency in CHILD cohort CBMCs

CBMC samples obtained from the child cohort were categorized into four phenotypes; H – healthy, W – wheeze (kids the develop a wheeze), A – atopy (kids positive for skin prick test), WA – wheeze and atopy (kids with wheeze and atopy). The most striking difference was found between the H and WA group with WA having significantly more naïve $CD8^+$ T cells. The WA group also had proportionally more pDCs compared to the other groups. **A** Frequency of total T cells and naïve CD8 T cells of CD45+ cells **B** Frequency of total pDCs of CD45+ cells. H= healthy, W= wheeze, A = Atopy, WA = wheeze and atopy

1.6 Hematopoiesis in early life

Hematopoiesis the process by which blood cells are formed. Hematopoiesis starts in the embryo and continues throughout life to replenish and maintain blood cells. In the prenatal period the primary site of hematopoiesis changes from the yolk sac to the foetal liver and finally to the bone marrow (figure 1.10). All blood cells (red and white) originate from hematopoietic stem cell (HSC) progenitors (figure 1.10). Lineage commitment by HSCs is determined through a tight regulation of transcription. Hematopoietic skewing occurs when different factors (immune activation, hormones, cytokines etc) cause an increase in the differentiation potential of a specific cell lineage. This phenomenon is believed impact disease susceptibility like in allergy where T cell and pDC development may influence disease.

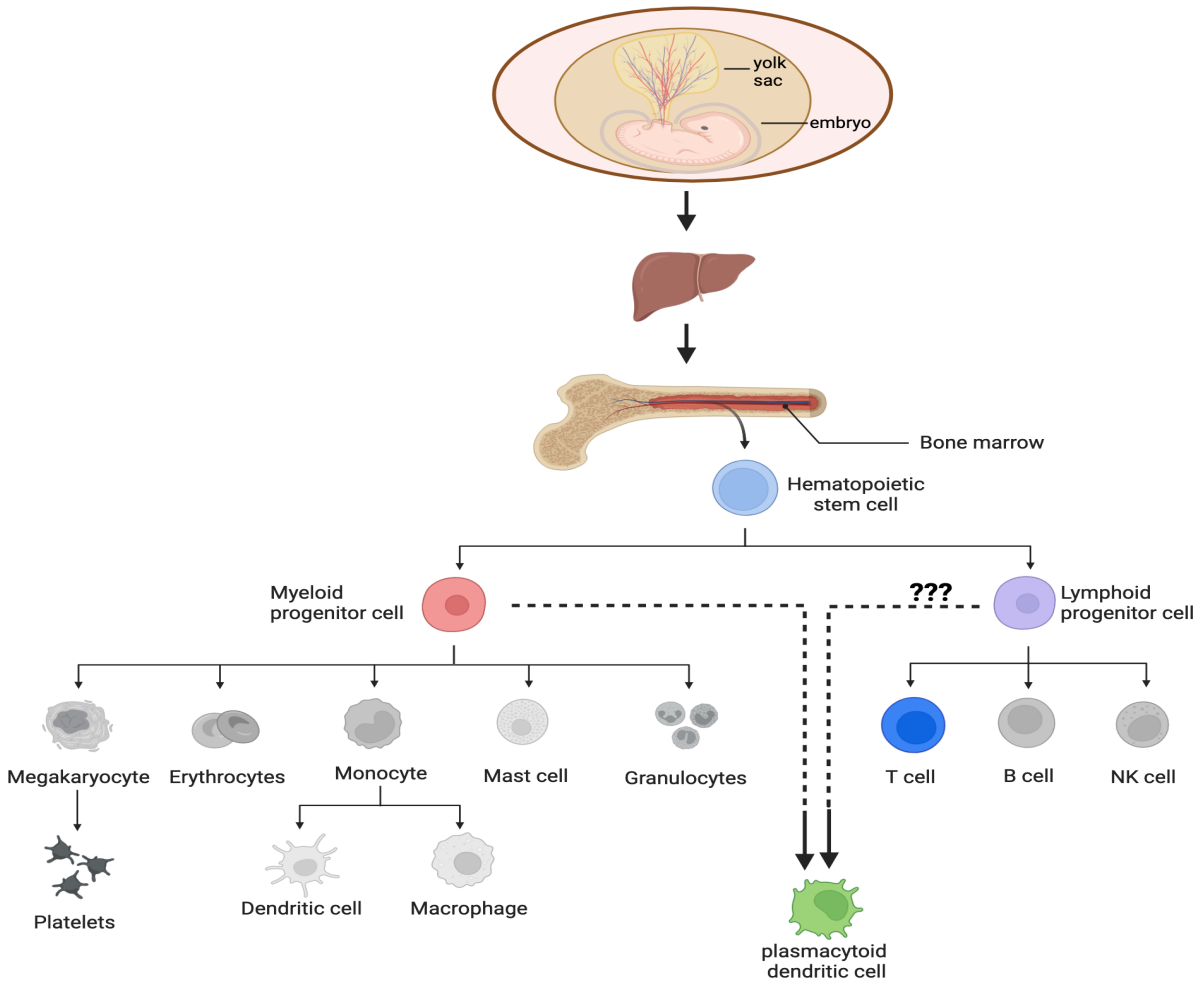

Figure 1.10 T cell and pDC hematopoiesis in early life

In neonates hematopoiesis starts in the yolk sac of the embryo and as the foetus matures blood cell production moves to the foetal liver and then the bone marrow. HSCs branch off into two major lineages myeloid and lymphoid cells. Myeloid progenitor cells give rise to red blood cells, granulocytes, monocytes, mast cells and pDCs. Lymphoid progenitor cells give rise to T cell, B cells and NK cells and a subset of pDCs (ongoing debate in the field).

1.6.1 Early T cell development and marker expression

In early life T cell progenitors migrate from the bone marrow to the thymus where they undergo selection and maturation before entry into the periphery. T cell ontogeny is strikingly different in human and mice. In humans T cell development occurs in the first trimester with mature T cells detected in the spleen and lymph nodes by the second trimester[172]. So, by birth humans have a fully functional and developed T cell compartment. Whereas in mice mature T cells only start to appear just before birth (1-2 days) and expand rapidly in the first week of life[173]. This difference highlights the limitations in using murine models to study immune development[174]. However, this difference is beneficial for this study as we may be able to assess T cell development at a time point in mice where the T cell compartment is still evolving.

In murine studies naive T cells have been broadly defined as $CD44^{lo}$, they are also considered to be $CD62L^{+}CD127^{+}CCR7^{+}$[64,175]. These surface proteins are involved in migration and survival during various biological processes. CD44 is a widely distributed transmembrane glycoprotein expressed by leukocytes and non-leukocytes and in T cells it facilitates cell adhesion, migration homing and proliferation. It supports T cells extravasation through tethering and rolling interactions with vascular endothelial cells expressing hyaluronic acid (HA) which is the main CD44 ligand[176]. HA is also expressed on other somatic and immune cells suggesting a potential role for T cells in regulating cell function via CD44. For example, DCs express HA and T cell CD44 ligation with DCs was shown to impact T cell antigen responsiveness and to be important

for DC-T cell interactions and cell clustering which are critical to immune responses[177]. CD44 deficiency may also affect the survival of Th1 cells, promote Th2 polarization of naïve cells and diminish Treg function through reduced TGFβ and IL-10 production[176,178]. CD44 is upregulated in activated T cells and may be more involved in effector cell migration in response to inflammation but its expression even at low levels in naïve T cells may be impactful during development. Unlike CD44, CD62L (L-selectin) is downregulated in newly activated T cells. CD62L is also a cell adhesion molecule that is expressed by leukocytes. It facilitates immune surveillance by supporting T cell recirculation between the blood and lymph node[179]. Furthermore, T cell homing to lymphoid organs is orchestrated through the action of CD62L and CCR7 a chemokine receptor[180,181]. Naïve T cells are found primarily recirculate within the blood and lymph nodes whereas activated T cell are more widely distributed due to having a more diverse expression of migration molecules[182,183]. Naïve T cell survival and maintenance is dependent on several factors one of which is IL-7[184]. CD127(IL-7Rα) ligation on T cells is required for T cells survival and its expression or lack thereof is used to distinguish subsets of activated T cells[184]. Overall, the expression profile of these key markers and localization of theses naïve T cells in early life may be predictive immune perturbations driving disease.

1.6.2 Early pDC development and marker expression

Plasmacytoid DCs are lineage-negative, type 1 interferon-producing cells that are capable of sensing intracellular viral DNA, RNA, and self-nucleic acid through TLR7 and TLR9[185]. In mice and humans respectively pDCs are distinguished by the expression of CD45R(B220), PDCA-1(CD317), CD45RA, Ly-6C, Siglec-H and CD45RA, IL-3R, CD303(BDCA2), CD304(BDCA4)[186]. At steady state following development in the bone morrow mature pDCs

traffic through the blood to lymphoid tissues. Besides the intestine and kidney very few are detected in non-lymphoid tissues[187]. They are however readily recruited to sites of inflammation. pDCs like T cells are known to express CD62L which facilitates lymphoid homing together with other migration molecules. In mice, most pDCs are localized in lymphoid tissues, specifically the spleen, making up about 0.1-0.5% of mononucleated cells[188]. Activated pDCs in addition to the secreting large amount of IFNα express T and B cell costimulatory molecules CD80, CD86, CD40, CD70, PD-L1 etc[189]. These costimulatory molecules have been involved in effector and helper T cell activation and polarization, generation of natural Tregs in the thymus and in B cell activation and antibody production[105,190–192]. Activated pDCs also upregulate MHC-II but they are not as robust APCs as the more classical cDCs. However, they have been shown to present autoantigens as a mechanism of inducing self-tolerance[193,194]. As previously discussed pDCs have also been implicated in allergic disease hence their localization and surface marker profile in early life may be important in allergy susceptibility.

Chapter 2: Perinatal antibiotics exposure and cell signatures

2.1 Introduction and hypothesis

The prevalence of allergic disease and atopy in the developed world continues create a growing health emergency, that is further driven by a poor understanding of the mechanisms inducing disease susceptibility and initiation[195]. The management of disease symptoms by current treatments rather than addressing the underlying immune dysregulation is not enough in many cases to provide patients with proper relief and a better quality of life[196]. In childhood especially allergic reactions can be very severe and may lead to death[57]. Hence having a better understanding of the how immune development in early life may drive and predict allergy may help with the creation of better allergy therapeutics. To that end in-depth characterization of factors and mechanisms known to impact immune development during the "window of opportunity/vulnerability" are useful to discovering non-trivial changes impacting allergy development.

Exploring the link between exposures during the perinatal period and allergy is especially important. For example, most of our microbiome in early life is acquired from maternal sources and changes to our internal microbial diversity can enhance or ameliorate allergy susceptibility[166]. So more in-depth knowledge on feto-maternal cross talk and microbiome development in children is neccessary[197,198]. As we have shown in mice antibiotic exposure specifically to Vancomycin (VANC) in utero or in the first 3 weeks of life results in dysbiosis and enhances allergy susceptibility whereas antibiotic exposure in adult mice has no impact on allergy development[163]. With this knowledge our murine model for systemic allergy is generated through VANC exposure. Since VANC exposed pups go on to develop allergy following antigenic challenge later in life it is postulated that the effects of VANC-induced dysbiosis may

be apparent through changes in immune cell signatures in these mice in the first couple weeks after birth which may correlates to the first 100 days after birth in humans providing a malleable system to better characterize these early life changes.

We hypothesized that in-utero antibiotic exposure will affect immune cell localization, signatures and IgE production in naïve mice. Preliminary human studies from our lab showed differences in T cells and pDCs signatures of CBMCs from healthy kids and kids with wheeze and atopy (predictor of future allergy) from the CHILD cohort (figure 1.9)[171]. Additionally in early life lymphoid tissues are among the first to be colonized. Hence our primary focus for this study was evaluating cell signatures of pDCs and naïve T cells in lymphoid tissues of mice 0-14 days after birth by VANC exposed or non-VANC exposed dams. We found that differences in the composition of T cells at specific time points in some tissue may play a role in hematopoietic skewing in VANC exposed pups (VANC mice) and that pDC phenotype may be more important for hematopoietic skewing than pDC proportion in specific lymphoid tissues.

2.2 Materials and methods

2.2.1 Mice

C57BL/6J (originally from The Jackson Laboratories, Bar Harbor, ME) were all maintained in a pathogen-free environment at the Biomedical Research Centre. All experiments were performed according to the institutional and Canadian Council on Animal Care guidelines approved by the University of British Columbia Animal Care Committee. At experimental end points mice were humanely sacrificed by avertin (2,2,2-tribromoethanol) overdose.

2.2.2 Antibiotic treatment and tissue harvest

Breeding pairs (7–10-week-old mice) were administered regular water or Vancomycin (Sigma-Aldrich, St Louis, MO) at 200mgL^{-1} in their drinking water. Following a successful plug dams were kept on their respective regular or vancomycin drinking water, all through gestation and weaning. At Day7, Day10 and Day14 after birth three (3) male and three (3) female pups from the two groups (12 pups total at each time point) were weaned. Blood, bone marrow, inguinal lymph nodes, spleen and thymus were harvested from each pup at the three time points.

2.2.3 Single cell isolation

Blood was collected in 1ml tubes containing 20ul ethylenediaminetetraacetic acid (EDTA). Tubes were centrifuged at 1500 Xg for 10mins and white blood cells (WBCs) in the plasma layer were removed and stored on ice in FACS Buffer (1X PBS, 2% FBS, 2mM EDTA). The tibia and femur of both hind legs were collected and stored in phosphate-buffered saline (PBS) on ice. Due to the bones being very brittle only one tibia or femur was obtained in some mice. The cartilage was removed from both ends of the bone, and they were flushed with PBS. Single cell suspension was then put through a 40µm cell strainer, and the collected cell suspension was put on ice. Inguinal lymph nodes, spleen and thymus were collected in cold PBS and then crushed with the base of a syringe and single cells were flushed through a 40µm cell strainer with PBS. Red blood cell lysis was performed with ACK lysis buffer (Gibco) for the spleen and BM cell suspensions. The collected cell suspensions were put on ice. This process was repeated for 12 pups at each time point. Each single cell suspension was split in two, one half for lymphocyte staining and the other half for myeloid staining. Cell numbers obtained from the BM at Day 7 were limited so the BM was excluded from analysis at Day 7.

2.2.4 Flow Cytometry

The freshly isolated single cells from the blood, bone marrow, inguinal lymph nodes, spleen and thymus were stained and analysed. Staining and antibody dilutions were prepared in FACs buffer. Isolated cells were incubated for 20 minutes at 4°C in FACS buffer containing 1:1000 Fc Receptor-blocking antibody, anti-CD16/32 (clone 2.4G2) and stained for 30 minutes at 4°C with antibody cocktails. Staining antibodies used were as follows: T cell panel; Alexa Fluor 700–conjugated CD45(30-F11), PE-Cy7–conjugated CD28(37.51), allophycocyanin (APC) CD62L(MEL-14), APC Cy-7-conjugated CD4(GK1.5), Brilliant Violet 421–conjugated CD8(53-6.7), Brilliant Violet 711–conjugated CD127(A7R34), Brilliant Violet–conjugated 785 CD3(17A2), BV 605 CD25(PC61), Brilliant Violet 510–conjugated CD27(LG.3A10), PerCP-cy5.5 CD69(H1.2F3), PE CCR7(4B12) and Fluorescein isothiocyanate conjugated(FITC) CD44(IM7). Myeloid panel- Alexa Fluor 700 conjugated CD45(30-F11), phycoerythrin-Cy7 conjugated B220(RA3-6B2), APC CD62L(MEL-14), APC Cy-7 MHC-II(M5/114.15.2), Pacific Blue conjugated CD19(1D3), CD90(53-2.1), NK1.1(PK136) and CD3(17A2), Brilliant Violet 711–conjugated PDCA-1(927), Brilliant Violet 785–conjugated CD86(GL-1), Brilliant Violet 605 conjugated PD-L1(10F9G2), Brilliant Violet 510 conjugated Ly-6C(HK1.4), PerCP-Cy5.5 CD11b(M1/70), PE conjugated F4-80, FITC CD11c(N418). Note the following markers were not analysed in 7-, 10- and 14-day old mice respectively CD80, CD86 and PDL-1 and CD80. Viable cells were identified using the Live/Dead™ fixable Near IR viability dye from Thermofisher scientific(L34981). Samples were run on a Beckman Coulter Cytoflex, and data analysis was performed with Flowjo software.

2.2.5 ELISA

Serum IgE concentration from 14-day old mice and adult mice exposed to and not exposed to antibiotics (Vancomycin) in early life was assessed using the protocol and reagents provided in the BD OptEIA Mouse IgE ELISA kit. A microplate reader was then used to read absorbance at 405nm. Results were manually analysed.

2.2.6 Statistical Analysis

All data and statistical analysis were carried out in GradPad Prism Software v.9. Differences between tissues due to treatment were compared using unpaired Student's t-test. Differences in tissues due to treatment and the age of mice was compared using two-way anova. A test was considered statistically significant at a probability of $< 5\%$ ($p < 0.05$). Data are mean ± SD.

2.3 Results

2.3.1 Study design

This study investigated the role of in-utero antibiotic exposure in naïve T cell and pDC signature, localization and IgE production in naïve mice. VANC was administered in the drinking water of dams after a successful plug all the way to weaning, control dams were put on regular water. Pups from these two groups were weaned and sacrificed at Day7(D7), Day10(D10) and Day14(D14) after birth and blood, lymph, spleen, BM, and thymus were harvested (figure 2.1A). T cells and pDCs were isolated from these tissues and analysed by flow cytometry. T cell subtypes and pDCS were assessed based on the analysis hierarchy in figure 2.1B and 2.1C. Naïve T cells were then assessed for expression of some migration and survival markers and

pDCs were assessed for activation marker upregulation. Finally, total serum IgE in 14-day old mice were analysed for by an IgE ELISA.

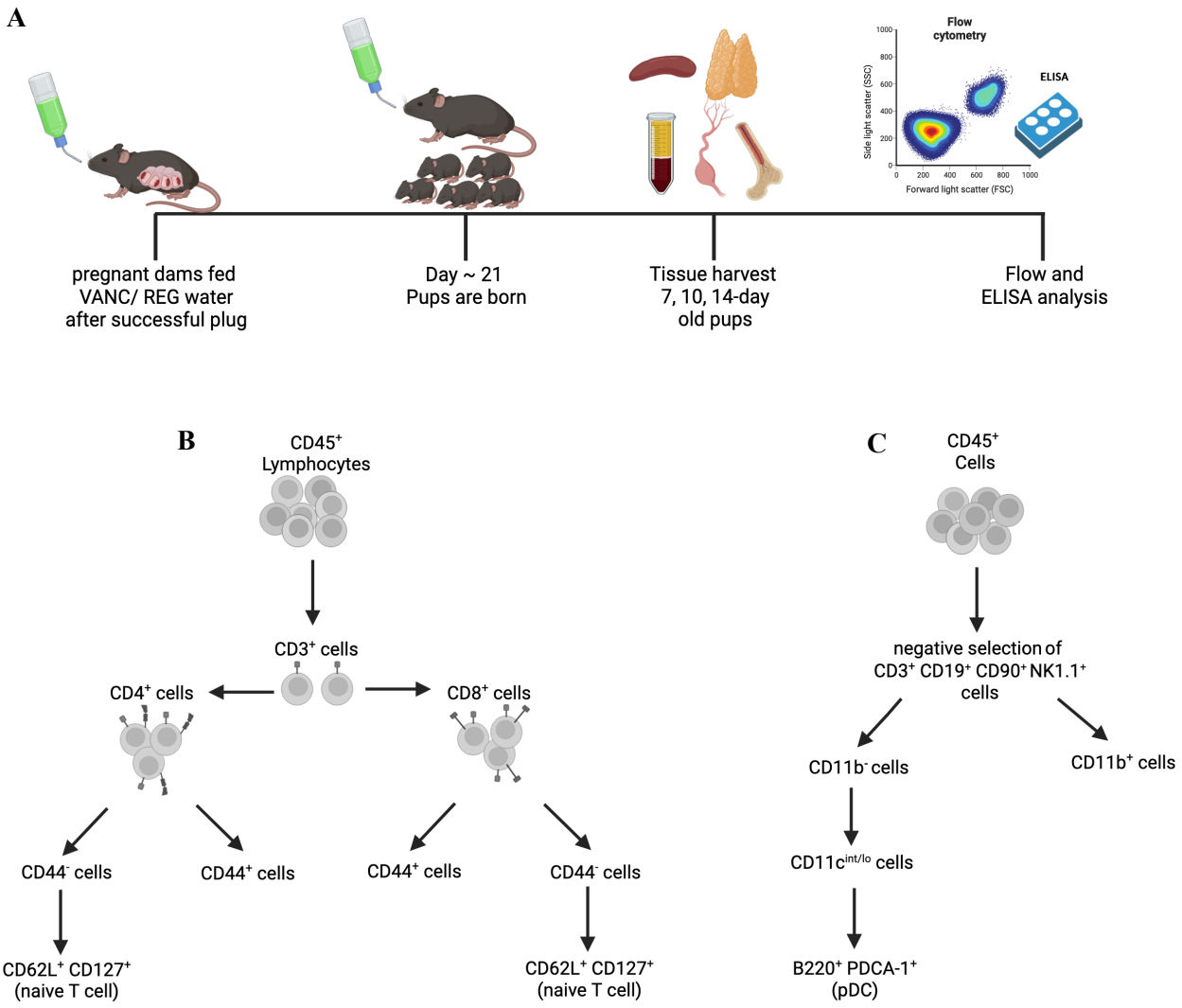

Figure 2.1 Study design and gating strategy

A Pregnant dams were administered regular water or vancomycin in their drinking water throughout gestation up to weaning. Pups from these dams were weaned 7,10 and 14 days after birth. Blood and lymphoid tissue were then harvested from these pups and single cell suspensions and sera were analysed by flow cytometry and ELISA. **B** T cell analysis was carried out using this gating strategy. **C** pDC analysis was carried out using this gating strategy

2.3.2 In-utero antibiotic exposure affects $CD4^+$ and $CD8^+$ T cell proportions in lymphoid tissues of neonates

To address how in-utero antibiotic exposure might impact T cell development and the subsequent development of allergy we harvested lymphoid tissue from naïve mice and assessed T cell proportions by flow cytometry. Cells were harvested from 7-, 10-, and 14-day old mice that were weaned shortly before sacrifice from dams administered regular water (REG mice) or vancomycin treated water (VANC mice) after a successful plug through to weaning.

The proportion of total $CD3^+$ cells localized in the blood, spleen, lymph node and thymus at D7, D10 and D14 in REG versus VANC mice was similar (figure 2.2A). In both groups the lymph nodes have the highest proportion of $CD3^+$ cells followed by the blood, spleen and thymus with the BM having the least number of cells. These observed proportions are different from those expected in adult mice as these tissues are still developing and being seeded by cells[199,200].

When comparing $CD3^+$ cells in the BM the only significant difference was observed at D10. VANC mice had a higher frequency of $CD3^+$ cells compared to REG mice and this trend was maintained at D14 though, likely due to the small number of animals used in this study it did not reach statistical significance at the latter time point. This suggests that in-utero antibiotic exposure may affect $CD3^+$ localization in the BM in the first two weeks after birth, hence a characterization of the BM $CD3^+$ cell heterogeneity may provide more insight on the role of these cells in the BM. No measurements were made for D7 in the BM due limited cell numbers isolated from 7-day old neonates.

Some intriguing differences were also observed in the BM and spleen when comparing VANC and REG $CD4^+$ and $CD8^+$ T cells. In the BM at D10 VANC mice had a higher frequency of $CD4^+$ T cells and less $CD8^+$ T cells and this trend was only perceived at D10 (figure 2.2B). The

high proportion of $CD4^+$ T cells in the BM of VANC mice may have implications for hematopoietic development at this time point as these cells elaborate a plethora of cytokines designed to expand select hematopoietic subsets. The D10 observations in the spleen were opposite to those in the BM when considering $CD4^+$ T cells (figure 2.2C). The proportion of splenic $CD4^+$ T cells in VANC mice were significantly less at D10 and significantly more at D14 in spleen highlighting differences between BM and spleen T cell heterogeneity (figure 2.2C). The differences observed in splenic $CD4^+$ T cell proportions were not robust when VANC and REG mice were compared at individual timepoints. No significant differences were perceived in the $CD8^+$ T cell population in spleen at all time points. Together these results may suggest that antibiotics and associated changes in the microbiome alter the temporal colonization of different tissues with T cells and that changes in ratio of $CD4^+$ to $CD8^+$ T cells may be consequential to the ongoing hematopoietic development of these primary hematopoietic tissues.

When considering the proportion of activated vs non-activated cells defined as $CD44^+$ or $CD44^-$, respectively, the most compelling results were observed at D14 in $CD8^+$ T cells. In the spleen, lymph, and thymus $CD8^+CD44^-$ T cells were proportionally more abundant than $CD8^+CD44^+$ T cells in both REG and VANC mice whereas majority of the cells in BM and blood were $CD44^+$ in both REG and VANC mice (figure 2.2D). So, majority of the $CD8^+$ T cells in the spleen, lymph, and thymus at D14 were naïve or non-activated and the opposite was observed in the BM and blood. So, upon maturation in the thymus and activation in the lymph node $CD8^+$ T cells migrate to the BM up to 14 days after birth. Outside of these broad trends in BM, lymph, and spleen VANC mice had significantly less $CD8^+CD44^+$ T cells and more $CD8^+CD44^-$ T cells in comparison to REG mice. So overall, there are more $CD44^-$ cells in the VANC mice in these 3 tissues. Similar trends were observed in the $CD4^+$ population however the difference did not

reach statistical significance (data not shown). VANC mice having a larger proportion of non-activated $CD8^+$ T cells at D14 in comparison to REG mice in the BM, lymph, and spleen may be critical to the developing immune landscape.

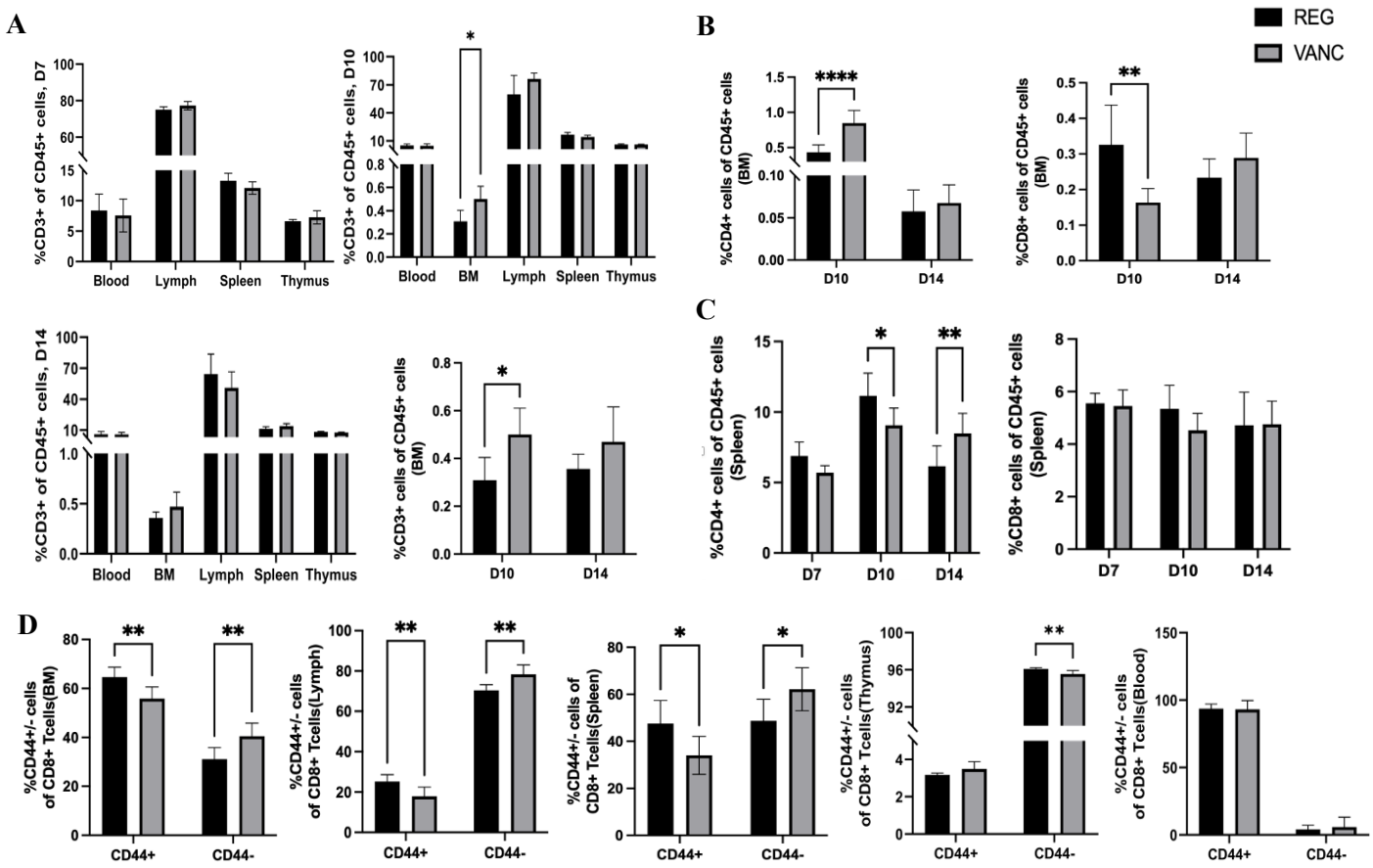

Figure 2.2 $CD3^+$, $CD4^+$, $CD8^+$, $CD44^+$ and $CD44^-$ T cell frequency in lymphoid tissues

A Quantification of the frequency of $CD3^+$ T cells of $CD45^+$ lymphocytes at D7, D10 and D14 in blood, BM, lymph, spleen and thymus and the frequency of $CD3^+$ T cells of $CD45^+$ lymphocytes in BM at D10 and D14 of VANC and REG mice. **B** Quantification of the frequency of $CD4^+$ and $CD8^+$ T cells of $CD45^+$ lymphocytes at D10 and D14 in the BM of VANC and REG mice. **C** Quantification of the frequency of $CD4^+$ and $CD8^+$ T cells of $CD45^+$ lymphocytes at D10 and D14 in the spleen of VANC and REG mice **D** Frequency of $CD44^+$ and $CD44^-$ $CD8^+$ T cells at D14 in blood, BM, lymph, spleen, and thymus of VANC and REG mice. Each bar represents 6 neonates, 3 male and 3 female. Dark bar = pups from dams administered regular water, grey bar = pups from dams administered vancomycin in drinking water. Bars indicate mean value ± SD.*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

2.2.3 Naïve $CD8^+$ T cells may be crucial to BM hematopoiesis in neonates

The bone marrow is a major site of hematopoiesis throughout life. In this study some differences were observed in the BM at D14 but were not as striking as at D10 when comparing VANC and REG mice. As shown in figure 2.2B, the increased proportion of $CD4^+$ T cells in the BM at D10 may be the driver of overall $CD3^+$ T cell increase in the VANC mice BM (figure 2.2A). At D14 however it seems there are proportionally more $CD8^+$ T cells in the BM of VANC mice even though this difference failed to reach statistical significance (figure 2.2B). To get a better understanding of the phenotype of these cells further analysis on their activation state and naïve (migration and survival) maker expression was carried out.

Among $CD4^+$ T cells no significant trends were observed between REG and VANC mice at the D10 and D14 in the proportion of cells expressing CD44 (figure 2.3A). $CD44^+CD8^+$ and $CD44^-CD8^+$ T cells have similar proportions in REG and VANC mice at D10 however at D14 VANC mice have less $CD44^+$ cells and more $CD44^-$ cells (figure 2.3B). This follows from the observations in figure 2.2D showing significantly more naïve $CD8^+$ T cells in VANC mice. VANC mice having more $CD44^-CD8^+$ T cells at day 14 may suggest changes in the BM microenvironment in early life from a $CD4^+$ T cell dominated environment at D10 to a $CD8^+$ T cell dominated environment at D14.

Naïve T cells were defined as $CD44^-CD62L^+CD127^+$ and the proportions of these cells within BM lymphocytes was assessed, since preliminary human data from CBMC studies showed that $CD8^+$ naïve T cells where significantly different in CBMCs of healthy kids versus kids that developed allergy later in life[171]. In agreement with this preliminary data no significant differences were observed in the proportion of $CD4^+$ naïve T cells but some differences were observed within the $CD8^+$ naïve T population at D10 and D14. At D10 the proportion of $CD8^+$

naïve T cell were significantly less in VANC mice and more at D14 (figure 2.3C). This increase in the frequency of naïve $CD8^+$ T cells in the BM of VANC mice may have consequences for the ongoing BM hematopoiesis. To get better insight on the function of these cells in the BM, we compared the level of expression of CD62L and CD127 in $CD4^+$ and $CD8^+$ naïve T cells. We found that expression trends in these cells are similar at D10 and D14. At D10 VANC mice have a lower expression of CD127 and higher expression of CD62L and at D14 no differences were perceived (figure 2.3D and E). The only outstanding difference is that the number of CCR7 expressing cells within the $CD4^+$ population was significantly higher in VANC mice at D10 (figure 2.3F). This may suggest that these T cells in VANC and REG mice may have different lymphoid homing potentials at D10 with $CD4^+$ naïve T cells localized in the BM having more migratory potential than $CD8^+$ naïve T cells. So, regardless of the immune events that have occurred before and up to D10 in the BM, these $CD4^+$ naïve T cells have the potential to migrate to other lymphoid tissues leaving a $CD8^+$ dominated environment in the BM as the pups age.

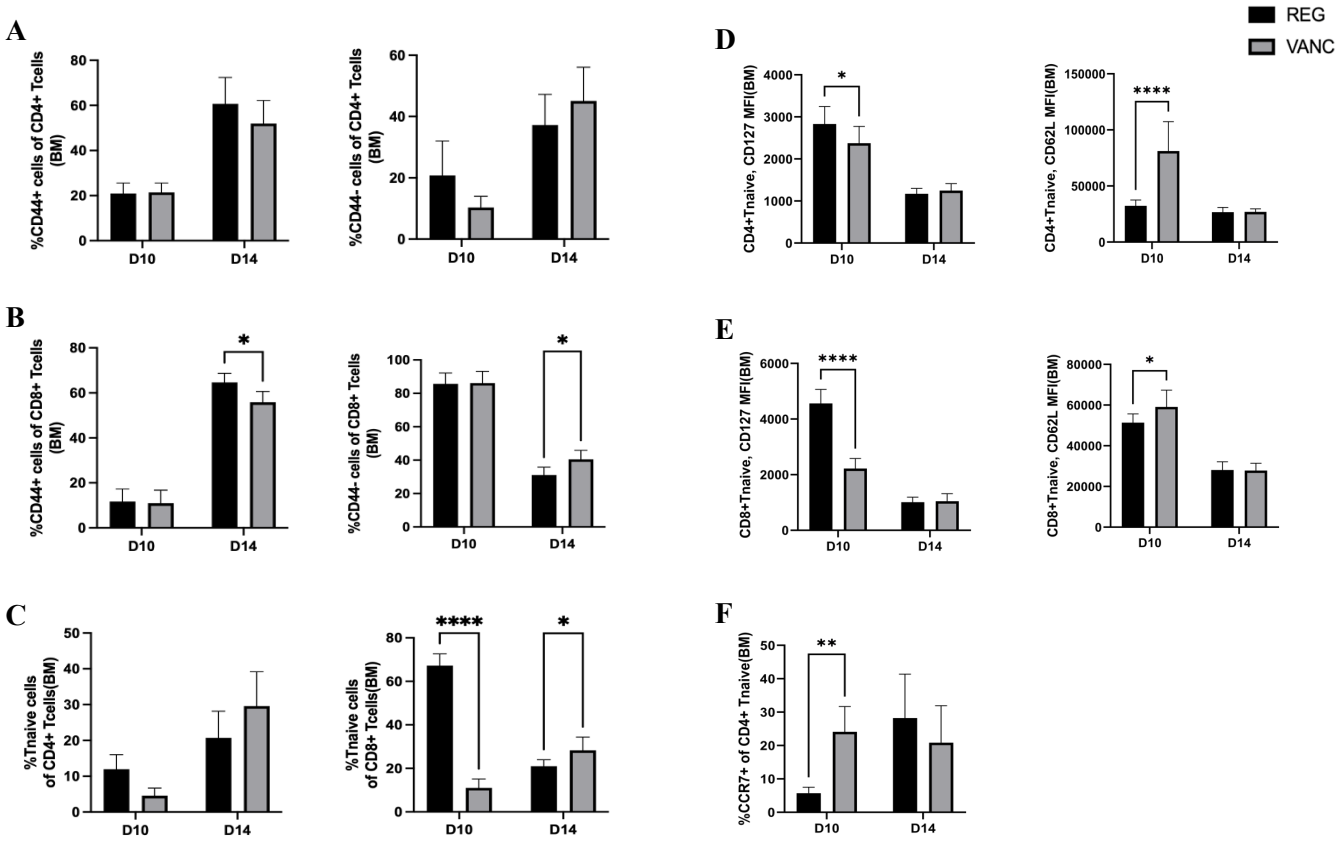

Figure 2.3 BM naïve T cell proportions and surface marker expression

A Quantification of the frequency of $CD44^+$ and $CD44^-$ $CD4^+$ T cells at D10 and D14 in VANC and REG mice **B** Quantification of the frequency of $CD44^+$ and $CD44^-$ $CD8^+$ T cells at D10 and D14 in VANC and REG mice **C** Quantification of the frequency of naïve $CD4^+$ T cells and naïve $CD8^+$ T cells in the BM at D10 and D14 in VANC and REG mice **D** MFI Mean fluorescence intensity measuring CD127 and CD62L expression in naïve $CD4^+$ T cells in VANC and REG mice **E** MFI measuring CD127 and CD62L expression in naïve $CD8^+$ T cells in VANC and REG mice **F** Quantification of the frequency of $CCR7^+$ naïve $CD4^+$ T cells at D10 and D14 in VANC and REG mice. Each bar represents 6 neonates, 3 male and 3 female. Dark bar = pups from dams administered regular water, grey bar = pups from dams administered vancomycin in drinking water. Bars indicate mean value ± SD.*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, MFI=Mean fluorescence intensity

2.3.4 In-utero antibiotic exposure may support $CD8^+$ T cell accumulation in the spleen but not surface marker expression

In early life the spleen is seeded by BM and thymic emigrants and is also a site for primary hematopoiesis, hence splenocytes may hold information on early life immune development. As shown in figure 2.2C the significant differences observed at D10 and D14 in the proportion of $CD4^+$ T cells in the spleen when comparing the different time points was not reflected in the tissue comparison at individual timepoints and no major trends were observed either in the proportion of splenic $CD8^+$ T cells. This suggests that differences in the proportion of $CD8^+$ and $CD4^+$ T cells in the spleen may not be as crucial to hematopoiesis.

Further analysis of CD44 expression on splenic $CD4^+$ T cells revealed no noteworthy differences (data not shown). However, at D14 VANC mice had less splenic $CD44^+CD8^+$ T cells and more $CD44^-CD8^+$ T cells than REG mice and as result more naïve T cells (figure 2.4A). The dominance of naïve $CD8^+$ T cells resembles results observed in the BM at D14. However no significant differences were observed in naïve T cell migration and survival markers at D14. Instead CD62L and CD27 expression seems to fluctuate at D7 and D10 but evens out between VANC and REG mice at D14 (figure 2.4B). Suggesting that just the presence of these cells in the

spleen at D14 may be important regardless of surface marker expression. Unlike the spleen and BM, no significant trends were observed in lymph node and thymus suggesting that at the selected time points they may not be as relevant to antibiotic driven T cell hematopoiesis.

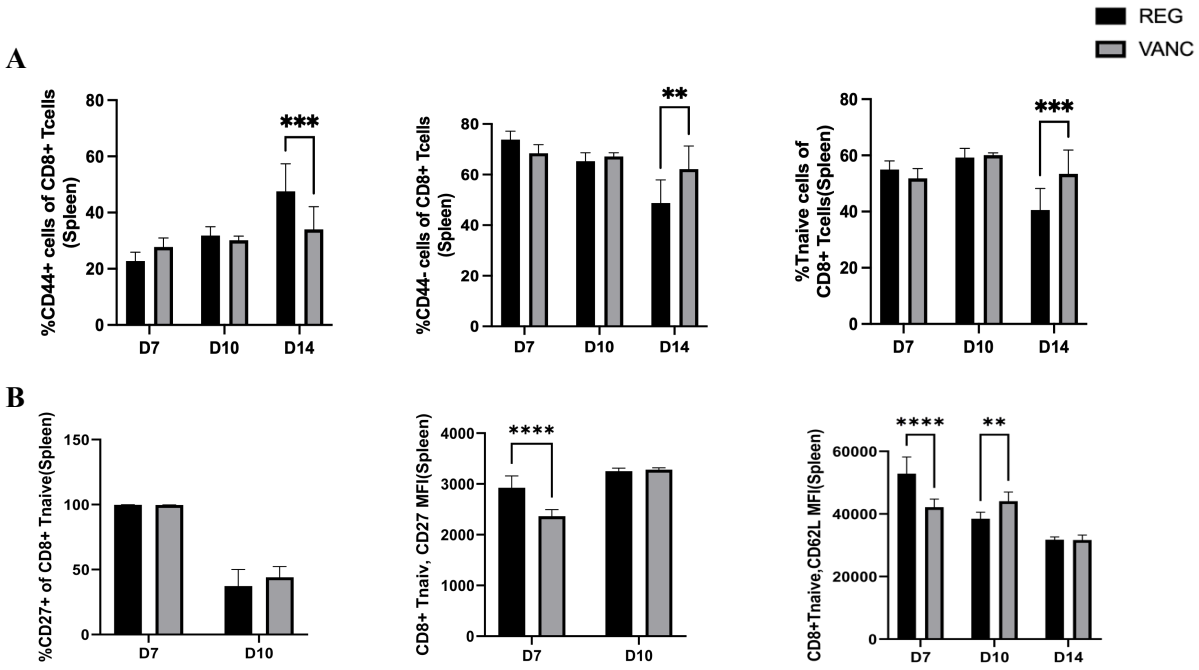

Figure 2.4 Spleen naïve T cell proportions and surface marker expression.

A Quantification of the frequency of $CD44^+$ and $CD44^-$ $CD8^+$ T cells and naïve $CD8^+$ T cells at D7, D10 and D14 in VANC and REG mice. **B** Quantification of the frequency of naïve $CD8^+$ T cell surface markers and marker expression in the spleen at D7, D10 and D14. Each bar represents 6 neonates, 3 male and 3 female. Dark bar = pups from dams administered regular water, grey bar = pups from dams administered vancomycin in drinking water. Bars indicate mean value ± SD.*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

2.3.5 Thymic plasmacytoid dendritic cells in antibiotic exposed mice may be functional effector cells

pDCs are well established as anti-viral type 1 interferon producing cells and they have also been shown to ameliorate allergic disease development. Furthermore, in our human CBMC study

pDCs emerged as one of the main cells of interest even though further analysis revealed they were not significantly different in healthy vs kids with wheeze and atopy (figure 1.9).

To address whether pDCs have a role in early life hematopoietic skewing we assessed pDC proportions and activation marker expression in the blood, BM, spleen, lymph, and thymus. pDCs were defined by flow cytometry as PDCA-1^{+} $B220^{+}$ $CD11c^{int/lo}$ cells. The most interesting observations were made in the spleen and thymus. In the spleen total pDCs proportions were similar between REG and VANC mice at D7, D10 and D14 (figure 2.5A). However, at D7 fewer VANC pDCs expressed CD86, and this trend continued at D10 and D14 but was not significant (figure 2.5B). MHCII expression follows a similar trend to CD86 at D10, but no significant trends were observed in CD80 and PD-L1 expressing pDCs (figure 2.5B). This suggests that in the spleen between D7 and D10, even though VANC pDCs may be activated, they might not be as robust effector cells compared to REG pDCs due to lower expression of costimulatory molecules like CD86 and MHCII.

In the thymus VANC mice had significantly more pDCs at D7 but the number decreases at D10 and D14 compared to REG mice even though not significantly (figure 2.5C). Figure 2.5D showed that at D10 VANC mice had significantly more cells expressing CD86 and CD80 which are both activating T cell co-stimulators via their interaction with CD28 expressed on T cells. PD-L1 expressing cells were also significantly higher and may be involved in T cell inhibition or anergy. Hence the upregulation of CD86, CD80 and PD-L1 at D10 may point to these thymic pDCs being involved in T cell activation or promoting T cell anergy or tolerance through co-stimulation. Overall, these results suggest that thymic pDCs may be more influential during the perinatal period than splenic pDCs and may play a role in immune development (potentially T cell function) in antibiotic expose mice.

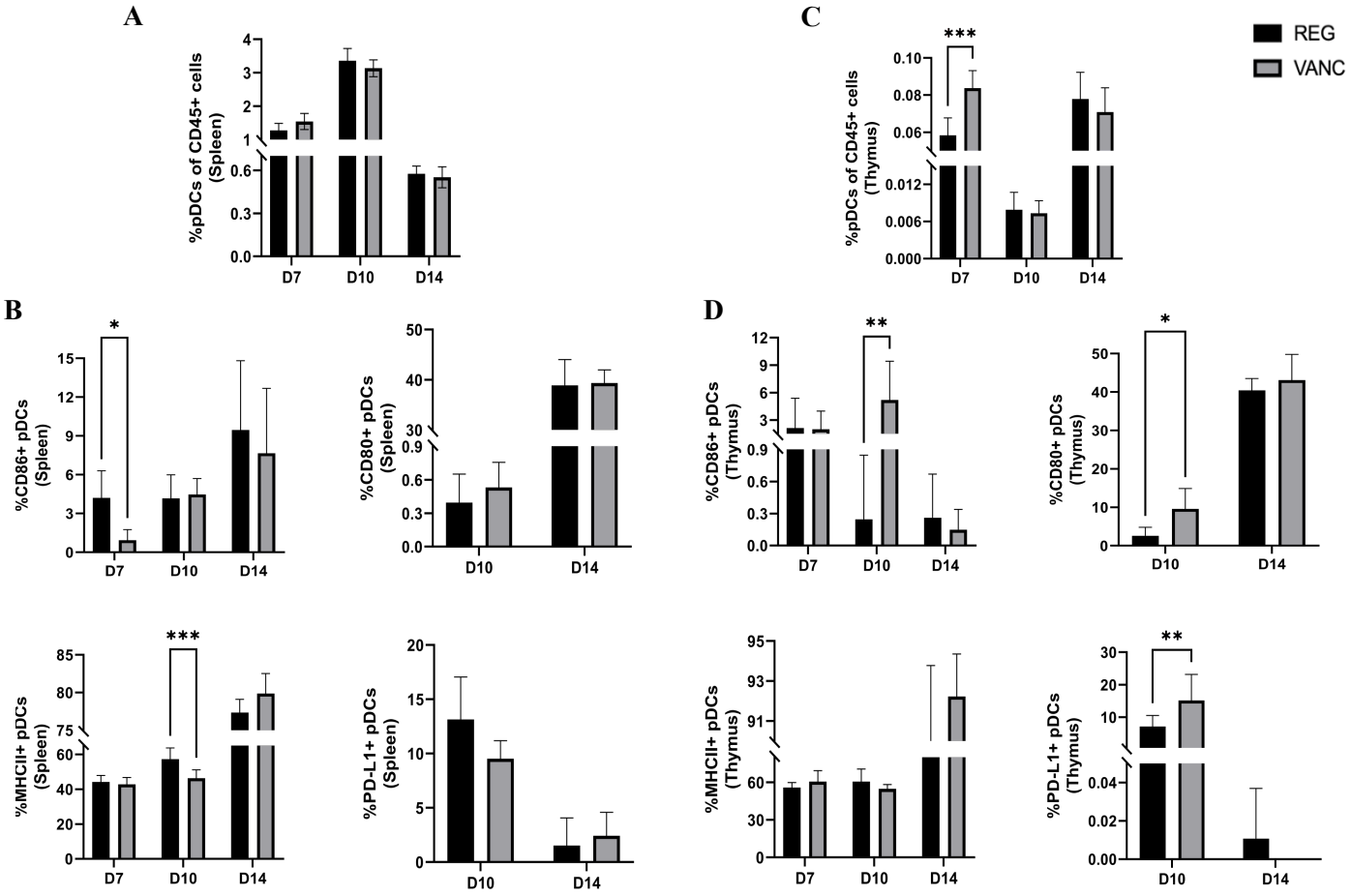

Figure 2.5 pDC proportions and activation marker expression in the spleen and thymus.

A Frequency of pDCs of $CD45^+$ cells at D7, D10 and D14 in spleen **B** Frequency of CD80, CD86, MHCII, and PD-L1 expressing pDCs in the spleen. **C** Frequency of pDCs of $CD45^+$ cells at D7, D10 and D14 in thymus **D** Frequency of CD80, CD86, MHCII, and PD-L1 expressing pDCs in the thymus. Each bar represents 6 neonates, 3 male and 3 female. Dark bar = pups from dams administered regular water, grey bar = pups from dams administered vancomycin in drinking water. Bars indicate mean value ± SD.*P<0.05, **P<0.01, ***P<0.001.

2.3.6 Total serum IgE was increased in antibiotic exposed 14-day old neonates

Atopy diagnosis is carried out by measuring total serum IgE and the skin prick test. So, we were curious about IgE production in these neonates. From our experimental group an IgE ELISA was used to assess neonates' serum at D14 as D7 and D10 may be too early for IgE detection due to the time required to adequately induce isotype switching in the Ig locus and subsequent gene and

protein expression and cell maturation. We also assessed total serum IgE in adult mice administered vancomycin for comparison.

We found that overall VANC mice have higher total serum IgE levels in both adults and neonates (figure 2.6). The relative difference in IgE expression in adults versus neonates was great with neonates producing very little IgE in comparison (figure 2.6A). This is expected since neonates have had little exposure to external antigens. Unlike VANC exposure, sex differences were not significant to IgE secretion in neonates. Since these neonates are sacrificed before much exposure to the external environment and antigens, we expected there to be minimal IgE production but expected overall more IgE in VANC than REG mice due the immune skewing because of VANC exposure. Given that the difference in total serum IgE between VANC and REG mice, it can be assumed that immune skewing due to antibiotics is well on its way at D14.

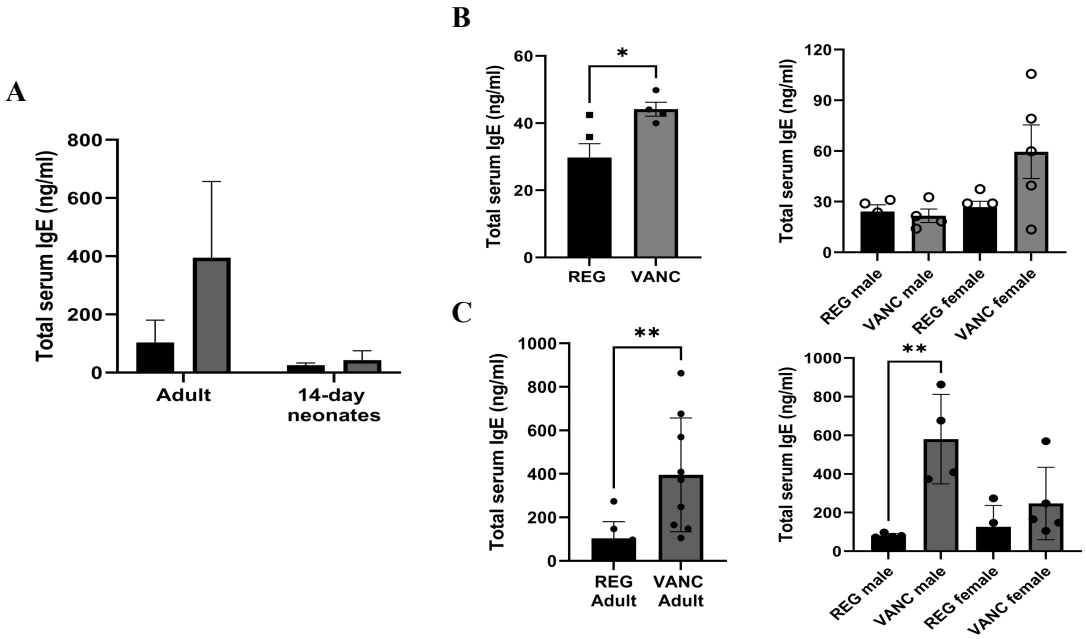

Figure 2.6 Quantification of total serum IgE in neonates and adult mice

A Total serum IgE in adults vs 14-day old neonates **B** Total serum IgE in VANC vs REG 14-day old neonates and sex differences. **C** Total serum IgE in VANC vs REG adult mice and sex differences. Bars indicate mean value ± SD.*P<0.05, **P<0.01, ***P<0.001.

2.4 Discussion

The results from this study show that in-utero antibiotic exposure may exert different effects on the development of immune cells in lymphoid organs. These differences are apparent through changes in the proportion of T cell subsets localized in a particular microenvironment, the upregulation of specific markers on pDCs and increased serum IgE in antibiotic exposed pups. T cells are critical to the development and progression of allergic disease through the secretion of T2 cytokines and B cell stimulation. They are also critical in inducing tolerance and decreasing disease severity in allergy. So, whether T cells have a have a proinflammatory or anti-inflammatory effect on allergic disease is dependent on several factors including the types of T cells subsets involved in the disease mechanism. From quantifying overall T cell frequencies at D7, D10 and D14 in the blood, spleen, lymph node and thymus in VANC and REG mice we found that these tissues had similar $CD3^+$ T cell frequencies when comparing the two groups at the three time points even though they were not yet seeded to adult levels[199,200]. This suggests that antibiotic driven hematopoietic skewing may not necessarily affect overall T cell proportions in different tissues. Rather the differences in individual T cell subsets may be more consequential. Within the $CD4^+$ and $CD8^-$ T cells subsets the most intriguing differences were observed in the spleen and BM.

The BM niche is critical for the maintenance of hematopoiesis throughout life. Molecular signals that affect cell composition and localization can promote alterations that may deregulate normal hematopoiesis leading to transient or sustained changes in cells within the BM[201]. These changes may have negative effects like increasing susceptibility to allergic disease. Hence VANC mice having significantly more $CD4^+$ T cells and less $CD8^+$ T cells in the BM at D10 may suggest that

at this time point the difference in T cell proportion in the BM may facilitate hematopoietic skewing of BM niche cells. The phenotype of these $CD4^+$ cells in BM of VANC mice also suggests that they have higher lymphoid migratory potential as they have increased expression of the lymphoid homing markers CCR7 and CD62L. So, they may have other effects later in other lymphoid organs. The different subsets of $CD4^+$ T cells (Th1, Th2, Th17, Tregs etc.) can produce a plethora of cytokines (e.g., IL-4, IL-13, IFNγ, IL-10, GM-CSF, IL-3, IL-17, etc.) which have wide ranging effects on various cell types including BM stromal cells[52,202]. In mouse BM activated $CD4^+$ T were shown to produce cytokines without external stimulation and were crucial for normal hematopoiesis[203]. $CD4^+$ T cells specifically within the BM have been shown to be required for the terminal differentiation of myeloid progenitors[204]. Hence an increased frequency of $CD4^+$ T cells in the BM of VANC mice compared to REG mice at D10 may induce changes to normal hematopoiesis favouring increased allergy susceptibility through changes in myeloid cell (DC, monocytes etc) development. In addition to cytokine effects, cell-cell interaction is another way T cells can contribute to hematopoiesis[205]. A high proportion of BM T cells expressed CD44 which is involved in various immune processes by binding hyaluronan which is expressed by myeloid DCs, macrophages etc. An increase in this cell-cell interaction may also promote allergy by inducing changes in myeloid hematopoiesis. Thus, a better characterization of cytokine secretion, transcriptome, and surface protein expression of these BM $CD4^+$ T cells at this time point and earlier may provide more insight into the mechanism driving changes in the BM and their effects on allergy development.

Contrary to D10 in the BM at D14, when comparing the naïve $CD4^+$ and $CD8^+$ T cell populations in VANC and REG mice the most outstanding differences were observed within the

$CD8^+$ naïve T cells. VANC mice had more $CD8^+$ naïve T cells. These T cells depending on the BM cytokine milieu have the potential to develop into type 2 cytotoxic $CD8^+$ T cells (Tc2) which can be maintained by BM stroma cytokine secretion further driving allergy susceptibility[85]. These cells may induce changes to hematopoiesis through similar mechanisms to $CD4^+$ T cells since they have similar cytokine profiles. Overall, this data suggests early on in VANC mice D10 (and maybe prior) $CD4^+$ T cells prep the BM microenvironment to support T2 skewing of other cells including $CD8^+$ T cells at D14 and the action of these cells coupled with exposure to viral infection in early life may drive allergy development. One piece of data we believe will be useful to acquire in future studies is the BM analysis data from D7. This data will be crucial to discerning how early $CD4^+$ T cell frequencies increase in the BM and will help determine which time point to probe further in future studies.

In humans and mice hematopoiesis primarily occurs in the BM throughout life but in early life and in some pathological cases hematopoiesis occurs in the foetal liver and spleen[206,207]. Additionally, in mice the spleen has been shown to contribute to hematopoiesis under physiological conditions hence hematopoietic skewing of splenic HSCs may contribute to allergy susceptibility. From this study the difference observed between REG and VANC mice in terms of splenic $CD3^+$ cells, $CD4^+$ and $CD8^+$ T cells were not significant so no strong conclusion can be made based on overall T cells proportions. However, a deeper dive into the naïve T cell population revealed differences at D14 in $CD8^+$ T cells. Like in the BM, VANC mice had more $CD8^+$ naïve T cells even though no significant differences were observed in their migration and survival surface markers. This suggests that even though the spleen and BM may have similar T cell frequency at D14 (naïve $CD8^+$ T cells dominant) the impact of these tissues to the ongoing

hematopoiesis may be different. A more in-depth characterization of BM and splenic $CD8^+$ T cells at this time point may help highlight those differences further. It would be useful to know whether these $CD8^+$ T cells are of the Tc2 subtype and where they were previously localized as they could be recent BM emigrants.

Opposite to the BM and spleen, the lack of perceived differences in the blood, lymph and thymus may suggest that they might not be as critical to antibiotic induced hematopoiesis at the selected time points highlighting tissue differences. An alternate explanation may also be a larger sample size may be required to discern diminutive differences in these tissues.

pDCs typically make up 0.1-0.5% of all nucleated cells and one study showed that a fully developed DC system is not observed until about 5 weeks after birth and that at earlier time points pDCs are more abundant in the spleen and thymus[208,209]. In this study the analysis of pDCs in the spleen and thymus emerged as tissues with the most interesting differences between REG and VANC mice. In both spleen and thymus, pDC proportions were not affected much by antibiotic exposure except at D7 in the thymus, which suggested that the maturation state of these cell may be more relevant than their overall numbers. Non-activated pDCs in neonates secrete comparable amounts of type1 interferons to adult mice and upon activation (through TLR signalling or CD40 ligation) upregulate costimulatory molecules important for T cell function[109]. In the spleen overall, fewer cells in VANC mice seem to be activated at the different time points suggesting the spleen may not be as critical to antibiotic driven pDC development. In the thymus, even though pDCs at D10 in VANC mice are fewer compared to REG mice, they have proportionally more cells expressing CD80, CD86 and PD-L1. CD80 and CD86 are involved in T cell co-stimulation as a signal for activation whereas PD-L1 is known to induce T cell anergy

or promote development of tolerance[97]. So, depending on the cytokine environment in the thymus at D10 they could skew T cell development towards tolerance or inflammation. Higher number of pDCs have been associated with less severe allergy however at this early stage it is not clear whether fewer thymic and splenic pDC may be directly impacting hematopoiesis. To determine this, a more thorough analysis of cytokine secretion by these cells and the cytokine milieu at the different time point may be useful.

pDC localization in the blood, BM, and lymph up to D14 in neonates was not significantly affected by antibiotic exposure. Assessing pDC proportions in these tissues with more samples may be necessary to verify this observation as cell numbers from individual mice in this project may not be sufficient to fully elucidate differences within tissues with small pDC numbers because of the age of the mice. It should be noted that pDC proportions decrease with age up to 5 weeks when adult levels are reached. This will be an important consideration when choosing time points to explore in follow up experiments[209].

Finally, observing increased serum IgE in VANC mice at D14 suggest that at this early time point the hematopoietic changes driving allergy development are ongoing since IgE is a pivotal effector molecule in eosinophilic allergy.

Overall, this study suggests that T cell development in the BM and spleen and pDC development in the spleen and thymus may be affected by in utero antibiotic exposure. These cells, through cytokine production and cell-cell interactions, may facilitate changes to these microenvironments in early life that promote allergy susceptibility through changes in the development trajectory of other important allergy effector cells. Sample size limitations and the rapidly changing lymphoid

tissue environment in early life may have confounded some of the important underlying biology but regardless, this study provides new insight into possible cell targets and time points for further study. However, the full implications of these results cannot be assessed until more mechanist studies are carried out to determine how hematopoietic changes are induced by VANC in these cells.

Chapter 3: Future Directions and Concluding Remarks

This study aimed to the determine the role of in-utero antibiotic exposure on hematopoiesis leading to increased allergy susceptibility with a focus on T cell and pDC development in neonatal mice. Based on the data it can be concluded that by D14 hematopoietic skewing favouring allergy development in antibiotic exposed pups has already begun, due to their increased serum IgE. Furthermore, the increased frequency of $CD4^+$ T cells in the BM suggests they may be the main driver of antibiotic induced hematopoietic skewing early on and this process is supported by Tc2 cells (type 2 $CD8^+$ T cells) as the mice age. In addition to T cell activity in the BM, pDC-T cell co-stimulation in the thymus may also be impacting T cell maturation and, by extension, hematopoiesis, and allergy development.

The CHILD study project from our lab provided preliminary data and rationale for embarking on this project. So, finding a significant difference in the proportion of naïve $CD8^+$ T cells between REG and VANC mice at D14 was very reassuring. It suggests that the timepoint selected closely mirrors changes captured in human CBMCs and that, at least early in life, human and murine hematopoiesis that led to allergy follow similar immune perturbations.

One question raised however by the prominent role of naïve $CD8^+$ T cells in humans and mice that have increased allergy susceptibility is how this cell population exerts its effects. Future studies to better characterize the cell surface protein expression and overall transcription profile may provide more insights.

The increased $CD4^+$ T cells frequency in the BM at D10 was not an expected outcome. However, these cells are critical to allergy development and tolerance so their presence in the BM can induce a variety of immune changes especially if they are activated. Like the naïve $CD8^+$ T cells, characterization of these BM $CD4^+$ T cells' activation status and cognate antigen identity, surface protein expression and overall transcription profile will be important. Better characterization of these cells of interest will require increase in the sample size or sample polling.

Overall, this study adds to the knowledge on the cells impacting for increased allergy susceptibility. It provides specific time points at which hematopoiesis can be interrogated in simple allergy mouse model to further our understanding of allergy development.

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