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PREFACE

This thesis is based on a collaboration between the Copenhagen Prospective Studies on Asthma at Copenhagen University Hospital Gentofte and the Technical University of Denmark from Dec 2015 to April 2019. The immunological data used in this thesis was generated by Susanne Brix Pedersen's group at PSB-Disease Systems Immunology based on biological samples from $COPSAC_{2000}$ cohort prior to initiation of this thesis work.

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Last but not least, I am extremely thankful to my husband Ying He for his understanding and encouragement. Thanks to my son for his love and understanding when mom can't spend too much time with him. In the end, I also want to thank my parents and my parents in law, for their countless and unconditional help whenever needed.

Ni Wang

Contents

SUMMARY

The prevalence of non-communicable diseases (NCDs) such as diabetes, asthma and mental disorders have been rising worldwide during the last decades. Those NCDs are suggested to be based on a common reason, as they can all be explained as the “altered reactivity” of the human body to the mismatched total modern environment. Among all forms of NCDs, childhood atopic diseases including asthma, allergic rhinitis and food allergy have attracted a lot of attention and have been extensively studied as they manifest during early life and may serve as an indicator of the altered immune system and forerunner of other forms of NCDs.

This PhD study aimed to investigate potential early life immune mechanisms underlying the development of asthma and other forms of allergic disorders. We hypothesized that a deregulated immune response to specific microorganisms in early life may result in inefficient removal of these infectious agents and subsequently result in exaggerated immune activity, in continuous or recurrent infections, and in development of asthma or allergic sensitization.

The hypothesis was studied by using data based on ex vivo stimulated cytokine release from peripheral blood mononuclear samples collected at 6-month-of age from children followed consecutively from birth to school age for collection of environmental exposure and clinical data. Immune response data were based on stimulation of immune cells with whole airway bacteria or pathogen associated components activating different adaptive or innate immune responses.

From examining cytokine production in response to three selected airway bacteria, we identified a reduced IL-2 production to link with increased levels of total IgE at the age of 6 years. Moreover, we also found that reduced IL-2 in combination with elevated IL-5 associated with increased risk of allergic rhinitis at the age of 7 years. These results provided evidence to support an association between a deregulated immune response against certain bacteria in early life and later allergic sensitization.

We also identified an IL-23-biased innate immune response against viral and bacterial derived nucleic acids to underline the association between asthma risk SNPs and childhood asthma. This result suggested that the link between the 17q21 asthma risk and asthma development is partly mediated via a deficient innate immune response to intracellular pathogens in early life. This result did not only provide evidence to support the hypothesis about deregulated innate immune function, but also pointed to a mechanism for how the 17q21 locus variants may increase the risk of developing childhood asthma.

Altogether, we here provide evidence for two different deregulated immune mechanisms in infants which couple pathogenic colonization in the airways in early life to later asthma development or allergic sensitization at school age.

1 INTRODUCTION

This PhD thesis is based on data from $COPSAC_{2000}$ which is an ongoing selected high risk birth cohort study of 411 children enrolled at age 1 month and born to mothers with active or previous doctor-diagnosed asthma in the Copenhagen metropolitan area during the period 1998-2001. This cohort study was designed to elucidate the effect of genetic and environmental interactions on the development of asthma, and other allergic disorders in these predisposed children, hoping to find preventive strategies before disease development.

One of the most influential findings from $COPSAC_{2000}$ is the identification of three bacteria strains as a risk factor for childhood asthma. Colonization of the upper airways (hypopharyngeal region) with any of the three bacterial strains (*H. influenzae, M. catarrhalis, and S. pneumoniae*) at one month of age increased the risk of asthma at age 5 years.[1] Subsequently, aberrant immune responses from 6 month PBMC against these bacteria were identified to associate with increased risk of asthma at 7 years[2] as well as the number of lower respiratory infections during the first 3 years of life.[3]

To elaborate the previous findings, in **paper I**, we went on to examine the association of the immune profile in response to the three airway bacteria and other types of allergic disorders (allergic rhinitis, allergic sensitization and total IgE level) during the first 7 years of life. In paper I, we also paid special attention to early production of IL-2 in relation to allergic disorders, as studies suggest for an important role of IL-2 in inhibition of allergic disorders.

Several associations between asthma risk SNPs and asthma have been recognized, but the underlying mechanisms are still uncovered. In **paper II**, we therefore aimed to conduct a mediation analysis to examine if certain early innate immune profiles could mediate the effect of asthma risk SNPs on development of asthma.

In the next section, I will introduce common background knowledge for both paper I and paper II, including the relevant information about immune responses, urgency and importance of studying the underlying mechanisms of asthma, as well as the risk factors of asthma and potential mechanisms.

2 BACKGROUND

The prevalence of non-communicable diseases (NCDs) have been spiking during the past decades. These NCDs are not isolated but share a common feature, the chronic low-grade inflammation.[4,5] Hence, it is suggested that different forms of NCDs may be driven by a common etiology.[6,7,8] One potential non-genetic associated reason is the alteration in the environmental and human gut microbiota.[9,10,11,12,13] The composition of gut microbiota has been suggested to contribute to NCDs through multiple ways, including immunologic, metabolic and physiologic pathways.[9,14] It was advocated that different forms of NCDs can all be explained as the "altered reactivity" of the total organism (the human body) to the mismatched total modern environment (the environment and life style).[6] Much effort has been given for studying allergy (asthma, allergic rhinitis, allergic dermatitis, and food allergy), as it manifests during early life and may serve as an indicator of the altered immune system and forerunner of other forms of NCDs.[4]

Like other chronic diseases, allergy also has a genetic component.[15] Many candidate risk loci for allergic disorders, including asthma,[16,17,18,19,20,21,22] atopic dermatitis,[23,24] total serum IgE,[25,26] eosinophil count,[27] and lung function,[28–30] have been identified by genome-wide association studies (GWASs). However, those encoded susceptibilities cannot explain the spike prevalence in allergic disorders in western societies and the global disparity, because genetic variants accumulate at a slower speed than the rise. Instead, environmental exposures are suggested to be the key player in such changes.[7,31,6,4] Environment exposures may imprint the susceptible individuals with epigenetic modifications,[6,32,33] regulating gene expression patterns by enhancing or silencing gene expression through various ways including DNA methylation,[34–38] histone modifications,[39–41] chromatin remodeling,[42] non-coding RNA[43–47] and RNA modification.[48,49]

Environmental exposures encountered both inside the womb and during early life are critical for later disease development.[6,50] Such identified environmental exposures include delivery mode (cesarean section),[51,52] exposure to antibiotics[34–36]and tobacco smoking,[56–58] older siblings,[59–62] dog or cat ownership,[63,64] living in farms,[65–67] and airway colonization of specific viruses [68–77] and bacteria.[78] Experiments with mouse models suggest that the decisive role of microbe exposure in immune development is only effective during early life.[79–81] Hence, the time-sensitive interaction between genetic components and environmental exposures during early life imprint and shape the immune system and subsequently lead to the development of disease.

The immune response of the prospective allergic children diverges from that of the controls in infancy, with selected evidence listed below. First, a deficient Treg response to microbial ligands in cord blood have been identified for children who later developed eczema.[82] Second, prospective asthmatic children showed an aberrant immune response to pathogenic airway bacteria in infancy.[83] Third, children who developed allergic disorders later had attenuated IFN-γ response in response to allergen and mitogen.[84] Fourth, allergic children normally have a distinct developmental trajectory of immune functions characterized by slower maturation of Th1 function. [85]

Given the divergent immune responses imprinted in early life and higher susceptibility to specific viruses (respiratory syncytial virus (RSV), human rhinovirus (HRV), influenza A) and certain bacteria (*Streptococcus pneumoniae, Haemophilus influenzae, and Moraxella catarrhalis*),[49-59] one possible mechanism underpinning allergic diseases has been proposed. It is proposed that the abnormal immune response is the common root contributing to the susceptibility of viral/bacterial infections and allergic diseases including asthma. [86,87 ,83,84,88]

The increasing incidence of allergy is thus suggested to be the result of a complex and dynamic interaction between genetics and environmental exposures rooted at an early age. One possible etiology for such association might be dysregulated immune pathways shaped by the early environmental exposures through epigenetic modifications in predisposed children. Such dysregulated immune pathways seem not to mount the correct immune response to efficiently contain and clear off the invading pathogen, thus giving rise to prolonged and recurrent infections, which gradually leads to development of asthma. Many environmental exposures, which positively associate with asthma have been identified, and the search for risk factors are still on going.

By fully acknowledging the critical impact of early exposures and genetics on later diseases and the development of the immune system, this thesis work aimed to characterize the early cytokine profiles of children who will have allergic disorders and asthma 7 years later, from the perspective of both the innate and adaptive immune response.

In the background, I will briefly introduce the general immune responses, followed by the clinical endpoints included in the thesis and the reason why they are chosen, and conclude the introduction with identified risk factors and possible mechanisms by which they modulate the immune system.

2.1 THE MAJOR CELLULAR COMPONENTS IN THE IMMUNE SYSTEM

The immune system helps us to combat the invading microbes when the first barriers such as the skin and mucosal surfaces are crossed. A functioning immune system comprises two interacting systems, the innate and the adaptive immune system. Innate immune cells are responsible for launching the first wave of immune attack by recognizing the common structures of those microbes or intrinsic danger signals and sending out immune mediators to train and recruit the adaptive immune cells to join the battle, which can target the invaders by recognizing specific antigens associated with the microbes.

To be more specific, in the battle against microbes, a range of innate immune cells such as macrophages, dendritic cells (DCs) and non-immune cells such as epithelial cells are activated in response to the encountered microbes by recognizing the conserved structures on the microbes (Pathogen Associated Molecular Patterns (PAMPs)) based on germ-line encoded pattern recognition receptors (PRR).[89] After recognition, DCs internalize the pathogen and migrate to the lymph node, where it subsequently presents antigen derived from the pathogen to naïve antigen-specific T cells. During the antigen presenting process, naïve Th cells are instructed to differentiate into specific lineages (Th1, Th2, Th17, Treg) through signals from co-stimulatory molecules and cytokines from the DCs.[89] Hence the PRR signaling pathways in DCs are the bridge between innate immunity and adaptive immunity, and it plays a critical role in deciding the type of immune responses elicited towards the invading microbes.

In the following part, PRRs and their associated signaling pathways in the innate immune cells and the cytokine environment required for T cell lineage commitment will be briefly introduced, with a focus on the PRRs pathways included in my PhD study.

2.1.1 Innate immune response – innate signaling pathways

As mentioned above, the innate immune system recognizes the invariant structures of invading microbes (i.e. the PAMPs) by genetically encoded receptors (the PRRs) expressed by innate immune cells. The innate immune cells include monocytes, dendritic cells (DC), tissue-resident macrophages (MØ), neutrophils, basophils, eosinophils, mast cells, γδ T cells, and innate lymphoid cells (ILCs).[90] ILCs resembles innate-like T cells in many aspects

although without T cell receptors (TCR), and produce a large amount of cytokines, similar in type to the T cell cytokines, in the initial stage of an immune response. ILCs can be classified into cytotoxic and helper lineages, in analogy to T cells. The cytotoxic lineage of ILCs is natural killer cells (NK), representing the cytotoxic T cell (Tc). Three subtypes of ILCs (ILC1, ILC2, ILC3) are the helper lineage and represent Th1, Th2, and Th17 correspondingly.[91]

The innate immune cells recognize and bind invading pathogens through their PRRs and ignite immune response by initiating different signaling pathways, resulting in secretion of various immune mediators. The PRRs exist in three different forms: secreted, membrane-bound and cytosolic form.

Since my project mainly focused on examining cytokines related to the signaling pathways initiated by membrane-bound PRRs, I will put an emphasis on them and only briefly introduce the other forms of PRRs.

2.1.1.1 Secreted PRRs

The secreted PRRs such as collectins, ficolins, and pentraxins tackle pathogen mainly through activation of the complement pathway, and by facilitating phagocytosis by neutrophils and macrophages via pathogen opsonization. [92]

2.1.1.2 Cytosolic PRRs

Cytosolic PRRs consist of two distinct receptor families, the retinoic acid inducible gene I like receptors (RLRs) and the nucleotide-binding domain and leucine-rich repeat containing receptors (NLRs). RLRs recognize viral RNA/DNA and are expressed in most cells.[92] RLRs are sensors of viral infection, and distinguish the non-self RNA through the modified 5' triphosphate end uniquely found in most viral RNA.[93,94] NLRs are a big family which contain about 22 identified receptors in humans, including NOD1, NOD2 and NLRP3.[92] NLRP3 is of specific interest because of its genetic variations and activation associated with many diseases including metabolic disorders and asthma.[95,96] Once activated, NLRP3 becomes part of the inflammasome together with apoptosis-associated speck-like protein containing a CARD (ASC) and activated caspase-1 with subsequent production of IL-1β and IL-18 from their inactive pro-forms. NLRP3 detects various molecules such as aluminum crystals, crystalline cholesterol as well as endogenous danger signals such as extracellular ATP, [97] and always works in concert with another activating pathway, e.g. Toll-like receptor 4 (TLR4) stimulated by lipopolysaccharide (LPS), as used in the current study setup.

2.1.1.3 Membrane-bound PRRs

The membrane-bound PRRs mainly include Toll like receptors (TLR) family and can be found on both plasma membrane and endosomal/lysosomal organelles.[92,98] The expression of TLRs are cell type-specific, and are primarily expressed in innate immune cells. Currently, 13 mammalian TLRs are known. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10, all expressed on the plasma membrane, are also known as cell-surface TLRs. They are mainly responsible for recognition of structural bacterial molecules, such as LPS from gram-negative bacteria, flagellin from motile bacteria and lipoprotein from bacterial cell walls, each recognized by TLR4, TLR5 and TLR2/TLR1 or TLR2/TLR6, respectively.

TLR3, TLR7, TLR8, TLR9, TLR11, TLR12 and TLR13 are expressed on the membrane of endosomal/lysosomal organelles in phagocytic cells and are known as intracellular TLRs. They primarily recognize nucleic acid (DNA and RNA) from viruses and bacteria. For example, TLR3 and TLR7 recognizes double-stranded RNA (dsRNA)[99,100] and single-stranded RNA

(ssRNA), respectively, both derived from the viral infection.[101,102] TLR9 detects unmethylated DNA with a CpG motif normally found in bacteria.[103]

The TLRs of highest relevance for this thesis are TLR4, TLR3, TLR7, and TLR9. These TLRs are expressed by various immune cells, including dendritic cells, macrophages, monocytes, B cells, T cells, and NK cells as well as non-immune cells such as epithelial cells and fibroblasts, in a cell type-specific expression pattern. Upon activation by their ligands (TLR4 ligand LPS, TLR9 ligand CpG DNA, TLR3 ligand polyriboinosinic:polyribocytidylic acid (poly I:C) and TLR7 ligand R848), these TLRs induce significant production of type I IFN and IL-12p70 and mediate Th1 responses.[104–112] Despite the seemingly converging cytokine response following activation by different ligands, differences between particular TLR pathways still exist due to specific combinations of various downstream adaptors and subsequently activation of specific transcription factors.

The signaling pathway of TLR3 is TRIF-dependent, which can be activated by the agonist poly(I:C). Engagement of TLR3 with dsRNA or poly(I:C) leads to the induction of pro-inflammatory cytokines, chemokines, and type I and III IFNs through NF-κB and IRF3 activation.[113–118] The expression of TLR3 was identified in fibroblasts, epithelial cells, macrophages and conventional DCs (cDCs) with different subcellular localization which lead to the production of different cytokines. [119–121] Both cell surface and intracellular expression of TLR3 were found in fibroblasts and epithelial cells, while in cDCs and macrophages, TLR3 is primarily identified in intracellular compartments. Different from TLR3 which is expressed in cDCs, TLR7 and TLR9 are primarily expressed in plasmacytoid dendritic cells (pDCs) and act as the major source of type I IFN in response to viral infection.[122] Besides pDCs, TLR7 is also detected in B cells, macrophages and monocytes.[123–128] TLR7 senses ssRNA and is expressed in intracellular compartments. Stimulation of TLR7 by ssRNA or R848 initiates the secretion of type I and III IFNs and other proinflammatory cytokines mediated by IRF7 and NF-κB in a MyD88 dependent manner. [129–131] As mentioned above, TLR9 and TLR7 are co-expressed at a very high level in pDCs, responsible for rigorous IFN production. Similarly as TLR7, TLR9 is also detected in other cell types, such as B cells, macrophages, mDCs and cDCs.[123–125,128] TLR9 recognizes unmethylated DNA and the ligand CpG, and the TLR9 signaling pathway is also MyD88 dependent. The activation of TLR9 by unmethylated DNA or CpG ligand results in the secretion of IFN mediated by IRF7 or production of TNF-α, IL-6 and IL-12 mediated by NF-κB, in a cell type dependent manner. For example, TLR9 activation in pDCs mainly produces IFN.[128,132]

2.1.1.4 Other receptors

Besides the aforementioned innate immune cells, an unconventional T cell subset, i.e, the γδ T cell, is also included in my study. The γδ T cells express γδ T cell receptors and is considered as an innate-like T cell subset. About 2-10% of T cells in peripheral blood are identified to be γδ T cells.[133] Vγ9Vδ2 T is the major γδ T cell subset found in the periphery and responds to a wide range of microbial-derived ligands, such as metabolites released by microbial infected antigen presenting cells (APCs) including bacterial 4-hydroxy-3-dimethylallyl pyrophosphate (HDMAPP).[134] They can eliminate pathogens by secreting a large amount of pro-inflammatory cytokines such as TNF-α and IFN-γ as well as antimicrobial compounds.[134] In addition to secreting cytokines, γδ T cells also provide help to B cells in antibody production,[135] promote the maturation of DCs,[136–139] participate macrophage recruitment,[140,141] presenting antigen to Th cells,[142] as well as supporting the integrity of epidermis.[143,144]

The cytokine production profile may be changed in patients with allergic disorders, for example, IL-4 produced by γδ T cells in bronchoalveolar lavage fluid of allergic asthmatic patients was previously identified.[145,146]

As we discussed before, early exposure can imprint the immune system and may drive the development of later disease. The effects of such imprinting on the immune system may be reflected by the immune response of each PRRs signaling pathway in asymptomatic children. Indeed, TLRs mediated responses have been investigated and an attenuated Th1 response was characterized for children with later allergy comparing to the controls. [85,82]

2.1.2 Major types of immune responses

In this part, I will briefly introduce the major types of immune responses tailored for clearance of distinct species of microbes, initiated by innate immune response through PRRs. The introduction will be focused on the cytokine profiles that contribute in induction and activation of each T cell lineage (Th effector, cells, Tc cells, ILCs) for two reasons: First, they are critical in shaping the direction of the immune response. Second, the rational of my study design and resultant interpretations are based on the cytokine milieu specifically related to each T cell lineage (**Figure 1**).

2.1.2.1 Type 1 immune response

The type 1 immune response is responsible for clearance of intracellular microbes (bacteria, protozoa, and viruses), and may cause autoimmune disorders if out of control. The major cellular components for type 1 immune response include Th1 cells, ILC1s, Tc1 cells and NK cells, all of which uniquely express transcription factor T-bet and produce IFN-γ.[147–149] In addition to T-bet, Tc1 and NK cells also express another T-box transcription factor Eomesodermin (Eomes).[148–151]

In response to intracellular microbes, IL-12 and IL-18 are released by DCs after activation of the PRRs signaling pathway.[152] The dendritic cell derived IL-12 and IL-18, in accompany with IFN-γ derived from ILC1/NK cells induce the differentiation of Th1 and Tc1 from naïve T cells. Macrophages would then be activated by IFN-γ derived from Th1, Tc1, and ILC1. The activated macrophages produce toxic antiviral molecules including the matrix metallopeptidase (MMPs), nitric oxide (NO), and cytokines promoting phagocytosis and clearance of the invaders.[153,154]

Th1

The cytokine environment created by IL-12, IFN-γ, and IFN-α, released by dendritic cells in response to PAMPs, is important for guiding the differentiation of Th1. A strong TCR stimulation may also favor Th1 lineage commitment.[155] The activation of STAT1 and STAT4 in response to IFN-γ and IL-12 are indispensable for activation of T-bet, which is considered as the signature transcription factor for Th1.[147,156] Besides the expression of T-bet and IFN-γ, specific chemokine receptors CXCR3A and CCR5 are also found on Th1 cells.[157] Th1 cells are recruited to the site of infection by ligands for CXCR3A (CXCL9, CXCL10, CXCL11) and CRR5 (CCL3, CCL4, CCL5). Th1 cells produce IFN-γ and Lymphotoxin alpha (LT-α) as key cytokines. [158,159]

Tc1

Similar to Th1 cells, Tc1 cells with cytolytic potential are induced in the same cytokine environment comprised by IL-12 and IFN-γ, and secret the same cytokines after lineage commitment via the activation of T-bet and Eomes.[160] Tc1 cells also express CXCR3A and CCR3 as their main chemokine receptors.[161] The major role of Tc1 cells are cytotoxic killing of the infected cells.

ILC1 and NK

ILC1 cells are defined as ILC cells expressing T-bet and producing IFN-γ, TNF-α, and GM-CSF in response to IL-15.[162–164] NK cells are the cytotoxic lineage of ILC1 whose induction is IL-15 dependent and characterized by IFN-γ production and T-bet and Eomes co-expression.[165] In peripheral blood, 5-15% of lymphocytes are NK cells.

2.1.2.2 Type 2 immune responses

The type 2 immune response is devoted to protection against extracellular helminths. It is also the immune response associated with allergy if dysregulated. The major immune cells in type 2 immune responses include mainly Th2 and ILC2 cells, which are characterized by expression of the transcription factor GATA3 and secretion of IL-4, IL-5, and IL-13.

During parasite infections, IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) are released by epithelial cells, which in turn activate a group of immune cells including mast cells, eosinophils, and ILC2s, resulting in the production of IL-5 and IL-13, and may also include a small amount of IL-4. IL-4 and IL-13 are critical in immunoglobulin class switching, favoring the production of IgG1 and IgE.[166] IL-5 is essential in eosinophil recruitment and supports the differentiation, activation, and survival of them.[159,159]

Th2

Unlike what happens for Th1 lineage commitment, in which the cytokines needed for initiating the differentiation are provided by activated DCs, the early cytokines guiding Th2 lineage commitment does not come from the DCs. NKT cells, naïve CD4 T cells, and basophils are all suggested as the important early source of IL-4, which initiate Th2 cell lineage commitment. NKT cells were identified to secrete IL-4 in response to IL-18 when IL-12 is not present.[167] Basophils are suggested as the early source of IL-4 because basophils are able to produce IL-4 in response to multiple stimuli including IL-18, IL-33 and proteases.[167] In addition, their potential for acting as APCs to induce Th2 was also suggested since besides IL-4 production, basophils can also express both MHCII and CD80/86.[168–170] Naïve T cells were also detected to secrete low amounts of IL-4 in vitro when cultured with low dosage antigen.[171]

Different Th2 differentiation pathway have been suggested. The default Th2 differentiation pathway suggests that in the absence of IL-12 and IFN-γ, Th2 differentiate from naïve Th0 by default.[172] In another model, an IL-4 dependent pathway suggested that in the presence of IL-4, naïve Th0 cells differentiate into Th2 cells by the help from activated DCs. No matter in which pathway the differentiation is initiated, GATA3 is the key transcription factor uniquely expressed during Th2 lineage commitment.[156,173–176] By binding to multiple sites of *IL4, IL13* gene locus and through epigenetic modifications, the direct role of GATA3 is to induce Th2 cytokines and regulate many Th2 specific genes such as the IL-33 receptor and CCR8.[177] In addition to GATA3, activation of STAT5 mediated by IL-2 is also necessary for Th2 cell lineage commitment. [178]

After lineage commitment, Th2 cells express specific chemokine receptors (CCR3, CCR4, CCR8) to assist recruitment, in response to their corresponding ligands CCL11, CCL17, CCL22, and CCL1.[157] Besides the chemokine receptors, chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) is also unique for Th2 cells.[179] In addition to those lineage-specific receptors, the β2 chain of IL-12 receptor is also found on Th2 cells and it has been reported that in the presence of IL-12, IFN-γ can be produced by differentiated Th2 cells,[147] suggesting for plasticity of Th2 cells.

Tc2

Tc2 cells are also characterized by the ability to produce IL4, IL-5, and IL-13. Tc2 cells possess less cytolytic potential compared to Tc1. The sequential signaling pathway leads to type 2 cytokine production in Tc2 is still unknown, but the fact that Tc2 differentiation requires IL-4, and subsequently result in STAT6 phosphorylation, may suggest for a GATA3 dependent pathway.[180]

ILC2

ILC2s play an essential role in type 2 immune responses, especially during the early stage.[181] The key transcription factors required for the development of ILC2s are GATA3 and[182] RORα,[183] while high expression level of Gfi-1 was also identified in ILC2s and may contribute to ILC2 development and their maintenance.[184] Besides conventional roles shared with Th2 cells such as the expulsion of parasites, involvement in allergic inflammation,[185] ILC2s also participate in the differentiation of adipocytes and energy metabolism.[186,187] ILC2s produce IL-5, IL-13, IL-9, and amphiregulin in response to IL-25, IL-33, and TSLP, the cytokines released by epithelial cells after parasite infection, in a TCR and TLR independent manner.[188,189] The distribution of ILC2s has been identified in many sites including lung, skin, gut and mesenteric fat. An extremely low amount of ILC2s has been detected in the peripheral blood.[189]

2.1.2.3 Type 17 immune responses

The type 17 immune responses are responsible for the eradication of extracellular microbes, and are associated with autoimmune disorders when out of control. Th17 cell, Tc17 cells, and ILC3 cells are the major cellular players specific for type 17 immune responses. Those cells uniquely express RORγt and produce IL-17 either alone or together with IL-22.

When infected by extracellular bacteria or fungi, human dendritic cells are induced to secret IL-1β and IL-23, which promotes the differentiation of Th17, Tc17 from naïve T cells and stimulate cytokine production by ILC3s. IL-17 (IL-17A and IL-17F) are the signature cytokines for type 17 immunity and plays an essential role in type 17 immune responses, due to its ability in promoting the recruitment of neutrophils, and in inducing the production of the antimicrobial peptides from epithelial cells. The other function of type 17 cells is to maintain the fine-tuned balance between the host and microbe at the barrier surface.

Th17

Although IL-6 and TGF-β were suggested to be essential for the differentiation of Th17 from naïve T cells in murine models,[190] recent studies indicate that both of them are not essential for human Th17 lineage commitment.[191,192] Instead, IL-23 and IL-1β have an indispensable role in guiding the differentiation of human Th17 cells from naïve Th cells.[191] Th17 precursors were isolated from human cord blood and newborn thymus, characterized by the expression of RORC, CCR6, and IL-23 receptor. Researchers showed that in the presence of IL-23 and IL-1β, those Th17 precursors differentiated into mature Th17 cells even when TGF-β was missing.[193] The key transcription factor in human Th17 is RORγt whose activation is mainly mediated by STAT3.[194]

The chemokine receptors expressed by human Th17 cells are CCR6 and CCR4.[195] Th17 cells are the most plastic Th effector cells and can shift to Th1/Th17 cells in the presence of IL-12.[196] Unlike Th1 and Th2 cells, only a tiny amount of Th17 can be found at sites of inflammation. Two possible reasons for such rarity are suggested: first, the population expansion of Th17 cells are limited due to its poor ability to produce IL-2 and impaired capacity

to replicate itself;[197,198] second, Th17 cells are very plastic, hence they can be easily converted into the other type of Th cells when the cytokine environment changes.

Tc17

In response to IL-1β, IL-23, IL-6, and TGF-β, naive Tc cells differentiate to Tc17 via STAT3 activation.[199] Tc17 is very plastic suggested by the observation that Tc17 produced both IFN and IL-17, with elevated RORγt and relatively weaker T-bet expression.[200] Tc17 expresses CCR6 and CCR5. [201]

ILC3

In line with Th17 and Tc17, ILC3s are also characterized by enhanced RORγt expression and elevated secretion of IL-17 and IL-22.[202] ILC3s primarily locate at mucosal surfaces and are critical for the development of lymph nodes and Peyer patches.[202] After lineage commitment, ILC3s are activated in response to IL-1, IL-7, and IL-23. [202,203]

2.1.2.4 Regulatory immune response

Maintenance of immune homeostasis is essential for health and is mediated by a group of CD4 T cells named T regulatory cells (Tregs). Deficiency in Tregs or inhibition in the function of Tregs results in autoimmune disease or allergic disorders.

The lineage-specific transcription factor for Tregs is Foxp3.[204] Stable and high amount of IL-2 receptor α chain (CD25) expression is also a unique identity for Tregs.[205] Tregs primarily consists of two subsets, i.e., the thymic-derived Treg (tTreg) induced by strong TCR and IL-2 receptor signaling,[206] and extrathymic-derived/ peripheral Treg (pTreg) induced by a high level of TGF-β and retinoic acid.[206,207] The biological functions of tTreg and pTreg are suggested to be different, with tTreg responsible for tolerance to self-antigens and pTreg responsible for tolerance to nonself-antigens.[208,209] Very recently, an ILC subset analogous to Tregs has been identified to contribute to intestinal homeostasis.[210]

Multiple suppressive functions employed by Tregs may be uncovered by its transcriptional profiles. Tregs express abundant amounts of ectoenzymes CD39 and CD73 which can inhibit the proliferation of effector T cells and activation of dendritic cells. It also produces IL-10, TGF-β, and granzymes. IL-10 is especially important for keeping homeostasis of immune functions in the gut.[206] CTLA-4 which is expressed on Tregs also facilitates the inhibition of T effector cells by competing with CD28 for their costimulatory receptors on APCs.[211] Studies revealed that TCR and IL-2 receptor signaling are indispensable for all of the variety of suppressive functions carried out by Treg.[205,212]

2.1.2.5 Other types of immune responses

In addition to the types of immune responses mentioned above, immune responses characterized by the involvement of Th9 or Th22 have also been identified.[213] In analogy to the other T cell subsets, Th9 and Th22 also associate with a unique cytokine profile. Th22 is distinct from Th17, although both of them express IL-22. Th22 is the major source of IL-22 and is also characterized by the expression of skin-homing receptors CCR4, CCR10 and chemokine receptor CCR6.[214] Th22 is suggested to play an important role in inflammatory skin disorders such as atopic dermatitis and psoriasis.[213,214]

Th9 is one of the most recently identified Th cell subsets. Th9 produces IL-9 as a signature cytokine and its lineage commitment is induced by the combination of IL-4 and TGF-β.[215] The definite functions of Th9 are still understudied, but a possible role in allergic disorders has

been suggested. Th9 is suggested to be an important player in allergic asthma which is supported by the evidence that allergic airway inflammation in recipient mice were induced as a result of DO11.10 Th9 cells transferring after ovalbumin (OVA) challenge. Subsequently, the application of anti-IL9 to these recipient mice lead to inhibited allergic inflammation.[216]

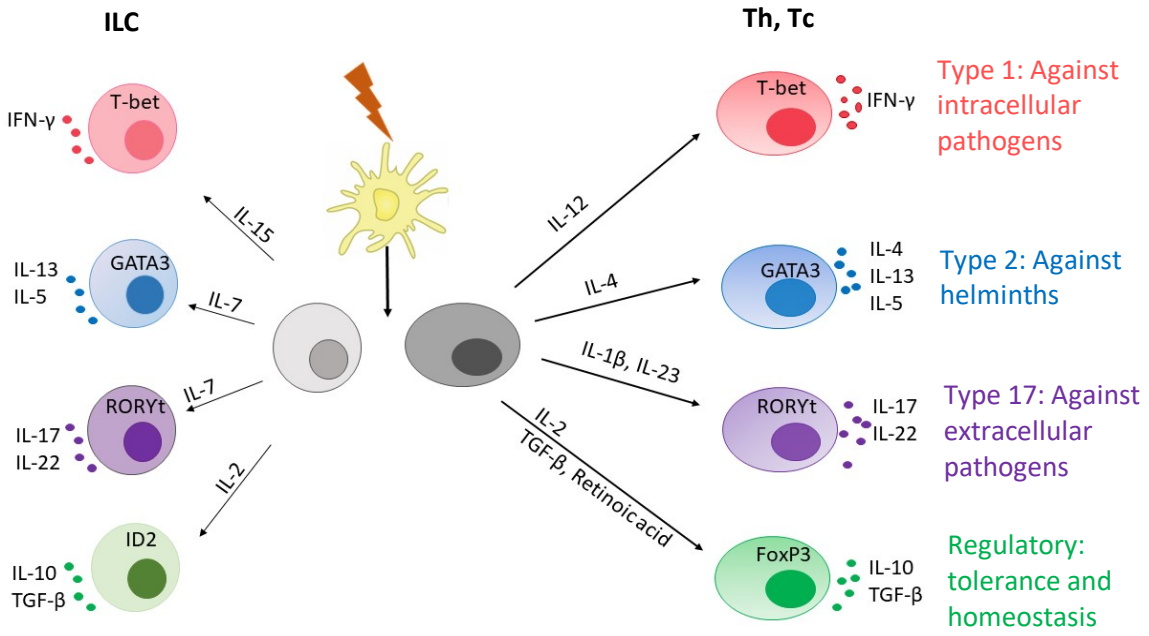

Figure 1. Major immune cell types.

Cellular components of major immune response types and lineage specific cytokines related to differentiation and activation.

2.2 ASTHMA AND ALLERGIC DISORDERS

Accompanying with the declining incidence of infectious diseases, due to the improved hygiene conditions and healthcare, is the climbing prevalence of allergic disorders and other forms of non-communicable diseases (NCDs) worldwide during the past five decades. As mentioned in the introduction, because allergy manifest at an early age, it is normally regarded as an indicator of the altered immune system and forerunner of other NCDs. [4]

Allergic disorders are the most prevalent non-communicable diseases and include asthma, allergic rhinitis, atopic dermatitis as well as food allergy. These disorders seriously affect the life quality of the patients and cause substantial economic loss for society. What is even worse is that there is no cure for allergic disorders and they can only be controlled by treatment. The difficulty in finding a universal treatment was because asthma including other allergic disorders are multifaceted disorders, with various phenotypes that originated from different pathophysiological mechanisms. [217,218]

Thus, it is ideal to find a way to prevent the establishment of asthma and other allergic diseases, rather than treat them. Several lines of research suggest that allergic disorders have an early onset and the trajectory of the disease can be traced back in early life.[3,31] Therefore, studying childhood asthma and other allergic disorders in childhood may help us getting a

better understanding of the mechanisms of those diseases and may eventually guide us to find way to prevent disease development.

Asthma is a chronic airway inflammation involving the cooperation of both the innate and adaptive immune system and epithelial cells, manifested by airway hyperresponsiveness (AHR), mucus hypersecretion, airway remodeling, and airway obstruction. The prevalence of asthma has doubled during the past decades, and it affects approximately 300 million people worldwide, with an increased mortality rate. [219,220] Asthma and other allergic disorders are not a single disease, but a syndrome, representing a heterogeneous group of multiple diseases sharing a similar clinical manifestation.[217,218,221] I will briefly introduce currently identified asthma endotypes and phenotypes in the following part.

2.2.1 Th2 endotype

Asthma is well known as a type-2 based immune disorder that depends on the orchestra of type 2 immune cells (Th2 cell, basophils, eosinophils, mast cells) and type 2 cytokines (Th2 cell derived cytokines: IL-4, IL-5, and IL-13; epithelial cells derived cytokines: IL-25, IL-31, IL-33, and thymic stromal lymphopoietin (TSLP)).[222] However, it becomes more and more clear that this is just one of the asthma endotypes, known as the Th2 endotype because of its dependence on Th2 cytokines.[221] Moreover, within this Th2 endotype, several subendotypes might also exist, such as IL-5 high, IL-13 high, or IgE high.[223] Hence, the definition of asthma based on Th2 immune responses is merely a simplification of the mechanisms.

About half of non-severe asthmatic patients have the Th2 endotype, and it is the endotype that most strongly relate to atopy.[224] Atopy refers to the ability to produce specific IgE against an allergen, known as allergic sensitization and generally assessed by either positive skin prick test (SPT) or the occurrence of specific IgE in the serum.[225] Sometimes, elevated total serum IgE is also used as a surrogate for atopy.[25,26] On the other hand, allergy refers to an unfavorable Th2 driven hypersensitivity response to harmless environmental substances known as allergens, resulting in one or more IgE mediated disease such as food allergy, atopic dermatitis, allergic rhinitis, and allergic asthma.[15] Children who are sensitized at an early age have a greater risk of developing eczema and food allergy, followed with higher susceptibility in allergic rhinitis and allergic asthma later in life. Such sequential development of atopic manifestation is well known as the “atopic march”.[226–231] The complexity of atopic asthma and allergic disorders cannot be simply explained by the Th2 endotype since not everyone with atopy subsequently follows the atopic march. For example, for children with atopic dermatitis, about 66% of them develop allergic sensitization and allergic rhinitis, and around 30% of them develop allergic asthma.[232] Allergic asthma and allergic rhinitis have the largest overlap, with 80% allergic asthmatic patients having allergic rhinitis. On the other hand, about 19% to 38% of patients with allergic rhinitis co-occur with asthma. [233–235]

Even though some studies suggested that allergic asthma and allergic rhinitis might be the same disease because they share common risk factors, pathophysiologic mechanisms as well as common treatment,[236–240] differences between them also exist.[241] Hence, in my study, besides childhood asthma, allergic sensitization and allergic rhinitis are also examined as single clinical outcomes.

2.2.2 Non-Th2 endotype

About half of severe asthmatic patients have a non-Th2 endotype and do not respond to conventional asthma treatment.[242] The non-Th2 endotype is also a heterogeneous group. The elevation of IL-17, IL-1β, TNF-α, and chemokine receptor CXCR2 are often observed in asthmatic patients having the non-Th2 endotype, indicating an association with neutrophil inflammation.[243] Two mechanisms explaining non-allergic asthma are suggested, the Th17

endotype and the neutrophil endotype.[222] The Th17 endotype is Th17-dependent and characterized by the production of IL-17A, IL-17F, and IL-22 and neutrophil infiltration. The Th17 endotype is strongly associated with severe asthma and steroid resistance. The neutrophil endotype is based on a dysregulated innate immune response. It is characterized by neutrophil inflammation, the enhanced expression of IL-1β, TNF-α, and NF-κB as well as altered TLR expression.[244]

2.2.3 Mixed Th2/Th17 endotype

A mixed Th2/Th17 endotype is also identified in asthma.[222,245,246] The plasticity of Th17 cells enables phenotype adjustment according to the microenvironment to further differentiate into dual-positive Th2/Th17 cells and secrete signature cytokines of both type 2 and type 17 (IL-4, IL-5, IL-17).[245,247] The interplay between Th2 and Th17 responses is very complicated. Studies suggest for a reciprocal regulation phenomenon in such mixed Th2/Th17 asthma, where IL-17 production by γδT cells and by Th17 in response to a breached epithelium promotes the production of IL-4 and IL-13 from ILC2 and Th2 cells, which suppress further IL-17 expression and neutrophilia in a negative feedback loop.[248] On the other hand, Th17 response may be amplified by IL-4 and IL-13, by upregulated expression of CD209a on dendritic cells.[249]

2.2.4 Asthma phenotypes and lung function

Childhood asthma can be classified into different subphenotypes by more precise phenotypic definitions. By doing this, a more homogeneous subgroup of asthma patients can be identified, which will further facilitate studies in finding the risk genetic variants and environmental exposures, as well as immune profile cataloging. Different definitions have been applied in further defining the subphenotypes. For example, childhood asthma was suggested to be classified into “transient wheeze”, “early persistent wheeze” and “late onset asthma” by the Tucson population based birth cohort.[221] In some cases, classification using intermediate phenotypes was also suggested because they can be more objectively measured. Such intermediate phenotypes include lung function and serum IgE.[15]

2.3 RISK FACTORS OF CHILDHOOD ASTHMA AND POSSIBLE MECHANISMS

Intensive studies during the last decades uncovered several risk factors for childhood asthma and other allergic disorders. Not surprising, as many other NCDs, childhood asthma and other allergic disorders are the outcome of a dynamic and complex interaction between genetic and environmental components.[4,6,7,31] In this part, the identified risk factors from both sources will be briefly introduced.

2.3.1 The genetic risk factor and mechanisms

The genetic background has a critical role in asthma and its importance has been suggested by several monozygotic twin studies since the year of 1971.[250] Those twin studies carried out in different countries during the last 50 years estimated that genetic background contributes 35% up to 95% to the risk of asthma.[250–260]

About 39 genetic variants have been associated to increased risk of asthma through genome wide association studies (GWAS) since the identification of the first genetic variant, ORMDL3 on chromosome 17q21, in 2007.[16] Subsequent studies also found a variant in the same 17q21 locus regulating the expression of another gene, GSDMB.[13,261,262] Because of the strong linkage disequilibrium (LD) and co-expression of genes in the same locus, gene variants clustered in this region is now recognized as 17q21 locus. Among all the identified asthma susceptible genes, the most robust ones are the 17q21 asthma locus (ORMDL3, GSDMB),

IL1RL1, IL-33, and TSLP.[15] A very recent meta-analysis based on multiple ancestry asthma GWAS found those asthma associated loci are enriched in enhancer markers in immune cells and may be involved in regulation of gene expression related to immune functions. [263]

Moreover, the biological functions of IL1RL1, TSLP, and IL-33 suggested that those genetic variants may work through the same pathway related to atopy. TSLP and IL-33 can promote the differentiation as well as activation of Th2 cells, via binding to IL-33 receptor encoded by IL1RL1 expressed on Th2 cells and other innate immune cells (mast cells, ILC2, macrophages, basophils, dendritic cells, and eosinophils).[264–267] Evidence from GWAS studies also support the associations between genetic variants in these gene locus and allergic diseases. For example, genetic variants in IL-33 and IL1RL1 have been reported to have a positive association with eosinophil count as well as asthma in a GWAS of eosinophil count.[27] Several candidate gene studies also confirmed that the loci around TSLP[268–270] and IL1RL1[271–273] may contribute to the susceptibility of asthma and atopy.

However, the role of the 17q21 locus on asthma susceptibility seems to be different and may not involve in atopic pathways. The association between the 17q21 locus and childhood early onset asthma and exacerbations has been reported as described above, but no associations with adult asthma, total serum IgE, and allergic sensitizations have ever been identified for the 17q21 locus. Some studies proposed a non-atopic pathway led by the 17q21 to childhood asthma and exacerbation.[274,275,276]

Of course, the functions of those risk variants are not limited to immune functions, their expression in airway epithelium also suggest their involvement in other biological functions including barrier integrity, cell adhesion, and airway remodeling.[263] All in all, the exact functions of those identified risk gene variants still need to be further elucidated.

2.3.2 The environmental risk factors and mechanisms

Besides the encoded genetic variance, many environmental factors have been identified as risk factors of asthma and other allergic disorders, partially due to their effects on the immune system.[6]

It is suggested that environmental exposures, especially the composition of the microbiota during early life have a tremendous and long-lasting influence on the development of the immune system and subsequent asthma and allergic disorders.

The first hypothesis proposed to connect microbial exposures and allergic disorders was the hygiene hypothesis, which suggests that limited early life exposure to microbes changes the early life immune system and results in enhanced susceptibility to allergic disorders.[277] This hypothesis was originally formulated to explain the sibling effect, i.e. having older siblings can protect the younger children against atopic disorders, because their exposure to environmental microbes would be inevitably promoted by having older siblings. Many environmental exposures result in altered microbial exposures of the infants, including household size, delivery mode (Cesarean section), antibiotic usage, pet ownership, daycare attendance, farming environment, viral infection, and bacterial colonization can all be covered by the umbrella of the hygiene hypothesis.

Moreover, a microflora hypothesis is proposed as an extension of the hygiene hypothesis, with a special focus on the role of microbes colonized inside and outside the human body. Evidences from germ free mice proved the effects of microbiota on supporting immune and metabolic homeostasis. Experiments in germ free mice showed that colonization of gut bacteria to germ free mice during early life can rescue their impaired immune function.[278,279] Furthermore, introduction of specific bacteria, such as *Bifidiobacterium infantis,* to germ free

mice, which are characterized by a biased Th2 response, can result in a rebalanced immune response.[280–285]

The critical impact of early life gut microbiota on the development of allergic disorders was also suggested to be in effect in human beings based on longitudinal studies. Gut microbial diversity in newborn (1 week and 1 month old) was identified to associate with asthma at school age.[286] The neonatal intestinal microbiome compositions of asthmatic and non-asthmatic children was distinct from each other in infancy (1 month after birth), with a lower abundance of specific bacterial and higher abundance of certain fungi in the high-risk group.[287] Moreover, the colonization of specific bacteria strains (*H. influenzae*, *M. catarrhalis*, *S. pneumoniae*) in the hypopharyngeal region of 1 month old infants resulted in increased risk of childhood asthma[78] and a subsequent study revealed that those prospective asthmatic children generally displayed an aberrant immune response against the three bacterial strains.[83]

Multiple mechanisms have been proposed regarding the association between early microbial exposure and later allergic disorders and asthma. One potential mechanism refers to the crosstalk between intestinal innate immune cells and microbes, i.e. pattern recognition receptors (PRRs) of the innate immune system recognizing pathogen-associated molecular patterns (PAMPs) such as the cell wall components LPS and peptidoglycan.[288] The function of dendritic cells in bridging innate immune response and adaptive immune response in asthma has been proposed.[289,290] By sensing the surrounding microbes with their equipped Toll like receptors (a group of PRRs), dendritic cells are able to train and decide the phenotype of the lymphocytes. Therefore, alterations in the microbial composition could also be transferred by the dendritic cells and influence the priming of the lymphocytes, which further affect the inflammatory response in the airways. It is now suggested that DCs may contribute in reducing the risk of allergy in a farming environment, which is rich in endotoxin (also known as LPS and recognized by TLR4). In a mouse experiment, chronic exposure to LPS protected the mice from house mite induced asthma, by inhibiting the recruitment of DCs to the lung through A20, which is an inhibitor of transcription nuclear factor κB (NF-κB). [291]

Besides the crosstalk between PRRs and PAMPs, microbial-derived metabolites can also interact with the immune system. One of the most studied metabolites from microbes is the short chain fatty acids (SCFAs), which are produced during bacterial fermentation and are used as primary energy sources for many gut microbes and host tissues.[292] The SCFAs contribute to both physiological and immune homeostasis.[293] SCFAs have an important role in supporting the integrity of mucosal barriers by inducing the ILC3s to secret IL-22, which in turn promotes the production of antimicrobial peptides and mucus by Paneth cells.[294] The SCFAs can also induce immune tolerance by inducing peripheral Tregs (pTregs), potentially through inhibition of histone deacetylases (HDACs).[295–297] Specific commensal bacterial species, i.e. *Clostridia* species, are identified to produce abundant levels of SCFAs, and are associated with pTregs induction in the colon.[298,299] The protective effect of the SCFAs on asthma was also supported in a HDM mouse model of asthma, in which cellular infiltration to the airways after HDM challenge was inhibited by the application of SCFAs.[300] Similar effects of SCFAs in humans was also suggested. Supplementation of *Lactobacillus rhamnosus* to infants with food allergy resulted in increased abundance of SCFA-producing bacterial strains and promoted the acquisition of tolerance.[301]

Another proposed mechanism is the microbe associated epigenetic modifications. Selected evidence supporting this mechanism is listed below. The transferring of conventional gut microbiota into germ-free neonatal mice led to increased methylation of CXCL16 and subsequently reduced recruitment of invariant natural killer T (iNKT) cells into the colon.[81] The methylation of asthma and allergy-related genes is influenced by early life exposure to farm microbes.[302,303] Moreover, the DNA methylation patterns of whole blood exposed to two

bacteria phyla (the Firmicutes and Bacteroidetes) were distinct from each other and may involve in many forms of NCDs.[304]

Other environmental risk factors for childhood asthma not related to microbes include maternal tobacco smoking and air pollution. Children are particular vulnerable to air pollution, partially because their lungs are under rapid development and their lung epithelial layers are highly permeable compared to adults.[305] Multiple mechanisms were also proposed for explaining the role of tobacco smoking and air pollution on asthma, including induced necrosis and apoptosis of airway epithelial cells,[306–309] promoted inflammation[310–312] and epigenetic modifications.[313–317] It is shown that the expression of pro-inflammatory cytokines (TNF-a, IL-6, IL-8) and chemokines was elevated in response to air pollution.[318] High levels of particulate matters (PM) were found to associate with hypermethylation of Foxp3, which is suggested to contribute to Tregs suppression, in peripheral blood of asthmatic children.[317]

In summary, both genetic variants and non-genetic risk factors have decisive effects on the risk of childhood asthma. However, when exploring the contributions of a risk factor on disease development, interaction with genetic background should also be considered. Environmental factors may only have an effect when the individuals have a specific genetic background. One such example is the interaction between 17q21 genetic variants and cat exposure. The protective effect of cat exposure on childhood asthma is suggested to be 17q21 genetic variants specific.[319,320] In line with this, more investigations are needed in order to elucidate the mechanisms causing asthma, given the complex interactions between the whole genome and overall environmental exposures.[6,31]

3 HYPOTHESIS AND AIMS

3.1 RESEARCH QUESTIONS AND HYPOTHESIS

It is well accepted that asthma and other allergic disorders have an early onset, which may be partially contributed by a dysregulated immune system imprinted by early life environmental exposures. Given the evidence that early life viral and bacterial infections are closely related to later asthma and other types of allergic disorders, we hypothesized that infants who go on to have allergic disorders might have deregulated immune responses against encountered microbes in early life.

The hypothesis of this thesis:

Children with a dysfunctional immune system cannot elicit the appropriate immune response against the invading pathogens, resulting in persistent colonization of pathogens, recurrent infections, and subsequent allergic disorders.

We tested the hypothesis using data collected from the birth cohort COPSAC2000, which is a high-risk longitudinal birth cohort study enrolling 411 children born between 1998 and 2001 at the age of 1 month, all with asthmatic mothers.

3.2 AIMS AND OBJECTIVES

This thesis aimed to examine and characterize the deregulated early life immune response of children in relation to development of later asthma and other allergic disorders.

The specific objectives were:

Paper I. The study aimed to examine the difference in bacteria-stimulated cytokine profiles from infant T cells between prospective allergic sensitized kids and healthy controls, with a particular interest in IL-2, which was suggested to be essential for development of Tregs.

Paper II. The study aimed to specify whether the effects of risk SNPs on asthma and other related clinical endpoints are mediated by any aberrant immune response related to specific innate immune signaling pathway in infancy.

4 METHODOLOGY

4.1 COPSAC2000

COPSAC2000 is an ongoing prospective clinical mother-child cohort of 411 children born in the Copenhagen metropolitan area during the period 1998-2001. The children were born to mothers with active or previous doctor-diagnosed asthma, enrollment of which is previously detailed. The children were enrolled at age 1 month and were prospectively followed at the COPSAC clinical research unit with regular follow-up visits every 6 months until age of 7 year and at the presentation of acute respiratory or skin symptoms. The clinical research unit was used as primary health care facility for the diagnosis and treatment of any airway or skin symptoms, strictly adhering to predefined, validated algorithms.

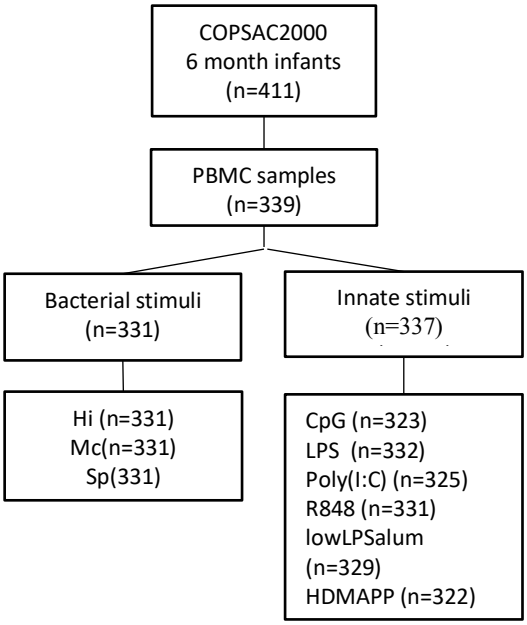

Figure 2. Flowchart of PBMC sampling and in vitro stimulation

This cohort study was approved by the Copenhagen Ethics Committee (KF 01-289/96), the Danish Data Protection Agency (2002-41-2434), and followed the principles of the Declaration of Helsinki. We followed Good Clinical Practice guidelines for data validation and quality control. Written and oral informed consent was obtained from the parents at enrolment prior to any study related procedures.

Study group

Peripheral blood was sampled from 339 infants. Peripheral blood mononuclear cells (PBMCs) were isolated from those sample and subsequently 337 of PBMCs samples were stimulated by innate ligands and 331 of them were treated with whole bacteria stimulation (***Figure2***).

Baseline characteristics of the entire cohort, the study group and the excluded group were calculated and displayed in ***Table 1,*** suggesting that there was no significant difference between the study cohort and the excluded group regarding the examined characteristics.

Table 1. Baseline characteristics of children with PBMC sampling

	COPSAC2000	Study group	Excluded	P value
N	**411**	**339**	**72**	
Sex(Boy)	49.4%(203)	50.4%(170)	44.6%(33)	0.43
Antibiotics in 3rd trimester	14.4%(59)	13.6%(46)	17.6%(13)	0.49
Cat in house in pregnancy	15.2%(60)	14.5%(48)	18.5%(12)	0.53
Delivery mode (Cesarean)	21.2%(87)	21.1%(71)	21.6%(16)	1
Any older siblings	40.4%(166)	39.2%(132)	45.9%(34)	0.34
Passive smoke in 3rd trimester	9.98%(41)	9.5%(32)	12.2%(9)	0.63
Smoking in 3rd trimester	15.3%(63)	14.5%(49)	18.9%(14)	0.44
Maternal dermatitis	42.6%(147)	42.1%(122)	45.5%(25)	0.75
Maternal allergy to inhaled allergen	86.9%(357)	87.8%(296)	82.4%(61)	0.29
Paternal asthma	14.2%(57)	13.7%(45)	16.4%(12)	0.68
Paternal dermatitis	10.7%(36)	11.7%(33)	5.56%(3)	0.27
Household income above 800K	8.64%(33)	8.76%(29)	7.84%(4)	1
Mother's highest education	15.2%(58)	15.1%(50)	15.7%(8)	1
Mother's age at birth (yr), mean(SD)	30.1(4.5)	30.18(4.5)	29.6(4.4)	0.42

4.2 INNATE AND ADAPTIVE CYTOKINE RESPONSES BY INFANT PBMC

The in vitro experiments for cytokine production and measurement was performed by others before I started my PhD project, hence I will briefly introduce the experimental procedure.

Peripheral blood mononuclear cell (PBMC) samples were isolated by density centrifugation from blood samples collected at 6 months of age and stored for up to 12 years at -140°C. After thawing, the cells were seeded in 96 well plates, followed by stimulation with one of six innate ligands or one of three whole bacteria for 40 hours, thereafter the supernatant cytokines were then measured by customized multiplex immunoassays from MesoScale Discovery read on a Sector Imager 6000 (MSD, Gaithersburg, MD, USA).

The innate ligands used in the experiment were lipopolysaccharide (ultrapure *E. coli* LPS), unmethylated cytosine poly guanosine DNA (CpG), polyinosinic:polycytidylic acid (poly(I:C)), resiquimod (R848),1-hydroxy-2-methyl-2-buten-4-yl 4-diphosphate (HDMAPP) and aluminum crystals (alum) in concert with low dose LPS (5 ng/mL) (lowLPSalum) or complete media alone (unstimulated), each corresponding to a specific innate signaling pathway whose activation results in expression of multiple cytokines.

The whole bacteria used in the experiments were UV-inactivated *H. influenzae*, *M. catarrhalis*, and *S. pneumonia.* The colonization of those bacteria were detected in the airways of the infants from the same cohort when they were 1 month old, and their occurrence was

suggested to be associated with increased risk of asthma at age 5 years. Besides the active cytokine response, composition of T cell subsets in the PBMC samples were also measured by flow cytometry.

The relevant immunological data from PBMC samples used in this PhD project are summarized in **Table 2**.

Table 2. Cytokine matrix and experiment setting for each paper

T cell composition	*In vitro* cytokine responses	
Paper 1 *T cell compartments*	*Paper 1* *Adaptive stimuli* *Hi, Mc, Sp*	*Paper 2* *Innate stimuli* *LPS, CpG, poly(I:C),R848, HDMAPP, lowLPS+alum*
Th	IFN-γ	IFN-γ, IL-12p70,IL-18,CXCL10
Tc	IL-4, IL-13	IL-4,IL-5,IL-13,IL-31,CCL17,CCL24
Treg	IL-17A	IL-1β,IL-6,IL-17A,IL-23,CXCL8,CCL20
γδT	TNF-α	IL-22,CCL27
iNKT	IL-2, IL-10	IL-10

Abbreviations: Hi: H. influenzae, Mc: M. catarrhalis, and Sp: S. pneumoniae.

4.3 CYTOKINE DATA TRANSFORMATION

Different sets of supernatant cytokines were collected and measured in response to various innate ligands and whole bacteria. Subsequently, varying transformation methods were applied for calculation purpose.

4.3.1 Cytokine secretion in response to innate ligands

The data derived from stimulation by innate ligands suffered 2 problems: inflation with 0 & populated with values below lower limit of detection (LLOD). For handling the 0 value, although we replaced them with half of the min concentrations of corresponding cytokines, other attempts were also made expecting to find a more analytical approach deal with 0 inflated data.

Attempts to recognize zero concentration

The level of cytokines were measured by a highly sensitive electrochemiluminescence method, in which the fluorescent signals were detected and converted to concentration with unit as pg/ml. When carefully compared the sigmoid curves plotted with concentration as x and fluorescent signal as y, we found that there was a cutoff value for each cytokine in each experiment, all signals smaller than that cutoff value were all converted to 0 pg/ml, indicating that although 0 pg/ml was a single value, it actually represented a range of signals (***Figure s1***). We then compared the signals associated with 0 pg/ml and the signals from blank samples by overlaying their density distributions (***Figure s2***). We further defined the 2.5% percentile of blank signals as lower bound and assigned all signals below it NAs. The rationale behind this was that those very signals might be due to technical error since signals smaller than blank would be very unusual. Those manually introduced NAs were then imputed by K-nearest neighbor algorithm (KNN). However, this method had a pitfall that cannot be neglected.

The imputed values from KNN were dependent on number of cytokines included in the dataframe (the number of neighbors), i.e., including different number of cytokines in the dataframe resulted in different result from KNN. Due to this inconsistency, we decided to treat all 0 in the same way by replacing 0 by the half of the minimum concentration of corresponding cytokine.

Although this signal based 0 differentiation method was not used in this PhD study, it still revealed the heterogeneity of signals behind 0, and may be improved by combining with other imputation method such as bayesian principal component analysis (bPCA), fuzzy K-means (FKM) and multiple imputations by chained equations (MICE).

Values below LLOD

To handle data below LLOD, the question we asked was: How reliable were they? If they were not reliable, can we find a better way to make them to be more precise? For the values below LLOD, we decided to keep them untouched after carefully investigation described below.

For each assay, LLOD was 2.5sd above the zero calibrator (the blank samples). If we adopt the usual way, such as to replace values bellow LLOD by a specific number, a manually introduced uniform pattern will appear and may have substantial impact on the later analysis because the large proportions of those small values. What we wanted was to reduce the noise but not enhance it. Hence, we need to find a better way to take care of those small values. The approach we decided to use was a weighted approach, i.e. assign each concentration with a corresponding weight, based on its fluorescent signal level. The concentrations were converted from the florescent signals, detected during the experiment, based on a 4 parameter logistic regression (4PL) model. Their association can be reflected by a sigmoid curve shown in ***Figure s3***. Smaller weights were assigned to concentrations below LLOD plotted as vertical lines, representing their bigger uncertainties. We used the segregation level of ligand specific cytokine patterns as the gold standard throughout the quality control process. We then compared the segregation of cytokine profiles generated by a weighted PCA with that of a normal PCA (**Figure 3**). The F statistics from the adonis test as well as the R^2 suggested that the weighted approach did not outperform the normal PCA in separating ligand specific cytokine profiles. On the other hand, this may also suggest that although the small values below LLOD have more uncertainty, they still contain unique ligand specific cytokine pattern, hence we keep the values below LLOD untouched.

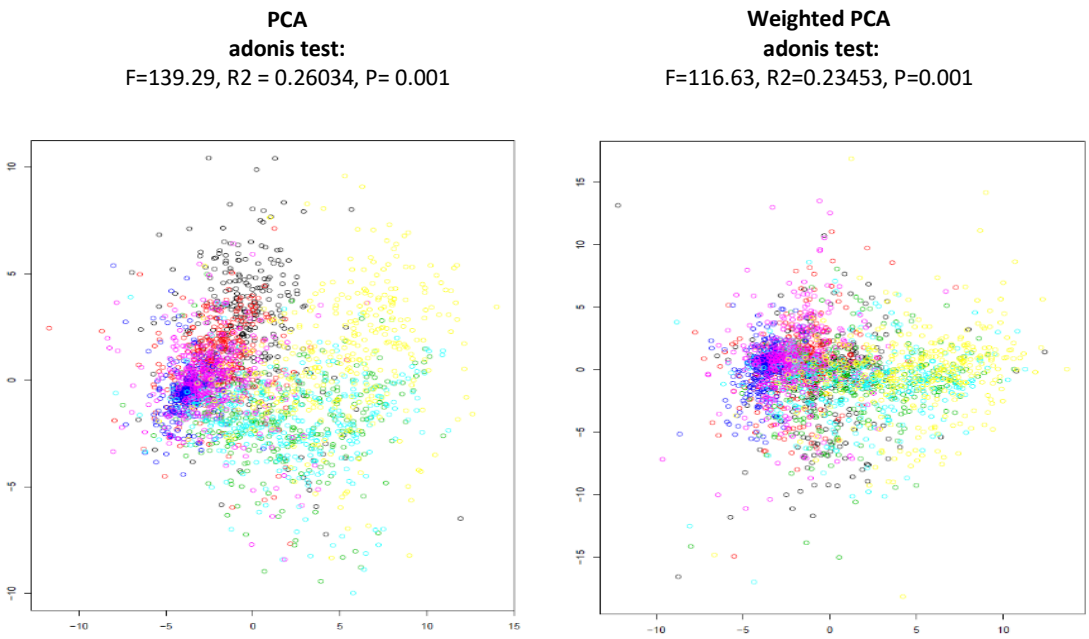

Figure 3. Comparison between PCA and weighted PCA

Transformation – cytokines in innate response

The supernatant levels of cytokines after 0 adjustment as described above were corrected for background levels by dividing stimulated cell values with the corresponding baseline level secreted from unstimulated PBMCs for each child. Thereafter, the levels were log-transformed to improve normal distribution prior to the statistical analysis. The distribution of each cytokine is shown in ***Figure 5***. PCA plot showing the segregation of cytokine profiles from each ligand can be found in ***Figure 4.***

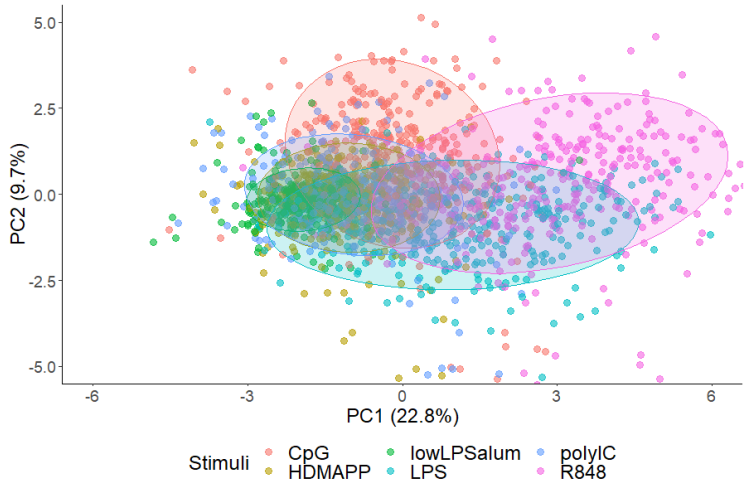

Figure 4. PCA score plot for cytokines from innate ligands stimulation

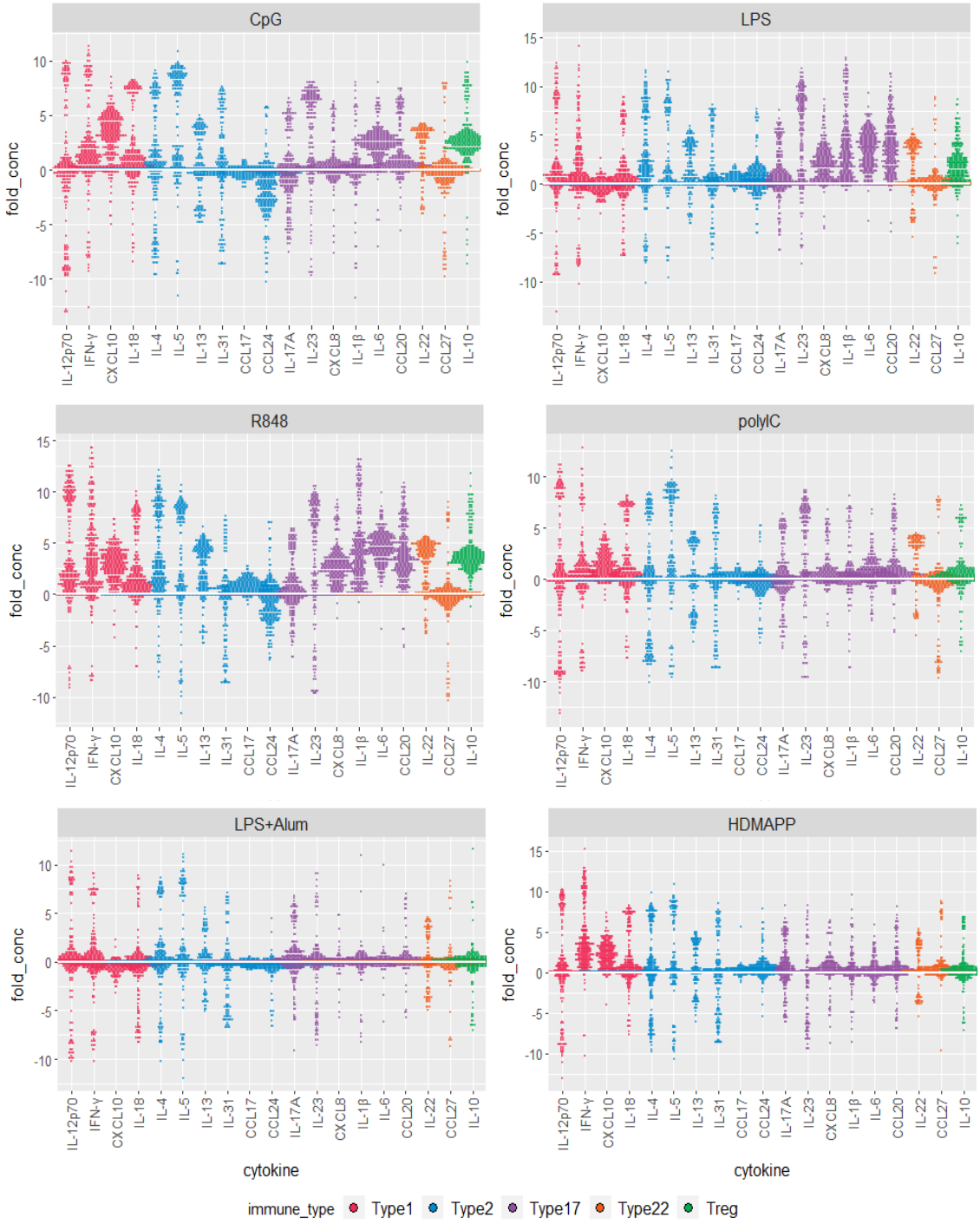

Figure 5. Dotplot for transformed cytokines derived from innate ligand stimulation of PBMCs collected at 6 month of age. N=337.

Abbreviations: LPS: lipopolysaccharide, polyIC: polyinosinic:polycytidylic acid,

R848: resiquimod, HDMAPP: 1-hydroxy-2-methyl-2-buten-4-yl 4-diphosphate,

LPS+Alum: LPS+ aluminum potassium sulfate,

CpG: unmethylated cytosine poly guanosine DNA

4.3.2 Cytokine secretion in response to whole bacteria

Whole bacteria are more potent in stimulating cytokine production compared with the innate ligands, hence this dataset didn't inherit as serious problems as the cytokines derived from innate ligands stimulation.

A different transformation method was applied for those cytokines. The supernatant levels of cytokines were adjusted for background levels by subtracting baseline level associated with unstimulated PBMCs from the corresponding stimulated cell values for each child. Afterwards, the levels were square root transformed to improve normal distribution prior to the statistical analysis. The distribution of each cytokine is shown in ***Figure 6*** and the PCA plot showing the separated cytokine profiles from different whole bacteria stimulations displayed in ***Figure 7***.

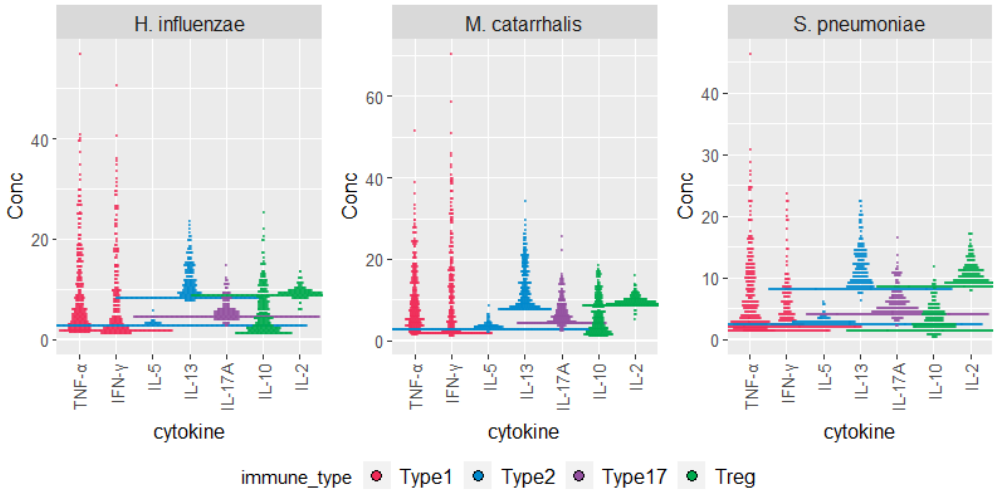

Figure 6. Dotplot for transformed cytokines derived from whole bacteria stimulation of PBMCs collected at 6 months of age. N=331.

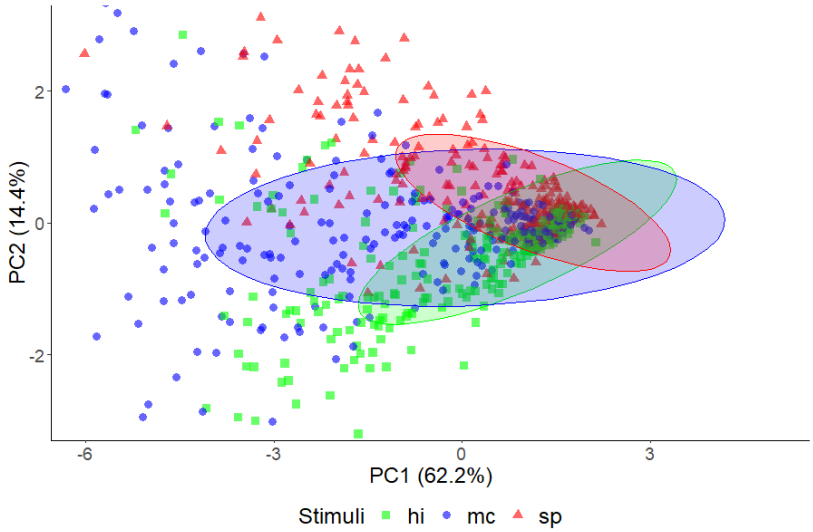

Figure 7. PCA score plot of cytokines from whole bacteria stimulated PBMCs collected at 6 months of age. Each dot represents one child. N= 331.

4.4 STATISTICAL APPROACHES

After data transformation, different statistical methods were employed for corresponding research question investigated in each paper. All the analyses were performed by R 3.4/ R3.5, including packages: xlsx, mice, mediation, survival, tidyverse, broom, ggrepel, reghelper, ggpubr, and compositions.

4.4.1 Statistical approach for paper I

The association between allergic disorders and the cytokine expression profiles (7 cytokines) in response to *H. influenzae*, *M. catarrhalis*, and *S. pneumoniae* were examined in this paper, with a special focus on the role of IL-2.

Stepwise regression

A stepwise multiple regression model with forced IL-2 retaining was used to find cytokines associated with the development of allergic disorders, in response to each of the whole bacteria. The final model was compared with a null model by ANOVA for goodness of fit

PCA regression

Besides the univariate analysis described above, a multivariate method was also employed, in which the immune patterns captured by a data-driven principal component analysis (PCA) on the 21 independent variables (3 bacterial stimulation x 7 different cytokines) was used for association analyses with the outcomes.

Regression for compositional data

Moreover, the associations between each of T cell compositions in the PBMC samples and outcomes were also investigated. T cell composition data was transformed by isometric log ratio (ilr) using sequential binary partitioning and their association with outcomes was analyzed by multiple regression models using a method recently described.[321]

The results are reported with 95% CI and p-values ≤0.05 were considered statistically significant.

4.4.2 Statistical approach for paper II

A casual mediation analysis was conducted aiming to find whether the early infant cytokine profiles associated with specific innate signaling pathway mediated the effect of selected asthma risk SNPs on development of asthma.

Associations by regression

Different regression models (linear, logistic, poisson, cox regression) were used to find the associations between SNPs & outcomes, SNPs & immune profiles captured by PCA, as well as the immune profiles captured by PCA & outcomes, based on the type of dependent variable.

Causal mediation analysis

A causal mediation analysis employing the potential outcome framework was then conducted for examining the mediation effect of immune profiles for specific pairs of SNP & outcome. This analysis was carried out by using the mediation package from R. This package was written according to the potential outcome framework using a simulation-based method

consisting of two regression model based equations for estimating the casual mediation effect.[322,323]

Multiple imputation and mediation analysis

As there were more than 30% of data missing from father dermatitis, mediation analysis with complete cases would lead to a biased estimate and reduced power. Thus multiple imputation using MICE (Multiple Imputation based on Chained Equation) procedure by mice package in R was applied for this variable before including it in the adjusted mediation analysis.[324] The point estimate of the mediation effect through multiple adjusted mediation analysis was the average of the estimates reported by each mediation analysis and its 95% confidence interval (CI) was calculated by taking quantiles of the stacked vector consisting of simulated unit mediation effect. [323,325]

4.5 SUPPLEMENTARY PLOTS FOR METHODOLOGY

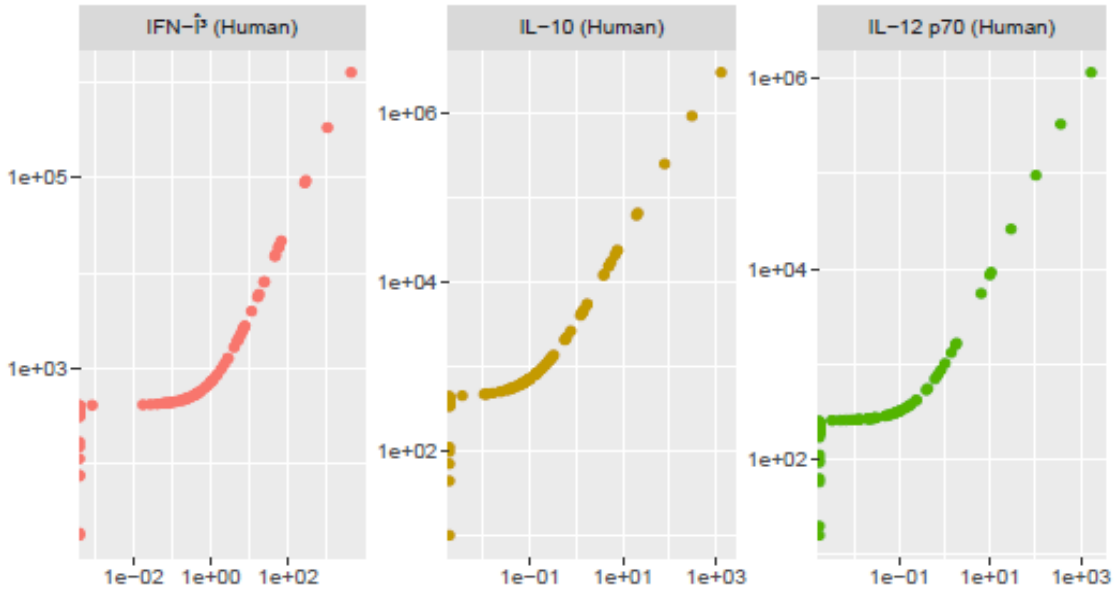

Figure s1. Plot for signal and concentration (Concentration as x, signal as y).

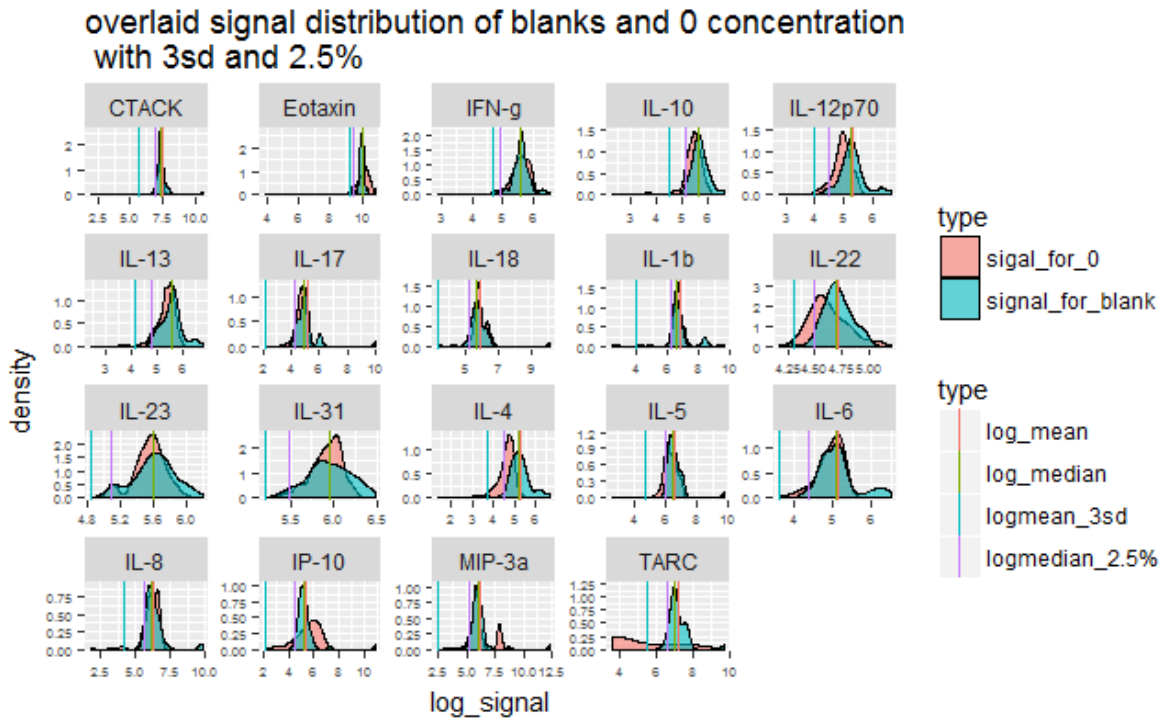

Figure s2. Density plot for signals from blank samples and signals from zero concentrations.

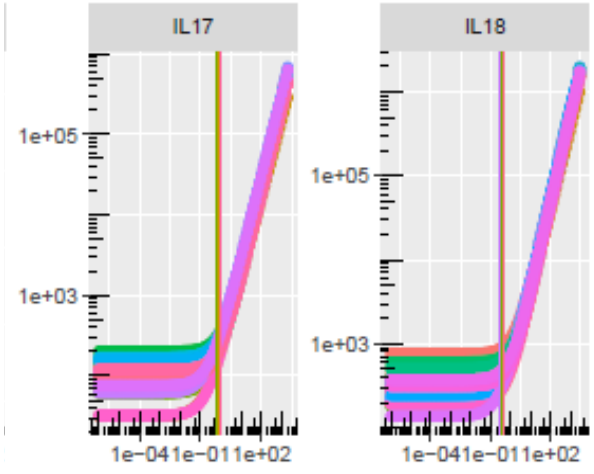

Figure s3. Overlaid standard sigmoid curves with LLOD as vertical line from multiple assays

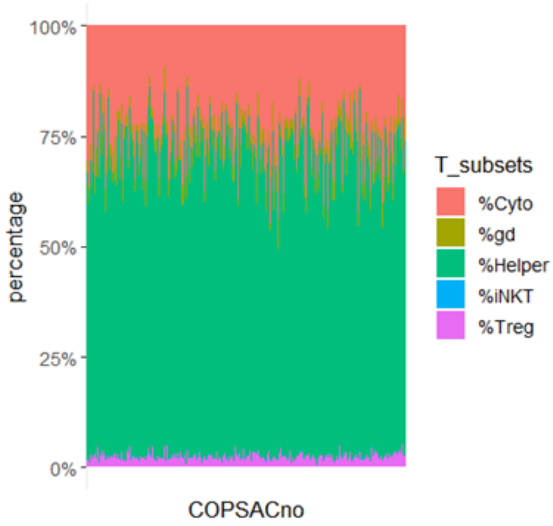

Figure s4. Frequency of each T cell subsets in PBMC samples

5 REDUCED IL-2 RESPONSE FROM PERIPHERAL BLOOD MONONUCLEAR CELLS EXPOSED TO BACTERIA AT 6 MONTHS OF AGE IS ASSOCIATED WITH ELEVATED TOTAL-IGE AND ALLERGIC RHINITIS DURING THE FIRST 7 YEARS OF LIFE

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Revised and Resubmitted to EBioMedicine. April 2019

5.1 ABSTRACT

Background: Autoimmunity and allergy have been associated with decreased number and function of regulatory T-cells and low interleukin-2 (IL-2) levels. We aimed to investigate if release of IL-2 from peripheral blood mononuclear cells (PBMCs) stimulated with pathogenic airway bacteria was associated with development of allergy-outcomes in early childhood.

Methods: PBMCs were isolated at age 6 months in 331 infants from the Copenhagen Prospective Studies on Asthma in Childhood 2000 ($COPSAC_{2000}$) mother-child cohort, and subsequently stimulated with *H. influenzae*, *M. catarrhalis* and *S. pneumoniae* in *in vitro* cultures. Levels of cytokines (IL-2, IL-10, IFN-γ, TNF-α, IL-5, IL-13 and IL-17A) were determined in the supernatant by electrochemiluminescence immunoassays. The immune profiles were analyzed for association with development of total-IgE, allergic sensitization and rhinitis during the first 7 years of life using regression models and principal component analysis (PCA).

Findings: An attenuated IL-2 response to stimulation with *H. influenzae* (p=0·011) and *M. catarrhalis* (p=0·027) was associated with elevated total-IgE at age 7. These findings were confirmed in a multivariate PCA model (PC2, p=0·032). An immune profile with both reduced IL-2 and elevated IL-5 was associated with increased risk of allergic rhinitis (PC3, p=0·038). We found no associations with development of allergic sensitization.

Interpretation: A reduced IL-2 response from PBMCs exposed to common pathogenic airway bacteria at age 6 months was associated with elevated total-IgE and allergic rhinitis during the first 7 years of life. These findings suggest that suppressed Treg activity in early life may herald onset of allergy in early childhood, which could be a target for low-dose IL-2 trials in the future.

5.2 INTRODUCTION

Reduced numbers and function of regulatory T-cells (Tregs) have been demonstrated in autoimmune and allergic diseases, suggesting that a dysregulated Treg system contributes to the pathogenesis of these conditions.[326–329] Interleukin 2 (IL-2) is critical for the development and survival of Tregs[330–332] and a possible role of the IL-2/Treg interplay in allergy has been

suggested by a study showing that reduced IL-2 expression by cord blood CD4(+) T cells associated with decreased Tregs and increased risk of food allergy in childhood.[333]

In the Copenhagen Prospective Studies on Asthma in Childhood 2000 ($COPSAC_{2000}$) mother-child cohort we conducted repeated clinical assessments including total-IgE, specific-IgE and allergic rhinitis through age 7 and biobanking of peripheral blood mononuclear cells (PBMCs) isolated from the infants at age 6 months. Recently, we investigated the PBMC response to *in vitro* stimulations with *H. influenzae*, *M. catarrhalis*, and *S. pneumoniae* measuring production of T cell-related cytokines of the Treg (IL-2, IL-10), Th1 (interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α)), Th2 (IL-5, IL-13) and Th17 (IL-17A) cells.[2,3]

The aim of the current study is to analyze the relationship between the PBMC response to bacterial stimulations and the development of allergy-related endpoints during the first 7 years of life, hypothesizing a protective role of IL-2.

5.3 MATERIALS AND METHODS

5.3.1 Study cohort

This study is part of the ongoing $COPSAC_{2000}$ prospective mother-child cohort of 411 children born to mothers with asthma.[334] The children were enrolled at age 1 month, excluded children born before gestational week 36 and children suffering from any respiratory disorder before enrollment. The children were followed prospectively at the COPSAC clinical research unit with regular follow-up visits every 6 months till age 7 and at episodes with respiratory symptoms. The clinical research unit was the primary health care facility for diagnosis and treatment of any respiratory symptoms, strictly adhering to validated algorithms.[335]

The study was approved by the Copenhagen Ethics Committee (KF 01-289/96), the Danish Data Protection Agency (2002-41-2434), and followed the principles of the Declaration of Helsinki. Written and oral informed consent was obtained from the parents at enrolment.

5.3.2 Bacterial stimulation of PBMCs

The bacterial stimulations of PBMCs was previously described in details.[2,3] Briefly, PBMCs were isolated by density centrifugation from blood samples collected at 6 months and stored for up to 12 years at -140°C. After thawing, the cells were stimulated with UV-inactivated *H. influenzae*, *M. catarrhalis*, and *S. pneumoniae* (50µg/mL) or blank sterile culture media alone in U-bottomed 96-well plates at $5x10^5$ cells/well (200µL total volume/well) for 40hours at 37ºC and 5% CO_2 in a humidified incubator. Supernatants were harvested and stored at -80ºC until quantification of IL-2, IL-10, IFN-γ, TNF-α, IL-5, IL-13 and IL-17A levels by customized multiplex immunoassays from MesoScale Discovery read on a Sector Imager 6000 (MSD, Gaithersburg, MD, USA). All assays were highly sensitive with a detection limit below 1pg/mL.

5.3.3 T cell immune phenotyping

The composition of the T cell compartment was analyzed on freshly thawed unstimulated PBMCs (5×10^5 cells) using flow cytometry. Staining and flow cytometry analysis were performed using the following antibody panel: CD3/eFlour450, CD8/FITC, TCRVa24-Ja18/PerCP-eFlour710, CD127/APC-eFlour780 (eBioscience, San Diego, CA), CD25/PC7, TCR γδ /PE (Beckman Coulter, Brea, CA) and CD4/V500 (BD Bioscience, San Jose, CA). T cell subsets were identified by a predefined gating strategy and analyzed in a blinded manner: Helper ($CD3^+CD4^+$), cytotoxic ($CD3^+CD8^+$), regulatory ($CD3^+CD4^+CD127^-CD25^+$), γδ ($CD3^+TCR\gamma\delta^+$) and invariant NK ($CD3^+TCRV\alpha24\text{-}J\alpha18^+$) T cells. All population frequencies were calculated relative to the $CD3^+$ T cell compartment.

5.3.4 Allergy-related endpoints

Total-IgE level was determined at 6 years by ImmunoCAP (Pharmacia Diagnostics AB, Uppsala, Sweden) and analyzed as a continuous variable.

Allergic sensitization was assessed at 6 years by skin prick tests (SPT) and measurements of specific-IgE against house dust mites, cat, dog, horse, birch, grass, mugwort, and molds as previous detailed.[336] SPT was done using allergen extracts (ALK Abello, Soluprick® SQ, Copenhagen, Denmark) defining a positive test as any wheal ≥3mm. Specific-IgE levels were measured with ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden), defining a positive test as any specific-IgE level ≥0·35kU_A/L. Sensitization was analyzed as a dichotomized variable, separately for SPT and specific-IgE as the overlap between test results is poor at young age.[337]

Allergic rhinitis at age 7 was diagnosed by the COPSAC pediatricians based on a parental interview on the child's history of symptoms.[338] Significant sneezing or blocked or runny nose affecting the wellbeing of the child in the past 12 months in periods without cold or flu defined rhinitis. The diagnosis required sensitization and congruence between symptoms and allergen exposure.

Statistical analysis

Initially, supernatant levels of cytokines in response to bacterial stimulations were adjusted by subtracting the baseline levels of cytokines secreted by PBMCs incubated with sterile media. Thereafter, the levels were square root-transformed. If an individual did not produce a measurable cytokine response in PBMCs after bacterial stimulation (non-responders), the cytokine level was set to zero.

First, we analyzed the association between the cytokines released upon bacterial stimulations at 6 months and total-IgE level (log-transformed), allergic sensitization by SPT (yes/no) and elevated specific-IgE (yes/no), and allergic rhinitis (yes/no) during the first 7 years of life using linear and logistic regression models. The associations with outcomes for cytokines from each bacterial stimulation were analyzed using forward stepwise selection while retaining IL-2. If the final model had multiple independent variables they were compared with a null model by ANOVA for goodness of fit. If only IL-2 was retained in the final model, the hypothesis test for its coefficient was used as assessment of goodness of fit. Only the models with significant overall p-values (≤0.05) are discussed in detail.

Thereafter, we conducted a data-driven principal component analysis (PCA) on the 21 independent variables (3 bacterial stimulation x 7 different cytokines) to capture immune patterns in the data, using principal component 1 to 4 (PC1-4) from the model for association analyses with the outcomes.

We also analyzed the association between T cell subsets and allergy-related outcomes. T cell composition data was transformed by isometric log ratio (ilr) using sequential binary partitioning and their association with outcomes was analyzed by multiple regression models.[321]

All analyses were conducted with R version 3.4.2.[339] The results are reported with 95% CI and p-values ≤0.05 were considered statistically significant.

5.4 RESULTS

5.4.1 Baseline characteristics

A total of 331 (81%) of the 411 infants in the cohort had PBMCs collected at age 6 months, which were subsequently exposed to *H. influenzae*, *M. catarrhalis,* and *S. pneumoniae* for assessment of the immune response by measuring supernatant cytokine levels. The T cell immune phenotyping and immune responses are described in **Tables 3-4**. Of the 331 infants, 259 (78%) had measurements of total-IgE (mean level, 117·6 IU/mL), 270 (82%) had SPT (N=251) and/or sIgE (N=259) with 100 (30%) being sensitized to one or more allergen either by SPT (N=40) and/or sIgE (N=94). Allergic rhinitis was assessed in 254 (77%) with a prevalence of 13% (N=34) by age 7 years.

A comparison between children with vs. without immune response data has previously been published showing no differences.[9,10]

Table 3. Supernatant cytokine levels in response to pathogenic airway bacteria at 6 months (n=331). IQR, Interquartile range.

	Control, median (IQR)	*H. influenzae, median (IQR)*	*M. catarrhalis, median (IQR)*	*S. pneumoniae, median (IQR)*
Cytokine supernatant				
IL-2 (pg/ml)	0.8 (0-4.2)	4.8 (1.2-14.6)	12.2 (3.2-28.5)	9.5 (1.4-41.8)
IL-10 (pg/ml)	0.0 (0-1)	15.0 (2.2-60.2)	22.5 (3.9-77.6)	1.8 (0-9.9)
IFN-γ (pg/ml)	0.4 (0-1)	4.5 (0.3-77.9)	19.9 (0.8-233.9)	1.4 (0.2-12.2)
TNF-α (pg/ml)	1.5 (0.5-4.1)	35.5 (8.2-169.2)	54.2 (17.7-201.7)	21.5 (5.2-101)
IL-5 (pg/ml)	0.0 (0.0-0.0)	0.0 (0.0-0.4)	0.4 (0.0-2.4)	0.0 (0.0-0.6)
IL-13 (pg/ml)	0.0 (0.0-0.0)	14.9 (0.0-75.5)	60.4 (5.7-203.3)	10.9 (0.0-71.6)
IL-17A (pg/ml)	0.0 (0.0-1.8)	1.9 (0.0-12.6)	9.4 (0.5-43.0)	2.2 (0.0-17.6)

Table 4. Composition of T cell compartment at 6 months (n=242). IQR, Interquartile range.

T cell profile	**Median(IQR)**
Helper T cells (%)	68.5(62.6-72.4)
Cytotoxic T cells (%)	24.1(20.6-29.8)
Regulatory T cells (%)	2.3(1.8-2.9)
γδ T cells (%)	3.0(2.2-3.9)
Invariant NK T cells (%)	0.1(0.1-0.1)

5.4.2 Bacteria-induced immune response and allergy-related outcomes

Total-IgE

Multiple regression analyses showed inverse associations between IL-2 production in response to *H. influenzae* and *M. catarrhalis* at age 6 months and total-IgE level at age 6 years: IL-2-coefficient$_{hi}$=-0·183 [95% CI; -0·324, -0·043], p=0·011 and IL-2-coefficient$_{mc}$=-0·150 [-0·282, -0·018], p=0·027, respectively. The IL-2 association was only seen in response to the Gram-negative bacteria *H. influenzae* (p_{Ftest}=0·028) and *M. catarrhalis* (P_{Ftest}=0·024), whereas

the model using cytokine data in response to the Gram-positive bacteria *S. pneumoniae* was not better than the null model (P_{Ftest}=0·139): IL-2-coefficient$_{sp}$=0·151 [0·001, 0·301], p=0·0503 (**Figure 8**).

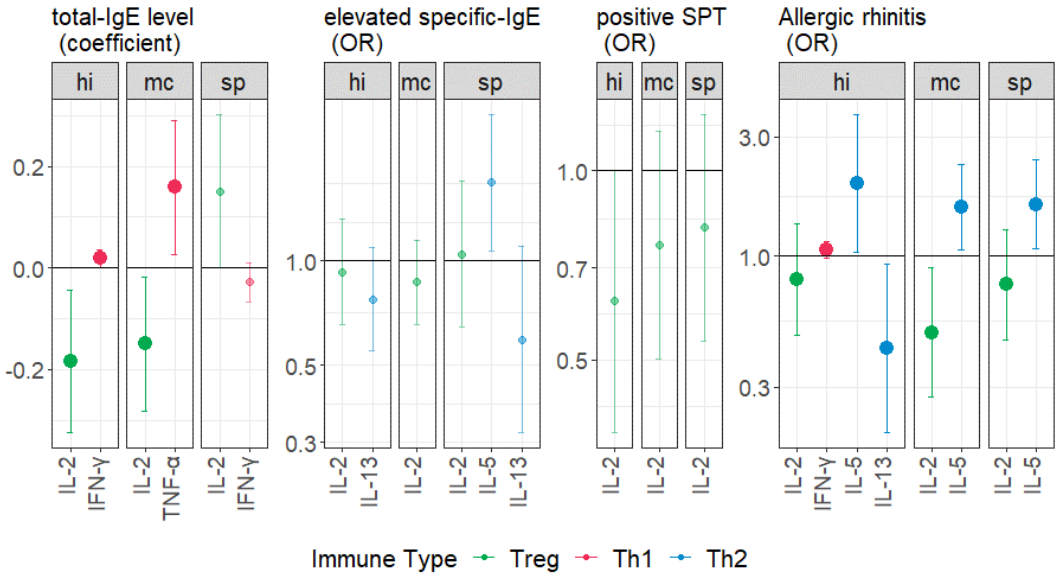

Figure 8. Association between cytokines upon stimulation with H. influenzae, M. catarrhalis and S. pneumoniae and log transformed total IgE, allergic sensitization determined by skin prick test and specific-IgE levelat 6 year, and allergic rhinitis at 7 year.

Besides IL-2, the production of IFN-ϒ and TNF-α in response to *H. influenzae* and *M. catarrhalis* were positively associated with total-IgE level: IFN-ϒ-coefficient$_{hi}$=0·019 [0·000, 0·0367], p=0·046, and TNF-α-coefficient$_{mc}$=0·159 [0·027, 0·290], p=0·019, respectively.

The result of a PCA analysis to identify individual cytokine profiles in response to the bacteria is depicted in **Figure 9** and **Figure e1**. The PCA association analysis for total-IgE confirmed the findings from the regression models. The cytokine pattern in PC2 from *M. catarrhalis* was inversely associated with total-IgE level (p=0·032) and the same trend was seen for PC2 from *H. influenzae* stimulation (p=0·051); i.e. higher total-IgE was associated with a decreasing score dominated by IL-2 (**Figure 9**).

Allergic sensitization

Higher IL-5 secretion in response to *S. pneumoniae* was significantly associated with sensitization diagnosed by sIgE at age 6: OR$_{sc}$=1·678 [1·069, 2·633], p=0·024, although the overall model with inclusion of all cytokines was not significantly better than the null model (overall p-value=0·080). No associations were observed between the immune response to the other bacterial stimulations and development of allergic sensitization determined by neither sIgE nor SPT (**Figures 8-9**).

Allergic rhinitis

IL-2 in response to *M. catarrhalis* and IL-13 in response to *H. influenzae* were inversely associated with allergic rhinitis by age 7: OR$_{mc}$=0·495 [0·273, 0·898], p=0·021, and OR$_{hi}$=0·429

[0·197, 0·933], p=0·033; i.e. higher IL-2 and IL-13 production reduced the risk of allergic rhinitis. We also found that elevated IL-5 in response to all the bacterial stimulations increased the risk of allergic rhinitis: OR_{hi}=1·954 [1·037, 3·681], p_{hi}=0·038, OR_{mc}=1·569 [1·056, 2·330], p_{mc}=0·026, and OR_{sp}=1·614 [1·070, 2·434], p_{sp}=0·022. The multiple regression models were all significantly better than the null models: p_{chisq}=0·048 for *H. influenzae*, p_{chisq}=0·017 for *M. catarrhalis*, and p_{chisq}=0·046 for *S. pneumoniae* (**Figure 8**).

The results from the PCA supported the main findings from the regression analyses regarding IL-5 and IL-2, but not for IL-13. We found an inverse association between development of allergic rhinitis and the immune pattern in PC3 upon *M. catarrhalis* stimulation (p=0·038), i.e. lower IL-2 and higher IL-5 from PBMCs at 6 months increased the risk of allergic rhinitis at age 7. In addition, a decreasing PC4 score from the *H. influenzae* stimulation was significantly associated with risk of allergic rhinitis, i.e. lower IL-17A and higher IFN-γ release increased the risk of allergic rhinitis (p=0·048) (**Figure 9**).

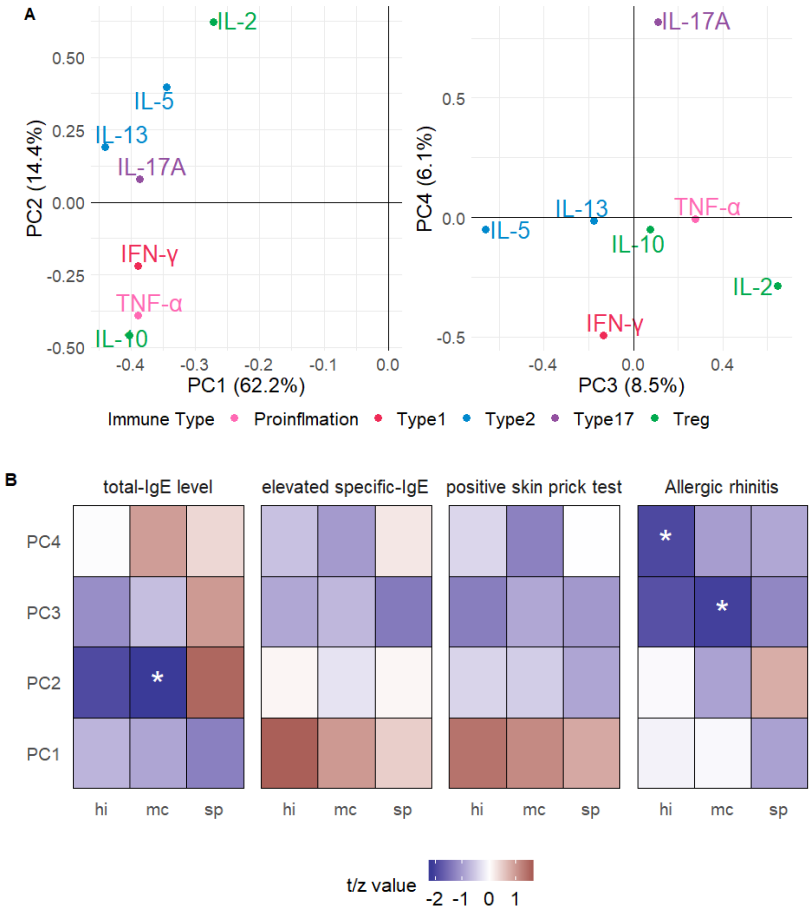

Figure 9. Association between cytokine profiles from 6 month (n=331) upon stimulation with H. influenzae, M. catarrhalis and S. pneumoniae and log transformed total IgE, allergic sensitization determined by skin prick test and specific-IgE level at age 6 and allergic rhinitis at age 7.

A. Cytokine loadings of PC1 to PC4 from principle components analysis (PCA).

B. Ordinary least square regression for log transformed total-IgE level and simple logistic regression for the rest clinical outcomes was each regressed on PC1-4.

Sensitivity analysis

As many of the children did not produce any IL-5 in PBMCs after bacterial stimulations (IL-5 non-responders), we conducted a sensitivity analysis excluding these (**Table e1**). This showed similar results for the univariate association between IL-5 and allergic rhinitis by age 7: OR_{hi}=2·532 [1·127, 5·690], p_{hi}=0·025, OR_{mc}=3·231 [1·274, 8·200], p_{mc}=0·014, and OR_{sp}=1·729 [1·084, 2·757], p_{sp}=0·021, and the multiple regression models were also significantly better than the null models: p_{chisq}=0·040 for *H. influenzae*, p_{chisq}=0·001 for *M. catarrhalis*, and p_{chisq}=0·048 for *S. pneumoniae* (**Figure e2**). The PCA results were consistent showing an inverse association between allergic rhinitis and the immune pattern in PC3 upon stimulations from both *M. catarrhalis* (p=0·036) and *H. influenzae* (p=0·038) (**Figure e3**).

5.4.3 T cell compartment composition

To study if the cytokine response profiles were related to an underlying difference in T cell subsets, we examined the T cell compartment composition in relation to the allergy-outcomes. The relative ratios of γδ T cells and iNKT cells amongst all T cells were associated with total-IgE level at age 6, but in opposite directions with an inverse association identified for iNKT cells (p=0·030), and positive association for γδ T cells (p=0·022) (**Figure 10**, **Figure e4**). No associations between the relative ratio of Tregs and development of allergy-related outcomes were observed (**Figure 10**).

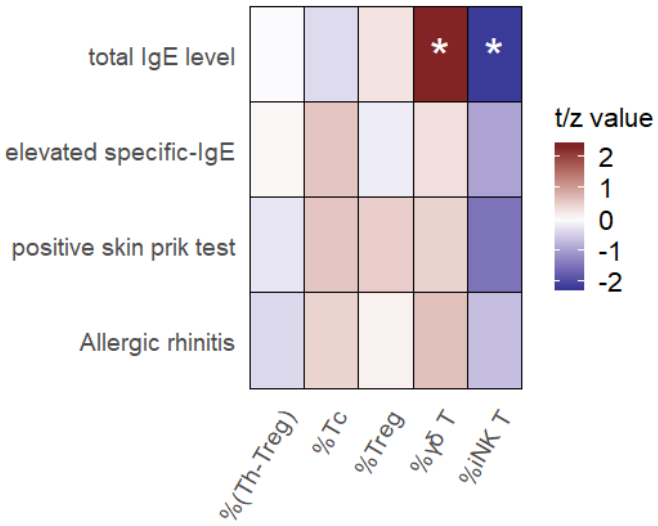

Figure 10. The association between T cell compartment at 6 month and allergy outcomes at age of 6 year.

5.5 DISCUSSION

Main findings

An attenuated IL-2 response to stimulation with the common Gram-negative pathogenic airway bacteria *H. influenzae* and *M. catarrhalis* in PBMCs collected at age 6 months was associated with elevated total-IgE at age 6 years. Furthermore, both reduced IL-2 and increased IL-5 production were associated with a higher risk of developing allergic rhinitis during the first 7 years of life. Altogether, these findings suggest a role of diminished IL-2 and increased IL-5 in development of allergy-related traits in early childhood.

Strengths and limitations

This is the first prospective cohort study to investigate infant's bacterial immune responses in PBMCs, which was done in a comprehensive manner by analyzing several cytokines of the Treg, Th1, Th2, and Th17 cells. The prospective nature of our study and the storage of PBMCs at age 6 months provide a unique possibility to investigate functional immunological properties in infancy before onset of allergy-related outcomes. As the PBMCs were collected in asymptomatic infants before onset of allergic disease, the bacterial immune response is unlikely to be affected by immune dysfunction caused by ongoing disease-driven inflammation.

It is an advantage of the study that the children participating in the $COPSAC_{2000}$ cohort solely used the COPSAC research pediatricians for diagnosis and treatment of any asthma and allergy-related disease. All diagnoses were done based on rigid standardized algorithms providing highly reliable homogeneous clinical outcomes.

It is a limitation that this was an *in vitro* study that may not reflect the complexity of the immune responses *in vivo*. Importantly, many of the infants stored PBMCs did not produce a measurable IL-5 response to any of the bacterial stimulations. However, analyses including the IL-5 non-responders as zeros as well as analyses excluding the non-responders showed similar associations with the clinical endpoints.

It is a limitation that the results are based on one concentration of antigen performed at one time point (40 hours culture). Therefore, we are not able to determine how the results would look like at different time points and by stimulating with different concentrations, which is particularly important for IL-2, which is consumed in the cultures. However, this would have been of greater importance if we had not detected an association between IL-2 PBMC responses and allergy-outcomes.

Finally, it is a limitation that all the children participating in the cohort were born to mothers with a history of asthma, which may hamper the external validity of our findings.

Interpretation

In this study, we aim to identify immune patterns in infancy in response to different bacteria in relation with development of allergic disorders in the first 7 years of life. The bacterial stimulations were chosen as we previously demonstrated that one-month-old infants colonized in the airways with *H. influenzae*, *M. catarrhalis*, and/or *S. pneumoniae* had an increased risk of asthma at age 5 years.[1] However, no direct link was shown between neonatal airway colonization with these bacteria and allergic outcomes, but studies indicate that environmental exposure to certain bacteria or bacterial components like lipopolysaccharide, which is contained in *H. influenzae* and *M. catarrhalis*, are associated with risk of allergic sensitization in childhood.[33,340,341]

We speculate that our finding of a reduced IL-2 production in concert with increased IL-5 to stimulations with the Gram-negative bacteria *H. influenzae* and *M. catarrhalis* may be caused by imbalanced Treg and Th2 populations and thereby increase the risk of developing allergic diseases. This finding may be of clinical importance as low-dose IL-2 therapy is being investigated with the purpose of expanding the Treg population in several diseases. We did not observe similar findings when analyzing the cytokine responses from the Gram-positive *S. pneumonia* stimulation, which may be due to the fact that this type of bacteria does not contain the innate immune ligand lipopolysaccharide.

Recent clinical trials have studied the tolerability and efficacy of low-dose IL-2 therapy in the treatment of autoimmune disorders and graft-versus-host disease.[342–346] All trials reported the

therapy to be safe and effective in mediating sustained expansion of systemic Tregs with clinically relevant improvement in patients with HCV-induced vasculitis,[342] alopecia areata,[346] and graft-versus-host disease.[345] We speculate that low-dose IL-2 therapy may have a beneficial role on allergy and propose initiation of clinical trials in children with allergy and subsequently trials investigating low-dose IL-2 as a possible therapeutic intervention or a prophylactic regimen in high-risk infants. Due to the proposed early window of immune programming for regulatory T cell development,[347] it might be speculated to be even more important to propagate Treg development in early infancy, once an impaired IL-2 production has been detected. We here addressed the question of IL-2 production in PBMCs collected at 6 months of age, as bacteria-specific T cells will have been expanded at this age if the child has been exposed to the given bacteria in vivo. This is in contrast to studies performed on cord blood. We selected a relatively short stimulation protocol of 40 hours with the aim of examining specifically the bacteria-specific memory and effector T cells present in the PBMC fraction that would be more readily activated for cytokine production than naïve T cells, which require a longer protocol. Although this study examines immune programming prior to disease development, it might also be valuable to use IL-2 therapy in already diseased individuals. Prior to trials in allergic diseases, it would valuable to investigate IL-2 PMBC responses in children with vs. without ongoing symptoms of allergic rhinitis.

Although IL-2 has pleiotropic effects and works on other types of Th effectors, the efficacy and specificity of low dosage IL-2 therapy may be supported by the selective responsiveness of Tregs against low does IL-2. It was reported that due to an enhanced expression of the IL-2 receptor (α and Ɣ chain) and promoted activity of endogenous serine/threonine phosphatase protein phosphates 1 and/or 2A in Tregs about 10-100 fold lower levels of IL-2 was required to activate STAT5 in Tregs compared to the levels needed for memory T cells or activated T cells. [348]

It is possible that reduced IL-2 production during the continuous immune activation induced by the host microbiome in infancy could affect the risk of developing allergic disease. It is recognized that the microbiome plays a key role in development of the immune system and the establishment of homeostatic tolerance by Tregs.[279,349,350] Thus, infants who respond to bacteria with lower IL-2 production could have an imbalanced immune system with blunted Treg function leading to proneness to develop higher circulating IgE levels, diminished control of tolerance to allergens, and subsequently develop symptomatic disease such as rhinitis.

We have previously within the same cohort reported abnormal bacterial immune response in PBMCs in infants developing asthma later in childhood.[2] The immune responses associated with asthma were dominated by increased Th2 cytokines (IL-5 and IL-13) in response to the same three pathogenic airway bacteria. However, in contrast to the current findings, no associations existed between IL-2 release and development of asthma, which underlines different immune patterns driving the heterogeneous phenotypes of allergy and asthma and suggests that low-dose IL-2 therapy might be targeted solely to the allergic phenotype. This fits well with a recent study from a food allergy mouse model showing that low dose IL-2 treatment could control the allergic inflammatory process by inducing expansion and activation of the Treg population. [344] Further experimental studies of i.e. allergen-specific stimulations of human PBMCs are needed to determine whether IL-2 therapy should solely be targeted to the allergic phenotype.

Higher IL-13 production upon bacterial stimulation of the PBMCs was observed to reduce the risk of allergic rhinitis, which was however not significant in the multivariate PCA approach. This is an unexpected finding as co-expression of IL-4, IL-13 and IL-5 are normally observed in patients with ongoing symptoms of rhinitis. A non-coordinate expression of IL-4, IL-13 and IL-5 can occur when distinct Th2 clones produce each cytokine individually. Thus, the discrepancy in expression of IL-5 and IL-13 may suggest a transient expression pattern

associated with the initial stage of Th2 priming as we investigated the response from stimulated PBMCs in 6-month-old asymptomatic infants long before onset of any symptoms of allergic rhinitis.

Our T cell compartment data showed an association between the composition of γδ T cells and total-IgE level suggesting a possible function of γδ T cells in the development of allergic diseases. Such association has previously been reported in children with atopic dermatitis aged 1 to 10 years, whereas no association was observed for total-IgE level within these children with atopic dermatitis.[351] Finally, we also identified an inverse association between the composition of iNKT cells and total-IgE, which is consistent with previous findings in adult asthmatics.[352]

Conclusion

Reduced IL-2 production and increased IL-5 from PBMCs in response to bacterial stimulations in early life were associated with development of elevated total-IgE and allergic rhinitis during the first 7 years of life. This finding is of possible clinical relevance as experimental studies have shown that low-dose IL-2 therapy can restore immune tolerance by expansion of Tregs.

5.6 SUPPLEMENTARY PLOTS AND FIGURES

Table e1. Supernatant IL-5 levels in response to pathogenic airway bacteria at 6 months (n=239).

	Control, median (IQR)	*H influenzae, median (IQR)*	*M catarrhalis, median (IQR)*	*S pneumoniae, median (IQR)*
Cytokine supernatant				
IL-5 (pg/ml)	0·0 (0-0·04)	0·12 (0-0·77)	1·29 (0·24-4·11)	0·14 (0-1·25)

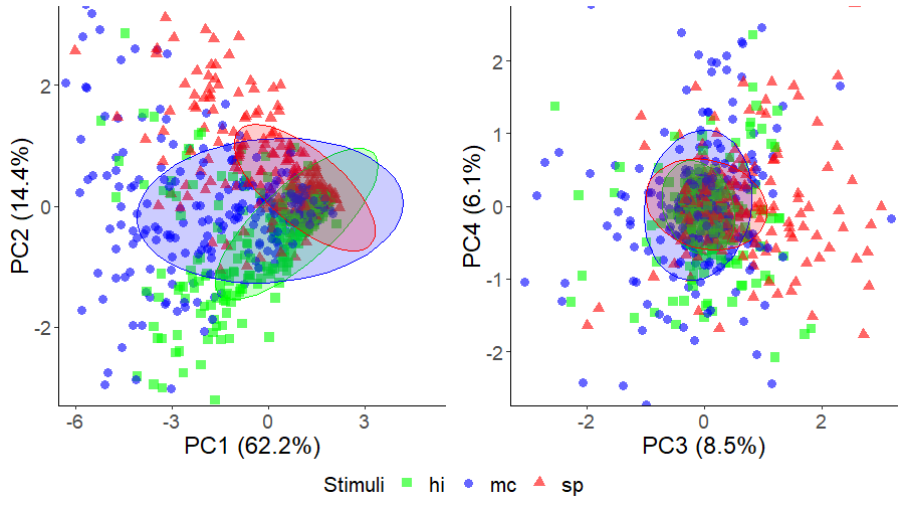

Figure e1. The score plots of principle components 1-4 (PC1-4) from principle components analysis (PCA) based on cytokine profiles from 6 month (n=331) upon stimulation with H. influenzae, M. catarrhalis and S. pneumoniae. The 90% confidence ellipse are added.

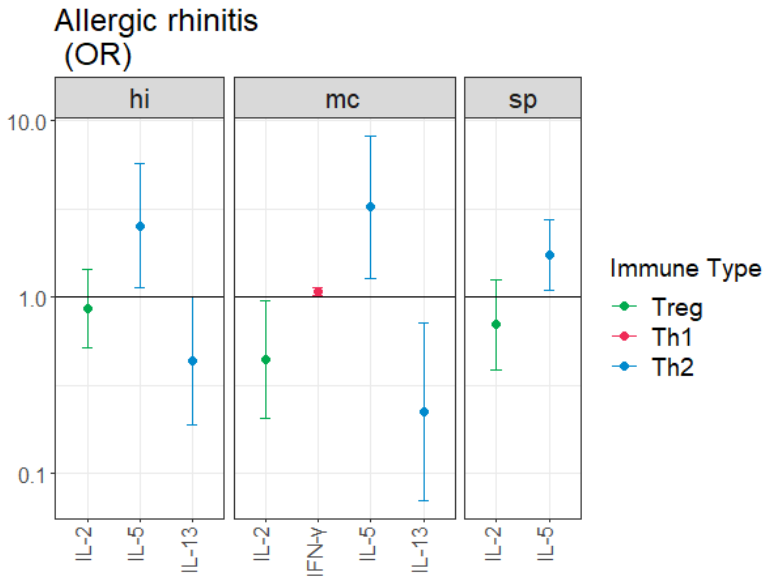

Figure e2. Sensitivity analyses of the association between selected cytokines and allergic rhinitis

Association between cytokines from age 6 month (n=239) after removal of the IL-5 non-responders upon stimulation with H. influenzae, M. catarrhalis and S. pneumoniae and allergic rhinitis (case/control=27/120) at 7 year

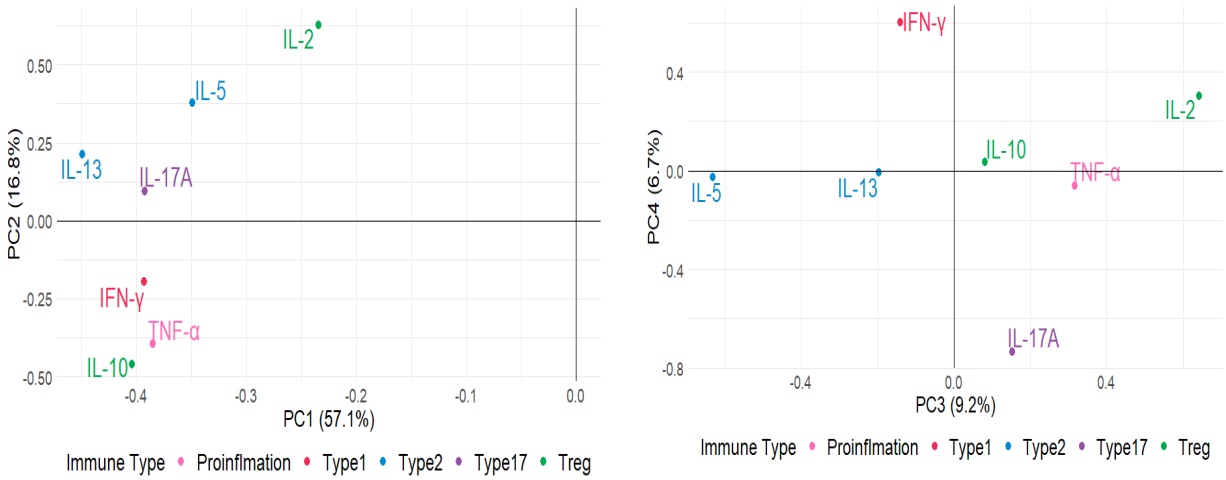

Figure e3. Cytokine loadings of PC1 to PC4 from principle component analysis (PCA) after removal of IL-5 non-responders.

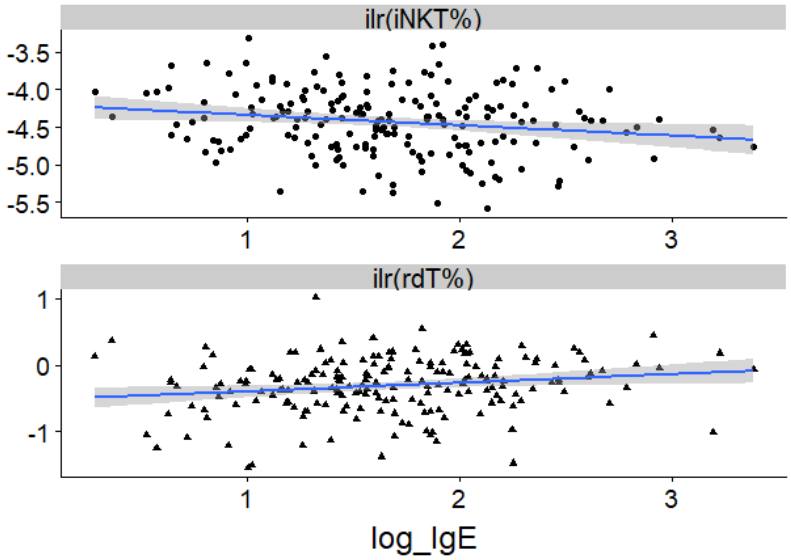

Figure e4. The scatter plot about association between T cell compartment and log transformed IgE level at age 6

6 INNATE IMMUNE RESPONSES TO BACTERIA AND VIRUSES MEDIATE THE EFFECT OF THE 17Q21 HIGH-RISK LOCUS ON CHILDHOOD ASTHMA

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Manuscript in preparation

6.1 ABSTRACT

Background: Several asthma risk loci that relate to immune functions have been identified by genome-wide association studies, but the underlying mechanisms have not been uncovered.

Objective: To examine whether perturbed innate immune responses are part of the causal pathway between identified risk variants and asthma using mediation analysis.

Methods: Peripheral blood mononuclear cells (PBMCs) collected from 336 infants at 6 months of age from the Copenhagen Prospective Studies on Asthma in Childhood 2000 (COPSAC2000) mother-child cohort were stimulated with six different innate ligands (LPS, CpG, poly(I:C), R848, HDMAPP, alum crystals together with low LPS) ex vivo followed by quantification of supernatant levels of 18 cytokines by immunoassays. The innate immune response profiles were decomposed by principal component (PC) analysis and represented by PC1-5, which were used in mediation analyses between 25 lead risk variants from a recent meta-genome-wide association study and investigator-diagnosed childhood asthma-endpoints through age 7.

Results: The effects of two variants from the 17q21 locus on asthma and asthma exacerbations were significantly mediated by the immune profiles in response to ligands mimicking bacterial DNA (CpG) and double-stranded viral RNA (poly(I:C)). PC3 from both CpG and poly(I:C) stimulations significantly mediated 18.4% and 12.1% of the increased risk of asthma through age 7 from ORMDL3 with p_{CpG}=0.03 and $p_{poly(I:C)}$=0.02. PC3 from CpG stimulation significantly mediated 15.4% and 18.1% of the increased risk of exacerbations before age 3 from GSDMB (p=0.04) and ORMDL3 (p=0.01). The most prominent cytokine in the significant immune profiles involved enhanced IL-23.

Conclusion: These results indicate that the 17q21 locus confer an increased risk of childhood asthma and exacerbations via deregulated production of IL-23 in response to intracellular bacterial and viral ligands. This response may promote ineffective neutrophilic recruitment to the lungs via innate lymphoid cell (ILC) 3 or Th17 activation.

6.2 INTRODUCTION

Heritability may explain as much as 25-80% of childhood asthma.[252] Several asthma risk loci and their lead single-nucleotide polymorphisms (SNPs) have been identified by independent genome-wide association studies (GWAS)[353 ,22,16,274] and a recent large consortium-based meta-GWAS study involving different ethnic groups confirmed multiple risk loci for asthma and

suggested their involvement in immune regulation by demonstrating enrichment in the enhancer regions of immune cells.[263] Possible biological mechanisms underlying these associations have been suggested by functional studies, including altered metabolic pathways and aberrant expression of immune mediators.[354] However, it remains to be elucidated how the replicated genetic variants, e.g.ORMDL3, GSDMB and CDHR3, affect immune regulation in early life and lead to an increased risk of asthma in childhood.

In the Copenhagen Prospective Studies on Asthma in Childhood 2000 ($COPSAC_{2000}$) birth cohort we harvested peripheral blood mononuclear cells (PBMCs) at age 6 months and subsequently quantified the innate immune response by measuring levels of cytokines secreted in response to in vitro stimulations with a panel of six different bacterial and viral ligands. In the current study, we aimed to investigate whether the early life innate immune response represented by these six different signaling pathways mediates the relationship between replicated risk alleles and development of childhood asthma and lung function traits in the first 7 years of life.

6.3 METHODS

6.3.1 Study cohort

This study is part of the $COPSAC_{2000}$ prospective clinical mother-child cohort study, which enrolled 411 children born to mothers with active or previous doctor-diagnosed asthma in the Copenhagen metropolitan area during the period 1998-2001, previously described in details.[355,334] In brief, the children were enrolled at age 1 month and were followed at the COPSAC clinical research unit with scheduled 6-monthly visits until age 7 years as well as at incidences of acute respiratory symptoms.[1,356] The families used COPSAC as their primary health care facility for the diagnosis and treatment of any airway, allergic or skin symptoms, strictly adhering to predefined, validated algorithms. [1,356]

The study was approved by the Copenhagen Ethics Committee (KF 01-289/96), the Danish Data Protection Agency (2002-41-2434), and followed the principles of the Declaration of Helsinki. We followed Good Clinical Practice guidelines for data validation and quality control. Written and oral informed consent was obtained from the parents at enrolment prior to any study related procedures.

6.3.2 Bacterial and viral ligand stimulation of PBMCs

The assessment of the innate immune response from PBMCs upon bacterial and viral ligand stimulations was similar to whole bacterial stimulations described previously.[83,3] PBMCs were isolated by density centrifugation from blood samples collected at 6 months of age and stored for up to 12 years at -140°C. After thawing, the cells were stimulated in U-bottomed 96-well plates at $5x10^5$ cells/well; 200µL total volume/well in complete media (RPMI 1640 medium completed with 2mM L-glutamine (Cambrex, East Rutherford, NJ), 0.1 M HEPES (Lonza), 100 U/ml penicillin/streptomycin (Lonza) and 10% heat-inactivated fetal bovine serum) for 40 hours at 37°C and 5% CO_2 in a humidified incubator with addition of one of 6 ligands activating different innate immune pathways (lipopolysaccharide (ultrapure *E. coli* LPS (tlrl-pelps, Invivogen, France), 200ng/mL)), unmethylated cytosine poly guanosine DNA (CpG (ODN2216, TIB MOLBIOL, Berlin), 30µg/mL), polyinosinic:polycytidylic acid (poly(I:C) (tlrl-picw-250, Invivogen, France), 50µg/mL), resiquimod (R848 (tlrl-r848-250, Invivogen, France), 2 µg/mL), 1-hydroxy-2-methyl-2-buten-4-yl 4-diphosphate (HDMAPP (I-M055, Echelon Biosciences, USA), 0.1 µg /mL) and aluminum potassium sulfate (alum crystals (tlrl-alk, Invivogen, France), 100 µg /mL) in concert with low dose LPS (5ng/mL) (lowLPSalum) or complete media alone (unstimulated). Supernatants were harvested and stored at -80°C until the quantification of the

following 18 cytokines: IFN-γ, IL-12p70, IL-18, IL-4, IL-5, IL-13, IL-31, CCL17, CCL24, IL-1β, IL-6, IL-17A, IL-23, CCL20, CXCL8, IL-22, CCL27 and IL-10 levels by customized multiplex immunoassays from MesoScale Discovery read on a Sector Imager 6000 (MSD, Gaithersburg, MD, USA). All assays were highly sensitive (Median and IQR of each cytokine are given in the **Online** Methods, Table E1).

6.3.3 Asthma risk loci

We selected 25 top SNPs (**Online Table E2**) from replicated asthma risk loci encompassing *RAD50, IL33, IL1R1, DENND1B, CDHR3, ORMDL3, GSDMB, NDFIP1/GNDPA1/SPRY4, GPX5/TRIM27, BACH2/GJA10/MAP3K7, STAT6/NAB2/LRP1, ZNF652/PHB, MICB/HCP5/MCCD1, GATA3/CELF2, TPD52/ZBTB10, CLEC16A/DEXI/SOCS1, IL1RL1/IL1RL2/IL18R1, TSLP/SLC25A46, IL4/IL13/RAD50, HLA-DRB1/HLA-DQA1, RANBP6/IL33, EMSY/LRRC32, RORA/NARG2/VPS13C, SMAD3/SMAD6/AAGAB, ERBB2/PGAP3/MIEN1* genes based on a recent meta-GWAS and other GWAS stuides.[353,22,263] Allelic discrimination of those SNPs in COPSAC2000 was investigated by genome-wide genotyping using the Infinium HumanOmniExpressExome BeadChip Kit (Illumina, San Diego, Calif). Sample and marker quality control were performed as previously reported.[263]

6.3.4 Clinical endpoints

Asthma was diagnosed based on a predefined, previously validated diagnostic algorithm,[1] requiring all of the following: (1) recurrent wheeze, i.e. diary recordings of at least 5 episodes of troublesome lung symptoms within the preceding 6 months, each lasting at least 3 days; (2) symptoms typical of asthma, e.g. exercise-induced symptoms, prolonged nocturnal cough, persistent cough outside common cold; (3) rescue use of inhaled short-acting bronchodilator; and (4) response to a 3-month trial of inhaled corticosteroids and relapse after end of treatment.

Persistent asthma was diagnosed in children fulfilling the above diagnostic criteria at any time point during childhood and still needing inhaled corticosteroids to control their symptoms in the 7th year of life, whereas transient asthma denotes children fulfilling the diagnostic criteria, but outgrowing their symptoms before age 7.

Asthma exacerbations were defined by symptoms requiring hospitalization, oral prednisolone (1-2mg/kg for 3 to 7 days) or high-dose ICS treatment (at least 1600mcg budesonide per day for 14 days).[275]If the child was admitted directly to the hospital without involvement of the COPSAC research unit, hospital records were retrieved and reviewed to confirm that symptom history and treatment fulfilled the above criteria.

Lung function at age 6: Spirometry was performed using a MasterScope Pneumoscreen spirometer (Erich Jäeger, Würzburg, Germany). A minimum of three tests with a within-test difference in forced expiratory volume in the first second (FEV1) of maximum 10% was completed. The best FEV1 and the corresponding maximal mid-expiratory flow (MMEF) were used in the analysis after calibrating the values for age, sex and height.

Whole-body plethysmography was done using a MasterScope sealed bodybox (Erich Jäeger, Würzburg, Germany). A minimum of two assessments of specific airway resistance (sRaw) with a within-test difference of maximum 0.3 kPa/s were obtained, using the mean of the two measurements in the analysis after calibrating the values for age, sex and height.

Bronchial responsiveness was assessed by measuring FEV1 after a saline inhalation and after subsequent inhalations of methacholine in quadrupling dose steps (APS Pro, CareFusion, 234

GmbH, Germany).[357] The provocative dose of methacholine producing a 20% fall in FEV1 (PD20) was estimated from the dose-response curve fitted with a logistic function.

Lower respiratory tract infections (LRTI) at age 0-3 years included pneumonia and bronchiolitis. Pneumonia was defined by significant cough, tachypnea, fever, and abnormal lung stethoscopy, whereas bronchiolitis was diagnosed in children with cough, tachypnea, chest retractions, auscultative widespread crepitation and/or rhonchi before age 1 year.[358–360]

6.3.5 Covariates

Covariates included a composite score for social circumstances (first principle component (PC) of PC analysis (PCA) of maternal educational level, annual family income, and maternal age); sex; older siblings; delivery mode; cat exposure at birth; parental smoking and passive smoking; antibiotic exposure during pregnancy; father history of eczema and asthma; mother history of eczema and allergy. Baseline characteristics of the covariates can be found in online **Table E3**.

6.3.6 Statistical analysis

Data transformation

Initially, the supernatant levels of cytokines in response to the innate ligand stimulations were adjusted for background levels by dividing stimulated cell values with the corresponding baseline level secreted from unstimulated PBMCs for each child. Thereafter, the levels were log-transformed to improve normal distribution prior to the statistical analysis. To consider the synergistic effects among the cytokines, a PCA for each ligand was employed to decompose the 18-dimensional cytokine data structure into fewer components utilizing the first five PCs in the analyses.

Models for SNPs

Associations between genotypes and the clinical phenotypes were examined using linear, logistic, quasi-Poisson, and a cox proportional hazard regression model based on the type of clinical endpoints. Genotypes with significant effect on clinical endpoints ($p<0.05$) were further studied by mediation analysis. All SNPs were analyzed by an additive model (0, 1 or 2 risk alleles) with the exception of the two lead SNPs from the 17q21 locus, which were examined using a recessive model.

Mediation analysis

Our current work was mainly built upon a causal mediation analysis generated by a mediation package from R.ref.[361,323] This package was written according to the potential outcome framework using a simulation-based method consisting of two regression model based equations for estimating the casual mediation effect. This approach was a generalized method and was applicable to both linear and non-linear model as well as non/semi-paramatric models, for different types of mediators and outcome variables.

The total effect of the selected SNPs on the specific clinical outcome was decomposed into a direct effect (ADE in the mediation output), which was independent of the immune responses, and indirect effect (ACME in the mediation output), which was mediated by the immune responses. Hence, the indirect effect (ACME) was the term we were interested in as this is the part of the SNP vs. clinical outcome effect mediated via the innate immune response. Ingrained with all types of mediation analysis is bias brought by confounders, which can be removed by adjusting for those confounders in the corresponding regression-based models

while performing the mediation analysis. We selected potential confounders based on the immune profiles, which showed a significant mediating effect in the unadjusted mediation analysis. Covariates associated with either the immune profile PCs and/or the clinical endpoints were subsequently included in the mediation analysis and an adjusted mediation effect was obtained in addition to the unadjusted mediation effect.

All analyses were performed in R with the packages: mediation.

6.4 RESULTS

6.4.1 Baseline characteristics

A total of 336(82%) of the 411 infants in the $COPSAC_{2000}$ cohort had both genotyping and PBMCs collected at age 6 months, which were subsequently stimulated with six different ligands mimicking various bacterial and viral pathogen-associated molecular patterns (PAMPs) for assessment of the secreted cytokine response by innate immune cells. Baseline results of the immune assessments are shown in Online **Table E1.**

Among the 336 infants, 62 (18.5%) developed asthma at some point in their first 7 years of life: 25 (40%) had transient asthma, and 37 (60%) had persistent asthma. At age 6 years, 225 (67%) of the 336 children had available PD20 data, 257 (76%) had spirometry assessments of FEV1 and 254 (76%) had MMEF data, and 255 (76%) had measurements of airway obstruction determined by sRaw. Acute asthma exacerbations occurred in 53 (19%) of the children before age 7 and LRTI before age 3 was recorded in 165 (56%) of children. Baseline characteristics and descriptive statistics of clinical endpoints are given in **Table E3**.

A comparison of the 336 children included in the study versus the 75 excluded children is outlined in **Table E3,** showing that the children included in the study did not suffer from selection bias.

6.4.2 SNPs, innate immune profiles and clinical phenotypes

First, we examined the association between the selected asthma risk SNPs and the clinical phenotypes (the SNP allele frequencies among 336 included children are shown in **Online Table E2)**. Several of the selected SNPs were associated with the clinical phenotypes (***Figure 11***).

Particularly, we observed that SNPs in the 17q21 locus, rs7216389 in the ORMDL3 gene and rs2305480 in GSDMB, were associated with an increased risk of developing asthma in the first 7 years of life: ORMDL3, HR=1.80[1.12, 2.90], p=0.01 and GSDMB, HR=1.73[1.04, 2.86], p=0.03. In particularly transient asthma: ORMDL3, OR=2.60[1.16, 5.82], p=0.02 and GSDMB, OR=2.78[1.15, 6.72], p=0.02, increased number of acute asthma exacerbations until age 7: ORMDL3, RR=2.23[1.17, 4.27], p=0.02 and GSDMB, RR=2.15[1.03, 4.47], p=0.04, and increased number of LRTI from 0-3 years of age: ORMDL3, RR=1.50[1.13, 1.99], p=0.01 were associated with the asthma risk alleles.

Second, we analyzed the associations between asthma SNPs and the ligand-stimulated immune profiles captured in PC1-5, restricting the analyses to SNPs showing effects on the clinical phenotypes. Several statistically significant relationships were observed as shown in **Figure 12A**. Of note, both ORMDL3 and GSDMB genotypes were significantly associated with the poly(I:C)-stimulated immune profile captured in PC3, and the ORMDL3 genotype was further associated with the CpG-stimulated immune profiles in PC3. These SNPs were also

both significantly associated with several of the asthma-related clinical phenotypes outlined in **Figure 11**.

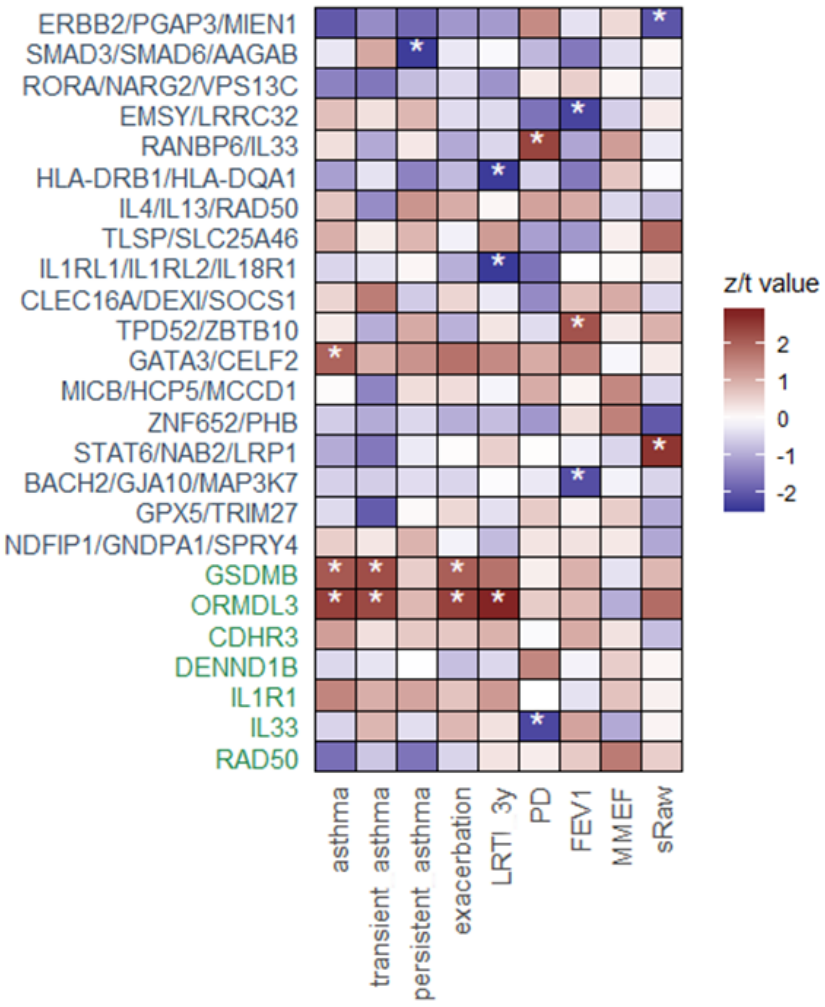

Figure 11. Total effect of risk SNPs on asthma, number of exacerbations, and lung function until age 7, and number of lower respiratory tract infections until 3 years of age.

SNP variants are designated by the names of their associated genes. The association of SNPs and clinical endpoints were examined by regression analysis, where z/t represents test statistics of the coefficients for logistic regression or linear regression. Significant associations ($p < 0.05$) are labeled with an asterisk.
Abbreviations: LRTI: Number of lower respiratory infections until 3 years of age. FEV1: A minimum of three tests with a within-test difference in forced expiratory volume in the first second. PD: The provocative dose of methacholine producing a 20% fall in FEV1. MMEF: maximal mid-expiratory flow. sRAW: A minimum of two assessments of specific airway resistance.

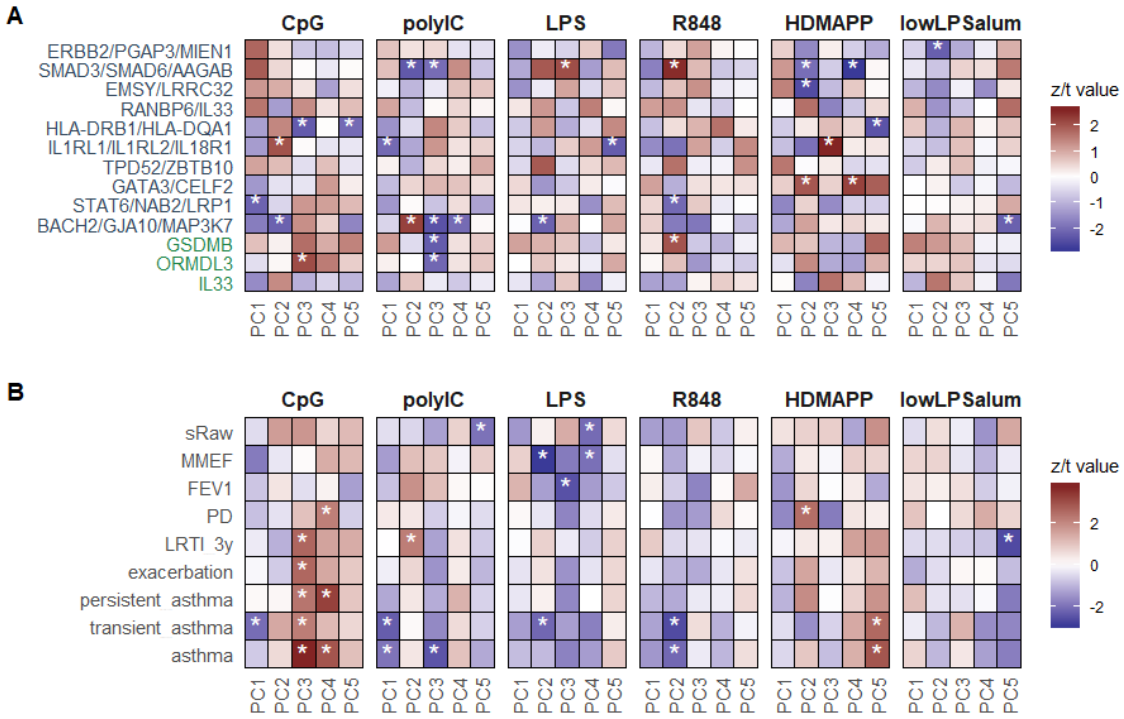

Figure 12. Associations between risk SNPs, clinical endpoints and immune profiles summarized by a principal component analysis.

Immune cytokine profiles were generated based on stimulation of PBMCs from 6-month-old infants with six different ligands activating different arm of the innate immune system.

- *A. The association between immune profiles and the clinically association SNPs (fig. 11) as examined by linear regression analysis. SNP variants are designated by the names of their associated genes*
- *B. Associations between immune profiles and clinical endpoints as examined by regression models. All significant associations are labeled with an asterisk. z/t represents test statistics of the coefficients for logistic regression or linear regression.*

Abbreviations: LRTI: Number of lower respiratory infections until 3 years of age. FEV1: A minimum of three tests with a within-test difference in forced expiratory volume in the first second. PD: The provocative dose of methacholine producing a 20% fall in FEV1. MMEF: maximal mid-expiratory flow. sRAW: A minimum of two assessments of specific airway resistance.

Third, we investigated associations between the ligand-stimulated innate immune profiles and the clinical phenotypes (**Figure 12B**). Amongst those immune profiles associated with ORMDL3 (**Figure 12A**), PC3 from CpG-stimulation and polyIC-stimulation were both strongly associated with development of asthma in the first 7 years of life: HR_{CpG}=1.50[1.21, 1.86], p_{CpG}=0.00; $HR_{poly(I:C)}$=0.76[0.61, 0.94], $p_{poly(I:C)}$=0.013, and CpG-PC3 was also associated with other asthma-related phenotypes (**Figure 12B**). The GSDMB linked immune profiles polyIC-PC3 and R848-PC2 (HR_{848}=0.84[0.71, 0.98], p_{R848}=0.03) both associated with asthma development (**Figure 12B**). In the following mediation analysis, we investigated the mediation effect of all the immune profile (PC1-5 from 6 ligands) for the significant pairs of genotype and clinical phenotypes.

6.4.3 Genotype-innate immune response-clinical phenotype mediation analysis

The potential outcome mediation analysis was based on regression models with 5000 bootstrap simulations and reported as the total effect, direct effect (ADE) and indirect effect (ACME) (**Figure 13A**).

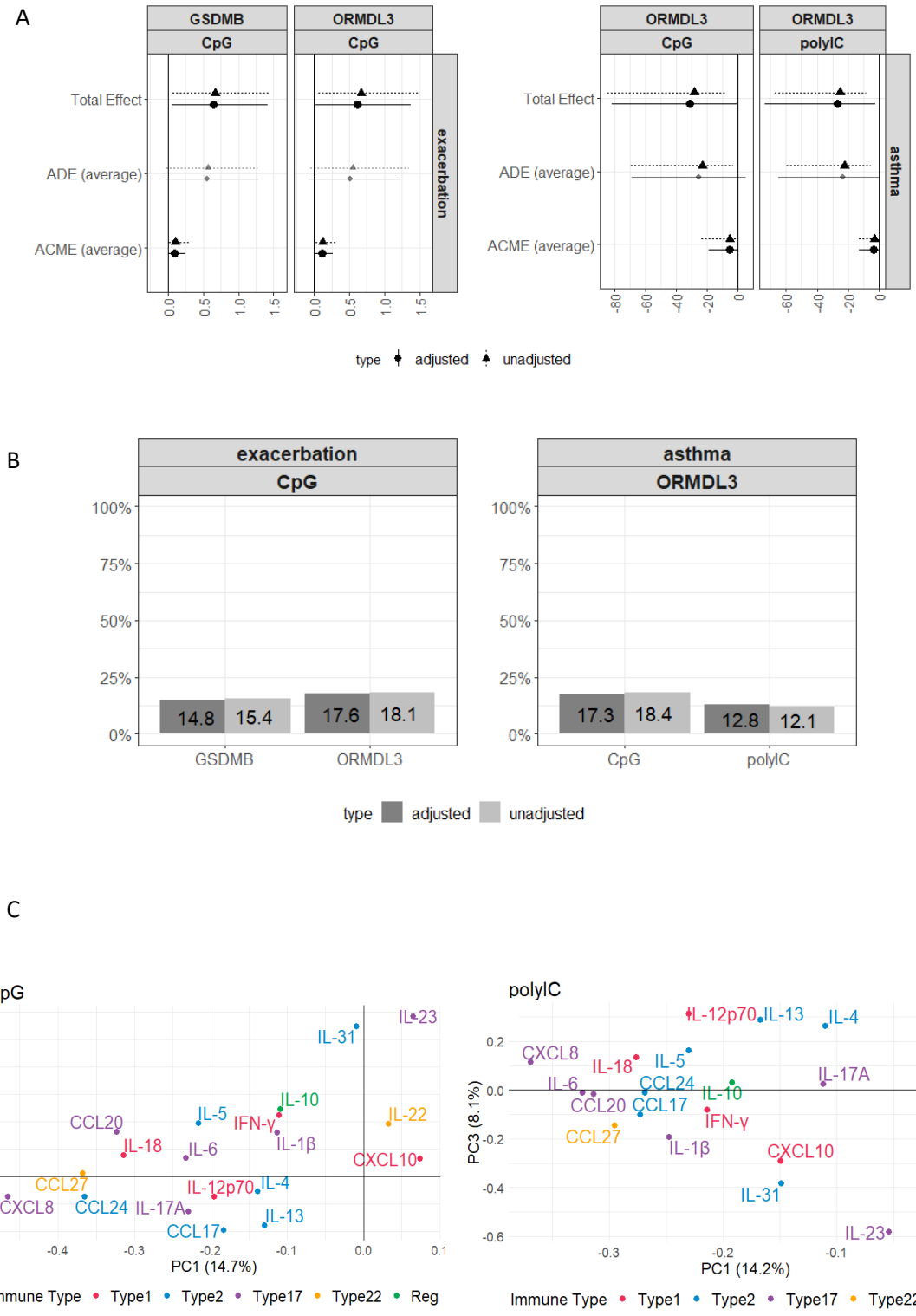

Figure 13. The significant mediation effect transmitted by innate immune responses to CpG and poly(I:C).

***A**: The average indirect effect (ADE), the direct effect and the total effect (ACME) from raw and confounder adjusted mediation analysis. The confounding variables adjusted for are delivery mode (Cesarean), older sibling at birth, smoking during 3rd trimester, and antibiotic usage during 3rd trimester.*
***B**: The proportion of variance in the clinical endpoints explained by the innate immune profiles for CpG*

and poly(I:C). ***C:*** *Loading plots of the PCA components found to significantly mediate the effect of the risk SNPs on asthma and number of asthma exacerbations.*

Genotype to asthma development mediated via the innate immune response

The mediation analysis for asthma until 7 years was based on an accelerated failure time (AFT) model in which a negative estimate suggests for a higher disease risk, i.e. less time needed to for development of asthma. Hence, the negative total effect mediated by CpG-PC3 ($Estimate_{CpG}$ = -5.20[-23.90, -1.28], p_{CpG} =0.03) and poly(I:C)-PC3 ($Estimate_{poly(I:C)}$ = -3.04[-13.00, -0.60], $p_{poly(I:C)}$ =-0.02), illustrated that children with the minor variant ORMDL3 (rs7216389) developed asthma faster as compared to those with the major variant (**Figure 13A** and **Table 5**) and that they each explained 18.4% and 12.1% of the increased risk from ORMDL3 (rs7216389) on asthma, respectively (**Figure 3B**). Adjusting the mediation analysis for maternal use of antibiotics in 3rd pregnancy trimester, smoking in 3rd trimester, delivery by Cesarean section, older siblings at birth and father's history of eczema, which were all associated with either immune profiles and/or clinical phenotypes (**appendix Figure E1**), did not change the outcome of the analysis (CpG-PC3: Estimate=-5.30[-18.70, -0.02] and poly(I:C)-PC3: Esitmate = -3.45[-12.70, -0.01]), explaining 17.3% and 12.8% of the increased risk of ORMDL3(rs7216389) on asthma, respectively (**Table 5**).

Table 5. Mediation analysis for asthma

Asthma			
ORMDL3			
Ligand	***Unadjusted***	***Adjusted***	
	Estimate(95%CI)	***Estimate(95%CI)***	***term***
CpG	***-5.20(-23.90, -1.28)***	***-5.36(-18.72, -0.02)***	Indirect effect (ACME)
	-23.09(-69.29, -3.15)	-25.61(-68.93, 5.15)	Direct effect (ADE)
	-28.3(-84.52, -8.28)	***-30.96(-81.39, -0.81)***	Total Effect
polyIC	***-3.04(-12.98, -0.6)***	***-3.45(-12.67, -0.01)***	Indirect effect (ACME)
	-22.06(-59.69, -5.26)	-23.58(-64.70, 0.77)	Direct effect (ADE)
	-25.10(-67.38, -8.12)	***-27.03(-73.40, -2.24)***	Total Effect

Genotype and development of exacerbations until school-age is mediated via specific innate immune response

The mediation analysis for exacerbations was based on a Poisson model in which the positive estimate suggested for a higher risk. For the number of asthma exacerbation until 7 years of age, the PC3 immune profile derived from CpG stimulated PBMCs was found to significantly mediate the effect of both rs7216389 (ORMDL3, Estimate=0.12 [0.03, 0.32], p=0.01) and rs2305480 (GSDMB, Estimate=0.10 [0.01, 0.30], p=0.04), detailed information can be found in **Figure 13A and Table 6**. The immune profile represented by CpG-PC3 accounts for 18.1% and 15.4%, respectively of the total effect of rs7216389 and rs2305480 on increased risk of exacerbation. Confounding adjustment did not change the outcome of the mediation analysis

(ORMDL3: Estimate=0.09 [0.01, 0.24] and GSDMB: Estimate=0.11 [0.02, 0.27]), contribution of 14.8% and 17.6% respectively.

The type of immune mediation effects

Focusing on the immune patterns mediated by CpG-PC3 and poly(I:C)-PC3, it appears from the loading plots (Figure 12C) that the immune mediation effect on childhood asthma and number of asthma exacerbations in children with the 17q21 locus risk variants (rs7216389 (ORMDL3) and rs2305480 (GSDMB)) was driven by synergistic interplay between several cytokines. Enhanced production of IL-23 and IL-31 seemed to main contributors. However, we found no significant mediation by single cytokines (data not show), emphasizing the importance of the interrelationship between multiple cytokines in the mediation aggravated via CpG-PC3 and poly(I:C)-PC3.

Table 6. Mediation analysis for Exacerbation

Exacerbation			
CpG			
SNP	***Unadjusted***	***Adjusted***	
ORMDL3	***Estimate(95%CI)***	***Estimate(95%CI)***	***term***
	0.12(0.03,0.32)	***0.11(0.02,0.27)***	Indirect effect (ACME)
	0.55(-0.05,1.34)	0.51(-0.08, 1.24)	Direct effect (ADE)
	0.67(0.07,1.49)	***0.61(0.02,1.37)***	Total Effect
GSDMB	***0.10(0.01,0.30)***	***0.09(0.01, 0.24)***	Indirect effect (ACME)
	0.57(-0.02, 1.27)	0.56(-0.03, 1.29)	Direct effect (ADE)
	0.67(0.06, 1.43)	***0.65 (0.05, 1.42)***	Total Effect

6.5 DISCUSSION

By investigating the mediation effect of innate immune responses in infants, we identified a specific deregulated immune response to intracellular microbial recognition to aggravate the link between development of childhood asthma and number of asthma exacerbations until 7 years of age and 17q21 locus SNP variants. The immune profile in stimulated PBMCs from affected children depended on an array of interacting cytokines, dominated by enhanced IL-23 secretion. **Advantages and limitations**

The prospective nature of our mother-child cohort study and the storage of PBMCs at age 6 months provide a unique possibility to investigate functional immunological properties in infancy before onset of asthma-related outcomes. As the PBMCs were collected in asymptomatic infants, the innate immune response is less likely to be affected by immunological dysfunction caused by ongoing disease-driven inflammation. Furthermore, this is to our knowledge the first prospective clinical cohort study to perform a comprehensive investigation of the innate immune response in infants PBMCs across multiple ligands and cytokines of the Type 1 (IFN-γ, IL-12p70, IL-18), Type 2 (IL-4, IL-5, IL-13, IL-31, CCL17, CCL24), Type 17 (IL-1β, IL-6, IL-17A, IL-23, CCL20, CXCL8), Type 22 (IL-22, CCL27) and

regulatory (IL-10) associated responses. However, a limitation is that this was an *in vitro* study that may not reflect the complexity of the immune responses happening *in vivo*.

Another advantage of this study is that mothers of all recruited infants were asthmatic and of Caucasian origin living in the Copenhagen area. This criterion ensures a relatively homogeneous genetic background of the study population thus eliminating the chance of possible confounders associated with population stratification. Moreover, another advantage of the study is that the children recruited by the $COPSAC_{2000}$ cohort were primarily diagnosed and treated by COPSAC research pediatricians for any allergy, airway or skin symptoms. This provides high reliability in the clinical outcomes based on the use of the same rigid standardized algorithms.

One limitation was that it was impossible to identify all confounders in the mediation analysis, thus caution is needed when explaining the mediation effect obtained here. One unmeasured confounder may relate to the airway microbiota. A great impact of microbiota on both the immune system and asthma has been suggested.[362,363] Hence, we should be conservative when presenting the identified mediation effect given that the sensitivity analysis for assessing some of the contingent variables of the mediation effect are missing. Moreover, due to an extensive linkage disequilibrium between rs7216389 (ORMDL3) and rs2305480 (GSDMB), it was hard to separate the effect from each other; hence it is safer to refer to them as 17q21 locus risk variants.

Main findings and interpretation

In this study, our primary aim was to find whether and how much validated asthma risk SNPs increased the susceptibility to asthma development until 7 years of age by modulating the innate immune response in asymptomatic infants. The mediators were innate immune responses against different PAMPs, represented by cytokines secreted from different activated immune pathways. The primary outcomes were time until asthma occurs by age 7 years and numbers of exacerbations before age 7. We found cytokine profiles upon activation by the ligands mimicking bacterial unmethylated DNA (CpG) and viral double-stranded RNA (poly(I:C)) significantly mediated the effect of two lead SNPs in the 17q21 locus (rs7216389 and rs2305480) on asthma. We did not detect significant effects of other SNPs on the clinical endpoints in our study, which was not surprising, since the effect sizes of those SNPs on asthma are quite small and larger sample sizes are usually needed in order to identify statistically significant associations.

The cytokine patterns in response to CpG and poly(I:C) with significant mediation effect were very similar, and both found to be mediated by an array of different cytokines dominated by elevated IL-23. The ligand ODN2206-CpG mimics the unmethylated DNA structure uniquely found in bacteria, and is a potent stimuli of human Toll like receptor 9 (TLR9).[92,98,103] The ligand poly(I:C) resembles dsRNA from viral infection, and is an agonist of human Toll like receptor 3 (TLR3).[98,100,99] Similar to TLR9, activation of the TLR3 pathway also leads to the release of type 1 interferons and pro-inflammatory cytokines.[100] Type 1 immune responses, as triggered by TLR9 and TLR3 pathways, enable elimination of intracellular infectious agents by cellular mediated killing of infected host cells via NK cells and cytotoxic T cells.

The association studies between risk SNPs and immune profiles shown in Figure 2a and loading plot of PCA analysis in Figure 3c suggested that in response to CpG and poly(I:C), instead of producing type 1 cytokines resembled by IFN-γ, IL-12p70, IL-18 and CXCL10, carriers of the risk alleles (rs7216389 and rs2305480) tended to express enhanced IL-23. For human immune cells, IL-23 is indispensable and pivotal in promoting expansion of Type-17-associated immune cells including Th17 and ILC3 from corresponding precursors as well as sustaining and expanding the memory Th17 cells.[201] By secreting Type-17 signature cytokines

such as IL-17, IL-22 and granulocyte–macrophage colony-stimulating factor (GM-CSF), those Type-17 cells are responsible for clearing off extracellular infection from both bacteria and fungi, mainly due to stimulation of CXCL8 production by nearby cells, resulting in recruitment of neutrophils and macrophages to the site of infection. Besides the aforementioned Type 17 immune cells, IL-23 in combination with IL-1β can also stimulate γδT cells and NKT cells to produce IL-17A.[364,365] Hence, both innate and adaptive Type 17 immune cells are all closely related to enhanced IL-23. Upregulation of IL-23 towards intracellular viral and bacterial ligands suggest for ineffective clearance of viruses and intracellular bacteria, which may lead to continuous exposure to these agents, concomitant with a cytokine environment in favoring the Type-17 immune response, and hence neutrophil recruitment. Combined, the innate immune response against CpG and poly(I:C) suggested for an incorrect immune response represented by a dampened Type-1 immune response against intracellular ligands (i.e. intracellular microbes) accompanied by a skewed Type-17 immune response mounted by carriers of 17q21 locus risk alleles (rs7216389 and rs2305480). Based on this we therefore speculate that carriers of the 17q21 locus risk alleles may hold a compromised Type-1 immune response accompanied by a predominant Type-17 immune response against viral and intra cellular bacterial infections in early life. Such dysfunctioning immune responsiveness would lead to recurrent infections and prolonged IL-23 secretion which further enhance the recruitment of neutrophils and macrophages by supporting Type-17 cells differentiation and activation, with progressive development of asthma exacerbations due to continued immune activation in the airways and asthma during childhood.[366,367] The role of the Type-17 skewed immune profiles suggested by our results are consistent with others and our previous report indicating a non-atopic pathway of 17q21 locus-associated childhood asthma.[274,275,262]The association between a Type-17 based immune profile and 17q21 SNPs suggested by our finding is also in line with a previous study by others in which they found children with risk alleles of rs7216389 had elevated IL-17A secretion in their cord blood.[368] We did not identify elevated IL-17A in our study, but this may mainly be due to the experimental design, as we focused on innate immune cell activation and harvested the supernatant already at 40 hours post-stimulation, which is a bit too early to stimulate IL-17A secretion.[369]

Altogether, these data point to a deregulated immune response towards intracellular microbes in infants to partly explain the enhanced risk of asthma exacerbations and development of childhood asthma in carriers of the 17q21 risk alleles.

Conclusion

Deregulated innate immune responses from PBMCs against viral and bacterial neucleic acid was identified to mediate the effect of 17q21 locus on childhood asthma.

6.6 SUPPLEMENTARY TABLES AND PLOTS

Table E1. Levels of secreted cytokines from PBMCs of 6 months old children stimulated with the indicated ligands. Median (IQR) in pg/mL is listed. N=337.

	Unstimulated	LPS	CpG	poly(I:C)	R848	HDMAPP	lowLPSalum
Cytokine							
IFN- γ	0.3(0.1,0.6)	0.4(0.2,1.4)	0.7(0.3,1.8)	0.4(0.1,0.8)	1.6(0.4,14)	2.9(1.1,9.0)	0.4(0.1,1.1)
IL-12p70	0.0(0.0,0.1)	0.1(0.0,0.2)	0.0(0.0,0.1)	0.0(0.0,0.1)	0.2(0.1,0.5)	0.0(0.0,0.1)	0.1(0.0,0.2)
IL-18	0.2(0.0,0.3)	0.2(0.1,0.5)	0.3(0.2,0.5)	0.3(0.2, 0.4)	0.5(0.2, 1.0)	0.2(0.1,0.4)	0.2(0.1, 0.4)
CXCL10	0.5(0.3,1.3)	0.5(0.2,1.1)	18.9(3.6,85)	1.7(0.5,8.3)	11.8(3.6,39.8)	4.0(0.8,17.7)	0.4(0.2,0.8)
IL-4	0(0,0)	0(0,0.1)	0(0,0)	0(0,0)	0.1(0,0.2)	0(0,0)	0(0,0.1)

IL-5	0(0,0)	0(0,0.1)	0.1(0,0.2)	0(0,0.1)	0(0,0.1)	0(0,0.1)	0(0,0.1)
IL-13	0(0,0.2)	0(0,0.8)	0(0,0.5)	0(0,0.3)	0.5(0, 1.3)	0(0,0.4)	0(0,0.9)
IL-31	0(0,0.1)	0(0,0.3)	0(0,0.2)	0(0,0.1)	0(0,0.1)	0(0,0.1)	0(0,0.1)
CCL17	3.4(2.3,5.3)	4.8(3.2,7.6)	2.7(1.8,3.9)	4.3(2.9,6.0)	7.4(4.4,12.5)	3.9(2.6,5.8)	4.4(3.0,6.8)
CCL24	51.9(15.6,441.0)	162.0(32.7,424.0)	19.2(11.2,30.8)	69.4(13.7,302)	42.8(18.7,89)	105.0(16.5,612.0)	113.0(21.8,370)
IL-1β	0.2(0,0.6)	2.4(0.7,11.0)	0.2(0,0.5)	0.2(0.1,0.6)	6.6(1.7,22.2)	0.2(0.1,0.7)	2.1(0.5,8.9)
IL-6	0.7(0.3,1.3)	18.8(3.0,130.0)	8.9(4.1,16.6)	1.2(0.6,2.6)	61.2(18.2,224.0)	0.8(0.4,1.7)	13.3(2.2,106.0)
IL-17A	0.2(0,0.6)	0.4(0.1,0.8)	0.2(0,0.4)	0.2(0,0.5)	0.4(0.1,0.7)	0.3(0,0.8)	0.4(0.1,0.7)
IL-23	0(0,0)	0.4(0,3.3)	0.1(0,0.6)	0(0,0.5)	0(0,1.7)	0(0,0)	0(0,2.0)
CCL20	0.3(0,0.6)	3.8(0.7,28.9)	0.5(0.3,0.9)	0.6(0.2,1.1)	4.7(1.1,19.8)	0.4(0.1,0.8)	2.6(0.6,21.9)
CXCL8	115.0(44.4,287.0)	552.0(136.0,4142.0)	128.0(70.5,278.0)	160.0(68.9,364.0)	1222.0(320.0,5333.0)	148(62.7,445.0)	435.0(127.0,3784.0)
IL-22	0(0,0)	0(0,8.4)	0(0,5.2)	0(0,6.2)	5.6(0,18.9)	0(0,0)	0(0,6)
CCL27	11.6(7.4,17,4)	13.7(7.8,17.5)	10.0(6.3,13.9)	10.2(5.7,16)	11.4(6.9,15.1)	14.8(9.8,21.2)	12.5(8.2,17.1)
IL-10	0.1(0,0.1)	0.2(0,1.3)	0.7(0.2,1.9)	0.1(0,0.2)	1.8(0.5,6)	0.1(0,0.2)	0.1(0,0.1)

Table E2. Frequency of SNPs for children having successful PBMC stimulation

Genes	rsID	value	n	Frequency
RAD50	rs6871536	0	178	0.6
		1	101	0.34
		2	19	0.06
IL33	rs928413	0	154	0.52
		1	124	0.42
		2	20	0.07
IL1R1	rs1558641	0	4	0.01
		1	73	0.24
		2	221	0.74
DENND1B	rs2786098	0	189	0.63
		1	96	0.32
		2	13	0.04
CDHR3	rs6967330	0	204	0.68
		1	80	0.27
		2	14	0.05
ORMDL3	rs7216389	0	230	0.69
		1	102	0.31
GSDMB	rs2305480	0	196	0.66
		1	102	0.34
NDFIP1/GNDPA1/SPRY4	rs7705042	0	144	0.48
		1	121	0.41
		2	33	0.11
GPX5/TRIM27	rs1233578	0	5	0.02
		1	77	0.26
		2	216	0.72
BACH2/GJA10/MAP3K7	rs2325291	0	123	0.41
		1	137	0.46
		2	38	0.13
STAT6/NAB2/LRP1	rs167769	0	52	0.17
		1	143	0.48

		2	103	0.35
ZNF652/PHB	rs17637472	0	58	0.19
		1	145	0.49
		2	95	0.32
MICB/HCP5/MCCD1	rs2855812	0	24	0.08
		1	123	0.41
		2	151	0.51
GATA3/CELF2	rs2589561	0	7	0.02
		1	85	0.29
		2	206	0.69
TPD52/ZBTB10	rs12543811	0	43	0.14
		1	125	0.42
		2	130	0.44
CLEC16A/DEXI/SOCS1	rs17806299	0	201	0.67
		1	90	0.3
		2	7	0.02
IL1RL1/IL1RL2/IL18R1	rs1420101	0	49	0.16
		1	142	0.48
		2	107	0.36
TLSP/SLC25A46	rs10455025	0	47	0.16
		1	132	0.44
		2	119	0.4
IL4/IL13/RAD50	rs20541	0	14	0.05
		1	107	0.36
		2	177	0.59
HLA-DRB1/HLA-DQA1	rs9272346	0	121	0.41
		1	132	0.44
		2	45	0.15
RANBP6/IL33	rs992969	0	20	0.07
		1	121	0.41
		2	157	0.53
EMSY/LRRC32	rs7927894	0	48	0.16
		1	149	0.5
		2	101	0.34
RORA/NARG2/VPS13C	rs11071558	0	228	0.77
		1	66	0.22
		2	4	0.01
SMAD3/SMAD6/AAGAB	rs2033784	0	34	0.11
		1	99	0.33
		2	165	0.55
ERBB2/PGAP3/MIEN1	rs2952156	0	33	0.11
		1	122	0.41
		2	143	0.48

Table E3. Baseline characteristics of covariates and clinical endpoints

Covariates	$COPSAC_{2000}$ 411	Study Cohort 336	Excluded children 75	P value
Antibiotics in 3rd trimester	14.4% (59)	13.4%(45)	18.7%(14)	0.32
Cat in house in pregnancy	15.2% (60)	14.2%(47)	19.7%(13)	0.35
Delivery mode (Cesarean)	21.2% (87)	21.1%(71)	21.3%(16)	1.00
Paternal asthma*	14.2% (57)	13.8%(45)	16.2%(12)	0.72
Paternal dermatitis*	10.7% (36)	11.7%(33)	5.5%(3)	0.26
Maternal dermatitis*	42.6% (147)	42.2%(122)	44.6%(25)	0.85
Maternal allergy to inhaled allergen*	86.9% (357)	87.8%(295)	82.7%(62)	0.32
Any older siblings	40.4% (166)	39.3%(132)	45.3%(34)	0.40
Passive smoke in 3rd trimester	10.0% (41)	9.5%(32)	12.0%(9)	0.66
Sex(Boy)	49.4% (203)	50.3%(169)	445.3%(34)	0.52
Smoking in 3rd trimester	15.3% (63)	14.3%(48)	20.0%(15)	0.29
Household income above 800K	8.7% (33)	8.8%(29)	7.7%(4)	1.00
Mother's age at birth (yr), mean(SD)	30.1 (4.5)	30.1(4.5)	29.6(4.4)	0.35^{t}
Mother's highest education**	15.2% (58)	15.2%(50)	15.4%(8)	1.00
Asthma until 7y*	17.5%(72)	18.5%(62)	13.3%(10)	0.38
Transient asthma*	9.3% (27)	9.7%(25)	6.5%(2)	0.80
Persistent asthma*	15.2%(47)	14.4%(39)	21.6%(8)	0.36
Exacerbation*	20.4%(64)	19.1%(53)	31.4%(11)	0.14
LRTI before 3y*	56.3%(188)	55.7%(165)	60%(23)	0.70
FEV1, mean(SD) *	-0.03(0.99)	-0.01(1.0)	-0.23(0.90)	0.20^{t}
MMEF, mean(SD) *	-0.11(1.04)	-0.11(1.04)	-0.06(1.07)	0.80^{t}
sRaw, mean(SD) *	0.11(1.01)	0.11(1.03)	0.18(0.88)	0.67^{t}
PD, mean(SD) *	-0.09(1.03)	-0.05(1.04)	-0.36(0.87)	0.10^{t}

Baseline comparisons between children in the study cohort and excluded. Except Household income above 800K which was compared by unpaired student's t-test, all the other comparisons were done by Pearson's chi-squared test.

*t : unpaired student's t-test. *Doctor diagnosed disease. **University education*

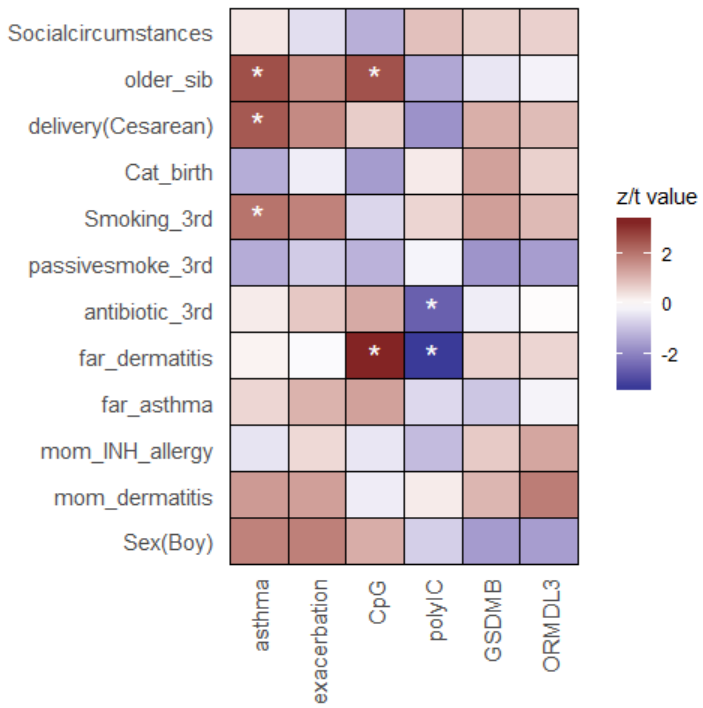

Figure E1. Associations between covariates and SNPs, clinical endpoints and immune profiles to find possible confounders.

The associations were examined by corresponding regression models and significant associations were labeled with an asterisk. Social circumstances describe the composite score based on mother's highest education level, mother's age at birth, and the annual family income. Abbreviation: mom_INH_allergy: allergy in the mother to inhalant allergens.

7 OVERALL DISCUSSION

We hypothesized that the dysregulated and improper immune response against environmental microbes in early life results in the onset of asthma and other allergic disorders. In this PhD study, we used an ex vivo experimental setup to study the functionality of the immune system, by which the intertwined innate and adaptive immune responses were studied separately.

In paper I, we examined the cytokine profiles, most likely associated with an adaptive immune response, elicited by each of the three common pathogenic airway bacteria (*H. influenzae*, *M. catarrhalis*, *S. pneumoniae*) in relation to later allergic disorders. Our purpose with this ex vivo stimulation protocol was to examine if early immune deregulation against certain bacteria was in play in vivo before disease debut. We therefore focused on using a relatively short ex vivo stimulation protocol (40 hours), shown in pre-study experiments to activate cells, and where most of the secreted T-cell cytokines would derive from bacteria-specific memory or effector T-cells being readily re-stimulated ex vivo. We know from other in-house and published protocols that naive human T cells require much longer ex vivo stimulation protocols (5 to 7 days) in concert with primed dendritic cells for production of cytokines. We identified an attenuated IL-2 response to stimulation with *H. influenzae* and *M. catarrhalis* in PBMCs collected at 6 months of age was associated with elevated total-IgE at age 6 years. We also found the combination of reduced IL-2 and increased IL-5 production was associated with a high risk of allergic rhinitis at 7 years of age. However, the finding was not applicable for cytokine profiles derived from the stimulation of *S. pneumoniae*. We speculated that the reason may be that *S. pneumoniae* does not have LPS in its cell wall, while the other two bacteria have. However, this idea cannot be tested in the current study. We also speculated that reduced IL-2 may lead to fewer Tregs, and in combination with elevated IL-5, the child may subsequently develop uncontrolled type 2 immune response clinically manifestated as elevated total serum IgE or allergic rhinitis. Again, we cannot test our speculation using currently available data, because no assessment was made about the activity and the quantity of the Treg population in stimulated PBMCs. Nevertheless, a dysregulated immune response against the common airway pathogens was uncovered in asymptomatic 6-month-old infants and associated with development of later allergic rhinitis and total IgE.

In paper II, we investigated whether innate immune responses mediated the effects of selected risk SNPs on asthma. In this study, the innate immune responses were reflected by cytokine profiles associated with the activation of each of six selected innate signaling pathways in ex vivo experiments. We identified type-17 biased cytokine profiles derived from two TLR signaling pathways, TLR3 and TLR9, to mediate the effect of the 17q21 risk locus on asthma.

Both TLR3 and TLR9 are nucleic-sensing TLRs and recognize viral or bacterial specific nucleic acid, i.e. TLR3 recognizes the double strand RNA (poly(I:C)) from virus and TLR9 recognizes unmethylated DNA components (CpG) primarily from bacteria. Generally speaking, the activation of both TLR3 and TLR9 normally result in copious production of type 1 cytokines characterized by IFN-γ, IL-12p70, CXCL10, and IL-18. The created cytokine environment would then contribute in activation of type 1 immune responses by induction and activation of many cellular components such as macrophages and Th1 cells. The immune patterns we discovered were mainly driven by IL-23, which suggested the involvement of a type 17 immune response as IL-23 hold an essential role in Th17 differentiation and ILC3 activation. They both lead to neutrophil recruitment via activation of CXCL8 production from IL-17-based activation of fibroblasts and epithelial cells. Clearly, the identified immune response deviated from the correct type 1 based immune response which should have been elicited in order for successful intracellular pathogen removal. This finding further supported the existence of a non-atopic pathway underlying the association between the 17q21 risk locus and childhood asthma,

overall revealing that a dysregulated innate immune response to intracellular ligands may mediate the effect of the 17q21 risk variants in relation to development of childhood asthma.

Collectively, the findings from both paper I and paper II supported our hypothesis about the dysregulated immune function in early life and onset of asthma due to inefficient pathogenic clearance.

8 OVERALL CONCLUSION

In conclusion, an early life deregulated immune response to environmental microbes in asymptomatic infants was identified to influence later development of allergic rhinitis, and to mediate the link between the 17q21 risk loci on childhood asthma development. The main conclusions from each paper are as following:

In **paper I**, we identified an attenuated IL-2 response in infancy against *H. influenzae* and *M. catarrhalis* to associate with elevated total-IgE at age 7. We also found the combination of reduced IL-2 and increased IL-5 production to associate with a higher risk of allergic rhinitis at school age.

In **paper II**, we identified aberrant innate immune responses associated with TLR3 and TLR9 to mediate the effect of the 17q21 risk locus on increased risk of childhood asthma.

In summary, this PhD study provides evidence that deregulated immune responses against pathogens in early life are associated with the development of asthma and other types of allergic disorders.

9 FUTURE STUDIES

This PhD study mainly focused on uncovering the deregulated immune function in relation to later disease, without investigating the impact of specific environmental exposures.

In the future, environmental exposures and multiple omics data (genomics, metabolomics, microbiome) may be integrated to elucidate their impacts on the functional immune responses in relation to later asthma.

It would be very interesting to investigate what kind of environmental factors imprinted the immune system and resulted in the identified aberrant immune responses. The environmental factors of interest include older sibling, cat or dog exposure, tobacco smoking, antibiotics exposure, season of birth. Many of the environmental factors lead to changes in microbiota. Given the essential role of microbiota in the development of the immune system, I believe it would be also fascinating to include data relating to the microbial composition of the gut and the airways from those children into the study.

After significant associations between specific environmental factors and functional immune responses have been identified, the pipeline constructed in paper II for causal mediation analysis can be applied for identifying the causal pathways from that environmental factor to asthma through innate or adaptive immune responses.

In the meantime, the interaction between environmental factors and genetic background should not be neglected when carrying out causal mediation analyses. For example, cat exposure was reported to significantly interact with the 17q21 locus in terms of childhood asthma. In this situation, the mediation effect of the functional immune responses may be genotype specific, hence, a moderated mediation analysis could be employed.

Moreover, to carry on with paper II, it would also be very interesting to examine if the identified dysregulated innate immune profile is the common reason for early respiratory infection and childhood asthma. This question can be addressed by the mediation analysis with some twist. For example, by adjusting for the number of lower respiratory infections in the mediation analysis.

Furthermore, the current study was based on immune profiles generated by an unsupervised data decomposition method (PCA). Using the unsupervised method for data decomposition can help us to identify immune patterns associated with multiple clinical outcomes, which was what happened in this PhD study. In future studies, we may focus on a specific clinical outcome and utilize supervised decomposition methods such as PLS-DA to generate immune patterns enriched with disease-specific variance.

10 REFERENCES

1. Bisgaard H, Hermansen MN, Buchvald F, et al. Childhood Asthma after Bacterial cololization of the Airway in Neonates. *N Engl J Med*. 2007;357:1487-1495. doi:10.1542/peds.2008-2139CCC

2. Birch S, Thysen AH, Brix S, Larsen JM, Bisgaard H, Rasmussen MA. Children with asthma by school age display aberrant immune responses to pathogenic airway bacteria as infants. *J Allergy Clin Immunol*. 2014;133(4):1008-1013.e4. doi:10.1016/j.jaci.2014.01.010

3. Vissing NH, Larsen JM, Rasmussen MA, et al. Susceptibility to Lower Respiratory Infections in Childhood is Associated with Perturbation of the Cytokine Response to Pathogenic Airway Bacteria. *Pediatr Infect Dis J*. 2016;35(5):561-566. doi:10.1097/INF.0000000000001092

4. Prescott SL. Early-life environmental determinants of allergic diseases and the wider pandemic of inflammatory noncommunicable diseases. *J Allergy Clin Immunol*. 2013;131:23-30. doi:10.1016/j.jaci.2012.11.019

5. Renz H, von Mutius E, Brandtzaeg P, Cookson WO, Autenrieth IB, Haller D. Gene-environment interactions in chronic inflammatory disease. *Nat Immunol*. 2011;12(4):273-277. doi:10.1038/ni0411-273

6. Renz H, Holt PG, Inouye M, Logan AC, Prescott SL, Sly PD. An exposome perspective: Early-life events and immune development in a changing world. *J Allergy Clin Immunol*. 2017;140(1):24-40. doi:10.1016/J.JACI.2017.05.015

7. Rappaport SM. Genetic Factors Are Not the Major Causes of Chronic Diseases. Scott RJ, ed. *PLoS One*. 2016;11(4):e0154387. doi:10.1371/journal.pone.0154387

8. Goodwin RD, Robinson M, Sly PD, Holt PG. Childhood atopy and mental health: a prospective, longitudinal investigation. *Psychol Med*. 2017;47(02):317-325. doi:10.1017/S0033291716001896

9. West CE, Renz H, Jenmalm MC, et al. The gut microbiota and inflammatory noncommunicable diseases : Associations and potentials for gut microbiota therapies. 2015;135:3-13. doi:10.1016/j.jaci.2014.11.012

10. Logan AC, Jacka FN, Craig JM, Prescott SL. The Microbiome and Mental Health: Looking Back, Moving Forward with Lessons from Allergic Diseases. *Clin Psychopharmacol Neurosci*. 2016;14(2):131-147. doi:10.9758/cpn.2016.14.2.131

11. Boroni Moreira a P, Fiche Salles Teixeira T, do C Gouveia Peluzio M, de Cássia Gonçalves Alfenas R. Gut microbiota and the development of obesity. *Nutr Hosp*. 2012. doi:10.3305/nh.2012.27.5.5887

12. Karlsson F, Tremaroli V, Nielsen J, Bäckhed F. Assessing the human gut microbiota in metabolic diseases. *Diabetes*. 2013. doi:10.2337/db13-0844

13. Bruce-Keller AJ, Salbaum JM, Berthoud HR. Harnessing Gut Microbes for Mental Health: Getting From Here to There. *Biol Psychiatry*. 2018. doi:10.1016/j.biopsych.2017.08.014

14. Sommer F, Bäckhed F. The gut microbiota-masters of host development and physiology. *Nat Rev Microbiol*. 2013;11(4):227-238. doi:10.1038/nrmicro2974

15. Carole Ober1 and Tsung-Chieh Yao. The Genetics of Asthma and Allergic Disease: A 21 st Century Prespective. *Immunol Rev*. 2011;242(1):10-30. doi:10.1111/j.1600-065X.2011.01029.x.The

16. Moffatt MF, Kabesch M, Liang L, et al. Genetic variants regulating ORMDL3 expression contribute to the risk of childhood asthma. *Nature*. 2007;448(7152):470-473. doi:10.1038/nature06014

17. Hancock DB, Romieu I, Shi M, et al. Genome-wide association study implicates chromosome 9q21.31 as a susceptibility locus for asthma in Mexican children. *PLoS Genet*. 2009;5(8). doi:10.1371/journal.pgen.1000623

18. Himes BE, Hunninghake GM, Baurley JW, et al. Genome-wide Association Analysis Identifies PDE4D as an Asthma-Susceptibility Gene. *Am J Hum Genet*. 2009;84(5):581-593. doi:10.1016/j.ajhg.2009.04.006

19. Bønnelykke K, Sleiman P, Nielsen K, et al. A genome-wide association study identifies CDHR3 as a susceptibility locus for early childhood asthma with severe exacerbations. *Nat Genet*. 2014;46(1):51-55. doi:10.1038/ng.2830

20. Li X, Howard TD, Zheng SL, et al. Genome-wide association study of asthma identifies RAD50-IL13 and HLA-DR/DQ regions. *J Allergy Clin Immunol*. 2010. doi:10.1016/j.jaci.2009.11.018

21. Mathias RA, Grant A V., Rafaels N, et al. A genome-wide association study on African-ancestry populations for asthma. *J Allergy Clin Immunol*. 2010;125(2). doi:10.1016/j.jaci.2009.08.031

22. Sleiman PMA, Flory J, Imielinski M, et al. Variants of DENND1B Associated with Asthma in Children . *N Engl J Med*. 2009;362(1):36-44. doi:10.1056/nejmoa0901867

23. Esparza-Gordillo J, Weidinger S, Fölster-Holst R, et al. A common variant on chromosome 11q13 is associated with atopic dermatitis. *Nat Genet*. 2009. doi:10.1038/ng.347

24. Ellinghaus D, Baurecht H, Esparza-Gordillo J, et al. High-density genotyping study identifies four new susceptibility loci for atopic dermatitis. *Nat Genet*. 2013;45(7):808-812. doi:10.1038/ng.2642

25. Weidinger S, Gieger C, Rodriguez E, et al. Genome-wide scan on total serum IgE levels identifies FCER1A as novel susceptibility locus. *PLoS Genet*. 2008. doi:10.1371/journal.pgen.1000166

26. Liang L, Willis-Owen SAG, Laprise C, et al. An epigenome-wide association study of total serum immunoglobulin e concentration. *Nature*. 2015. doi:10.1038/nature14125

27. Gudbjartsson DF, Bjornsdottir US, Halapi E, et al. Sequence variants affecting eosinophil numbers associate with asthma and myocardial infarction. *Nat Genet*. 2009;41(3):342-347. doi:10.1038/ng.323

28. Wilk JB, Chen TH, Gottlieb DJ, et al. A genome-wide association study of pulmonary function measures in the framingham heart study. *PLoS Genet*. 2009;5(3). doi:10.1371/journal.pgen.1000429

29. Loth DW, Artigas MS, Gharib SA, et al. Genome-wide association analysis identifies six new loci associated with forced vital capacity. *Nat Genet*. 2014;46(7):669-677. doi:10.1038/ng.3011

30. Repapi E, Sayers I, Wain L V., et al. Genome-wide association study identifies five loci associated with lung function. *Nat Genet*. 2010;42(1):36-44. doi:10.1038/ng.501

31. Wild CP. Complementing the Genome with an "Exposome": The Outstanding Challenge of Environmental Exposure Measurement in Molecular Epidemiology. *Cancer Epidemiol Biomarkers Prev*. 2005;14(8):1847-1850. doi:10.1158/1055-9965.EPI-05-0456

32. Lloyd CM, Saglani S. Development of allergic immunity in early life. *Immunol Rev*. 2017;278(1):101-115. doi:10.1111/imr.12562

33. Ege MJ, Mayer M, Normand A-C, et al. Exposure to Environmental Microorganisms and Childhood Asthma. *N Engl J Med*. 2011;364(8):387-401. doi:10.1056/NEJMoa1402121

34. Heyn H, Esteller M. An adenine code for DNA: A second life for N6-methyladenine. *Cell*. 2015;161(4):710-713. doi:10.1016/j.cell.2015.04.021

35. Tahiliani M, Koh KP, Shen Y, et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science (80-)*. 2009;324(5929):930-935. doi:10.1126/science.1170116

36. Jones PA. Functions of DNA methylation: Islands, start sites, gene bodies and beyond. *Nat Rev Genet*. 2012;13(7):484-492. doi:10.1038/nrg3230

37. Wu X, Zhang Y. TET-mediated active DNA demethylation: Mechanism, function and beyond. *Nat Rev Genet*. 2017;18(9):517-534. doi:10.1038/nrg.2017.33

38. Allis CD, Jenuwein T. The molecular hallmarks of epigenetic control. *Nat Rev Genet*. 2016;17(8):487-500. doi:10.1038/nrg.2016.59

39. Andrews FH, Strahl BD, Kutateladze TG. Insights into newly discovered marks and readers of epigenetic information. *Nat Chem Biol*. 2016;12(9):662-668. doi:10.1038/nchembio.2149

40. Strahl BD, Allis CD. The language of covalent histone modifications. *Nature*. 2000;403(6765):41-45. doi:10.1038/47412

41. Brownell JE, Zhou J, Ranalli T, et al. Tetrahymena histone acetyltransferase A: A homolog to yeast Gcn5p linking histone acetylation to gene activation. *Cell*. 1996;84(6):843-851. doi:10.1016/S0092-8674(00)81063-6

42. Clapier CR, Iwasa J, Cairns BR, Peterson CL. Mechanisms of action and regulation of ATP-dependent chromatin-remodelling complexes. *Nat Rev Mol Cell Biol*. 2017;18(7):407-422. doi:10.1038/nrm.2017.26

43. Zhang Y, Cao X. Long noncoding RNAs in innate immunity. *Cell Mol Immunol*. 2016;13(2):138-147. doi:10.1038/cmi.2015.68

44. Aune TM, Spurlock CF. Long non-coding RNAs in innate and adaptive immunity. *Virus Res*. 2016;212:146-160. doi:10.1016/j.virusres.2015.07.003

45. Chen YG, Satpathy AT, Chang HY. Gene regulation in the immune system by long noncoding RNAs. *Nat Immunol*. 2017;18(9):962-972. doi:10.1038/ni.3771

46. Ebbesen KK, Kjems J, Hansen TB. Circular RNAs: Identification, biogenesis and function. *Biochim Biophys Acta - Gene Regul Mech*. 2016;1859(1):163-168. doi:10.1016/j.bbagrm.2015.07.007

47. Quinn JJ, Chang HY. Unique features of long non-coding RNA biogenesis and function. *Nat Rev Genet*. 2016;17(1):47-62. doi:10.1038/nrg.2015.10

48. H C, JJ C, X W, et al. Human ADAR1 Prevents Endogenous RNA from Triggering Translational Shutdown. *Cell*. 2018;172:811-824.e14. doi:10.1016/j.cell.2017.12.038

49. Liddicoat BJ, Piskol R, Chalk AM, et al. RNA editing by ADAR1 prevents MDA5 sensing of endogenous dsRNA as nonself. *Science (80-)*. 2015;349(6252):1115-1120. doi:10.1126/science.aac7049

50. Rappaport SM. Implications of the exposome for exposure science. *J Expo Sci Environ Epidemiol*. 2011;21(1):5-9. doi:10.1038/jes.2010.50

51. Bager P, Wohlfahrt J, Westergaard T. Caesarean delivery and risk of atopy and allergic disesase: Meta-analyses. *Clin Exp Allergy*. 2008;38(4):634-642. doi:10.1111/j.1365-2222.2008.02939.x

52. Thavagnanam S, Fleming J, Bromley A, Shields MD, Cardwell CR. A meta-analysis of the association between Caesarean section and childhood asthma. *Clin Exp Allergy*. 2008;38(4):629-633. doi:10.1111/j.1365-2222.2007.02780.x

53. Kozyrskyj AL, Ernst P, Becker AB. Increased risk of childhood asthma from antibiotic use in early life. *Chest*. 2007;131(6):1753-1759. doi:10.1378/chest.06-3008

54. Metsälä J, Lundqvist A, Virta LJ, Kaila M, Gissler M, Virtanen SM. Prenatal and post-natal exposure to antibiotics and risk of asthma in childhood. *Clin Exp Allergy*. 2015;45(1):137-145. doi:10.1111/cea.12356

55. Russell SL, Gold MJ, Hartmann M, et al. Early life antibiotic-driven changes in microbiota enhance susceptibility to allergic asthma. *EMBO Rep*. 2012;13(5):440-447. doi:10.1038/embor.2012.32

56. Gonzalez-Barcala FJ, Pertega S, Sampedro M, et al. Impact of parental smoking on childhood asthma. *J Pediatr (Rio J)*. 2013;89(3):294-299. doi:10.1016/j.jped.2012.11.001

57. Mitchell EA, Beasley R, Keil U, Montefort S, Odhiambo J. The association between tobacco and the risk of asthma, rhinoconjunctivitis and eczema in children and adolescents: Analyses from phase three of the ISAAC programme. *Thorax*. 2012;67(11):941-949. doi:10.1136/thoraxjnl-2011-200901

58. Thomson NC, Chaudhuri R, Livingston E. Asthma and cigarette smoking. *Eur Respir J*. 2004;24(5):822-833. doi:10.1183/09031936.04.00039004

59. Strachan DP. Family size, infection and atopy: the first decade of the “hygiene hypothesis”. *Thorax*. 2000;55 Suppl 1:S2-10. http://www.ncbi.nlm.nih.gov/pubmed/10943631.

60. Strachan DP, Aït-Khaled N, Foliaki S, et al. Siblings, asthma, rhinoconjunctivitis and eczema: A

worldwide perspective from the International Study of Asthma and Allergies in Childhood. *Clin Exp Allergy*. 2015;45(1):126-136. doi:10.1111/cea.12349

61. Koppelman GH, Jansen DF, Schouten JP, et al. Sibling effect on atopy in children of patients with asthma. *Clin Exp Allergy*. 2003;33(2):170-175. doi:10.1046/j.1365-2222.2003.01591.x

62. Ball TM, Castro-Rodriguez JA, Griffith KA, Holberg CJ, Martinez FD, Wright AL. Siblings, Day-Care Attendance, and the Risk of Asthma and Wheezing during Childhood. *N Engl J Med*. 2002;343(8):538-543. doi:10.1056/nejm200008243430803

63. Chen CM, Tischer C, Schnappinger M, Heinrich J. The role of cats and dogs in asthma and allergy - a systematic review. *Int J Hyg Environ Health*. 2010. doi:10.1016/j.ijheh.2009.12.003

64. Perzanowski MS, Rönmark E, Platts-Mills TAE, Lundbäck B. Effect of cat and dog ownership on sensitization and development of asthma among preteenage children. *Am J Respir Crit Care Med*. 2002;166(5):696-702. doi:10.1164/rccm.2201035

65. Schenker MB. Farming and asthma. *Occup Environ Med*. 2005;62(4):211-212. doi:10.1136/oem.2004.019109

66. Kilpeläinen, Terho, Helenius, Koskenvuo. Farm environment in childhood prevents the development of allergies. *Clin Exp Allergy*. 2000;30(2):201-208. doi:10.1046/j.1365-2222.2000.00800.x

67. Von Ehrenstein OS, Von Mutius E, Illi S, Baumann L, Böhm O, Von Kries R. Reduced risk of hay fever and asthma among children of farmers. *Clin Exp Allergy*. 2000;30(2):187-193. doi:10.1046/j.1365-2222.2000.00801.x

68. Kloepfer KM, Olenec JP, Lee WM, et al. Increased H1N1 infection rate in children with asthma. *Am J Respir Crit Care Med*. 2012;185(12):1275-1279. doi:10.1164/rccm.201109-1635OC

69. Hasegawa S, Hirano R, Hashimoto K, Haneda Y, Shirabe K, Ichiyama T. Characteristics of atopic children with pandemic H1N1 influenza viral infection: Pandemic H1N1 influenza reveals "occult" asthma of childhood. *Pediatr Allergy Immunol*. 2011;22(1 PART 2). doi:10.1111/j.1399-3038.2010.01090.x

70. Nguyen-Van-Tam JS, Openshaw PJM, Hashim A, et al. Risk factors for hospitalisation and poor outcome with pandemic A/H1N1 influenza: United Kingdom first wave (May-September 2009). *Thorax*. 2010;65(7):645-651. doi:10.1136/thx.2010.135210

71. Mandelcwajg A, Moulin F, Menager C, Rozenberg F, Lebon P, Gendrel D. Underestimation of influenza viral infection in childhood asthma exacerbations. *J Pediatr*. 2010;157(3):505-506. doi:10.1016/j.jpeds.2010.04.067

72. Jartti T, Gern JE. Role of viral infections in the development and exacerbation of asthma in children. *J Allergy Clin Immunol*. 2017;140(4):895-906. doi:10.1016/j.jaci.2017.08.003

73. Message SD, Laza-Stanca V, Mallia P, et al. Rhinovirus-induced lower respiratory illness is increased in asthma and related to virus load and Th1/2 cytokine and IL-10 production. *Proc Natl Acad Sci*. 2008;105(36):13562-13567. doi:10.1073/pnas.0804181105

74. Copenhaver CC, Gern JE, Li Z, et al. Cytokine Response Patterns, Exposure to Viruses, and

Respiratory Infections in the First Year of Life. *Am J Respir Crit Care Med*. 2004;170(2):175-180. doi:10.1164/rccm.200312-1647oc

75. Jackson DJ, Evans MD, Gangnon RE, et al. Evidence for a causal relationship between allergic sensitization and rhinovirus wheezing in early life. *Am J Respir Crit Care Med*. 2012;185(3):281-285. doi:10.1164/rccm.201104-0660OC

76. Jackson DJ, Gangnon RE, Evans MD, et al. Wheezing rhinovirus illnesses in early life predict asthma development in high-risk children. *Am J Respir Crit Care Med*. 2008;178(7):667-672. doi:10.1164/rccm.200802-309OC

77. Kusel MMH, de Klerk NH, Kebadze T, et al. Early-life respiratory viral infections, atopic sensitization, and risk of subsequent development of persistent asthma. *J Allergy Clin Immunol*. 2007;119(5):1105-1110. doi:10.1016/j.jaci.2006.12.669

78. Bisgaard H, Hermansen MN, Buchvald F, et al. Childhood Asthma of the Airway in Neonates. *N Engl J Med*. 2007;357:1487-1495. doi:10.1542/peds.2008-2139CCC

79. Arnold IC, Dehzad N, Reuter S, et al. Helicobacter pylori infection prevents allergic asthma in mouse models through the induction of regulatory T cells. *J Clin Invest*. 2011;121(8):3088-3093. doi:10.1172/JCI45041

80. Cahenzli J, Köller Y, Wyss M, Geuking MB, McCoy KD. Intestinal microbial diversity during early-life colonization shapes long-term IgE levels. *Cell Host Microbe*. 2013;14(5):559-570. doi:10.1016/j.chom.2013.10.004

81. Olszak T, An D, Zeissig S, et al. Microbial exposure during early life has persistent effects on natural killer T cell function. *Science (80-)*. 2012;336(6080):489-493. doi:10.1126/science.1219328

82. Ismail IH, Boyle RJ, Mah LJ, Licciardi P V., Tang MLK. Reduced neonatal regulatory T cell response to microbial stimuli associates with subsequent eczema in high-risk infants. *Pediatr Allergy Immunol*. 2014;25(7):674-684. doi:10.1111/pai.12303

83. Larsen JM, Brix S, Thysen AH, Birch S, Rasmussen MA, Bisgaard H. Children with asthma by school age display aberrant immune responses to pathogenic airway bacteria as infants. *J Allergy Clin Immunol*. 2014;133(4). doi:10.1016/j.jaci.2014.01.010

84. Stern DA, Guerra S, Halonen M, Wright AL, Martinez FD. Low IFN-γ production in the first year of life as a predictor of wheeze during childhood. *J Allergy Clin Immunol*. 2007;120(4):835-841. doi:10.1016/j.jaci.2007.05.050

85. Tulic MK, Hodder M, Forsberg A, et al. Differences in innate immune function between allergic and nonallergic children: New insights into immune ontogeny. *J Allergy Clin Immunol*. 2011;127(2). doi:10.1016/j.jaci.2010.09.020

86. Stein RT, Martinez FD. Respiratory syncytial virus and asthma: Still no final answer. *Thorax*. 2010;65(12):1033-1034. doi:10.1136/thx.2009.133967

87. Baraldo S, Contoli M, Bazzan E, et al. Deficient antiviral immune responses in childhood: Distinct roles of atopy and asthma. *J Allergy Clin Immunol*. 2012;130(6):1307-1314. doi:10.1016/j.jaci.2012.08.005

88. Holtzman MJ. Asthma as a chronic disease of the innate and adaptive immune systems responding to viruses and allergens. *J Clin Invest*. 2012;122(8):2741-2748. doi:10.1172/JCI60325

89. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell*. 2006;124(4):783-801. doi:10.1016/j.cell.2006.02.015

90. Iwasaki A, Medzhitov R. Regulation of adaptive immunity by the innate immune system. *Science (80-)*. 2010;327(5963):291-295. doi:10.1126/science.1183021.Regulation

91. Diefenbach A, Colonna M, Romagnani C. The ILC World Revisited. *Immunity*. 2017;46(3):327-332. doi:10.1016/j.immuni.2017.03.008

92. Heine H. TLRs, NLRs and RLRs: Innate sensors and their impact on allergic diseases – A current view. *Immunol Lett*. 2011;139(1-2):14-24. doi:10.1016/J.IMLET.2011.04.010

93. Hornung V, Kato H, Poeck H, Akira S, Conzelmann K, Schlee M. 5¶-Triphosphate RNA Is the Ligand for RIG-I. *Science (80-)*. 2006;994(5801):994-997. doi:10.1126/science.1132505

94. Pichlmair A, Schulz O, Tan CP, Na¨slund TI. RIG-I- mediated antiviral responses to single-stranded RNA bearing 5¶-phosphates. 2006;314(10):997-1002.

95. Besnard AG, Guillou N, Tschopp J, et al. NLRP3 inflammasome is required in murine asthma in the absence of aluminum adjuvant. *Allergy Eur J Allergy Clin Immunol*. 2011;66(8):1047-1057. doi:10.1111/j.1398-9995.2011.02586.x

96. McGettrick AF, O'Neill LAJ. NLRP3 and IL-1beta in macrophages as critical regulators of metabolic diseases. *Diabetes, Obes Metab*. 2013;15(s3):1-21.

97. Jo EK, Kim JK, Shin DM, Sasakawa C. Molecular mechanisms regulating NLRP3 inflammasome activation. *Cell Mol Immunol*. 2016;13(2):148-159. doi:10.1038/cmi.2015.95

98. Hawn TR, Underhill DM. Toll-like Receptors in Innate Immunity. *Int Immunol*. 2005;17(1):1-14. doi:10.1016/B978-012455900-4/50268-3

99. Wang T, Town T, Alexopoulou L, Anderson JF, Fikrig E, Flavell RA. Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. *Nat Med*. 2004;10(12):1366-1373. doi:10.1038/nm1140

100. Alexopoulou L. Recognition of double tranded RNA and activaiton of NFkb by TLR 3. 2000;77(1985).

101. Diebold SS, Hemmi H, Kaisho T, Akira S, Reis e Sousa C. Innate antiviral resonse by means of TLR7-mediated recognition of signle-strandet RNA. *Science (80-)*. 2004;303(MARCH):1529-1531. https://www.ncbi.nlm.nih.gov/pubmed/8842040.

102. Heil F, Hemmi H, Hochrein H, et al. Species-Specific Recognition of Single-Stranded RNA via Toll-like Receptor 7 and 8. *Science (80-)*. 2004;303(5663):1526-1529.

103. Krieg AM, Yi A-K, Matson S, et al. CpG motigs in bavterial DNA trigger direct B-cell activation. *Nature*. 1995;374(6):546-549.

104. Agrawal S, Doughty B, Gerwitz A, Blenis J, Van Dyke T, Pulendran B. Cutting edge: different Toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos. *J Immunol*. 2003;171(10):4984-4989. http://www.ncbi.nlm.nih.gov/pubmed/14607893.

105. Dillon S, Agrawal A, Dyke T Van, et al. A Toll-like receptor 2 ligand stimulates Th2 responses in vivo, via induction of extracellular signal-regulated kinase mitogen-activated protein kinase and c-Fos in dendritic cells. *J Immunol*. 2004;172:4733-4743. doi:10.4049/jimmunol.172.8.4733

106. Pulendran B. Modulating Th1/Th2 responses with microbes, dendritic cells, and pathogen recognition receptors. *Immunol Res*. 2004;29(1-3):187-196. doi:10.1385/IR:29:1-3:187

107. Pulendran B. Modulating vaccine responses with dendritic cells and toll-like receptors. *Immunol Rev*. 2004;199:227-250. doi:10.1111/j.0105-2896.2004.00144.x

108. Pulendran B, Kumar P, Cutler CW, Mohamadzadeh M, Van Dyke T, Banchereau J. Lipopolysaccharides from Distinct Pathogens Induce Different Classes of Immune Responses In Vivo. *J Immunol*. 2001;167(9):5067-5076. doi:10.4049/jimmunol.167.9.5067

109. Pulendran B. Variegation of the Immune Response with Dendritic Cells and Pathogen Recognition Receptors. *J Immunol*. 2005;174:2457-2465. doi:10.4049/jimmunol.174.5.2457

110. Pulendran B. Variegation of the Immune Response with Dendritic Cells and Pathogen Recognition Receptors. *J Immunol*. 2014;174(5):2457-2465. doi:10.4049/jimmunol.174.5.2457

111. Pulendran B, Ahmed R. Translating innate immunity into immunological memory: Implications for vaccine development. *Cell*. 2006;124(4):849-863. doi:10.1016/j.cell.2006.02.019

112. Akira S, Takeda K, Kaisho T. Toll-like receptors: Critical proteins linking innate and acquired immunity. *Nat Immunol*. 2001;2(8):675-680. doi:10.1038/90609

113. Li K, Chen Z, Kato N, Gale M, Lemon SM. Distinct poly(I-C) and virus-activated signaling pathways leading to interferon-β production in hepatocytes. *J Biol Chem*. 2005;280(17):16739-16747. doi:10.1074/jbc.M414139200

114. Tissari J, Siren J, Meri S, Julkunen I, Matikainen S. IFN- Enhances TLR3-Mediated Antiviral Cytokine Expression in Human Endothelial and Epithelial Cells by Up-Regulating TLR3 Expression. *J Immunol*. 2005;174:4289-4294. doi:10.4049/jimmunol.174.7.4289

115. Park C, Lee S, Cho IH, et al. TLR3-mediated signal induces proinflammatory cytokine and chemokine gene expression in astrocytes: Differential signaling mechanisms of TLR3-induced IP-10 and IL-8 gene expression. *Glia*. 2006;53(3):248-256. doi:10.1002/glia.20278

116. Matsukura S, Kokubu F, Kurokawa M, et al. Synthetic double-stranded RNA induces multiple genes related to inflammation through Toll-like receptor 3 depending on NF-κB and/or IRF-3 in airway epithelial cells. *Clin Exp Allergy*. 2006;36(8):1049-1062. doi:10.1111/j.1365-2222.2006.02530.x

117. Kim H, Yang E, Lee J, et al. Double-stranded RNA mediates interferon regulatory factor 3 activation and interleukin-6 production by engaging Toll-like receptor 3 in human brain astrocytes. *Immunology*. 2008;124(4):480-488. doi:10.1111/j.1365-2567.2007.02799.x

118. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. *Nature*. 2001;413(6857):732-738. doi:10.1038/35099560

119. Visintin A, Mazzoni A, Spitzer JH, Wyllie DH, Dower SK, Segal DM. Regulation of Toll-Like Receptors in Human Monocytes and Dendritic Cells. *J Immunol*. 2001;166:249-255. doi:10.4049/jimmunol.166.1.249

120. Muzio M, Bosisio D, Polentarutti N, et al. Differential Expression and Regulation of Toll-Like Receptors (TLR) in Human Leukocytes: Selective Expression of TLR3 in Dendritic Cells. *J Immunol*. 2000;164(11):5998-6004. doi:10.4049/jimmunol.164.11.5998

121. Cario E, Podolsky DK. Differential alteration in intestinal epithelial cell expression of Toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. *Infect Immun*. 2000;68(12):7010-7017. doi:10.1128/IAI.68.12.7010-7017.2000

122. Diebold SS, Kaisho T, Hemmi H, Akira S, Reis E Sousa C. Innate Antiviral Responses by Means of TLR7-Mediated Recognition of Single-Stranded RNA. *Science (80-)*. 2004;303(5663):1529-1531. doi:10.1126/science.1093616

123. Colonna M, Trinchieri G, Liu YJ. Plasmacytoid dendritic cells in immunity. *Nat Immunol*. 2004;5(12):1219-1226. doi:10.1038/ni1141

124. Wagner H. The immunobiology of the TLR9 subfamily. *Trends Immunol*. 2004;25(7):381-386. doi:10.1016/j.it.2004.04.011

125. Kadowaki N, Ho S, Antonenko S, et al. Subsets of Human Dendritic Cell Precursors Express Different Toll-like Receptors and Respond to Different Microbial Antigens. *J Exp Med*. 2002;194(6):863-870. doi:10.1084/jem.194.6.863

126. Ito T, Amakawa R, Fukuhara S. Roles of toll-like receptors in natural interferon-producing cells as sensors in immune surveillance. *Hum Immunol*. 2002;63(12):1120-1125. doi:10.1016/S0198-8859(02)00750-4

127. Hornung V, Rothenfusser S, Britsch S, et al. Quantitative Expression of Toll-Like Receptor 1-10 mRNA in Cellular Subsets of Human Peripheral Blood Mononuclear Cells and Sensitivity to CpG Oligodeoxynucleotides. *J Immunol*. 2002;168(9):4531-4537. doi:10.4049/jimmunol.168.9.4531

128. Liu Y-J. IPC: Professional Type 1 Interferon-Producing Cells and Plasmacytoid Dendritic Cell Precursors. *Annu Rev Immunol*. 2005;23(1):275-306. doi:10.1146/annurev.immunol.23.021704.115633

129. Honda K, Yanai H, Negishi H, et al. IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature*. 2005;434(7034):772-777. doi:10.1038/nature03464

130. Hemmi H, Kaisho T, Takeuchi O, et al. Small-antiviral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat Immunol*. 2002;3(2):196-200. doi:10.1038/ni758

131. Kawai T, Sato S, Ishii KJ, et al. Interferon-α induction through Toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6. *Nat Immunol*. 2004;5(10):1061-1068. doi:10.1038/ni1118

132. Kawai T, Akira S. Toll-like Receptors and Their Crosstalk with Other Innate Receptors in Infection and Immunity. *Immunity*. 2011;34(5):637-650. doi:10.1016/j.immuni.2011.05.006

133. Born WK, Reardon CL, O'Brien RL. The function of gammadelta T cells in innate immunity. *Curr Opin Immunol*. 2006;18(1):31-38. doi:S0952-7915(05)00200-1 [pii] 10.1016/j.coi.2005.11.007

134. Bonneville M, Scotet E. Human Vγ9Vδ2 T cells: promising new leads for immunotherapy of infections and tumors. *Curr Opin Immunol*. 2006;18(5):539-546. doi:10.1016/j.coi.2006.07.002

135. Vantourout P, Hayday A. Six-of-the-best: Unique contributions of γδ T cells to immunology. *Nat Rev Immunol*. 2013;13(2):88-100. doi:10.1038/nri3384

136. Dieli F, Caccamo N, Meraviglia S, et al. Reciprocal stimulation of γδ T cells and dendritic cells during the anti-mycobacterial immune response. *Eur J Immunol*. 2004;34(11):3227-3235. doi:10.1002/eji.200425368

137. Martino A, Casetti R, D'Alessandri A, Sacchi A, Poccia F. Complementary function of γδ T-lymphocytes and dendritic cells in the response to isopentenyl-pyrophosphate and lipopolysaccharide antigens. *J Clin Immunol*. 2005;25(3):230-237. doi:10.1007/s10875-005-4080-8

138. Leslie DS, Vincent MS, Spada FM, et al. CD1-mediated gamma/delta T cell maturation of dendritic cells. *J Exp Med*. 2002;196(12):1575-1584. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12486100.

139. Ismaili J, Olislagers V, Poupot R, Fournié J-J, Goldman M. Human gamma delta T cells induce dendritic cell maturation. *Clin Immunol*. 2002;103(3 Pt 1):296-302. doi:10.1006/clim.2002.5218

140. Skeen MJ, Freeman MM, Ziegler HK. Changes in peritoneal myeloid populations and their proinflammatory cytokine expression during infection with Listeria monocytogenes are altered in the absence of γ/δ T cells . *J Leukoc Biol*. 2004;76(1):104-115. doi:10.1189/jlb.1103574

141. Ditirro J, Rhoades ER, Roberts AD, et al. Disruption of the cellular inflammatory response to Listeria monocytogenes infection in mice with disruptions in targeted genes. *Infect Immun*. 1998;66(5):2284-2289.

142. Brandes M. Professional Antigen-Presentation Function by Human gd T Cells. *Science (80-)*. 2005;309(5732):264-268. doi:10.1126/science.1110267

143. Laggner U, Di Meglio P, Perera GK, et al. Identification of a Novel Proinflammatory Human Skin-Homing V 9V 2 T Cell Subset with a Potential Role in Psoriasis. *J Immunol*. 2011;187(5):2783-2793. doi:10.4049/jimmunol.1100804

144. Sharp LL, Jameson JM, Cauvi G, Havran WL. Dendritic epidermal T cells regulate skin homeostasis through local production of insulin-like growth factor 1. *Nat Immunol*. 2005;6(1):73-79. doi:10.1038/ni1152

145. Spinozzi F, Agea E, Bistoni O, et al. Local expansion of allergen-specifric CD30+Th2-type gamma,delta T cells in bronchial asthma. *Mol Med*. 1995;1(7):821-826.

146. Beetz S, Wesch D, Marischen L, Welte S, Oberg H-HH, Kabelitz D. Innate immune functions of human gammadelta T cells. *Immunobiology*. 2008;213(3-4):173-182. doi:10.1016/j.imbio.2007.10.006

147. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell*. 2000;100(6):655-669. doi:10.1016/S0092-8674(00)80702-3

148. Sullivan BM, Juedes A, Szabo SJ, von Herrath M, Glimcher LH. Antigen-driven effector CD8 T cell function regulated by T-bet. *Proc Natl Acad Sci*. 2003;100(26):15818-15823. doi:10.1073/pnas.2636938100

149. Townsend MJ, Weinmann AS, Matsuda JL, et al. T-bet regulates the terminal maturation and homeostasis of NK and Vα14i NKT cells. *Immunity*. 2004;20(4):477-494. doi:10.1016/S1074-7613(04)00076-7

150. Pearce EL, Mullen AC, Martins GA, et al. Control of Effector CD8+ T Cell Function by the Transcription Factor Eomesodermin. *Science (80-)*. 2003;302(5647):1041-1043. doi:10.1126/science.1090148

151. Gordon SM, Chaix J, Rupp LJ, et al. The Transcription Factors T-bet and Eomes Control Key Checkpoints of Natural Killer Cell Maturation. *Immunity*. 2012;36(1):55-67. doi:10.1016/j.immuni.2011.11.016

152. Manetti R. Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. *J Exp Med*. 1993;177:1199-1204. doi:10.1084/jem.177.4.1199

153. Muraille E, Leo O, Moser M. Th1/Th2 paradigm extended: Macrophage polarization as an unappreciated pathogen-driven escape mechanism? *Front Immunol*. 2014;5(NOV). doi:10.3389/fimmu.2014.00603

154. Murray PJ, Allen JE, Biswas SK, et al. Macrophage Activation and Polarization: Nomenclature and Experimental Guidelines. *Immunity*. 2014;41(1):14-20. doi:10.1016/j.immuni.2014.06.008

155. Constant S, Pfeiffer C, Woodard A, Pasqualini T, Bottomly K. Extent of T cell receptor ligation can determine the functional differentiation of naive CD4+ T cells. *J Exp Med*. 1995;182(5):1591-1596. http://www.ncbi.nlm.nih.gov/pubmed/7595230.

156. David Farrar J, Asnagli H, Murphy KM. T helper subset development: Roles of instruction, selection, and transcription. *J Clin Invest*. 2002;109(4):431-435. doi:10.1172/JCI200215093

157. Bonecchi R, Bianchi G, Bordignon Pp, et al. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J Exp Med*. 1998;187(1):129-134.

158. Romagnani S. Human TH1 and TH2 subsets: doubt no more. *Immunol Today*. 1991;12(8):256-257. doi:10.1016/0167-5699(91)90120-I

159. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol*. 1986;136(7):2348-2357. http://www.ncbi.nlm.nih.gov/pubmed/2419430.

160. Carter LL, Murphy KM. Lineage-specific Requirement for Signal Transducer and Activator of Transcription (Stat)4 in Interferon γ Production from CD4 + Versus CD8 + T Cells. *J Exp Med*. 1999;189(8):1355-1360. doi:10.1084/jem.189.8.1355

161. Larrubia JR, Calvino M, Benito S, et al. The role of CCR5/CXCR3 expressing CD8+ cells in liver damage and viral control during persistent hepatitis C virus infection. *J Hepatol*. 2007;47(5):632-641. doi:10.1016/j.jhep.2007.04.009

162. Klose CSN, Flach M, Möhle L, et al. Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages. *Cell*. 2014;157(2):340-356. doi:10.1016/j.cell.2014.03.030

163. Tanriver Y, Diefenbach A. Transcription factors controlling development and function of innate lymphoid cells. *Int Immunol*. 2014;26(3):119-128. doi:10.1093/intimm/dxt063

164. Fuchs A, Vermi W, Lee JS, et al. Intraepithelial type 1 innate lymphoid cells are a unique subset of il-12- and il-15-responsive ifn-γ-producing cells. *Immunity*. 2013;38(4):769-781. doi:10.1016/j.immuni.2013.02.010

165. Sun JC, Lanier LL. NK cell development, homeostasis and function: Parallels with CD8 + T cells. *Nat Rev Immunol*. 2011;11(10):645-657. doi:10.1038/nri3044

166. Romagnani S. Lymphokine Production by Human T Cells in Disease States. *Annu Rev Immunol*. 1994;12(1):227-257. doi:10.1146/annurev.iy.12.040194.001303

167. Yoshimoto T. The hunt for the source of primary interleukin-4: How we discovered that natural killer T cells and basophils determine T helper type 2 cell differentiation in vivo. *Front Immunol*. 2018;9(APR). doi:10.3389/fimmu.2018.00716

168. Perrigoue JG, Saenz SA, Siracusa MC, et al. MHC class II-dependent basophil-CD4+ T cell interactions promote TH2 cytokine-dependent immunity. *Nat Immunol*. 2009;10(7):697-705. doi:10.1038/ni.1740

169. Sokol CL, Chu NQ, Yu S, Nish SA, Laufer TM, Medzhitov R. Basophils function as antigen-presenting cells for an allergen-induced T helper type 2 response. *Nat Immunol*. 2009;10(7):713-720. doi:10.1038/ni.1738

170. Yoshimoto T, Yasuda K, Tanaka H, et al. Basophils contribute to TH2-IgE responses in vivo via IL-4 production and presentation of peptide-MHC class II complexes to CD4+ T cells. *Nat Immunol*. 2009;10(7):706-712. doi:10.1038/ni.1737

171. Noben-Trauth N, Hu-Li J, Paul WE. Conventional, Naive CD4+ T Cells Provide an Initial Source of IL-4 During Th2 Differentiation. *J Immunol*. 2000;165(7):3620-3625. doi:10.4049/jimmunol.165.7.3620

172. Pulendran B, Artis D. New paradigms in type 2 immunity. *Science (80-)*. 2012;337(6093):431-435. doi:10.1126/science.1221064

173. Zhang DH, Yang L, Cohn L, et al. Inhibition of allergic inflammation in a murine model of asthma by expression of a dominant-negative mutant of GATA-3. *Immunity*. 1999;11(4):473-482. doi:10.1016/S1074-7613(00)80122-3

174. Pai S-Y, Truitt ML, Ho I-C. GATA-3 deficiency abrogates the development and maintenance of T helper type 2 cells. *Proc Natl Acad Sci*. 2004;101(7):1993-1998. doi:10.1073/pnas.0308697100

175. Zhu J, Min B, Hu-Li J, et al. Conditional deletion of Gata3 shows its essential function in TH1-TH2 responses. *Nat Immunol*. 2004;5(11):1157-1165. doi:10.1038/ni1128

176. Zheng WP, Flavell RA. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell*. 1997;89(4):587-596. doi:10.1016/S0092-8674(00)80240-8

177. Wei G, Abraham BJ, Yagi R, et al. Genome-wide Analyses of Transcription Factor GATA3-Mediated Gene Regulation in Distinct T Cell Types. *Immunity*. 2011;35(2):299-311. doi:10.1016/j.immuni.2011.08.007

178. Zhu J, Cote-Sierra J, Guo L, Paul WE. Stat5 activation plays a critical role in Th2 differentiation. *Immunity*. 2003;19(5):739-748. doi:10.1016/S1074-7613(03)00292-9

179. Cosmi L, Annunziato F, Iwasaki M, et al. CRTH2 is the most reliable marker for the detection of circulating human type 2 Th and type 2 T cytotoxic cells in health and disease. *Eur J Immunol*. 2000;30(10):2972-2979. doi:10.1002/1521-4141(200010)30:10<2972::AID-IMMU2972>3.0.CO;2-#

180. Acacia de Sa Pinheiro A, Morrot A, Chakravarty S, et al. IL-4 induces a wide-spectrum intracellular signaling cascade in CD8 + T cells . *J Leukoc Biol*. 2007;81(4):1102-1110. doi:10.1189/jlb.0906583

181. Neill DR, Wong SH, Bellosi A, et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature*. 2010;464(7293):1367-1370. doi:10.1038/nature08900

182. Hoyler T, Klose CSN, Souabni A, et al. The Transcription Factor GATA-3 Controls Cell Fate and Maintenance of Type 2 Innate Lymphoid Cells. *Immunity*. 2012;37(4):634-648. doi:10.1016/j.immuni.2012.06.020

183. Halim TYF, MacLaren A, Romanish MT, Gold MJ, McNagny KM, Takei F. Retinoic-Acid-Receptor-Related Orphan Nuclear Receptor Alpha Is Required for Natural Helper Cell Development and Allergic Inflammation. *Immunity*. 2012;37(3):463-474. doi:10.1016/j.immuni.2012.06.012

184. Spooner CJ, Lesch J, Yan D, et al. Specification of type 2 innate lymphocytes by the transcriptional determinant Gfi1. *Nat Immunol*. 2013;14(12):1229-1236. doi:10.1038/ni.2743

185. Chang YJ, Kim HY, Albacker LA, et al. Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity. *Nat Immunol*. 2011;12(7):631-638. doi:10.1038/ni.2045

186. Lee MW, Odegaard JI, Mukundan L, et al. Activated type 2 innate lymphoid cells regulate beige fat biogenesis. *Cell*. 2015;160(1-2):74-87. doi:10.1016/j.cell.2014.12.011

187. Brestoff JR, Kim BS, Saenz SA, et al. Group 2 innate lymphoid cells promote beiging of white adipose tissue and limit obesity. *Nature*. 2015;519(7542):242-246. doi:10.1038/nature14115

188. Walker JA, McKenzie ANJ. Development and function of group 2 innate lymphoid cells. *Curr Opin Immunol*. 2013;25(2):148-155. doi:10.1016/j.coi.2013.02.010

189. Mjösberg JM, Trifari S, Crellin NK, et al. Human IL-25-and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161. *Nat Immunol*. 2011;12(11):1055-1062. doi:10.1038/ni.2104

190. Harrington LE, Hatton RD, Mangan PR, et al. Interleukin 17-producing CD4 + effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol*. 2005;6(11):1123-1132. doi:10.1038/ni1254

191. Cosmi L, De Palma R, Santarlasci V, et al. Human interleukin 17–producing cells originate from a CD161 + CD4 + T cell precursor . *J Exp Med*. 2008;205(8):1903-1916. doi:10.1084/jem.20080397

192. Acosta-Rodriguez E V., Napolitani G, Lanzavecchia A, Sallusto F. Interleukins 1β and 6 but not transforming growth factor-β are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol*. 2007;8(9):942-949. doi:10.1038/ni1496

193. Ghoreschi K, Laurence A, Yang XP, et al. Generation of pathogenic T H 17 cells in the absence of TGF-β signalling. *Nature*. 2010;467(7318):967-971. doi:10.1038/nature09447

194. Wan C-K, Andraski AB, Spolski R, et al. Opposing roles of STAT1 and STAT3 in IL-21 function in CD4 + T cells . *Proc Natl Acad Sci*. 2015;112(30):9394-9399. doi:10.1073/pnas.1511711112

195. Acosta-Rodriguez E V., Rivino L, Geginat J, et al. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat Immunol*. 2007;8(6):639-646. doi:10.1038/ni1467

196. Annunziato F, Cosmi L, Santarlasci V, et al. Phenotypic and functional features of human Th17 cells. *J Exp Med*. 2007;204(8):1849-1861. doi:10.1084/jem.20070663

197. Santarlasci V, Maggi L, Mazzoni A, et al. IL-4-induced gene 1 maintains high Tob1 expression that contributes to TCR unresponsiveness in human T helper 17 cells. *Eur J Immunol*. 2014;44(3):654-661. doi:10.1002/eji.201344047

198. Santarlasci V, Maggi L, Capone M, et al. Rarity of Human T Helper 17 Cells Is due to Retinoic Acid Orphan Receptor-Dependent Mechanisms that Limit Their Expansion. *Immunity*. 2012;36(2):201-214. doi:10.1016/j.immuni.2011.12.013

199. Kondo T, Takata H, Matsuki F, Takiguchi M. Cutting Edge: Phenotypic Characterization and Differentiation of Human CD8+ T Cells Producing IL-17. *J Immunol*. 2009;182(4):1794-1798. doi:10.4049/jimmunol.0801347

200. Yen H-R, Harris TJ, Wada S, et al. Tc17 CD8 T Cells: Functional Plasticity and Subset Diversity. *J Immunol*. 2009;183(11):7161-7168. doi:10.4049/jimmunol.0900368

201. Annunziato F, Romagnani C, Romagnani S. The 3 major types of innate and adaptive cell-mediated effector immunity. *J Allergy Clin Immunol*. 2015;135(3):626-635. doi:10.1016/j.jaci.2014.11.001

202. Spits H, Artis D, Colonna M, et al. Innate lymphoid cells-a proposal for uniform nomenclature. *Nat Rev Immunol*. 2013;13(2):145-149. doi:10.1038/nri3365

203. Killig M, Glatzer T, Romagnani C. Recognition strategies of group 3 innate lymphoid cells. *Front*

Immunol. 2014;5(APR). doi:10.3389/fimmu.2014.00142

204. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *J Immunol*. 2003;4:330-336. doi:10.1038/ni904

205. Furtado GC, de Lafaille MAC, Kutchukhidze N, Lafaille JJ. Interleukin 2 Signaling Is Required for CD4 $^{+}$ Regulatory T Cell Function. *J Exp Med*. 2002;196(6):851-857. doi:10.1084/jem.20020190

206. Josefowicz SZ, Lu L-F, Rudensky AY. Regulatory T Cells: Mechanisms of Differentiation and Function. *Annu Rev Immunol*. 2012;30(1):531-564. doi:10.1146/annurev.immunol.25.022106.141623

207. Chen W, Jin W, Hardegen N, et al. Conversion of Peripheral CD4 + CD25 − Naive T Cells to CD4 + CD25 + Regulatory T Cells by TGF-β Induction of Transcription Factor Foxp3 . *J Exp Med*. 2003;198(12):1875-1886. doi:10.1084/jem.20030152

208. Samstein RM, Josefowicz SZ, Arvey A, Treuting PM, Rudensky AY. Extrathymic generation of regulatory T cells in placental mammals mitigates maternal-fetal conflict. *Cell*. 2012;150(1):29-38. doi:10.1016/j.cell.2012.05.031

209. Zheng Y, Josefowicz S, Chaudhry A, Peng XP, Forbush K, Rudensky AY. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. *Nature*. 2010;463(7282):808-812. doi:10.1038/nature08750

210. Wang S, Xia P, Chen Y, et al. Regulatory Innate Lymphoid Cells Control Innate Intestinal Inflammation. *Cell*. 2017;171(1):201-216.e18. doi:10.1016/j.cell.2017.07.027

211. Wing K, Onishi Y, Prieto-Martin P, et al. CTLA-4 control over Foxp3+ regulatory T cell function. *Science (80-)*. 2008;322(5899):271-275. doi:10.1126/science.1160062

212. Levine AG, Arvey A, Jin W, Rudensky AY. Continuous requirement for the TCR in regulatory T cell function. *Nat Immunol*. 2014;15(11):1070-1078. doi:10.1038/ni.3004

213. Plank MW, Kaiko GE, Maltby S, et al. Th22 Cells Form a Distinct Th Lineage from Th17 Cells In Vitro with Unique Transcriptional Properties and Tbet-Dependent Th1 Plasticity. *J Immunol*. 2017;198(5):2182-2190. doi:10.4049/jimmunol.1601480

214. Eyerich S, Eyerich K, Pennino D, et al. Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. *J Clin Invest*. 2009;119(12):3573-3585. doi:10.1172/JCI40202

215. Kaplan MH. Th9 cells: Differentiation and disease. *Immunol Rev*. 2013;252(1):104-115. doi:10.1111/imr.12028

216. Staudt V, Bothur E, Klein M, et al. Interferon-Regulatory Factor 4 Is Essential for the Developmental Program of T Helper 9 Cells. *Immunity*. 2010;33(2):192-202. doi:10.1016/j.immuni.2010.07.014

217. Lötvall J, Akdis CA, Bacharier LB, et al. Asthma endotypes: A new approach to classification of disease entities within the asthma syndrome. *J Allergy Clin Immunol*. 2011;127(2):355-360. doi:10.1016/j.jaci.2010.11.037

218. Carr TF, Bleecker E. Asthma heterogeneity and severity. *World Allergy Organ J*. 2016;9(1). doi:10.1186/s40413-016-0131-2

219. Asher I, Pearce N. Global burden of asthma among children. *Int J Tuberc Lung Dis*. 2014;18(11):1269-1278. doi:10.5588/ijtld.14.0170

220. Masoli M, Fabian D, Holt S, Beasley R. The global burden of asthma: Executive summary of the GINA Dissemination Committee Report. *Allergy Eur J Allergy Clin Immunol*. 2004;59(5):469-478. doi:10.1111/j.1398-9995.2004.00526.x

221. Guibas G V., Mathioudakis AG, Tsoumani M, Tsabouri S. Relationship of Allergy with Asthma: There Are More Than the Allergy "Eggs" in the Asthma "Basket." *Front Pediatr*. 2017;5:1-7. doi:10.3389/fped.2017.00092

222. Agache I, Akdis CA. Endotypes of allergic diseases and asthma: An important step in building blocks for the future of precision medicine. *Allergol Int*. 2016;65(3):243-252. doi:10.1016/j.alit.2016.04.011

223. Agache I, Sugita K, Morita H, Akdis M, Akdis CA. The Complex Type 2 Endotype in Allergy and Asthma: From Laboratory to Bedside. *Curr Allergy Asthma Rep*. 2015;15(6). doi:10.1007/s11882-015-0529-x

224. Woodruff PG, Modrek B, Choy DF, et al. T-helper type 2-driven inflammation defines major subphenotypes of asthma. *Am J Respir Crit Care Med*. 2009;180(5):388-395. doi:10.1164/rccm.200903-0392OC

225. Jarvis D, Burney P. ABC of allergies: The epidemiology of allergic disease. *BMJ*. 2011;316(7131):607-610. doi:10.1136/bmj.316.7131.607

226. Lau S, Nickel R, Niggemann B, et al. The development of childhood asthma: lessons from the German Multicentre Allergy Study (MAS). *Paediatr Respir Rev*. 2002;3(3):265-272. http://www.ncbi.nlm.nih.gov/pubmed/12376064.

227. Gustafsson D, Sjöberg O, Foucard T. Development of allergies and asthma in infants and young children with atopic dermatitis - A prospective follow-up to 7 years age. *Allergy Eur J Allergy Clin Immunol*. 2000;55(3):240-245. doi:10.1034/j.1398-9995.2000.00391.x

228. Kulig M, Bergmann R, Klettke U, Wahn V, Tacke U, Wahn U. Natural course of sensitization to food and inhalant allergens during the first 6 years of life. *J Allergy Clin Immunol*. 1999;103(6):1173-1179. doi:10.1016/S0091-6749(99)70195-8

229. Ohshima Y, Yamada A, Hiraoka M, et al. Early sensitization to house dust mite is a major risk factor for subsequent development of bronchial asthma in Japanese infants with atopic dermatitis: Results of a 4-year followup study. *Ann Allergy, Asthma Immunol*. 2002;89(3):265-270. doi:10.1016/S1081-1206(10)61953-9

230. Rhodes HL, Thomas P, Sporik R, Holgate ST, Cogswell JJ. A birth cohort study of subjects at risk of atopy: Twenty-two-year follow-up of wheeze and atopic status. *Am J Respir Crit Care Med*. 2002;165(2):176-180. doi:10.1164/ajrccm.165.2.2104032

231. Rhodes HL, Sporik R, Thomas P, Holgate ST, Cogswell JJ. Early life risk factors for adult asthma: A birth cohort study of subjects at risk. *J Allergy Clin Immunol*. 2001;108(5):720-725.

doi:10.1067/mai.2001.119151

232. Illi S, Von Mutius E, Lau S, et al. The natural course of atopic dermatitis from birth to age 7 years and the association with asthma. *J Allergy Clin Immunol*. 2004;113(5):925-931. doi:10.1016/j.jaci.2004.01.778

233. Corren J. Allergic rhinitis and asthma: How important is the link? *J Allergy Clin Immunol*. 1997;99(2). doi:10.1016/S0091-6749(97)70127-1

234. Settipane RJ, Hagy GW, Settipane GA. Long-Term Risk Factors for Developing Asthma and Allergic Rhinitis: A 23-Year Follow-Up Study of College Students. *Allergy Asthma Proc*. 2006;15(1):21-25. doi:10.2500/108854194778816634

235. Pawankar R. Allergic rhinitis and asthma: Are they manifestations of one syndrome? *Clin Exp Allergy*. 2006;36(1):1-4. doi:10.1111/j.1365-2222.2006.02420.x

236. Compalati E, Ridolo E, Passalacqua G, Braido F, Villa E, Canonica GW. The link between allergic rhinitis and asthma: The united airways disease. *Expert Rev Clin Immunol*. 2010;6(3):413-423. doi:10.1586/eci.10.15

237. Fokkens W, Van Drunen CM. Nose and lung, two of a kind? *Allergy Eur J Allergy Clin Immunol*. 2006;61(6):653-655. doi:10.1111/j.1398-9995.2006.01169.x

238. Rowe-Jones JM. The link between the nose and lung, perennial rhinitis and asthma - Is it the same disease? In: *Allergy: European Journal of Allergy and Clinical Immunology*. Vol 52. ; 1997:20-28. doi:10.1111/j.1398-9995.1997.tb04818.x

239. Spector SL. Overview of comorbid associations of allergic rhinitis. *J Allergy Clin Immunol*. 1997;99(2). doi:10.1016/S0091-6749(97)70126-X

240. Wallace D V., Dykewicz MS, Bernstein DI, et al. The diagnosis and management of rhinitis: An updated practice parameter. *J Allergy Clin Immunol*. 2008;122(2 SUPPL.). doi:10.1016/j.jaci.2008.06.003

241. Bachert C, Vignola AM, Gevaert P, Leynaert B, Van Cauwenberge P, Bousquet J. Allergic rhinitis, rhinosinusitis, and asthma: One airway disease. *Immunol Allergy Clin North Am*. 2004;24(1):19-43. doi:10.1016/S0889-8561(03)00104-8

242. Chung KF. Asthma phenotyping: A necessity for improved therapeutic precision and new targeted therapies. *J Intern Med*. 2016;279(2):192-204. doi:10.1111/joim.12382

243. Wesolowska-Andersen A, Seibold MA. Airway molecular endotypes of asthma: Dissecting the heterogeneity. *Curr Opin Allergy Clin Immunol*. 2015;15(2):163-168. doi:10.1097/ACI.0000000000000148

244. Simpson JL, Grissell T V., Douwes J, Scott RJ, Boyle MJ, Gibson PG. Innate immune activation in neutrophilic asthma and bronchiectasis. *Thorax*. 2007;62(3):211-218. doi:10.1136/thx.2006.061358

245. Cosmi L, Maggi L, Santarlasci V, et al. Identification of a novel subset of human circulating memory CD4+ T cells that produce both IL-17A and IL-4. *J Allergy Clin Immunol*. 2010;125(1-3). doi:10.1016/j.jaci.2009.10.012

246. Liu W, Liu S, Verma M, et al. Mechanism of TH2/TH17-predominant and neutrophilic TH2/TH17-low subtypes of asthma. *J Allergy Clin Immunol*. 2017;139(5):1548-1558.e4. doi:10.1016/j.jaci.2016.08.032

247. Raymond M, Van VQ, Wakahara K, Rubio M, Sarfati M. Lung dendritic cells induce TH17 cells that produce T H2 cytokines, express GATA-3, and promote airway inflammation. *J Allergy Clin Immunol*. 2011;128(1):192-201.e6. doi:10.1016/j.jaci.2011.04.029

248. Nakajima S, Kitoh A, Egawa G, et al. IL-17A as an inducer for Th2 immune responses in murine atopic dermatitis models. *J Invest Dermatol*. 2014;134(8):2122-2130. doi:10.1038/jid.2014.51

249. Ponichtera HE, Shainheit MG, Liu BC, et al. CD209a Expression on Dendritic Cells Is Critical for the Development of Pathogenic Th17 Cell Responses in Murine Schistosomiasis. *J Immunol*. 2014;192(10):4655-4665. doi:10.4049/jimmunol.1400121

250. EDFORS-LUBS M -L. ALLERGY IN 7000 TWIN PAIRS. *Allergy*. 1971;26(4):249-285. doi:10.1111/j.1398-9995.1971.tb01300.x

251. Fagnani C, Annesi-Maesano I, Brescianini S, et al. Heritability and Shared Genetic Effects of Asthma and Hay Fever: An Italian Study of Young Twins. *Twin Res Hum Genet*. 2008;11(02):121-131. doi:10.1375/twin.11.2.121

252. Duffy DL, Martin NG, Battistutta D, Hopper JL, Mathews JD. Genetics of Asthma and Hay Fever in Australian Twins. *Am Rev Respir Dis*. 1990;142:1351-1358. doi:10.1164/ajrccm/142.6_pt_1.1351

253. van Beijsterveldt CEM, Boomsma DI. Genetics of parentally reported asthma, eczema and rhinitis in 5-yr-old twins. *Eur Respir J*. 2007;29(3):516-521. doi:10.1183/09031936.00065706

254. Nystad W, Røysamb E, Magnus P, Tambs K, Harris JR. A comparison of genetic and environmental variance structures for asthma, hay fever and eczema with symptoms of the same diseases: A study of Norwegian twins. *Int J Epidemiol*. 2005;34(6):1302-1309. doi:10.1093/ije/dyi061

255. Thomsen SF, Van Der Sluis S, Kyvik KO, Skytthe A, Backer V. Estimates of asthma heritability in a large twin sample. *Clin Exp Allergy*. 2010;40(7):1054-1061. doi:10.1111/j.1365-2222.2010.03525.x

256. Hallstrand TS, Fischer ME, Wurfel MM, Afari N, Buchwald D, Goldberg J. Genetic pleiotropy between asthma and obesity in a community-based sample of twins. *J Allergy Clin Immunol*. 2005;116(6):1235-1241. doi:10.1016/j.jaci.2005.09.016

257. Koeppen-Schomerus G, Stevenson J, Plomin R. Genes and environment in asthma: A study of 4 year old twins. *Arch Dis Child*. 2001;85(5):398-400. doi:10.1136/adc.85.5.398

258. Skadhauge LR, Christensen K, Kyvik KO, Sigsgaard T. Genetic and environmental influence on asthma: A population-based study of 11,688 Danish twin pairs. *Eur Respir J*. 1999;13(1):8-14. doi:10.1183/09031936.99.13100899

259. Laitinen T, Räsänen M, Kaprio J, Koskenvuo M, Laitinen LA. Importance of genetic factors in adolescent asthma: A population-based twin-family study. *Am J Respir Crit Care Med*. 1998;157(4 PART I):1073-1078.

260. Nieminen MM, Kaprio J, Koskenvuo M. A population-based study of bronchial asthma in adult twin pairs. *Chest*. 1991;100(1):70-75. doi:10.1378/chest.100.1.70

261. Verlaan DJ, Berlivet S, Hunninghake GM, et al. Allele-Specific Chromatin Remodeling in the ZPBP2/GSDMB/ORMDL3 Locus Associated with the Risk of Asthma and Autoimmune Disease. *Am J Hum Genet*. 2009;85(3):377-393. doi:10.1016/j.ajhg.2009.08.007

262. Halapi E, Gudbjartsson DF, Jonsdottir GM, et al. A sequence variant on 17q21 is associated with age at onset and severity of asthma. *Eur J Hum Genet*. 2010;18(8):902-908. doi:10.1038/ejhg.2010.38

263. Demenais F, Margaritte-Jeannin P, Barnes KC, et al. Multiancestry association study identifies new asthma risk loci that colocalize with immune-cell enhancer marks. *Nat Genet*. 2018;50:42-53. doi:10.1038/s41588-017-0014-7

264. Ziegler SF. The role of thymic stromal lymphopoietin (TSLP) in allergic disorders. *Curr Opin Immunol*. 2010;22(6):795-799. doi:10.1016/j.coi.2010.10.020

265. Smith DE. IL-33: A tissue derived cytokine pathway involved in allergic inflammation and asthma. *Clin Exp Allergy*. 2010;40(2):200-208. doi:10.1111/j.1365-2222.2009.03384.x

266. Borish L, Steinke JW. Interleukin-33 in asthma: How big of a role does it play? *Curr Allergy Asthma Rep*. 2011;11(1):7-11. doi:10.1007/s11882-010-0153-8

267. Lloyd CM. IL-33 family members and asthma - bridging innate and adaptive immune responses. *Curr Opin Immunol*. 2010;22(6):800-806. doi:10.1016/j.coi.2010.10.006

268. Harada M, Hirota T, Jodo AI, et al. Thymic stromal lymphopoietin gene promoter polymorphisms are associated with susceptibility to bronchial asthma. *Am J Respir Cell Mol Biol*. 2011;44(6):787-793. doi:10.1165/rcmb.2009-0418OC

269. Hunninghake GM, Soto-Quirós ME, Avila L, et al. TSLP polymorphisms are associated with asthma in a sex-specific fashion. *Allergy Eur J Allergy Clin Immunol*. 2010;65(12):1566-1575. doi:10.1111/j.1398-9995.2010.02415.x

270. Bunyavanich S, Melen E, Wilk JB, et al. Thymic stromal lymphopoietin (TSLP) is associated with allergic rhinitis in children with asthma. *Clin Mol Allergy*. 2011;9. doi:10.1186/1476-7961-9-1

271. Savenije OEM, Kerkhof M, Reijmerink NE, et al. Interleukin-1 receptor-like 1 polymorphisms are associated with serum IL1RL1-a, eosinophils, and asthma in childhood. *J Allergy Clin Immunol*. 2011. doi:10.1016/j.jaci.2010.12.014

272. Savenije OE, Mahachie John JM, Granell R, et al. Association of IL33-IL-1 receptor-like 1 (IL1RL1) pathway polymorphisms with wheezing phenotypes and asthma in childhood. *J Allergy Clin Immunol*. 2014;134(1):170-177. doi:10.1016/j.jaci.2013.12.1080

273. Reijmerink NE, Postma DS, Bruinenberg M, et al. Association of IL1RL1, IL18R1, and IL18RAP gene cluster polymorphisms with asthma and atopy. *J Allergy Clin Immunol*. 2008;122(3):651-654.e8. doi:10.1016/j.jaci.2008.06.030

274. Moffatt MF, Phil D, Gut IG, Demenais F, Strachan DP. A Large-Scale, Consortium-Based Genomewide Association Study of Asthma. *N Engl J Med*. 2010;363(13):1211-1221.

doi:10.1056/NEJMoa0906312.A

275. Bisgaard H, Bønnelykke K, Sleiman PMA, et al. Chromosome 17q21 gene variants are associated with asthma and exacerbations but not atopy in early childhood. *Am J Respir Crit Care Med*. 2009. doi:10.1164/rccm.200809-1436OC

276. Halapi E, Gudbjartsson DF, Jonsdottir GM, et al. A sequence variant on 17q21 is associated with age at onset and severity of asthma. *Eur J Hum Genet*. 2010;18(8):902-908. doi:10.1038/ejhg.2010.38

277. Stiemsma L, Reynolds L, Turvey S, Finlay B. The hygiene hypothesis: current perspectives and future therapies. *ImmunoTargets Ther*. July 2015:143. doi:10.2147/ITT.S61528

278. Macpherson AJ, Harris NL. Interactions between commensal intestinal bacteria and the immune system. *Nat Rev Immunol*. 2004;4(6):478-485. doi:10.1038/nri1373

279. Round JL, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol*. 2009;9(5):313-323. doi:10.1038/nri2515

280. Herbst T, Sichelstiel A, Schär C, et al. Dysregulation of allergic airway inflammation in the absence of microbial colonization. *Am J Respir Crit Care Med*. 2011;184(2):198-205. doi:10.1164/rccm.201010-1574OC

281. Cario E, Gerken G, Podolsky DK. Toll-Like Receptor 2 Controls Mucosal Inflammation by Regulating Epithelial Barrier Function. *Gastroenterology*. 2007;132(4):1359-1374. doi:10.1053/j.gastro.2007.02.056

282. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell*. 2004;118(2):229-241. doi:10.1016/j.cell.2004.07.002

283. Rakoff-Nahoum S, Hao L, Medzhitov R. Role of Toll-like Receptors in Spontaneous Commensal-Dependent Colitis. *Immunity*. 2006;25(2):319-329. doi:10.1016/j.immuni.2006.06.010

284. Sellon RK, Tonkonogy S, Schultz M, et al. Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. *Infect Immun*. 1998;66(11):5224-5231.

285. Sudo N, Sawarnura S, Tanaka K, Aiba Y, Kubo C, Koga Y. The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. *J Immunol*. 1997;159:1739-1745.

286. Abrahamsson TR, Jakobsson HE, Andersson AF, Björkstén B, Engstrand L, Jenmalm MC. Low gut microbiota diversity in early infancy precedes asthma at school age. *Clin Exp Allergy*. 2014;44(6):842-850. doi:10.1111/cea.12253

287. K.E. F, A.R. S, S. H, et al. Neonatal gut microbiota associates with childhood multisensitized atopy and T cell differentiation. *Nat Med*. 2016;22(10):1187-1191. doi:10.1038/nm.4176

288. Chieppa M, Rescigno M, Huang AYC, Germain RN. Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement. *J Exp Med*. 2006;203(13):2841-2852. doi:10.1084/jem.20061884

289. Gill MA. The role of dendritic cells in asthma. *J Allergy Clin Immunol*. 2012;129(4):889-901. doi:10.1016/j.jaci.2012.02.028

290. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. *Nat Immunol*. 2004;5(10):987-995. doi:10.1038/ni1112

291. Schuijs MJ, Willart MA, Vergote K, et al. Farm dust and endotoxin protect against allergy through A20 induction in lung epithelial cells. *Science (80-)*. 2015;349(6252):1106-1110. doi:10.1126/science.aac6623

292. Wong JMW, De Souza R, Kendall CWC, Emam A, Jenkins DJA. Colonic health: Fermentation and short chain fatty acids. In: *Journal of Clinical Gastroenterology*. Vol 40. ; 2006:235-243. doi:10.1097/00004836-200603000-00015

293. Macia L, Tan J, Vieira AT, et al. Metabolite-sensing receptors GPR43 and GPR109A facilitate dietary fibre-induced gut homeostasis through regulation of the inflammasome. *Nat Commun*. 2015;6. doi:10.1038/ncomms7734

294. Plunkett CH, Nagler CR. The Influence of the Microbiome on Allergic Sensitization to Food. *J Immunol*. 2017;198(2):581-589. doi:10.4049/jimmunol.1601266

295. Corrêa-Oliveira R, Fachi JL, Vieira A, Sato FT, Vinolo MAR. Regulation of immune cell function by short-chain fatty acids. *Clin Transl Immunol*. 2016;5(4). doi:10.1038/cti.2016.17

296. Smith PM, Howitt MR, Panikov N, et al. The microbial metabolites, short-chain fatty acids, regulate colonic Tregcell homeostasis. *Science (80-)*. 2013;341(6145):569-573. doi:10.1126/science.1241165

297. Martin-Gallausiaux C, Béguet-Crespel F, Marinelli L, et al. Butyrate produced by gut commensal bacteria activates TGF-beta1 expression through the transcription factor SP1 in human intestinal epithelial cells. *Sci Rep*. 2018;8(1). doi:10.1038/s41598-018-28048-y

298. Furusawa Y, Obata Y, Fukuda S, et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature*. 2013;504(7480):446-450. doi:10.1038/nature12721

299. Arpaia N, Campbell C, Fan X, et al. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature*. 2013;504(7480):451-455. doi:10.1038/nature12726

300. Trompette A, Gollwitzer ES, Yadava K, et al. Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. *Nat Med*. 2014;20(2):159-166. doi:10.1038/nm.3444

301. Canani RB, Sangwan N, Stefka AT, et al. Lactobacillus rhamnosus GG-supplemented formula expands butyrate-producing bacterial strains in food allergic infants. *ISME J*. 2016;10(3):742-750. doi:10.1038/ismej.2015.151

302. Lluis A, Depner M, Gaugler B, et al. Increased regulatory T-cell numbers are associated with farm milk exposure and lower atopic sensitization and asthma in childhood. *J Allergy Clin Immunol*. 2014;133(2). doi:10.1016/j.jaci.2013.06.034

303. S. M, F. B, J. G, et al. Farm exposure and time trends in early childhood may influence DNA methylation in genes related to asthma and allergy. *Allergy Eur J Allergy Clin Immunol*. 2013;68(3):355-364. doi:10.1111/all.12097

304. Kumar H, Lund R, Laiho A, et al. Gut Microbiota as an Epigenetic Regulator: Pilot Study Based on Whole-Genome Methylation Analysis. *MBio*. 2014;5(6). doi:10.1128/mbio.02113-14

305. Ji H, Khurana Hershey GK. Genetic and epigenetic influence on the response to environmental particulate matter. *J Allergy Clin Immunol*. 2012;129(1):33-41. doi:10.1016/j.jaci.2011.11.008

306. Gualtieri M, Mantecca P, Cetta F, Camatini M. Organic compounds in tire particle induce reactive oxygen species and heat-shock proteins in the human alveolar cell line A549. *Environ Int*. 2008;34(4):437-442. doi:10.1016/j.envint.2007.09.010

307. Franco R, Sánchez-Olea R, Reyes-Reyes EM, Panayiotidis MI. Environmental toxicity, oxidative stress and apoptosis: Ménage à Trois. *Mutat Res - Genet Toxicol Environ Mutagen*. 2009;674(1-2):3-22. doi:10.1016/j.mrgentox.2008.11.012

308. Zhang J, Ghio AJ, Chang W, Kamdar O, Rosen GD, Upadhyay D. Bim mediates mitochondria-regulated particulate matter-induced apoptosis in alveolar epithelial cells. *FEBS Lett*. 2007;581(22):4148-4152. doi:10.1016/j.febslet.2007.07.080

309. Dagher Z, Garçon G, Billet S, et al. Activation of different pathways of apoptosis by air pollution particulate matter (PM2.5) in human epithelial lung cells (L132) in culture. *Toxicology*. 2006;225(1):12-24. doi:10.1016/j.tox.2006.04.038

310. Marano F. Effects of fine atmospheric particles on human respiratory epithelium: The role of organic compounds in oxidative stress, inflammatory and repair responses. *Toxicol Lett*. 2009;189:S43-S44. doi:10.1016/j.toxlet.2009.06.116

311. Baeza-Squiban A, Bonvallot V, Boland S, Marano F. Airborne particles evoke an inflammatory response in human airway epithelium. Activation of transcription factors. In: *Cell Biology and Toxicology*. Vol 15. ; 1999:375-380. doi:10.1023/A:1007653900063

312. Lodovici M, Bigagli E. Oxidative stress and air pollution exposure. *J Toxicol*. 2011;2011. doi:10.1155/2011/487074

313. Baccarelli A, Wright RO, Bollati V, et al. Rapid DNA methylation changes after exposure to traffic particles. *Am J Respir Crit Care Med*. 2009;179(7):572-578. doi:10.1164/rccm.200807-1097OC

314. Carmona JJ, Sofer T, Hutchinson J, et al. Short-Term airborne particulate matter exposure alters the epigenetic landscape of human genes associated with the mitogen-Activated protein kinase network: A cross-sectional study. *Environ Heal A Glob Access Sci Source*. 2014;13(1). doi:10.1186/1476-069X-13-94

315. Madrigano J, Baccarelli A, Mittleman MA, et al. Prolonged exposure to particulate pollution, genes associated with glutathione pathways, and DNA methylation in a cohort of older men. *Environ Health Perspect*. 2011;119(7):977-982. doi:10.1289/ehp.1002773

316. Tarantini L, Bonzini M, Apostoli P, et al. Effects of particulate matter on genomic DNA methylation content and iNOS promoter methylation. *Environ Health Perspect*. 2009;117(2):217-222. doi:10.1289/ehp.11898

317. Nadeau K, McDonald-Hyman C, Noth EM, et al. Ambient air pollution impairs regulatory T-cell function in asthma. *J Allergy Clin Immunol*. 2010;126(4). doi:10.1016/j.jaci.2010.08.008

318. Maier KL, Alessandrini F, Beck-Speier I, et al. Health effects of ambient particulate matter - Biological mechanisms and inflammatory responses to in vitro and in vivo particle exposures. *Inhal Toxicol*. 2008;20(3):319-337. doi:10.1080/08958370701866313

319. Stokholm J, Chawes BL, Vissing N, Bønnelykke K, Bisgaard H. Cat exposure in early life decreases asthma risk from the 17q21 high-risk variant. *J Allergy Clin Immunol*. 2018;141(5):1598-1606. doi:10.1016/j.jaci.2017.07.044

320. Bräuner E V., Loft S, Raaschou-Nielsen O, Vogel U, Andersen PS, Sørensen M. Effects of a 17q21 chromosome gene variant, tobacco smoke and furred pets on infant wheeze. *Genes Immun*. 2012;13(1):94-97. doi:10.1038/gene.2011.51

321. Hron K, Filzmoser P, Thompson K. Linear regression with compositional explanatory variables. *J Appl Stat*. 2009;36(7):769-778. doi:10.1080/0266476YYxxxxxxxx

322. Imai K, Keele L, Tingley D. A General Approach to Causal Mediation Analysis. *Psychol Methods*. 2010;15(4):309-334. doi:10.1037/a0020761

323. Tingley D, Yamamoto T, Hirose K, Keele L, Imai K. Mediation: R Package for Causal Mediation Analysis. *J Stat Softw*. 2014;59(5):1-38. doi:10.18637/jss.v059.i05

324. Van Buuren S, Groothuis-Oudshoom K. mice: Multivariate Imputation by Chained Equations in R. *J Stat*. 2011;45(3):1-67. doi:10.18637/jss.v045.i03>.

325. Zhang Z, Wang L, Tong X. Mediation analysis with missing data through multiple imputation and bootstrap. *Quant Psychol Res*. 2014;140:341-355. doi:10.1007/978-3-319-19977-1_24

326. Illi S, von Mutius E, Liu J, et al. Impairment of T-regulatory cells in cord blood of atopic mothers. *J Allergy Clin Immunol*. 2008;121(6):1491-1499.e13. doi:10.1016/j.jaci.2008.04.010

327. Sakaguchi S, Ono M, Setoguchi R, et al. Foxp3+CD25+CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol Rev*. 2006;212:8-27. doi:10.1111/j.0105-2896.2006.00427.x

328. Kelchtermans H, De Klerck B, Mitera T, et al. Defective CD4+CD25+ regulatory T cell functioning in collagen-induced arthritis: an important factor in pathogenesis, counter-regulated by endogenous IFN. *Arthritis Res Ther*. 2005;7(2):R402-R415. doi:10.1186/ar1500

329. Grindebacke H, Wing K, Andersson AC, Suri-Payer E, Rak S, Rudin A. Defective suppression of Th2 cytokines by CD4+CD25+ regulatory T cells in birch allergies during birch pollen season. *Clin Exp Allergy*. 2004;34(9):1364-1372. doi:10.1111/j.1365-2222.2004.02067.x

330. Zheng SG, Wang P, Gray JD, Wang J, Horwitz DA. IL-2 Is Essential for TGF- to Convert Naive CD4+CD25- Cells to CD25+Foxp3+ Regulatory T Cells and for Expansion of These Cells. *J Immunol*. 2014;178(4):2018-2027. doi:10.4049/jimmunol.178.4.2018

331. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol*. 2005;6(11):1142-1151. doi:10.1038/ni1263

332. Yu A, Zhu L, Altman NH, Malek TR. A Low Interleukin-2 Receptor Signaling Threshold Supports the Development and Homeostasis of T Regulatory Cells. *Immunity*. 2009;30(2):204-217. doi:10.1016/j.immuni.2008.11.014

333. Zhang Y, Collier F, Naselli G, et al. Cord blood monocyte-derived inflammatory cytokines suppress IL-2 and induce nonclassic "TH2-type" immunity associated with development of food allergy. *Sci Transl Med*. 2016;8(321). doi:10.1126/scitranslmed.aad4322

334. Bisgaard H. The Copenhagen Prospective Study on Asthma in Childhood (COPSAC): Design, rationale, and baseline data from a longitudinal birth cohort study. *Ann Allergy, Asthma Immunol*. 2004;93(4):381-389. doi:10.1016/S1081-1206(10)61398-1

335. Bisgaard H, Pipper CB, Bønnelykke K. Endotyping early childhood asthma by quantitative symptom assessment. *J Allergy Clin Immunol*. 2011;127(5):1155-1164.e2. doi:10.1016/j.jaci.2011.02.007

336. Schoos AMM, Chawes BL, Rasmussen MA, Bloch J, Bønnelykke K, Bisgaard H. Atopic endotype in childhood. *J Allergy Clin Immunol*. 2016;137(3):844-851.e4. doi:10.1016/j.jaci.2015.10.004

337. Schoos AMM, Chawes BLK, Følsgaard N V., Samandari N, Bønnelykke K, Bisgaard H. Disagreement between skin prick test and specific IgE in young children. *Allergy Eur J Allergy Clin Immunol*. 2015;70(1):41-48. doi:10.1111/all.12523

338. Chawes BLK, Kreiner-Møller E, Bisgaard H. Objective assessments of allergic and nonallergic rhinitis in young children. *Allergy Eur J Allergy Clin Immunol*. 2009;64(10):1547-1553. doi:10.1111/j.1398-9995.2009.02085.x

339. Team RC. R: A Language and Environment for Statistical Computing. 2018. https://www.r-project.org/.

340. Braun-Fahrländer C, Riedler J, Herz U, et al. Environmental Exposure to Endotoxin and Its Relation to Asthma in School-Age Children. *N Engl J Med*. 2002;347(12):869-877. doi:10.1056/nejmoa020057

341. Vatanen T, Kostic AD, D'Hennezel E, et al. Variation in Microbiome LPS Immunogenicity Contributes to Autoimmunity in Humans. *Cell*. 2016;165(4):842-853. doi:10.1016/j.cell.2016.04.007

342. Saadoun D, Rosenzwajg M, Joly F, et al. Regulatory T-Cell Responses to Low-Dose Interleukin-2 in HCV-Induced Vasculitis. *N Engl J Med*. 2011;365:2067-2077.

343. Hartemann A, Bensimon G, Payan CA, et al. Low-dose interleukin 2 in patients with type 1 diabetes: A phase 1/2 randomised, double-blind, placebo-controlled trial. *Lancet Diabetes Endocrinol*. 2013;1(4):295-305. doi:10.1016/S2213-8587(13)70113-X

344. Vigneron J, Brimaud F, Churlaud G, et al. Low-Dose IL-2 Induces Regulatory T Cell–Mediated Control of Experimental Food Allergy. *J Immunol*. 2016;197(1):188-198. doi:10.4049/jimmunol.1501271

345. Koreth J, Matsuoka K, Kim HT. Interleukin-2 and Regulatory T Cells in Graft-versus-Host Disease. *N Engl J Med*. 2010;362(5):387-401. doi:10.1056/NEJMoa1402121

346. Hofman P, Bahadoran P, Passeron T, et al. Effects of Low-Dose Recombinant Interleukin 2 to Promote T-Regulatory Cells in Alopecia Areata. *JAMA Dermatology*. 2014;150(7):748. doi:10.1001/jamadermatol.2014.504

347. Al Nabhani Z, Dulauroy S, Marques R, et al. A Weaning Reaction to Microbiota Is Required for Resistance to Immunopathologies in the Adult. *Immunity*. 2019. doi:10.1016/j.immuni.2019.02.014

348. Yu A, Snowhite I, Vendrame F, Rosenzwajg M. Selective IL-2 Responsiveness of Regulatory T Cells Through Multiple Intrinsic Mechanisms Supports the Use of Low-Dose IL-2 Therapy in Type 1 Diabetes. *Diabetes*. 2015;64:2172-2183. doi:10.2337/db14-1322

349. Atarashi K, Shima TTT, Imaoka A, et al. Induction of Colonic Regulatory T Cells by Indigenous Clostridium Species. *Science (80-)*. 2011;331(21):337-341.

350. Smith K, McCoy KD, Macpherson AJ. Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. *Semin Immunol*. 2007;19(2):59-69. doi:10.1016/j.smim.2006.10.002

351. Brunetti E, Landi F, Casati A, et al. Analysis of circulating gammadelta+ T cells in children affected by IgE-associated and non-IgE-associated allergic atopic eczema/dermatitis syndrome. *Clin Exp Immunol*. 2005;141(1):116-121. doi:10.1111/j.1365-2249.2005.02813.x

352. Wi J-O, Shim J-U, Park CK, et al. Inverse association of peripheral blood CD4+ invariant natural killer T cells with atopy in human asthma. *Hum Immunol*. 2010;71(2):186-191. doi:10.1016/j.humimm.2009.10.011

353. Bønnelykke K, Sleiman P, Nielsen K, Kreiner-Møller E. A genome-wide association study identifies CDHR3 as a susceptibility locus for early childhood asthma with severe exacerbations. *Nat Genet*. 2014;46(1):51-55. doi:10.1038/ng.2830

354. Stein MM, Thompson EE, Schoettler N, et al. A decade of research on the 17q12-21 asthma locus: Piecing together the puzzle. *J Allergy Clin Immunol*. 2018;142(3):749-764. doi:10.1016/j.jaci.2017.12.974.A

355. Bisgaard H, Pipper CB, Bønnelykke K. Endotyping early childhood asthma by quantitative symptom assessment. *J Allergy Clin Immunol*. 2011;127(5):1155-1164.e2. doi:10.1016/j.jaci.2011.02.007

356. Bisgaard H, Hermansen MN, Loland L, Halkjaer LB, Buchvald F. Intermittent Inhaled Corticosteroids in Infants With Episodic Wheezing. *N Engl J Med*. 2006;354:1998-2005. doi:10.1542/peds.2007-0846nnn

357. Hallas HW, Chawes BL, Rasmussen MA, et al. Airway obstruction and bronchial reactivity from age 1 month until 13 years in children with asthma: A prospective birth cohort study. *PLoS Med*. 2019;16(1). doi:10.1371/journal.pmed.1002722

358. Chawes BLK, Poorisrisak P, Johnston SL, Bisgaard H. Neonatal bronchial hyperresponsiveness precedes acute severe viral bronchiolitis in infants. *J Allergy Clin Immunol*. 2012;130(2). doi:10.1016/j.jaci.2012.04.045

359. Vissing NH, Chawes BLK, Bisgaard H. Increased risk of pneumonia and bronchiolitis after

bacterial colonization of the airways as neonates. *Am J Respir Crit Care Med*. 2013;188(10):1246-1252. doi:10.1164/rccm.201302-0215OC

360. Bisgaard H, Hermansen MN, Bønnelykke K, et al. Association of bacteria and viruses with wheezy episodes in young children: Prospective birth cohort study. *BMJ*. 2010;341(7776):770. doi:10.1136/bmj.c4978

361. VanderWeele T. Mediation Analysis: A Practitioner's Guide. *Ssrn*. 2016. doi:10.1146/annurev-publhealth-032315-021402

362. Macpherson AJ, Hooper L V., Littman DR. Interactions Between the Microbiota and the Immune System. *Science (80-)*. 2012;336(June):1268-1273. doi:10.1126/science.1223490

363. Belkaid Y, Hand TW. Role of the microbiota in immunity and inflammation. *Cell*. 2014;157(1):121-141. doi:10.1016/j.cell.2014.03.011

364. Sutton CE, Lalor SJ, Sweeney CM, Brereton CF, Lavelle EC, Mills KHG. Interleukin-1 and IL-23 Induce Innate IL-17 Production from γδ T Cells, Amplifying Th17 Responses and Autoimmunity. *Immunity*. 2009;31(2):331-341. doi:10.1016/j.immuni.2009.08.001

365. Soulard V, Cavaillon J-M, Marie JC, et al. Cutting Edge: Crucial Role of IL-1 and IL-23 in the Innate IL-17 Response of Peripheral Lymph Node NK1.1- Invariant NKT Cells to Bacteria. *J Immunol*. 2010;186(2):662-666. doi:10.4049/jimmunol.1002725

366. Chao C-C, Yearley JH, Kastelein RA, et al. IL-23 induces spondyloarthropathy by acting on ROR-γt+ CD3+CD4−CD8− entheseal resident T cells. *Nat Med*. 2012;18(7):1069-1076. doi:10.1038/nm.2817

367. Gaffen SL, Jain R, Garg AV CD. IL-23-IL-17 immune axis: Discovery, Mechanistic Understanding, and Clinical Testing. *Nat Rev Immunol*. 2014;14(9):585-600. doi:10.1038/nri3707.IL-23-IL-17

368. Lluis A, Schedel M, Liu J, et al. Asthma-associated polymorphisms in 17q21 influence cord blood ORMDL3 and GSDMA gene expression and IL-17 secretion. *J Allergy Clin Immunol*. 2011;127(6):1587-1594.e6. doi:10.1016/j.jaci.2011.03.015

369. Larkin J, Bedoya SK, Collins EL, Wilson TD, Lau K. Isolation and Th17 Differentiation of Naïve CD4 T Lymphocytes. *J Vis Exp*. 2013;(79). doi:10.3791/50765

11 SUBMITTED PAPER

Title: Reduced IL-2 response from peripheral blood mononuclear cells exposed to bacteria at 6 months of age is associated with elevated total-IgE and allergic rhinitis during the first 7 years of life

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Abbreviations:

COPSAC = Copenhagen Prospective Studies on Asthma in Childhood

PBMC = Peripheral Blood Mononuclear Cell

PCA = Principal Component Analysis

Treg = regulatory T cell

SPT = Skin prick test

Research in context:

Evidence before this study

It is well known that T regulatory cell (Tregs) play important roles in maintaining homeostasis of the immune system. Interleukin 2 (IL-2) is crucial for the development and survival of Tregs acting via its high affinity receptor CD25 and it has been demonstrated that low-dose IL-2 therapy results in significant expansion and activation of Tregs. It has been shown in randomized placebo-controlled trials that low dose IL-2 therapy is safe in humans and has the capacity to successfully control the manifestation of several diseases related to imbalanced immune responses, including hepatitis C virus-mediated vasculitis, graft-versus-host disease, alopecia areata and type I diabetes. Recently, it has been shown that a disturbed IL-2/Treg interplay may be off importance in childhood allergy development as reduced IL-2 expression by cord blood CD4(+) T cells was associated with decreased numbers of Tregs and increased risk of subsequent food allergy in the Barwon Infant study. Further, findings from a mouse model showed that low dose IL-2 treatment could dampen the allergic inflammatory process by inducing expansion and activation of Tregs. These findings suggest a potential application of low dose IL-2 therapy for prevention and treatment of childhood allergy, but further human studies are needed to disentangle the role of IL-2 expression in early life in relation to development of allergic outcomes.

Added value of this study

In this study we investigated the functional immune properties of peripheral blood mononuclear cells (PBMCs) from 6-month-old healthy infants stimulated with pathogenic airway bacteria and show that immune responses characterized by reduced production of IL-2 are associated with development of elevated total-IgE levels and allergic rhinitis during the first 7 years of life. This suggests the existence of suppressed Treg activity in early life heralding the onset of allergy outcomes during early childhood, which may be reverted by low dose IL-2 therapy.

Implications of all the available evidence

These findings support the initiation of low dose IL-2 therapy trials for preventing and treating childhood allergic airway disorders.

ABSTRACT (261 words)

Background: Autoimmunity and allergy have been associated with decreased number and function of regulatory T-cells and low interleukin-2 (IL-2) levels. We aimed to investigate if release of IL-2 from peripheral blood mononuclear cells (PBMCs) stimulated with pathogenic airway bacteria was associated with development of allergy-outcomes in early childhood.

Methods: PBMCs were isolated at age 6 months in 331 infants from the Copenhagen Prospective Studies on Asthma in Childhood 2000 ($COPSAC_{2000}$) mother-child cohort, and subsequently stimulated with *H. influenzae*, *M. catarrhalis* and *S. pneumoniae* in *in vitro* cultures. Levels of cytokines (IL-2, IL-10, IFN-γ, TNF-α, IL-5, IL-13 and IL-17A) were determined in the supernatant by electrochemiluminescence immunoassays. The immune profiles were analyzed for association with development of total-IgE, allergic sensitization and rhinitis during the first 7 years of life using regression models and principal component analysis (PCA).

Findings: An attenuated IL-2 response to stimulation with *H. influenzae* (p=0·011) and *M. catarrhalis* (p=0·027) was associated with elevated total-IgE at age 7. These findings were confirmed in a multivariate PCA model (PC2, p=0·032). An immune profile with both reduced IL-2 and elevated IL-5 was associated with increased risk of allergic rhinitis (PC3, p=0·038). We found no associations with development of allergic sensitization.

Interpretation: A reduced IL-2 response from PBMCs exposed to common pathogenic airway bacteria at age 6 months was associated with elevated total-IgE and allergic rhinitis during the first 7 years of life. These findings suggest that suppressed Treg activity in early life may herald onset of allergy in early childhood, which could be a target for low-dose IL-2 trials in the future.

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Key words: IL-2; airway bacteria; birth cohort; IgE; sensitization.

INTRODUCTION

Reduced numbers and function of regulatory T-cells (Tregs) have been demonstrated in autoimmune and allergic diseases, suggesting that a dysregulated Treg system contributes to the pathogenesis of these conditions.[1–4] Interleukin 2 (IL-2) is critical for the development and survival of Tregs[5–7] and a possible role of the IL-2/Treg interplay in allergy has been suggested by a study showing that reduced IL-2 expression by cord blood CD4(+) T cells associated with decreased Tregs and increased risk of food allergy in childhood.[8]

In the Copenhagen Prospective Studies on Asthma in Childhood 2000 ($COPSAC_{2000}$) mother-child cohort we conducted repeated clinical assessments including total-IgE, specific-IgE and allergic rhinitis through age 7 and biobanking of peripheral blood mononuclear cells (PBMCs) isolated from the infants at age 6 months. Recently, we investigated the PBMC response to *in vitro* stimulations with *H. influenzae*, *M. catarrhalis*, and *S. pneumoniae* measuring production of T cell-related cytokines of the Treg (IL-2, IL-10), Th1 (interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α)), Th2 (IL-5, IL-13) and Th17 (IL-17A) cells.[9,10]

The aim of the current study is to analyze the relationship between the PBMC response to bacterial stimulations and the development of allergy-related endpoints during the first 7 years of life, hypothesizing a protective role of IL-2.

MATERIALS AND METHODS

Study cohort

This study is part of the ongoing $COPSAC_{2000}$ prospective mother-child cohort of 411 children born to mothers with asthma.[11] The children were enrolled at age 1 month, excluded children born before gestational week 36 and children suffering from any respiratory disorder before enrollment. The children were followed prospectively at the COPSAC clinical research unit with regular follow-up visits every 6 months till age 7 and at episodes with respiratory symptoms. The clinical research unit was the primary health care facility for diagnosis and treatment of any respiratory symptoms, strictly adhering to validated algorithms.[12]

The study was approved by the Copenhagen Ethics Committee (KF 01-289/96), the Danish Data Protection Agency (2002-41-2434), and followed the principles of the Declaration of Helsinki. Written and oral informed consent was obtained from the parents at enrolment.

Bacterial stimulation of PBMCs

The bacterial stimulations of PBMCs was previously described in details.[9,10] Briefly, PBMCs were isolated by density centrifugation from blood samples collected at 6 months and stored for up to 12 years at -140°C. After thawing, the cells were stimulated with UV-inactivated *H. influenzae*, *M. catarrhalis*, and *S. pneumoniae* (50µg/mL) or blank sterile culture media alone in U-bottomed 96-well plates at $5x10^5$ cells/well (200µL total volume/well) for 40hours at 37°C and 5% CO_2 in a humidified incubator. Supernatants were harvested and stored at -80°C until quantification of IL-2, IL-10, IFN-γ, TNF-α, IL-5, IL-13 and IL-17A levels by customized multiplex immunoassays from MesoScale Discovery read on a Sector Imager 6000 (MSD, Gaithersburg, MD, USA). All assays were highly sensitive with a detection limit below 1pg/mL.

T cell immune phenotyping

The composition of the T cell compartment was analyzed on freshly thawed unstimulated PBMCs (5 x 10^5 cells) using flow cytometry. Staining and flow cytometry analysis were performed using the following antibody panel: CD3/eFlour450, CD8/FITC, TCRVa24-Ja18/PerCP-eFlour710, CD127/APC-eFlour780 (eBioscience, San Diego, CA), CD25/PC7, TCRγδ/PE (Beckman Coulter, Brea, CA) and CD4/V500 (BD Bioscience, San Jose, CA). T cell subsets were identified by a predefined gating strategy and analyzed in a blinded manner: Helper ($CD3^+CD4^+$), cytotoxic ($CD3^+CD8^+$), regulatory ($CD3^+CD4^+CD127^-CD25^+$), γδ ($CD3^+TCR\gamma\delta^+$) and invariant NK ($CD3^+TCRV\alpha24\text{-}J\alpha18^+$) T cells. All population frequencies were calculated relative to the $CD3^+$ T cell compartment.

Allergy-related endpoints

Total-IgE level was determined at 6 years by ImmunoCAP (Pharmacia Diagnostics AB, Uppsala, Sweden) and analyzed as a continuous variable.

Allergic sensitization was assessed at 6 years by skin prick tests (SPT) and measurements of specific-IgE against house dust mites, cat, dog, horse, birch, grass, mugwort, and molds as previous detailed.[13] SPT was done using allergen extracts (ALK Abello, Soluprick® SQ, Copenhagen, Denmark) defining a positive test as any wheal ≥3mm. Specific-IgE levels were measured with ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden), defining a positive test as any specific-IgE level ≥0·35kU_A/L. Sensitization was analyzed as a dichotomized variable, separately for SPT and specific-IgE as the overlap between test results is poor at young age.[14]

Allergic rhinitis at age 7 was diagnosed by the COPSAC pediatricians based on a parental interview on the child's history of symptoms.[15] Significant sneezing or blocked or runny nose affecting the wellbeing of the child in the past 12 months in periods without cold or flu defined rhinitis. The diagnosis required sensitization and congruence between symptoms and allergen exposure.

Statistical analysis

Initially, supernatant levels of cytokines in response to bacterial stimulations were adjusted by subtracting the baseline levels of cytokines secreted by PBMCs incubated with sterile media. Thereafter, the levels were square root-transformed. If an individual did not produce a measurable cytokine response in PBMCs after bacterial stimulation (non-responders), the cytokine level was set to zero.

First, we analyzed the association between the cytokines released upon bacterial stimulations at 6 months and total-IgE level (log-transformed), allergic sensitization by SPT (yes/no) and elevated

specific-IgE (yes/no), and allergic rhinitis (yes/no) during the first 7 years of life using linear and logistic regression models. The associations with outcomes for cytokines from each bacterial stimulation were analyzed using forward stepwise selection while retaining IL-2. If the final model had multiple independent variables they were compared with a null model by ANOVA for goodness of fit. If only IL-2 was retained in the final model, the hypothesis test for its coefficient was used as assessment of goodness of fit. Only the models with significant overall p-values (≤0.05) are discussed in detail.

Thereafter, we conducted a data-driven principal component analysis (PCA) on the 21 independent variables (3 bacterial stimulation x 7 different cytokines) to capture immune patterns in the data, using principal component 1 to 4 (PC1-4) from the model for association analyses with the outcomes.

We also analyzed the association between T cell subsets and allergy-related outcomes. T cell composition data was transformed by isometric log ratio (ilr) using sequential binary partitioning and their association with outcomes was analyzed by multiple regression models.[16]

All analyses were conducted with R version 3.4.2.[17] The results are reported with 95% CI and p-values ≤0.05 were considered statistically significant.

RESULTS

Baseline characteristics

A total of 331 (81%) of the 411 infants in the cohort had PBMCs collected at age 6 months, which were subsequently exposed to *H. influenzae*, *M. catarrhalis,* and *S. pneumoniae* for assessment of the immune response by measuring supernatant cytokine levels. The T cell immune phenotyping and immune responses are described in **Tables 1-2**. Of the 331 infants, 259 (78%) had measurements of total-IgE (mean level, 117·6 IU/mL), 270 (82%) had SPT (N=251) and/or sIgE (N=259) with 100 (30%) being sensitized to one or more allergen either by SPT (N=40) and/or sIgE (N=94). Allergic rhinitis was assessed in 254 (77%) with a prevalence of 13% (N=34) by age 7 years.

A comparison between children with vs. without immune response data has previously been published showing no differences.[9,10]

Bacteria-induced immune response and allergy-related outcomes

Total-IgE

Multiple regression analyses showed inverse associations between IL-2 production in response to *H. influenzae* and *M. catarrhalis* at age 6 months and total-IgE level at age 6 years: IL-2-coefficient$_{hi}$=-0·183 [95% CI; -0·324, -0·043], p=0·011 and IL-2-coefficient$_{mc}$=-0·150 [-0·282, -0·018], p=0·027, respectively. The IL-2 association was only seen in response to the Gram-negative bacteria *H. influenzae* (p_{Ftest}=0·028) and *M. catarrhalis* (P_{Ftest}=0·024), whereas the model using cytokine data in response to the Gram-positive bacteria *S. pneumoniae* was not better than the null model (P_{Ftest}=0·139): IL-2-coefficient$_{sp}$=0·151 [0·001, 0·301], p=0·0503 (**Figure 1**).

Besides IL-2, the production of IFN-Y and TNF-α in response to *H. influenzae* and *M. catarrhalis* were positively associated with total-IgE level: IFN-Y-coefficient$_{hi}$=0·019 [0·000, 0·0367], p=0·046, and TNF-α-coefficient$_{mc}$=0·159 [0·027, 0·290], p=0·019, respectively.

The result of a PCA analysis to identify individual cytokine profiles in response to the bacteria is depicted in **Figure 2** and **Figure e1**. The PCA association analysis for total-IgE confirmed the findings from the regression models. The cytokine pattern in PC2 from *M. catarrhalis* was inversely associated

with total-IgE level (p=0·032) and the same trend was seen for PC2 from *H. influenzae* stimulation (p=0·051); i.e. higher total-IgE was associated with a decreasing score dominated by IL-2 (**Figure 2**).

Allergic sensitization

Higher IL-5 secretion in response to *S. pneumoniae* was significantly associated with sensitization diagnosed by sIgE at age 6: OR_{sc}=1·678 [1·069, 2·633], p=0·024, although the overall model with inclusion of all cytokines was not significantly better than the null model (overall p-value=0·080). No associations were observed between the immune response to the other bacterial stimulations and development of allergic sensitization determined by neither sIgE nor SPT (**Figures 1-2**).

Allergic rhinitis

IL-2 in response to *M. catarrhalis* and IL-13 in response to *H. influenzae* were inversely associated with allergic rhinitis by age 7: OR_{mc}=0·495 [0·273, 0·898], p=0·021, and OR_{hi}=0·429 [0·197, 0·933], p=0·033; i.e. higher IL-2 and IL-13 production reduced the risk of allergic rhinitis. We also found that elevated IL-5 in response to all the bacterial stimulations increased the risk of allergic rhinitis: OR_{hi}=1·954 [1·037, 3·681], p_{hi}=0·038, OR_{mc}=1·569 [1·056, 2·330], p_{mc}=0·026, and OR_{sp}=1·614 [1·070, 2·434], p_{sp}=0·022. The multiple regression models were all significantly better than the null models: p_{chisq}=0·048 for *H. influenzae*, p_{chisq}=0·017 for *M. catarrhalis*, and p_{chisq}=0·046 for *S. pneumoniae* (**Figure 1**).

The results from the PCA supported the main findings from the regression analyses regarding IL-5 and IL-2, but not for IL-13. We found an inverse association between development of allergic rhinitis and the immune pattern in PC3 upon *M. catarrhalis* stimulation (p=0·038), i.e. lower IL-2 and higher IL-5 from PBMCs at 6 months increased the risk of allergic rhinitis at age 7. In addition, a decreasing PC4 score from the *H. influenzae* stimulation was significantly associated with risk of allergic rhinitis, i.e. lower IL-17A and higher IFN-γ release increased the risk of allergic rhinitis (p=0·048) (**Figure 2**).

Sensitivity analysis

As many of the children did not produce any IL-5 in PBMCs after bacterial stimulations (IL-5 non-responders), we conducted a sensitivity analysis excluding these (**Table e1**). This showed similar results for the univariate association between IL-5 and allergic rhinitis by age 7: OR_{hi}=2·532 [1·127, 5·690], p_{hi}=0·025, OR_{mc}=3·231 [1·274, 8·200], p_{mc}=0·014, and OR_{sp}=1·729 [1·084, 2·757], p_{sp}=0·021, and the multiple regression models were also significantly better than the null models: p_{chisq}=0·040 for *H. influenzae*, p_{chisq}=0·001 for *M. catarrhalis*, and p_{chisq}=0·048 for *S. pneumoniae* (**Figure e2**). The PCA results were consistent showing an inverse association between allergic rhinitis and the immune pattern in PC3 upon stimulations from both *M. catarrhalis* (p=0·036) and *H. influenzae* (p=0·038) (**Figure e3**).

T cell compartment composition

To study if the cytokine response profiles were related to an underlying difference in T cell subsets, we examined the T cell compartment composition in relation to the allergy-outcomes. The relative ratios of γδ T cells and iNKT cells amongst all T cells were associated with total-IgE level at age 6, but in opposite directions with an inverse association identified for iNKT cells (p=0·030), and positive association for γδ T cells (p=0·022) (**Figure 3**, **Figure e4**). No associations between the relative ratio of Tregs and development of allergy-related outcomes were observed (**Figure 3**).

DISCUSSION

Main findings

An attenuated IL-2 response to stimulation with the common Gram-negative pathogenic airway bacteria *H. influenzae* and *M. catarrhalis* in PBMCs collected at age 6 months was associated with elevated total-IgE at age 6 years. Furthermore, both reduced IL-2 and increased IL-5 production were associated with a higher risk of developing allergic rhinitis during the first 7 years of life. Altogether, these findings suggest a role of diminished IL-2 and increased IL-5 in development of allergy-related traits in early childhood.

Strengths and limitations

This is the first prospective cohort study to investigate infant's bacterial immune responses in PBMCs, which was done in a comprehensive manner by analyzing several cytokines of the Treg, Th1, Th2, and Th17 cells. The prospective nature of our study and the storage of PBMCs at age 6 months provide a unique possibility to investigate functional immunological properties in infancy before onset of allergy-related outcomes. As the PBMCs were collected in asymptomatic infants before onset of allergic disease, the bacterial immune response is unlikely to be affected by immune dysfunction caused by ongoing disease-driven inflammation.

It is an advantage of the study that the children participating in the $COPSAC_{2000}$ cohort solely used the COPSAC research pediatricians for diagnosis and treatment of any asthma and allergy-related disease. All diagnoses were done based on rigid standardized algorithms providing highly reliable homogeneous clinical outcomes.

It is a limitation that this was an *in vitro* study that may not reflect the complexity of the immune responses *in vivo*. Importantly, many of the infants stored PBMCs did not produce a measurable IL-5 response to any of the bacterial stimulations. However, analyses including the IL-5 non-responders as zeros as well as analyses excluding the non-responders showed similar associations with the clinical endpoints.

It is a limitation that the results are based on one concentration of antigen performed at one time point (40 hours culture). Therefore, we are not able to determine how the results would look like at different time points and by stimulating with different concentrations, which is particularly important for IL-2, which is consumed in the cultures. However, this would have been of greater importance if we had not detected an association between IL-2 PBMC responses and allergy-outcomes.

Finally, it is a limitation that all the children participating in the cohort were born to mothers with a history of asthma, which may hamper the external validity of our findings.

Interpretation

In this study, we aim to identify immune patterns in infancy in response to different bacteria in relation with development of allergic disorders in the first 7 years of life. The bacterial stimulations were chosen as we previously demonstrated that one-month-old infants colonized in the airways with *H. influenzae*, *M. catarrhalis*, and/or *S. pneumoniae* had an increased risk of asthma at age 5 years.[18] However, no direct link was shown between neonatal airway colonization with these bacteria and allergic outcomes, but studies indicate that environmental exposure to certain bacteria or bacterial components like lipopolysaccharide, which is contained in *H. influenzae* and *M. catarrhalis*, are associated with risk of allergic sensitization in childhood.[19–21]

We speculate that our finding of a reduced IL-2 production in concert with increased IL-5 to stimulations with the Gram-negative bacteria *H. influenzae* and *M. catarrhalis* may be caused by imbalanced Treg and Th2 populations and thereby increase the risk of developing allergic diseases.

This finding may be of clinical importance as low-dose IL-2 therapy is being investigated with the purpose of expanding the Treg population in several diseases. We did not observe similar findings when analyzing the cytokine responses from the Gram-positive *S. pneumonia* stimulation, which may be due to the fact that these bacteria do not contain the innate immune ligand lipopolysaccharide.

Recent clinical trials have studied the tolerability and efficacy of low-dose IL-2 therapy in the treatment of autoimmune disorders and graft-versus-host disease.[22–26] All trials reported the therapy to be safe and effective in mediating sustained expansion of systemic Tregs with clinically relevant improvement in patients with HCV-induced vasculitis,[22] alopecia areata,[26] and graft-versus-host disease.[25] We speculate that low-dose IL-2 therapy may have a beneficial role on allergy and propose initiation of clinical trials in children with allergy and subsequently trials investigating low-dose IL-2 as a possible therapeutic intervention or a prophylactic regimen in high-risk infants. Due to the proposed early window of immune programming for regulatory T cell development,[27] it might be speculated to be even more important to propagate Treg development in early infancy, once an impaired IL-2 production has been detected. We here addressed the question of IL-2 production in PBMCs collected at 6 months of age, as bacteria-specific T cells will have been expanded at this age if the child has been exposed to the given bacteria in vivo. This is in contrast to studies performed on cord blood. We selected a relatively short stimulation protocol of 40 hours with the aim of examining specifically the bacteria-specific memory and effector T cells present in the PBMC fraction that would be more readily activated for cytokine production than naïve T cells, which require a longer protocol. Although this study examines immune programming prior to disease development, it might also be valuable to use IL-2 therapy in already diseased individuals. Prior to trials in allergic diseases, it would valuable to investigate IL-2 PMBC responses in children with vs. without ongoing symptoms of allergic rhinitis.

Although IL-2 has pleiotropic effects and works on other types of Th effectors, the efficacy and specificity of low dosage IL-2 therapy may be supported by the selective responsiveness of Tregs against low does IL-2. It was reported that due to an enhanced expression of the IL-2 receptor (α and Y chain) and promoted activity of endogenous serine/threonine phosphatase protein phosphates 1 and/or 2A in Tregs about 10-100 fold lower levels of IL-2 was required to activate STAT5 in Tregs compared to the levels needed for memory T cells or activated T cells. [28]

It is possible that reduced IL-2 production during the continuous immune activation induced by the host microbiome in infancy could affect the risk of developing allergic disease. It is recognized that the microbiome plays a key role in development of the immune system and the establishment of homeostatic tolerance by Tregs.[29–31] Thus, infants who respond to bacteria with lower IL-2 production could have an imbalanced immune system with blunted Treg function leading to proneness to develop higher circulating IgE levels, diminished control of tolerance to allergens, and subsequently develop symptomatic disease such as rhinitis.

We have previously within the same cohort reported abnormal bacterial immune response in PBMCs in infants developing asthma later in childhood.[9] The immune responses associated with asthma were dominated by increased Th2 cytokines (IL-5 and IL-13) in response to the same three pathogenic airway bacteria. However, in contrast to the current findings, no associations existed between IL-2 release and development of asthma, which underlines different immune patterns driving the heterogeneous phenotypes of allergy and asthma and suggests that low-dose IL-2 therapy might be targeted solely to the allergic phenotype. This fits well with a recent study from a food allergy mouse model showing that low dose IL-2 treatment could control the allergic inflammatory process by inducing expansion and activation of the Treg population. [24] Further experimental studies of i.e. allergen-specific stimulations of human PBMCs are needed to determine whether IL-2 therapy should solely be targeted to the allergic phenotype.

Higher IL-13 production upon bacterial stimulation of the PBMCs was observed to reduce the risk of allergic rhinitis, which was however not significant in the multivariate PCA approach. This is an unexpected finding as co-expression of IL-4, IL-13 and IL-5 are normally observed in patients with ongoing symptoms of rhinitis. A non-coordinate expression of IL-4, IL-13 and IL-5 can occur when distinct Th2 clones produce each cytokine individually. Thus, the discrepancy in expression of IL-5 and IL-13 may suggest a transient expression pattern associated with the initial stage of Th2 priming as we investigated the response from stimulated PBMCs in 6-month-old asymptomatic infants long before onset of any symptoms of allergic rhinitis.

Our T cell compartment data showed an association between the composition of γδ T cells and total-IgE level suggesting a possible function of γδ T cells in the development of allergic diseases. Such association has previously been reported in children with atopic dermatitis aged 1 to 10 years, whereas no association was observed for total-IgE level within these children with atopic dermatitis.[32] Finally, we also identified an inverse association between the composition of iNKT cells and total-IgE, which is consistent with previous findings in adult asthmatics.[33]

Conclusion

Reduced IL-2 production and increased IL-5 from PBMCs in response to bacterial stimulations in early life were associated with development of elevated total-IgE and allergic rhinitis during the first 7 years of life. This finding is of possible clinical relevance as experimental studies have shown that low-dose IL-2 therapy can restore immune tolerance by expansion of Tregs.

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Conflict of interests:

The authors declare no conflicts of interest.

Author contributions:

The guarantor of the study is HB who is responsible for the integrity of the work as a whole, from conception and design to conduct of the study and acquisition of data, analysis and interpretation of data and writing of the manuscript. HB had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. BC and NW were responsible for data analysis and wrote the first draft of the manuscript. AMS, JML, AT, SBP, MAR, JS and KB contributed to design of the study, interpretation of data and writing of the manuscript. JML, AT and SBP were responsible for generating the PMBC immune response data. All co-authors have contributed substantially to the analyses and/or interpretation of the data and have provided

important intellectual input and approval of the final version of the manuscript. No honorarium, grant, or other form of payment was given to anyone to produce the manuscript.

Tables

Table 1. Supernatant cytokine levels in response to pathogenic airway bacteria at 6 months (n=331).

IQR=Interquartile range.

Cytokine supernatant	Control, median (IQR)	*H. influenzae*, median (IQR)	*M. catarrhalis*, median (IQR)	*S. pneumoniae*, median (IQR)
IL-2 (pg/ml)	0·8 (0-4·2)	4·8 (1·2-14·6)	12·2 (3·2-28·5)	9·5 (1·4-41·8)
IL-10 (pg/ml)	0·0 (0-1)	15·0 (2·2-60·2)	22·5 (3·9-77·6)	1·8 (0-9·9)
IFN-γ (pg/ml)	0·4 (0-1)	4·5 (0·3-77·9)	19·9 (0·8-233·9)	1·4 (0·2-12·2)
TNF-α (pg/ml)	1·5 (0·5-4·1)	35·5 (8·2-169·2)	54·2 (17·7-201·7)	21·5 (5·2-101)
IL-5 (pg/ml)	0·0 (0·0-0·0)	0·0 (0·0-0·4)	0·4 (0·0-2·4)	0·0 (0·0-0·6)
IL-13 (pg/ml)	0·0 (0·0-0·0)	14·9 (0·0-75·5)	60·4 (5·7-203·3)	10·9 (0·0-71·6)
IL-17A (pg/ml)	0·0 (0·0-1·8)	1·9 (0·0-12·6)	9·4 (0·5-43·0)	2·2 (0·0-17·6)

Table 2. Composition of T cell compartment at 6 age months (n=242).

IQR=Interquartile range.

T cell profile	**Median (IQR)**
Helper T cells (%)	68·5(62·6-72·4)
Cytotoxic T cells (%)	24·1(20·6-29·8)
Regulatory T cells (%)	2·3(1·8-2·9)
γδ T cells (%)	3·0(2·2-3·9)
Invariant NK T cells (%)	0·1(0·1-0·1)

Values are percentage of all CD3+ T cells

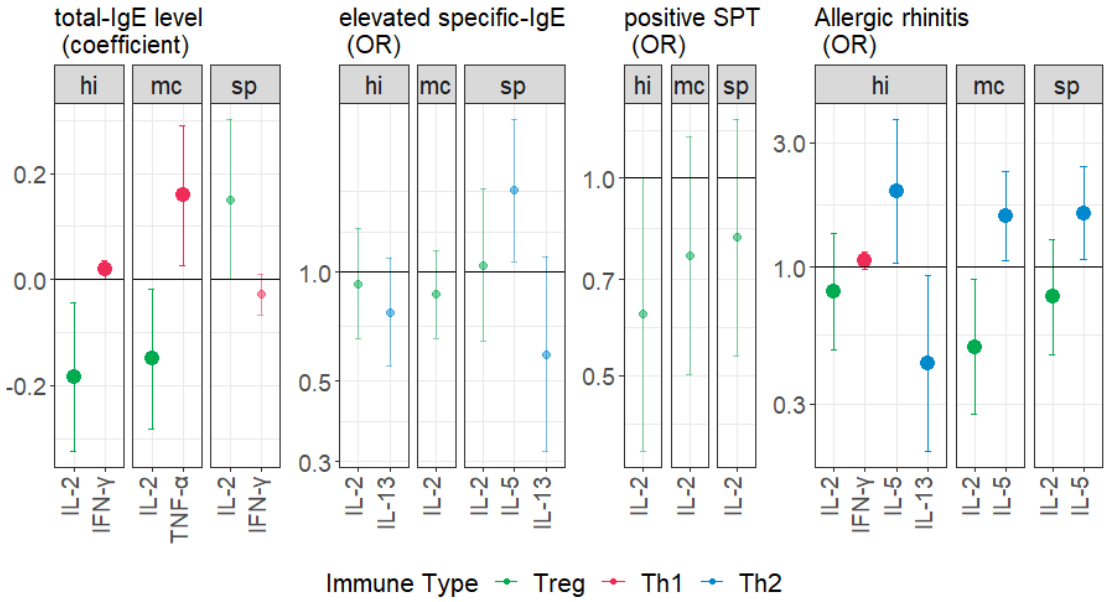

Figure 1. Association between selected cytokines and allergic outcomes

Association between cytokines from 6 month (n=331) upon stimulation with H. influenzae, M. catarrhalis and S. pneumoniae and log transformed total IgE (mean=1.505, SD=1.115), allergic sensitization determined by skin prick test (case/control=44/173) and specific-IgE level (case/control=93/173) at 6 year, and allergic rhinitis (case/control=38/185) at 7 year. The shown cytokines were from the best fitted models, which were defined by forward stepwise regression in relation to each clinical outcome.

Ordinary least square regression for log transformed total-IgE level and logistic regression for the rest of allergy outcomes was each regressed on cytokines from each stimulation in a forward stepwise fashion by retaining IL-2 in the selection process. The overall fitness of selected models was accessed and relevant model statistics (Odds Ratio (OR) for binary outcomes, coefficients for continuous outcome and their associated 95% confidence intervals) for significant ones were shown in solid colors and bigger dot size, otherwise the colors were faint and the dots were smaller.

Positive SPT = positive skin prick test, hi=H. influenzae, mc= M. catarrhalis, sp= S. pneumoniae.

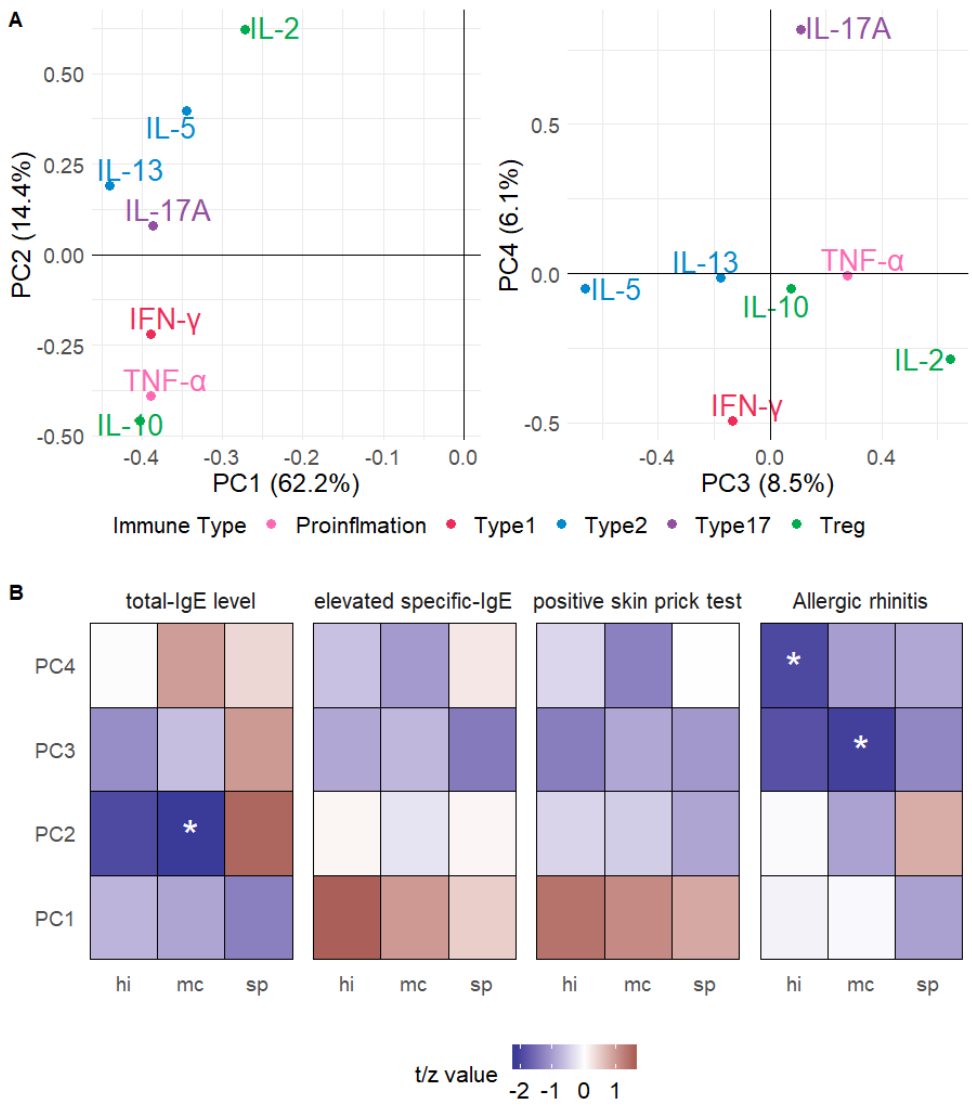

Figure 2. Association between cytokine profiles from 6 month (n=331) upon stimulation with H. influenzae, M. catarrhalis and S. pneumoniae and log transformed total IgE, allergic sensitization determined by skin prick test and specific-IgE level at age 6 and allergic rhinitis at age 7

A. Cytokine loadings of PC1 to PC4 from principle components analysis (PCA).

B. Ordinary least square regression for log-transformed total-IgE level and simple logistic regression for the rest clinical outcomes was each regressed on PC1-4. The values of test statistic of regression coefficients are plotted in the heatmaps. Blue indicates negative association and red indicate positive association.

Asterixis (*) indicates p<0.05. hi=H. influenzae. mc= M. catarrhalis. sp= S. pneumoniae. Log transformed total IgE (mean=1.505, sd=1.115). Number of allergic rhinitis and control (38/185). Number of Kids with allergic sensitization assessed by level of specific IgE and healthy control (93/173). Number of Kids with allergic sensitization assessed by skin prick test (SPT) and healthy control (44/173).

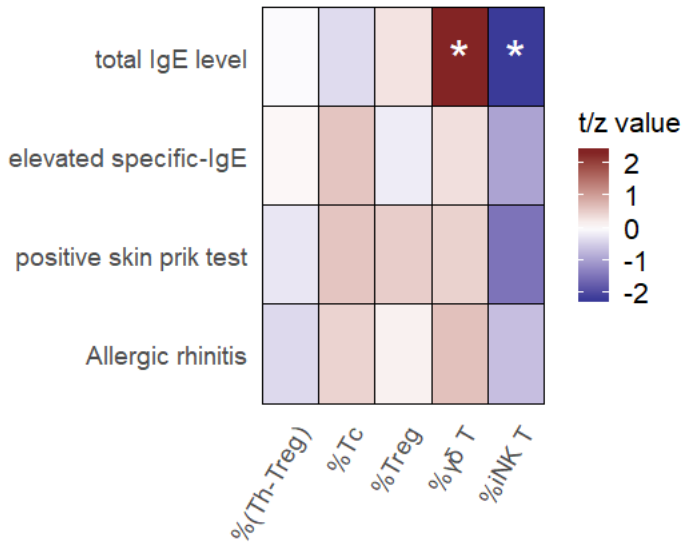

Figure 3. The association between T cell compartment at 6 month and allergy outcomes at age of 6 year.

Multiple linear regression for log transformed total-IgE level and multiple logistic regression for the rest of allergy outcomes was regressed on isometric transformed T cell compartments. The values of test statistic of regression coefficients are plotted in the heatmaps.

Blue indicates negative association and red indicate positive association. Asterixis (*) indicates p<0.05.

Online table and figures

Table e1. Supernatant IL-5 levels in response to pathogenic airway bacteria at 6 months (n=239).

IQR=Interquartile range

	Control, median (IQR)	***H influenzae, median (IQR)***	***M catarrhalis, median (IQR)***	***S pneumoniae, median (IQR)***
Cytokine supernatant				
IL-5 (pg/ml)	0·0 (0-0·04)	0·12 (0-0·77)	1·29 (0·24-4·11)	0·14 (0-1·25)

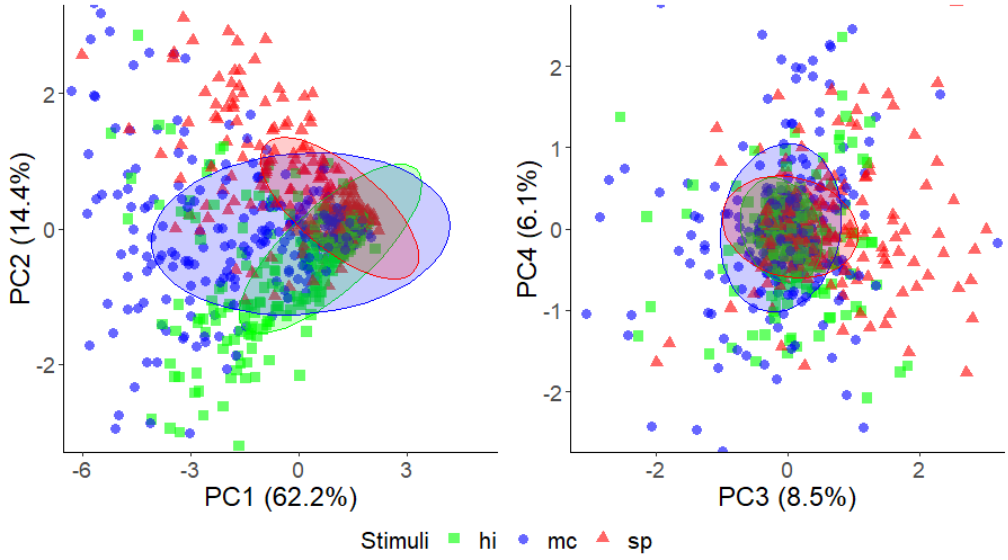

Figure e1. The score plots of principle components 1-4 (PC1-4) from principle components analysis (PCA) based on cytokine profiles from 6 month (n=331) upon stimulation with H. influenzae, M. catarrhalis and S. pneumoniae. The 90% confidence ellipse are added.

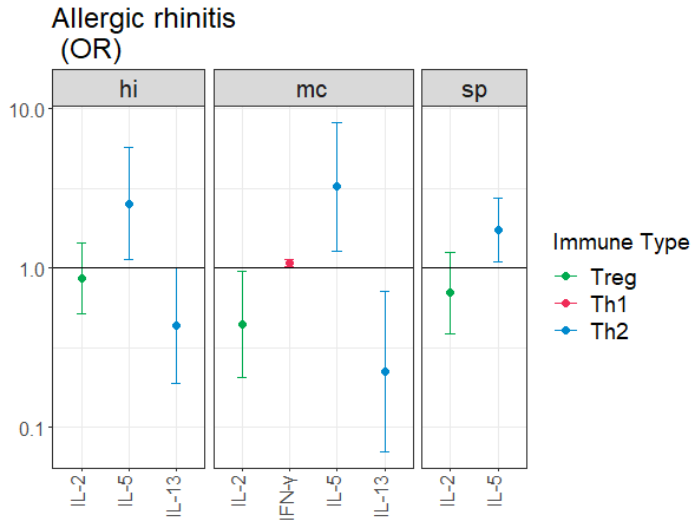

Figure e2. Sensitivity analyses of the association between selected cytokines and allergic rhinitis

Association between cytokines from age 6 month (n=239) after removal of the IL-5 non-responders upon stimulation with H. influenzae, M. catarrhalis and S. pneumoniae and allergic rhinitis (case/control=27/120) at 7 year. The shown cytokines are derived from the best fitted models defined by forward stepwise regression in relation to allergic rhinitis.

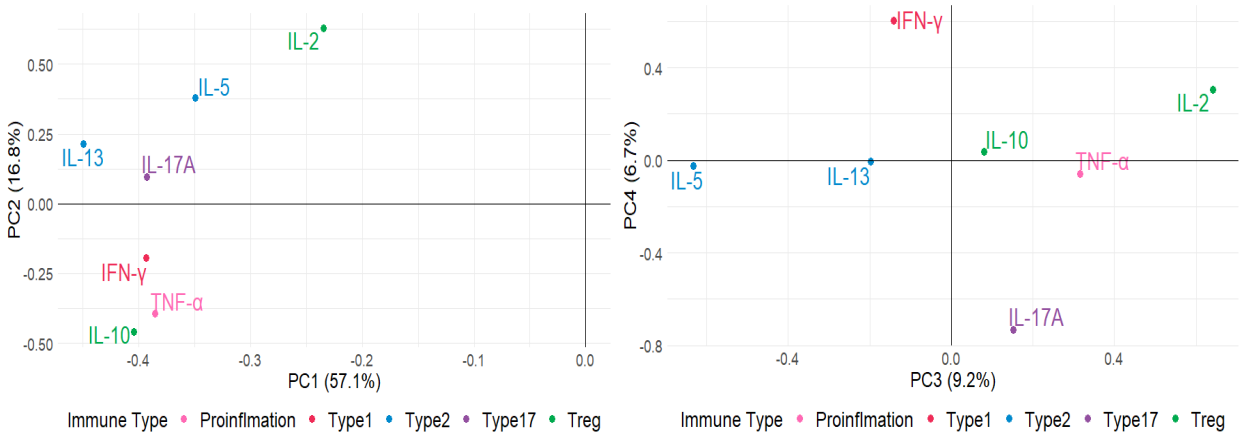

Figure e3. Cytokine loadings of PC1 to PC4 from principle component analysis (PCA)

Loading plots from PCA based on cytokines from 239 children, after removal of IL-5 non-responders.

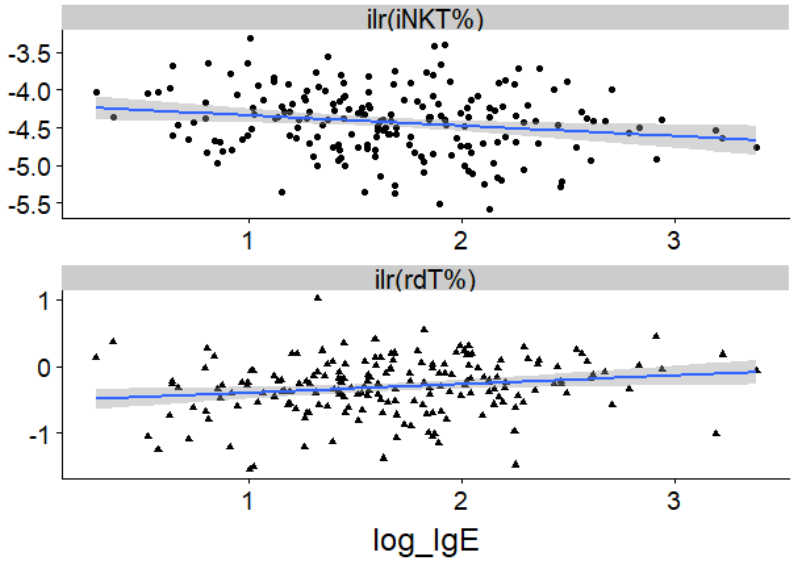

Figure e4. The scatter plot about association between T cell compartment and log transformed IgE level at age 6.

REFERENCES

1. Illi S, von Mutius E, Liu J, et al. Impairment of T-regulatory cells in cord blood of atopic mothers. *J Allergy Clin Immunol*. 2008;121(6):1491-1499.e13. doi:10.1016/j.jaci.2008.04.010

2. Sakaguchi S, Ono M, Setoguchi R, et al. Foxp3+CD25+CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol Rev*. 2006;212:8-27. doi:10.1111/j.0105-2896.2006.00427.x

3. Kelchtermans H, De Klerck B, Mitera T, et al. Defective CD4+CD25+ regulatory T cell functioning in collagen-induced arthritis: an important factor in pathogenesis, counter-regulated by endogenous IFN. *Arthritis Res Ther*. 2005;7(2):R402-R415. doi:10.1186/ar1500

4. Grindebacke H, Wing K, Andersson AC, Suri-Payer E, Rak S, Rudin A. Defective suppression of Th2 cytokines by CD4+CD25+ regulatory T cells in birch allergies during birch pollen season. *Clin Exp Allergy*. 2004;34(9):1364-1372. doi:10.1111/j.1365-2222.2004.02067.x

5. Zheng SG, Wang P, Gray JD, Wang J, Horwitz DA. IL-2 Is Essential for TGF- to Convert Naive CD4+CD25- Cells to CD25+Foxp3+ Regulatory T Cells and for Expansion of These Cells. *J Immunol*. 2014;178(4):2018-2027. doi:10.4049/jimmunol.178.4.2018

6. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol*. 2005;6(11):1142-1151. doi:10.1038/ni1263

7. Yu A, Zhu L, Altman NH, Malek TR. A Low Interleukin-2 Receptor Signaling Threshold Supports the Development and Homeostasis of T Regulatory Cells. *Immunity*. 2009;30(2):204-217. doi:10.1016/j.immuni.2008.11.014

8. Zhang Y, Collier F, Naselli G, et al. Cord blood monocyte-derived inflammatory cytokines suppress IL-2 and induce nonclassic "TH2-type" immunity associated with development of food allergy. *Sci Transl Med*. 2016;8(321). doi:10.1126/scitranslmed.aad4322

9. Birch S, Thysen AH, Brix S, Larsen JM, Bisgaard H, Rasmussen MA. Children with asthma by school age display aberrant immune responses to pathogenic airway bacteria as infants. *J Allergy Clin Immunol*. 2014;133(4):1008-1013.e4. doi:10.1016/j.jaci.2014.01.010

10. Vissing NH, Larsen JM, Rasmussen MA, et al. Susceptibility to Lower Respiratory Infections in Childhood is Associated with Perturbation of the Cytokine Response to Pathogenic Airway Bacteria. *Pediatr Infect Dis J*. 2016;35(5):561-566. doi:10.1097/INF.0000000000001092

11. Bisgaard H. The Copenhagen Prospective Study on Asthma in Childhood (COPSAC): Design, rationale, and baseline data from a longitudinal birth cohort study. *Ann Allergy, Asthma Immunol*. 2004;93(4):381-389. doi:10.1016/S1081-1206(10)61398-1

12. Bisgaard H, Pipper CB, Bønnelykke K. Endotyping early childhood asthma by quantitative symptom assessment. *J Allergy Clin Immunol*. 2011;127(5):1155-1164.e2. doi:10.1016/j.jaci.2011.02.007

13. Schoos AMM, Chawes BL, Rasmussen MA, Bloch J, Bønnelykke K, Bisgaard H. Atopic endotype in childhood. *J Allergy Clin Immunol*. 2016;137(3):844-851.e4. doi:10.1016/j.jaci.2015.10.004

14. Schoos AMM, Chawes BLK, Følsgaard N V., Samandari N, Bønnelykke K, Bisgaard H. Disagreement between skin prick test and specific IgE in young children. *Allergy Eur J Allergy Clin Immunol*. 2015;70(1):41-48. doi:10.1111/all.12523

15. Chawes BLK, Kreiner-Møller E, Bisgaard H. Objective assessments of allergic and nonallergic rhinitis in young children. *Allergy Eur J Allergy Clin Immunol*. 2009;64(10):1547-1553. doi:10.1111/j.1398-9995.2009.02085.x

16. Hron K, Filzmoser P, Thompson K. Linear regression with compositional explanatory variables. *J Appl Stat*. 2009;36(7):769-778. doi:10.1080/0266476YYxxxxxxxx

17. Team RC. R: A Language and Environment for Statistical Computing. 2018. https://www.r-project.org/.

18. Bisgaard H, Hermansen MN, Buchvald F, et al. Childhood Asthma after Bacterial cololization of the Airway in Neonates. *N Engl J Med*. 2007;357:1487-1495. doi:10.1542/peds.2008-2139CCC

19. Ege MJ, Mayer M, Normand A-C, et al. Exposure to Environmental Microorganisms and Childhood Asthma. *N Engl J Med*. 2011;364(8):387-401. doi:10.1056/NEJMoa1402121

20. Braun-Fahrländer C, Riedler J, Herz U, et al. Environmental Exposure to Endotoxin and Its Relation to Asthma in School-Age Children. *N Engl J Med*. 2002;347(12):869-877. doi:10.1056/nejmoa020057

21. Vatanen T, Kostic AD, D'Hennezel E, et al. Variation in Microbiome LPS Immunogenicity

Contributes to Autoimmunity in Humans. *Cell*. 2016;165(4):842-853. doi:10.1016/j.cell.2016.04.007

22. Saadoun D, Rosenzwajg M, Joly F, et al. Regulatory T-Cell Responses to Low-Dose Interleukin-2 in HCV-Induced Vasculitis. *N Engl J Med*. 2011;365:2067-2077.

23. Hartemann A, Bensimon G, Payan CA, et al. Low-dose interleukin 2 in patients with type 1 diabetes: A phase 1/2 randomised, double-blind, placebo-controlled trial. *Lancet Diabetes Endocrinol*. 2013;1(4):295-305. doi:10.1016/S2213-8587(13)70113-X

24. Vigneron J, Brimaud F, Churlaud G, et al. Low-Dose IL-2 Induces Regulatory T Cell–Mediated Control of Experimental Food Allergy. *J Immunol*. 2016;197(1):188-198. doi:10.4049/jimmunol.1501271

25. Koreth J, Matsuoka K, Kim HT. Interleukin-2 and Regulatory T Cells in Graft-versus-Host Disease. *N Engl J Med*. 2010;362(5):387-401. doi:10.1056/NEJMoa1402121

26. Hofman P, Bahadoran P, Passeron T, et al. Effects of Low-Dose Recombinant Interleukin 2 to Promote T-Regulatory Cells in Alopecia Areata. *JAMA Dermatology*. 2014;150(7):748. doi:10.1001/jamadermatol.2014.504

27. Al Nabhani Z, Dulauroy S, Marques R, et al. A Weaning Reaction to Microbiota Is Required for Resistance to Immunopathologies in the Adult. *Immunity*. 2019. doi:10.1016/j.immuni.2019.02.014

28. Yu A, Snowhite I, Vendrame F, Rosenzwajg M. Selective IL-2 Responsiveness of Regulatory T Cells Through Multiple Intrinsic Mechanisms Supports the Use of Low-Dose IL-2 Therapy in Type 1 Diabetes. *Diabetes*. 2015;64:2172-2183. doi:10.2337/db14-1322

29. Round JL, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol*. 2009;9(5):313-323. doi:10.1038/nri2515

30. Atarashi K, Shima TTT, Imaoka A, et al. Induction of Colonic Regulatory T Cells by Indigenous Clostridium Species. *Science (80-)*. 2011;331(21):337-341.

31. Smith K, McCoy KD, Macpherson AJ. Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. *Semin Immunol*. 2007;19(2):59-69. doi:10.1016/j.smim.2006.10.002

32. Brunetti E, Landi F, Casati A, et al. Analysis of circulating gammadelta+ T cells in children affected by IgE-associated and non-IgE-associated allergic atopic eczema/dermatitis syndrome. *Clin Exp Immunol*. 2005;141(1):116-121. doi:10.1111/j.1365-2249.2005.02813.x

33. Wi J-O, Shim J-U, Park CK, et al. Inverse association of peripheral blood CD4+ invariant natural killer T cells with atopy in human asthma. *Hum Immunol*. 2010;71(2):186-191. doi:10.1016/j.humimm.2009.10.011