Kirsten Ilse Mylène Looman

THE EARLY LIFE ADAPTIVE IMMUNITY

Determinants and immune-mediated diseases

The Early Life Adaptive Immunity: Determinants and immune-mediated diseases

Kirsten Ilse Mylène Looman

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The Early Life Adaptive Immunity: Determinants and immune-mediated diseases

De adaptieve immuniteit in het vroege leven:
determinanten en immuungemedieerde ziekten

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MANUSCRIPTS THAT FORM THE BASIS OF THIS THESIS

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*Denotes equal contribution

1 General introduction

INTRODUCTION

The immune system

The immune system has a major role in health and disease. The immune system is composed of a crucial balance between an adequate defense against harmful pathogens and a sufficient immune regulation to prevent self-harm.[1] In this balance a close cooperation between the innate and adaptive immunity is essential.[2]

The first line in the defense against pathogens is formed by the skin, the respiratory tract, the gastrointestinal tract and the urogenital tract which stand in constant connection with the outside world.[3] Chemical, mechanical and microbial barriers within these tracts protect against pathogen invasions.[3] When a pathogen enters the human body, the immune reaction is initiated by the innate immune cells and components.[2] The innate immunity consists of various cells that contain pattern-recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs).[4] This recognition enables a rapid response to invading antigens.[2, 4, 5] In addition, innate immune cells trigger responses by the adaptive arm of the immune system.[2, 5]

The adaptive immunity is unique in the generation of a long-lasting immunological memory after exposure to a specific antigen.[2, 5] The two main cell lineages within the adaptive immunity are the T and B cells.[6]

T cells

The development of T cells starts in the bone marrow where pluripotent hematopoietic stem cells develop into common lymphoid progenitor cells.[7-9] After migration to the thymus, these progenitor cells get committed to the T-cell lineage (prothymocytes) and develop in a sequential manner to $CD4^+$ helper T cells or $CD8^+$ cytotoxic T cells that recognize processed antigens presented on major histocompatibility complex (MHC) II and I, respectively.[7, 8] The cells enter the thymus through high endothelial venules and first migrate to the subscapular region.[10] Here, diversity in the T cell receptors (TCR) is constructed through V(D)J-recombination of first the TCRB and subsequently the TCRA loci, encoding the β chain and the α chain of the TCR.[11] These gene rearrangements in the TCR loci lead to a diverse set of mature TCRs to enable the recognition of various antigens.[11] The cells with functional-expressed TCRs migrate to the cortex of the thymus to develop into double positive ($CD4^+CD8^+$) cells that undergo positive and negative selection.[10, 12] In positive selection, the cells that contain TCRs that bind MHC complexes differentiate further. This applies to approximately 10% of the cells.[7, 12] The cells that survive will subsequently undergo negative selection, i.e. those cells with high affinity for MHC complexes presenting autoantigens

undergo apoptosis to prevent auto-reactivity.[7, 12] Following selection, the mature naive $CD4^+$ and $CD8^+$ T cells will migrate to lymphoid organs.[7, 8]

$CD4^+$ T cells are the helper T cells and support the immune response of other immune cells including B cells and $CD8^+$ T cells.[13-15] Helper T cells recognize the MHC-II complex that is expressed by immune cells.[14, 15] This is in contrast to the $CD8^+$ T cells that recognize MHC-I, present on all cells with a nucleus.[15, 16] This enables the $CD8^+$ T cells to directly respond to cells that present aberrant proteins such as in infections or cancer.[16, 17] Within the $CD4^+$ T cell lineage, distinct subsets can be formed depending on the signals present during the immune response. These subsets are characterized by the production of different combinations of cytokines, which are small, soluble proteins important in intercellular signaling.[18] Within the $CD4^+$ T cells, amongst other cells, the T helper (Th) 1, Th2, Th17, Th22 and regulatory T cells (Treg) can be distinguished.[19-21] The Th1 cells are producers of interleukin (IL)-2, interferon-gamma (IFN-ɣ) and tumor necrosis factor alpha (TNF-α).[19] Th1 cells are involved in the host's defense against intracellular pathogens.[19] However, excessive Th1 responses have been associated with autoimmune diseases. In contrast, Th2 cells are producers of IL-4, IL-5 and IL-13.[22] Th2 cells are involved in the defense against extracellular pathogens. However, excessive Th2 responses have been associates with allergies and atopy.[22, 23] Th17 cells fight extracellular pathogens and fungi by production of IL-17, IL-20 and IL-21.[19, 20] However, an excessive Th17 response has been observed in immune-mediated diseases such as atopic dermatitis and rheumatic disease.[20] Th22 cells produce IL-22, a cytokine contributing to the production of antimicrobials and cell survival, and have chemokine receptors (CCR4, CCR6 and CCR10) that mediate migration to the skin to preserve barrier homeostasis.[21] The Tregs are producers of IL-10 and are important in regulating the immune responses to maintain homeostasis.[19]

Next, both the $CD4^+$ and $CD8^+$ T cells can be classified into naive, central memory and effector memory cells.[6, 24] Naive and central memory cells contain the CD62L and CCR7 homing receptors, which are absent on the effector memory cells.[24] Consequently, the naive and central memory T cells have the capability to migrate to the primary and secondary lymphoid organs, whereas the effector T cells migrate to the peripheral tissue to execute their effector function.[24, 25] A small fraction of the effector memory T cells belongs to the long-living memory and resides in the peripheral tissue after an infection to execute their effector function directly in case of re-infection with a pathogen.[25] The effector memory T cells can be separated into those expressing CD45RO (TemRO) and those expressing CD45RA (TemRA) from alternative splicing of the *PTPRC* gene.[26] Expression of CD45RA marks the most differentiated state, which displays more effector activity.[24] $CD8^+$ T cells have the ability to eliminate infected cells by bacteria and viruses and previous studies have observed that these cells are important in the defense against infections, including

the defense against herpes viruses such as cytomegalovirus (CMV) and Epstein Barr virus (EBV).[6, 25] The effector memory T cells carry CCR4 and CCR5, which allows them to infiltrate in inflamed tissue.[24]

Next to these previously described T cells expressing TCRαβ receptors (αβT cells), which are the most prevalent in the human blood, there is also a T-cell subset expressing a γδTCR. The γδ T-cell subset consists for the largest part of $V\delta2^+V\gamma9^+$ T cells in humans.[27] In contrast to αβ T cells, the $V\delta2^+V\gamma9^+$ T cells do not recognize HLA but butyrophilin 3A molecules that present phosphoantigens.[28-30] Phosphoantigens are phosphate-bearing antigens produced by various microbes and cancers.[31] An example of a phosphoantigen is the microbial metabolite (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) that is present in mycobacterium tuberculosis and malaria.[28, 29] $V\delta2^+V\gamma9^+$ cells produce IFN-γ after activation and have the ability to develop into a long-lasting immunological memory.[30]

B cells

The development of B cells takes place in the bone marrow where pluripotent hematopoietic stem cells develop into common lymphoid progenitor cells.[9, 32] In the bone marrow, these lymphoid progenitor cells first undergo VDJ recombination of the immunoglobulin (Ig) heavy chain to form precursor B cells and next VJ recombination of the Ig light chain to develop into transitional B cells.[33-36] These transitional B cells will migrate to the lymph nodes where they will further develop into mature naive B cells (IgM^+IgD^+) and can get activated after direct recognition of whole antigens or antigens presented by $CD4^+$ T cells or other antigen presenting cells.[37-39] B cells recognize whole antigens through the B cell receptor (BCR) which is composed of a membrane-bound immunoglobulin (Ig) and CD79a (Igα) and CD79b (Igβ) which function as signal transduction molecules.[40] After recognition of an antigen in the lymph node by the BCR, the activated B cell undergoes clonal expansion within the germinal center of the lymph node.[41] Activation-induced cytidine deaminase (AID) is an enzyme that causes mutations in the variable regions of the Ig genes.[42, 43] Introduction of somatic hypermutation by AID in the Ig variable domains induces variation in antigen-binding strength. Following selection, only those daughter cells with high affinity for the specific antigen will survive.[42, 43] In addition, AID mediates Ig class switch recombination, enabling B cells to change from an IgM to IgG, IgA or IgE isotypes.[44] Ig class switching does not affect the antigen recognition but affects the effector function of the B cell as each Ig isotype has its own characteristics.[45-47] Finally, daughter cells will develop into memory B cells and plasma cells.[34, 35]

The response to form memory B cells can occur in a T-cell dependent or T-cell independent manner.[33] The T-cell dependent responses in the germinal center can generate $CD27^+IgA^+$, $CD27^+IgD^+$, $CD27^+IgE^+$, $CD27^{+/-}IgG^+$, $CD27^+IgM^+$ memory B cells, whereas T-cell inde-

pendent responses outside the germinal center generate $CD27^-IgA^+$, $CD27^+IgM^+IgD^+$ and $CD27^-IgE^+$ memory B cells.[33] In addition to the antigen-BCR interaction, T cell dependent activation of B cells is for a large part based on CD40-CD40 ligand binding between the B and T follicular helper cells.[48] B cells engage cognate T cells through presentation of antigen on MHC class II. Furthermore, the T cells produce cytokines such as IL-4 and IL-21 that stimulate B cell differentiation and expansion.[49] T cell independent B memory cell development occurs within the intestine for the $CD27^-IgA^+$ B memory cells and within the marginal zone of the spleen for the natural effector ($CD27^+IgM^+IgD^+$) B cells.[33] Molecular determination has shown that the B cells which develop through a T cell dependent pathway undergo more somatic hypermutation of the variable domains in the Ig heavy and light chains of the BCR for antigen recognition.[33] Finally, the B cells can develop into the plasma cells which spontaneously release soluble immunoglobulins.[33] Figure 1.1 provides an overview of the studied memory T- and B-cell subsets in this thesis.

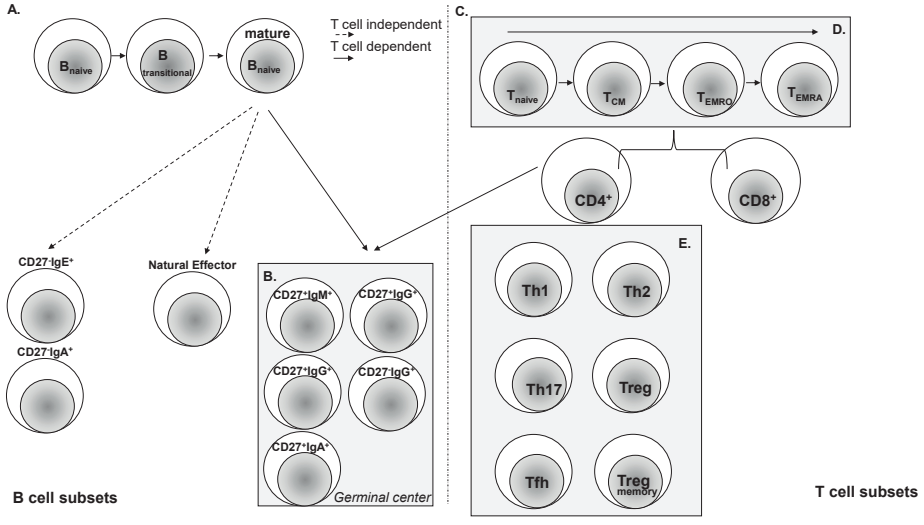

Figure 1.1. Overview of the studied T and B memory cell subsets

A. The differentiation of the naive B cell to mature naive mature B cell is represented. B cells play a crucial role in humoral immunity through the production of antibodies. The naive mature B cells develop into memory B cells upon antigen exposure. **B.** The cells represented in box B develop within the germinal center and their development into memory B cells is T cell dependent. The other memory B cells have a T cell independent memory formation. **C.** The T cells, also called the cellular immunity, can be divided into $CD4^+$, helper T cells that recognize processed antigens presented on MHC-II by antigen presenting cells, and $CD8^+$, cytotoxic T cells that recognize processed antigens presented on MHC-I by antigen presenting cells. **D.** The differentiation of naive T cells into memory T cells is represented. After a $CD4^+$ or $CD8^+$ naive T cell is exposed to a processed antigen by an antigen presenting cell, the naive T cell develops into a central memory T (Tcm) cell or effector memory (Tem) RO^+ or RA^+ T cell. **E.** Within the $CD4^+$ memory T cells, the T helper 1 (Th1), Th2, Th17, and regulatory T cells can be detected.[33, 50] The $CD4^+$ T cells help in the T cell dependent differentiation of B cells.

The maturation of the immune system during childhood

The maturation of the immune system starts during fetal life and continues during childhood, along with the general development of the child. Various genetic, environmental and metabolic factors (such as vitamin D and adiposity) during childhood affect the immune system maturation starting from fetal life.[51-53] Due to these factors, that also likely interact with each other, and the continuous progress of the child's development, the study of the maturation of the immune system in childhood is complex.[54]

It is assumed that specific time intervals, also known as susceptibility windows, exist during the fetal period and childhood in which the developing child is particularly sensitive to environmental factors.[54-56] The exact time of these susceptibility windows in the development is unknown and could also differ inter-individually.[55] However, it is hypothesized that these susceptibility windows are correlated with the different phases of growth and organ development.[55, 56] The child's development can be divided into different phases that each consist of consecutive physiological and psychological changes.[56, 57] Each phase is unique in terms of developmental processes and exposures but interacts with previous and future phases. Increasing knowledge on genetic and non-genetic factors that affect the child's immune development is important to increase children's health with possibly life-long consequences.[54, 57, 58] The Generation R cohort is a population-based pediatric cohort that studies these developmental processes in a general population of which one aspect is immune maturation.[59]

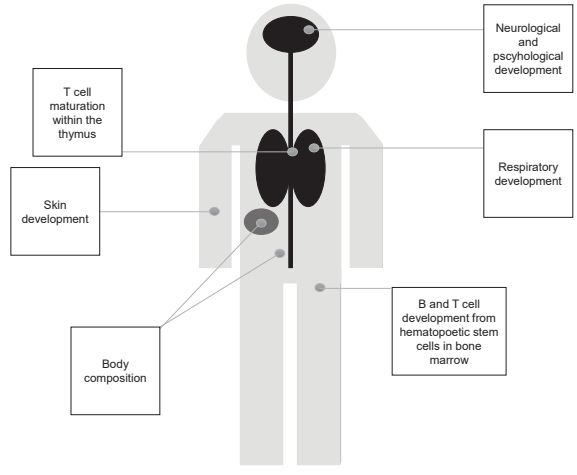

Figure 1.2. Overview of the developmental processes in the child possibly associated with the adaptive immunity.

The immune system is shaped during early life under the influence of inherited genetic factors and the exposure to various environmental factors. Skewing of the adaptive immune response and immune memory is a risk factor of childhood disease which can be visualized.

Immune-mediated childhood disease

The combination of genetic and non-genetic factors determines the risk of childhood disease.[60-62] First, the genetic heritability determines a child's baseline risk for disease.[60, 63, 64] However, it has been shown that children with a similar genotype have different phenotypes, meaning different expressions of disease are possible despite genetic similarities.[65] This indicates that there also exists a role for non-genetic factors in the risk of disease onset.[53, 61, 62]

Atopy is common in childhood and it is defined as a genetic predisposition to develop an IgE response to environmental particles (allergens), which might lead to atopic disease.[66] Atopic diseases are the most common chronic childhood diseases and include atopic dermatitis, food and inhalant allergies and allergic asthma.[60, 67] The corresponding prevalence among Dutch children is 5-15% for atopic dermatitis, 26.4% for allergic sensitization (measured among children aged 7-14 years) and 8.1% for asthma (measured among children aged 5-18 years).[68-70] The prevalence of atopic disease continues to rise, and prediction models indicate that more than 50% of the European population will suffer from any allergic disease by 2025.[71] The pathophysiology of these atopic diseases consists of a combination of various genetic and non-genetic factors. These factors together drive the child's immune system into an increased type 2 immunity with an increase in Th2 and IgE^+ memory B cells.[60, 72-77] In turn, the clinical presence of these atopic diseases might also in itself affect the cellular composition of the immune system.[78, 79] However, studies on the composition of the adaptive immunity with adjustment for confounders have not been performed previously in a large cohort of children.

Attention deficit hyperactivity disorder (ADHD) is the most prevalent childhood behavioral disorder with an estimated prevalence of 3-5% within Europe.[80, 81] Previous studies have observed that ADHD is associated with immune-mediated diseases such as asthma and type 1 diabetes mellitus.[82, 83] The exact pathophysiology of ADHD is unknown. Yet, it has been hypothesized that also in ADHD immunological dysregulation might contribute to the pathogenesis of this behavioral disorder.[82-84]

Besides diseases that have an onset during childhood, it has been hypothesized that certain genetic and non-genetic risk factors during childhood might affect the risk of disease in later life. One example is multiple sclerosis, a neurologic auto-immune disease with a peak onset between 20 and 40 years of age, which is characterized by T-cell mediated demyelination of the central nervous system.[85-87] It is unknown if a genetic predisposition for MS already causes alterations in T cell composition during childhood.

Genetic and non-genetic factors in immune-mediated childhood diseases

The immune system could be seen as a mediator between genetic and non-genetic childhood determinants and the onset of various childhood conditions. The first months of life, the child is passively protected by maternal immunoglobulin G (IgG) antibodies that have crossed the placenta during the third trimester.[5] In addition, maternal IgA is transferred to the child through breastfeeding.[88] After birth, the adaptive immunity starts to generate memory B and T cells after antigen exposure.[5] The development of the adaptive immunity is affected by both genetic and non-genetic factors.[5, 6, 89, 90] These factors each take account for approximately 50% in the shaping of the adaptive immunity.[6, 89, 90] Previously, various maternal, child and environmental factors have been identified as determinants involved in the shaping of the adaptive immunity during the first years of life.[6] Many of these determinants have also been associated with childhood disease like asthma, atopic dermatitis and allergies.[61]

First, the genetic determinants in relation to the immune system and immune-mediated diseases will be discussed. From previous genetic studies, such as genome wide association studies, various single nucleotide polymorphisms (SNPs) have been related with immune-mediated diseases.[64, 91, 92] A SNP is a genetic variation in a single nucleotide of the genome and can occur in both non-coding and coding regions of the genome.[92, 93] Within the coding regions, the SNPs can be classified in synonymous substitutions and nonsynonymous substitutions.[93] Synonymous substitutions do not lead to amino acid changes.[93] In contrast, a nonsynonymous substitution is a base pair mutation, such as a missense mutation or nonsense mutation, that leads to amino acid sequence changes.[93] Such genetic variants with a minor allele frequency (MAF) of more than 5% are common and genetic variants with a MAF of less than 1% are rare. Genetic variants with a MAF between 1% and 5% are classified as low-frequency variants.[94] An example of a nonsynonymous substitution is the mutation in the filaggrin gene (*FLG*). *FLG* mutations are the strongest genetic risk factor for atopic dermatitis and a common genetic variant with approximately 10% of the European population being heterozygote for this mutation.[95] These mutations lead to an increased permeability of the skin for allergens, possibly contributing to the altered immune composition in individuals with atopic dermatitis.[95-97] Subsequently, these *FLG* mutations also increase the risk for other atopic diseases: allergies and allergic asthma.[95, 98-100] In MS, various risk SNPs have been identified.[101] These SNPs can be combined into a polygenic risk score (PRS) to study the genetic risk of MS as a whole.[102, 103]

Second, the environmental determinants relating to the immune system and immune-mediated disease will be discussed. Higher levels of vitamin D have been associated with a protection against asthma and respiratory infections possibly through effects of vitamin D on T-cell subsets.[104-107] However, the effect of vitamin D on T cell shaping has not been

studied previously in children. Another increasingly prevalent childhood condition is obesity.[108, 109] Chronic low-grade inflammation in adipose tissue in individuals with obesity increases the risk for chronic disease such as metabolic syndrome and asthma.[108, 109] This pro-inflammatory state is also reflected in the adipose tissue and blood with increased pro-inflammatory immune cells such as macrophages and cytotoxic $CD8^+$ T cells and pro-inflammatory cytokines such as interleukin IL-1, IL-6 and TNF-α.[110-113] However, limited knowledge is available on the presence of these inflammatory processes in childhood.

In summary, it is possible that the immune system acts as a mediator between environmental determinants and various childhood diseases (Figure 1.3).

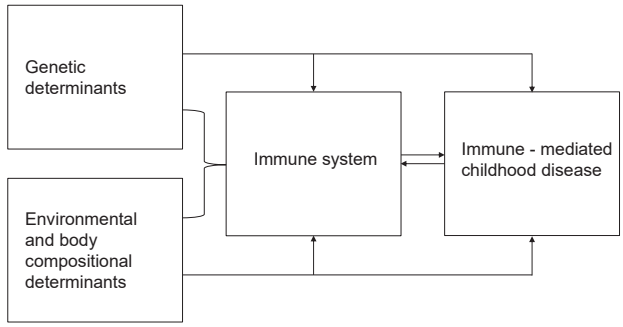

Figure 1.3 Hypothesis: the immune system as mediator in immune-mediated childhood disease.

Hypothesis

This thesis is based on the hypothesis that the immune system is shaped during early life under the influence of inherited genetic factors and the exposure to various environmental factors and that skewing of the adaptive immune response and immune memory is a risk factor of childhood disease which can be visualized.

THIS THESIS

This thesis is built upon three main aims, which consist of subsequent research questions:

1. **To examine the association between the adaptive immunity and health outcomes in school-aged children.**

- What is the role of T helper, T regulatory and memory B- and T-cell subsets in atopic diseases like atopic dermatitis, asthma and allergic sensitization in a large cohort of children?
- Is a pro-inflammatory differentiated immune system involved in the pathophysiology of attention hyperactivity/deficit disorder (ADHD)?

2. **To identify the role of serum 25-hydroxyvitamin D (25(OH)D) and adiposity on the shaping of the adaptive immunity in school-aged children.**
- What is the association between serum 25(OH)D and circulating T cell numbers in children?
- Do children with adiposity already have an inflammatory immune profile compared to non-adipose children?

3. **To identify the role of specific genetic variants associated with immune-mediated diseases on the shaping of the immune system.**
- Does the presence of filaggrin haploinsufficiency affect B and T cell composition in children with and without atopic dermatitis?
- Do children with a higher SNP-defined genetic risk score for multiple sclerosis already show alterations in T memory cell numbers?

The Generation R Study

This thesis is based on studies that are embedded within the Generation R Study.[59, 114] The Generation R Study is a multi-ethnic prospective birth cohort study located in Rotterdam, the Netherlands.[59] The objective of the Generation R Study is to identify the origins of health and disease from fetal life onwards in a multidisciplinary setting.[59] At baseline, the study included 9,778 women living in Rotterdam with a delivery date between April 2002 and January 2006.[115]

Within the current thesis, determinants and outcomes were measured at either the child's age of 6 years or the child's age of 10 years. In addition, the studies within this thesis use data on circulating $CD19^+$ B, $CD3^+$ T cell or monocyte numbers which were obtained at the research center by venipuncture at 6 and 10 years.[6, 114] At 6 years, in 3,465 children total T and B cell counts were determined by the use of diagnostic lyse-no-wash protocol (BD Biosciences).[6, 23, 114]. Additionally, at 6 years, detailed immune phenotyping was performed in 1,121 children by the use of 6-color flow cytometry on a 3-laser LSRII (BD Biosciences).[6] At 10 years, in 950 children detailed immune phenotyping was performed by the use of 11-color flow cytometry on 4-laser LSR Fortessa (BD Biosciences).[23, 114] The smaller sample numbers for detailed immune phenotyping were the result of logistics and time constraints for the extensive procedure. Maternal and child characteristics were obtained from pregnancy onwards by prenatal and postnatal questionnaires and by measurements at local health centers and midwife practices.[59, 115] Prenatal and postnatal questionnaires during the first four years of life and at 6 and 10 years of age were used to determine environmental factors that could potentially affect the associations we studied. Information on children's genotype was collected from cord blood and analyzed by Illumina 610K and 660K SNP arrays (Illumina, San Diego, California, USA). The Medical Ethical Committee (MEC) of

the Erasmus Medical Center approved the Generation R Study (MEC-2012-165).[59] Written informed consent was obtained from parents or legal representatives of all participants included.[59]

Outline

Part I of this thesis aims to study immune cell composition in specific childhood diseases. **Chapter 2** studies helper and regulatory T cells and $CD27^+$ and $CD27^-$ IgG^+, IgA^+, IgE^+ memory B cells in children with any atopic disease compared to children without atopic disease. In addition, the individual atopic conditions defined as atopic dermatitis, food- or inhalant-allergic sensitization, and asthma are studied in relation to B- and T-cell subsets. **Chapter 3** focuses on the associations between peripheral circulating helper and regulatory T, $CD4^+$ and $CD8^+$ T-cell subsets and $CD19^+$ memory B cells with attention problems in children.

Part II of this thesis aims to identify early life environmental and body compositional determinants of immune cell composition in children.

Chapter 4 describes the association between serum vitamin 25(OH)D and $CD4^+$ and $CD8^+$ central and effector memory T-cell subsets. In addition, this chapter describes the relation between serum 25(OH)D and herpes virus seropositivity and upper respiratory tract infections. **Chapter 5** describes the associations between child's fat mass measurements and monocytes and T-cell subsets. These fat mass measurements include total fat mass index, android-to-gynoid fat mass ratio, visceral fat index and liver fat fraction.

Part III of this thesis aims to identify genetic determinants of immune cell composition in children. **Chapter 6** studies the association between filaggrin gene (*FLG*) mutations and T cell composition in children of the general population and in children with atopic dermatitis. **Chapter 7** is devoted to the study of multiple sclerosis genetic risk scores and T cell numbers in children.

Finally, **Chapter 8** encompasses a general discussion of the thesis and future prospects.

Table 1.1. Overview of determinants and outcomes within each thesis chapter.

Chapter	Age	Childhood determinant or immune-mediated disease	Immunological data
2	10 years	Ever doctor-diagnosed atopic dermatitis *Questionnaires* Ever doctor-diagnosed asthma *Questionnaires* Food- and inhalant-allergic sensitization *Skin prick tests*	**T-cell subsets** § CD4 & CD8 **total** § CD4 & CD8 **naive** § CD4 & CD8 **central** and **effector memory** § Th1, Th2, Th17, Treg **B-cell subsets** § Total § Naive § IgG, IgA, IgE memory
3	10 years	Attention problems score *Child and Behavior Checklist answered by mother*	**T-cell subsets** § CD4 & CD8 **total** § CD4 & CD8 **naive** § CD4 & CD8 **central** and **effector memory** § Th1, Th2, Th17, Treg **B-cell subsets** § Total § Transitional and naive § IgG, IgA memory
4	6 years	Serum 25(OH)D *Venous blood samples*	**T-cell subsets** § CD4 & CD8 **total** § CD4 & CD8 **naive** § CD4 & CD8 **central** and **effector memory**
5	10 years	BMI *Measured at research center* FMI and android-to-gynoid fat ratio *DXA* Visceral FMI and liver fat fraction *MRI*	**T-cell subsets** § CD4 & CD8 **total** § CD4 & CD8 **naive** § CD4 & CD8 **central** and **effector memory** § Th1, Th2, Th17, Treg § $v\delta2^{+}\gamma9^{+}$ T subsets **Monocytes** § Classical, intermediate, non-classical
6	10 years	Filaggrin gene mutation status *Cord blood*	**T-cell subsets** § CD4 & CD8 **total** § CD4 & CD8 **naive** § CD4 & CD8 **central** and **effector memory** § Th1, Th2, Th17, Th22 Treg **B-cell subsets** § Total § Transitional and naive § IgG, IgA, IgE memory § IgM only
7	6 years	MS-PRS *Cord blood*	**T-cell subsets** § CD4 & CD8 **total** § CD4 & CD8 **naive** CD4 & CD8 **central** and **effector memory**

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; BMI, body mass index; DXA, Dual-energy X-ray absorptiometry; FMI, fat mass index;Ig, Immunoglobulin; MRI, Magnetic resonance imaging; MS-PRS, multiple sclerosis polygenic risk score; Th, helper T cell; Treg, regulatory T cell.; Tcm, central memory T lymphocytes; TemRA, effector memory RA-positive T cells; TemRO, effector memory RO-positive T cells.

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Immune cell composition in immune-mediated disease

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Associations of Th2, Th17, Treg cells, and IgA^{+} memory B cells with atopic disease in children.

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ABSTRACT

Background

New insights into immune cells could contribute to treatment and monitoring of atopic disease. Because nongenetic factors shape the human immune system, we here studied these immune cells in a large cohort with atopic children with adjustment for prenatal and postnatal confounders.

Methods

Information on atopic dermatitis, inhalant- and food-allergic sensitization, asthma lung function scores was obtained from 855 10-year-old children within the Generation R cohort. 11-color flow cytometry was performed to determine $CD27^+$ and $CD27^-IgG^+$, IgE^+ and IgA^+ memory B cells, Th1, Th2, Th17, and Treg memory cells from venous blood. Associations between any atopic disease, the individual atopic diseases, and immune cell numbers were determined.

Results

Children with any atopic disease had higher Th2, Treg, Treg memory, and $CD27^+IgA^+$ memory B-cell numbers compared to children without atopic disease. When studying the individual diseases compared to children without the individual diseases, children with atopic dermatitis, inhalant-, and food-allergic sensitization had higher memory Treg cell numbers 12.3% (95% CI 4.2; 21.0), (11.1% (95% CI 3.0; 19.8), (23.7% (95% CI 7.9; 41.8), respectively. Children with food-allergic sensitization had higher total B and $CD27^+IgA^+$ memory B-cell numbers (15.2% [95% CI 3.2; 28.7], 22.5% [95% CI 3.9; 44.3], respectively). No associations were observed between asthma and B- or T-cell numbers.

Conclusion

Children with any atopic disease and children with inhalant- and food-allergic sensitization or atopic dermatitis had higher circulating memory Treg cells, but not higher IgE^+ B-cell numbers. The associations of higher Treg and $CD27^+IgA^+$ B-cell numbers in children with food-allergic sensitization are suggestive of TGF-β-mediated compensation for chronic inflammation.

INTRODUCTION

Atopic dermatitis, food allergy, allergic rhinitis and asthma are atopic conditions with a high prevalence in children.[1] These diseases often co-exist leading to high comorbidity of disease at young age.[2] This may be due to a shared immunopathophysiology.[1-3] Atopic diseases share a T helper 2 (Th2) cell driven pathogenesis.[4-6] This Th2-driven response is characterized by skewed cytokine responses including IL-4, IL-5 and IL-13, which in turn drive eosinophilic inflammation and immunoglobulin (Ig) class switching to IgE in B cells.[4-7] Soluble IgE binds to high affinity receptors, FcεR1, on mast cells and basophils, and subsequent exposure to the initial allergen will induce IgE-mediated FcεR1 receptor crosslinking and instant release of inflammatory mediators responsible for allergic symptoms present in atopic diseases.[8, 9] Regulatory T (Treg) cells have the ability to suppress this inflammatory response.[10] Treg cells can be subdivided in two distinct subsets: naive and memory Tregs. While naive Tregs mostly originate from the thymus, memory Tregs are induced in the periphery and display the highest suppressive ability.[11, 12]

Insights into the biology of the cells that produce IgE are limited. In contrast to B cells expressing other Ig isotypes (IgM, IgD, IgA or IgG), IgE-expressing B cells are scarce and surface expression of IgE on these cells is low.[13, 14] Our group has developed a method to enable detection of IgE-expressing B cells and plasma cells.[13] In addition to the classical $CD27^+$ memory B cells and plasma cells that are derived from T-cell dependent responses in the germinal center, $CD27^-IgE^+$ B cells can be derived independent of cognate T-cell help.[13, 15] These $CD27^-IgE^+$ memory B cells were increased in adults and children with atopic dermatitis and displayed increased levels of somatic hypermutations, a sign of enhanced antibody maturation.[13-17] However, these previous results did not adjust for confounders and are based on limited numbers of hospital-based patient populations (largest study n=164).[16]

Therefore, we now quantified circulating memory B cell, Treg and Th cell subsets with 11-color flow cytometry in a large birth cohort of ten-year-old children and assessed the association of atopic dermatitis, food- and inhalant-allergic sensitization and asthma with these cells following adjustment for confounders.

METHODS

Study Design

This cross-sectional study was embedded in the Generation R Study, a population-based prospective birth cohort study located in Rotterdam, the Netherlands.[18] The Medical Ethical Committee of the Erasmus University Medical Center in Rotterdam has approved the study

protocol (MEC-2012-165). Written informed consent was obtained from parents or legal representatives of all participants. For the present study, we selected ten-year-old children with at least one atopic disease measurement and with at least one immune cell outcome. This resulted in the inclusion of 855 children. The number of children included in the individual analyses is dependent on the combination of exposure and outcome and ranges from 648 to 776 children (Supplementary Table 2.1).

Atopic dermatitis measurements

Physician-diagnosed atopic dermatitis ever was diagnosed from parental-reported questionnaires obtained at the child's age of 10 years ('Was your child ever diagnosed by a physician with atopic dermatitis', 'yes;no').[19-21]

Allergic sensitization measurements

We used skin prick tests using the scanned area method to determine the presence of total, inhalant- and food-allergic sensitization at the age of 10 years.[22] The skin prick test included a positive control (histamine dihydrochloride 10 mg/mL) in duplicate and a negative control (NaCl 0.9%). We included house dust mite, 5-grass mixture, birch, cat and dog (ALK-Abelló B.V., Almere, the Netherlands) as inhalant allergens and hazelnut, cashew nut, peanut and peach as food allergens (homemade food allergen extracts).[20] A positive skin test was defined as an area ≥40% of the histamine response.[19] Information on physician-diagnosed allergy was obtained from questions adapted from the International Study on Asthma and Allergy in Childhood (ISAAC).[20]

Asthma and lung function measurements

At ten years of age, we measured Forced Expiratory Volume in 1 second (FEV_1), FEV_1/Forced Vital Capacity (FVC), and Forced Expiratory Flow after exhaling 75% of the FVC (FEF_{75}) by spirometry (MasterScreen-Pneumo, Jaeger Toennies (Viasys) CareFusion Netherlands), according to ERS/ATS guidelines.[23-25] Lung function measurements were converted into z-scores adjusted for sex, age, height and ethnicity according to Global Lung Initiative reference data.[26] Information on wheezing in the past 12 months and physician-diagnosed asthma ever were obtained from questions adapted from ISAAC.[23,24] Current asthma was defined as physician-diagnosed asthma ever together with use of asthma medication or wheezing in the past 12 months.

Immune cell outcomes

Peripheral blood samples were obtained from children at 10 years. Absolute counts of peripheral blood $CD19^+$ B cells and $CD3^+$ T cells were obtained using a diagnostic lyse-no-wash protocol (BD Biosciences, San Jose, CA). In addition, detailed immune-phenotyping

of B and T cells using 11-color flow cytometry was performed on LSRII Fortessa (BD Biosciences) with standardized configuration according to Euroflow protocols.[27]

In addition to total $CD19^+$ B cells, the following $CD19^+CD38^{dim}IgD^-$ memory B-cell subsets were defined: $CD27^+IgG^+$, $CD27^-IgG^+$, $CD27^+IgE^+$, $CD27^-IgE^+$, $CD27^+IgA^+$ and $CD27^-IgA^+$ memory B cells.[16] Within total $CD3^+$ T cells, $CD4^+$ and $CD8^+$ lineages were defined, as well as $CD4^+CD127^{dim}CD25^{high}$ Treg cells and their naive ($CD45RA^+$) and memory ($CD45RA^-$) subsets. After exclusion of Tregs, Th cell subsets within $CD4^+CD45RA^-$ cells were defined as Th1 $CCR6^-CXCR3^+CCR4^-$, Th2 $CCR6^-CXCR3^-CCR4^+$ and Th17 $CCR6^+CXCR3^-CCR4^+$ cells.[16] Finally, within both the $CD4^+$ and $CD8^+$ lineages, naive ($CD45RO^-CCR7^+$), central memory T cells (Tcm;$CD4RO^+CCR7^+$), effector memory RO-positive T cells (TemRO;$CD45RO^+CCR7^-$) and effector memory RA-positive cells (TemRA; $CD45RO^-CCR7^-$) were defined.[28-30] The data were analyzed with the software packages FacsDIVA software v8 (BD Biosciences), Infinicyt software (Cytognos, Salamanca, Spain) and FlowJo software v10 (FlowJo LLC, Ashland, Oregon). Cell numbers were presented per μL blood. The gating strategies are represented in Supplementary Figure 2.1-2.3.

Covariates

Information on maternal age, prepregnancy body mass index, smoking during pregnancy, breastfeeding during the first 4 months, family history of atopy and asthma and net household income was derived from parental questionnaires.[19, 20, 23] Child's sex and birth weight were obtained from midwife and hospital registries.[28] Child's weight and height were measured at the research center and body mass index (BMI) was calculated. Information on child's ethnicity and number of children in household was obtained from parental questionnaires.[19, 20, 23, 28]

Statistical analysis

To obtain a normal distribution, the cell numbers were natural log transformed. Multivariable linear regression analyses were conducted to determine associations between any atopic condition, atopic dermatitis, allergic sensitization, asthma, lung function and immune cell numbers to limit bias due to confounding. Covariates were added if they were associated with immune cells based on our previous study and if they met the epidemiological criteria for confounders in our study and finally, if the addition of the covariate resulted in an effect estimate alteration of at least 10%.[31, 32] We adjusted for the following covariates: sex, gestational age, maternal age, maternal prepregnancy BMI, breastfeeding during the first 4 months, number of children in the household, child ethnicity, family history of atopy or asthma, cesarean section, child's BMI at 9 years.[32] In the nonimputed dataset, percentages of missing values within the covariates ranged from 0 to 24% and for child ethnicity 32.6%. Tenfold multiple imputation was performed. Pooled regression coefficients are presented in our study. We did not adjust for multiple testing because of strong correlations between

immune cell numbers. A p-value <0.05 was considered significant. Statistical analyses were performed using SPSS version 21.0 (IBM Corp., Armonk, NY, USA) and R version 3.3.3 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Study population characteristics

Our study population consisted of 855 children with a median age of 9.8 years (Table 2.1). 230 (29.6%) children had allergic sensitization, of which 50 (6.5%) to food allergens and 225 (29.0%) to inhalant allergens. 63 children (8.5%) were ever diagnosed with asthma. A total of 163 (22.1%) had ever atopic dermatitis. The concomitant atopic diseases in the study population are shown in Supplementary Figure 2.4.

Table 2.1. Characteristics of the study population.

Child characteristics	**(N=855)**	**Missing** (%)
Sex (N,%)		0.0
Girls	460 (53.8)	
Birth weight, g	3510 [3150;3830]	0.1
BMI at 9 years, kg/m^2	17.08 [15.81;18.49]	0.0
Ethnicity (N,%)		32.6
Dutch or Western	484 (84.0)	
Siblings in household (N,%)		12.9
0	103 (13.8)	
1	406 (54.5)	
2 or more	194 (26.0)	
Ever diagnosed with atopic dermatitis (N,%)	163 (22.1)	13.7
Ever diagnosed with asthma (physician) (N,%)	63 (8.5)	13.1
Current asthma within the past 12 months at 9 years (N,%)	34 (4.6)	13.1
Positive allergy skin prick test (N,%)	230 (29.6)	9.2
Positive food allergy skin prick test (N,%)	50 (6.5)	9.4
Positive inhalant skin prick test (N,%)	225 (29.0)	9.1
Maternal characteristics		
Age, years	31.80 [29.12, 34.34]	0.0
Prepregnancy BMI, kg/m^2	22.60 [20.72, 24.91]	24.0
Smoking during pregnancy (N,%)	176 (23.8)	13.6
Breastfeeding during first 4 months (N,%)	606 (90.3)	21.5
Family history of atopy and asthma (N,%)	397 (49.9)	6.9
Net household income, euro (N,%)		16.4
>2200	525 (73.4)	

Abbreviations: BMI, body mass index; g, gram; kg, kilogram; m, meter; N, number. Values are based on the nonimputed dataset. Values are presented as median [25%-75% range] or numbers (%)

Th and Treg cell subsets

Children with any atopic condition compared to no atopic condition had 13.8% (95%CI 4.1; 24.4) higher Th2, 7.1% (95% 1.0; 13.5) higher Treg and 18.4% (95%CI 5.1; 33.3) higher Th17 cell numbers (Table 2.2). Next, we studied the T-cell subsets per atopic condition. No associations were observed between atopic conditions and Th1 cell numbers.

Table 2.2. Associations between any atopic condition B- and T-cell subsets.

Outcome / Exposure	Any atopic condition (n=729; yes=343, no=386)
T-cell subsets	
Th1	3.29 (-8.54; 16.64)
Th2	**13.82 (4.12; 24.44)**
Treg	**7.10 (1.03; 13.54)**
Treg naive	2.76 (-5.01; 11.16)
Treg memory	**17.03 (8.79; 25.91)**
Th2/Th1 ratio	10.10 (-4.88; 27.45)
Th17	**18.37 (5.11; 33.31)**
B-cell subsets	
B total	**9.79 (3.60; 16.36)**
B naive	**9.28 (2.34; 16.69)**
IgG^+ $CD27^-$	5.52 (-3.64; 15.55)
IgG^+ $CD27^+$	9.21 (-0.94; 20.39)
IgE^+ $CD27^-$	-0.93 (-2.25; 2.16)
IgE^+ $CD27^+$	0.11 (-3.56; 3.93)
IgA^+ $CD27^-$	**14.20 (2.29; 27.49)**
IgA^+ $CD27^+$	**12.35 (3.15; 22.37)**

Abbreviations: CI, confidence interval; Ig, immunoglobulin; Th, helper T cell; Treg, regulatory T cell. The numbers represent % cell increase or decrease (95%CI) for the children with any atopic condition compared to children without any atopic condition. Numbers are based on multivariable linear regressions and adjusted for the following covariates: sex, gestational age at birth, maternal age, maternal prepregnancy BMI, breastfeeding during the first 4 months, number of children in the household, child ethnicity, family history of atopy or asthma, cesarean section, child BMI at 9 years. The bold values represent the statistically significant ($P < .05$) results

14.5% (95%CI 4.4; 25.5) higher Th2 cell numbers were observed in children with allergic sensitization compared to those without allergic sensitization. Specifically, 12.5% (95%CI 2.5; 23.5) and 28.7% (95%CI 8.8; 52.3) higher Th2 cell numbers were observed in inhalant and food allergic sensitization, respectively. Th2 cells were not associated with atopic dermatitis, asthma or lung function (Table 2.3-2.4). To further study Th2 cells in relation to intrinsic and extrinsic atopic dermatitis, we stratified for food-allergic sensitization. Although higher effect estimates were observed in children with food-allergic sensitization, no significant associations were observed (Supplementary Table 2.3). Children with food-allergic sensitization had 17.2% (95%CI 4.8; 31.1) higher total Tregs. Following separation into naive ($CD45RA^+$) and memory ($CD45RA^-$) Treg, we found that the difference in total Treg was fully explained by 23.7% (95%CI 7.9; 41.8) higher memory Treg numbers. In ad-

dition, memory Tregs were 12.3% (95%CI 4.2; 21.0) and 11.1% (95%CI 3.0; 19.8) higher in children with any or inhalant-allergic sensitization, respectively. Children with atopic dermatitis had 12.0% (95%CI 4.8; 19.8) higher total Tregs, 11.0% (95%CI 1.5; 21.5) higher naive Treg and 17.2% (95%CI 7.5; 27.8) higher memory Treg cells. In addition, children with 1SD increase in FEV_1 or FEF_{75} had 3.7% (95%CI 0.3; 7.3) and 3.6% (95%CI 0.1; 7.2) higher total Tregs, respectively. For FEF_{75}, the difference in Tregs was completely dependent on naive Tregs with 4.8% (95%CI 0.1; 9.8) higher naive Tregs for children with 1SD increase in FEF_{75}. T-cell subsets were not associated with asthma. Finally, children with atopic dermatitis had 15% (95%CI 0.2; 32.0) higher Th17 and children with food-allergic sensitization had 26.2% (95%CI 0.7; 58.2) higher Th17 cell numbers, whereas there was no association for any and inhalant-allergic sensitization or asthma (Table 2.3-2.4). No associations were observed between physician-diagnosed allergy and T cells (Supplementary Table 2.4).

Memory B cells

Children with any atopic conditions compared to no atopic condition had 9.8% (95%CI 3.6; 16.4) higher total and 9.3% (95%CI 2.3;16.7) higher naive B cell numbers. Furthermore, they had 14.2% (95%CI 2.3; 27.5) higher IgA^+CD27^- and 12.4% (95%CI 3.2; 22.4) higher IgA^+CD27^+B cell numbers. No associations were observed for IgE^+ and IgG^+ memory B cells. When studying the individual atopic conditions, children with food-allergic sensitization had 15.2% (95%CI 3.2; 28.7) higher total B cells and 16.2% (95%CI 2.6; 31.5) higher naive B cells compared to children without food-allergic sensitization. Children with 1 SD increase in FEV_1 z-score had 3.9% (95%CI 0.6; 7.4) higher total B cell numbers. However, lung function was not associated with B-cell subsets (Table 2.2-2.4). Numbers of $CD27^+$ and $CD27^-$ IgG^+, IgA^+ and IgE^+ memory B cells were not associated with any or inhalant-allergic sensitization, atopic dermatitis or asthma. Food-allergic sensitization was associated with 22.5% (95%CI 3.9; 44.3) higher $CD27^+IgA^+$ B cell numbers but not with $CD27^+IgE^+$, $CD27^-IgE^+$, $CD27^+IgG^+$, $CD27^-IgG^+$ or $CD27^-IgA^+$ B cells (Table 2.3). To further analyze the immune cells in children with asthma, we stratified children with information on asthma diagnoses by inhalant-allergic sensitization. However, no differences were observed (data not shown). No associations were observed for physician-diagnosed allergy and B cells (Supplementary Table 2.4).

Memory T-cell subsets and atopic diseases

Children with atopic dermatitis had 5.8% (95%CI 0.1; 11.7) higher $CD4^+$ total and 11.4% (95%CI 1.1; 22.8) higher $CD4^+$ Tcm cell numbers. Children with any allergic sensitization had 10.9% (95%CI 1.5; 21.2) higher $CD4^+$ TemRO cell numbers. This was for inhalant-allergic sensitization 10.2% (95%CI 0.8; 20.5) higher and food-allergic sensitization 22.1% (95%CI 3.7; 43.8) higher. Furthermore, children with food-allergic sensitization had 11.2% (95%CI 0.9; 22.4) higher $CD4^+$ total cell numbers. No associations were observed between $CD8^+$ T-cell subsets and atopic conditions (Supplementary Table 2.2).

Table 2.3. Associations between allergic sensitization, atopic dermatitis, asthma and B- and T-cell subsets.

Exposure / Outcome	Any allergic sensitization	Inhalant-allergic sensitization	Food-allergic sensitization	Atopic dermatitis	Asthma ever	Asthma current
T-cell subsets						
Th1	-2.11 (-13.64;10.97)	-3.28 (-14.75;9.73)	-1.25 (-21.54;24.27)	11.56 (-2.97;28.26)	-16.19 (-31.91;3.15)	-9.40 (-31.19;19.30)
Th2	**14.49 (4.42;25.53)**	**12.52 (2.52;23.49)**	**28.70 (8.77;52.27)**	5.70 (-4.53;17.02)	13.05 (-3.07;31.86)	14.19 (-6.78;39.89)
Treg	5.89 (-0.41;12.58)	5.88 (-0.46;12.63)	**17.2 (4.79;31.07)**	**12.02 (4.78;19.76)**	4.80 (-5.21;15.86)	2.13 (-10.43;16.45)
Treg naive	3.56 (-4.66;12.48)	4.61 (-3.74;13.69)	14.28 (-1.72;32.89)	**11.02 (1.45;21.51)**	1.61 (-11.18;16.25)	1.90 (-14.54;21.52)
Treg memory	**12.27 (4.17;21.00)**	**11.06 (2.98;19.77)**	**23.70 (7.91;41.79)**	**17.17 (7.45;27.78)**	2.30 (-10.31;16.67)	2.68 (-13.46;21.83)
Th2/Th1 ratio	**16.89 (0.48;35.97)**	16.28 (-0.16;35.43)	30.28 (-1.29;71.94)	-5.30 (-20.10;12.23)	**34.84 (4.82;73.46)**	25.97 (-9.78;75.90)
Th17	12.79 (-0.21;27.48)	10.90 (-1.94;25.43)	**26.20 (0.68;58.19)**	**15.00 (0.22;31.97)**	-6.03 (-23.58;15.55)	-5.41 (-28.16;24.55)
B-cell subsets						
B total	4.67 (-1.46;11.18)	3.66 (-2.46;10.17)	**15.24 (3.16;28.74)**	3.29 (-3.47;10.52)	2.01 (-7.80;12.86)	-2.67 (-14.96;11.39)
B naive	5.24 (-1.64;12.61)	4.26 (-2.61;11.63)	**16.15 (2.57;31.54)**	1.46 (-6.02;9.53)	-0.43 (-11.11;11.52)	-5.99 (-19.22;9.40)
IgG^+ $CD27^-$	0.55 (-8.50;10.49)	0.03 (-9.04;9.99)	5.40 (-11.41;25.37)	-1.09 (-11.03;9.94)	-1.89 (-16.48;15.22)	-6.60 (-24.56;15.57)
IgG^+ $CD27^+$	4.21 (-5.80;15.28)	3.34 (-6.65;14.40)	16.98 (-2.91;40.95)	0.26 (-10.62;12.46)	7.14 (-9.82;27.29)	-3.54 (-23.31;21.33)
IgE^+ $CD27^-$	-0.54 (-2.75;1.88)	-0.51 (-2.79;1.86)	2.48 (-1.80;6.94)	-0.60 (-2.92;2.18)	-2.92 (-6.55;-0.84)	-2.18 (-6.99;2.88)
IgE^+ $CD27^+$	1.01 (-2.83;5.00)	0.55 (-3.30;4.55)	6.25 (-1.05;14.10)	-3.64 (-7.69;0.58)	-2.97 (-8.98;3.43)	-2.37 (-10.33;6.28)
IgA^+ $CD27^-$	4.07 (-7.21;16.72)	2.65 (-8.55;15.23)	20.14 (-2.76;48.44)	6.97 (-5.90;21.60)	1.04 (-16.80;22.70)	2.51 (-20.76;32.62)
IgA^+ $CD27^+$	8.29 (-0.95;18.40)	6.69 (-2.48;16.73)	**22.47 (3.91;44.33)**	1.40 (-8.23;12.04)	6.05 (-8.69;23.17)	-3.76 (-21.14;17.46)

Abbreviations: CI, confidence interval; Ig, immunoglobulin; Th, helper T cell; Treg, regulatory T cell. The numbers represent % cell increase or decrease (95%CI) for children with allergic sensitization, atopic dermatitis or asthma versus children without these respective diseases. Numbers are based on multivariable linear regressions and adjusted for the following covariates: sex, gestational age at birth, maternal age, maternal prepregnancy BMI, breastfeeding during the first 4 months, number of children in the household, child ethnicity, family history of atopy or asthma, cesarean section, child BMI at 9 years. The bold values represent the statistically significant ($P < .05$) results.

Table 2.4. Associations between lung function and B- and T-cell subsets.

Outcome / Exposure	FEV_1	FEV_1/FVC	FEF_{75}
T-cell subsets			
Th1	5.50 (-1.28;12.74)	0.03 (-6.37;6.88)	2.76 (-3.92;9.89)
Th2	2.90 (-2.14;8.19)	1.51 (-3.46;6.73)	1.91 (-3.15;7.24)
Treg	**3.73 (0.32;7.26)**	2.39 (-0.95;5.84)	**3.59 (0.14;7.15)**
Treg naive	4.51 (-0.20;9.45)	3.14 (-1.47;7.97)	**4.83 (0.05;9.83)**
Treg memory	1.75 (-2.36;6.03)	1.23 (-2.87;5.52)	0.88 (-3.31;5.25)
Th2/Th1 ratio	-2.49 (-9.96;5.60)	1.46 (-6.30;9.87)	-0.80 (-8.51;7.55)
Th17	4.05 (-2.51;11.05)	3.04 (-3.43;9.94)	4.32 (-2.30;11.38)
B-cell subsets			
B total	**3.93 (0.58;7.40)**	-0.24 (-3.46;3.10)	0.90 (-2.42;4.33)
B naive	3.40 (-0.35;7.29)	-0.27 (-3.88;3.47)	0.66 (-3.05;4.51)
IgG^+ $CD27^-$	1.35 (-3.73;6.70)	0.11 (-4.90;5.38)	1.20 (-3.96;6.63)
IgG^+ $CD27^+$	3.60 (-2.09;9.62)	0.11 (-5.37;5.90)	1.22 (-4.44;7.21)
IgE^+ $CD27^-$	-0.65 (-1.59;0.91)	-0.22 (-2.02;0.47)	-0.26 (-2.00;0.53)
IgE^+ $CD27^+$	-0.04 (-2.93;1.04)	-1.13 (-3.09;0.87)	-1.20 (-3.19;0.84)
IgA^+ $CD27^-$	1.66 (-4.52;8.24)	1.09 (-5.03;7.62)	0.56 (-5.66;7.19)
IgA^+ $CD27^+$	3.27 (-1.69;8.47)	-0.86 (-5.60;4.13)	-0.20 (-5.08;4.94)

Abbreviations: CI, confidence interval; FEF75, Forced expiratory flow at 75% of FVC; FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; Ig, immunoglobulin; Th, helper T cell; Treg, regulatory T cell; SD, standard deviation. The numbers represent % cell increase or decrease (95%CI) per SD increase in lung function. Numbers are based on multivariable linear regressions and adjusted for the following covariates: sex, gestational age at birth, maternal age, maternal prepregnancy BMI, breastfeeding during the first 4 months, number of children in the household, child ethnicity, family history of atopy or asthma, cesarean section, child BMI at 9 years. The bold values represent the statistically significant ($P < .05$) results

DISCUSSION

In this population-based cross-sectional study, we demonstrated that children with any atopic disease had higher Th2, Treg, Treg memory, Th17 and $CD27^-$ and $CD27^+IgA^+$B cell numbers. More specifically, children with allergic sensitization had higher Th2 and Th17 cell numbers compared to children without allergic sensitization. Furthermore, we observed that food- and inhalant-allergic sensitization and atopic dermatitis were associated with higher Treg numbers These associations were mainly explained by higher memory Treg numbers, in the presence of normal naive Treg numbers. In addition, we observed that $CD27^+IgE^+$ and $CD27^-IgE^+$ B cells were similar in children with and without atopic conditions. In contrast, children with food-allergic sensitization had higher numbers of $CD27^+IgA^+$ memory B cells than children without. Finally, children with allergic sensitization or atopic dermatitis had higher $CD4^+$ memory T cell numbers but not $CD8^+$ memory T cell numbers. No differences were observed between ever or current asthma diagnosis and B- and T-cell subsets.

Previous literature and interpretation

We expanded our previous studies on immunological phenotype in atopic children and extended previous literature by studying memory Treg and IgA^+ and IgE^+ B cells in a large population-based cohort with adjustment for confounders. In accordance with previous findings, we observed higher Th2 cell numbers in children with inhalant- and food-allergic sensitization compared to children without these conditions.[4-6] Previous studies showed that this Th2-driven pathogenesis in atopic disease may result in higher Treg cell numbers.[10, 33] On the other hand, Treg activation may skew the Th1/Th2 balance towards Th2.[33, 34] We indeed observed higher memory Treg cell numbers in children with inhalant- and food-allergic sensitization or atopic dermatitis, but not in those with asthma.[35] Interestingly, when studying Tregs divided in naive and memory Tregs, the association was mainly explained by memory Treg cells. The naive and memory Tregs have been shown to be two distinct subsets.[11] Naive Treg cells derive from the thymus, whereas the production of memory Treg cells is induced peripherally.[12] Therefore, higher memory Treg cell numbers in children with allergic sensitization or atopic dermatitis may not be due to an intrinsic T cell abnormality but to peripheral inflammatory responses that result in the induction of Treg differentiation.[12, 36] It has been hypothesized that memory Treg cells are important to reduce pro-inflammatory immune responses in atopic disease by anti-inflammatory cytokine production such as IL-10 and TGF-β.[12, 36, 37] Therefore, higher memory Treg cell numbers in our population may indicate that children with allergic sensitization and atopic dermatitis experience a chronic inflammatory immune response that needs to be suppressed.[33, 38, 39] Numbers of Treg cells were highest in food-allergic sensitization. A possible explanation is the production of the cytokine TGF-β. TGF-β is produced within the gut-associated lymphoid tissues, which are important in the intestinal homeostasis and thus most likely involved in food-allergic sensitization.[10] By the induction of TGF-β, conventional T cells differentiate to peripheral Treg cells.[12] Peripheral Treg cells, also known as memory Treg cells, are important in the recognition of non-self-antigens.[10, 12] We speculate that chronic inflammation as seen in atopic diseases contributes to the production of TGF-β and thereby the increase in peripheral Treg cell numbers.[3, 10] Additionally, peripheral Tregs can be produced in specialized non-thymic niches located in the lungs.[10] This could explain our observation of higher Treg cell numbers in inhalant-allergic sensitization.

In addition to higher Treg cell numbers in children with food-allergic sensitization, we observed higher $CD27^+IgA^+$ memory B cells in this group. $CD27^+IgA^+$ memory B cells develop in germinal center responses following T cell co-stimulation, whereas $CD27^-IgA^+$ cells can be produced independently of T cell responses.[13, 40, 41] TGF-β, is also essential in IgA production by mediating IgA class switch recombination.[42] Secretory IgA is important in maintaining the intestinal function.[43] Previous studies suggest that IgA contributes to the discrimination of commensal bacteria and food allergens from invading pathogens.[13, 43] Our

finding that children with food-allergic sensitization have more IgA^+ memory B cells may suggest that these children have an increased IgA-response compared to children without food-allergic sensitization. In addition, Treg cells have been shown to maintain IgA^+ B cell numbers.[42] This might explain why we specifically observed the association with $CD27^+IgA^+$ memory B cells in food-allergic sensitization.

We observed increased Th17 in food-allergic sensitization and atopic dermatitis but not in asthma. Th17 induces the production of pro-inflammatory cytokines and chemokines causing acute inflammatory circumstances.[44, 45] In addition, Th17 cell differentiation is dependent on TGF-β produced specifically by T cells.[44, 45] The associations for Treg, $CD27^+IgA^+$ memory B cells and Th17 might indicate that production of TGF-β is enhanced in chronic inflammatory processes of allergic disease.[44] Possibly, this may not drive the Th2-mediated inflammation, but represents a compensation mechanism to dampen responses.

The findings in the Th and Treg cell subsets are supported by our sub-analyses on $CD4^+$ and $CD8^+$ memory subsets. We observed higher $CD4^+$Tcm and $CD4^+$TemRO cell numbers in children with inhalant- and food-allergic sensitization. No differences between allergic and non-allergic children were observed in $CD8^+$ T-cell subsets. These findings support our observation that the number of Th2 and Treg cells are higher in children with allergic sensitization compared to children without allergic sensitization because both cell types are characterized by $CD4^+$ surface molecules.[12, 33]

In contrast with previous studies, we did not observe associations between IgE^+ B cells and atopic diseases.[13, 14, 16, 17, 46] Similarly, a small study (n=23) did not observe differences in IgE^+ memory B cells in patients with and without atopic dermatitis.[47] Most likely the difference could be explained by differences in study populations: tertiary referral center versus population-based.[16] Also, in contrast with our study, these previous studies studied current atopic disease instead of ever atopic disease, which probably is inherent to a more severe and persistent atopic condition. This suggests that IgE^+ B cells are more involved in severe atopic disease. Also, the lack of associations could be due to an attenuation of disease activity of atopic dermatitis and asthma ever over time.[3]

We observed higher overall Treg cell numbers in children with higher FEV_1 or FEF_{75}, mainly explained by higher naive Tregs. In children with allergic sensitization or atopic dermatitis we found higher memory Treg cells. As higher memory Treg cells represent chronic inflammation, naive cells have no memory of sensitization to specific antigens.[33, 48, 49] Children with better lung functions are less likely to have asthma,[50, 51] and indeed high naive Tregs suggest that these children had no chronic immune activation compared to children with allergic sensitization and atopic dermatitis.[33] However, we did not observe lower Treg memory cells

in children with better lung function. This might be explained by differences in systemic immune responses, but could also be a chance finding.[6]

Eosinophilic asthma is most likely Th2 and IgE-mediated.[3, 6] However, other types of asthma such as virally induced asthma or neutrophilic asthma have different underlying pathophysiologies.[6, 52] This might dilute any association between asthma and immune parameters. Also, local immune responses in the airways may not be detectable in peripheral blood.[53, 54]

Methodological considerations

A major strength of our study is the detailed, extensive data on determinant and outcome. We used 11-color flow cytometry to obtain detailed information on an extensive set of immune cell numbers. We used skin prick tests and lung function test besides questionnaires to obtain objective data and limit response bias. Furthermore, this study was embedded in a large population-based prospective cohort with detailed data on potential confounders. Previous studies on this topic did not adjust for confounders.[13, 14, 38] The addition of this adjustment in a large cohort is crucial to extend unbiased knowledge on immune cell composition in children with atopic disease. However, the following limitations apply. First, because atopic diseases share a common underlying immunopathophysiology,[3] we had high percentages of concomitant atopic diseases within the individual atopic disease groups studied. We were unable to perform sensitivity analyses in children with one atopic disease because the exclusion of children with multiple atopic diseases would cause a loss of statistical power. Second, the number of current asthma was low in our population. Therefore, we also studied children with asthma ever and determined lung function at the age of 10 years. Consequently, our study is underpowered to test the underlying hypothesis that IgE^+ positive B cells may be a feature of more severe persistent conditions, defined as current atopic disease. Also, the definition of the diagnosis asthma did not limit to IgE-mediated asthma. Therefore, we stratified asthma diagnosis by inhalant-allergic sensitization. This led to low subgroup numbers. Third, for measuring allergic sensitization we selected common allergens in 10-year-old children. Consequently, allergens with low sensitization prevalence at the age of ten years, such as milk and egg, were not included.

Conclusion

Children with any atopic disease have higher Th2, Treg, Treg memory and $CD27^+IgA^+$ memory B-cell numbers compared to children without atopic disease. Specifically, food- or inhalant-allergic sensitization and atopic dermatitis had $CD4^+$ effector memory T cell numbers, explained by a Th2 and Th17-mediated immune response and higher Treg memory cell numbers. Furthermore, children with food-allergic sensitization had higher $CD27^+IgA^+$ memory B cells, suggestive of TGF-β mediated compensation for chronic inflammation. B and T cells did not differ in children with and without asthma.

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SUPPLEMENTARY MATERIALS

Supplementary Table 2.1. Numbers of children included in the individual analyses performed.

Outcome \ Exposure	Any allergic sensitization	Inhalant-allergic sensitization	Food-allergic sensitization	Atopic dermatitis	Asthma ever or current	Lung function
T-cell subsets						
Th1	747	748	746	709	714	661
Th2	746	747	745	708	713	660
Treg	736	737	735	700	706	651
Treg naive	733	734	732	697	703	648
Treg memory	733	734	732	697	703	648
Th2/Th1 ratio	746	747	745	708	713	656
Th17	742	743	741	705	709	656
B-cell subsets						
B total	775	776	774	737	742	689
B naive	768	769	767	729	734	685
IgG^+ $CD27^-$	767	768	766	728	733	684
IgG^+ $CD27^+$	767	768	766	728	733	684
IgE^+ $CD27^-$	767	768	766	728	733	684
IgE^+ $CD27^+$	767	768	766	728	733	684
IgA^+ $CD27^-$	767	768	766	728	733	684
IgA^+ $CD27^+$	767	768	766	728	733	684

Abbreviations: Ig, immunoglobulin; Th, helper T cell; Treg, regulatory T cell. The numbers represent the number of children included in the individual analyses. The analyses are based on a total number of 855 different children.

Supplementary Table 2.2. Associations between allergic sensitization, atopic dermatitis and asthma and memory T cell subsets.

Exposure / Outcome	Any allergic sensitization	Inhalant-allergic ensitization	Food-allergic sensitization	Atopic dermatitis	Asthma ever	Asthma current
$CD4^+$	2.58 (-2.69;8.13)	1.80 (-3.47;7.37)	**11.16 (0.93;22.42)**	**5.77 (0.11;11.74)**	1.38 (-6.63;10.08)	-4.89 (-14.69;6.04)
$CD4^+$ naive	1.66 (-4.82;8.58)	0.91 (-5.58;7.85)	11.55 (-1.16;25.88)	7.01 (-0.25;14.80)	-0.73 (-10.70;10.36)	-7.98 (-19.99;5.85)
$CD4^+$ Tcm	3.06 (-5.67;12.59)	1.29 (-7.34;10.73)	4.77 (-11.00;23.34)	**11.39 (1.07;22.77)**	1.35 (-12.53;17.44)	-6.81 (-23.26;13.16)
$CD4^+$ TemRO	**10.89 (1.49;21.15)**	**10.21 (0.81;20.48)**	**22.12 (3.74;43.75)**	6.19 (-3.69;17.07)	10.38 (-4.98;28.22)	8.42 (-10.91;31.95)
$CD4^+$ TemRA	16.12 (-1.56;36.99)	15.91 (-1.85;36.88)	30.84 (-3.51;77.41)	0.23 (-16.74;20.66)	9.35 (-17.34;44.67)	3.91 (-28.00;49.96)
$CD8^+$	2.41 (-3.53; 8.72)	2.17 (-3.80;8.51)	4.67 (-6.19;16.80)	-0.12 (-6.34;6.51)	5.05 (-4.57;15.63)	1.40 (-10.68;15.12)
$CD8^+$ naive	5.52 (-2.32;13.98)	4.42 (-3.38;12.86)	10.49 (-4.13;27.34)	2.13 (-6.18;11.18)	6.40 (-6.40;20.96)	4.03 (-12.10;23.11)
$CD8^+$ Tcm	2.07 (-11.48;17.69)	0.98 (-12.51;16.54)	-10.40 (-31.06;16.47)	1.14 (-13.51;18.29)	-0.94 (-21.77;25.45)	1.31 (-25.67;38.08)
$CD8^+$ TemRO	1.28 (-8.10;11.62)	1.52 (-7.94;11.95)	4.36 (-12.74;24.82)	-0.49 (-10.35;10.46)	2.64 (-12.45;20.34)	-8.02 (-25.33;13.30)
$CD8^+$ TemRA	-3.28 (-13.57;8.23)	-2.84 (-13.25;8.82)	11.69 (-9.16;37.34)	-4.85 (-16.08;7.88)	15.28 (-4.90;39.74)	16.82 (-9.04;50.02)

Abbreviations: CI, confidence interval; Tcm, central memory T cell; Tem, effector memory T cell. The numbers represent % cell increase or decrease (95%CI) per SD increase in lung function or for the children with asthma compared to children without asthma. Numbers are based on multivariable linear regressions and adjusted for the following covariates: sex, gestational age at birth, maternal age, maternal prepregnancy BMI, breastfeeding during the first 4 months, number of children in the household, child ethnicity, family history of atopy or asthma, cesarean section, child BMI at 9 years. The bold values represent the statistically significant ($P < .05$) results

Supplementary Table 2.3. Associations between atopic dermatitis and immune cells stratified for allergic sensitization and food-allergic sensitization.

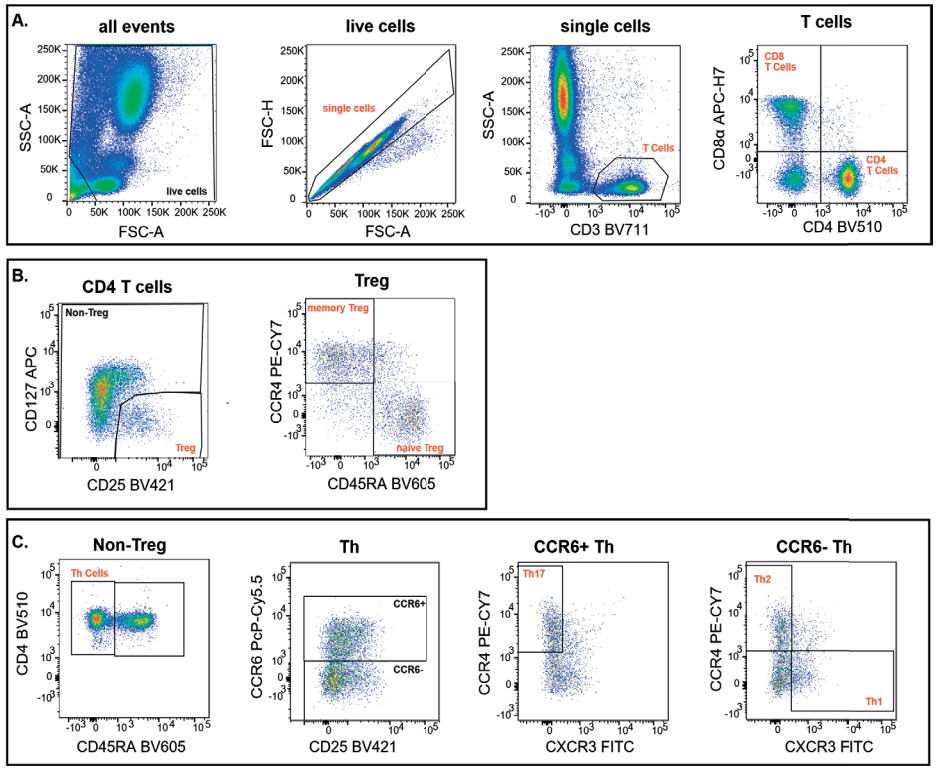
Exposure / Outcome	With allergic sensitization (n_{total}=198, $n_{atopicdermatitis}$= 70)	Without allergic sensitization (n_{total}=469, $n_{atopicdermatitis}$= 70)	With food-allergic sensitization (n_{total}=43, $n_{atopicdermatitis}$= 20)	Without food-allergic sensitization (n_{total}=623, $n_{atopicdermatitis}$= 119)
T cell-subsets				
Th1	19.17 (-7.05; 52.78)	8.74 (-11.44; 33.53)	26.44 (-34.21; 143.01)	12.73 (-4.0; 32.38)
Th2	-0.45 (-15.91; 17.85)	4.78 (-10.04; 22.05)	-23.31 (-47.50; 12.01)	5.97 (-5.87; 19.29)
Treg	**19.08 (6.88; 32.68)**	4.41 (-5.45 ;15.30)	20.09 (-5.11; 51.97)	10.70 (2.55; 19.50)
Treg naive	**27.92 (11.00; 47.41)**	-0.08 (-12.86; 14.58)	35.51 (-3.90; 91.09)	9.56 (-1.26; 21.57)
Treg memory	10.32 (-4.14; 26.96)	**16.07 (2.37; 31.60)**	2.05 (-22.45; 34.29)	**16.86 (5.97; 28.87)**
Th2/Th1 ratio	-16.47 (-38.62; 13.69)	-3.71 (-24.76; 23.23)	-39.35 (-74.41; 43.76)	-6.04 (-22.62; 14.08)
Th17	-1.10 (-19.42; 21.40)	24.00 (-0.45; 54.46)	112.44 (-11.18; 408.12)	16.78 (-0.91; 37.64)
B cell-subsets				
B total	**-8.11 (-18.79; 3.98)**	**11.60 (1.25; 23.01)**	**43.80 (8.98; 89.74)**	3.82 (-3.95; 12.22)
B naive	-11.28 (-22.71; 1.84)	**11.85 (0.01; 25.09)**	**52.99 (7.82; 117.09)**	3.02 (-5.68; 12.53)
IgG^+ $CD27^-$	**-17.90 (-31.75; -1.25)**	6.16 (-8.87; 23.67)	-23.04 (-48.82; 15.74)	-1.22 (-12.42; 11.41)
IgG^+ $CD27^+$	-14.71 (-31.33; 5.92)	-1.21 (-15.90; 16.04)	-6.27 (-39.08; 44.20)	-4.12 (-16.00; 9.45)
IgE^+ $CD27^-$	-0.70 (-5.05; 3.85)	1.82 (-1.88; 5.66)	0.84 (-11.45;14.83)	0.12 (-2.71; 3.04)
IgE^+ $CD27^+$	-5.60 (-12.82; 2.21)	-0.71 (-6.59; 5.54)	10.37 (-11.24; 37.23)	-4.35 (-8.85; 0.37)
IgA^+ $CD27^-$	-17.78 (-35.31; 4.49)	17.64 (-1.80; 40.92)	-9.08 (-46.43; 54.33)	4.26 (-9.93; 20.67)
IgA^+ $CD27^+$	-12.84 (-26.80; 3.77)	0.61 (-13.02; 16.38)	11.17 (-27.54; 70.55)	-2.56 (-13.04; 9.19)

Abbreviations: CI, confidence interval; Ig, immunoglobulin; n, number; Th, helper T cell; Treg, regulatory T cell. The numbers represent % cell increase or decrease (95%CI) for children with, atopic dermatitis compared to children without atopic dermatitis stratified for (food) allergic sensitization. Numbers are based on multivariable linear regressions and adjusted for the following covariates: sex, gestational age at birth, maternal age, maternal prepregnancy BMI, breastfeeding during the first 4 months, number of children in the household, child ethnicity, family history of atopy or asthma, cesarean section, child BMI at 9 years. The bold values represent the statistically significant (P < .05) results

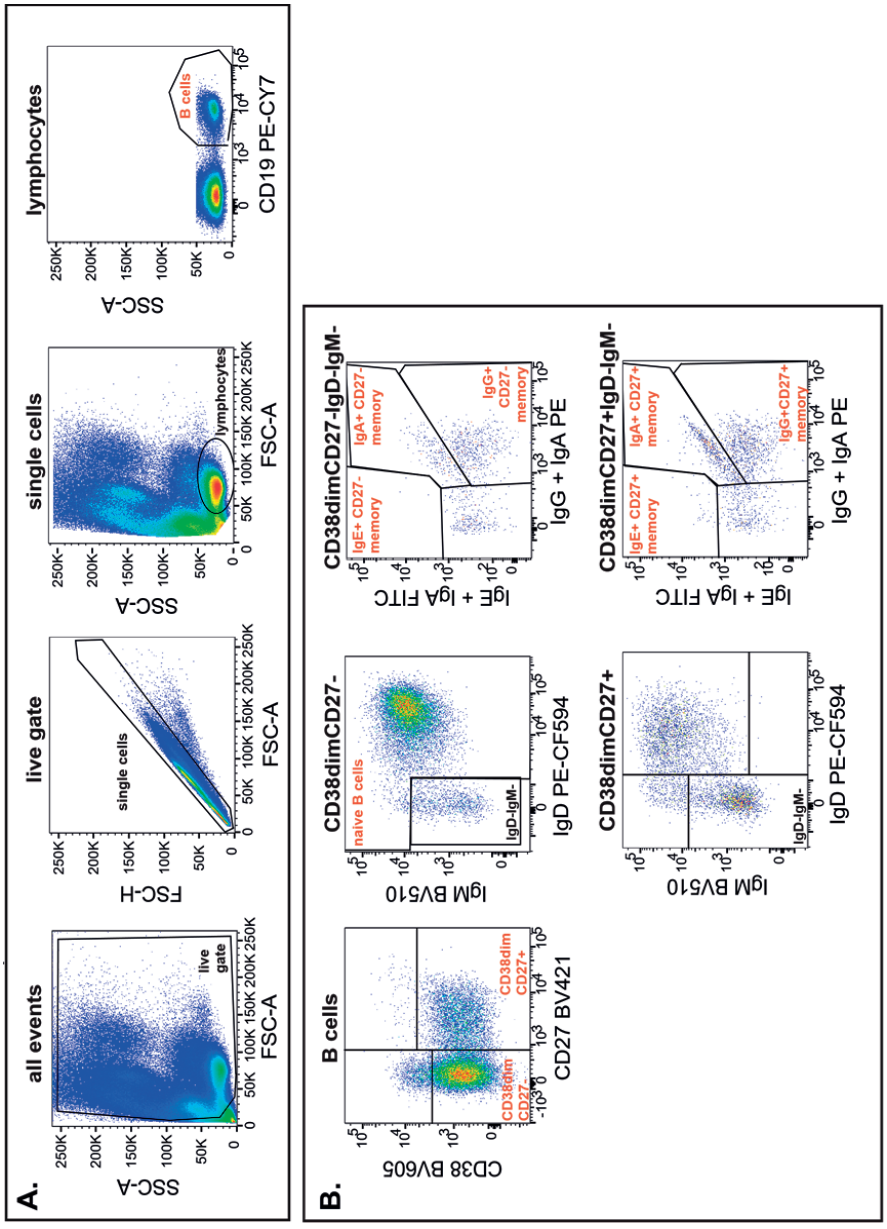
Supplementary Table 2.4. Associations between physician-diagnosed allergy and B- and T-cell subsets.

Exposure / Outcome	**Physician-diagnosed allergy (yes, n=79; no, n=649)**
T-cell subsets	
Th1	13.13 (-6.46 ;36.82)
Th2	12.54 (-2.22; 29.53)
Treg	7.89 (-1.45; 18.12)
Treg naive	5.87 (-6.30; 19.62)
Treg memory	10.73 (-1.58; 24.57)
Th2/Th1 ratio	-0.56 (-21.07; 25.28)
Th17	2.66 (-14.94; 23.90)
B-cell subsets	
B total	**10.45 (0.82; 21.01)**
B naive	10.02 (-0.68; 21.88)
IgG^+ $CD27^-$	0.65 (-12.84; 16.20)
IgG^+ $CD27^+$	10.99 (-4.94; 29.60)
IgE^+ $CD27^-$	-2.84 (-6.08; 0.52)
IgE^+ $CD27^+$	-3.94 (-9.29; 1.73)
IgA^+ $CD27^-$	-2.20 (-17.86; 16.44)
IgA^+ $CD27^+$	12.83 (-1.44; 29.16)

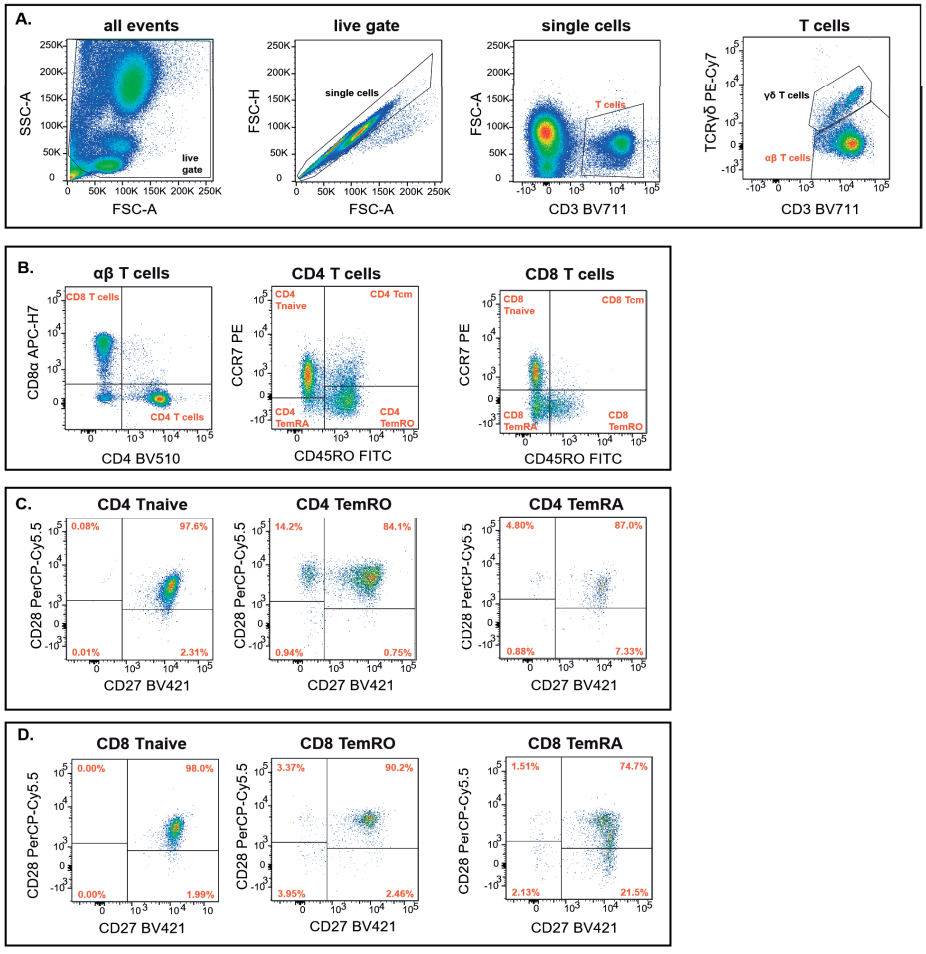
Abbreviations: CI, confidence interval; Ig, immunoglobulin; Th, helper T cell; Treg, regulatory T cell. The numbers represent % cell increase or decrease (95%CI) for children with physician-diagnosed allergy versus children without allergy diagnosis. Numbers are based on multivariable linear regressions and adjusted for the following covariates: sex, gestational age at birth, maternal age, maternal prepregnancy BMI, breastfeeding during the first 4 months, number of children in the household, child ethnicity, family history of atopy or asthma, cesarean section, child BMI at 9 years. The bold values represent the statistically significant ($P < .05$) results



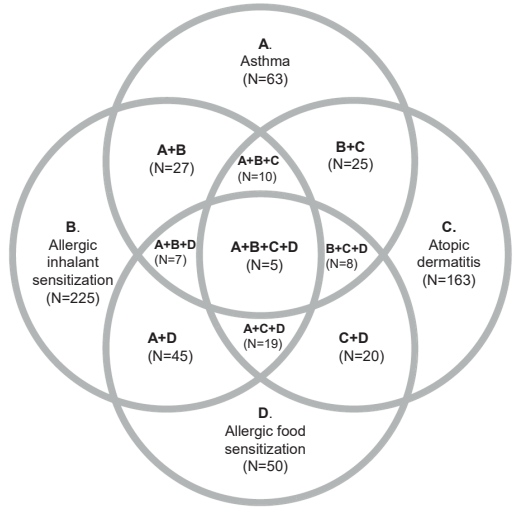
Supplementary Figure 2.1. Gating strategy of T helper subsets.



Supplementary Figure 2.2. Gating strategy B cell subsets.



Supplementary Figure 2.3. Gating strategy naive and memory T cells.



Supplementary Figure 2.4. Overview of concomitant atopic disease in the study population.

3 Associations between T cells and attention problems in the general pediatric population.

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ABSTRACT

Objective

The pathogenesis of attention-deficit/hyperactivity disorder (ADHD) is currently unclear. We hypothesized that chronic immune activation, as indexed by T and B cells, plays a role in the pathophysiology of attention problems. Therefore, we examined T- and B-cell subsets in a general pediatric population with information on attention problems.

Methods

We included 756 ten-year-old children from the Generation R population-based cohort. Eleven-color flow cytometry was performed on peripheral blood samples to determine T- and B-cell subsets. The Child Behavior Checklist rated by parents was used to measure attention problems. Data were analyzed using linear regression analyses, adjusting for maternal and child covariates and co-occurring childhood psychopathology.

Results

For T helper 1 (Th1) cells, 1 standard deviation (SD) increase was associated with 5.3% (95%CI 0.3;10.5) higher attention problem scores. Furthermore, 1SD increase in $CD8^+$ T cells was associated with 7.5% (95%CI 2.4; 12.7) higher attention problem scores. Within total $CD8^+$ T cells, 1SD increase in naive or central memory cells was associated with 6.9% (95%CI 2.0; 12.1) and 6.4% (95%CI 1.5; 11.6) higher attention problem scores, respectively. No associations between Th2, Treg or B memory cells and attention problem scores were observed.

Conclusion

Higher Th1 and cytotoxic T cell numbers are associated with higher attention problem scores independent of co-occurring psychopathology. This might indicate a possible role of a pro-inflammatory immune profile in childhood attention problems.

INTRODUCTION

Attention-deficit/hyperactivity disorder (ADHD) is the most prevalent child behavioral disorder with a prevalence of 5% and an estimated yearly burden of 491,500 disability adjusted life years globally.[1, 2] ADHD is characterized by hyperactivity, impulsivity and inattention inappropriate for the child's age.[1] The clinical diagnosis of ADHD is based on the *Diagnostic and Statistical Manual of Mental Disorders*–5 Task Force criteria in which children should have minimally 6 of the formulated ADHD criteria before the age of 12 years old.[3]

The pathophysiology of ADHD is complex and suggestive of an interplay between genetic and environmental factors that impact neurobiological processes.[4] Growing evidence points to a role of immunological processes as possible additional underlying biological mechanism in the pathophysiology of ADHD.[5] Currently, the evidence for the contribution of immunological processes in the pathophysiology of ADHD can be divided in three categories: 1) genetic studies, 2) studies on associations between ADHD and immune-mediated diseases 3) cytokine studies.[5] Namely, susceptibility genes for autoimmune disease, such as human leukocyte antigen (HLA)-DR4, HLA-DRB and complement C4B, have been associated with ADHD.[6] Previous meta-analyses have linked ADHD to both autoimmune and atopic diseases including atopic dermatitis, asthma and allergies.[7, 8] Finally, some studies have found that children with attention problems have increased levels of pro-inflammatory cytokines such as interleukin (IL)-2, IL-5, IL-6, interferon-gamma (IFN-ɣ), and tumor necrosis factor (TNF)-α, suggesting a pro-inflammatory state.[4, 9, 10] However, this observation has not been confirmed by all studies [11], and in some cases the opposite direction of associations has been identified. For example, Yu et al. (2020) found that higher IL-8 was positively associated with children's ability to control or regulate attention, emotion, and behavior. Correlations between a pro-inflammatory state and onset of attention problems form the basis of the hypothesis that there could be a causal relationship between these.[4] The brain is connected to the peripheral immune system by the lymphatic system.[12, 13] Previously, both $CD4^+$ helper and $CD8^+$ cytotoxic T cells have been shown to be involved in brain development and functioning.[12] $CD4^+$ T cells are thought to contribute to memory development, whereas infiltration of $CD8^+$ T cells in the central nervous system disturbs homeostasis in microglial and neuronal activity.[12, 14] Such infiltration can occur in a chronic inflammatory state such as in auto-immune and atopic diseases.[15] $CD4^+$ and $CD8^+$ T cells belong together with B cells to the adaptive immunity.[16] $CD4^+$ and $CD8^+$ T cells develop from naive to central memory and effector memory cells.[16, 17] $CD4^+$ memory cells include Th1, which are involved in autoimmunity, and Th2 and Th17, which are involved in allergic disease.[16] B and T cells are closely linked to each other as described in more extent previously.[16]

Despite the reproducible finding of an association between chronic immune disease and attention problems, insight into the immunological basis is limited. Specifically, immune studies thus far have been restricted to cytokine analyses in small preselected subsamples and do not include T and B cell numbers in the general pediatric population. Furthermore, previous studies did not account for various confounding factors during the development of the child that might affect the association between immunological mechanisms and attention problems. Finally, co-occurring psychopathology has not been considered and therefore it is currently unclear whether associations are unique to attention problems or more broadly related to mental health problems.

More insight into the potential neuro-immunological involvement in attention problems could inform future research directions, the understanding of the pathogenesis of attention problems and the development of therapeutic options. Therefore, we here examined the association between B- and T-cell subsets and attention problem scores dimensionally, based on data from over 700 10-year-old children from the general population, with adjustment for confounders and consideration of effect modification by sex.

METHODS

Design and ethical considerations

The study is part of the Generation R Study, a population-based prospective birth cohort study located in Rotterdam, the Netherlands.[18] The Medical Ethical Committee of the Erasmus University Medical Center in Rotterdam approved the study (MEC-2012-165). Written informed consent from legal representatives was obtained from all participants. At child's age of 10 years, data collection was performed in 7,393 children. 5,862 children visited the research center (response rate 79%), of which 4,593 children provided blood samples. In 950 children detailed immune phenotyping was performed. The sample number for detailed phenotyping was restricted as a result of a maximum of 3 samples that could be processed in the laboratory per day. For the CBCL, mothers of 5,398 children answered the questionnaire at 10 years (response rate 73%).[18] For the present study, we selected all children with information on at least one of the measured immune cells and with information on the attention problem score by means of the Child Behavior Checklist (CBCL/6-18). This resulted in a number of 756 children for the analyses (Figure 3.1).

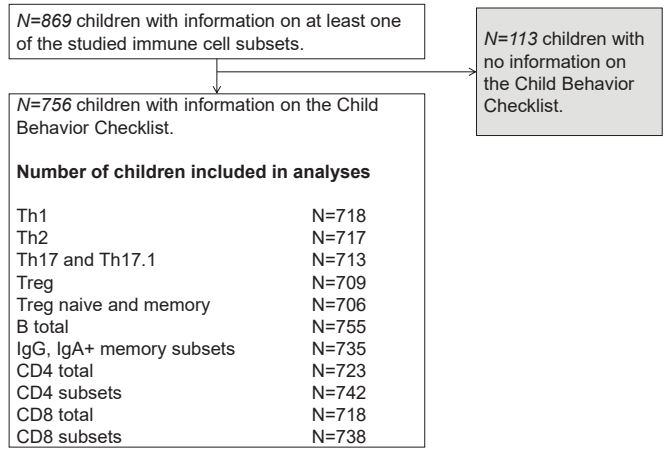

Figure 3.1. Flowchart of participants included in the study.

Assessment of immune cells

Peripheral blood samples were obtained at a median age of 9.8 [25-75% range 9.6; 9.9]. First, a diagnostic lyse-no-wash protocol (BD Biosciences, San Jose, CA) was used to obtain absolute counts of peripheral blood B and T cells from peripheral blood samples. Next, 11-color immunophenotyping was performed on the 4-laser LSR Fortessa (BD Biosciences).[19, 20] Within the total $CD19^+$ B cells, we obtained the following subsets of $CD19^+CD21^+D38^{dim}IgD^-$ memory B cells: $CD27^+IgA^+$ and $CD27^-IgA^+$.[21] In addition, transitional B cells ($CD19^+CD27^-CD38^{high}$) and CD21low B cells ($CD19^+CD21^{low}CD38^{dim}CD27^-$) were determined. Within the total $CD3^+$ T cells, we obtained $CD4^+$ and $CD8^+$ lineages defined as the following subsets: naive ($CD45RO^-CCR7^+$), central memory T cells (Tcm;$CD4RO^+CCR7^+$), effector memory RO-positive T cells (TemRO;$CD45RO^+CCR7^-$) and effector memory RA-positive cells (TemRA; $CD45RO^-CCR7^-$). We determined the naive ($CD45RA^+$) and memory ($CD45RA^-$) subsets of $CD4^+CD127^-CD25^+$ regulatory T (Treg) cells. After we excluded the Treg cells, we determined T helper (Th) cell subsets within $CD4^+CD45RA^-$ cells: Th1 $CCR6^-CXCR3^+CCR4^-$, Th2 $CCR6^-CXCR3^-CCR4^+$ and Th17 $CCR6^+CXCR3^-CCR4^+$. We analyzed the data with FacsDIVA software v8 (BD Biosciences), Infinicyt software (Cytognos, Salamanca, Spain) and FlowJo software v10 (FlowJo LLC, Ashland, Oregon). Cell numbers were presented per µL in blood.[20, 22]

Assessment of attention problem scores

Attention problems were assessed at a median age of 9.8 [25-75% range 9.6; 9.9] by means of parent-rated CBCL/6-18, a questionnaire aimed for children between 6 and 18 years. The CBCL/6-18 is a well-validated standard measure of behavioral problems in children.[23-25] The questionnaire consists of 99 behavioral problem items that can be rated on a three-point

scale: 0 (not true), 1 (somewhat or sometimes true), 2 (very or often true). We used the CBCL attention score that is widely used in epidemiological studies.[23-25] The following 10 times are included in the CBCL attention problem scale: "acts too young for his/her age", "fails to finish things", "cannot concentrate and cannot pay attention for long", "cannot sit still, restless or hyperactive", "confused or seems to be in a daze", "daydreams or gets lost in his/her thoughts", "impulsive or acts without thinking", "poor school work", "inattentive or easily distracted", "stares blankly". The internal consistency of the CBCL attention score in this study is 0.80.

Covariates

We obtained information on child's sex, gestational age and birthweight from midwife and hospital registries.[26] Child ethnicity was determined based on birth country of parents and categorized as Western and non-Western, as previously described.[27] Child's length and weight were determined at our research center at 10 years and BMI was calculated. Information on maternal age at enrollment, prepregnancy body mass index, smoking during pregnancy, and highest maternal education was derived from parental questionnaires.[22, 23, 25, 27] The Brief Symptom Inventory (BSI) was used to measure maternal psychiatric symptoms during pregnancy at a child's gestational age of 20-25 weeks. The BSI is a self-reported questionnaire with high validity and reliability to measure the psychological state of an individual in the preceding 7 days.[28-30] The total score of the BSI was calculated to create the General Symptom Index (GSI), a subscale used to measure maternal psychological symptoms during pregnancy. A total problem scale was created including all 99 items from the CBCL-6/18, with the exception of the 10 items measuring attention problems to assess other co-occurring mental health problems.

Statistical Analyses

Because of the presence of the value zero in the attention scores and to approximate a normal distribution of the residuals of our models, we used a LN+1 transformation on the attention scores (outcome)(Supplementary Figure 3.1). Cell numbers (determinant) were converted to z-scores for easier interpretation. Multivariable linear regression analyses were used to determine associations between individual immune cell numbers and attention problem scores. Confounder selection was based on previous studies.[22] Confounders were added to the regression model if addition of the confounder resulted in at least 10% change in the beta of the independent variable.[31, 32] The following covariates were added to the model: child's sex, birth weight, gestational age, child's BMI at 10 years, ethnicity and maternal age at enrollment, highest maternal education, maternal prepregnancy BMI and maternal smoking during pregnancy. To establish whether identified associations are independent of co-occurring child neurodevelopmental problems we adjusted our models for this total score. Next, we tested possible effect modification by sex by adding an interaction term

of sex with cell numbers.[30, 33, 34] The percentage of missing data in the covariates ranged from 0.0 to 4.1% with exception for maternal smoking during pregnancy (12.7%), GSI score during pregnancy (15.6%) and maternal prepregnancy BMI (23.1%). We performed multiple imputation by chained equations (n=20) to minimalize potential bias due to missing information on covariates (R package "Mice version 3.6.0"). Determinants and outcome were not imputed. A wide range of predictors, including variables that were not included as confounders, were used as is generally recommended in case of multiple imputation procedures.[35] To check the process of multiple imputation, characteristics of the imputed and non-imputed dataset were compared and univariate analyses, multivariate analyses before and after imputation were compared. Because of the natural log transformation for our outcome, pooled regression coefficients were back transformed and can be interpreted as % increase or decrease in attention problem score per 1SD increase in cell number/μL blood. Non-linearity of the observed associations was considered and tested by adding splines but was not present. No adjustment for multiple testing was performed because of strong correlations between immune cell numbers, leading to dependent tests (Supplementary Figure 3.2) .[36, 37] Correlations between covariates are presented in Supplementary Figure 3.2. A two-tailed p-value of <0.05 was considered significant. Statistical analyses were performed using SPSS version 25.0 for Windows (IBM Corp., Armonk, NY, USA) and R version 3.6.1.

RESULTS

Study population characteristics

Characteristics of the study population are presented in Table 3.1. Of the 756 children included, 404 were girls. Highest maternal education was classified as higher in 62.5%, as secondary in 34.5% and as primary in 3.0%. Median GSI during pregnancy was 6.0 (25-75% range 3.0;12.0). Median total CBCL problem score was 9.0 (25-75% range 4.0;18.7). Characteristics of the study population were comparable in the imputed dataset and overall effect estimates were comparable before and after multiple imputation (Supplementary Table 3.1-3.3). Non-responder analyses showed that included children had a higher socioeconomic status, were more often Western, had higher birth weight, had less often mothers that smoked and had mothers with a lower GSI (Supplementary Table 3.2). Median cell numbers are presented in Table 3.2.

Table 3.1. Characteristics of the study population.

Child characteristics (n=756)	General group	Missing in study population (%)
Sex (N, %)		0.0
Boy	352 (46.6)	
Girl	404 (53.4)	
Gestational age, weeks	40.1 [39.3;41.0]	0.7
Birth weight, grams	3510.0 [3155.0;3827.5]	0.1
BMI, kg/m^2	17.0 [15.7;18.4]	0.0
Ethnicity (N, %)		1.3
Western	649 (87.0)	
Non-western	97 (13.0)	
Attention score CBCL	2.0 [1.0;5.0]	0.0
Maternal characteristics		
Education (N, %)		4.1
Primary	22 (3.0)	
Secondary	251 (34.5)	
Higher	455 (62.5)	
Prepregnancy BMI, kg/m^2	22.6 [20.8;24.9]	23.1
GSI during pregnancy	0.12 [0.06;0.2]	15.6
Smoking during pregnancy		12.7
Never	511 (77.4)	
Until pregnancy was known	74 (11.2)	
Continued during pregnancy	75 (11.4)	

Abbreviations: BMI, body mass index; CBCL, Child Behavior Checklist; g, grams; GSI, global severity index; N, number; wk, weeks.

Table 3.1 represents the child and maternal characteristics for the study population in the non-imputed dataset and are represented as number (%) or median [25-75% range].

Table 3.2. Median cell numbers in the study population.

T cells	Median cell number per microliter blood (Interquartile range)
$CD4^+$	
$CD4^+$total	1077.8 [880.9;1288.4]
$CD4^+$naive	679.2 [533.6;869.8]
$CD4^+$Tcm	168.6 [110.8;234.9]
$CD4^+$TemRO	16.9 [7.8;37.0]
$CD4^+$TemRA	131.0 [87.1;202.9]
Th1	31.0 [18.8;46.3]
Th2	54.7 [38.3;79.5]
Th17	30.9 [19.8;43.2]
Th17.1	11.9 [5.6;20.0]
Treg	78.0 [59.8;98.1]
Treg naive	45.6 [32.7;61.9]
Treg memory	29.4 [22.5;38.7]
$CD8^+$	
$CD8^+$total	594.1 [483.4;747.5]
$CD8^+$naive	319.0 [240.7;438.8]
$CD8^+$Tcm	22.9 [11.5;37.5]
$CD8^+$TemRO	79.2 [50.7;115.7]
$CD8^+$TemRA	85.1 [49.6;138.5]
B cells	
B total	482.0 [374.0;625.9]
B naive	304.8 [231.9;416.2]
IgG^+CD27^-	4.3 [2.4;7.2]
IgG^+CD27^+	15.7 [9.8;23.4]
IgA^+CD27^-	2.0 [1.2;3.2]
IgA^+CD27^+	11.3 [7.9;15.9]
Transitional	49.1 [31.0;70.5]
$CD21^{low}$	9.7 [6.4;14.1]

Abbreviations: Tcm, central memory T cells; TemRA, effector memory RA-positive T cells; TemRO, effector memory RO-positive T cells. Th, helper T cell; Treg, regulatory T cell, Ig, immunoglobulin.

Th and Treg cell subsets and attention problems

Children with 1SD higher Th1 cell numbers had 5.3% (95%CI 0.3;10.5) higher attention problem scores (Table 3.3). No associations were observed between attention problem score and Treg cell numbers. No associations were observed between either Th2, Th17, naive Treg or memory Treg cells and attention problem scores (Table 3.3).

Table 3.3. Associations between T helper and T regulatory cells and attention problems.

	Attention score CBCL		
T cells	***β***	**95%CI**	**P-value**
Th1	5.28	(0.33;10.49)	0.037
Th2	4.49	(-0.55;9.77)	0.08
Th17	-0.049	(-5.21;4.47)	0.84
Th17.1	2.14	(-2.17;7.24)	0.39
Treg	3.98	(-0.96;9.16)	0.12
Treg naive	2.75	(-2.15;7.89)	0.28
Treg memory	4.36	(-0.67; 9.65)	0.09
B cells			
B total	0.68	(-3.98;5.57)	0.78
B naive	1.67	(-3.09;6.66)	0.50
IgG^+CD27^-	-1.17	(-5.89;3.78)	0.64
IgG^+CD27^+	-2.32	(-6.88;2.47)	0.34
IgA^+CD27^-	-1.68	(-6.37;3.25)	0.50
IgA^+CD27^+	-1.86	(-6.43;2.93)	0.44
Transitional	-0.63	(-5.23;4.18)	0.79
$CD21^{low}$	-1.83	(-6.47;3.04)	0.45

Abbreviations: CI, confidence interval; CBCL, Child Behavior Checklist; Th, helper T cell; Treg, regulatory T cell, Ig, immunoglobulin.
Betas (*βs)* represent % attention problem score increase or decrease per 1SD increase in cell number/uL blood. Associations are based on multivariable linear regression analyses performed within the imputed dataset and adjusted for child (sex, gestational age, birth weight, BMI, ethnicity, highest maternal education, total problem score) and maternal (prepregnancy BMI, maternal smoking during pregnancy and maternal global severity index during pregnancy) covariates.

T effector memory cells and attention problems

1SD increase in total $CD8^+$ cell numbers was associated with 7.5% (95%CI 2.4;12.7) higher attention problem scores (Table 3.4). This increase was mainly explained by an increase in $CD8^+$ naive and Tcm cell numbers. Namely, 1SD increase in $CD8^+$naive cells was associated with 6.9% (95%CI 2.0;12.1) higher attention problem scores. 1SD increase in $CD8^+$Tcm was associated with 6.4% (95%CI 1.5;11.6) higher attention scores. Across $CD8^+$ T cells no associations were observed with $CD8^+$TemRO or $CD8^+$TemRA cell numbers and attention problem scores. Within $CD4^+$ T cell numbers, a positive association was observed for $CD4^+$Tcm cell numbers and attention problems (5.0% (95%CI 0.2;10.1)). No associations were observed with other $CD4^+$-subset cell numbers (Table 3.4).

B-cell subsets and attention problems

No associations were observed between total or naive B cells and attention problems. No associations were observed for $CD27^+$ or $CD27^-$ IgG^+ and IgA^+ memory B cells, CD21low or transitional B cells and attention problem scores (Table 3.3).

Table 3.4. Associations between T effector memory cells and attention problems.

	Attention score CBCL		
	β	95%CI	P-value
CD4$^+$			
CD4$^+$total	4.34	(-0.60;9.53)	0.09
CD4$^+$naive	2.67	(-2.09;7.65)	0.28
CD4$^+$Tcm	4.99	(0.16;10.06)	0.04
CD4$^+$TemRO	0.78	(-4.01;5.80)	0.76
CD4$^+$TemRA	-1.49	(-6.04;3.28)	0.53
CD8$^+$			
CD8$^+$total	7.45	(2.42;12.72)	0.003
CD8$^+$naive	6.90	(1.96;12.07)	0.006
CD8$^+$Tcm	6.41	(1.51;11.55)	0.01
CD8$^+$TemRO	1.34	(-3.42;6.33)	0.59
CD8$^+$TemRA	-0.79	(-5.48;4.15)	0.75

Abbreviations: CBCL, Child Behavior Checklist; CI, confidence interval; Tcm, central memory T cells; TemRA, effector memory RA-positive T cells; TemRO, effector memory RO-positive T cells.
Betas (*βs)* represent % attention problem score increase or decrease per 1SD increase in cell number/uL blood. Associations are based on multivariable linear regression analyses performed within the imputed dataset and adjusted for child (sex, gestational age, birth weight, BMI, ethnicity, highest maternal education, total problem score) and maternal (prepregnancy BMI, maternal smoking during pregnancy and maternal global severity index during pregnancy) covariates.

Subgroup and sensitivity analyses

Statistical interaction by sex was only observed for CD8$^+$ TemRO cell numbers. When the analyses were stratified for sex, the association was slightly stronger in boys than in girls. Boys had per 1SD increase in CD8$^+$ TemRO cell numbers 8.2% (95%CI 1.0;16.0) higher attention problem scores. (Supplementary Table 3.4). Results did not change when adjusting for depressive symptoms instead of adjustment for total neurodevelopmental problem score (Supplementary Table 3.5).

DISCUSSION

This population-based cohort observed that higher numbers of Th1 and CD8$^+$, CD8$^+$ naive and CD8$^+$ Tcm cells were associated with higher attention problem scores independent of co-occurring child neurodevelopmental problems. No associations were observed between Th2, Th17 and CD4$^+$ effector memory subsets and attention problem scores. No associations were observed between memory B cells and attention problem scores.

Our study contributed to literature by the determination of associations between B and T cell numbers and attention problems with adjustment for important possible confounders.

The possibility of an altered adaptive immune cell composition has been suggested previously but not yet been studied.[38] The availability of data from a large-scale population-based cohort of children was a unique setting to determine this. Previous studies on associations between attention problems and the immune system are highly heterogeneous in study population and study design. However, a systematic review reported associations between immunological inflammation and attention problems.[4] We here extended previous literature by studying peripheral circulating immune cells in relation to attention problems in children from a population-based birth cohort study. Because of the high heterogeneity in studies on immunological markers and attention problems, direct comparison of our results to previous literature is not straightforward. However, in accordance with previous evidence pointing towards a pro-inflammatory mediated immune system, we observed higher Th1 cells in children with higher attention problem scores.[4, 39] No studies on T and B cells in attention problems have been reported previously. Yet, IL-1β, IL-2, IFN-ɣ and TNF-α, pro-inflammatory cytokines produced by Th1 cells as well as other immune cells such as other lymphocytes and macrophages, have been linked to ADHD or ADHD symptoms in previous studies.[4, 5, 9] In addition, ADHD genetic risk has been associated with Th1-mediated diseases such as psoriasis, rheumatoid arthritis and ADHD diagnosis with type 1 diabetes and autoimmune thyroiditis.[5, 40, 41]

In contrast with previous studies that suggested increased Th2 cells because of the association of attention problems with atopic disease, we did not observe an association between attention problems and Th2 cell numbers.[8, 39, 42] Previously, it has been shown that in chronic atopic dermatitis immunological inflammation could cause a shift towards increased Th1 and $CD8^+$ T cell numbers.[43, 44] This might explain the association of attention problems with both Th1 and Th2-mediated disease as well as our study results with increased Th1, $CD8^+$ T cells, but not Th2 cells. In addition, another explanation could be the presence of different ADHD phenotypes which has been suggested previously because of inter-individual differences.[4, 5] Moreover, we here studied attention problems as a continuous spectrum within the general population, as opposed to ADHD in clinical samples. Hence, it is important to test to what extent our findings can be generalized at more extreme ends of symptom severity.

Next to higher Th1 cells, we observed higher total $CD8^+$, $CD8^+$ naive and $CD8^+$ Tcm cell numbers in children with more attention problems. Tcm cells express CCR7 and L-selectin, leading to circulation in blood and the primary and secondary lymphoid organs.[17] Despite previous research hypothesizing an association between $CD8^+$ T cells and attention problems, this is the first study to examine and support such an association.[38]

Various underlying mechanisms could be speculated. First, Th1 and $CD8^+$ cells could contribute to the pathogenesis of attention problems. In other neuropsychiatric dis-

eases a cross-talk between the peripheral immune system and cerebral immunity has been shown.[45, 46] $CD8^+$ T cells are thought to be involved in neuroinflammation by blood-brain barrier disruption [47]. However, these neuroinflammatory processes with alterations in immune cell numbers have never been studied in relation to specifically attention problems and require further study. Besides the adaptive immunity, a role for the innate immunity in the pathophysiology of attention problems is possible.[48] Second, it could be speculated that altered neuronal processes as observed in attention problems cause changes in the peripheral immunity. Protocadherins (Pcdhs) are involved in neuronal development and have been implicated in the pathogenesis of ADHD.[13, 49] Pcdhs also have immunomodulatory functions and specifically PCDH18 is an activation marker of $CD8^+$ T cells and inhibitor of the effector $CD8^+$ T cells.[13, 50] This might explain our observation of higher $CD8^+$ naive and $CD8^+$ Tcm cells but not effector memory cells. Third, attention problems have been associated with oxidative stress through reactive oxygen species, psychological stress and diet.[23, 39, 51] Previous studies show that children with attention problems have higher risks for unhealthy diets and obesity.[9, 23] Thus, increased levels of Th1 and $CD8^+$ could reflect oxidative stress.[52] More studies are needed to further unravel the possible role of an immunological pathophysiology in attention problems and to determine if there exists a causal relationship between adaptive immunity and attention problems.

Because multiple psychiatric conditions have been associated with inflammation it could be argued that the observed associations are due to other psychiatric conditions. To account for other behavioral or neurodevelopmental problems, we adjusted for concurrent neurodevelopmental problems. Interestingly, the observed associations were independent of total CBCL problem score and depressive symptom score, supporting an association independent of other psychiatric conditions.[53]

Finally, we observed differences in associations between boys and girls. While the direction of associations observed for total $CD8^+$, $CD8^+$ naive and $CD8^+$ Tcm with attention problems were consistent across sex, slightly stronger associations were observed in boys, in particular for $CD8^+$ TemRO. Previous studies on ADHD report sex differences in ADHD symptoms across childhood.[33, 34] Girls tend to have lower scores on hyperactivity, inattention, impulsivity and externalizing problems than boys which could explain the stronger associations in boys.[33, 34]

An important strength of our study is the extensive assessment of immune cell numbers in relation to dimensional attention problems in a population-based setting. We had the ability to adjust the associations for confounders including other child psychopathologies in contrast to previous studies. However, the following limitations should be taken into consideration. First, no information on clinical diagnosis of ADHD in children was available.

This limits the generalizability of our findings to a clinical setting in children with ADHD diagnosis. Instead, we used the well-validated parental-reported CBCL questionnaires to measure attention problems dimensionally, as used previously in epidemiological studies.[23, 25, 30, 54] This method has a higher feasibility than daily ADHD symptom measurements but might be less accurate.[23] In addition, we were unable to study the subgroup of children on ADHD medication as the number of children with medication was too low. Residual confounding and the possibility of chance finding due to multiple tests cannot be ruled out and the present exploratory findings should be considered as hypothesis-generating.[37] Non-responder analyses showed that responders differed in characteristics compared with non-responders. This might limit the generalizability of the study to other populations such as populations with a lower socio-economic status. Finally, cause-effect relationships between immune cells and attention problems cannot be established because of the cross-sectional design of this study.

Conclusion

Higher Th1, total $CD8^+$, $CD8^+$ naive, $CD8^+$ Tcm cell numbers are observed in children with more attention problems, independent of other childhood psychopathology. The results suggest that children with attention problems have a skewed balance towards Th1 cells leading to higher cytotoxic T cell numbers.

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SUPPLEMENTARY MATERIALS

Supplementary Table 3.1. Characteristics of the study population in the imputed dataset.

Child characteristics (n=756)	**General group**
Sex (N, %)	
Boy	353 (46.6)
Girl	404 (53.4)
Gestational age, wk	40.1 [39.3;41.0]
Birth weight, g	3510.0 [3150.0; 3826.3]
BMI, kg/m^2	17.0 [15.8; 18.4]
Ethnicity (N, %)	
Western	655 (86.6)
Non-western	101(13.4)
Attention score CBCL	2.0 [1.0;5.0]
Maternal characteristics	
Education (N, %)	
Primary	25 (3.3)
Secondary	264 (34.9)
Higher	467 (61.8)
Prepregnancy BMI, kg/m^2	22.7 [20.8; 25.0]
GSI during pregnancy	0.1 [0.06;0.23]
Smoking during pregnancy	
Never	580 (76.7)
Until pregnancy was known	92 (12.2)
Continued during pregnancy	84 (11.1)

Abbreviations: BMI, body mass index; CBCL, Child Behavior Checklist; g, grams; GSI, global severity index; N, number; wk, weeks.

Supplementary Table 3.1 represents the child and maternal characteristics for the study population. Values are based on the imputed dataset (analyzed sample) and represented as number (%) or median [25-75% range].

Supplementary 3.2. Non-responder analyses

Child characteristics	Responders (n=756)	Non-responders (n=5106)
Sex (N, %)		
Boy	352 (46.6)	2561 (50.2)
Girl	404 (53.4)	2544 (49.8)
Gestational age, wk	40.1 [39.3; 41.0]	40.1 [39.0; 41.0]
Birth weight, g	3510.0 [3155.0; 3827.5]	3420.0 [3050;3768.8]
BMI, kg/m^2	17.0 [15.7;18.4]	16.9 [15.7;18.8]
Ethnicity (N, %)		
Western	649 (87.0)	3267 (65.8)
Non-western	97 (13.0)	1700 (34.2)
Attention score CBCL	2.0 [1.0;5.0]	2.0 [1.0;5.0]
Maternal characteristics		
Education (N, %)		
Primary	22 (3.0)	386 (8.3)
Secondary	251 (34.5)	2008 (34.2)
Higher	455 (62.5)	2251 (48.5)
Prepregnancy BMI, kg/m^2	22.6 [20.8; 24.9]	22.6 [20.8;25.3]
GSI during pregnancy	0.12 [0.06;0.2]	0.15 [0.08;0.35]
Smoking during pregnancy		
Never	511 (77.4)	3391 (76.2)
Until pregnancy was known	74 (11.2)	376 (8.5)
Continued during pregnancy	75 (11.4)	681 (15.3)

Abbreviations: BMI, body mass index; CBCL, Child Behavior Checklist; g, grams; GSI, global severity index; N, number; wk, weeks.

Supplementary Table 3.2 compares the characteristics of the study population (n=756) versus the children that visited the research center but were not included in the current study because of no data on the immune cell numbers or attention problem score at 10 years of age. Values are based on the non-imputed dataset and represented as number (%) or median [25-75% range].

Supplementary Table 3.3. Analyses before multiple imputation.

	Attention score CBCL	
CD4$^+$	***β***	**95%CI**
CD4$^+$total	5.2	(-1.0;11.9)
CD4$^+$naive	4.4	(-1.6; 10.7)
CD4$^+$Tcm	4.3	(-1.6; 10.6)
CD4$^+$TemRO	-0.3	(-6.4; 6.1)
CD4$^+$TemRA	-0.7	(-5.9; 4.9)
Th1	10.9	(4.5; 17.7)
Th2	1.3	(-4.5; 7.6)
Th17	-4.9	(-10.7; 1.3)
Th17.1	4.8	(-1.2; 11.2)
Treg	3.4	(-2.7; 9.9)
Treg naive	2.4	(-3.5; 8.6)
Treg memory	3.9	(-2.5; 10.7)
CD8$^+$		
CD8$^+$total	4.4	(-1.3; 11.3)
CD8$^+$naive	8.0	(1.6; 14.8)
CD8$^+$Tcm	4.8	(-1.5; 11.5)
CD8$^+$TemRO	-0.5	(-6.5; 6.0)
CD8$^+$TemRA	0.7	(-5.3; 6.1)
B cells		
B total	0.8	(-5.0; 6.7)
B naive	1.9	(-4.0; 8.2)
IgG$^+$ CD27$^-$	-2.7	(-8.3; 3.3)
IgG$^+$ CD27$^+$	-3.5	(-9.1; 2.6)
IgA$^+$ CD27$^-$	-2.6	(-8.2; 3.4)
IgA$^+$ CD27$^+$	-1.8	(-7.5; 4.3)
Transitional	-2.3	(-7.6; 3.3)
CD21^{low}	-0.3	(-5.9; 5.8)

Abbreviations: CI, confidence interval; CBCL, Child Behavior Checklist; Tcm, central memory T cells; TemRA, effector memory RA-positive T cells; TemRO, effector memory RO-positive T cells; Th, helper T cell; Treg, regulatory T cell, Ig, immunoglobulin. Betas (*βs)* represent % attention problem score increase or decrease per 1SD increase in cell number/uL blood. The associations are based on multivariable linear regression analyses and adjusted for child (sex, gestational age, birth weight, BMI, ethnicity, highest maternal education, total problem score) and maternal (prepregnancy BMI, maternal smoking during pregnancy and maternal global severity index during pregnancy) covariates.
Number of children included is 486 to 505 dependent on the individual because of the exclusion of children with any missing on any covariate.

Supplementary Table 3.4. Associations between T cells and attention problem scores stratified by child's sex.

	Boys (N=352)			Girls (N=404)		
	β	**95%CI**	**P-value**	***β***	**95%CI**	**P-value**
Th1	10.86[a]	(2.27;20.17)	0.01	3.64 [g]	(-2.61; 10.29)	0.26
Th2	6.89[a]	(-1.56;15.63)	0.11	2.86 [h]	(-3.48; 9.62)	0.39
Th17	-0.36 [b]	(-8.21; 8.16)	0.93	-0.60 [i]	(-6.52; .570)	0.85
Th17.1	3.67 [b]	(-3.98;11.93)	0.36	1.96 [i]	(-4.38; 8.74)	0.55
Treg	6.83 [c]	(-0.54; 14.75)	0.07	1.80 [i]	(-4.80;8.85)	0.60
Treg naive	4.70 [d]	(-2.51; 12.43)	0.21	1.06 [k]	(-5.57; 8.16)	0.76
Treg memory	6.84 [d]	(-0.57 14.80)	0.07	2.45 [k]	(-4.36; 9.73)	0.49
$CD8^+$						
$CD8^+$ total	12.86 [e]	(5.38; 20.87)	0.001	3.84 [l]	(-2.99; 11.15)	0.28
$CD8^+$ naive	8.24 [f]	(1.18;15.80)	0.02	6.14 [m]	(-0.81; 13.57)	0.09
$CD8^+$Tcm	10.09 [f]	(2.58; 18.14)	0.008	4.73 [m]	(-1.84; 11.74)	0.16
$CD8^+$TemRO	8.22 [f]	(0.96; 16.01)	0.03	-3.27 [m]	(-9.59; 3.49)	0.34
$CD8^+$TemRA	2.98 [f]	(-3.28; 9.65)	0.36	-5.27 [m]	(-12.22; 2.23)	0.16

Abbreviations: CI, confidence interval; Tcm, central memory T cells; TemRA, effector memory RA-positive T-cells; TemRO, effector memory RO-positive T cells.

Betas (*βs)* represent % attention problem score increase or decrease per 1SD increase in cell number/uL blood. The associations are based on multivariable linear regression analyses and adjusted for child (gestational age, birth weight, BMI, ethnicity, highest maternal education, total problem score) and maternal (prepregnancy BMI, maternal smoking during pregnancy and maternal global severity index during pregnancy) covariates.

[a] n=335, [b] n=334, [c] n=330, [d] n=329, [e] n=333, [f] n=343
[g] n=383, [h] n=382, [i] n= 379, [k] n=377, [l] n=385, [m] n=396

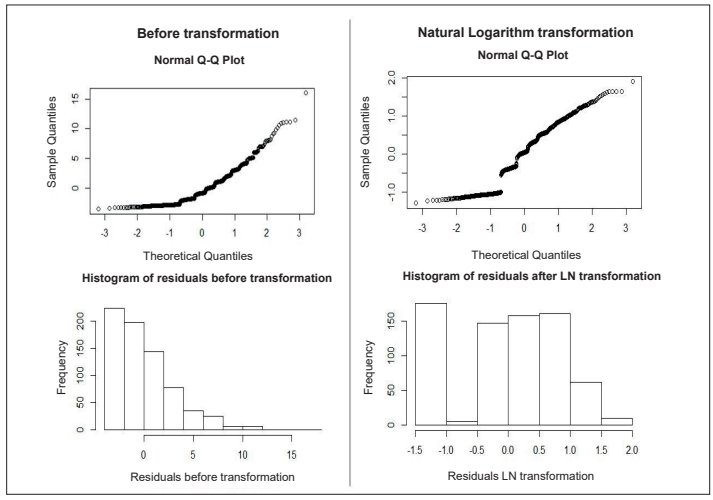
Supplementary Table 3.5. Analyses adjusted for depression instead of total CBCL.

	Attention score CBCL		
$CD4^+$	***β***	**95%CI**	**P-value**
$CD4^+$total	3.61	(-1.87;9.39)	0.20
$CD4^+$naive	1.65	(-3.60;7.18)	0.55
$CD4^+$Tcm	4.77	(-0.61;10.43)	0.08
$CD4^+$TemRO	1.73	(-3.65;7.42)	0.54
$CD4^+$TemRA	-1.23	(-6.31;4.13)	0.65
Th1	5.67	(0.10;11.56)	0.046
Th2	4.45	(-1.12;10.34)	0.12
Th17	-1.43	(-6.66;4.11)	0.61
Th17.1	2.74	(-2.71;8.50)	0.33
Treg	4.37	(-1.13;10.18)	0.12
Treg naive	2.75	(-2.69;8.49)	0.33
Treg memory	5.01	(-0.61;10.94)	0.08
$CD8^+$			
$CD8^+$total	7.59	(1.96;13.51)	0.008
$CD8^+$naive	6.31	(0.83;12.09)	0.02
$CD8^+$Tcm	6.52	(1.04;12.29)	0.02
$CD8^+$TemRO	2.67	(-2.70;8.34)	0.34
$CD8^+$TemRA	-1.31	(-6.52;4.19)	0.63
B cells			
B total	0.12	(-5.06;5.58)	0.97
B naive	1.27	(-4.03;6.86)	0.65
IgG^+CD27^-	-0.25	(-5.58;5.38)	0.93
IgG^+CD27^+	-2.22	(-7.26;3.22)	0.43
IgA^+CD27^-	-0.98	(-6.26;4.61)	0.73
IgA^+CD27^+	-1.99	(-7.09;3.38)	0.46
Transitional	1.42	(-6.52;3.95)	0.60
$CD21^{low}$	-2.93	(-8.04;2.46)	0.28

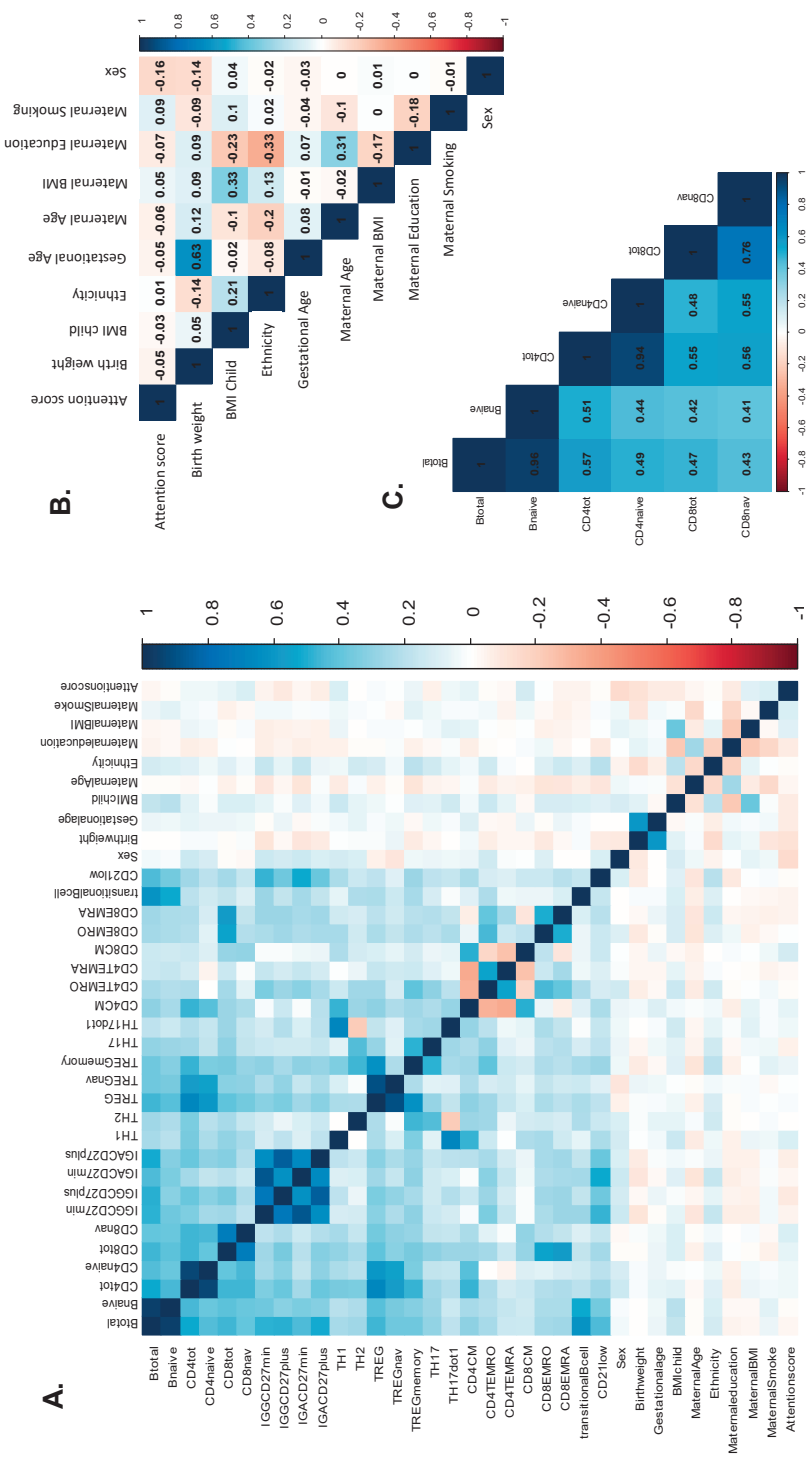
Abbreviations: CI, confidence interval; CBCL, Child Behavior Checklist; Tcm, central memory T lymphocytes; TemRA, effector memory RA-positive T lymphocytes; TemRO, effector memory RO-positive T lymphocytes; Th, helper T cell; Treg, regulatory T cell, Ig, immunoglobulin.

Betas (*βs) represent %* attention problem score increase or decrease per 1SD increase in cell number/uL blood. The associations are based on multivariable linear regression analyses and adjusted for child (sex, gestational age, birth weight, BMI, ethnicity, depression problem score) and maternal (prepregnancy BMI, maternal smoking during pregnancy, maternal global severity index during pregnancy, highest maternal education) covariates.

The following questions are included in the 13 item depressive/anxious symptom CBCL score: fears he/she might think or do something bad, he/she has to be perfect, feels or complains no one loves him/her, feels worthless or inferior, nervous/high-strung/tense, too fearful or anxious, feels too guilty, self-conscious or easily embarrassed, talks about killing self, worries, fears he/she might think or do something bad, fears going to school, fears certain animals/ situations/ or places other than school (Cronbach's alpha=0.77).



Supplementary Figure 3.1. Information on natural logarithm transformation and model fit.
Supplementary Figure 3.1 shows the model in which Th1 is used as determinant. The model before transformation has a skewness of 1.34 and a kurtosis of 5.3. The model after natural logarithm transformation has a skewness of -0.04 and a kurtosis of 1.9, corresponding to a platykurt model.



Supplementary Figure 3.2. Correlation Plot of dependent and independent variables

Supplementary Figure 3.2 shows the Pearson correlation coefficients between **A.** all included variables and **B.** confounding factors and attention problem score **C.** main B and T cell lineages.

II

Vitamin D and adiposity in relation to immune cell composition

4 The role of vitamin D on circulating memory T cells in children: The Generation R Study.

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ABSTRACT

Background

Previous studies have demonstrated that vitamin D affects T-cell function and maturation via the vitamin D receptor. However, no studies in children have been performed on this topic. Because most of the T-cell memory is formed in the first 5 years of life, we aimed to determine the association between serum 25-hydroxyvitamin D (25(OH)D) levels and numbers of circulatory naive, central memory (Tcm), and effector memory (Tem) T lymphocytes in a large population of healthy children.

Methods

Among 3189 children participating in a population-based prospective cohort, we measured 25(OH)D levels and performed detailed immunophenotyping of naive and memory T lymphocytes at a median age of 6.0 years (95% range 5.7-7.9). Detailed lymphocyte subsets were available in 986 children. Multivariable linear regression analyses were performed to determine the association between 25(OH)D and the maturation of T lymphocytes in children adjusted for cord blood 25(OH)D levels, herpes seropositivity, sociodemographic and lifestyle confounders. Furthermore, multivariable logistic regression analyses were performed to determine associations between 25(OH)D and childhood infections.

Results

Higher 25(OH)D levels were associated with higher numbers of Tem lymphocytes. Every 10 nmol/L higher 25(OH)D was associated with 2.20% (95% CI 0.54-3.89; *P*=.009) higher CD4TemRA, 1.50% (95% CI 0.38-2.62; *P*=0.008) higher CD4TemRO, and 1.82% (95% CI 0.11-3.56; *P*=0.037) higher CD8TemRA cell numbers. Generally, stronger associations were observed among boys. 25(OH)D levels were not significantly associated with naive, Tcm cell numbers, herpes seropositivity, or URTIs.

Conclusion

Our results suggest that vitamin D enhances cellular immunity in young children.

INTRODUCTION

The active metabolite of vitamin D, also known as calcitriol, has preventive effects on several diseases, including bone diseases, cancers, infections and autoimmune diseases.[1] Due to its effect on the control of infections and development of autoimmune diseases, vitamin D is thought to affect T lymphocytes: the $CD4^+$ helper T cells and the $CD8^+$ cytotoxic T cells.[2] $CD4^+$ T cells regulate both the activation of $CD8^+$ T cells and the maturation of B cells into memory B cells and Ig-producing plasma cells, whereas $CD8^+$ T cells induce cell death of pathogen-infected or malignant cells.[2] Memory T cells can be classified into central memory cells, circulating in blood and lymphoid tissues, and effector memory cells, which migrate to affected tissues.[3] Within effector memory T cells (Tem), a further distinction can be made based on the expression of either the CD45RO or the CD45RA splice variants, where the latter ones are more differentiated.[3]

Vitamin D may play a role in the formation of memory T cells, because activated T cells express the vitamin D receptor (VDR).[4, 5] In cooperation with chromatin modification enzymes as histone acetyltransferases and histone deacetylases, the VDR is important in the regulation of genes that regulate cellular differentiation, apoptosis and proliferation of (non-) cancer cells.[4, 6, 7] VDR knock-out mouse models showed increased proliferation of T cells with limited differentiation.[8] This is in line with observations from adult studies.[9, 10] The effects of vitamin D on T cell activation are likely to affect the formation of immunological memory.[9, 10]

No studies on 25-hydroxyvitamin D (25(OH)D) and the maturation of T memory cells in children have been performed. Because T cell numbers are highly dynamic in the first five years of life after which these stabilize, we examined the association between 25(OH)D and circulatory naive, central memory and effector memory T lymphocyte numbers in 3,189 six-year-old children.

METHODS

Study Design

This study was embedded in the Generation R Study, a population-based prospective cohort in the Netherlands, and has been described elsewhere.[11] For all children, written informed consent was obtained. The Medical Ethical Committee of Erasmus Medical Center approved the Generation R Study.[11] Peripheral non-fasting blood samples were obtained by antecubital venipuncture to determine serum 25(OH)D and blood T-cell subsets at six years.[12] We included six-year-old children with available measurements of serum 25(OH)D and T, B, NK cells (n=3,189). In this population, lymphocyte subsets were available in 986-990 children.

Assessment of vitamin D

Serum was extracted from peripheral blood samples and was stored at -80°C until quantification of 25(OH)D levels using isotope dilution on-line solid phase extraction liquid chromatography-tandem mass spectrometry (ID-XLC-MS/MS).[12] The lower detection limit of 25(OH)D was 4.0 nmol/L. Intra-assay and inter-assay coefficients of variation of the 25(OH)D analyses for concentrations between 25-180 nmol/L were <6% and <8%, respectively.[12] Children were categorized into three groups based on vitamin D status: deficient (<50 nmol/L), sufficient (50 to <75 nmol/L), or optimal (≥75 nmol/L).[12]

Assessment of basic blood lymphocyte subsets

Lymphocyte subsets were determined within 24 hours following blood sampling. Absolute numbers of $CD3^+$ T cells, $CD19^+$ B cells and $CD16^+/CD56^+$NK cells were obtained with a routine diagnostic lyse-no-wash protocol on a BD FACSCalibur (BD Biosciences, San Jose, CA, USA).

Assessment of detailed blood lymphocyte subsets

Detailed analysis of T-cell subsets was performed with 6-color flow cytometry.[13, 14] T cells were categorized into $CD4^+$ and $CD8^+$ lineages, in which a further distinction was made between naive cells ($CD45RO^-CCR7^+$), central memory T cells (Tcm; $CD45RO^+CCR7^+$), effector memory RO positive T cells (TemRO; $CD45RO^+CCR7^-$) and effector memory RA positive cells (TemRA; $CD45RO^-CCR7^-$).

Covariates

Information on sex, birth weight and date of birth was obtained from midwife and hospital registries.[14] To take previous 25(OH)D levels into account, cord blood 25(OH)D levels were defined using ID-XLC-MS/MS. Information on siblings in household, ethnicity, and history of upper respiratory tract infections (URTI) at six years were obtained from medical records and questionnaires.[12] Sex-specific standard deviation scores (SDS) for body mass index (BMI, kg/m^2) were calculated.[15] Seropositivity of CMV (IgG-CMV), herpes simplex virus 1 (IgG-HSV-1) and/or EBV (IgG-EBV viral capsid antigen (VCA)) were determined by ELISA from serum samples at six years.[16] Child ethnicity was determined as previously described and categorized into Western (Dutch, European, American, Oceanian) or non-Western (Turkish, Moroccan, African, Asian).[13, 15, 16] Child lifestyle characteristics as playing sports and time spent watching television, using a computer and/or playing outside at daytime were determined using questionnaires administered to the parents at the child age of six years.[12] Season of blood drawn was obtained from time of research center visit.[12] Household income, folic acid supplement use and smoking during pregnancy, were obtained with parental derived questionnaires.[14] Delivery reports and questionnaires derived at multiple consecutive time points provided information on maternal breastfeeding duration and exclusivity.[14]

Statistical Analyses

Kruskal-Wallis rank-sum tests, one-way ANOVA and chi-squared tests were conducted to determine differences in baseline characteristics between deficient, sufficient and optimal 25(OH)D levels. Natural log-scale transformations on lymphocyte numbers (cells per μL blood) were performed. Multivariable linear regression analyses were performed to assess associations between 25(OH)D and immune cell numbers. Covariates were included in the final multivariable model if the covariate resulted in an alteration in effect estimate of at least 10%, for at least one of the outcomes.[17] Based on this criterion, we adjusted for the following child characteristics: sex, age, ethnicity, birth weight, number of children in household, IgG-CMV, IgG-HSV-1 and/or IgG-EBV-VCA, season of blood draw, BMI-SDS, playing sports, watching television and using a computer, playing outside at daytime, serum 25(OH)D at birth and URTIs at six years of age. Additionally, we adjusted for the parental characteristics household income, exclusive breastfeeding, start of folic acid use and maternal smoking during pregnancy. Effect modification by sex, ethnicity and age was evaluated by adding the interaction terms of each of these variables with 25(OH)D to our multivariable model. Binary logistic regression analyses were performed to determine associations between 25(OH)D and IgG-CMV, IgG-HSV-1 and/or IgG-EBV-VCA and a multinomial regression analysis was performed to determine the association between 25(OH)D and URTI numbers at six years old, with no infections as reference category. We adjusted for the same confounders as in the linear regression analyses. Because of strong correlations between our outcomes (Pearson's correlations >0.80), we did not perform adjustments for multiple testing. To reduce attrition bias, multiple imputation of the covariates was performed (n=5 imputations), using the Fully Conditional Specification method (predictive mean matching). The percentage of missing values in the original database ranged from 0.2%-45%. Pooled regression coefficients of the five imputed datasets were reported in this paper as regression coefficients (ßs) or odds ratios (ORs) with 95% confidence intervals (CI). Statistical analyses were performed using SPSS version 21.0 (IBM Corp., Armonk, NY, USA) and R version 3.3.3 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Study population characteristics

The study population consisted of 3,189 children (48.4% boys) with a median age of 6.0 years (95%CI 5.7 to 7.9). The median 25(OH)D level was 63.8 nmol/L (95%CI 17.0-131.0). Children were classified into three groups based on the serum 25(OH)D levels: deficient (<50 nmol/L) in 31.5%, sufficient (50-75 nmol/L) in 33.6% and optimal (≥75 nmol/L) in 34.9% (Table 4.1). Supplementary Table 4.1 shows the characteristics based on the original data.

Table 4.1 Child and Maternal Characteristics of the Study Population stratified by 25(OH)D status.

Study population characteristics	Serum 25(OH)D			P-value
	Deficient (N=1005) (<50 nmol/L)	Sufficient (N=1129) (50 to <75 nmol/L)	Optimal (N=1055) (≥75 nmol/L)	
Child Characteristics				
Sex				0.17
Boy	535 (53.2)	591 (52.3)	520 (49.3)	
Girl	470 (46.8)	538 (47.7)	535 (50.7)	
Age, years	6.07 [5.73, 8.24]	5.98 [5.69, 7.71]	5.96 [5.68, 7.55]	**<0.001**
Birth weight (g)	3383 (575)	3435 (580)	3424 (561)	0.09
Ethnicity (N,%)				**<0.001**
Dutch or other Western	446 (44.4)	853 (75.6)	914 (86.6)	
Non-Western	559 (55.6)	276 (24.4)	141 (13.4)	
BMI at 6 years, kg/m^2	16.1[13.6,22.6]	15.9[13.9,20.6]	15.7 [13.7,19.9]	**<0.001**
>1 child in household	799 (79.5)	933 (82.6)	900 (85.3)	**0.002**
Herpes Virus Seropositivity, IgG (N,%)				
CMV	454 (45.2)	393 (34.8)	344 (32.6)	**<0.001**
EBV	587 (58.4)	566 (50.1)	462 (43.8)	**<0.001**
HSV-1	274 (27.3)	203 (18.0)	157 (14.9)	**<0.001**
Season of blood sampling (N,%)				**<0.001**
Winter	391 (38.9)	242 (21.4)	117 (11.1)	
Spring	341 (33.9)	353 (31.3)	227 (21.5)	
Summer	75 (7.5)	258 (22.9)	409 (38.8)	
Fall	198 (19.7)	276 (24.4)	302 (28.6)	
Playing sports (N,%)	378 (37.6)	540 (47.8)	499 (47.3)	**<0.001**
Watching television using computer, hours/day	1.82 [0.26;6.21]	1.32 [0.29, 4.49]	1.29 [0.25, 4.07]	**<0.001**
Playing outside at daytime, hours/day	0.93 [0.00;4.22]	1.23 [0.14;4.30]	1.57 [0.23;4.49]	**<0.001**
Upper Respiratory Tract Infections over previous year at 6 years				0.27
Never	723 (71.9)	822 (72.8)	777 (73.6)	
1-2 times	176 (17.5)	194 (17.2)	195 (18.5)	
> 2 times	106 (10.5)	113 (10.0)	83 (7.9)	
25(OH)D level at 6 years, nmol/L	36.0 [12.9;50.0]	63.0 [51.0;75.0]	89.0 [76.0;157.4]	**<0.001**
Maternal Characteristics				
Folic Acid supplement use during pregnancy (N,%)				0.66
Start Preconceptionally	303 (30.1)	318 (28.2)	325 (30.8)	
Start in first 10 weeks of pregnancy	304 (30.2)	365 (32.3)	323 (30.6)	
None	398 (39.6)	446 (39.5)	407 (38.6)	
Breastfeeding during first 4 months (%)				**<0.001**
Exclusively Breastfed	174 (17.3)	295 (26.1)	266 (25.2)	
Partially Breastfed	727 (72.3)	723 (64.0)	676 (64.1)	
Never Breastfed	104 (10.3)	111 (9.8)	113 (10.7)	
Smoking during pregnancy (%)				**0.009**
Never	720 (71.6)	856 (75.8)	812 (77.0)	
Until pregnancy was known	92 (9.2)	111 (9.8)	95 (9.0)	
Continued during pregnancy	193 (19.2)	162 (14.3)	148 (14.0)	

Table 4.1 Child and Maternal Characteristics of the Study Population stratified by 25(OH)D status. (*continued*)

Study population characteristics	Serum 25(OH)D			P-value
	Deficient (N=1005) (<50 nmol/L)	Sufficient (N=1129) (50 to <75 nmol/L)	Optimal (N=1055) (≥75 nmol/L)	
Net monthly household income, euro				**<0.001**
< 2200	664 (66.1)	446 (41.3)	316 (30.0)	
≥ 2200	341 (33.9)	663 (58.7)	739 (70.0)	

Abbreviations: CMV, cytomegalovirus; EBV, Epstein Barr Virus, HSV-1, Herpes Simplex Virus type 1; 25(OH)D, 25-hydroxyvitamin D. Values are mean ± SD, median (95% range), or percentages and based on the imputed data. Kruskal Wallis rank sum tests, one-way ANOVA and chi-squared tests were conducted to examine possible differences in baseline characteristics between deficient, sufficient and optimal 25(OH)D levels.

Associations between 25(OH)D levels and T-cell numbers

To study the effects of 25(OH)D levels on blood lymphocytes, we performed multivariable linear regressions on each lineage as a whole (Table 4.2). Neither total T, B and NK cell numbers, nor naive $CD4^+$ and $CD8^+$ T cells were associated with differences in 25(OH)D levels. Subsequently, memory T-cell subsets were analyzed. This showed positive associations between 25(OH)D levels and absolute numbers of $CD4^+$ TemRO, $CD4^+$ TemRA and

Table 4.2. Associations of 25(OH)D levels (per 10 nmol/L) with blood lymphocyte cell numbers at 6 years of age.

Blood Lymphocytes	ß	(95% CI)	P-value
Total T[a]	0.718	(-0.176;1.620)	0.12
Total B[a]	-0.068	(-1.166;1.042)	0.90
Total NK[a]	0.300	(1.022;1.622)	0.66
$CD4^+$ T[b]	0.626	(-0.293;1.554)	0.18
$CD4^+$ naive[c]	0.399	(-0.873;1.687)	0.54
$CD4^+$ Tcm[c]	-0.752	(-1.940;0.450)	0.22
$CD4^+$ TemRO[c]	1.496	(0.381;2.624)	**0.008**
$CD4^+$ TemRA[c]	2.197	(0.535;3.885)	**0.009**
$CD8^+$ T[b]	0.891	(-0.175;1.964)	0.10
$CD8^+$ naive[c]	0.570	(-0.806;1.964)	0.42
$CD8^+$ Tcm[c]	-1.066	(-2.899;0.801)	0.26
$CD8^+$ TemRO[c]	0.099	(-1.542;1.757)	0.91
$CD8^+$ TemRA[c]	1.819	(0.108;3.560)	**0.04**

[a] N= 3,189, [b] N= 990, [c] N= 986.

Abbreviations: Tcm, Central Memory T lymphocytes; TemRA, Effector Memory RA positive T lymphocytes; TemRO, Effector Memory RO positive T lymphocytes; CI, confidence interval.

Betas (ßs) represent regression coefficients derived from multivariable linear regression, based on multiple imputed dataset. The regression coefficients represent the percentage difference in cell number per 10 nmol/L higher 25(OH)D levels. Associations are adjusted for sex, age, birth weight, ethnicity, number of children in household, CMV seropositivity, HSV-1 seropositivity, EBV seropositivity, upper respiratory tract infections (URTIs) over the previous year at 6 years, season of blood draw, BMI-SDS, playing sports, watching television or computer, playing outside at daytime, serum 25(OH)D at birth, household income, exclusive breast feeding, maternal folic acid supplement use and maternal smoking during pregnancy. Bold values indicate statistically significant (P<0.05) associations.

$CD8^+$ TemRA cells. A 10 nmol/L higher serum 25(OH)D was associated with 2.20% (95%CI 0.54 to 3.89, p=0.009) higher $CD4^+$ TemRA numbers, 1.50% (95%CI 0.38;2.62, p=0.008) higher $CD4^+$ TemRO numbers and 1.82% (95% CI 0.11;3.56, p=0.037) higher $CD8^+$ TemRA numbers. No significant associations were observed between 25(OH)D and the Tcm subsets or between 25(OH)D and the $CD8^+$ TemRO subsets. Figures 4.1 and 4.2 show the predicted natural log T cell numbers for 25(OH)D levels. Supplementary Table 4.2 shows the results stratified by 25(OH)D status.

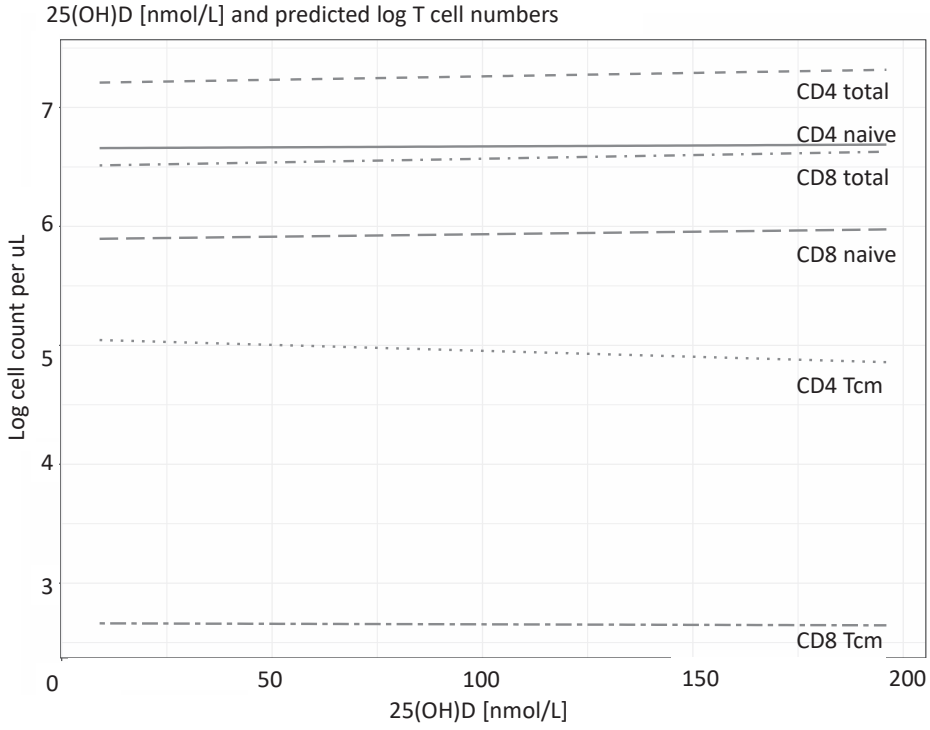

Figure 4.1. The association between 25(OH)D [nmol/L] and predicted log T memory cell numbers.
Abbreviations: Tcm, Central Memory T lymphocytes. Figure 4.1 presents the association between 25(OH)D and predicted log cell count per µL. The Figure is adjusted for the following confounders: sex, birth weight, age, ethnicity, birth weight, number of children in household, CMV seropositivity, HSV-1 seropositivity, EBV seropositivity, URTIs over the previous year at 6 years, season of blood draw, BMI-SDS, playing sports, watching television or computer, playing outside at daytime, serum 25(OH)D at birth, household income, exclusive breast feeding, maternal folic acid supplement use and maternal smoking during pregnancy. No significant associations were observed between these T cell numbers and 25(OH)D (p>0.05).

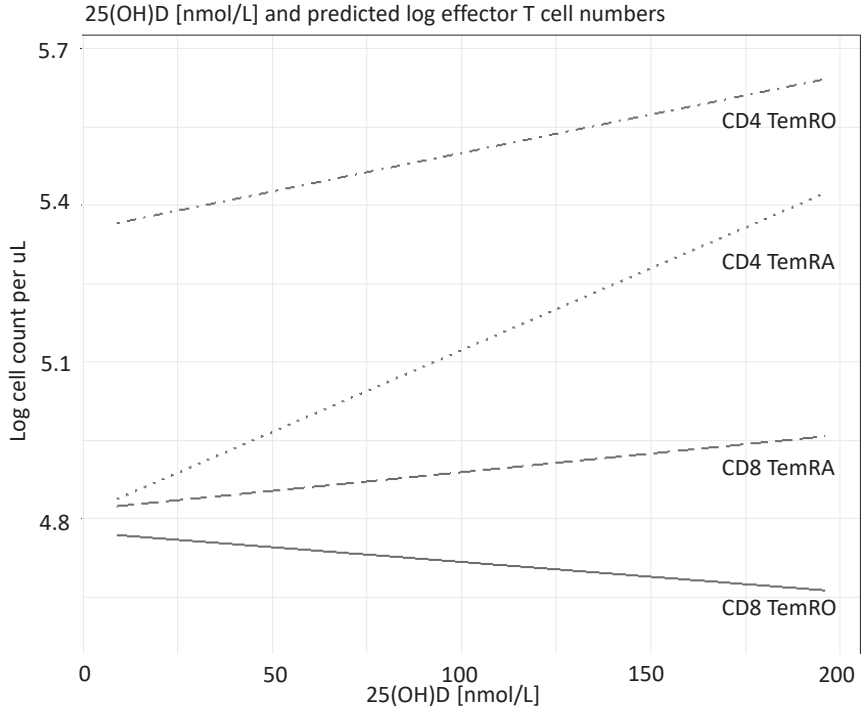

Figure 4.2. The association between 25(OH)D [nmol/L] and predicted log T memory cell numbers.
Abbreviations: TemRA, Effector Memory RA positive T lymphocytes; TemRO, Effector Memory RO positive T lymphocytes. Figure 4.2 presents the association between 25(OH)D and predicted log cell count per µL. The Figure is adjusted for the following confounders: sex, birth weight, age, ethnicity, birth weight, number of children in household, CMV seropositivity, HSV-1 seropositivity, EBV seropositivity, URTIs over the previous year at 6 years, season of blood draw, BMI-SDS, playing sports, watching television or computer, playing outside at daytime, serum 25(OH)D at birth, household income, exclusive breast feeding, maternal folic acid supplement use and maternal smoking during pregnancy.

Analyses stratified by sex

Because sex is likely to modify associations between vitamin D and lymphocyte numbers, we evaluated whether associations were different in boys and girls.[18] Indeed, the interaction term between sex and 25(OH)D was significant ($p<0.030$) for multiple lymphocyte outcomes. Therefore, we stratified our analyses for sex. In general, stronger associations between 25(OH)D and blood lymphocytes were observed in boys than in girls (Table 4.3). Associations between 25(OH)D levels and $CD4^+$ TemRA and $CD8^+$ TemRA were stronger in boys, whereas the association between 25(OH)D levels and $CD8^+$ TemRO was stronger in girls.

Table 4.3. Associations of 25(OH)D levels (per 10 nmol/L) with blood lymphocyte cell numbers at 6 years of age stratified by sex.

	Boys (N=1,646)		Girls (N=1,543)	
Blood lymphocytes	**ß (95% CI)**	**P-value**	**ß (95% CI)**	**P-value**
Total T	**1.596 (0.323;2.884)**[a]	**0.014**	-0.386 (-1.686;0.929)[d]	0.56
Total B	0.750 (-0.845;2.370)[a]	0.36	-1.195 (-2.731;0.366)[d]	0.13
Total NK	0.728 (-1.158;2.649)[a]	0.45	0.155 (-1.702;2.047)[d]	0.87
CD4$^+$ T	**1.673 (0.372;2.992)**[b]	**0.012**	-0.616 (-1.959;0.744)[e]	0.37
CD4$^+$ naive	**1.889 (0.058;3.754)**[c]	**0.043**	-1.349 (-3.175;0.512)[f]	0.15
CD4$^+$ Tcm	-0.365 (-2.084;1.383)[c]	0.68	-1.312 (-3.015;0.419)[f]	0.14
CD4$^+$ TemRO	0.932 (-0.653;2.544)[c]	0.25	**2.078 (0.448;3.734)**[f]	**0.012**
CD4$^+$ TemRA	**4.035 (1.760;6.362)**[c]	**<0.001**	0.191 (-2.258;2.703)[f]	0.88
CD8$^+$ T	**1.364 (-0.155;2.905)**[b]	**0.08**	0.140 (-1.384;1.688)[e]	0.86
CD8$^+$ naive	0.838 (-1.112;2.828)[c]	0.40	0.310 (-1.898;1.998)[f]	0.98
CD8$^+$ Tcm	-0.554 (-3.193;2.157)[c]	0.69	-1.665 (-4.307;1.005)[f]	0.22
CD8$^+$ TemRO	0.257 (-1.981;2.546)[c]	0.82	-0.322 (-2.589;2.594)[f]	0.80
CD8$^+$ TemRA	**3.150 (0.846;5.506)**[c]	**0.007**	-0.309 (-2.596;2.143)[f]	0.98

[a] N= 1,646, [b] N= 509, [c] N= 506, [d] N= 1,543, [e] N= 481, [f] N= 480

Abbreviations: Tcm, Central Memory T lymphocytes; TemRA, Effector Memory RA positive T lymphocytes; TemRO, Effector Memory RO positive T lymphocytes; CI, confidence interval.

Betas (ßs) represent regression coefficients derived from multivariable linear regression, based on multiple imputed dataset. The regression coefficients represent the percentage difference in cell number per 10 nmol/L higher 25(OH)D levels. Associations are adjusted for age, birth weight, ethnicity, number of children in household, CMV seropositivity, HSV-1 seropositivity, EBV seropositivity, upper respiratory tract infections (URTIs) over the previous year at 6 years, season of blood draw, BMI-SDS, playing sports, watching television or computer, playing outside at daytime, serum 25(OH)D at birth, household income, exclusive breast feeding, maternal folic acid supplement use and maternal smoking during pregnancy. Bold values indicate statistically significant (<0.05) associations.

Associations between 25(OH)D levels and infections

To examine whether the observed associations between serum 25(OH)D and memory T cells could be explained by previous childhood infections, we determined the associations between 25(OH)D and IgG-CMV, IgG-HSV1 and IgG-EBV-VCA serpositivity and URTIs at 6 years of age. However, 25(OH)D was not significantly associated with herpesvirus seropositivity nor number of URTIs after adjustment for confounding variables (Table 4.4).

Table 4.4. Associations of 25(OH)D levels (per 10nmol/L) at 6 years of age with childhood infections.

	Total N	OR (95% CI)	P-value
Herpesvirus seropositivity			
CMV	1,185	1.001 (0.969;1.034)	0.95
EBV	1,606	0.994 (0.962;1.028)	0.72
HSV-1	630	0.982 (0.944;1.023)	0.39
Upper respiratory tract infection at 6 years			
0	2,017	REF	REF
1-2	343	1.057 (0.999;1.119)	0.06
>2	272	0.956 (0.903;1.012)	0.12

Abbreviations: CMV, cytomegalovirus; EBV, Epstein Barr Virus, HSV-1, Herpes Simplex Virus type 1; OR, odds ratio; CI, confidence interval.

The odds ratios (ORs) are derived from logistic regression analyses, based on multiple imputed dataset. The ORs of herpesvirus seropositivity or upper respiratory tract infections (URTIs) per 10 nmol/L increase in 25(OH)D levels are represented. The following covariates are added to the logistic regression models: sex, birth weight, age, ethnicity, birth weight, number of children in household, CMV seropositivity, HSV-1 seropositivity, EBV seropositivity, URTIs over the previous year at 6 years, season of blood draw, BMI-SDS, playing sports, watching television or computer, playing outside at daytime, serum 25(OH)D at birth, household income, exclusive breast feeding, maternal folic acid supplement use and maternal smoking during pregnancy. Bold values indicate statistically significant (<0.05) associations.

DISCUSSION

In this large prospective cohort study, we observed that higher serum 25(OH)D levels were associated with higher memory T-cell numbers in six-year-old children. The associations were affected by sex: 25(OH)D had a pronounced positive effect on $CD4^+$ TemRO numbers in girls, whereas in boys $CD4^+$ TemRA and $CD8^+$ TemRA were higher.

Previous literature and interpretation

This is the first study that determined associations between 25(OH)D and memory T cell numbers in a large population of healthy children. This study in children is relevant because early life exposures are crucial in the maturation of the immune system and disease susceptibility.[19] Since T cell memory is long lasting, the conditions under which T cell memory is formed may have long-lasting effects on immunity.[19]

In line with our observations, previous studies reported positive associations between 25(OH)D and T lymphocyte differentiation into Tem cells, and between 25(OH)D and activated T cells.[8-10] However, these studies were limited by their study population (small sample size, adult or mouse populations) or lack of adjustment for possible confounders as season of blood draw, herpes seropositivity, and lifestyle determinants. Our results therefore extend observations from previous studies.

Vitamin D levels might have direct effects on memory T cells, because naive T lymphocytes contain 1α-Hydroxylase (CYP27B) and upregulate VDR expression upon stimulation via the T-cell receptor.[5] The mitochondrial cytochrome P450 enzyme 1α-Hydroxylase catalyzes the transformation from the inactive form of vitamin D to the active form of vitamin D.[20] Binding of this active form of vitamin D to the VDR leads to upregulation of PLC-γ1, which is a key enzyme in the T cell receptor-signaling pathway.[21] The VDR can bind to promoter regions of various genes, inducing recruitment of transcription factors and co-regulatory molecules on promoters that regulate gene transcription to control cell proliferation, differentiation, apoptosis and angiogenesis.[4] Via these epigenetic mechanisms, vitamin D can enhance antigen responses and maturation of naive T cells.[4]Additionally, vitamin D affects T-cell homing via induction of chemokine receptor CCR10, resulting in increased responsiveness to chemokine CCL27 and the ability for T cells to migrate to sites of infection.[22] We did not observe an association between serum 25(OH)D and $CD8^+$ TemRO numbers, whereas we did observe these associations for $CD4^+$ TemRA, $CD4^+$ TemRO and $CD8^+$ TemRA. One explanation might be that vitamin D mainly enhances the function of regulatory $CD4^+$ T cells.[23] The observed association between 25(OH)D and memory $CD4^+$ T cells may therefore partly rely on increased numbers of regulatory $CD4^+$T cells.[23]

Higher serum 25(OH)D levels have been reported to be protective against autoimmune diseases.[1] This could be explained by inhibition of IL-2-gene transcription by the VDR.[24] The cytokine IL-2 is needed by T cells to proliferate.[24] Therefore, immune responses can be regulated by limiting the presence of IL-2.[24] This mechanism could contribute to explain the observed preventive effects of vitamin D on autoimmune diseases. Additionally, vitamin D suppresses cell-mediated Th1 immunity and promotes synthesis of $CD4^+CD25^+$ T regulatory lymphocytes that produce the anti-inflammatory cytokine IL-10.[22]

In line, we observed different associations between 25(OH)D and memory T cells for boys and girls, which extends previous literature that describes sex differences in the immune system and immune development.[18] Additionally, it has been reported that males experience infections more frequently than females.[25] Furthermore, sex differences in body composition could be an explanation because vitamin D is fat-soluble.[26] However, upon correction for URTI and total fat and fat-free mass, no changes in effect estimates were observed. Hence, hormonal differences between boys and girls that already exist in utero and before puberty might explain the observed sex-specific differences of vitamin D on memory T cells.[27]

We did not observe significant associations between 25(OH)D and URTIs at the age of six years. A recent meta-analysis concludes preventive effects of vitamin D supplementation on respiratory infections.[28] Similarly, observational studies have found that higher 25(OH)

D levels protect against respiratory infections.[1] These different results could be explained by various reasons. First, our observational design only captures one-year of URTIs preceding vitamin D levels at the age of six years. Second, information on URTIs in our study is obtained from questionnaires, which could introduce bias. Third, the meta-analysis discusses effects of vitamin D supplementation, whereas our study determines the association between serum 25(OH)D and URTIs. Interestingly, this meta-analysis observed the strongest effect in individuals with profound deficient 25(OH)D levels (<25 nmol/L) at baseline.[28] Also, the size of the effect varies according to the causative agent.[1]

In addition, no associations between herpesvirus seropositivity and 25(OH)D were observed in our study. Previous studies on this topic are scarce: an in-vitro study observed down-regulation of the VDR by CMV and it has been hypothesized that EBV is linked to vitamin D status.[29] However, no in-vivo studies in a healthy population of children have been performed on this association.

Methodological considerations

The strength of this study is the large number of children and the embedment in a large population-based prospective study. The prospective design of the Generation R cohort enabled us to adjust for a large number of confounders. Another strength is the accurate measurement of exposure and outcome. ID-XLC-MS/MS is highly sensitive and specific in measuring 25(OH)D levels and therefore the standard for epidemiological studies.[12] Serum 25(OH)D was measured because this provides the best estimate of vitamin D status in individuals.[30] Furthermore, extensive and detailed immune phenotyping enabled us to evaluate functionally distinct subsets of naive and memory T lymphocytes. IgG-CMV and IgG-EBV-VCA were associated with significant expansions of the $CD8^+$ memory compartment (approximately 2-fold), and to a lesser extent (approximately 1.1 to 1.3-fold) with expansions of $CD4^+$memory T cells in our previous study.[13] These $CD4^+$ memory T cells included both $CD4^+$ TemRO and $CD4^+$ TemRA cells. Therefore, in our study, we included herpes seropositivity as confounders in the analyses. Additionally, we included relevant sociodemographic, lifestyle confounders. However, residual confounding cannot be excluded.

Conclusion

We observed positive associations between serum 25(OH)D and Tem numbers in children. This indicates that vitamin D may enhance cellular immunity in young children. Since T cell memory is long lasting, these effects of vitamin D in young children might affect the risk of infectious and inflammatory diseases at later age.

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SUPPLEMENTARY MATERIALS

Supplementary Table 4.1. Characteristics of the study population based on the original dataset.

Child Characteristics (N=3,189)	**Total N (%) or Median [95% range]**	**Missing (%)**
Sex (N,%)		0 (0.0)
Boy	1534 (48.4)	
Girl	1646 (51.6)	
Age, years	6.0 [5.7-7.9]	0 (0.0)
Birth weight (g)	3415 (573)	6 (0.2)
Ethnicity (N,%)		118 (3.7)
Dutch or other Western	2155 (67.6)	
Non-Western	916 (32.4)	
BMI at 6 years, kg/m^2	15.9 [13.8-21.2]	5 (0.2)
More than 1 child in household	2240 (83.2)	496 (15.6)
Herpes Virus Seropositivity, IgG (N,%)		
CMV	1185 (37.4)	18 (0.6)
EBV	1606 (50.7)	21 (0.7)
HSV-1	630 (19.9)	18 (0.6)
Season of blood sampling (N,%)		0 (0.0)
Winter	750 (23.5)	
Spring	921 (28.9)	
Summer	742 (23.3)	
Fall	776 (24.3)	
Playing sports (N,%)	1233 (28.7)	457 (14.3)
Watching television using computer, hours/day	1.7 [0.3-5.0]	825 (25.9)
Playing outside at daytime, hours/day	1.1 [0.1-3.6]	819 (25.7)
Upper Respiratory Tract Infections over previous year at 6 years		557 (17.5)
Never	2017 (76.6)	
1-2 times	343 (13.0)	
> 2 times	272 (10.3)	
25(OH)D level at 6 years, nmol/L	63.8 [17.0-131.0]	0 (0.0)
25(OH)D status at 6 years, nmol/L (N,%)		0 (0.0)
Deficient (<50 nmol/L)	1005 (31.5)	
Sufficient (50 to <75 nmol/L)	1129 (35.4)	
Optimal (≥75 nmol/L)	1055 (33.1)	
Maternal Characteristics (N=3,189)	**Total N (%) or Median (95% range)**	**Missing (%)**
Folic Acid supplement use during pregnancy (N,%)		1123 (35.2)
Start Preconceptionally	831 (40.2)	
Start in first 10 weeks of pregnancy	618 (29.9)	

Supplementary Table 4.1. Characteristics of the study population based on the original dataset. (*continued*)

Child Characteristics (N=3,189)	**Total N (%) or Median [95% range]**	**Missing (%)**
None	617 (29.9)	
Breastfeeding during first 4 months (%)		527 (30.5)
Exclusively Breastfed	523 (25.0)	
Partially Breastfed	1371 (65.6)	
Never Breastfed	195 (9.3)	
Smoking during pregnancy (%)		183 (10.6)
Never	1150 (77.4)	
Until pregnancy was known	143 (9.2)	
Continued during pregnancy	254 (13.4)	
Net monthly household income, euro		782 (24.5)
< 2200	982 (40.8)	
≥ 2200	1425 (59.2)	

Abbreviations: CMV, cytomegalovirus; EBV, Epstein Barr Virus, HSV-1, Herpes Simplex Virus type 1; 25(OH)D, 25-hydroxyvitamin D. Values are based on the non-imputed data.
Values are mean ± SD, median (95% range), or number (percentages) and based on the non-imputed data.

Supplementary Table 4.2. Characteristics of the study population stratified by 25(OH)D status.

Blood Lymphocytes	**Deficient 25(OH)D** (<50 nmol/L)		**Sufficient 25(OH)D** (50 to <75 nmol/L)		**Optimal 25(OH)D** (≥75 nmol/L)	
	ß (95% CI)	**P-value**	**ß (95% CI)**	**P-value**	**ß (95% CI)**	**P-value**
Total T	-0.029 (0.090;0.033)[a]	0.36	-0.043 (-0.090;0.005)[d]	0.078	REF	REF
Total B	0.010 (0.066;0.086)[a]	0.80	-0.023 (-0.082;0.035)[d]	0.44	REF	REF
Total NK	-0.008 (-0.099;0.082)[a]	0.86	-0.007 (-0.077;0.063)[d]	0.84	REF	REF
CD4$^+$	-0.023 (-0.086;0.040)[b]	0.48	**-0.057 (-0.106;-0.007)**[e]	**0.024**	REF	REF
CD4$^+$ naive	0.001 (-0.087;0.088)[c]	0.99	**-0.084 (-0.152;-0.016)**[f]	**0.015**	REF	REF
CD4$^+$ Tcm	0.068 (-0.015;0.150)[c]	0.11	-0.056 (-0.120;0.008)[f]	0.089	REF	REF
CD4$^+$ TemRO	**-0.078 (-0.154;-0.002)**[c]	**0.044**	-0.014 (-0.073;0.045)[f]	0.64	REF	REF
CD4$^+$ TemRA	**-0.163 (-0.276;-0.051)**[c]	**0.005**	-0.017 (-0.104;0.07)[f]	0.71	REF	REF
CD8$^+$	-0.039 (-0.112;0.034)[b]	0.30	-0.015 (-0.071;0.042)[e]	0.60	REF	REF
CD8$^+$ naive	-0.017 (-0.112;0.077)[c]	0.72	-0.027 (-0.099;0.046)[f]	0.47	REF	REF
CD8$^+$ Tcm	0.114 (-0.014;0.234)[c]	0.08	-0.092 (-0.191;0.006)[f]	0.06	REF	REF
CD8$^+$ TemRO	0.20 (-0.093;0.134)[c]	0.73	0.042 (-0.047;0.130)[f]	0.35	REF	REF
CD8$^+$ TemRA	**-0.162 (-0.725;-0.045)**[c]	**0.006**	0.029 (-0.061;0.119)[f]	0.53	REF	REF

[a] N= 1005, [b] N= 246, [c] N= 244, [d] N= 1129, [e] N= 351, [f] N= 351.

Abbreviations: Tcm, Central Memory T lymphocytes; TemRA, Effector Memory CD45$^+$RA positive T lymphocytes; TemRO, Effector Memory CD45$^+$RO positive T lymphocytes; CI, confidence interval.

Betas (ßs) represent regression coefficients derived from multivariable linear regression, based on multiple imputed dataset. The regression coefficients represent the percentage difference in cell number with an optimal 25(OH)D status as reference. Associations are adjusted for gender, birth weight, age, ethnicity, number of children in household, CMV/HSV-1/EBV seropositivity at the age of 6 year, upper respiratory tract infections (URTIs) over the previous year at 6 years, season of blood draw, BMI-SDS, playing sports, watching television or computer, playing outside at daytime, serum 25(OH)D at birth, household income, exclusive breast feeding, maternal folic acid supplement use and maternal smoking during pregnancy. Bold values indicate statistically significant (P<0.05) associations.

5

Childhood adiposity associated with expanded effector memory $CD8^+$ and $V\delta2^+V\gamma9^+$ T cells.

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ABSTRACT

Background

Adult obesity is associated with chronic low-grade inflammation and may give rise to future chronic disease. However, it is unclear whether adiposity-related inflammation is already apparent in childhood. We aimed to study associations between child adiposity measures with circulating monocytes and naive and memory subsets in $CD4^+$, $CD8^+$, and γδ T cell lineages.

Methods

Ten-year-old children (n = 890) from the Generation R Cohort underwent dual-energy x-ray absorptiometry and magnetic resonance imaging for body composition (body mass index [BMI], fat mass index [FMI], android-to-gynoid fat mass ratio, visceral fat index, liver fat fraction). Blood samples were taken for detailed immunophenotyping of leukocytes by 11-color flow cytometry.

Results

Several statistically significant associations were observed. A 1SD increase in total FMI was associated with +8.4% (95% CI 2.0, 15.2) $V\delta2^+V\gamma9^+$ and +7.4% (95% CI 2.4, 12.5) $CD8^+$TemRO cell numbers. A 1SD increase in visceral fat index was associated with +10.7% (95% CI 3.3, 18.7) $V\delta2^+V\gamma9^+$ and +8.3% (95% CI 2.6, 14.4) $CD8^+$ TemRO cell numbers. Higher android-to-gynoid fat mass ratio was only associated with higher $V\delta2^+V\gamma9^+$ T cells. Liver fat was associated with higher $CD8^+$TemRO cells but not with $V\delta2^+V\gamma9^+$ T cells. Only liver fat was associated with lower Th17 cell numbers: a 1-SD increase was associated with -8.9% (95% CI -13.7, -3.7) Th17 cells. No associations for total $CD8^+$, $CD4^+$ T cells, or monocytes were observed. BMI was not associated with immune cells.

Conclusion

Higher $V\delta2^+V\gamma9^+$ and $CD8^+$TemRO cell numbers in children with higher visceral fat index could reflect presence of adiposity-related inflammation in children with adiposity of a general population.

INTRODUCTION

Childhood overweight and obesity are major public health concerns.[1] High childhood body mass index (BMI) predisposes to high BMI in adulthood.[2-4] In school-aged children and adolescents, high BMI increases the risk of adverse health outcomes associated with overweight and obesity in adulthood.[3, 4] These adverse health outcomes include asthma, insulin resistance, coronary heart disease and metabolic syndrome.[3, 5, 6] In addition, obesity might increase the risk of respiratory tract infections,[7] and increases the morbidity and mortality in coronavirus disease-19 (COVID-19).[8]

Adiposity is associated with chronic low-grade inflammation which predominantly originates in visceral adipose tissue.[9-12] Fat biopsies of adults with overweight or obesity have been shown to be infiltrated with Th1, Th17 and $CD8^+$ T cells, which might be a reflection of this chronic low-grade inflammation.[12] Positive associations have been observed between human fat mass and intermediate and non-classical monocytes in blood.[13, 14] In adolescents with overweight or obesity, increased circulating effector memory $CD4^+$ and $CD8^+$ T cells have been observed compared to adolescents without overweight or obesity.[15]

Adiposity-related inflammation is thought to contribute to the onset of obesity-related morbidity such as insulin resistance.[13, 16] $CD8^+$ T cells are probably contributors to this adiposity-related inflammation in an early phase by production of pro-inflammatory cytokines such as IL-2 which promote T cell proliferation and adipogenesis.[9] In a later phase, the intermediate and non-classical monocytes are thought to contribute to the adiposity-related inflammation through increased TNF-α production.[13, 17, 18] Th1 and Th17 are producers of various pro-inflammatory cytokines, potentially contributing to the pro-inflammatory state in overweight and obesity as the presence of these pro-inflammatory cytokines, such as TNF-α, has previously been associated with the onset of obesity-related morbidity.[12, 16, 19, 20] Regarding γδ T cells, less studies are present but increased differentiation into effector memory γδ T with a decreased antiviral response has been observed in adults with overweight or obesity.[21, 22] Within the γδ T cells, the $V\delta2^+V\gamma9^+$ subset is the most dominant in human blood.[23-25]

The majority of studies on studies adiposity-associated inflammation have been performed in mouse models or human adults. The effects of adipose tissue on the immune system in school-aged children and studies with detailed immune phenotyping and information fat mass distribution in children are not present. Previous studies on the associations between immune cell numbers and adiposity in adults did not account for confounders such as sex, ethnicity, lifestyle factors and socio-economic status.[24]

We hypothesized that higher fat mass in childhood is associated with higher numbers of pro-inflammatory monocytes and higher T effector memory cells in peripheral blood. We here related body composition determinants (BMI, FMI) and body fat distribution determinants (android-to-gynoid fat mass ratio, visceral fat index and liver fat fraction) as determined by DXA and MRI with a detailed set of monocyte and T-cell subsets in 890 10-year-old children of a population-based cohort.

METHODS

Study Design

This cross-sectional study was performed within the Generation R Study, a population-based cohort study from early pregnancy onwards located in Rotterdam, the Netherlands. The design of the study has been previously described in detail.[26] The medical ethical committee of Erasmus University Medical Center approved the study (MEC-2012–165). Written informed consent was collected from all parents or legal guardians of the children. We selected children of whom information on BMI or fat mass measurements by dual-energy X-ray absorptiometry (DXA) and immune cell numbers at 10 years of age were available. The number of children included in the individual analyses is dependent on the availability of information on both exposure and outcome and ranges from 535 to 881 children (Table 5.1).

Body composition measurements

BMI (kg/m^2) was calculated from height and weight measurements at our research center. Next, age- and sex-adjusted BMI standard deviation scores (SDS) were calculated based on Dutch reference growth charts.[27] Children were categorized into BMI categories (underweight (n=44, 4.8%), healthy weight (n=719, 80.9%), overweight (n=107, 12.0%) and obesity (n=20, 2.3%)) based on cutoffs of the International Obesity Task Force.[28] Total fat mass (grams), android fat mass and gynoid fat mass were determined with DXA measurements (GE Lunar iDXA, enCORE software version 12.6; GE Healthcare).[29, 30] A fat mass index (FMI, kg/m^4) was calculated.[31] Android-to-gynoid fat mass ratio was determined by the division of android fat by gynoid fat. The android fat is the central fat mass in the abdomen whereas the gynoid fat mass reflects the fat mass distribution within the hip.[29] MRI was performed to determine visceral fat mass and liver fat fraction. From these measurements, a visceral fat index was calculated by division of visceral fat mass by height^3.[31]

Immune cell measurements

Peripheral blood samples were obtained and stained to obtain absolute numbers of peripheral blood CD45$^+$ and CD3$^+$ T cells using a diagnostic lyse-no-wash protocol (BD Biosciences).[24] Next, 11-color-flow cytometry was performed on LSRII Fortessa (BD Biosciences)

Table 5.1. Number of children included in the individual analyses.

	BMI	FMI	Android-to-gynoid fat mass ratio	Visceral fat index	Liver fat fraction
Monocyte subsets					
Monocytes	881	872	872	576	651
Classical monocytes	878	869	869	575	649
Intermediate monocytes	878	869	869	575	649
Non-classical monocytes	878	869	869	575	649
T-cell subsets					
TCR $\gamma\delta^+$ T cells	855	846	846	557	630
$V\delta2^+V\gamma9^+$ T cells	879	870	870	575	651
$V\delta2^+V\gamma9^+$ T naive	874	866	866	574	649
$V\delta2^+V\gamma9^+$ Tcm	874	866	866	574	649
$V\delta2^+V\gamma9^+$ Tem	874	866	866	574	649
$V\delta2^+V\gamma9^+$ TemRA	874	866	866	574	649
$CD4^+$ T cells	834	825	825	541	614
$CD4^+$ T naive	852	843	843	555	628
$CD4^+$ Tcm	852	843	843	555	628
$CD4^+$ TemRO	852	843	843	555	628
$CD4^+$ TemRA	852	843	843	555	628
Th1 cells	829	821	821	538	611
Th2 cells	828	820	820	537	610
Th17 cells	824	816	816	535	607
Treg cells	818	809	809	537	609
$CD8^+$ T cells	828	820	820	539	612
$CD8^+$ T naive	848	840	840	554	626
$CD8^+$ Tcm	848	840	840	554	626
$CD8^+$ TemRO	848	840	840	554	626
$CD8^+$ TemRA	848	840	840	554	626

The numbers represent the number of children included in the individual analyses.
Abbreviations: BMI, body mass index; FMI, fat mass index; TCR; T cell receptor; Tcm, T central memory; Tem, T effector memory; TemRA, T effector memory RA-positive; TemRO, T effector memory RO-positive; Th, T helper; Treg, T regulatory

with standardize configuration according to Euroflow protocols for detailed immunophenotyping of monocyte and T-cell subset cell numbers.[32, 33]

Monocytes and T cells were defined within the $SSC^{low}CD45^{dim}$ population of $CD45^+$ leukocytes (Figures 5.1-5.2). For monocyte gating, $CD3^+$ T cells and $CD19^+$ B cells were excluded prior to selection for $CD123^-$IREM-2^+HLA-DR^+ cells. Within $CD123^-$IREM-2^+HLA-DR^+ cells, classical ($CD14^+CD16^-$), intermediate ($CD14^+CD16^+$) and non-classical monocytes ($CD14^-CD16^+$) were defined (Figure 5.1).[18]

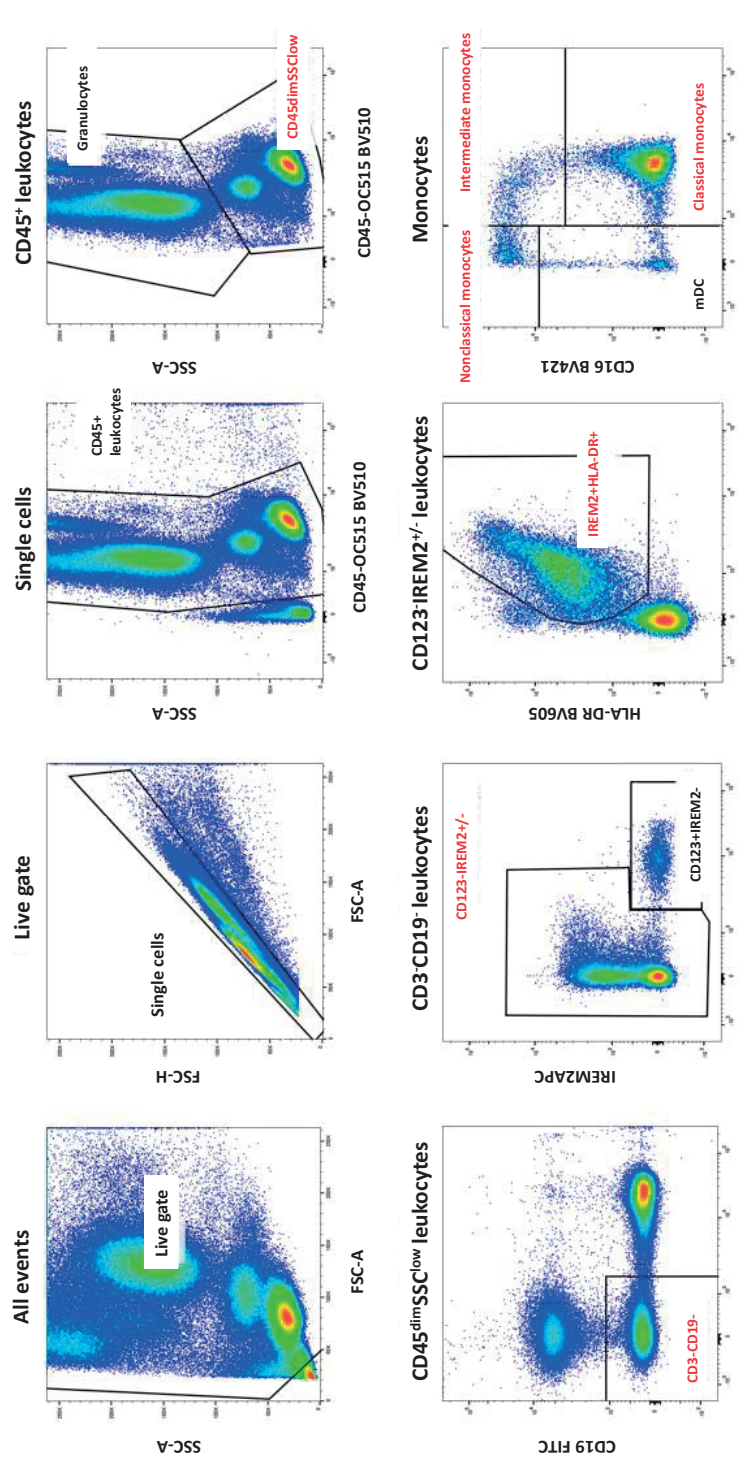

Figure 5.1. Representative sample illustrating the gating strategy used to identify monocyte subsets in peripheral blood using 11-color flow cytometry. Within $CD45^+$ leukocytes $SSC^{low}CD45^{dim}$ populations were defined. After exclusion of $CD3^+$ T cells and $CD19^+$ B cells, $CD123^-IREM\text{-}2^{+/-}$ cells and later $IREM\text{-}2^+HLA\text{-}DR^+$ cells were defined. $IREM\text{-}2^+HLA\text{-}DR^+$ cells included the following monocytes: classical monocytes ($CD14^+CD16^-$), intermediate monocytes ($CD14^+CD16^+$) and non-classical monocytes ($CD14^-CD16^+$). Abbreviations: dim, dimmer; FSC-A, forward scatter area; FSC-H, forward scatter height; IREM, immune receptor expressed on myeloid cells; mDC, myeloid dendritic cells; SSC-A, side scatter area

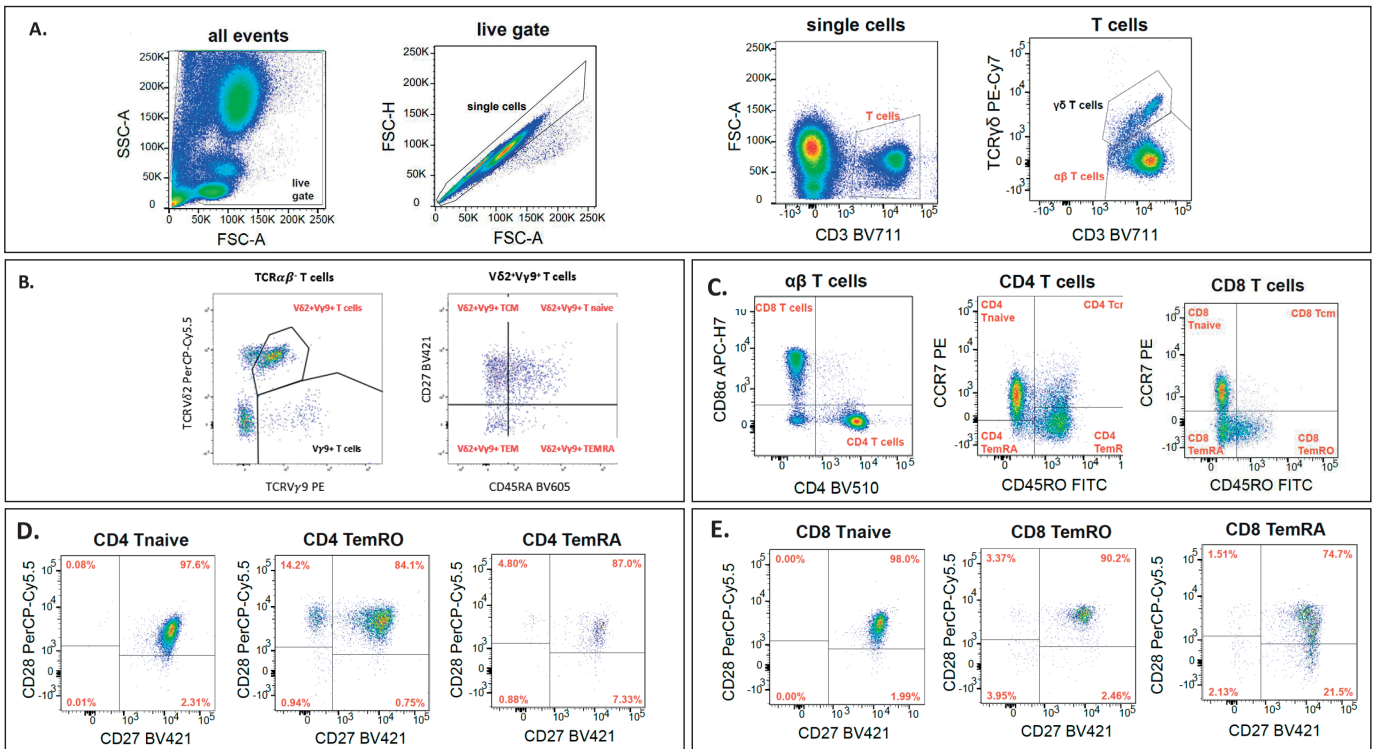

Figure 5.2. Representative sample illustrating the gating strategy used to identify Vδ2^{+}Vγ9^{+} T cell and CD4^{+} and CD8^{+} memory subsets in peripheral blood using 11-color flow cytometry. After exclusion of TCRαβ^{+} T cells, TCRαβ^{-} Vδ2^{+}Vγ9^{+} T cells were defined. Within TCRαβ^{-} Vδ^{2+}Vγ9^{+} T cells, T naive (CD45RA^{+}CD27^{+}) cells, T central memory (Tcm; CD45RA^{-}CD27^{+}) cells, T effector memory (Tem; CD45RA^{-}CD27^{-}) cells and T effector memory RA-positive (TemRA; CD45RA^{+}CD27^{-}) cells were defined. After exclusion of TCRαβ^{-} T cells, the CD4^{+} and CD8^{+} memory Tcm and TemRO and TemRA cells were defined. Abbreviations: FSC-A, forward scatter area; FSC-H, forward scatter height; SSC-A, side scatter area; Tcm, central memory T cell; Tem, effector memory T cell

$V\delta2^+V\gamma9^+$ T cells were defined within the $CD3^+$ $TCR\alpha\beta^-$ cells on the basis of Vδ2 and Vγ9 positivity (Figure 5.2). Within total $V\delta2^+V\gamma9^+$ T cells, naive ($CD45RA^+CD27^+$), central memory (Tcm; $CD45RA^-CD27^+$) cells, effector memory (TemRO; $CD45RA^-CD27^-$) and effector memory RA-positive (TemRA; $CD45RA^+CD27^-$) cell subsets were defined.[34]

Within $TCR\alpha\beta^+CD3^+$ T cells, $CD4^+$ and $CD8^+$ T cell numbers were determined. For each lineage, T naive ($CD45RO^-CCR7^+$) cells, T central memory (Tcm; $CD45RO^+CCR7^+$) cells, T effector memory RO-positive (TemRO; $CD45RO^+CCR7^-$) cells and T effector memory RA-positive (TemRA; $CD45RO^-CCR7^-$) were defined. Within the $CD4^+$ lineage, T regulatory (Treg; $CD4^+CD127^{dim}CD25^{high}$), T helper (Th) 1 ($CCR6^-CXCR3^+CCR4^-$), Th2 ($CCR6^-CXCR3^-CCR4^+$) cells and Th17 ($CCR6^+CXCR3^-CCR4^+$) cell numbers were determined.[33] The used antibodies for the flowcytometry analyses are presented in Table 5.2.

Table 5.2. Antibodies used in flow cytometric analyses.

Antibody	Clone	Fluorochrome	Supplier	Identifier RRID
CD3	OKT3	BV711	BioLegend, San Diego, CA	AB_2875052
CD4	OKT4	BV510	BioLegend, San Diego, CA	AB_2561866
CD8	SK1	APC-H7	BD Biosciences	AB_1645482
CD14	MO-P9	APC-H7	BD Biosciences	AB_1645464
CD16	3G8	BV421	Biolegend, San Diego, CA	AB_2561578
CD19	HIB19	FITC	BD Biosciences	AB_395812
CD21	B-ly4	APC	BD Biosciences	AB_2085309
CD25	BC96	BV421	BioLegend, San Diego, CA	AB_11126749
CD27	O323	BV421	BioLegend, San Diego, CA	AB_11150782
CD28	CD28.2	PerCP-Cy5.5	BioLegend, San Diego, CA	AB_2073718
CD38	HIT2	BV605	BioLegend, San Diego, CA	AB_2562915
CD45	GA90	OC515	Cytognos	AB_2848147
CD45RA	HI100	BV605	BioLegend	AB_2563814
CD45RO	UCHL1	FITC	DAKO, Glostrup, Denmark	AB_578677
CD127	A019D5	APC	BioLegend, San Diego, CA	AB_10900804
CCR4	TG6/CCR4	PECy7	BioLegend, San Diego, CA	AB_2244410
CCR6	G034E3	PerCP-Cy5.5	BioLegend, San Diego, CA	AB_10918437
CCR7	3D13	PE	ThermoFisher Scientific	AB_10670625
CCR10	69036	PE	R&D systems, Minneapolis, MN	AB_2204787
CXCR3	G025H7	FITC	BioLegend, San Diego, CA	AB_10983066
CXCR5	51505	APC	R&D systems, Minneapolis, MN	AB_357109
HLA-DR	L243	BV605	BioLegend San Diego, CA	AB_11219187
IREM2	UP-H2	APC	Immunostep	AB_11140615
TCRαβ	IP26	PE-Cy7	BioLegend San Diego, CA	AB_10639947
TCRγδ	11F2	PECy7	BD Biosciences	AB_2870377
TCRVδ2	B6	PerCP-Cy5.5	BioLegend, San Diego, CA	AB_1877263
TCRVγ9	B3	PE	BioLegend, San Diego, CA	AB_1236408

FacsDIVA software v8 (BD Biosciences, San Jose, USA), Infinicyt software (Cytognos, Salamanca, Spain), and FlowJo software v10 (FlowJo LLC, USA) were used for data analyses. The gating strategies are presented in Figure 5.1-5.2 and in our previous study.[33] Absolute cells per microliter of blood (cells/µl) were calculated by multiplying the percentage of cells of interest by the total leukocyte and T cell numbers obtained from the Trucount analyses.

Covariates

Information on maternal characteristics including prepregnancy BMI (kg/m^2) and educational level (higher versus secondary and primary) was obtained by means of questionnaires obtained during pregnancy.[5, 24] Information on child's sex was obtained from midwife and hospital registries. Postpartum questionnaires retrieved information on ever breastfeeding during the first four months.[33] Child's ethnic background (Western versus non-Western) was defined based on each parent's country of birth.[5, 24, 26, 35] Information on diet quality score and doing sports (>2 versus <2 hours per week) was obtained from questionnaires at respectively ages 8 and 10 years.[35, 36] The food-based diet quality score is the sum of 10 diet components ("fruit", "vegetables", "whole grains", "fish", "legumes", "nuts, "dairy", "oils and soft or liquid margarines", "sugar-containing beverages", "high fat and processed meat") obtained from food frequency questionnaires. The score ranges from 0 to 10 in which a higher score refers to a healthier diet.[36]

Statistical analyses

Characteristics of the study population were determined and represented as median with interquartile range (IQR, 25-75% range) for continuous non-normally distributed variables and mean with standard deviation for continuous normally distributed variables. To gain more insight in the distribution of the characteristics between children without overweight or obesity versus children with overweight or obesity we presented the data for the total group and for those two specific weight groups. For standardization and interpretation purposes, z-scores for the body composition determinants (BMI, FMI) and body fat distribution determinants (android-to-gynoid fat mass ratio, visceral fat index and liver fat fraction) were used. All children had the same age and therefore standardization by age was not necessary. Immune cell numbers were recalculated by adding one because of values equaling zero and natural log-transformed afterwards to obtain a normal distribution.

Multivariable linear regression analyses were conducted to determine associations between body composition and fat distribution and immune cell numbers adjusted for child and maternal confounders. Potential confounders were added if they were associated with immune cells based on our previous study and if they met the epidemiological criteria for confounders in our study, and finally, if the effect estimate changed at least by 10% after addition of the potential confounder.[24] The models were adjusted for maternal prepregnancy BMI, maternal educational level, child's sex, child's ethnicity, breastfeeding during

the first 4 months, food-based diet quality score at 8 years and doing sports. We tested but did not include the following variables because these did not affect the effect estimates of the models: birth weight and gestational age.

Multiple imputation (n=20) using predictive mean matching was performed for missing values on confounders. All measures of association are presented as pooled estimates from the imputed data sets and represent the percentage cell increase or decrease. No differences in value distribution or direction of associations were observed between the non-imputed and imputed dataset. To account for potential multiple testing, a two-sided p-value lower than 0.0125 was considered significant using the Bonferroni method (p=0.05 divided by the four immune cell categories: monocytes, TCRγδ T, $CD4^+$ and $CD8^+$ T cells). The fat mass and immune cell measures are highly correlated and therefore no additional correction for multiple testing was performed. Effect modification by sex and BMI was tested by adding interaction terms to the regression models but not statistically significant (p>0.05) and therefore stratification by sex or BMI was not performed. Statistical analyses were performed using SPSS version 25.0 for Windows (IBM Corp., Armonk, NY, USA) and R version 3.6.1.

RESULTS

Study population characteristics

Children had a median age of 9.8 [IQR 9.7;10.0] years. Children with overweight or obesity were more often girl (63.8% versus 52.6%), had lower food-based diet quality scores (4.4±1.1SD versus 4.64±1.1SD), performed more often <2 hours sports/week (38.5% versus 27.2%) and had less often a Western ethnicity (66.9% versus 86.2%) (all statistically significant with p<0.05) compared to children without overweight or obesity. Mothers of children with overweight or obesity had a higher median prepregnancy BMI (kg/m^2) compared to mothers of children without overweight or obesity (25.2 [IQR 22.1;30.1] versus 22.5 [IQR 20.7;24.4] and had more often a lower educational level (62.2% versus 39.1%). Percentages of missing values within the covariates ranged from 0% to 24.7%. The study population characteristics are presented in Table 5.3.

Adiposity measures do not correlate with changes in monocyte subsets

No associations were observed between BMI, FMI, visceral fat index, android-to-gynoid fat mass ratio or liver fat fraction with total monocyte numbers. When studying the monocyte subsets, namely classical, intermediate and non-classical monocytes, no associations were observed with the adiposity measures (Table 5.4-5.6).

Table 5.3. Details of the study population.

	Total group (N = 890)	Healthy weight (N = 719)*	Overweight/obese (N = 127)*	Missing (%)
Birth and infant characteristics				
Female sex, N (%)	481 (54.0)	378 (52.6)	81 (63.8)	0.0
Western Ethnicity, N (%)	729 (83.3)	610 (86.2)	83 (66.9)	1.7
Breastfeeding during first 4 months, N (%)	631 (90.3)	516 (90.5)	80 (87.0)	21.5
Child characteristics				
Age at follow-up measurements (y)	9.8 [9.7;10.0]	9.8 [9.7;10.0]	9.9 [9.7;10.0]	0.0
Height (cm)	142.4 [138.4; 146.9]	142.2 [138.2; 146.5]	145.7 [140.9; 150.2]	0.0
Food-based diet quality score at 8 years(36)	4.6 (1.1)	4.6 (1.1)	4.4 (1.1)	24.6
Doing sports ≥2 h/wk, N (%)	526 (71.4)	444 (72.8)	56 (61.5)	17.2
Child's body composition				
BMI (kg/m^2)	17.0 [15.8;18.5]	16.8 [15.8;17.8]	21.2 [20.5;23.3]	0.0
Weight status (N, %)				0.0
Underweight*	44 (4.9)	-	-	
Healthy weight*	719 (80.8)	-	-	
Overweight*	107 (12.0)	-	-	
Obese*	20 (2.2)	-	-	
Total fat mass (g)	8464 [6812;11325]	8162 [6749;9925]	16386 [14147;19381]	1.0
Fat mass index (kg/m^4)	2.1 [1.7;2.7]	2.0 [1.7;2.4]	3.7 [3.3;4.2]	1.0
Visceral fat mass (g)	386.7 [306.4;516.2]	373.2 [294.4;477.2]	709.1 [540.1;872.6]	34.7
Liver fat fraction (%)	1.97 [1.69;2.39]	1.94 [1.68; 2.33]	2.54 [2.01; 3.14]	26.1
Android-to-gynoid fat ratio	0.24 [0.20;0.29]	0.23 [0.20;0.27]	0.38 [0.33;0.44]	1.0
Maternal characteristics				
Prepregnancy BMI (kg/m^2)	22.6 [20.7;24.9]	22.5 [20.7;24.4]	25.2 [22.1;30.1]	24.4
Higher educational level (N, %)	500 (59.4)	426 (62.2)	45 (39.1)	5.4

Values are based on the non-imputed dataset and presented as mean (SD) for normally distributed numerical characteristics and as median [25%-75% range] for non-normally distributed numerical characteristics or as numbers (%) for categorical characteristics.

Abbreviations: BMI, body mass index; g, grams; h, hour; kg, kilogram; m, meter; N, number; SD, standard deviation; wk, week.
*Clinical categories based on the International Obesity Task Force age and sex specific BMI cut-offs.(28) Children with underweight were excluded.

Vδ2$^+$Vγ9$^+$ T-cell subsets were higher in children with higher fat measures

1SD higher FMI, android-to-gynoid fat mass ratio or visceral fat index were statistically significantly associated with 8.4% (95%CI 2.0; 15.2), 8.2% (95%CI 2.4; 14.4) and 10.7% (95%CI 3.3; 18.7) higher total Vδ2$^+$Vγ9$^+$T cell number, respectively. No associations for BMI and liver fat mass ratio with Vδ2$^+$Vγ9$^+$ T cells were observed. When studying Vδ2$^+$Vγ9$^+$ T cells, the same directions of associations were observed, but the associations for total Vδ2$^+$Vγ9$^+$ T cells were not driven by a specific subgroup (Table 5.4-5.6).

Table 5.4. Associations between body composition measurements and monocyte and T-cell subsets.

	Body mass index*		**Fat mass index**		**Android-to-gynoid fat mass ratio**	
	% cell change (95% CI)	**p-value**	% cell change (95% CI)	**p-value**	% cell change (95% CI)	**p-value**
Monocyte subsets						
Monocytes	0.66 (-0.54; 1.87)	0.28	2.26 (-1.00; 5.63)	0.18	0.34 (-2.56; 3.32)	0.82
Classical monocytes	0.66 (-0.60; 1.93)	0.31	2.45 (-0.99;6.00)	0.17	0.26 (-2.79; 3.41)	0.87
Intermediate monocytes	0.48 (-1.19; 2.19)	0.57	0.74 (-3.75; 5.43)	0.75	-1.31 (-5.28; 2.82)	0.53
Non-classical monocytes	1.33 (-0.62; 3.33)	0.18	0.72 (-4.45; 6.17)	0.79	1.83 (-2.93; 6.83)	0.46
T-cell subsets						
TCRγδ$^+$ T cells	0.88 (-0.77; 2.55)	0.30	5.58 (1.10; 10.26)	0.014	4.51 (-0.48; 8.71)	0.03
Vδ2$^+$Vγ9$^+$ T cells	1.14 (-1.17; 3.51)	0.34	**8.41 (1.99; 15.23)**	**0.010**$^{\#}$	**8.23 (2.42; 14.37)**	**0.005**$^{\#}$
Vδ2$^+$Vγ9$^+$ T naive cells	2.67 (-0.20; 5.62)	0.07	7.94 (0.16; 16.31)	0.046	8.49 (1.35; 16.13)	0.02
Vδ2$^+$Vγ9$^+$ Tcm cells	-0.081 (-2.96; 2.88)	0.96	7.50 (-0.42; 16.06)	0.06	5.03 (-2.04; 12.61)	0.17
Vδ2$^+$Vγ9$^+$ Tem cells	-0.63 (-2.62; 1.40)	0.54	2.28 (-3.01; 7.86)	0.41	3.10 (-1.76; 8.20)	0.22
Vδ2$^+$Vγ9$^+$ TemRA cells	1.36 (-0.53; 3.28)	0.16	4.22 (-0.83; 9.52)	0.10	5.05 (0.40; 9.90)	0.03
CD4$^+$ T cells	0.04 (-0.99; 1.07)	0.94	0.97 (-1.74; 3.76)	0.49	-0.23 (-2.65; 2.25)	0.86
CD4$^+$ T naive cells	-0.58 (-1.82; 0.67)	0.36	-1.19 (-4.43; 2.17)	0.48	-1.84 (-4.77; 1.18)	0.23
CD4$^+$ Tcm cells	0.12 (-1.56; 1.84)	0.89	2.68 (-1.85; 7.43)	0.25	1.79 (-2.28; 6.04)	0.39
CD4$^+$ TemRO cells	0.74 (-1.01; 2.53)	0.41	3.06 (-1.64; 7.97)	0.21	-0.05 (-4.11; 4.18)	0.98
CD4$^+$ TemRA cells	2.31 (-0.73; 5.45)	0.14	6.49 (-1.69; 15.36)	0.12	2.61 (-4.49; 10.24)	0.48
Th1 cells	1.49 (-0.74; 3.78)	0.19	5.28 (-0.70; 11.63)	0.09	3.31 (-2.03; 8.94)	0.23
Th2 cells	0.62 (-1.10; 2.36)	0.48	3.24 (-1.32; 8.00)	0.17	-1.28 (-5.23; 2.83)	0.54
Th17 cells	0.70 (-1.38; 2.83)	0.51	2.67 (-2.82; 8.47)	0.35	-2.01 (-6.77; 2.99)	0.42
Treg cells	-0.12 (-1.31; 1.09)	0.85	1.02 (-2.13; 4.28)	0.53	0.07 (-2.74; 2.97)	0.96
CD8$^+$ T cells	0.98 (-0.13; 2.10)	0.09	3.75 (0.70; 6.89)	0.02	1.24 (-1.46; 4.02)	0.37
CD8$^+$ T naive cells	0.62 (-0.81; 2.07)	0.40	2.84 (-1.10; 6.94)	0.16	0.32 (-3.19; 3.95)	0.86
CD8$^+$ Tcm cells	-0.59 (-1.85; 3.10)	0.63	3.99 (-2.69; 1.12)	0.25	2.38 (-3.56; 8.68)	0.44
CD8$^+$ TemRO cells	1.79 (-0.13; 3.76)	0.07	**7.35 (2.40; 12.54)**	**0.003**$^{\#}$	3.95 (-0.42; 8.52)	0.08
CD8$^+$ TemRA cells	2.31 (0.22; 4.45)	0.03	5.91 (0.26; 11.89)	0.04	3.15 (-1.90; 8.46)	0.23

Abbreviations: CI, confidence interval; SD, standard deviation; TCR; T cell receptor; Tcm, T central memory; Tem, T effector memory; TemRA, T effector memory RA-positive; TemRO, T effector memory RO-positive; Th, T helper; Treg, T regulatory. The numbers represent the percentage increase or decrease in absolute cell number per microliter of blood (95% CI) per SD increase in body composition measurements. Numbers are based on multivariable linear regressions and adjusted for the following covariates: maternal prepregnancy BMI, maternal educational level, child's sex, child's ethnicity, breastfeeding during the first 4 months, food-based diet quality score at 8 y and playing sports.

* BMI is sex and age adjusted. $^{\#}$ Statistically significant after correction for multiple testing (four independent tests), p<0.0125

Table 5.5. Associations between visceral fat mass and liver fat fraction and monocyte and T-cell subsets.

	Visceral fat index		Liver fat fraction	
	% cell change (95% CI)	p-value	% cell change (95% CI)	p-value
Monocyte subsets				
Monocytes	3.54 (-0.07; 7.27)	0.06	0.41 (-2.80; 3.73)	0.81
Classical monocytes	3.63 (-0.17; 7.58)	0.06	0.50 (-2.89; 4.01)	0.78
Intermediate monocytes	3.82 (-1.17; 9.06)	0.14	-2.55 (-6.80; 1.89)	0.26
Non-classical monocytes	4.24 (-1.63; 10.45)	0.16	0.80 (-4.35; 6.21)	0.77
T-cell subsets				
TCRγδ$^+$ T cells	5.31 (0.21; 10.68)	0.04	4.89 (0.35; 9.64)	0.04
Vδ2$^+$Vγ9$^+$ T cells	**10.72 (3.29; 18.69)**	**0.004**$^{\#}$	7.51 (1.07; 14.36)	0.02
Vδ2$^+$Vγ9$^+$ T naive cells	11.13 (2.19; 20.86)	0.014	8.42 (0.66; 16.78)	0.03
Vδ2$^+$Vγ9$^+$ Tcm cells	10.21 (1.03; 20.22)	0.03	3.16 (-4.46; 11.38)	0.43
Vδ2$^+$Vγ9$^+$ Tem cells	3.64 (-2.38; 10.03)	0.24	1.50 (-3.77; 7.05)	0.59
Vδ2$^+$Vγ9$^+$ TemRA cells	6.78 (0.89; 13.01)	0.02	3.97 (-1.16; 9.38)	0.13
CD4$^+$ T cells	0.80 (-2.26; 3.96)	0.61	1.73 (-1.07; 4.61)	0.23
CD4$^+$ T naive cells	-0.33 (-4.08; 3.57)	0.87	0.41 (-3.00; 3.93)	0.82
CD4$^+$ Tcm cells	0.46 (-4.44; 5.61)	0.86	1.21 (-3.30; 5.94)	0.61
CD4$^+$ TemRO cells	4.33 (-0.82; 9.74)	0.10	2.55 (-2.04; 7.35)	0.28
CD4$^+$ TemRA cells	2.30 (-6.26; 11.65)	0.61	0.92 (-6.84; 9.33)	0.82
Th1 cells	6.07 (-0.38; 12.93)	0.07	-0.31 (-5.92; 5.64)	0.92
Th2 cells	0.19 (-4.72; 5.35)	0.94	1.53 (-3.03; 6.31)	0.52
Th17 cells	-0.70 (-6.51; 5.47)	0.82	**-8.88 (-13.75; -3.73)**	**0.001**$^{\#}$
Treg cells	2.24 (-1.22; 5.83)	0.21	1.15 (-2.04; 4.45)	0.48
CD8$^+$ T cells	4.42 (0.87; 8.09)	0.014	1.24 (-1.84; 4.41)	0.44
CD8$^+$ T naive cells	3.79 (-0.72; 8.49)	0.10	1.57 (-2.46; -2.46)	0.45
CD8$^+$ Tcm cells	5.54 (-1.91; 13.55)	0.15	0.94 (-5.48; 7.79)	0.78
CD8$^+$ TemRO cells	**8.33 (2.59; 14.38)**	**0.004**$^{\#}$	**6.60 (1.63; 11.82)**	**0.009**$^{\#}$
CD8$^+$ TemRA cells	2.59 (-3.45; 9.00)	0.41	3.39 (-2.14; 9.23)	0.24

Abbreviations: CI, confidence interval; SD, standard deviation; TCR; T cell receptor; Tcm, T central memory; Tem, T effector memory; TemRA, T effector memory RA-positive; TemRO, T effector memory RO-positive; Th, T helper; Treg, T regulatory

The numbers represent the percentage increase or decrease in absolute cell number per microliter of blood (95% CI) per SD increase in fat mass measurements. Numbers are based on multivariable linear regressions and adjusted for the following co-variates: maternal prepregnancy BMI, maternal educational level, child's sex, child's ethnicity, breastfeeding during the first 4 months, food-based diet quality score at 8 y and playing sports.

$^{\#}$ Statistically significant after correction for multiple testing (four independent tests), p<0.0125

CD8$^+$ TemRO cells are increased in children with higher FMI, visceral fat index and liver fat fraction

None of the fat mass measures were correlated with total CD8$^+$ T cells. Yet, higher FMI, visceral fat index and liver fat fraction were all statistically significantly associated with higher CD8$^+$TemRO cells: 7.4% (95%CI 2.4;12.5), 8.3% (95%CI 2.6;14.4) and 6.6% (95%CI 1.6;11.8) higher for each SD increase in the respective fat measure. Android-to-gynoid fat mass ratio was not associated with CD8$^+$ T cell numbers. 1SD increase in liver fat fraction

was associated with 8.8% (95%CI -13.8;-3.7) lower Th17 cell numbers. Other adiposity measures were not associated with Th17. No associations were observed between memory $CD4^+$ T cell numbers and adiposity measures (Table 5.4-5.6).

Table 5.6. Associations children with overweight and obese and monocyte and T-cell subsets compared to children with normal weight.

	Healthy weight* (N=719)	**Overweight and obese* (N=127)**	
	% cell change (95% CI)	% cell change (95% CI)	**p-value**
Monocyte subsets			
Monocytes	REF	5.20 (-3.16; 14.27)	0.23
Classical monocytes	REF	5.52 (-3.31;15.15)	0.23
Intermediate monocytes	REF	3.48 (-7.95; 16.20)	0.56
Non-classical monocytes	REF	5.48 (-7.85; 20.73)	0.44
T-cell subsets			
TCRγδ$^+$ T cells	REF	6.83 (-4.43; 19.41)	0.25
Vδ2$^+$Vγ9$^+$ T cells	REF	14.51 (-2.15; 33.99)	0.09
Vδ2$^+$Vγ9$^+$ T naive cells	REF	13.51 (-6.52; 37.69)	0.20
Vδ2$^+$Vγ9$^+$ Tcm cells	REF	14.07 (-6.38; 38.97)	0.19
Vδ2$^+$Vγ9$^+$ Tem cells	REF	6.10 (-7.44; 21.62)	0.40
Vδ2$^+$Vγ9$^+$ TemRA cells	REF	12.36 (-1.20; 27.79)	0.08
CD4$^+$ T cells	REF	-4.11 (-10.53; 2.77)	0.24
CD4$^+$ T naive cells	REF	-7.52 (-15.11; 0.75)	0.07
CD4$^+$ Tcm cells	REF	1.49 (-9.63; 13.98)	0.80
CD4$^+$ TemRO cells	REF	-5.13 (-15.50; 6.52)	0.37
CD4$^+$ TemRA cells	REF	-3.44 (-21.00; 18.03)	0.73
Th1 cells	REF	0.031 (-14.02; 16.38)	1.00
Th2 cells	REF	0.52 (-10.53; 12.93)	0.93
Th17 cells	REF	3.70 (-9.96; 19.43)	0.61
Treg cells	REF	-3.11 (-10.71; 5.13)	0.45
CD8$^+$ T cells	REF	1.99 (-5.49; 10.08)	0.61
CD8$^+$ T naive cells	REF	-0.76 (-10.23; 9.71)	0.88
CD8$^+$ Tcm cells	REF	10.97 (-6.11; 31.16)	0.22
CD8$^+$ TemRO cells	REF	5.20 (-7.10; 19.13)	0.42
CD8$^+$ TemRA cells	REF	-1.00 (-13.98; 13.92)	0.89

Abbreviations: CI, confidence interval; SD, standard deviation; TCR; T cell receptor; Tcm, T central memory; Tem, T effector memory; TemRA, T effector memory RA-positive; TemRO, T effector memory RO-positive; Th, T helper; Treg, T regulatory

The numbers represent the percentage increase or decrease in absolute cell number per microliter of blood (95% CI) when comparing children with overweight or obesity to children with normal weight. Numbers are based on multivariable linear regressions and adjusted for the following covariates: maternal prepregnancy BMI, maternal educational level, child's sex, child's ethnicity, breastfeeding during the first 4 months, food-based diet quality score at 8 y and playing sports.

*Clinical categories based on the International Obesity Task Force age and sex specific BMI cut-offs.(28) Children with underweight were excluded.

DISCUSSION

The present study determined the association between adiposity and immune cell numbers in a general population of school-aged children with adjustment for several confounders. In this population, we showed that higher total FMI, visceral fat index and android-to-gynoid fat mass ratio were associated with higher numbers of circulating $V\delta2^+V\gamma9^+$ T cells. Higher total FMI, visceral fat index and liver fat fraction were associated with higher $CD8^+$ T_{EMRO} cell numbers. Only higher liver fat fraction was associated with lower peripherally circulating Th17 cell numbers. No associations were observed between adiposity and monocytes or memory $CD4^+$ T cell numbers in children of the general population.

Previous literature and interpretation

Previous studies on fat mass and $CD8^+$ T cells showed that mice with obesity had higher $CD8^+$ T cells in adipose tissue.[9, 37] This increase in $CD8^+$ T cell numbers in adipose tissue and blood has also been observed in adults and adolescents who are overweight or obese.[12, 15, 37-39] However, we did not observe associations between BMI and immune cell numbers. We extended previous literature by determining fat mass by DXA and MRI as these are more accurate in determining fat mass composition and fat mass distribution compared to anthropometric measures.[31] Indeed, we did observe associations for fat mass: positive associations were observed for FMI, visceral fat index and liver fat fraction with total $CD8^+$ and $CD8^+_{TEMRO}$ cell numbers. The observed higher number of $CD8^+$ T_{EM} cells in our study and previous studies could be a direct effect of the increased fat mass. A previous study in adipose tissue of adults showed that $CD8^+$ T cell infiltration, and especially the effector memory subset, might be the first immune cell appearance in adiposity-induced inflammation.[9, 38] The effector $CD8^+$ T cells lack CCR7 expression and are able to directly migrate towards infected or inflamed tissues to execute their effector function.[40] It is thought that this effector function of the $CD8^+$ T_{EMRO} cells further triggers the inflammatory cascade that is observed in adipose tissue by production of pro-inflammatory cytokines and the subsequent attraction of monocytes.[38] Based on mice studies, this initiation and maintenance of inflammation by effector $CD8^+$ T cell is thought to play a role in obesity-related morbidity such as insulin resistance.[38, 41, 42] In adults with metabolic syndrome, increased differentiation of $CD8^+$ T cells has been observed.[43] Yet, the persistency and dynamics of the observed immune profile over time in relation to adiposity in children and the long-term effects of these immune alterations are topics for future study.

In contrast to previous studies, we did not observe associations between BMI or other fat mass measures and monocytes.[9, 13, 14, 16, 38, 44-46] Previous studies showed that monocyte infiltration and macrophage accumulation in adipose tissue occurred after the $CD8^+$ T cell recruitment in adipose tissue.[9, 38] Therefore, it could be hypothesized that we here observed

an earlier phase of adiposity-related inflammation as the population of this study is comprised of school-aged children with only 2.3% children with obesity. However, some studies also observed monocyte increase in children.[14, 45, 46] Age could explain this difference as two studies included children up to 16 years old and one study children up to 18 years old, whereas our study included 10-year-old children. The relatively low number of children with obesity might also be an explanation as one study only observed the higher monocyte number in children with obesity.[46]

In addition to the classical αβT cells, we examined $\gamma\delta^+$ T cell numbers and the predominant $V\delta2^+V\gamma9^+$ subset.[25] These $V\delta2^+V\gamma9^+$ T cells have innate-like features and are activated by phosphoantigens through butyrophilin 3A, independent of MHC.[25] Following activation, $V\delta2^+V\gamma9^+$ cells contribute to the host's defense against pathogens by the production of IFN-γ. However, increased numbers of $\gamma\delta^+$ T cells in adipose tissue have been associated with inflammation and insulin resistance in mice.[21] Still, insights into $\gamma\delta^+$ T cells, specifically the $V\delta2^+V\gamma9^+$ subset in relation to adiposity is scarce. A study in 15 adults with obesity showed a lower percentage of $V\delta2^+V\gamma9^+$ T cells than a non-obese control group.[22] We observed in contrast to this previous study higher numbers of $V\delta2^+V\gamma9^+$ T cells in adipose children of the general population.[22] This can possibly be explained by the fact that this previous study studied relative immune cell numbers in contrast to absolute numbers as was used in our study. Yet, this previous study did observe higher numbers of effector memory $V\delta2^+V\gamma9^+$ T cells.[22] The exact role of $V\delta2^+V\gamma9^+$ T in metabolic diseases remains to be studied.[47] Increased differentiation of $V\delta2^+V\gamma9^+$ T cells has been associated with decreased IFN-γ responses and thereby a reduced host's defense against viral antigens.[22, 48] This might contribute to the increased susceptibility of overweight individuals to severe or persistent viral infection, e.g. with SARS-CoV2 in COVID-19 patients.[8]

Overall, we observed the strongest associations for visceral fat index with immune cells and no associations between BMI and immune cells. These observations are similar to previous literature that stresses the importance of determining fat mass distribution rather than body mass composition in defining populations at risk for adverse health effects of high fat mass.[12, 49, 50] Namely, it has been shown that adipose tissue is actively involved in various metabolic processes which vary upon the location of the tissue.[10, 51] Especially the viscerally located white adipose tissue, has been associated with systemic low-grade inflammation.[12, 51] Measures of fat mass distribution that we used were android-to-gynoid fat mass ratio, visceral fat index and liver fat fraction, all indicators of centrally located fat mass Previously, higher android-to-gynoid fat mass ratio in children was shown to be a risk for developing metabolic syndrome independent of BMI status.[52] Likewise, both visceral and liver fat have been associated with adverse metabolic outcomes in children.[53, 54] Therefore, measuring fat

mass quantity and distribution might be more specific indicators for low-grade inflammation in adiposity.

Overall, we observed consistent results for $V\delta2^+V\gamma9^+$ T and $CD8^+_{TEMRO}$ across different fat measurements. However, specifically for liver fat, decreased Th17 cells were observed. This might reflect an increased recruitment of Th17 towards liver fat, which has been observed previously in non-alcohol fatty liver disease (NAFLD).[55, 56] Possibly, excess liver fat mainly causes local inflammation in an early stage, ultimately leading to systemic inflammation.[55] In NAFLD it has been shown that especially Th17 cells cause liver damage and fibrosis progression.[57] Further studies are needed because conflicting results about Th17 are present as there are also studies that have shown increased circulating Th17 in adult patients with NAFLD.[56] It is currently unknown what the clinical relevance of our Th17 observation in relation to adiposity could be already at child age.

The pathophysiology of adiposity-related inflammation likely comprises of a combination of factors: initiation of adipocytes cell death caused by hypertrophy , hypoxia due to insufficient vascularization of expanded adipose tissue, oxidative stress of the adipocytes leading to production of pro-inflammatory adipokines such as leptin.[58] It is thought that these chronic inflammatory triggers result in T cell exhaustion and premature immunosenescence.[59] However, this is an area that needs further study, especially in children with adiposity. The reduced functionality of exhausted T cells could underlie the increased incidence of infection and cancer observed in patients with obesity.[48, 59] Importantly, weight loss following bariatric surgery was observed to reverse the premature immunosenescence in patients with obesity.[43] This T-cell plasticity following reversibility of adiposity-related inflammation underscores the importance of early detection of adiposity in children with appropriate lifestyle intervention.

Methodological considerations

This study included a large cohort of school-aged children with comprehensive datasets of body composition measures and immune cell numbers and detailed information on important confounders such as socio-economic status and lifestyle factors. Adiposity was studied in detail by both BMI and fat mass measures determined by DXA and MRI, both accepted as accurate measures for determining body fat distribution.[31, 60]

Despite this unique setting, we still encountered several limitations and considerations. First, we did not study fat mass and immune cells over time, which prevented us from drawing conclusions about the direction of the associations or about the effect of fat mass change over time on immune cell numbers. Second, validation of MRI and DXA as fat mass composition and fat distribution measures is needed in large populations of children.[60] Although

both MRI and DXA are increasingly used and generally accepted in determining fat mass distribution in children, most validation studies have been performed in adults.[60] Third, although we used extensive multiparameter flow cytometry, we were unable to include the surface marker CCR7 into the γδT cell subset tube to more accurately distinguish between naive and T_{EMRA} subsets of $V\delta2^+V\gamma9^+$ T cells.[61, 62] Fourth, we could not study the adiposity measures in children with obesity specifically because of the limited number of children within this group. Fifth, the variability of fat mass amounts and BMI was limited due to the small number of children with obesity within our cohort. The low variability of BMI is relatively unique and could explain the lack of observations for BMI within our study. Lastly, because this is an observational study, there is a chance of residual confounding for example by unmeasured dietary or lifestyle factors.

Conclusion

Adiposity in school-aged children of a general population was associated with higher $V\delta2^+V\gamma9^+$ and $CD8^+$ T cells, whereas specifically higher liver fat was associated with lower Th17 cell numbers. These results might indicate that fat-associated inflammation is already present at young age.

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III

Genetic determinants of immune cell composition

6 Increased Th22 cell numbers in a general pediatric population with filaggrin haploinsufficiency.

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ABSTRACT

Background

Mutations in the filaggrin gene (FLG) affect epidermal barrier function and increase the risk of atopic dermatitis (AD). We hypothesized that FLG mutations affect immune cell composition in a general pediatric population. Therefore, we investigated if school-aged children with and without FLG mutations have differences in in T- and B-cells subsets.

Methods

This study was embedded in a population-based prospective cohort study, the Generation R Study, and included 523 children of European genetic ancestry aged 10 years. The most common FLG mutations in the European population (R501X, S1085CfsX36, R2447X and S3247X) were genotyped. Additionally, 11-color flow cytometry was performed on peripheral blood samples to determine helper T (Th), regulatory T (Treg) and CD27+ and CD27- memory B cells. Subset analysis was performed in 358 non-AD and 102 AD cases, assessed by parental questionnaires.

Results

FLG mutations were observed in 8.4% of the total population and in 15.7% of the AD cases. Children with any *FLG* mutation had higher Th22 cell numbers compared to *FLG* wild-type children in the general and non-AD population. Children with and without *FLG* mutations had no difference in Th1, Th2, Th17, Treg or memory B cell numbers. Furthermore, in children with AD, *FLG* mutation carriership was not associated with differences in T- and B-cell subsets.

Conclusion

School-aged children of a general population with *FLG* mutations have higher Th22 cell numbers, which reflects the immunological response to the skin barrier dysfunction. *FLG* mutations did not otherwise affect the composition of the adaptive immunity in this general pediatric population.

INTRODUCTION

Filaggrin is a filament-associated protein that is encoded by the filaggrin gene (*FLG*) and is an important contributor to the preservation of the skin barrier.[1,2] Approximately 10% of the European population is a heterozygote carrier of a disrupting mutation in *FLG*.[3] Both complete loss-of-function and reduced functional activity of filaggrin lead to destruction of the stratum corneum (SC) and consequently skin barrier dysfunction.[1,4] This barrier dysfunction due to *FLG* mutations is presumed to be caused by lower numbers of tight junctions, reduced density of the protein corneodesmosin and impaired maturation and excretion of lamellar bodies in the epidermis which are important in maintaining cell-to-cell integrity.[2]

Failure in barrier function through mutations in *FLG* results in increased skin permeability for percutaneous transfer of exogenous particles including allergens and pollutants.[1,2,4] Accordingly, *FLG* mutations are the strongest genetic risk factor for atopic dermatitis (AD).[1,3,5] A previous meta-analysis showed that *FLG* haploinsuffiency results in an odds ratio (OR) of 3.12 for the incidence of AD.[6] In addition, *FLG* mutations are associated with a form of AD that starts in early infancy and persists into adulthood, a higher incidence of skin infections and a higher likelihood of having asthma, inhalant or food allergies.[2,7-9]

The increased permeability of the skin as a result of *FLG* mutations is thought to affect immune responses and maturation of adaptive immune cells. Filaggrin is also expressed in the thymus, the primary lymphoid organ in which T cells are formed.[10] Hence, *FLG* mutations potentially affect the peripheral immune cell compartment through effects in skin and thymus, and previous studies observed higher γδT17 and T helper (Th) 17 in filaggrin-deficient flaky tail (ft/ft) mice.[10] In addition, a case study reported higher numbers of circulating thymus-emigrated regulatory T (Treg) cells and Th2 in 6 AD patients with a heterozygote *FLG* mutation.[11] Another study, including 2 heterozygous, 2 homozygous and 1 compound heterozygous AD patient, showed increased Th17 cells in *FLG*-mutation group.[12] On the other hand, literature on the role of B cell dysregulation in AD is scarce and conflicting.[13-16]. It can be hypothesized that mutations in *FLG* can affect B-cell numbers due to skewing of the Th cell populations.

We hypothesized that *FLG* mutations affect T- and B-cell maturation in children through effects on the skin and thymus. Until now, no studies on this association have been performed in the general pediatric population and only case studies have been performed in AD patients.[11,12] It is of interest to examine the role of *FLG* mutations in adaptive immune maturation in both a general population, a non-AD, and AD population to further understand the role of *FLG* in the immune maturation. Therefore, we here studied the associations

between common *FLG* mutations in the European population and immune cell numbers, as determined by 11-color flow cytometry, within a population-based birth cohort study including a subgroup non-AD and AD patients.

METHODS

Study design

This study was embedded within the Generation R Study, a prospective birth cohort study conducted in Rotterdam, the Netherlands. The Medical Ethical Committee of the Erasmus MC, University Medical Center Rotterdam approved the study (MEC-2012-165).[17] Written informed consent was obtained from parents or legal representatives of all children. We included all children with European genetic ancestry [18] with information on *FLG* mutation (homozygous, compound heterozygous, heterozygous or wild type) and information on at least one of the immune cell outcomes. This resulted in a total number of 523 children (Figure 6.1). AD was defined as physician-diagnosed eczema from parental questionnaires obtained at the child's age of 10 years ('Was your child ever diagnosed by a physician with atopic dermatitis', 'yes; no'). This information was available for 470 children, including 102 subjects with AD.[16]

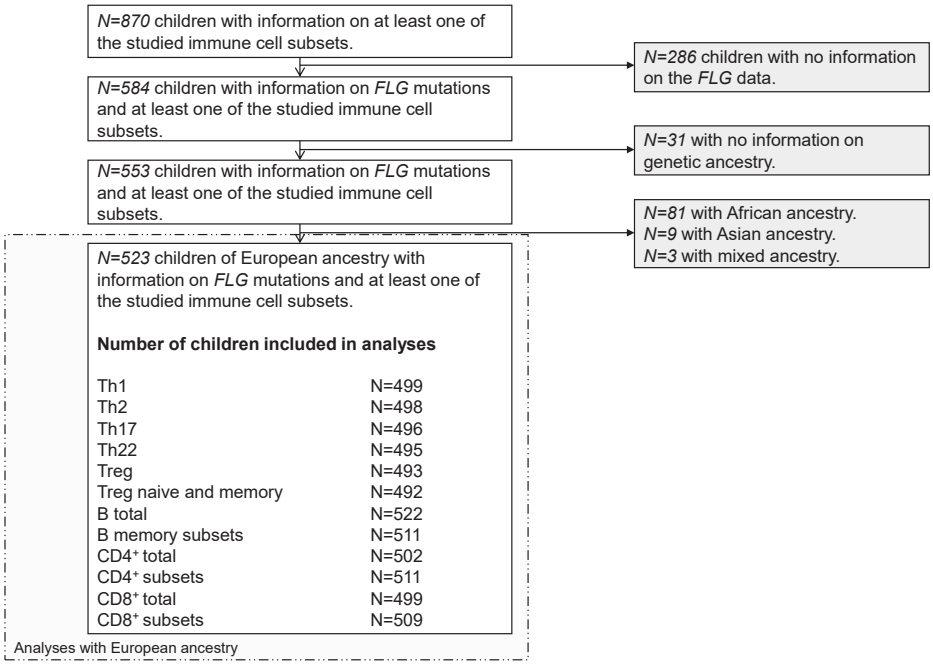

Figure 6.1. Flowchart of study population selection
Abbreviations: FLG, filaggrin gene; N, number; Th, helper T cell; Treg, regulatory T cell.

FLG genotype

DNA samples obtained from umbilical cord blood were genotyped by modified Taqman allelic discrimination assays for common European mutations in *FLG* (R501X (rs61816761), S1085CfsX36 (rs41370446), R2447X (rs138726443), and S3247X (rs150597413) with the use of primers as described previously.[19,20] The distribution of the *FLG* mutations was as follows: 4.2% had R501X (rs61816761), 1.1% had S1085CfsX36 (rs41370446), 3.5% had R2447X (rs138726443), and 0.0% had S3247X (rs150597413). Because S3247X (rs150597413) was not present within our study population, this mutation was not included in the analyses. Children were classified as having a *FLG* mutation if they were homozygous, compound heterozygous or heterozygous for any of the three mutations that were present in our study population. Children without any of the mutations were classified as wild type.

Immune cell numbers

Peripheral blood samples from children were obtained at the age of 10 years.[16] The analyses were performed on fresh blood cells within 24 hours of sampling. Absolute counts of $CD3^+$ T cells and $CD19^+$ B cells per µLblood were determined with diagnostic lyse-no-wash protocol and detailed immunophenotyping was performed with 11-color flow cytometry (LSR Fortessa, BD Biosciences). We determined naive ($CD45RO^-CCR7^+$), effector memory RO-positive T cells (TemRO; $CD45RO^+CCR7^-$) and effector memory RA-positive T cells (TemRA; $CD45RO^-CCR7^-$) within $CD4^+$ and $CD8^+$ lineages.[16,21,22] Within Treg cells, the differentiation in naive ($CD45RA^+$) and memory ($CD45RA^-$) was determined.[16] Finally, the following T helper (Th) cell subsets ($CD4^+CD45RA^-$) were determined after exclusion of Treg cells on the basis of chemokine receptor profiles as defined previously:[16,23-27] Th1 ($CCR6^-CXCR3^+CCR4^-$), Th2 ($CCR6^-CXCR3^-CCR4^+$), Th17 ($CCR6^+CXCR3^-CCR4^+CCR10^-$), Th17.1 ($CCR6^+CXCR3^+CCR4^-$) and Th22 ($CCR6^+CXCR3^-CCR4^+CCR10^+$). In addition $CD27^+$ and $CD27^-$ IgG^+, IgA^+, IgE^+ $CD19^+CD38^{dim}IgD^-$ memory B-cell subsets were defined.[16] Gating strategies for Th cell determination are presented in Supplementary Figure 6.3

Statistical Analyses

First, characteristics of the study population were determined, stratified for *FLG* mutation status and AD diagnosis. P-values for determining differences between the categorical variables of both groups were calculated with chi-squared tests. Next, median cell numbers with interquartile range (IQR) were determined. Differences in cell numbers between children with and without *FLG* mutations were determined with the non-parametric Mann-Whitney U tests. Subset analyses on the associations of *FLG* genotype with immune cell numbers were performed within non-AD and AD children. The possibility of effect modification by AD diagnosis was tested by performing linear regression analyses between immune cell numbers and *FLG* mutation status with the addition of an interaction term between *FLG* mutation status and AD diagnosis. To assure a normal distribution of the outcome in

the linear regression analysis, a natural-log transformation for the immune cell outcomes was used. No adjustment for multiple testing was performed because of strong correlation between the immune cells studied. Statistical analyses were performed with SPSS version 21.0 (IBM Corp.) and R version 3.6.1 (R Foundation for Statistical Computing).

RESULTS

Study population characteristics

Characteristics of the study population are presented in Table 6.1. Within the total group of 523 children with European ancestry, *FLG* mutations were detected in 44 (8.4%) children, includ-

Table 6.1. Study population characteristics.

		Total population				Subset analyses	
Child characteristics	**Total (n=523)**	**Wildtype population (n=479)**	***FLG* mutation population (n=44)**	**P-value**	**Missing in total study population (N, %)**	**Atopic dermatitis (n=102)**	**Non-atopic dermatitis (n=358)**
Sex (N, %)				1.0	0.0		
Female	280 (53.5)	256 (53.4)	24 (54.5)			48 (47.1)	192 (53.6)
Male	243 (46.5)	223 (46.6)	20 (45.5)			54 (52.9)	166 (46.4)
FLG mutations (N, %)				--	0.0		
Wildtype	479 (91.6)	479 (100.0)	--			86 (84.3)	336 (93.9)
1 or more mutations	44 (8.4) †	--	44 (100.0)			16 (15.7)[2]	22 (6.1)[1]
Type *FLG* mutations (N, %)							
S1085CfsX36 (rs41370446)	22 (4.2)	--	22 (4.2)	--	0.0	7 (6.9)	12 (3.4)
R2447X (rs138726443)	6 (1.1)	--	6 (1.1)	--	0.2	1 (1.0)	4 (1.1)
R501X (rs61816761)	18 (3.5)	--	18 (3.5)	--	0.4	9 (9.0) ‡	7 (2.0)
S3247X (rs150597413)	0 (0.0)	--	0 (0.0)	--	0.4	0 (0.0)	0 (0.0)
Ever physician diagnosed atopic dermatitis (N, %)§	102 (22.2)	86 (20.4)	16 (42.1)	**0.004**	12.0	102 (100.0)	0 (0.0)

Table 6.1 represents the child and maternal characteristics for the study population stratified for *FLG* mutations. Values are based on the non-imputed dataset and represented as number (%). Chi-squared tests were conducted to examine possible differences in baseline characteristics between the different genotypes.

Abbreviations: *FLG*, filaggrin gene; N, number;

† Including 3 biallelic mutations (2 compound heterozygous and 1 homozygous)

‡ Including one biallelic mutations (homozygous)

§ Based on parental-reported questionnaires obtained at the child's age of 10 years: ever atopic dermatitis (no or yes)

[1] 1 compound heterozygous biallelic mutation

[2] 1 compound heterozygous and 1 homozygous mutation

ing 3 biallelic mutations (2 compound heterozygous and 1 homozygous). The proportion of patients with AD was lower in the wild-type group compared to the group with *FLG* mutations (20% versus 42%; p<0.01). Within the non-AD population, 6.1% of the children had a *FLG* mutation, including one compound heterozygous. Within the AD population, 15.7% of the children had a *FLG* mutation, including one homozygous and one compound heterozygous.

Higher Th22 cell numbers in children of the general population with *FLG* mutations

Children of the general population with a *FLG* mutation had higher Th22 cell numbers compared to children of the wild-type population (Figure 6.2A, Supplementary Table 6.1). The median cell number within the *FLG* mutation group was 5.60/μL (IQR 4.04;8.94) and 4.5/μL (IQR 2.5;7.4, p=0.03) within the wild-type group. To determine if this association between Th22 and *FLG* was different between children with and without AD, we performed a linear regression analysis with the following interaction term: *FLG* mutation status*AD. This interaction term was non-significant (p=0.13) and therefore effect modification by AD in this association is not likely. However, when the analyses were stratified, the association between *FLG* and Th22 was slightly stronger in the non-AD group with a median cell number of 6.8/μL (IQR 4.9;11.4) in non-AD children with *FLG* mutations compared to 4.5/μL (IQR 2.4;7.5, p=0.006) in non-AD children without *FLG* mutations (Supplementary Table 6.2). In contrast, when studying the differences in absolute Th22 cell numbers between children with and without *FLG* mutations in the AD population, no significant differences were observed (median 5.0/μL (IQR 3.6;6.4) and 4.6/μL (IQR 2.9;7.4) respectively (p=0.64, Supplementary Figure 6.1A)).

No associations between Th1, Th2, Th17 and Treg and *FLG* mutations

No differences in median cell numbers between *FLG* mutation and wild-type group were observed for Th1, Th2, Th17 and Treg (Figure 6.2A, Supplementary Table 6.1). This was similar in the subset analyses that were stratified for AD diagnosis (Supplementary Figure 6.1A, Supplementary Table 6.2). No differences in median cell numbers between the *FLG* mutation and the wild-type group were observed for the effector memory $CD4^+$ and $CD8^+$ T-cell subsets: naive, Tcm, TemRA, TemRO (Figure 6.2B, Supplementary Figure 6.1B).

No associations between memory B cells and *FLG* mutations

No differences in median cell numbers between the *FLG* mutation and the wild-type group were observed for total B cells and naive mature B cells (Supplementary Table 6.1). In addition, no associations between *FLG* mutations and the following $CD27^+$ and $CD27^-$ memory B-cell subsets were observed: IgA^+, IgE^+, IgG^+, IgM^+ (Figure 6.3). Similarly, no changes were observed in the subset analyses that stratified the analyses for AD diagnosis (Supplementary Figure 6.2, Supplementary Table 6.2).

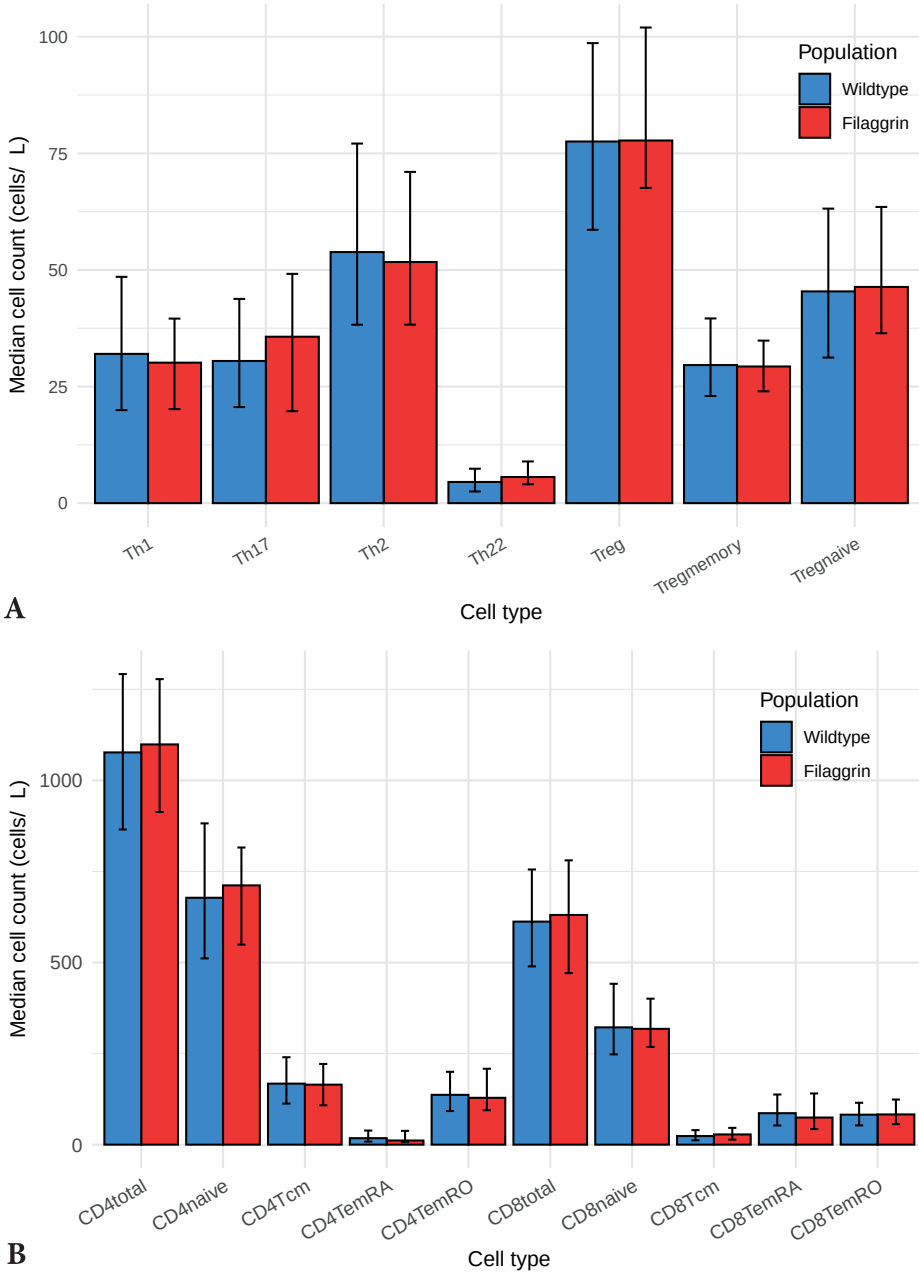

Figure 6.2. Absolute numbers of blood T-cell subsets stratified by FLG mutation status.

A. The median (IQR) cell count per µL blood for Th and Treg cell numbers stratified for FLG mutation.

B. The median (IQR) cell count per µL blood for $CD4^+$ and $CD8^+$ effector memory T-cell numbers stratified for FLG mutation. Abbreviations: IQR, interquartile range; Tcm, central memory T lymphocytes; TemRA, effector memory RA-positive T lymphocytes; TemRO, effector memory RO-positive T lymphocytes; Th, helper T cell; Treg, regulatory T cell. * denotes a two-sided P-value <0.05. Supplementary Table 6.1 shows the absolute numbers and P-values.

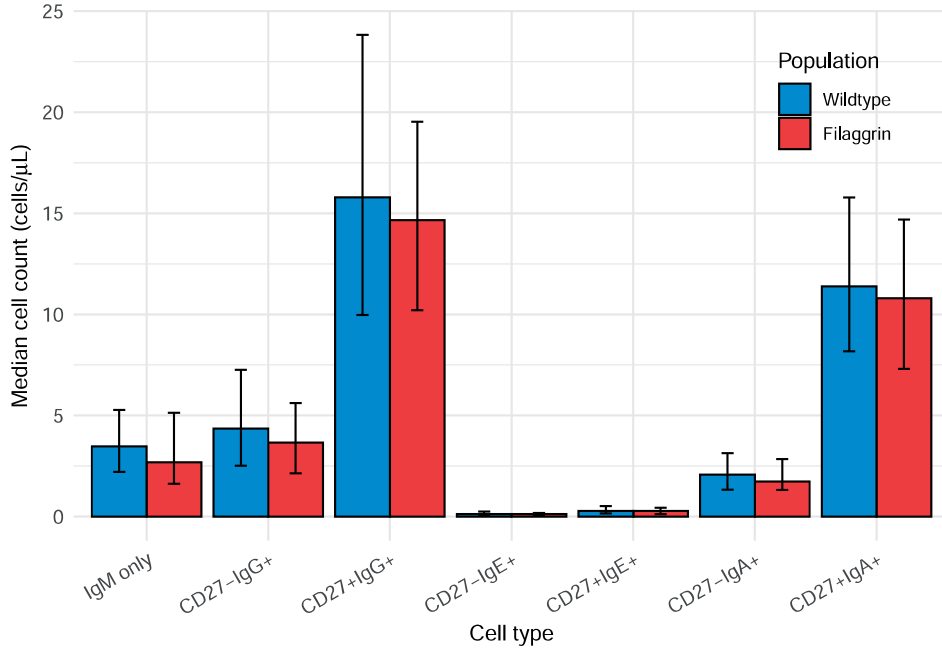

Figure 6.3. Absolute numbers of blood memory B-cell subsets stratified by FLG mutation status.
The median (IQR) cell count per µL blood for B memory cell numbers stratified for FLG mutation is shown. Abbreviations: IQR, interquartile range; Ig, Immunoglobulin. Supplementary Table 6.1 shows the absolute numbers and P-values.

DISCUSSION

In this population-based study among children of European genetic ancestry, we observed a prevalence of 8.4% for *FLG* mutations. In addition, we demonstrated that children with *FLG* mutations had higher Th22 cell numbers than children without *FLG* mutations. In contrast, the Th1, Th2, Th17, Treg and memory B cell numbers were comparable between children with and without *FLG* mutations. In addition, among children with AD, those with or without *FLG* mutations had no differences in B- or T-cell subsets.

Comparison with literature and interpretation

All previous studies on *FLG* mutations and immune cell numbers have been performed within mice models or smaller numbers of AD patients.[10-12,28] This is the first study that provides insight in the role of *FLG* mutations on immune cell numbers in school-aged children of a general population. The setting of this study within a population-based pediatric cohort study is unique to study the association of *FLG* on immune cell numbers in a general population.

We observed higher Th22 cell numbers in children with *FLG* mutations in the general populations, with a slightly higher median Th22 cell number in non-AD children with a mutation in *FLG*. No previous studies that assess the association between *FLG* mutations and Th22 have been performed within a general population. Interestingly, in contrast to our findings, several studies have observed increased Th22 cell numbers in the skin and circulation of patients with AD.[29,30] Within AD patients, the role of Th22 is still not fully elucidated. Current literature suggests both protective and pro-inflammatory roles for Th22 by the production of IL-22.[31] IL-22 contributes to skin integrity and is known for its role in the defense against different pathogens in the skin by the production of antimicrobial proteins.[32,33] However, the combined secretion of IL- 22 and TNF-α is thought to have an pro-inflammatory effect as observed in AD.[31] Possible explanations for the higher number of Th22 cell numbers in children without AD but with *FLG* mutations could be the following. First, the increase in Th22 could represent some level of inflammation due to *FLG* mutations without apparent clinical symptoms. Second, hypothetically, the increase in Th22 could contribute to skin homeostasis in children without AD to prevent further inflammatory processes leading to AD. However, further studies are needed to elaborate on the Th22 cell function in children with and without AD.

In contrast to previous studies investigating the effect of *FLG* mutation status, we did not observe differences in Th2, Th17 and Treg cell numbers between children with and without *FLG* mutations both in the total study population and in the subgroup of patients with AD. [10-12,28] The discrepancies between previous studies and our current study could be explained by differences in investigated populations and species. Previous mice studies and skin equivalents studied the effect of complete absence of filaggrin, compared to the filaggrin haploinsufficiency in our study population which leads to 50% reduction in filaggrin expression.[2,10,28] In addition, these studies could represent a different immunological setting than is present in human skin. [28] It is also expected that previous results on immune cell numbers in AD populations are affected by disease severity. Namely, the presence of different immune cells is dependent on disease state, including disease flare and chronic AD.[29,30] In turn, immune cells in active AD skin can induce downregulation of filaggrin protein expression in the skin independent of *FLG* mutations, subsequently affecting immune cell composition.[20] Although we do not have information on disease severity in our AD population, this study included a population-based, relatively healthy cohort in which we expect most children to have mild AD. Therefore, alteration in immune cell numbers is probably not only dependent on *FLG* mutation genotype, but also on AD severity and epigenetic and environmental factors.

In addition, we did not observe differences in memory B cell numbers between children with and without *FLG* mutations. This is in line with our previous study in which we did

not observe any association between B cells and AD.[16] No previous studies have investigated B-cell subsets in relation to *FLG* mutations.

Finally, within our study, we studied the associations between *FLG* mutations and the adaptive immunity. It can be speculated that *FLG* mutations cause alterations in the innate immunity such as eosinophilic granulocytes and ILC2 cell numbers. Future studies are needed to determine whether *FLG* mutations are associated with altered innate immunity cell numbers.

Methodological considerations

A major strength is that this study investigated the association between *FLG* genotype and a large panel of B and T cells in the general population for the first time. We had detailed and extensive information on immune cell numbers from 11-color flow cytometry and obtained objective information on genetic ancestry. However, the following four limitations need to be addressed. First, we used chemokine receptor profiles which are surrogate markers of Th cells. Due to the large scale of our study and the need to process fresh blood within 24 hours, in vitro activation and cytokine staining were not feasible. Importantly, multiple studies have shown that the use of surface chemokine receptors is a robust approach to define Th subsets with the corresponding cytokine profiles.[23,24] Second, the AD population for the subset analyses was relatively small which could have limited the power in the statistical analyses. Nevertheless, in comparison to previous studies, only including a maximum of 6 AD patients with *FLG* mutations, this is the largest study on *FLG* mutations in both the general population and AD patients. Third, our AD population was defined by ever-having physician-diagnosed AD before or at the age of 10 years and no information on current disease activity was available. Therefore, it is likely that a subset of the children has outgrown AD at the age of 10 and this might affect their immunophenotype. Fourth, as mentioned previously, our study included the four most common *FLG* mutations in the European population. To prevent misclassification, we selected children with genetic European ancestry for the current study. Although the choice for including the most common *FLG* mutations in European populations is in line with previous studies [11,12], other less frequent *FLG* mutations could exist in low numbers since up to 113 *FLG* mutations resulting in premature protein termination have been described. A recent study including patients with AD and Ichthyosis Vulgaris (IV), showed that screening the entire encoding region of *FLG* for mutations led to an improvement of the diagnostic yield. [34] As this is the first study in a general cohort addressing the association between *FLG* mutation and immune cell numbers, future studies are needed for validation of our results.

Conclusion

In conclusion, school-aged children of a general population with *FLG* mutations have higher Th22 cell numbers, which might reflect the skin barrier dysfunction that is caused by decreased filaggrin expression in the epidermis. In our study population, *FLG* mutations do not otherwise affect the composition of T and B cells in a general pediatric population, nor in the children with AD.

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SUPPLEMENTARY MATERIALS

Supplementary Table 6.1. The median (IQR) cell counts per µL blood for T and B cell numbers stratified by *Filaggrin Gene* (*FLG*) mutation status.

	Total study population (n=523)		
	Wild type (n=479)	***FLG* mutation (n=44)**	**P-value[1]**
Th1	32 (20.0-48.5)	30.1 (20.2-39.6)	0.31
Th2	53.8 (38.3-77.1)	51.7 (38.3-71.0)	0.99
Th17	30.5 (20.6-43.8)	35.7 (19.7-49.2)	0.13
Th22	4.5 (2.5-7.4)	5.6 (4.04-8.9)	**0.03**
Treg	77.5 (58.6-98.6)	77.8 (67.6-102.0)	0.48
Treg naive	45.4 (31.2-63.2)	46.4 (36.4-63.5)	0.25
Treg memory	29.6 (23.0-39.6)	29.3 (24.0-34.9)	0.74
Total B cells	478.5 (370.0-632.0)	493.0 (389.5-575.8)	0.69
B naive	303.1 (228.9-416.6)	323.2 (245.6-366.9)	0.90
IgM only	3.5 (2.2-5.27)	2.7 (1.6-5.1)	0.06
$CD27^-IgG^+$	4.4 (2.5-7.3)	3.7 (2.1-5.6)	0.14
$CD27^+IgG^+$	15.8 (10.0-23.8)	14.7 (10.2-19.5)	0.29
$CD27^-IgE^+$	0.1 (0.1-0.3)	0.1 (0.1-0.2)	0.69
$CD27^+IgE^+$	0.3 (0.2-0.5)	0.3 (0.1-0.4)	0.33
$CD27^-IgA^+$	2.1 (1.3-3.1)	1.7 (1.3-2.8)	0.26
$CD27^+IgA^+$	11.4 (8.2-15.8)	10.8 (7.3-14.7)	0.30
$CD4^+$ total	1076.8 (865.2-1291.9)	1098.8 (913.2-1278.1)	0.76
$CD4^+$ naive	677.8 (511.3-882.0)	711.8 (549.2-815.8)	0.49
$CD4^+$ Tcm	167.5 (112.8-240.0)	164.8 (108.1-221.5)	0.41
$CD4^+$ TemRO	136.8 (92.4-200.0)	128.6 (94.6-208.4)	0.75
$CD4^+$ TemRA	17.8 (8.3-38.8)	11.4 (7.3-37.9)	0.39
$CD8^+$ total	612.3 (489.4-755.5)	630.5 (471.1-780.6)	0.96
$CD8^+$ naive	322.1 (247.9-441.6)	317.9 (268.3-400.8)	0.94
$CD8^+$ Tcm	23.6 (11.8-39.9)	28.1 (14.0-46.2)	0.37
$CD8^+$ TemRO	82.3 (52.9-115.0)	83.0 (56.3-123.9)	0.45
$CD8^+$ TemRA	86.4 (52.7-137.8)	74.5 (42.8-140.6)	0.45

[1] Two-sided P-value determined by Mann-Withney U test · Abbreviations: Ig, immunoglobulin;IQR, interquartile range; Tcm, central memory T lymphocytes; TemRA, effector memory RA-positive T lymphocytes; TemRO, effector memory RO-positive T lymphocytes; Th, helper T cell; Treg, regulatory T cell.

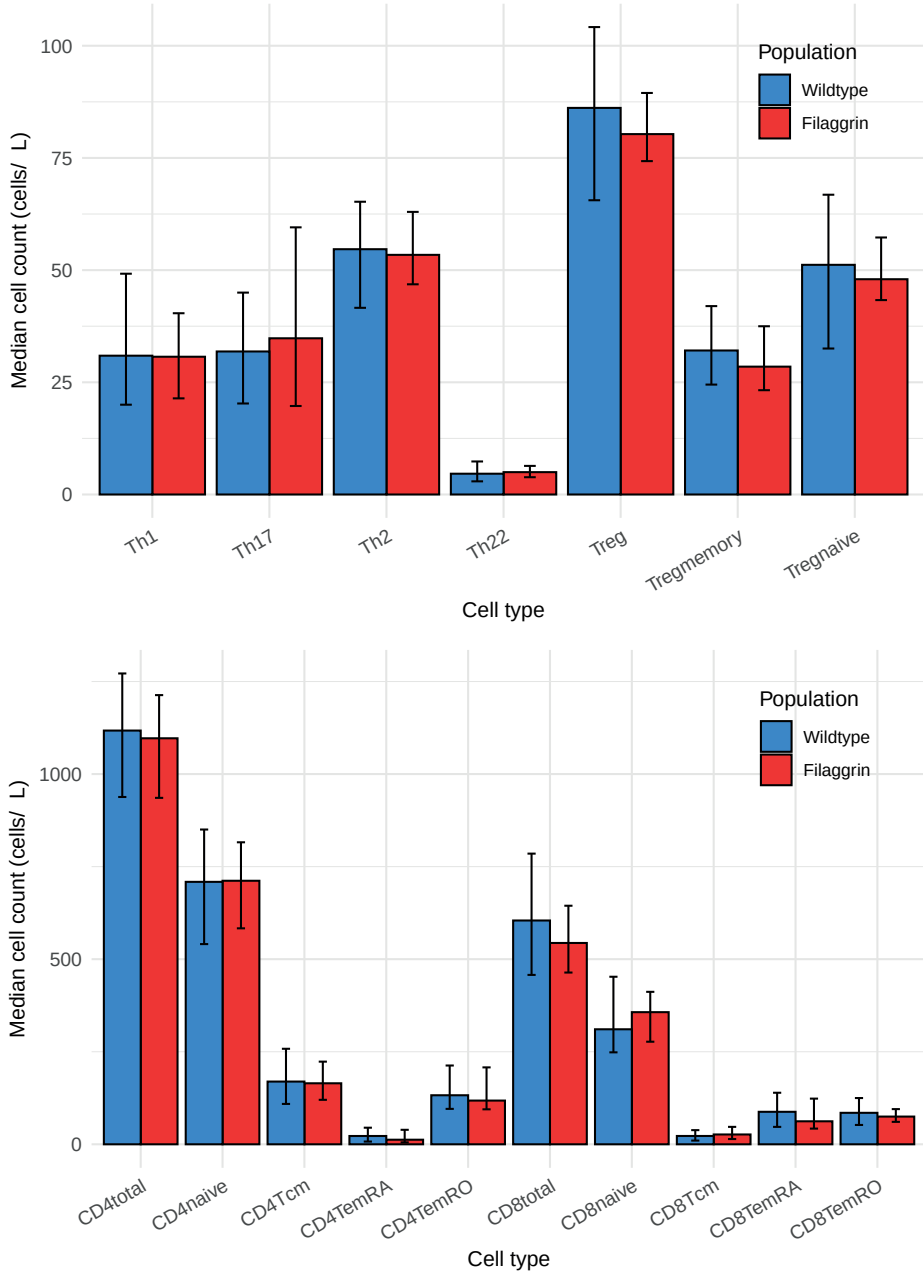
Supplementary Table 6.2. The median (IQR) cell counts per µL blood for T and B cell numbers stratified by *Filaggrin Gene* (*FLG*) mutation status divided into the non-atopic dermatitis and atopic dermatitis subset.

	Non atopic dermatitis population (n=358)			Atopic dermatitis (n=102)		
	Wild type (n=393)	***FLG* mutation (n=28)**	**P-value[1]**	**Wild type (n=86)**	***FLG* mutation (n=16)**	**P-value[1]**
Th1	31.3 (18.3-47.3)	31.6 (24.3-44.5)	0.79	30.9 (20.0-49.2)	30.7 (21.4-40.4)	0.43
Th2	54.0 (37.1-78.9)	44.5 (37.4-69.0)	0.42	54.7 (41.6-65.24)	53.4 (46.8-63.0)	0.76
Th17	30.4 (20.7-42.9)	34.6 (23.3-47.0)	0.24	31.9 (20.3-45.0)	34.8 (19.7-59.5)	0.51
Th22	4.50 (2.4-7.5)	6.8 (4.9-11.4)	**0.006**	4.6 (2.9-7.4)	5.0 (3.6-6.4)	0.64
Treg	76.0 (57.0; 95.8)	75.4 (66.3-107.0)	0.36	86.2 (65.6-104.2)	80.3 (74.3-89.5)	0.68
Treg naive	44.8 (31.2-61.7)	46.1 (36.2-68.0)	0.24	51.2 (32.5-66.8)	48.0 (43.3-57.3)	0.80
Treg memory	28.4 (21.9-37.5)	29.6 (26.1-33.7)	0.67	32.1 (24.5-42.0)	28.5 (23.2-37.5)	0.43
Total B cells	481.0 (375.5-628.5)	505.0 (407.3-590.8)	0.75	463.0 (360.0-604.3)	446.5 (332.5-545.0)	0.51
B naive	300.9 (231.1-416.9)	333.5 (255.3-403.2)	0.55	306.4 (212.4-407.4)	280.9 (198.0-363.3)	0.50
IgM only	3.4 (2.1-5.3)	2.5 (2.0-4.8)	0.24	3.4 (2.3-5.1)	3.0 (1.9-5.2)	0.52
$CD27^-IgG^+$	4.4 (2.5-7.4)	3.8(2.3-5.8)	0.49	3.4 (2.3-5.5)	3.3 (2.3-5.5)	0.93
$CD27^+IgG^+$	15.7 (9.9-23.6)	15.3 (11.5-21.6)	0.94	14.9 (10.3-20.0)	15.1 (10.5-18.9)	0.78
$CD27^-IgE^+$	0.1 (0.1-0.3)	0.1 (0.1-0.2)	0.64	0.1 (0.1-0.2)	0.1 (0.1-0.2)	0.63
$CD27^+IgE^+$	0.3 (0.2-0.5)	0.3 (0.1-0.5)	0.63	0.3 (0.1-0.4)	0.3 (0.2-0.4)	0.94
$CD27^-IgA^+$	2.1 (1.4-3.1)	1.8 (1.2-2.8)	0.28	1.9 (1.9-2.8)	1.6 (1.4-3.5)	0.95
$CD27^+IgA^+$	2.1 (1.4-3.1)	1.8 (1.2-2.8)	0.81	11.3 (8.4-15.1)	11.1 (7.8-14.9)	0.76
$CD4^+$ total	1073.3 (866.1-1288.3)	1098.9 (901.8-1309.2)	0.49	1117.5 (938.1-1271.8)	1096.6 (935.8-1213.3)	0.82
$CD4^+$ naive	667.0 (512.1-864.9)	773.5 (587.8-832.7)	0.25	708.9 (540.8-850.2)	711.8 (583.3-815.8)	0.92
$CD4^+$ Tcm	166.6 (111.0-237.0)	158.1 (107.1-218.5)	0.65	169.4 (109.0-258.0)	164.8 (120.1-223.3)	0.85
$CD4^+$ TemRO	136.8 (89.1-199.2)	149.7 (91.2-216.1)	0.47	132.5 (95.7-212.8)	118.1 (94.5-207.7)	0.78
$CD4^+$ TemRA	16.8 (8.7-36.5)	11.4 (8.4-37.4)	0.54	22.4 (7.4-44.9)	12.4 (5.3-39.2)	0.54
$CD8^+$ total	597.2 (507.3-747.3)	681.9 (587.5;824.6)	0.19	604.5 (457.5-785.1)	543.8 (463.9-644.4)	0.53
$CD8^+$ naive	321.1 (246.1-431.6)	300.6 (267.9-369.6)	0.93	310.7 (248.4-452.6)	356.9 (277.1-411.8)	0.72
$CD8^+$ Tcm	24.2 (11.9-40.3)	26.3 (14.2-44.6)	0.79	22.5 (10.0-38.2)	26.6 (14.2-47.1)	0.38
$CD8^+$ TemRO	81.1 (52.5-112.2)	96.1 (56.3-133.9)	0.25	85.0 (52.6-125.0)	74.9 (60.6-95.0)	0.69
$CD8^+$ TemRA	84.4 (50.2-136.1)	79.6 (60.9-135.2)	0.72	87.8 (47.1-139.2)	62.1 (42.4-123.4)	0.30

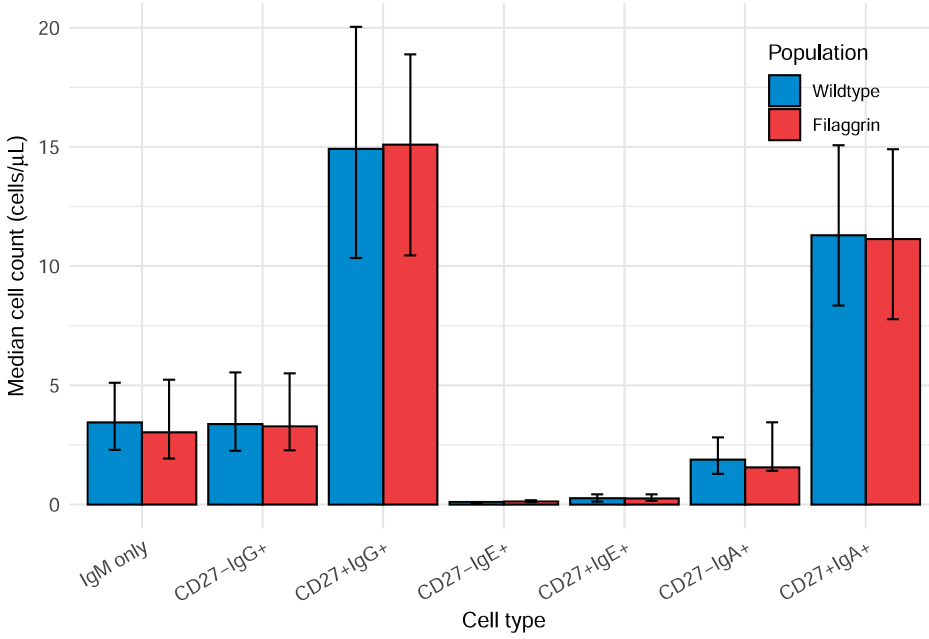
[1] Two-sided P-value determined by Mann-Withney U test · *Abbreviations*: Ig, immunoglobulin; IQR, interquartile range; Tcm, central memory T lymphocytes; TemRA, effector memory RA-positive T lymphocytes; TemRO, effector memory RO-positive T lymphocytes; Th, helper T cell; Treg, regulatory T cell.

Supplementary Table 6.3. Number of children included in the individual analyses.

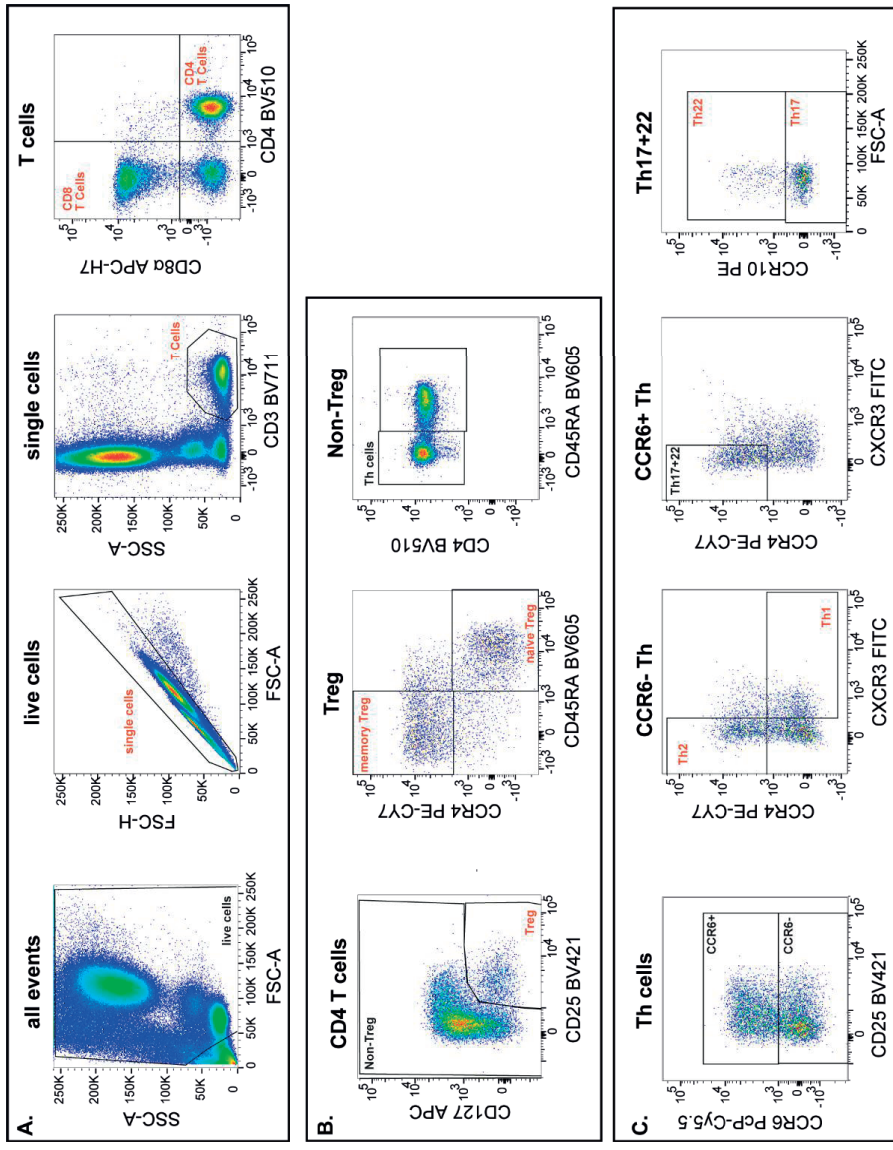
	Total population (n=523)	Atopic dermatitis (n=102)	Non-atopic dermatitis (=358)
$CD4^+$ T cells	502	101	339
$CD4^+$ T naive	511	102	347
$CD4^+$ Tcm	511	102	347
$CD4^+$ TemRO	511	102	347
$CD4^+$ TemRA	511	102	347
Th1 cells	499	101	336
Th2 cells	498	101	335
Th17 cells	496	100	335
Th22 cells	495	100	334
Treg cells	493	98	334
Treg naive	492	98	334
Treg memory	492	98	334
$CD8^+$ T cells	499	101	337
$CD8^+$ T naive	509	102	345
$CD8^+$ Tcm	509	102	345
$CD8^+$ TemRO	509	102	345
$CD8^+$ TemRA	509	102	345
Total B cells	522	102	357
B naive	512	102	347
IgM only	511	102	346
$CD27^-IgG^+$	511	102	346
$CD27^+IgG^+$	511	102	346
$CD27^-IgE^+$	511	102	346
$CD27^+IgE^+$	511	102	346
$CD27^-IgA^+$	511	102	346
$CD27^+IgA^+$	511	102	346



Supplementary Figure 6.1A-B. Absolute numbers of blood T cell subsets stratified by *FLG* mutation status in children with atopic dermatitis. (A-B) The median (IQR) Th, Treg, $CD4^+$ and $CD8^+$ Tem cell count per µL blood stratified for *FLG* mutation. Abbreviations: Tcm, central memory T-lymphocytes; Tem, effector memory T-lymphocytes; Th, helper T-cell; Treg, regulatory T-cell. *P-value<0.05. Supplementary Table 6.2 shows the absolute numbers and P-values.



Supplementary Figure 6.2. Absolute numbers of blood memory B cell subsets stratified by *Filaggrin Gene* mutation status in children with atopic dermatitis. The median (IQR) cell count per µL blood for B memory cell numbers stratified for *FLG* mutation. Abbreviations: IQR, interquartile range. Supplementary Table 6.2 shows the absolute numbers and P-values.



Supplementary Figure 6.3: Gating strategy T helper subsets

7

T cell composition and polygenic multiple sclerosis risk: A population-based study in children.

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**Denotes equal contribution*

ABSTRACT

Background

Patients with multiple sclerosis (MS) have altered T cell function and composition. Common genetic risk variants for MS affect proteins that function in the immune system. It is currently unclear to what extent T cell composition is affected by genetic risk factors for MS, and how this may precede a possible disease onset. Here, we aim to assess whether an MS polygenic risk score (PRS) is associated with an altered T cell composition in a large cohort of children from the general population.

Methods

We included genotyped participants from the population-based Generation R study in whom immunophenotyping of blood T cells was performed at the age of 6 years. Analyses of variance were used to determine the impact of MS-PRSs on total T cell numbers (n = 1261), $CD4^+$ and $CD8^+$ lineages, and subsets therein (n= 675). In addition, T-cell-specific PRSs were constructed based on functional pathway data.

Results

The MS-PRS negatively correlated with $CD8^+$ T cell frequencies ($p = 2.92\times10^{-3}$), which resulted in a positive association with $CD4^+/CD8^+$ T cell ratios ($p = 8.27\times10^{-9}$). These associations were mainly driven by two of 195 genome-wide significant MS risk variants: the main genetic risk variant for MS, HLA-DRB1*15:01 and an HLA-B risk variant. We observed no significant associations for the T-cell-specific PRSs.

Conclusion

Our results suggest that MS-associated genetic variants affect T cell composition during childhood in the general population.

INTRODUCTION

The exact pathophysiology of the autoimmune response in MS remains to be elucidated, and results from a complex interplay between genetic and environmental risk factors that contribute to disease risk. Migration studies suggest a pivotal time-window during childhood in which environmental risk factors (e.g. Epstein-Barr virus [EBV] or cytomegalovirus [CMV] seropositivity, and serum 25-hydroxyvitamin-D [25(OH)D]) contribute to MS risk.[1,2]

Genome-wide association studies (GWAS) have identified 233 genetic variants (single nucleotide polymorphisms, SNPs) that significantly affect disease susceptibility in adult patients.[3] These studies have shown that the genetic risk of MS is polygenic, with a large number of genetic variants that each have a small effect on disease risk.[3] However, the majority of people with high genetic risk for MS are never diagnosed with the disease.[4] How these risk variants together affect biological mechanisms culminating in a higher risk of developing MS and at which time points during the lifespan are unknown. To capture this combined effect of genetic risk variants, polygenic risk score (PRS) analyses can be used.[5]

T cells are suggested to be the main immune lineage that are directly or indirectly influenced by genetic MS risk variants.[3] Several studies have described altered T cell homeostasis in MS patients, with increased $CD4^+/CD8^+$ ratios that decrease during treatment.[6,7]

Here, we aimed to determine how PRSs for MS are associated with the distribution of naive, memory and activated $CD4^+$ and $CD8^+$ T cell populations in 6-year-old children from the general population. We assessed the main genetic variants driving these associations and the relationship between overall genetic MS risk and EBV and CMV seropositivity, as well as serum 25(OH)D levels.

METHODS

Study design

This study was performed in the context of the population-based Generation R study (n=9,749), located in the Netherlands, which investigates various aspects of childhood development.[8] At the age of 6-years, absolute numbers of total T cells were measured in 3,465 participants, and detailed phenotyping of T-cell subsets was performed in 1,079 participants.[9] In the current study, we included unrelated participants of European ancestry, who had high-quality genotype data available (Figure 7.1).

The study protocol of the Generation R study has been approved by the Medical Ethical Committee of the Erasmus Medical Center in Rotterdam. The legal representatives of the children provided written informed consent.

Genotype data

Sample collection of the genetic data and genotype calling procedures have been reported in previous work.[10] Genotype data were collected at birth, derived from cord blood, or during a visit to the research center, and genotyped on Illumina 610K and 660K SNP arrays. Information on the quality control of the genetic data, the imputation method and the calculation of principal components can be found elsewhere.[11] To summarize, we selected subjects of European ancestry based upon the first four principal components inside the range of the HapMap Phase II Northwestern European founder population.[12] Furthermore, we imputed the genotype data using 1000 Genomes (Phase I version 3) data to calculate our PRSs.[13]

Polygenic scoring

We used a large discovery GWAS for MS (N=41,505; 14,802 cases/26,703 controls), carried out by the International Multiple Sclerosis Genetics Consortium (IMSGC: http://imsgc.net/publications/), using imputed genotype data to estimate weighted PRSs.[3] We used PRSice 2,[14] an R-script to calculate clumped ($r^2 < 0.10$, kB window = 250kB) and p-value thresholded polygenic scores in PLINK (v1.9),[15] for the computation of our PRSs. We used various p-value thresholds (P_T<0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1) in the calculation of the PRSs to include the effect of suggestive variants and capture the polygenic architecture of MS.[3] In addition, we computed a MS-PRS only incorporating the genome-wide risk variants ($P_T<5\times10^{-8}$) from the final meta-analysis of the IMSGC (47,429 cases/68,374 controls).

Because of the strong effect of the major histocompatibility complex (MHC) region on MS pathophysiology,[3] three sets of PRSs were calculated: one with all SNPs included, one excluding SNPs from the MHC region and one only including SNPs from the MHC. To characterize the presence of *HLA-DRB1*15:01,* we used rs3135388 as tag SNP.[16] Rs9266629 was included in our analyses as tag SNP for *HLA-B* genetic risk variant rs3819284. In addition, we used rs9268839, rs1057149 and rs2187688 as tag SNPs for *HLA-DRB* variants of importance in rheumatoid arthritis, diabetes mellitus type 1 and systemic lupus erythematosus to investigate possible confounding of the T cell distribution.[17,18]

Due to the low minor allele frequency (<0.20) of rs3135388 and rs9266629, we dichotomized their carriership into 2 groups, homozygous non-risk and at-risk participants. At-risk participants consisted of heterozygous and homozygous carriers of the effect allele.

Construction of T cell specific PRSs

In a final analysis, we used cis expression quantitative trait loci effect data (cis-eQTL) to associate the MS genome-wide risk variants with particular T-cell subsets, providing us with the possibility to construct PRSs specific for these subsets.

$CD4^+$ and $CD8^+$ T-cell-specific PRSs were constructed by assessing whether a locus implicated by a SNP[3] had high mRNA expression in a specific subset.[19–22] Additionally, expression quantitative trait loci were assessed for all MS risk SNPs and assigned to specific T-cell subsets.[20] Lastly, gene ontology analyses were performed on all MS risk SNPs to assign loci to functional pathways in the aforementioned lymphocyte subsets.[23–25]

EBV and CMV serology

To identify the presence of IgG antibodies against CMV and EBV-viral capsid antigen (VCA), enzyme-linked immunoassays (ELISA) were used, as described before.[9] Seropositivity was defined by a sample-threshold ratio above 0.6 (CMV) and 0.8 (EBV capsid antigen).

Vitamin D assessment

Measurements of 25(OH)D were conducted in blood sera of children at the age of 6 years (110 µL per sample) using the liquid chromatography/tandem mass spectrometry (LC-MS/MS) method, which is a highly sensitive method for measuring 25(OH)D levels, commonly used in epidemiological studies.[26] Serum 25 (OH)D levels were residualized for season of blood draw to adjust for the non-linear effect of season on serum 25(OH)D levels.

T cell phenotyping

The presence and phenotype of $CD4^+$ and $CD8^+$ T cell populations were analyzed in whole blood using 6-color flow cytometry.[9] Absolute T cell numbers were determined with a routine diagnostic lyse-no-wash protocol.[9] We analyzed the following subsets within the $CD4^+$ and $CD8^+$ T cell lineage[9]: naive ($CD45RO^-CCR7^+$), central memory (Tcm; $CD45RO^+CCR7^+$), $CD45RO^+$ effector memory (TemRO; $CD45RO^+CCR7^-$), CD45RA-positive effector memory (TemRA; $CD45RO^-CCR7^-$), terminally differentiated ($CD57^+$) and activated ($HLA\text{-}DR^+$) T cells. $CD56^+$ natural killer T cells were excluded from our analysis. Above subsets were analyzed as part of the Generation R study.[6,7] $CD4^+/CD8^+$ T cell ratios were calculated by dividing the total number of $CD4^+$ and $CD8^+$ T cells. Flow cytometric data were obtained using an LSRII flow cytometer (BD Biosciences) with standardized instrument settings and analyzed using FACSDiva analysis software.[27,28]

Statistical analyses

We performed our analyses using R statistical software (version 3.5.1).[29] Before our analyses we divided the MS-PRSs into quartiles. In the first analyses we analyzed the association

between environmental risk factors for MS (EBV, CMV and vitamin D) and our PRSs, to investigate possible confounding in our subsequent T cell analyses. To determine the association between the MS-PRSs' quartiles and EBV and CMV seropositivity, logistic regression was performed across the different P-value thresholds. We used analyses of variance (ANOVAs) to investigate the relationship between the MS-PRSs' quartiles and serological 25(OH)D levels.

All T cell outcomes of interest were log-transformed to ensure normally distributed residuals of our ANOVA models in the subsequent T cell analyses. At first, the MS-PRSs' quartiles, based on different thresholds, were all associated with absolute T cell counts using ANOVAs. Next, the PRS threshold with the strongest association (lowest P-value) was used in subsequent ANOVA analyses investigating the associations with different T cell subpopulations of interest. In addition, we performed ANOVA analyses with the MS-PRS including only the genome-wide significant risk variants, to be able to compare the effects of these variants on T cell populations with the effects of suggestive variants. Tukey tests were used to investigate post hoc differences in significant ANOVA associations.

In all our analyses we adjusted for age at blood withdrawal, sex and the first ten genetic principal components. Effect modification by sex was tested in a separate sensitivity analysis. We used False Discovery Rate (FDR) per research question to account for multiple testing.[30]

RESULTS

Study population characteristics

Of the participants for whom absolute T cell counts were determined, 2,286 had genotype data available. After selection on relatedness, genotype quality and European ancestry, 1,261 participants remained eligible for analyses using absolute T cell numbers (Figure 7.1). Participants had an even distribution of sex (50.8% male) and a median age of 6.0 years (IQR: 5.9-6.2) (Table 7.1). No difference was found in mean PRS for MS compared to the participants who had no absolute T cell counts available (n=1,569) (p=0.62).

Detailed immunophenotyping data were available from 1,079 participants. Selection on relatedness, genotype quality and ancestry left us with 675 participants eligible for the detailed T-cell subset analyses (Figure 7.1). Descriptive characteristics of these participants were comparable to the participants with absolute T cell counts available (Table 7.1), and the MS-PRS did not differ with participants who did not take part in the detailed immunophenotyping (n=2,155) (p=0.42).

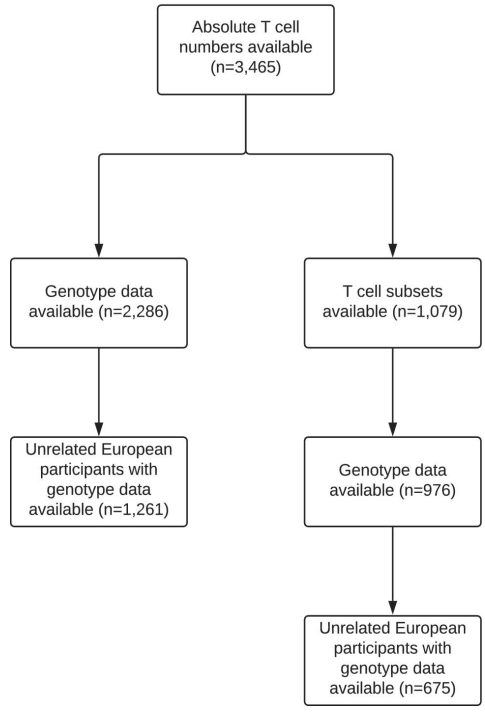

Figure 7.1 Flowchart of the participant selection in the current study

Table 7.1. Descriptive characteristics of study samples.

	Absolute T-cell counts (n=1,261)	**T-cell phenotyping (n=675)**
Age, median (IQR)	6.0 (5.9-6.2)	6.0 (5.9-6.2)
Male, n (%)	641 (50.8)	340 (50.4)
Level of maternal education, n (%)		
• High	818 (64.8)	419 (62.1)
• Middle	405 (32.1)	239 (35.4)
• Low	17 (1.3)	9 (1.3)
• Unknown	21 (1.7)	8 (1.2)
Season of blood draw, n (%)		
• Spring	282 (22.4)	171 (25.3)
• Summer	290 (23.0)	150 (22.2)
• Autumn	351 (27.8)	174 (25.8)
• Winter	338 (26.8)	180 (26.7)
EBV-VCA seropositivity, n (%)	512/1,225 (41.8)	270/649 (41.6)
CMV seropositivity, n (%)	353/1,227 (28.8)	191/649 (29.4)
Serum 25(OH)D levels, nmol/L, median (IQR)	74.0 (58.7-88.0)	74.0 (57.0-88.0)
Reported presence of paternal MS, n (%)	2/1,1036 (0.2)	0/572 (0.0)
Reported presence of maternal MS, n (%)	2/1,144 (0.2)	0/611 (0.0)

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; CMV, cytomegalovirus; EBV-VCA, Epstein-Barr virus viral capsid antigen; IQR, interquartile range; MS, multiple sclerosis; n, number; nmol, nanomol.

EBV and CMV serology and serum 25(OH)D levels

EBV and CMV serology were measured in 4,464 participants. Out of these participants, 1,551 had good-quality genotype data available. We found no relationship in these participants between the PRSs' quartiles for MS and EBV and CMV seropositivity (Supplementary Table 7.1-7.2).

Serum 25(OH)D levels were measured in 3,983 participants, of whom 1,442 had good-quality genotype data available. The known association of the rs7041 A-allele with lower 25(OH)D levels was replicated in our dataset ($p=5.19\times10^{-10}$).[31] The genotype data did not include rs5688. The MS risk SNP located in the CYP24A1 gene (rs2248137), as well as the MS-PRSs' quartiles across all thresholds were not associated with the level of serum 25(OH)D in these participants (Supplementary Table 7.3).

Effects on absolute T cell counts

We found no significant association between the MS-PRSs' quartiles and absolute T cell counts across all thresholds (Supplementary Table 7.4). The PRS with a threshold of $P_T<0.005$ revealed the strongest association with absolute total T cell counts (p=0.29) and was used in our subsequent detailed immunophenotyping analyses.

Associations with T cell subpopulations

The MS-PRS ($P_T<0.005$) had a significant association with total ($p=2.92\times10^{-3}$) and naive ($p=1.85\times10^{-4}$) $CD8^+$ T cell numbers and $CD4^+/CD8^+$ T cell ratios ($p=8.27\times10^{-9}$). A complete overview of the associations between the MS-PRS ($P_T<0.005$) and T cell populations can be found in Table 7.2. Post hoc Tukey tests revealed significant negative associations of the first and second MS-PRSs' quartiles with the fourth quartile considering both total and naive $CD8^+$ T cell numbers (Figure 7.2A-B). Accordingly, this resulted in a significant positive association of the PRS with the $CD4^+/CD8^+$ ratio across multiple quartiles (Figure 7.2C). In the sensitivity analyses, we found no evidence for interaction effects by sex (Supplementary Table 7.5).

To investigate the MHC dependency of these significant associations, we excluded the *HLA-DRB1*15:01* tag variant rs3135388 and the MHC region from our MS-PRS (Supplementary Tables 7.6-7.9). The removal of rs3135388 (and variants with high linkage disequilibrium to this variant within a 1-Mb region) attenuated the significant $CD8^+$ results, but these associations remained significant and the association involving the $CD4^+/CD8^+$ ratio increased in significance. Additional adjustment for *HLA-DRB1*15:01* status within this model still resulted in a significant association between the MS-PRS and the $CD4^+/CD8^+$ ratio (Supplementary Table 7.7). When adding the status of several other *HLA-DRB* alleles to our model as covariates, we again observed a significant association of the MS-PRS with the $CD4^+/$

$CD8^+$ ratio (Supplementary Table 7.8). After removal of the total MHC region, the association between the MS-PRS and $CD4^+/CD8^+$ ratio became weaker, but remained significant (Supplementary Table 7.9). The PRS with only MHC risk variants showed comparable results to our PRS including both MHC and non-MHC risk variants (Supplementary Table 7.10). Due to the different linkage-disequilibrium (LD) structure of the MHC region, we constructed several PRSs using various clumping parameters for the MHC region. However, altered clumping of the MHC did not significantly change our results (Supplementary Table 7.11).

In addition, we calculated an MS-PRS only incorporating risk variants that were genome-wide significant ($P_T<5\times10^{-8}$) in the MS GWAS performed by the IMSGC,[3] and analyzed the association with the T-cell subsets (Supplementary Table 7.12). We observed a similar positive association of the first and second quartiles of the MS-PRS with the fourth quartile concerning the $CD4^+/CD8^+$ ratio (Figure 7.2D and Supplementary Table 7.12).

Table 7.2. Analysis of variance results of the multiple sclerosis polygenic risk score (PT < 0.005) and T-cell subsets of interest.

T-cell subset	Unadjusted P-value	FDR-adjusted P-value
$CD4^+$	0.17	0.71
$CD4^+$ naive	0.14	0.70
$CD4^+$ Tcm	0.54	0.77
$CD4^+$ TemRO	0.49	0.77
$CD4^+$ TemRA	0.46	0.77
$CD8^+$	$2.92\text{x}10^{-3}$	**0.02***
$CD8^+$ naive	$1.85\text{x}10^{-4}$	**$3.14\text{x}10^{-3}$***
$CD8^+$ Tcm	0.25	0.72
$CD8^+$ TemRO	0.32	0.72
$CD8^+$ TemRA	0.61	0.77
$CD3^+CD8^-HLADR^+$	0.53	0.77
$CD3^+CD8^+$ $HLADR^+$	0.27	0.72
$CD3^+CD8^+CD57^-CD56^-$	0.55	0.77
$CD3^+CD8^+CD57^+CD56^-$	0.56	0.77
$CD3^+CD8^-CD57^-CD56^-$	0.59	0.77
$CD3^+CD8^-CD57^+CD56^-$	0.83	0.91
$CD4^+/CD8^+$ ratio	$8.27\text{x}10^{-9}$	**$2.81\text{x}10^{-7}$****

Included: n = 675 children

Abbreviations: FDR, false discovery rate; Tcm, central memory T cells; TemRA, RA-positive effector memory T cells; TemRO, RO-positive effector memory T cells.

Analyses are adjusted for age, sex, and 10 genetic principal components.

Significant values after FDR multiple testing correction are highlighted in bold.

*p < 0.05, **p < 0.001.

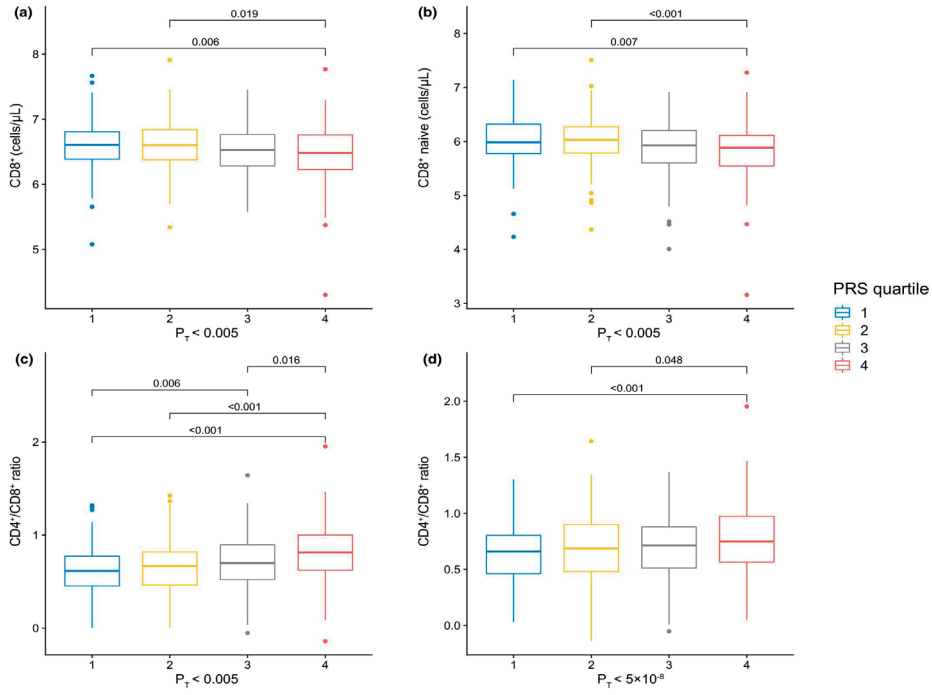

Figure 7.2. Post hoc Tukey test results of the different polygenic risk scores (PRSs) associated with the CD4+/CD8+ ratio and other T cell subsets.

Numbers on the x-axis correspond to the different quartiles of the multiple sclerosis (MS)-PRS. Based on increasing MS-PRS, the participants were divided into four quartiles of similar sample size.

(a) Association between total $CD8^+$ cells and the quartiles of the MS-PRS (P_T <0.005).

(b) Association between naive $CD8^+$ cells and the quartiles of the MS-PRS (P_T <0.005).

(c) Association between the $CD4^+/CD8^+$ ratio and the quartiles of the MS-PRS (P^T <0.005).

(d) Association between the $CD4^+/CD8^+$ ratio and the quartiles of the MS-PRS (P^T <5×10^{-8})

Individual risk variant analyses

Subsequently, we investigated the relationship between an increased $CD4^+/CD8^+$ ratio and individual genetic MS risk variants. Of the 195 out of 233 MS genome-wide significant risk SNPs that were available in our study population, two risk variants showed a significant association with the $CD4^+/CD8^+$ ratio after FDR correction (Supplementary Table 7.13, Figure 7.3A). A positive association was found for rs3135388 (*HLA-DRA*, a tag variant for *HLA-DRB1*15:01*), whereas a negative association was observed for rs9266629 (intergenic, between *ZDHHC20P2* and *FGFR3P1*, a tag variant for HLA-B variant rs3819284).

Next, we associated these two risk variants with the previous T-cell subsets of interest (Supplementary Table 7.14-7.15). Rs3135388 status (MHC class II) was positively associated with $CD4^+$ total and naive numbers, whereas rs9266629 (MHC class I) showed positive associa-

tions with various $CD8^+$ subsets (e.g. total $CD8^+$, $CD8^+$ naive and $CD8^+$ Tcm) (Figure 7.3A). Figure 7.3B shows the combined effects of the two risk variants on the $CD4^+/CD8^+$ ratio.

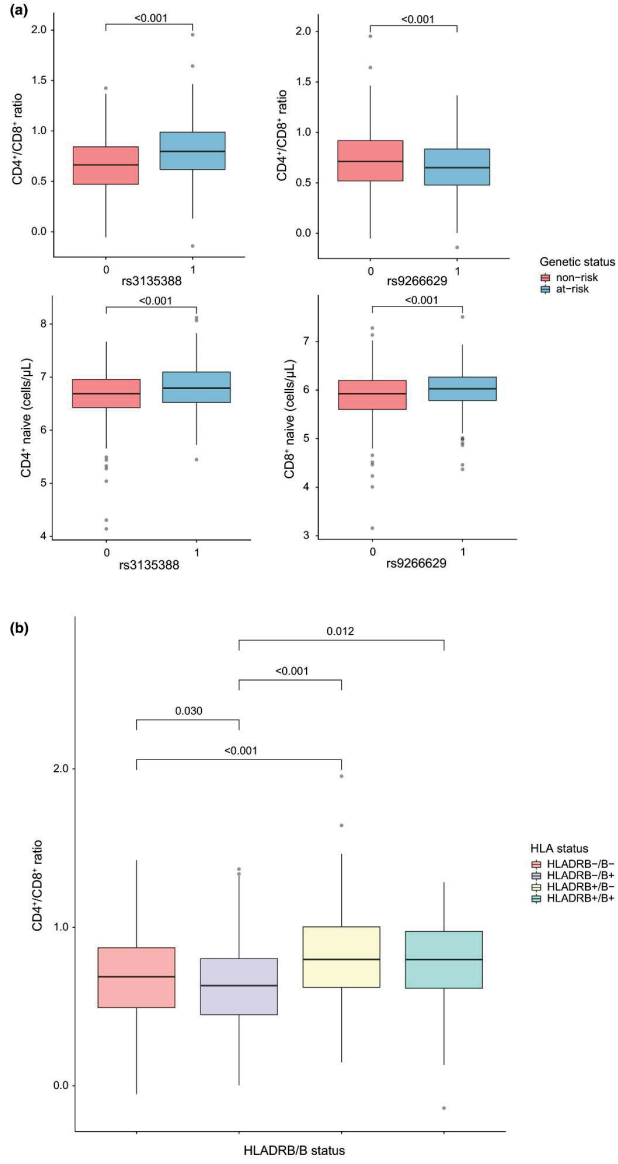

Figure 7.3. Associations of rs3135388 and rs9266629 with T cell subsets and the $CD4^+/CD8^+$ ratio.
Carriership of rs3135388 and rs9266629 was dichotomized into two groups, homozygous non-risk and at-risk. At-risk participants consisted of heterozygous and homozygous carriers of the effect allele. (a) Left: Associations of rs3135388 with the $CD4^+/CD8^+$ ratio and $CD4^+$ naive cells. Right: Associations of rs9266629 with the $CD4^+/CD8^+$ ratio and $CD8^+$ naive cells. (b) Combined associations of rs3135388 and rs9266629 with the $CD4^+/CD8^+$ ratio

T cell PRS

In a final analysis, the autosomal genome-wide significant MS risk variants from the IMSGC were classified into specific MS associated T-cell PRSs (Figure 7.4, Supplementary Table 7.16). We observed no significant associations between the different PRSs and the T cell outcomes, including the $CD4^+/CD8^+$ ratio.

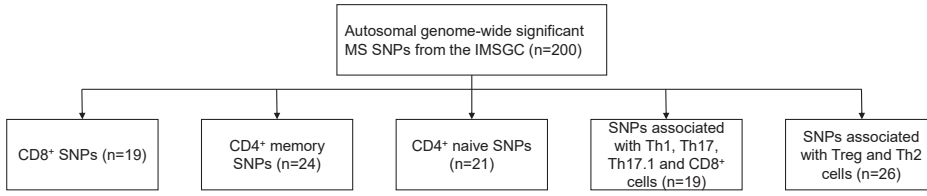

Figure 7.4. Flowchart describing the selection process of specific T cell polygenic risk scores using cis expression quantitative trait loci data. Risk variants were grouped using a combination of mRNA expression data, expression quantitative trait loci, and gene ontology analyses as described in the Materials and Methods section. IMSGC, International Multiple Sclerosis Genetics Consortium; MS, multiple sclerosis; SNP, single nucleotide polymorphism.

When incorporating rs3135388 and rs9266629 status into the T cell PRSs, we observed several dose-dependent significant associations (Supplementary Table 7.17-7.19). A higher $CD8^+$ PRS quartile was associated with a lower count of $CD8^+$ T-cell subsets and a higher $CD4^+/CD8^+$ ratio ($p=8.55\times10^{-7}$). The PRS involving Th1, Th17, Th17.1 and $CD8^+$ associated variants showed positive associations with $CD4^+$ TemRA counts (p=0.01) and the $CD4^+/CD8^+$ ratio ($p=2.34\times10^{-6}$)(Supplementary Table 7.18). Similar associations were observed for the PRS incorporating variants associated with Treg and Th2 cells (Supplementary Table 7.19). All these associations were driven by rs3135388 and rs9266629 status.

DISCUSSION

In this study, we found that PRSs for MS are associated with T cell numbers in the peripheral blood at the age of 6 years in children from the general population. A higher genetic risk for MS was associated with an increased $CD4^+/CD8^+$ ratio, which was the result of a negative association between the MS-PRS and total and naive $CD8^+$ T cell numbers. Two genome-wide significant MS risk variants, rs3135388 and rs9266629, were individually associated with the changes in the $CD4^+/CD8^+$ ratio and absolute numbers of several $CD4^+$ and $CD8^+$ T-cell subsets. No associations were found between T cell specific MS-PRSs and T cell numbers.

We found no associations between MS-PRSs and seropositivity for EBV and CMV, and serum 25(OH)D levels. We conclude that MS-PRSs are associated with T cell composition

in children of a general population, and that this mechanism likely contributes to the overall risk of developing MS later in life.

Our findings are consistent with immunological alterations reported in adult MS patients. An increased $CD4^+/CD8^+$ ratio has been described in MS, as well as in other autoimmune disease patients and their relatives.[6,7,32] We now found that genetic predisposition for MS alters the $CD4^+/CD8^+$ ratio in children from the general population. This is relevant because the $CD4^+/CD8^+$ ratio is a relatively stable immunological parameter from child- to young adulthood.[33] Our reported observed negative correlations of the MS-PRS with $CD8^+$ and not $CD4^+$ T cells are consistent with previously described reductions of $CD8^+$ and not $CD4^+$ T cells in the peripheral blood of MS patients.[6] In late-stage adult MS, there is a local enrichment of memory $CD8^+$ T cells in the CNS.[34,35] Accordingly, in MS-discordant monozygotic twins, a clonally expanded memory $CD8^+$ T cell population was found in the CSF of twins asymptomatic for MS.[36] In children, however, it is not likely that genetically determined reductions in $CD8^+$ T cells are the indirect result of an increased influx of memory cells into the CNS, since both central and effector memory T-cell populations did not correlate with the MS-PRS. Instead, genetic variation may impair thymic output of naive $CD8^+$ T cells, a phenomenon described in MS patients at an early age possibly before disease onset, which is supported by our observed negative association between the MS-PRS and naive $CD8^+$ T cells.[6,37] The fact that the positive correlation of the MS-PRS with the $CD4^+/CD8^+$ ratio is mainly driven by major-MHC risk alleles could imply that in children, the development of CD4 and CD8 double- into single-positive thymocytes is influenced by genetically altered HLA class I and II-expressing thymic epithelial cells.[38,39]

The major risk variant for MS, *HLA-DRB1*15:01*, was found to be associated with higher $CD4^+$ T cell numbers, whereas the rs9266629 variant that is protective for MS and a tag-variant for *HLA-B* SNP rs3819284 associated positively with $CD8^+$ T cell numbers. The same associations were observed when incorporating these two variants in our MS-associated T cell PRSs. For $CD4^+$ T cells, it has been reported that naive populations are genetically affected and more activated and thus prone to differentiate into effector subsets during early MS.[40] Additionaly, it was found that *HLA-DRB1*15:01* status increases autoproliferation and brain-homing of the $CD4^+$ T cells.[41] Disturbed T cell homeostasis in favour of $CD4^+$ T cells, influenced by rs3135388, the absence of rs9266629 and overall genetic risk for MS, may consequently be one of the first steps in the immunopathogenesis before possible onset of symptoms in MS. An increased proportion of these cells in peripheral blood could lay the foundation for the activation of auto-reactive $CD4^+$ T cells later in life under the influence of environmental risk factors and *HLA-DRB1*15:01*, as suggested in the immunopathogenesis of MS.[42] Subsequent migration across the blood-brain barrier could initiate the MS disease process, where auto-reactive T cells cause inflammatory demyelination of the CNS.[42]

The entire MHC region contributed substantially to our results. When investigating a MS-PRS with only MHC risk variants our results were comparable to our PRS with all variants. Excluding the MHC region from the MS-PRS attenuated our results, however they remained significant. This finding is in line with the widely known substantial role of the MHC region in MS pathogenesis. Due to this strong association of MHC alleles with MS (especially *HLA-DRB1*15:01*), we kept these variants in our PRSs, as this is the closest reflection of overall genetic MS risk. However PRSs are not able to fully capture the effect of the MHC due to the epistatic interactions within this region of the genome.

We did not find an association between MS-PRSs and known environmental risk factors for MS (i.e. EBV and CMV serology and serum 25(OH)D). Low levels of serum 25(OH)D have been associated with increased MS risk in adults and children.[43,44] Our results suggest that serum 25(OH)D is not correlated with polygenic risk scores for MS at this age. This environmental risk factor could exert its effect on MS pathophysiology independently, a hypothesis also demonstrated by Mendelian randomization studies.[45]

Persistent EBV and CMV seropositivity affect MS susceptibility in adults, but also in children.[46,47] The observed non-significant association in our study between polygenic risk for MS and EBV status could be explained by the EBV-status assessment used in our study. EBV-seropositivity in the Generation R study was tested by using EBV-VCA antibodies, which are not associated with MS in adults.[48] However, in pediatric MS EBV-VCA positivity is significantly more present compared to controls, pleading for the validity of this marker in children.[49] Another possible explanation is that genetic MS risk does not associate with seropositivity for these viruses at this young age, and that EBV and CMV exert their effect at a later age in MS pathophysiology, as supported by earlier work.[50]

Our study has several strengths. First, we were able to include a large number of young children, allowing us to detect robust effects of genetic MS risk even before possible disease onset. Furthermore, we incorporated several suggestive genetic risk variants for MS in our PRSs to reflect the polygenic architecture of MS as much as possible. Additionally, we tested for CMV seropostivity and serum 25(OH)D levels using accurate methods. Lastly, we performed additional analyses incorporating specific T cell PRSs constructed with functional pathway data.

Limitations are also present. Due to the cross-sectional design of our study we are unable to investigate the temporal dynamics between genetic risk for MS and the T cell compartment of the immune system. Secondly, it was not possible to investigate the functional properties of T cells in this study, such as their pro-inflammatory and transmigration capacity. Future studies are needed to validate our results, test the reported relationships longitudinally and

incorporate environmental risk factors for MS to address their effect on T cells and to better understand the pathophysiology of MS. Longitudinal well-powered population-based study designs could potentially translate the altered $CD4^+/CD8^+$ ratio to a clinically-usable biomarker for MS.

Conclusion

In conclusion, we report an association between genetic risk scores for MS and alterations in the T cell lineages in school-aged children from the general population. This shows that MS genetics change the composition of the adaptive immunity during childhood, which possibly contributes to overall risk to develop MS in later life.

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SUPPLEMENTARY MATERIALS

Supplementary Table 7.1. Logistic regression results of the different polygenic risk scores (PRS) and serum-positivity for the Epstein-Barr virus (EBV). (n=1,551)

EBV-positivity	Determinant	β	SE	P-value
	PRS 5×10^{-8}	-0.006	0.052	0.91
	PRS 0.001	0.028	0.052	0.59
	PRS 0.005	0.007	0.052	0.90
	PRS 0.01	0.019	0.053	0.72
	PRS 0.05	-0.010	0.051	0.84
	PRS 0.1	-0.001	0.052	0.99
	PRS 0.5	-0.057	0.052	0.28
	PRS 1	-0.069	0.052	0.19

Abbreviations: β, beta; EBV, Epstein-barr virus; PRS, polygenic risk score; SE, standard error.
Analyses are adjusted for age, sex and ten genetic principal components (PC's).

Supplementary Table 7.2. Logistic regression results of the different polygenic risk scores (PRS) and serum-positivity for the Cytomegalovirus (CMV). (n=1,551)

CMV-positivity	Determinant	β	SE	p
	PRS 5×10^{-8}	-0.043	0.056	0.45
	PRS 0.001	-0.007	0.056	0.90
	PRS 0.005	-0.007	0.056	0.90
	PRS 0.01	0.013	0.057	0.82
	PRS 0.05	-0.039	0.056	0.48
	PRS 0.1	-0.053	0.056	0.35
	PRS 0.5	-0.020	0.056	0.72
	PRS 1	-0.026	0.057	0.64

Abbreviations: β, beta; CMV, cytomegalovirus; PRS, polygenic risk score; SE, standard error.
Analyses are adjusted for age, sex and ten genetic principal components (PC's).

Supplementary Table 7.3. ANOVA results of the different polygenic risk scores (PRS) and serological vitamin D levels. (n=1,442)

Vitamin D	Determinant	P-value
	PRS 5×10^{-8}	0.50
	PRS 0.001	0.45
	PRS 0.005	0.40
	PRS 0.01	0.84
	PRS 0.05	0.39
	PRS 0.1	0.45
	PRS 0.5	0.67
	PRS 1	0.52

Abbreviations: PRS, polygenic risk score
Analyses are adjusted for age, sex and ten genetic principal components (PC's).

Supplementary Table 7.4. ANOVA results of the different polygenic risk scores (PRS) and the absolute total T cell numbers. (n=1,261)

Absolute total T cell numbers	Determinant	P-value
	PRS 0.001	0.92
	PRS 0.005	0.29
	PRS 0.01	0.65
	PRS 0.05	0.45
	PRS 0.1	0.64
	PRS 0.5	0.70
	PRS 1	0.50

Abbreviations: PRS, polygenic risk score
Analyses are adjusted for age, sex and ten genetic principal components (PC's).

Supplementary Table 7.5. ANOVA results of the polygenic risk score (P_T<0.05), with an interaction term for sex, and the different T-cell subsets of interest. (n=675)

$PRS_{(p0.005)}$*Sex	T-cell subset	P-value
	$CD4^+$	0.77
	$CD4^+$ Naive	0.86
	$CD4^+$ Tcm	0.08
	$CD4^+$ TemRO	0.59
	$CD4^+$ TemRA	0.77
	$CD8^+$	0.28
	$CD8^+$ Naive	0.28
	$CD8^+$ Tcm	0.10
	$CD8^+$ TemRO	0.22
	$CD8^+$ TemRA	0.64
	$CD3^+CD8^-$ $HLADR^+$	0.78
	$CD3^+CD8^+$ $HLADR^+$	0.75
	$CD3^+CD8^+CD57^-CD56^-$	0.07
	$CD3^+CD8^+CD57^+CD56^-$	0.19
	$CD3^+CD8^-CD57^-CD56^-$	0.22
	$CD3^+CD8^-CD57^+CD56^-$	0.38
	$CD4^+/CD8^+$ ratio	0.53

Abbreviations: PRS, polygenic risk score; Tcm, central memory T cell; TemRA, effector memory RA^+ T cell; TemRO, effector memory RO^+ T cell.

Analyses are adjusted for age and ten genetic principal components (PC's).

Supplementary Table 7.6. ANOVA results of the polygenic risk score (P_T<0.005) and the significant T-cell subsets of interest, after excluding HLA-DRB1*15:01 tag variant rs3135388 (and variants with high linkage disequilibrium to this variant within a 1 Mb region). (n=675)

$PRS_{(p0.005)}$	T-cell subset	P-value
	$CD8^+$	**0.01***
	$CD8^+$ Naive	**1.77×10^{-3}***
	$CD4^+/CD8^+$ ratio	**1.34×10^{-9}****

Abbreviations: HLA, human leukocyte antigen; PRS, polygenic risk score.
Analyses are adjusted for age, sex and ten genetic principal components (PC's). Significant values are highlighted in bold.
*: p<0.05
**: p<0.001

Supplementary Table 7.7. ANOVA results of the polygenic risk score (P_T<0.005) and the significant T-cell subsets of interest, after excluding HLA-DRB1*15:01 tag variant rs3135388, with additional adjustment for HLA-DRB1*15:01 status. (n=675)

$PRS_{(p0.005)}$	T-cell subset	P-value
	$CD8^+$	**0.04***
	$CD8^+$ Naive	**0.04***
	$CD4^+/CD8^+$ ratio	**2.58×10^{-4}****

Abbreviations: HLA, human leukocyte antigen; PRS, polygenic risk score.
Analyses are adjusted for age, sex and ten genetic principal components (PC's). Significant values are highlighted in bold.
*: p<0.05
**: p<0.001

Supplementary Table 7.8. ANOVA results of the polygenic risk score (P_T<0.005) and the significant T-cell subsets of interest, after excluding HLA-DRB tag variants (rs9268839, rs1057149, rs2187688 and rs3135388), with additional adjustment for their status in the model. (n=675)

$PRS_{(p0.005)}$	T-cell subset	P-value
	$CD8^+$	**0.04***
	$CD8^+$ Naive	0.05
	$CD4^+/CD8^+$ ratio	**2.97×10^{-4}****

Abbreviations: HLA, human leukocyte antigen; PRS, polygenic risk score.
Analyses are adjusted for age, sex and ten genetic principal components (PC's). Significant values are highlighted in bold.
*: p<0.05
**: p<0.001

Supplementary Table 7.9. ANOVA results of the polygenic risk score (P_T<0.005) and the significant T-cell subsets of interest, after excluding variants from the MHC region. (n=675)

$PRS_{(p0.005)}$	T-cell subset	P-value
	$CD8^+$	0.40
	$CD8^+$ Naive	0.07
	$CD4^+/CD8^+$ ratio	**0.03***

Abbreviations: MHC, major histocompatibility complex; PRS, polygenic risk score.
Analyses are adjusted for age, sex and ten genetic principal components (PC's). Significant values are highlighted in bold.
*: p<0.05

Supplementary Table 7.10. ANOVA results of the polygenic risk score (P_T<0.005), only including variants from the MHC region, and the different T-cell subsets of interest. (n=675)

$PRS_{(p0.005)}$	Immunological subset	Unadjusted P-value	FDR-adjusted P-value
	$CD4^+$	0.19	0.47
	$CD4^+$ Naive	0.1	0.33
	$CD4^+$ Tcm	0.48	0.58
	$CD4^+$ TemRO	0.79	0.79
	$CD4^+$ TemRA	0.17	0.47
	$CD8^+$	4.50×10^{-2}	0.26
	$CD8^+$ Naive	1.67×10^{-3}	**0.01***
	$CD8^+$ Tcm	0.09	0.33
	$CD8^+$ TemRO	0.53	0.60
	$CD8^+$ TemRA	0.66	0.70
	$CD3^+CD8^-HLADR^+$	0.46	0.58
	$CD3^+CD8^+$ $HLADR^+$	0.23	0.50
	$CD3^+CD8^+CD57^-CD56^-$	0.34	0.58
	$CD3^+CD8^+CD57^+CD56^-$	0.46	0.58
	$CD3^+CD8^-CD57^-CD56^-$	0.34	0.58
	$CD3^+CD8^-CD57^+CD56^-$	0.43	0.58
	$CD4^+/CD8^+$ ratio	2.27×10^{-8}	**3.90×10^{-7}****

Abbreviations: FDR, false discovery rate; HLA, human leukocyte antigen; MHC, major histocompatibility complex; PRS, polygenic risk score; Tcm, central memory T cell; TemRA, effector memory RA^+ T cell; TemRO, effector memory RO^+ T cell
Analyses are adjusted for age, sex and ten genetic principal components (PC's). Significant values after multiple testing correction are highlighted in bold.
*:p<0.05
**: p<0.001

Supplementary Table 7.11. ANOVA results (unadjusted p-value) of the MHC polygenic risk scores (P_T<0.005) associated with the $CD4^+/CD8^+$ ratio, using different LD-window sizes and clumping r^2 values. (n=675)

	LD-window (kb)					
Clumping r^2	10	100	250	500	1000	2000
0.1	9.96×10^{-10}	9.00×10^{-9}	8.27×10^{-9}	2.48×10^{-7}	3.36×10^{-9}	1.16×10^{-9}
0.01	2.20×10^{-9}	1.27×10^{-9}	1.56×10^{-9}	2.38×10^{-10}	6.66×10^{-11}	1.06×10^{-8}
0.001	1.82×10^{-7}	4.77×10^{-6}	5.93×10^{-6}	4.31×10^{-5}	6.77×10^{-6}	2.08×10^{-3}

Abbreviations: kb, kilobase; LD, linkage disequilibrium; MHC, major histocompatibility complex.
Analyses are adjusted for age, sex and ten genetic principal components (PC's).

Supplementary Table 7.12. ANOVA results of the polygenic risk score (P_T<5×10^{-8}) and the different T-cell subsets of interest. (n=675)

$PRS_{(p5E-8)}$	Immunological subset	Unadjusted p-value	FDR-adjusted p-value
	$CD4^+$	0.28	0.72
	$CD4^+$ Naive	0.31	0.72
	$CD4^+$ Tcm	0.91	0.94
	$CD4^+$ TemRO	0.36	0.76
	$CD4^+$ TemRA	0.14	0.70
	$CD8^+$	0.54	0.77
	$CD8^+$ Naive	0.10	0.69
	$CD8^+$ Tcm	0.22	0.72
	$CD8^+$ TemRO	0.79	0.90
	$CD8^+$ TemRA	0.43	0.77
	$CD3^+CD8^-HLADR^+$	0.99	0.99
	$CD3^+CD8^+$ $HLADR^+$	0.77	0.90
	$CD3^+CD8^+CD57^-CD56^-$	0.87	0.92
	$CD3^+CD8^+CD57^+CD56^-$	0.67	0.82
	$CD3^+CD8^-CD57^-CD56^-$	0.28	0.72
	$CD3^+CD8^-CD57^+CD56^-$	0.57	0.77
	$CD4^+/CD8^+$ ratio	8.60×10^{-4}	**0.01***

Abbreviations: FDR, false discovery rate; HLA, human leukocyte antigen; Tcm, central memory T cell; TemRA, effector memory RA^+ T cell; TemRO, effector memory RO^+ T cell
Analyses are adjusted for age, sex and ten genetic principal components (PC's). Significant values after multiple testing correction are highlighted in bold.
*:p<0.05, **: p<0.001

Supplementary Table 7.13. MS risk variants associated with the $CD4^+/CD8^+$ ratio

SNP	Chr	Position (hg19)	Effect allele	β	SE	ΔR^2	P-value	FDR-adjusted P-value
rs3135388	6	32413051	A	0.132	0.021	0.054	1.13×10^{-9}	**2.20×10^{-7}**
rs9266629	6	31346822	C	-0.073	0.019	0.020	3.64×10^{-4}	**3.55×10^{-2}**

Abbreviations: Chr, chromosome; FDR, false discovery rate; HLA, human leukocyte antigen; MS, multiple sclerosis; SE, standard error; SNP, single nucleotide polymorphism.

Risk variants with a significant association are shown after applying FDR multiple testing correction. Linear regression analyses are adjusted for age, sex and ten genetic principal components (PC's). Significant values are highlighted in bold.

*:p<0.05

**: p<0.001

Supplementary Table 7.14. Regression results of risk variant rs3135388 on the different T-cell subsets of interest. (n=675)

rs3135388	T-cell subset	β	SE	ΔR^2	Unadjusted P-value	FDR-adjusted P-value
	$CD4^+$	0.08	0.029	0.012	3.70×10^{-3}	**0.02***
	$CD4^+$ Naive	0.13	0.039	0.017	8.36×10^{-4}	**0.01***
	$CD4^+$ Tcm	0.09	0.038	0.008	0.02	0.07
	$CD4^+$ TemRO	-0.01	0.036	1.204×10^{-4}	0.78	0.85
	$CD4^+$ TemRA	3.09×10^{-4}	0.052	5.230×10^{-6}	0.95	0.95
	$CD8^+$	-0.06	0.034	0.004	0.10	0.22
	$CD8^+$ Naive	-0.11	0.042	0.010	0.01	**0.04***
	$CD8^+$ Tcm	-0.07	0.059	0.002	0.21	0.35
	$CD8^+$ TemRO	-0.05	0.057	0.001	0.37	0.53
	$CD8^+$ TemRA	0.04	0.057	0.001	0.51	0.64
	$CD3^+CD8^-HLADR^+$	-0.04	0.048	0.001	0.41	0.55
	$CD3^+CD8^+$ $HLADR^+$	-0.14	0.075	0.005	0.07	0.18
	$CD3^+CD8^+CD57^-CD56^-$	-0.05	0.087	4.704×10^{-4}	0.58	0.69
	$CD3^+CD8^+CD57^+CD56^-$	0.19	0.118	0.004	0.11	0.22
	$CD3^+CD8^-CD57^-CD56^-$	0.12	0.087	0.003	0.16	0.28
	$CD3^+CD8^-CD57^+CD56^-$	0.08	0.103	0.001	0.42	0.55
	$CD4^+/CD8^+$ ratio	0.13	0.021	0.054	1.13×10^{-9}	**9.45×10^{-7}****

Abbreviations: FDR, false discovery rate; HLA, human leukocyte antigen; SE, standard error; Tcm, central memory T cell; TemRA, effector memory RA^+ T cell

Analyses are adjusted for age, sex and ten genetic principal components (PC's). Significant values are highlighted in bold.

*:p<0.05

**: p<0.001

Supplementary Table 7.15. Regression results of risk variant rs9266629 on the different T-cell subsets of interest. (n=675)

rs9266629	T-cell subset	β	SE	ΔR^2	Unadjusted P-value	FDR-adjusted P-value
	$CD4^+$	3.73×10^{-3}	0.026	2.982×10^{-5}	0.89	0.91
	$CD4^+$ Naive	0.01	0.036	3.068×10^{-5}	0.89	0.91
	$CD4^+$ Tcm	0.09	0.035	0.009	0.01	**0.04***
	$CD4^+$ TemRO	0.02	0.032	4.457×10^{-4}	0.59	0.69
	$CD4^+$ TemRA	-0.18	0.047	0.021	1.61×10^{-4}	**2.74×10^{-3}***
	$CD8^+$	0.08	0.031	0.010	0.01	**0.04***
	$CD8^+$ Naive	0.13	0.038	0.016	8.84×10^{-4}	**0.01***
	$CD8^+$ Tcm	0.16	0.053	0.014	1.91×10^{-3}	**0.01***
	$CD8^+$ TemRO	0.08	0.052	0.003	0.14	0.27
	$CD8^+$ TemRA	-0.08	0.051	0.004	0.10	0.22
	$CD3^+CD8^-HLADR^+$	0.02	0.044	3.032×10^{-4}	0.65	0.74
	$CD3^+CD8^+$ $HLADR^+$	0.11	0.068	0.004	0.10	0.22
	$CD3^+CD8^+CD57^-CD56^-$	0.16	0.079	0.006	0.04	0.11
	$CD3^+CD8^+CD57^+CD56^-$	0.10	0.108	0.001	0.38	0.53
	$CD3^+CD8^-CD57^-CD56^-$	0.07	0.079	0.001	0.37	0.53
	$CD3^+CD8^-CD57^+CD56^-$	0.12	0.094	0.003	0.20	0.34
	$CD4^+/CD8^+$ ratio	-0.07	0.019	0.020	3.64×10^{-4}	**4.13×10^{-3}***

Abbreviations: FDR, false discovery rate; HLA, human leukocyte antigen; PRS, polygenic risk score; SE, standard error; Tcm, central memory T cell; TemRA, effector memory RA^+ T cell.

Analyses are adjusted for age, sex and ten genetic principal components (PC's). Significant values after multiple testing correction are highlighted in bold.

*:$p<0.05$

Supplementary Table 7.16. Different SNPs included in the T-cell specific PRSs.

$CD8^+$ SNPs	$CD4^+$ memory SNPs	$CD4^+$ naive SNPs	SNPs associated with Th1, Th17, Th17.1 and $CD8^+$	SNPs associated with Treg and Th2
rs10271373	rs10271373	rs10245867	rs10271373	rs10245867
rs1076928	rs1076928	rs1026916	rs1076928	rs10271373
rs10951154	rs10951154	rs10271373	rs10951154	rs1076928
rs11079784	rs11079784	rs1076928	rs11079784	rs10951154
rs2150879	rs1177228	rs11079784	rs1177228	rs11079784
rs2331964	rs12147246	rs2150879	rs12622670	rs1177228
rs4409785	rs12622670	rs2331964	rs2150879	rs12147246
rs4896153	rs2150879	rs34681760	rs2269434	rs2150879
rs58166386	rs2269434	rs35540610	rs2331964	rs2269434
rs6533052	rs2331964	rs4409785	rs4796224	rs2331964
rs6670198	rs354033	rs4796224	rs4896153	rs354033
rs701006	rs4796224	rs4896153	rs6533052	rs4409785
rs72928038	rs4896153	rs6533052	rs701006	rs4796224
rs7731626	rs6533052	rs701006	rs7731626	rs4896153
rs7855251	rs6742	rs72928038	rs7855251	rs6533052
rs7977720	rs701006	rs7731626	rs7975763	rs6742
rs883871	rs7731626	rs7855251	rs883871	rs701006
rs9808753	rs7855251	rs7977720	rs9900529	rs72928038
rs9909593	rs7975763	rs883871	rs9909593	rs7731626
	rs7977720	rs9900529		rs7855251
	rs883871	rs9909593		rs7975763
	rs9610458			rs7977720
	rs9900529			rs883871
	rs9909593			rs9610458
				rs9900529
				rs9909593

Abbreviations: PRS, polygenic risk score; SNP, single nucleotide polymorphism; Th, T helper cell; Treg, regulatory T cell

Supplementary Table 7.17. ANOVA results of the $CD8^+$ polygenic risk score, including rs3135388 and rs9266629 status, and different $CD8^+$ T-cell subsets. (n=675)

$CD8^+$ PRS	T-cell subset	P-value
	$CD8^+$	0.07
	$CD8^+$ Naive	**5.22×10^{-3}***
	$CD8^+$ Tcm	**0.02***
	$CD8^+$ TemRO	0.15
	$CD8^+$ TemRA	0.69
	$CD4^+/CD8^+$ ratio	**8.55×10^{-7}****

Abbreviations: PRS, polygenic risk score; Tcm, central memory T cell; TemRA, effector memory RA+ T cell; TemRO, effector memory RO+ T cell; Treg, regulatory T cell
Analyses are adjusted for age and ten genetic principal components (PC's). Significant values are highlighted in bold.
*:p<0.05
**: p<0.001

Supplementary Table 7.18. ANOVA results of the polygenic risk score, incorporating Th1, Th17, Th17.1 and $CD8^+$ associated variants, and different $CD4^+$ T-cell subsets, including rs3135388 and rs9266629 status. (n=675)

PRS incorporating Th1, Th17, Th17.1 and $CD8^+$ associated variants	T-cell subset	P-value
	$CD4^+$ TemRA	**0.01***
	$CD4^+/CD8^+$ ratio	**2.34×10^{-6}****

Abbreviations: PRS, polygenic risk score; TemRA, effector memory RA+ T cell; Th, T helper cell; Treg, regulatory T cell
Analyses are adjusted for age, sex and ten genetic principal components (PC's).
*:p<0.05
**: p<0.001

Supplementary Table 7.19. ANOVA results of the polygenic risk score, incorporating Treg and Th2 associated variants, and different $CD4^+$ T-cell subsets, including rs3135388 and rs9266629 status. (n=675)

PRS incorporating Treg and Th2 associated variants	T-cell subset	P-value
	$CD4^+$ TemRA	**7.92×10^{-3}***
	$CD4^+/CD8^+$ ratio	**3.63×10^{-8}****

Abbreviations: PRS, polygenic risk score; TemRA, effector memory RA+ T cell; Th, T helper cell; Treg, regulatory T cell
Analyses are adjusted for age, sex and ten genetic principal components (PC's).
*:p<0.05
**: p<0.001

8 General Discussion

SUMMARY OF MAIN FINDINGS AND DISCUSSION

Rationale and overview

This thesis is based on the hypothesis that the immune system is shaped during early life under the influence of genetic variants and the exposure to various environmental factors. Skewing of the adaptive immune response and immune memory by these factors is a risk factor for childhood disease which can be visualized. In order to study this, we formulated the following three aims: **I)** To examine the association between adaptive immunity and health outcomes in children, **II)** To identify the role of serum 25-hydroxyvitamin D (25(OH) D) and adiposity on the shaping of the adaptive immunity in healthy children, **III)** To identify the role of specific genetic variants associated with immune-mediated diseases on the shaping of the immune system. This chapter discusses the results of this thesis in a broader perspective including the methodological considerations of our studies and future implications.

Immune composition in immune-mediated childhood diseases

Why do some children develop immune-mediated diseases during childhood, whereas other children do not? This is a crucial question in the understanding of the pathophysiology of immune-mediated diseases. Differences in the immune system between children with and without immune-mediated childhood diseases could provide further understanding of the pathophysiology of childhood immune-mediated disease as well as new possibilities for monitoring and treatment of these diseases.

In **Chapter 2**, I showed that 10-year-old children with any atopic disease had higher T helper (Th)2, Th17 and memory regulatory T (Treg) cell numbers than children without any atopic disease. In addition, children with any atopic disease had higher total B cell numbers, mainly explained by higher $CD27^{+}IgA^{+}$ and $CD27^{-}IgA^{+}$ memory B cells. Additionally, in **Chapter 2** atopic dermatitis, asthma and allergic sensitization were studied as individual entities. Children with atopic dermatitis or allergic sensitization had higher naive and memory Treg and Th17 numbers. Higher IgA^{+} B memory cell numbers were only observed in children with food-allergic sensitization. No alterations in circulating T or B cells were observed in children with asthma compared to the non-asthmatic population. I concluded that the observations of higher Treg memory and IgA^{+} B cell numbers could be a representation of a transforming growth factor beta (TGF-β) mediated compensation for chronic inflammation observed in atopic disease.

In **Chapter 3**, I showed that 10-year-old children with higher attention problems had higher Th1 and cytotoxic T cell numbers which might indicate a role of a pro-inflammatory immune profile in attention problems in childhood.

The link between environmental and metabolic factors and the immune system

Previous studies have linked various environmental factors and body compositional measures to the immune-mediated diseases in childhood that we studied in this thesis.[1-6] It has been hypothesized that these factors might contribute to the susceptibility of disease through effects on the immune system. My studies focused on serum 25(OH)D and adiposity.

In **Chapter 4**, I concluded that serum 25(OH)D might enhance cellular immunity in children as we observed higher $CD4^+$ TemRO, $CD4^+$ TemRA and $CD8^+$ TemRA cell numbers in children with higher levels of serum 25(OH)D.

In **Chapter 5**, I showed that children with higher total fat mass index and higher visceral fat mass index had higher $CD8^+$TemRO T cell numbers and higher $V\delta2^+V\gamma9^+$ T cell numbers. Only higher liver fat was associated with lower Th17 cell numbers. These results might suggest that children with adiposity already have a pro-inflammatory immune profile.

A role for genetics in skewing the immune system with an increased risk of disease?

In **Chapter 6,** I observed that children of a general pediatric population with any of the most common European *FLG* mutations (R501X, S1085CfsX36, R2447X, and S3247X) had higher Th22 cell numbers. I suggested that this might be an immunological response to the altered skin barrier. In a subpopulation of children with ever doctor-diagnosed atopic dermatitis no differences in immune cells were observed, possibly due to a decreased filaggrin protein expression within the atopic dermatitis population regardless of *FLG* mutations.[7] It could be speculated that there exists a spectrum of phenotypes within atopic dermatitis dependent on underlying immune composition alterations in children and *FLG* mutations.[8]

Chapter 7 defined a polygenic risk score (PRS) for multiple sclerosis (MS), the MS-PRS. I observed that children with a higher MS-PRS had a higher ratio of $CD4^+/CD8^+$ cells which was mainly explained by a decrease in total and naive $CD8^+$ T cells in 6-year-old children.

Table 8.1. Overview of changes in circulating immune cell subset numbers and their relation to immune-mediated disease, serum 25(OH)D, adiposity and genetic variants.

	IMMUNE-MEDIATED DISEASE					VITAMIN D AND BODY COMPOSITION		GENETIC VARIANTS	
	Atopic dermatitis	Food allergy	Inhalant allergy	Asthma	ADHD	Vitamin D	Adiposity	*FLG* mutations	MS-PRS
CD4$^+$ T	↑	↑	=	=	=	=	=	=	=
Naive	=	=	=	=	=	=	=	=	=
Tcm	↑	=	=	=	=	=	=	=	=
TemRO	=	↑	↑	=	=	↑	=	=	=
TemRA	=	=	=	=	=	↑	=	=	=
Th1	=	=	=	=	↑	n/a	=	=	n/a
Th2	↑	↑	↑	=	=	n/a	=	=	n/a
Th17	↑	↑	=	=	=	n/a	↓ Liver fat	=	n/a
Th17.1	n/a	n/a	n/a	n/a	n/a	n/a	n/a	=	n/a
Th22	n/a	n/a	n/a	n/a	n/a	n/a	n/a	↑	n/a
Treg memory	↑	↑	↑	=	=	n/a	n/a	=	n/a
CD8$^+$ T	=	=	=	=	↑	=	=	=	↓
Naive	=	=	=	=	↑	=	=	=	↓
Tcm	=	=	=	=	↑	=	=	=	=
TemRO	=	=	=	=	=	=	↑	=	=
TemRA	=	=	=	=	=	↑	=	=	=
CD19$^+$ B	=	↑	=	=	=	n/a	n/a	=	n/a
Transitional	n/a	n/a	n/a	n/a	=	n/a	n/a	=	n/a
Naive	=	↑	=	=	=	n/a	n/a	=	n/a
IgM	n/a	n/a	n/a	n/a	n/a	n/a	n/a	=	n/a
IgG	=	=	=	=	=	n/a	n/a	=	n/a
IgA	=	↑	=	=	=	n/a	n/a	=	n/a
IgE	=	=	=	=	n/a	n/a	n/a	=	n/a
Monocytes	n/a	n/a	n/a	n/a	n/a	n/a	=	n/a	n/a
Vδ2$^+$Vγ9$^+$Tcells	n/a	n/a	n/a	n/a	n/a	n/a	↑	n/a	n/a

Green represents a positive association, red represents a negative association, grey represents no association and white represents that the specific association was not studied.

Abbreviations: ADHD, attention deficit disorder; *FLG*, filaggrin gene; Ig, immunoglobulin; MS, multiple sclerosis; n/a, not applicable; Tcm, central memory T cell; TemRA, effector memory RA$^+$ T cell; TemRO, effector memory RO$^+$ T cells; Th, helper T cell; Treg, regulatory T cell.

Overlapping epidemiology and immunology?

The presence of immune-mediated diseases in childhood is accompanied by a higher likelihood of other immune-mediated disease.[9, 10] Atopic dermatitis, food and inhalant allergies and eosinophilic asthma often co-occur as these conditions are all a manifestation of atopy.[11] Reasons for this co-occurrence include overlap in genetics, environmental exposures and immunological pathways.[12, 13] A previous study within twins showed a correlation estimate of 0.55 between atopic dermatitis and asthma and a correlation estimate of 0.62 between atopic dermatitis and inhalant allergies.[12, 14-16] The atopic immunological pathophysiology comprises of an increased Th2-mediated immune response with interleukin (IL)-4, IL-5 and IL-13 leading to eosinophilic inflammation and class-switching to IgE^+ in B cells with sensitization to certain allergens.[17, 18] Childhood asthma has previously been associated with childhood ADHD in several studies.[19-22] Yet, despite the co-occurrence of these immune-mediated diseases within children we did not observe a specific or related immune cell composition pattern for attention problems and asthma (Table 8.1).

Immune cell composition changes before disease onset and temporality

Examination of those environmental exposures associated with later life diseases in a healthy pediatric population could provide insights in early pathological changes.[23] We showed that serum 25(OH)D and adiposity affect T cell composition in children (Table 8.1, Figure 8.2). Adiposity has been associated with immune-mediated diseases.[24, 25] Future studies should determine if the observed T cell alterations within our study on adiposity contribute to the onset of immune-mediated disease. Determining temporality and reversibility of effects is of interest when speculating about interventions and reversibility of earlier unfavorable exposures. During development, children might attain temporal vulnerability to certain environmental exposures which might imply that strengths of associations differ

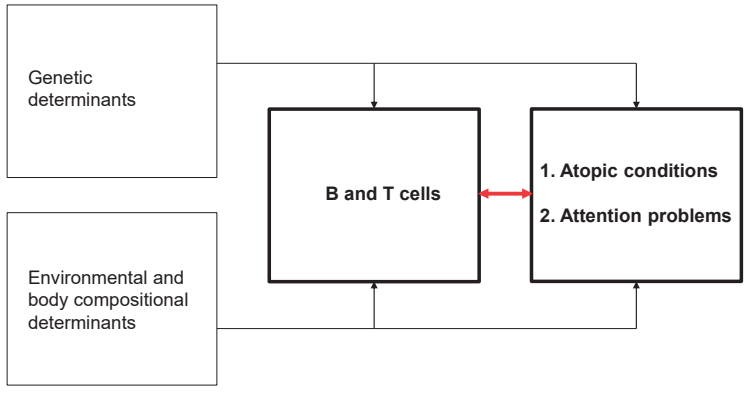

Figure 8.1. Schematic overview highlighting the studies in Chapters 2 and 3 examining atopic diseases and attention problems in relation to immune composition, respectively.

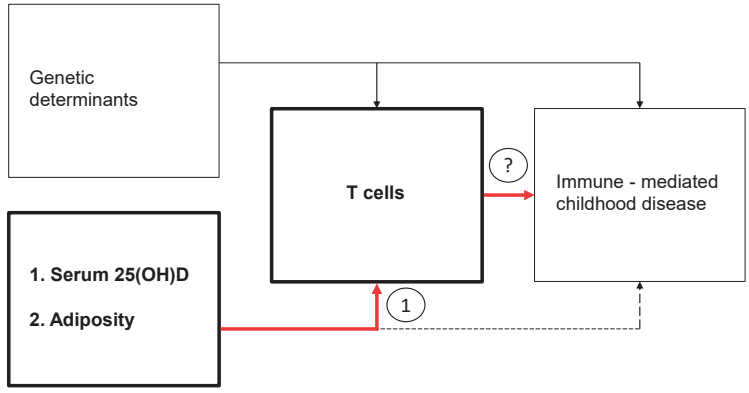

Figure 8.2 Schematic overview highlighting the studies in Chapters 4 and 5 examining serum 25(OH)D and adiposity in relation to the immune composition, respectively. Abbreviation: 25(OH)D, 25-hydroxyvitamin D.

by age.[23, 26] It has been shown that increase in BMI at early age is associated with higher rates of cardiovascular diseases and asthma.[27, 28] The timing of the increase in BMI in children is called the adiposity rebound.[27, 28] Unfortunately, within our study of 10-year-old children we did not have a sufficient number of time points with measurements of body mass index to determine the age of adiposity rebound.[27-29] Previous studies have shown that the immune system composition is highly dynamic during the first six years of life.[30-32] At birth, a relative peak in naive cells is observed after which immune memory, e.g. B and T cells, is built.[31, 33] After the age of six years old, the overall immune composition has been observed to be relatively stable.[30-34]

It has been suggested that immune composition is affected by seasonal changes in environmental exposures such as infections.[35, 36] A previous study that measured B and T cells within individuals across four seasons observed that season does not affect the immune composition in adults.[37] In addition, it has been shown that season of birth does not affect the child's immune cell composition or the immune maturation patterns.[31, 38] Another study showed that challenging the immune system with the influenza vaccination leads to an immune response to the influenza antigen after which the immune system returns to its own balanced state in which the inter-individual differences within the B and T cell composition remain comparable to the B and T composition as was present before vaccination.[34] This suggests that the overall B and T cell composition can be regarded as stable with possible short-lasting variations due to short-lasting exposures that reflect the elasticity of the B and T cell landscape.[34] Therefore, it is unlikely that the time of immune cell measurement within our studies affects the immune cell composition of the children at the same age. Future studies could determine if early life interventions also affect immune cell composition and ultimately severity or onset of disease.[39-41]

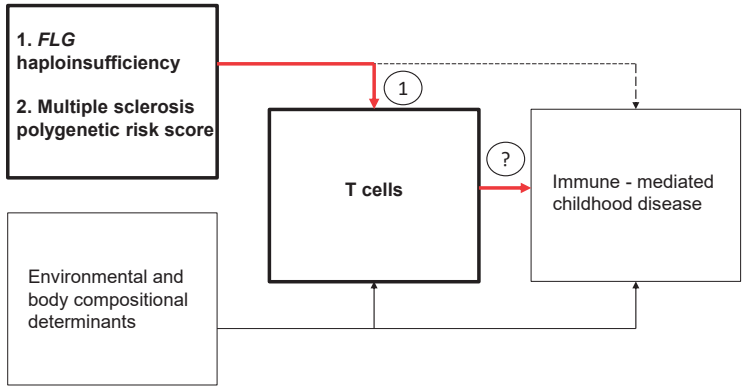

Figure 8.3 Schematic overview highlighting the studies in Chapters 6 and 7 examining *FLG* mutations and MS-PRS in relation to the immune composition, respectively. Abbreviation: *FLG*, Filaggrin gene.

Genetic susceptibility and development to immune-mediated disease

The penetrance of a genetic variant refers to the proportion of individuals with a certain genotype that will develop the corresponding phenotype.[42] Complete penetrance invariably leads to clinical symptoms.[43] Reduced or incomplete penetrance means that carriage of a gene does not always lead to clinical symptoms.[43] In case of reduced or incomplete penetrance, environmental factors, other genetic traits and epigenetics can contribute to the phenotype.[42, 44] For *FLG* haploinsufficiency there is incomplete penetrance.[45] In a previous German cohort among school-aged children, 38.5% of the school-aged children with *FLG* haploinsufficiency had a diagnosis of atopic dermatitis.[46] In this German cohort, the prevalence of having any *FLG* mutation was 7.4% which is comparable to the study on *FLG* haploinsufficiency in this thesis that showed a prevalence of 8.4%.[46] In contrast to the study on *FLG* haploinsufficiency where stratification by atopic dermatitis was possible, no stratification by multiple sclerosis diagnosis was possible in the study on MS-PRS and T cell composition.

From genome-wide association studies, 233 SNPs have been identified as susceptible genetic variants in MS.[47, 48] These SNPS each have a small contribution to developing MS with an incomplete genetic penetrance.[48] Of interest for future studies would be to identify the children within this cohort that eventually develop MS. In this way, we could determine if the immune cell composition alterations that we observed at early age in children with a high MS-PRS affect MS onset in later life. This could be hypothesized as a previous study showed good discriminative performance of the MS-PRS for predicting MS.[49] Examining this in future studies could be challenging due to the relatively low prevalence of MS and the long follow-up needed for diagnosis of MS.[50] Potentially, case-control studies could be used for this within large cohorts such as the UK Biobank Study.[51, 52] Recently, comparable

research was performed within the UK Biobank Study that provided evidence that childhood adiposity potentiated the genetic risk for MS.[49] Increasing knowledge on gene-gene interactions and environment-gene interactions and their effect on immune composition and MS risk could provide further understanding of the MS pathogenesis and ultimately help in the development of preventive or treatment options.[49]

METHODOLOGICAL CONSIDERATIONS

Internal validity

In order for studies to have external validity, these should first guarantee internal validity.[53] Internal validity determines the validity of the conclusions made within the study population and is assessed by three main pillars: selection bias, information bias and confounding.[53]

Selection bias in studies occurs due to factors that determine an individual's participation to a study at baseline or factors that determine an individual's loss-to-follow-up. The studies within this thesis were performed within the Generation R Study.[54] At baseline, the study included 9,778 mothers (61% response rate).[54] For the visit at the research center at 6 and 10 years, there was a response rate of 81% and 79%, respectively.[54] The number of children with detailed immune phenotyping was based on a random maximum sample of 3 children per day due to feasibility reasons. Although we studied the whole spectrum of socio-economic status, in general, the participants within the Generation R studies tended to live in a more prosperous environment with higher net household income and higher parental education compared to the non-responders.[54] In addition, the responders were more often reported as the Dutch ethnicity.[54] However, this selection only threatens the internal validation if the non-responders differ in the associations studied compared to the responders.[53, 55] It is difficult to determine to what extent associations could differ in the non-responders compared to the responders and therefore it is best to interpret the results with the caution of a possible selection bias by socio-economic status.

Socio-economic status is negatively associated with adiposity and it could be speculated that the less health conscious children have a higher risk of loss-to-follow-up.[56] Hypothetically, if the children with obesity within our study are all health conscious, possibly due to a higher socio-economic status, and these children compensate for their obesity by having a healthy lifestyle limiting the T cell mediated inflammation, the associations could be biased to the more extreme end. The selection in the study could lead to change in direction of the observed associations. Yet, it has been previously shown that selective inclusion at baseline of a study does not strongly affect the studied associations and therefore large differences in the studied associations between the responders and non-responders are not expected.[57] In

some cases selection bias and confounding may overlap, e.g. if differential study participation is based on a factor that is measured within a cohort study, the analyses could adjust for this factor.[53, 55] In this case, adjusting for the factor associated with the selection such as socio-economic status could alleviate this.[53, 55]

Second, information bias occurs due to erroneous measurements of determinants, outcomes or confounders.[53] This might lead to misclassification of the study subjects. Misclassification can be categorized into differential and non-differential misclassification.[53] Differential misclassification is non-random, meaning that the number of misclassified determinants, outcome or confounders is non-equally divided between exposed and non-exposed participants. The effect of differential misclassification on the studied associations can lead to underestimation and overestimation of the effect estimates. On the contrary, non-differential misclassification is random with regard to the studied variables, meaning that the number of misclassified determinants, outcome or confounders is equally divided between exposed and non-exposed participants. The effect of this type of misclassification biases towards the null.[53] Within our studies it is possible that determinants or confounders have been misclassified such as the determinants and confounders that were retrieved by parental-reported questionnaires. However, this is most likely independent of their immune cell outcomes, and hence non-differential, because misreporting of environmental exposures or disease outcomes is independent of laboratory measurements. Differential misclassification could be argued for the attention problem questionnaires in which it could be hypothesized that mothers of children are more focused on the attention problems of the child than mothers of children without attention problems leading to differential recall bias.[58]

Third, confounding is defined as a factor that causes erroneous associations in observational studies due to its association with both the exposure and the outcome without being involved in the causal pathway of the studied exposure and outcome.[53] The strength of the Generation R Study is the information on many possible confounders over time. Adjusting for confounders is of importance in studies covering the shaping of the immune system as at least 50% of the immune shaping during childhood is affected by non-genetic factors.[31] However, a chance of residual confounding within our studies cannot be excluded due to the possibility of limited measurements of certain factors or missing factors.[59] Unmeasured confounding factors could be unmeasured lifestyle factors, air pollution or microbial diversity for the study on atopic diseases and immune composition.[60-62] An example of limited measurement or misreporting could be within our study covering adiposity where we adjusted for diet quality of the children.[63] Although this diet score includes various aspect of the child's diet, it is based on food frequency questionnaires in which not all individual foods can be measured and the recall of quantity of food intake might lead to some measurement errors.[63, 64]

External validity

External validity determines to what extent our results are valid in other populations. External validity is important in determining the implications of studies in other populations than the study population.[53] In determining the external validity, the characteristics of the study population should be compared to the characteristics of these other populations. Differences in study population could imply that the results of a specific study are not generalizable to another setting. This could be argued regarding our studies that are embedded within the Generation R Study with inclusion of a higher proportion of individuals with a higher socio-economic status at baseline.[54] Yet, differences in study populations do not necessarily mean that results cannot be extrapolated. For example, if the difference in study population does not affect the biological mechanisms underlying the observed association, an association might still be valid in a different population. Although our results should be extrapolated to other populations with caution, it should be noted that we had a considerable diverse population regarding various factors, including socio-economic status.[54] It is complex to state what the general external validity of our studies is, as this also depends on the individual studies and the possible other settings (i.e. geographically setting or severity of disease) to which the studies are compared.[30]

From an immunological perspective the studies within this thesis have a unique setting for several reasons: the ability to study immune maturation in young children of a general population, the ability to adjust for various confounding variables during childhood and to study immune maturation in a relatively large population. The inclusion of a healthy pediatric population provides insight in early disease pathophysiology which might be ideal to reverse the immune composition alterations before irreversibility is present with onset of disease.[65] In the study on MS-PRS, the absence of the disease MS also had the advantage that purely the association between genetic variants and immune alterations could be studied without effects of presence of MS on immune cell outcomes. Our results are likely to have a higher external validity for general populations compared to a setting with children that have severe phenotypes of immune-mediated disease. For example, we did not observe increased circulating IgE^+ memory B cells in children with atopic disease, in contrast to a previous study that included children with a severe atopic disease.[18] It can be speculated that the differences in immune subsets in the other study were greater because that study involved a group of children with a more severe disease phenotype and more severe chronic inflammation than those in the Generation R Study. In addition, we observed minimal B and T cell changes in children with *FLG* mutations from a general population. This is in contrast to previous studies that studied *FLG* mutations in patients with active atopic dermatitis and observed an increased Th2-mediated immunity.[66, 67] This suggests that there are differences in immunological composition between populations with different disease severities.

Immune maturation in the first years of life has been observed in various cohorts across the world.[30, 31, 68-70] The conclusion of these geographically diverse cohorts is that although some differences exist between these cohorts for individual cell type numbers, generally a common pattern in immune maturation can be observed. The general common pattern of immune maturation comprises of a steady build-up of memory B cell and T cell along with a decrease in neutrophils.[38] Yet, the immunological subsets of children from Tanzania have been compared to children of the Generation R Study which showed that the children in Tanzania had higher numbers of $CD4^+$ effector T cells during the first year of life compared to the Generation R population.[30] In addition, these children had higher memory B cell numbers with a shift to the IgM isotype.[30] In contrast, the Generation R Study population had higher numbers of IgA memory B cells compared to the Tanzanian cohort.[30] This suggests that the underlying biological pathogenesis of immune maturation is shared across various populations and is probably comparable between countries with comparable surroundings, yet heterogeneity in individual cell numbers might exist between countries such as The Netherlands and Tanzania due to multiple genetic and environmental differences such as infection rate, microbial environment and vaccination status.[30] Therefore, validation of our results in other pediatric cohorts is needed. It could be speculated that differences in immune maturation also exist within the Netherlands dependent on living area, as also within the Netherlands neighborhoods differ in environmental exposures such as pollution, food quality and socio-economic status.[61, 71, 72]

Finally, during the first years of life, the adaptive immunity is dynamic and has been shown to mature according to an age-specific pattern.[30, 31, 38, 68, 69] It is likely that there exist susceptibility windows for immune maturation which are defined as periods in time during the child's development in which the child is especially vulnerable to certain exposures.[23, 26, 31, 73, 74] Therefore, extrapolation of our results to other ages should be performed with caution and longitudinal studies are necessary.

FUTURE PERSPECTIVES

Future directions from a system biology perspective

It is likely that individual exposures do not contribute as a sole factor to immune maturation or onset of immune-mediated diseases.[61, 75-77] To facilitate the visualization of the interactions between various environmental and genetic factors a system biology approach could be used in future studies on the immune maturation in childhood.[78] This system biology approach, also referred to as multi-omics research, is an interdisciplinary statistical technique to construct highly detailed models to visualize complex multiple associations between all environmental and genetic factors an individual is exposed to.[78, 79] Within the multi-omics

studies, individual omics studies are present such as exposome studies and studies including genomics.[80-82] Exposome studies include all non-genetic exposures an individual is exposed to including environmental factors and metabolic factors.[83] An example of an exposome study is the human early life exposome project that studies a spectrum of environmental exposures in pregnancy and childhood.[76, 84] Studying the exposome may, for example, provide answers to the question why one individual develops atopic disease upon certain antigen exposures whereas other individuals do not by incorporating the cumulative effect of certain exposures and interactions between exposures.[76, 77, 80, 84] A challenge for this exposome approach is the large study population needed to guarantee sufficient power of statistical analyses and the measurement of a large number of potentially important environmental exposures.[85, 86] The genomics studies include all genetic factors an individual is exposed to.[82] In contrast to the studies within this thesis, a system biology approach is more hypothesis generating than hypothesis-driven research.[78, 83] This system biology approach has also been suggested for studies on immune maturation to discover new drivers of immune maturation.[77, 80, 83] It has been shown that a multi-omics approach could provide new insights into the immune pathophysiology of disease with relatively little prior knowledge.[79, 83] The method of system biology could be an interesting addition to current hypothesis-driven research by approaching a real life situation and possibly providing new insights useful in personalized medicine.

Determination of causality in future studies

A challenge for future studies is to determine causality in studies on the maturation of the immune system in childhood. Current literature on childhood immune maturation is mainly observational and cross-sectional and cannot determine causality due to various factors such as the possibility of reversed causation, residual confounding.[31, 77, 87] Likewise, the studies in this thesis cannot make conclusions about causality. Often both directions of the association could be argued as is also represented in Figure 8.1. We reasoned that a Th2-mediated immune system contributes to eosinophilic inflammation as is observed in atopic diseases.[18] Yet, it can also be speculated that atopic diseases cause immune cell composition changes. For example, in atopic dermatitis it has been shown that the skin inflammation causes a decrease in the protein filaggrin within keratinocytes which in turn causes a further dysfunction of the skin barrier with an increase in pro-inflammatory cytokines such as IL-1.[88-90] The Bradford Hill criteria can be used to consider the likelihood of cause and effect in associations determined within observational studies.[87, 91, 92] Furthermore, Mendelian randomization could be an even more interesting method to determine if previously observed associations on adaptive immune maturation within observational studies are causal.[93, 94] Mendelian randomization is a methodological approach that uses specific genetic variants that have previously been associated with the exposure of interest and subsequently determines the association of these genes with the outcome of interest.[93, 94]

Mendelian randomization is based on the assumption that the possession of certain genes is randomly allocated and can therefore be compared to a randomized controlled trial.[93, 94] It is important that the genetic allocation that is used in Mendelian randomization is not directly associated with the outcome of interest but only through its effect of the exposure of interest. Previously, Mendelian randomization has been used for various studies including studies on vitamin D.[95] A previous Mendelian randomized study showed that low 25(OH)D levels are causally related to the risk of bacterial pneumonia.[96] This might be reflected within our study that showed that higher serum 25(OH)D is associated with enhanced cellular immunity. In addition, various SNPs that are associated with adiposity, body mass index and obesity have been identified through genome-wide association studies.[97, 98] Previous studies using these genetic variants in order to perform Mendelian randomization determined that the association between adiposity and asthma and adiposity and inflammatory conditions such as type II diabetes and cardiovascular disease is causal.[5, 99] The increasing availability of data from genome-wide association studies increases possibilities for the use of Mendelian randomization in future studies.[95, 100]

Future changing environments and immunity

It can be expected that the future environment for children is composed differently compared to the environment illustrated in the current thesis.[59, 101] Urbanization and pollution in the coming years are expected to be accompanied by a decrease in environmental microbial diversity.[62, 102, 103] The biodiversity hypothesis states that contact with the natural environment increases a microbial diversity which stimulates a balanced immunity development and limits the chances of immune-mediated diseases such as atopic diseases.[62] Previously, it has been shown that children with atopic conditions have a less diverse immune microbiome.[62, 104] Many studies could not make conclusions about causality in these associations.[62] However, a recent trial in children showed that measures to increase biodiversity at daycares increased the diversity of child's skin and gut microbiome and caused a shift towards a more immune-regulatory cell numbers with an increase in Treg cell numbers and an increase in IL-10.[105] This suggests a causal relationship between biodiversity and immune regulation and could explain how biodiversity might have an impact on immune-mediated disease. Previous studies suggest that the rise in atopic conditions in the coming years, to a prevalence of more than 50% of all Europeans by 2025, might be due to these biodiversity changes.[43, 103, 104] Therefore, it has been postulated that the rise of allergies might be accompanied by a rise in other immune-mediated diseases that have been associated with the microbiome.[73, 103]

GENERAL CONCLUSIONS

The current thesis provided further knowledge on the adaptive immunity as intermediate between environmental, genetic variants and immune-mediated disease in healthy children from a population-based cohort. We compared immune composition between children with and without immune-mediated diseases. Additionally, we discussed genetic and environmental pathways that might affect the shaping of the immune system. Although effects of the studied environmental and genetic factors on immunity might be small and no causal relations can be established, the results of this thesis contribute to the understanding of immune maturation in children with and without immune-mediated disease.

Part I concluded that various atopic conditions show similarities in an altered underlying adaptive immune composition including a more Th2-mediated immunity and increase in memory regulatory T cells and IgA^+-memory B cells. In addition, attention problems were associated with a Th1-mediated immunity. Although immune-mediated childhood diseases such as asthma and attention problems co-occur within individuals in epidemiological studies, no comparable clusters in immune alterations could be observed within our studies. **Part II** of this thesis concluded that 25(OH)D and childhood adiposity affect T-cell subsets within healthy children. **Part III** showed that the *FLG* mutations and MS-PRS affect T-cell composition in healthy children. Part II and III showed that T cell composition alterations associated with genetic variants, environmental exposures or body compositional variations are present before possible onset of disease. Overall, due to small effects of individual exposures, it is likely that a broad spectrum of environmental exposures and genetic variants contributes to the final immune phenotype of the child.

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A APPENDICES

SAMENVATTING

Het immuunsysteem speelt een cruciale rol in gezondheid en ziekte. Binnen het immuunsysteem moet er een voldoende balans zijn tussen de verdediging tegen ziekteverwekkers en immuunregulatie om schade van reacties tegen onschadelijke stoffen (allergie) of het eigen lichaam (auto-immuniteit) te voorkomen. In deze balans is er een nauwe samenwerking tussen het aangeboren en de verworven, ook wel adaptieve, immuniteit. De adaptieve immuniteit wordt met name gevormd door T- en B-cellen. Dit proefschrift is gebaseerd op de hypothese dat de balans in het immuunsysteem wordt gevormd gedurende de kindertijd onder invloed van genetische en niet-genetische factoren en dat een disbalans in de vorming van de adaptieve immuunrespons en het immunologisch geheugen een risicofactor is voor de ontwikkeling van bepaalde ziekten op de kinderleeftijd.

Om dit te onderzoeken heb ik drie doelen opgesteld: I) het bestuderen van de associatie tussen de adaptieve immuniteit en gezondheidsuitkomsten bij kinderen, II) het identificeren van de rol van vitamine D en adipositas op de adaptieve immuniteit bij kinderen en III) het bepalen van de rol van specifieke genetische varianten geassocieerd met immuungemedieerde ziekten op de vorming van de adaptieve immuniteit.

Adaptieve immuniteit en immuungemedieerde ziekten op de kinderleeftijd

Deel I van dit proefschrift behandelt de associatie tussen de adaptieve immuniteit en immuungemedieerde ziekten op de kinderleeftijd. Nieuwe inzichten in deze associaties zouden kunnen bijdragen aan het begrip van de pathogenese van deze ziekten op de kinderleeftijd en de kennis voor het monitoren en behandelen van immuungemedieerde ziekten op de kinderleeftijd.

In hoofdstuk 2 heb ik de associatie tussen B- en T-cel subpopulaties en atopische ziekten onderzocht bij kinderen op de leeftijd van 10 jaar oud. Kinderen met een atopische ziekte hebben hogere aantallen T helper 2 (Th2), regulatoire T (Treg), geheugen Treg en B-geheugencellen, die immunoglobuline (Ig)A tot expressie brengen. Bij het onderzoeken van de individuele atopische ziekten zag ik dat kinderen met eczeem of sensitisatie voor inhalatie- of voedselallergenen hogere aantallen geheugen Treg cellen hebben. Deze Treg cellen hebben een rol in de immuunregulatie. Alleen de kinderen met sensitisatie voor voedselallergenen hadden hogere aantallen $CD27^{+}IgA^{+}$-geheugen B-cellen, een subset van de B-cellen die zich ontwikkelt met behulp van de T-cellen. IgA heeft een rol in de mucosale immuniteit van onder andere de darm. Er werden geen associaties tussen astma en B- of T-cellen gevonden. De hogere aantallen Treg en $CD27^{+}IgA^{+}$ B-geheugencellen bij kinderen met sensitisatie voor voedselallergenen is suggestief voor een TGF-β gemedieerde compensatie voor chronische inflammatie.

In hoofdstuk 3 heb ik de relatie tussen de adaptieve immuniteit en aandachtsproblemen bij kinderen op 10-jarige leeftijd onderzocht. De pathogenese van ADHD is momenteel nog onduidelijk. Op basis van voorgaande literatuur en eerder aangetoonde associaties tussen de aanwezigheid van ADHD en immuungemedieerde ziekten formuleerde ik de hypothese dat chronische immuunactiviteit een rol in de pathofysiologie van ADHD zou kunnen spelen. Ik concludeerde dat kinderen met hogere aantallen van circulerende Th1 en cytotoxische T-cellen meer aandachtsproblemen hebben. Dit zou kunnen wijzen op een mogelijke rol voor een pro-inflammatoir immuunprofiel in de pathogenese van aandachtsproblemen bij kinderen.

Vitamine D en adipositas en adaptieve immuniteit

In deel II van dit proefschrift worden serum 25-hydroxyvitamine D (25(OH)D) en adipositas in relatie tot de B- en T-cellen bestudeerd. Voorgaande studies hebben aangetoond dat vitamine D effect heeft op de T-cel functie via de vitamine D receptor die aanwezig is op geactiveerde T-cellen. Er waren echter geen studies aanwezig die de relatie tussen vitamine D en T-cellen bij kinderen hebben bepaald. In hoofdstuk 4 heb ik bij kinderen van 6 jaar oud serum 25(OH)D spiegels bepaald in relatie tot T-cel aantallen. Hierbij concludeer ik dat kinderen met hogere vitamine D spiegels, hogere aantallen effector geheugen T-cellen hebben (specifiek $CD4^+$TemRO, $CD4^+$TemRA en $CD8^+$TemRA). Dit suggereert dat vitamine D de cellulaire immuniteit van kinderen stimuleert.

In hoofdstuk 5 heb ik de relatie tussen vetmassa en monocyten, $CD4^+$ en $CD8^+$ en γδ T-cellen bij tienjarige kinderen bepaald. Bij volwassenen is de aanwezigheid van obesitas geassocieerd met chronische laaggradige inflammatie wat mogelijk bijdraagt aan toekomstige chronische ziekten. Het is onduidelijk of deze adipositas-gerelateerde inflammatie al aanwezig is bij kinderen. In dit hoofdstuk beschrijf ik onder andere dat de aanwezigheid van meer visceraal vet bij kinderen geassocieerd is met meer circulerende $V\delta2^+V\gamma9^+$ T-cellen en $CD8^+$TemRO cellen. Dit impliceert mogelijk dat adipositas-gerelateerde inflammatie al aanwezig is bij kinderen met adipositas binnen een algemene populatie.

Genetische varianten en adaptieve immuniteit

Deel III van dit proefschrift bespreekt de associatie tussen genetische varianten en de adaptieve immuniteit. In hoofdstuk 6 hebben we de relatie onderzocht tussen genetische varianten, ook wel single nucleotide polymorphisms (SNPs), in het filaggrine gen (*FLG*) en de adaptieve immuniteit. Mutaties in het *FLG* veroorzaken een defect in de barrièrefunctie van de epidermis en leiden daarmee tot een verhoogd risico op het ontwikkelen van eczeem. De eerste studie van deel III van dit proefschrift heeft de meest voorkomende SNPs binnen de Europese populatie (R501X, S1085CfsX36, R2447X, en S3247X) onderzocht. Een variant in *FLG* was aanwezig bij 8,4% van de kinderen van de totale populatie en bij 15,7% van de

kinderen binnen de kinderen met eczeem. Binnen de totale algemene populatie zag ik dat kinderen met een *FLG* variant meer Th22 cellen hebben. Dit zou een uiting van de immunologische reactie op de veranderde huidbarrière kunnen zijn. Er waren geen verschillen in Th1, Th2, Th17, Treg of de B-geheugencellen. Binnen de kinderen met eczeem was er geen verschil in cel-aantallen tussen kinderen met en zonder een *FLG* variant.

In hoofdstuk 7 hebben we bij 6-jarige kinderen polygenetische risico scores (MS-PRS) voor multiple sclerose (MS) bepaald op basis van bekende genetische risicovarianten voor MS. We bepaalden de associaties tussen deze MS-PRS en T-cellen. We observeerden dat een hogere MS-PRS gecorreleerd is met lagere aantallen van $CD8^+$ T-cellen. Op basis van deze correlatie was een hogere MS-PRS gecorreleerd met een hogere $CD4^+/CD8^+$ T-cel ratio. Deze associaties werden met name gedreven door HLA-DRB1*15:01 en HLA-B, genetische risico varianten die in de literatuur het meest geassocieerd zijn met de ziekte MS. Onze studie suggereert dat genetische risico varianten van MS al op kinderleeftijd een verandering veroorzaken in de T-cel compositie in een algemene populatie.

Samenvattend draagt het huidige proefschrift bij aan de kennis over de relatie en interacties van de adaptieve immuniteit met omgevingsfactoren, genetische factoren en immuungemedieerde ziekten op de kinderleeftijd.

In deel I concludeer ik dat er binnen de atopische ziekten een overlap bestaat in het onderliggende immuunprofiel van kinderen. Echter, ondanks dat er een overlap in de epidemiologie van ADHD en astma is, was er geen overlap in immuuncel compositie tussen deze twee aandoeningen. In deel II concludeer ik dat vitamine D status en adipositas bijdragen aan T-cel compositie op kinderleeftijd. In deel III concludeer ik dat de *FLG*-mutaties en de MS-PRS geassocieerd zijn met T-cel compositie in gezonde kinderen. Gezamenlijk tonen we in deel II en III van dit proefschrift aan dat genetische varianten, omgevingsfactoren en lichaamssamenstelling effect hebben op de onderliggende adaptieve immuniteit van kinderen. Dit is mogelijk nog voor het optreden van ziekte. Ondanks de individueel kleine effecten van de gevonden associaties is het waarschijnlijk dat een breed spectrum van omgevings- en genetische factoren bijdraagt aan het uiteindelijke immuunfenotype van het kind.

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LIST OF ABBREVIATIONS

25(OH)D	25- hydroxyvitamin D
α	alfa
AD	Atopic dermatitis
ADHD	Attention-deficit/hyperactivity disorder
AID	Activation-induced cytidine deaminase
ANOVA	Analysis of variance
ATS	American Thoracic Society
β	Beta; regression coefficient
BCR	B cell receptor
BMI	Body Mass Index
BSI	Brief Symptom Inventory
CBCL	Child Behavior Checklist
CCL	Chemokine ligand
CCR	CC-type chemokine receptor
CD	Cluster of differentiation
Chr	Chromosome
CI	Confidence Interval
cis-eQTL	Cis expression quantitative trait loci
CMV	Cytomegalovirus
CXCR	CXC-type chemokine receptor
DAMP	Danger-associated molecular pattern
dim	Dimmer
DXA	Dual-energy x-ray absorptiometry
EBV	Epstein Barr Virus
ELISA	Enzyme-linked immunosorbent assay
eQTL	Expression quantitative trait loci
ERS	European Respiratory Society
FDR	False discovery rate
FEF_{75}	Forced expiratory flow after exhaling 75% of the FVC
FEV1	Forced expiratory volume in 1 second
FLG	Filaggrin gene
FMI	Fat mass index
FSC-A	Forward scatter area
FSC-H	Forward scatter height
ft/ft	Filaggrin deficient flaky tail
FVC	Forced vital capacity
γ	Gamma
g	Gram
GSI	General Symptom Index
GWAS	Genome-wide association study

HMB-PP	(E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate
HLA	Human leukocyte antigen
HSV- 1	herpes simplex virus type 1
ID- XLC- MS/MS	Isotope dilution online solid-phase extraction liquid chromatography- tandem mass spectrometry
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IMSGC	International Multiple Sclerosis Genetics Consortium
INF	Interferon
IQR	Interquartile range
IREM	Immune receptor expressed on myeloid cells
ISAAC	International Study on Asthma and Allergy in Childhood
IV	Ichthyosis Vulgaris
kb	Kilobase
kg	Kilogram
L	Liter
LD	Linkage disequilibrium
LN	Natural logaritm
log	Logaritm
m	Meter
MAF	Minor allele frequency
mDC	Myeloid dendritic cells
MEC	Medical Ethical Committee
MHC	Major histocompatibility complex
mo	Months
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
mTEC	Medullary thymic epithelial cell
n	Number (sample size)
NaCl	Natrium chloride
NAFLD	Nonalcoholic fatty liver disease
nmol	nanomol
NK cell	Natural killer cell
OR	Odds ratio
PLC	*Phospholipase C*
PAMP	Pathogen-associated molecular patterns
PC	Principle component

PRR	Pattern recognition receptor
PRS	Polygenic risk score
PTPRC	Protein Tyrosine Phosphatase Receptor Type C
SC	Stratum corneum
SD	Standard deviation
SDS	Standard deviation score
SE	Standard error
SNP	Single nucleotide polymorphism
SPSS	Statistical Package Social Sciences
SSC-A	Side scatter area
Tcm	Central memory T cell
TCR	T cell receptor
Tem	Effector memory T cell
TemRA	$CD45RO^-$ effector memory T cell
TemRO	$CD45RO^+$ effector memory T cell
Th cell	T helper cell
TGF	Transforming growth factor
TNF	Tumor necrosis factor
Treg	Regulatory T cell
µ	micro
URTI	Upper respiratory tract infections
VCA	Viral capsid antigen
VDR	Vitamin D receptor
y	Years

PHD PORTFOLIO

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Research master's degree Health Sciences, specialization Medicals Clinical Epidemiology, NIHES, Erasmus University Rotterdam	2015-2019	120
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Study Design (CC01)	2015	4.3
Biostatistical Methods I: Basic Principles	2015	5.7
Biostatistical Methods II: Classical	2015	4.3
Development Research Proposal (DRP)	2016	2.5
Oral Research Presentation (PRES)	2016	1.4
English Language (SC01)	2015	1.4
Scientific Writing in English for Publication (SC07)	2017	2.0
Research Seminars (SEM)	2015-2017	0.8
Principles of Research in Medicine and Epidemiology (ESP01)	2015	0.7
Introduction to Public Health (ESP41)	2015	0.7
Primary and Secondary Prevention Research (ESP45)	2015	0.7
Fundamentals of Medical Decision Making (ESP70)	2015	0.7
Pharmaco-epidemiology and Drug Safety (EWP03)	2017	1.9
Advanced Topics in Clinical Trials (EWP10)	2017	1.9
Advanced Analysis of Prognosis Studies (EWP13)	2017	0.9
Principles of Epidemiologic Data-analysis (EWP25)	2017	0.7
Clinical Epidemiology (CE02)	2015	5.7
Methodologic Topics in Epidemiologic Research (EP02)	2015	1.4
Methods of Clinical Research (ESP10)	2015	0.7
Research Symposium (SYMP)	2017	1.4
Clinical Practice-relevant Therapeutic Trials (ESP73)	2015	0.7
Final Exam (FE)	2019	2.0
Elective courses		
Repeated Measurements in Clinical Studies (CE08)	2016	1.4
Missing Values in Clinical Research (EP16)	2016	0.7

Causal Inference (ESP48)	2016	0.7
Causal Mediation Analysis (ESP69)	2016	0.7
Joint Models for Longitudinal and Survival Data (ESP72)	2016	0.7
Quality of Life Measurement (HS11)	2016	0.9
Courses for the Quantitative Researcher (SC17)	2016	0.0
Health Economics (ESP25)	2016	0.7
Exchange courses at Harvard T.H. Chan School of Public Health		
Study Design in Clinical Epidemiology	2016	2.1
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Advanced Molecular Immunology, Postgraduate Course, Molmed Erasmus MC	2016	1.4
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Basic course for clinical investigators (BROK®)	2020	1.0
Advanced Teaching Skills – Harvard Medical School	2021	1.0
Seminars, workshops and symposia		
Research meetings Generation R	2016-2017, 2019-2021	1.0
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Looman KIM, Jansen MAE, Voortman T, van den Heuvel D, Jaddoe VWV, Franco OH, van Zelm MC, Moll HA. The role of vitamin D on circulating memory T cells in children: The Generation R Study. *Pediatr Allergy Immunol. 2017;28(6):579-587.*

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