The Immune System Out of Shape?

Shaping of adaptive immunity by persistent viral infections in young children

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Shaping of adaptive immunity by persistent viral infections in young children

Het immuunsysteem uit vorm?

Het vormen van de verworven afweer door persisterende virale infecties in jonge kinderen

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Chapter 1

GENERAL INTRODUCTION

Protection of the human body: the immune response

The human body is continuously exposed to various pathogens. To protect itself against these potential threats, the human body possesses a combination of mechanisms to either inhibit the entrance of the pathogens, or to neutralize and quickly remove pathogens that do enter the human body. These defense mechanisms can be subdivided into three different layers of protection.

The first layer of protection: physical and chemical barriers

The first layer of protection, is composed of a physical and chemical barrier, which prevent the initial entrance of pathogens into the human system. The major physical barrier is the epithelial lining on the outside of our body, i.e. skin tissue and the mucosal membranes of our gut and lungs. The cells in this epithelial lining are tightly connected (e.g. via tight junctions, gap junctions or adherens junctions), thereby preventing pathogens to penetrate through the epithelium.[1,2] In addition, air flow around the skin, peristaltic movement of the bowel, and movement of cilia on the apical side of epithelial cells mechanically remove pathogens from the human body. Thirdly, in addition to these physical barriers, chemical barriers are formed, which include mucus, sweat, saliva and tears. These fluids contain antimicrobial proteins, contain a high salt concentration and have a low pH, which together inhibit attachment and proliferation of pathogens.[3] Finally, the human body is colonized with commensal microbiota. These bacteria do not only occupy potential niches and thereby prevent the attachment of harmful pathogens, but are also essential for epithelial cell proliferation and differentiation, and support the second and third layers of protection.[4] (Figure 1).

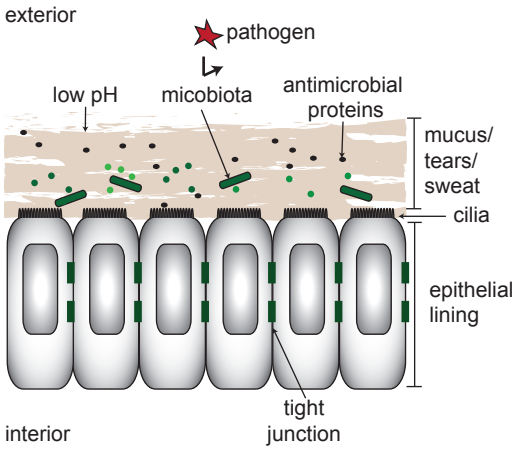


Figure 1. First layer of protection: A physical and chemical barrier.
The physical barrier is composed of a tight epithelial lining near the exterior of the human body, closely connected via structures including tight junctions. The chemical barrier is composed of mucus, tears and sweat containing low pH, high salt concentration and containing antimicrobial proteins. The commensal microbiota and cilia on top of epithelial cells further prevent pathogen adherence.

The second layer of protection: the innate immune response

In case a pathogen does enter the human body, an immune response is needed to eliminate it.[5] Cells and molecules of the vertebrate immune system are able to recognize foreign particles and to initiate a cascade of responses for pathogen neutralization and removal. Important mediators of the second layer of protection are soluble proteins that form the complement system,[6] and innate immune cells. These innate cells carry receptors that recognize conserved pathogenic structures, such as pathogen-associated, microbe-associated or damage-associated molecular

patterns on bacteria, fungi, parasites or damaged host cells.[7-9] Various innate cell populations can be discriminated, each with a distinct role in the immune response (Figure 2).[10,11] First of all, pathogenic threats can be fought directly by two types of innate cells, i.e natural killer (NK) cells and eosinophils. NK cells recognize and kill pathogen-infected cells,[12,13] whereas eosinophils locally release anti-pathogenic toxins upon infiltration of extracellular pathogens.[14] Secondly, mast cells can release chemokines and pro-inflammatory cytokines that induce the dilation of blood vessels, thereby mediating the recruitment of additional innate immune cells from blood, including monocytes, neutrophils, basophils and dendritic cells (DCs).[15] Basophils start to release their granule content, which stimulates further recruitment of innate cells.[15] Monocytes migrate into tissue and differentiate into macrophages that, together with the newly recruited neutrophils, take up pathogens by phagocytosis and destroy the pathogens internally in endosomal structures.[16,17] Finally, DCs take up the pathogen, and migrate through the lymphatic system into secondary lymphoid tissues. In these tissues, B and T lymphocytes are activated and form the third layer of protection.[18] DCs thereby perform a crucial bridging function between the second and third layers of protection.

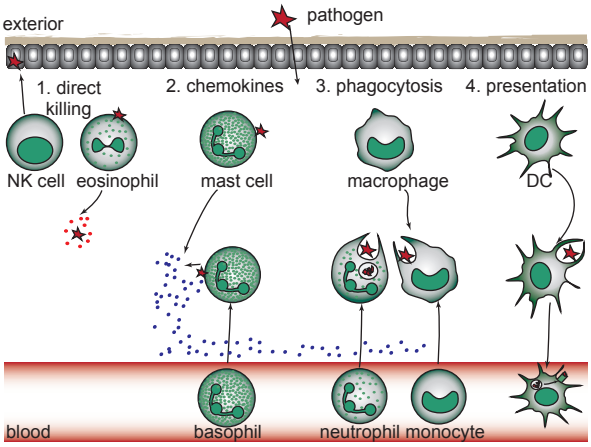


Figure 2. Second layer of protection: Innate immune responses.
Innate leukocytes possess four basic mechanisms to fight pathogen infections:
1) direct killing of pathogen-infected cells by NK cells; or removal of free pathogen in the local tissue by eosinophils;
2) production of chemokines by mast cells and basophils;
3) phagocytosis and internal pathogen killing by macrophages and neutrophils;
4) phagocytosis and antigen presentation by dendritic cells (DCs) to initiate adaptive immune responses.

The third layer of protection: the adaptive immune response

B and T lymphocytes form the third line of defense: the adaptive immune response (Figure 3). These cells carry specialized receptors, i.e. the B-cell receptor (BCR) or T-cell receptor (TCR). In contrast to other proteins, these receptors differ between each newly generated B and T cell, and together these cells can recognize an enormous diversity of structures, i.e. epitopes. Mature B and T cells circulate through blood and lymphatic vessels between lymphoid tissues. B cells recognize soluble antigens dispersed into the lymph nodes via the lymphatic system. T cells, however, only recognize an antigen when it is presented on specialized protein complexes called the human leukocyte antigens (HLA's; also known as the major histocompatibility complex (MHC) proteins), by DCs that migrated from local tissue into the lymph nodes. Upon recognition of specific antigen, a B or T cell becomes activated, resulting in extensive clonal proliferation and differentiation into effector cells.

Two major types of T lymphocytes exist: cytotoxic and helper T cells. Cytotoxic T cells recognize antigens presented on HLA proteins A, B or C (HLA-class I), which

are expressed on all cells of the body except for red blood cells. Presented in the groove of these HLA-class I molecules are intracellular peptides.[19] By scanning the antigens presented in these HLA-class I molecules, cytotoxic T cells are able to scan the content of a cell. In case of a viral infection, intracellular viral peptides will also be presented in HLA-class I molecules on the cell membrane and can be recognized by cytotoxic T cells. Local recognition of virus-infected cells by activated cytotoxic T cells subsequently triggers the release of cytotoxic molecules resulting in the direct killing of the infected cells.[20] The TCR – HLA-class I interaction is stabilized by the co-receptor molecule cluster of differentiation 8 (CD8), expressed on the cytotoxic T cell, which is therefore also referred to as $CD8^+$ T cell. Helper T cells recognize peptides presented on HLA proteins DP, DQ or DR (HLA-class II). Different from HLA-class I molecules, HLA-class II molecules present peptides taken up from the cell's exterior, such as bacterial antigens. As antigen uptake and presentation in the groove of HLA-class II proteins can only be performed by specialized cells called antigen-presenting cells (APCs), helper T-cell interactions are limited mainly to cells of the immune system.[20,21] Activated helper T cells function predominantly in stimulating the activation of surrounding antigen-specific B and cytotoxic T lymphocytes.[22] The TCR – HLA-class II interaction is stabilized by the co-receptor CD4, and helper T cells are therefore also referred to as $CD4^+$ T cells.

Upon antigen recognition, and proper stimulation by helper T cells, B cells undergo extensive clonal expansion and can differentiate into plasma cells, which secrete large amounts of soluble variants of the BCR: immunoglobulins (Igs). These Igs can bind the pathogen and thereby prevent it from binding and infecting cells, thereby neutralizing the pathogenic threat. Furthermore, they can stimulate phagocytosis of the pathogen by phagocytic cells, such as neutrophils and macrophages, or they can induce antibody-dependent cytotoxicity, both processes resulting in the destruction of the pathogen.[20]

The antigen-specific B- and T-cell responses are highly effective in controlling an infection. However, upon primary infection, the selective activation followed by extensive proliferation and maturation of lymphocytes takes several days. Therefore, the host depends largely on the immediate innate immune response in the first days after primary infection. Importantly, after primary infection with a pathogen and subsequent clearance by the immune system, a selected set of antigen-experience lymphocytes involved in this response remain present in the circulation as "memory" lymphocytes. In case of a subsequent encounter with the same pathogen, these memory lymphocytes are able to recognize the pathogen and to respond more quickly than naive B and T cells. The adaptive immune responses thereby provide immunological memory. Consequently, the pathogenic threat can be removed before the development of clinical symptoms and the host is functionally immune for this specific pathogen.

Hematopoiesis

All immune cells, or leukocytes, are continuously generated throughout life from tissue-specific, pluripotent hematopoietic stem cell (HSC) in the bone marrow. This process is called hematopoiesis and involves a branched multi-step differentiation process in which the HSC proliferates and differentiates into each of the leukocyte lineages. First, the progenitor cells becomes restricted towards either the myeloid or lymphoid lineage. Myeloid progenitors can still differentiate into mast cells, monocytes, granulocyte subsets (i.e. neutrophilic, basophilic and eosinophilic

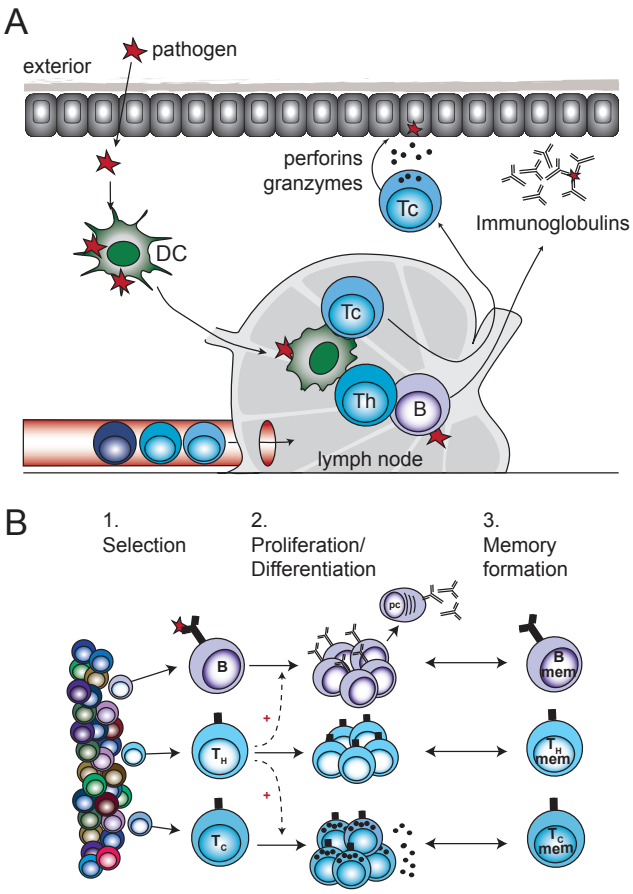


Figure 3. Third layer of protection: Adaptive immune responses.
A) Soluble antigen and antigen-loaded DCs migrate to secondary lymphoid structures, including the lymph nodes. Lymphocytes enter the lymph node via the blood circulation. DCs activate helper T cells (T_H). Together they activate cytotoxic T cells (T_C) and B cells. Cytotoxic T cells migrate into the tissue, where they induce death of virus-infected cells. B cells differentiate in immunoglobulin-producing plasma cells. Immunoglobulins disperse into tissue where they neutralize antigens. **B)** Initiation of adaptive immune responses follows three phases: *1.* Selection of antigen-specific B cells, T_H cells and T_C cells. *2.* Proliferation and differentiation of antigen-specific lymphocytes. T_H cells stimulate B-cell and T_C-cell proliferation and differentiation. B cells differentiate into immunoglobulin producing plasma cells. Activated cytotoxic T cells release cytotoxic granules upon antigenic trigger. *3.* Memory formation. A selection of lymphocytes remain after pathogen clearance to provide immunological memory.

granulocytes), certain DC subsets, erythrocytes (red blood cells) and thrombocytes (platelets). Mast cells originate directly from myeloid progenitors. Monocytes and granulocyte subsets develop via an intermediate myeloblast cell stage. Monocytes leave the bone marrow into the circulation, but upon their migration into tissue differentiate further into macrophages.

Lymphoid progenitors can differentiate into B, T and NK lymphocytes, and certain DC subsets (Figure 4). Commitment and differentiation into the B- or NK-cell lineages takes place in bone marrow, whereas early progenitors exit the bone marrow and migrate into the thymus where they develop into T cells (reviewed in 23).

Antigen-independent B-cell and T-cell antigen receptor formation

In contrast to myeloid cell development, B- and T-cell differentiation is a lengthy process in which the cells undergo various stages of differentiation and proliferation.[24-28] These stages are required to ensure regulated rearrangement of genetic elements in the genes encoding the BCRs and TCRs.[27,29] The BCR is composed of four peptide chains; two identical Ig heavy chains (IgH) and two identical Ig light chains (IgL). The latter are either of the Lambda (Igλ) or Kappa (Igκ) isotype (Figure 5A).[29] The TCR is composed of only two TCR chains, being

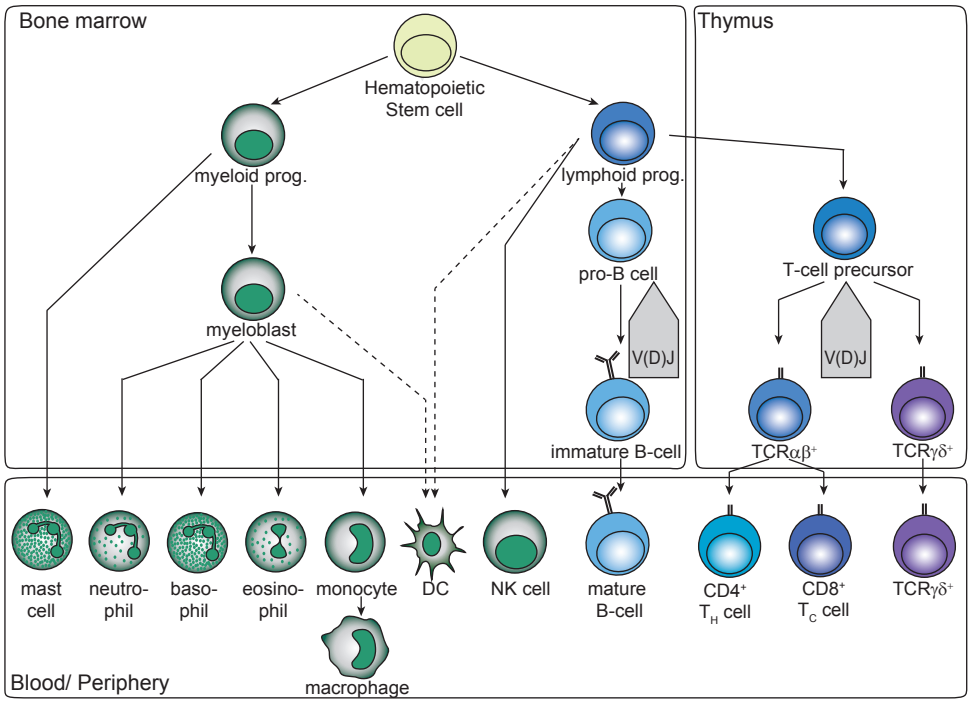


Figure 4. Schematic representation of the hematopoietic differentiation pathways.
Hematopoietic stem cells differentiate into a myeloid progenitor or lymphoid progenitor. Myeloid progenitors give rise to innate immune cells. They differentiate directly into mast cells, or via a myeloblast stage into neutrophils, basophils, eosinophils, monocytes and selected dendritic cell (DC) subsets (dashed line). Monocytes differentiate into macrophages upon migration into tissue. Lymphoid progenitors give rise to selected DC subsets (dashed line), NK cells and B cells in bone marrow, and T cells in the thymus. T-cell precursors differentiate further into TCRαβ$^+$ T cells or TCRγδ$^+$ T cells. All lymphocytes undergo V(D)J recombination (V(D)J) to develop a functional antigen receptor. TCRαβ$^+$ T cells further differentiate into CD4-expressing helper T cells (T_H) and CD8-expressing cytotxic T cells (T_C). For simplicity, erythrocytes and thrombocytes are excluded from the figure. Figure adapted from Rieger *et al.*[23]

either composed of one TCRα and one TCRβ chain (TCRαβ$^+$ T cells; comprising ~95% of all T cells), or of one TCRγ paired with one TCRδ chain (TCRγδ$^+$ T cells; comprising ~5% of all T cells) (Figure 5B). Each BCR and TCR contains a constant domain (or backbone), providing stability to the receptor and anchoring it in the cell membrane, and a variable domain, which is unique in each B or T cell and is responsible for the specific recognition of antigens. An adult adaptive human immune system contains approximately 10^{12} different lymphocytes. As it is not possible to code this large number of TCRs or BCRs in the human genome, the variable domains of BCRs and TCRs are formed in a process called V(D)J-recombination (Figure 5C).

V(D)J recombination is a highly structured and step-wise process in which the BCR and TCR are developed via a number of maturation steps.[24-28,30,31] In the human genome, the gene *IGH* (coding for the B-cell IgH chain), and the genes *TCRB* and *TCRD* (coding for the TCRβ and TCRδ proteins, respectively) are all composed of multiple non-identical copies of three different gene segments; a Variable (V) gene segment, a Diversity (D) gene segment and a Joining (J) gene segment.[32-34] For each of the *IGH, TCRB* and *TCRD* loci, V(D)J recombination starts with the random selection of one D and one J gene segment, which are then brought in close

proximity. Next, the DNA in between the two segments is removed and excised in the form of a circular excision product and the D and J segments are rearranged into a DJ recombination product. Subsequently, this DJ segment is recombined to one V segment in a similar process, resulting in a VDJ recombination product. Together this VDJ segment forms a first exon of the IgH, TCRβ or TCRδ proteins. The resulting V(D)J exon is subsequently transcribed, spliced towards a downstream constant (C) region and translated into a functional protein (Figure 5C). A similar process occurs for the *IGL*, *TCRA* and *TCRG* gene loci encoding the IgL, TCRα and TCRγ proteins respectively, though these loci contain only V and J gene segments and consequently require only V to J recombination.[34,35] This largely random selection of V, (D) and J gene segments ensures high combinatorial diversity of BCRs and TCRs. Still, to even further increase the variability in BCRs and TCRs, random nucleotides are inserted or deleted at the sites of V(D)J recombination, resulting in a high junctional diversity.[36-38] Together, the combinatorial diversity and junctional diversity enable the formation of a repertoire of more than 10^{12} unique lymphocytes.[29]

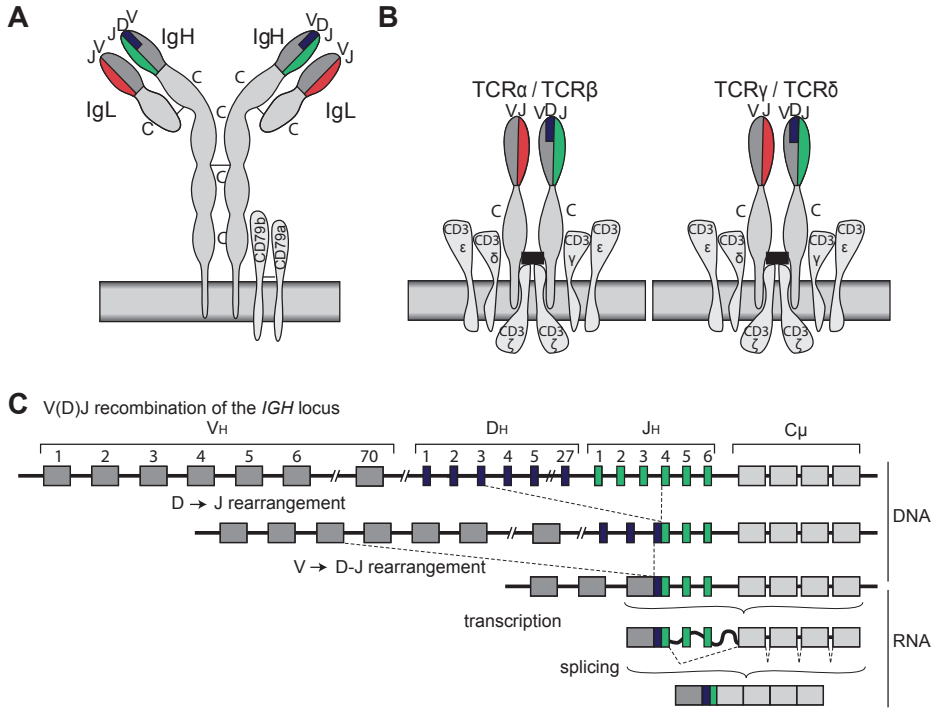


Figure 5. B-cell receptor and T-cell receptor formation.
A) The B-cell receptor (BCR) is composed of two identical Ig heavy (IgH) and two identical Ig light (IgL) peptide chains. Each IgH chain is composed of a Variable (V), Diversity (D), Joining (J) and Constant (C) region (the latter composed of four domains). Each IgL is composed of a V, J and C region. The BCR is anchored in the cell membrane by anchoring molecules CD79a and CD79b. **B)** The T-cell receptor (TCR) is composed of a combination of a TCRα and a TCRβ peptide chain (left), or a combination of a TCRγ and TCRδ peptide chain (right). The TCRβ and TCRδ chains are composed of V, D, J and C regions. The TCRα and TCRγ chains are composed of V, J and C regions. The TCR is anchored in the cell membrane by anchoring molecules CD3ε, CD3δ, CD3γ and CD3ζ. **C)** V(D)J recombination starts with the rearrangement of a D to a J segment, followed by a V to DJ rearrangement, resulting in a V(D)J exon. Upon functional rearrangement, the DNA is transcribed, the VDJ exon is spliced towards the C region, and translated into a functional protein.

Antigen-dependent B-cell maturation

Affinity maturation and class-switch recombination

After V(D)J recombination, a functional BCR is produced and expressed on the cell membrane of the naive B cell, which exits the bone marrow and migrates to secondary lymphoid organs. Upon antigen encounter, the naive B cell becomes activated and, in case of a protein antigen, recruits antigen-specific $CD4^+$ helper T cells through the presentation of antigenic peptides in HLA-class II proteins. Interactions between B cells and $CD4^+$ helper T cells trigger clonal proliferation and formation of germinal centers. Germinal centers are specialized lymphoid tissue structures that support two important B-cell maturation processes: BCR affinity maturation and class switch recombination (CSR). BCR affinity maturation is a process to optimize the BCR variable domain for more optimal recognition of the antigen (Figure 6). During extensive proliferation, B cells induce point mutations, i.e. somatic hypermutations (SHM), particularly targeting the V(D)J exon of their IgH and IgL chains, under the control of activation-induced cytidine deaminase (AID).[39,40] Subsequently, B cells expressing BCRs with higher affinity for the antigen are positively selected, resulting in increased overall affinity of the B-cell pool responding to a specific antigen.[41-44]

CSR is a process to change the constant region of the BCR and thereby adapt its effector functions. In naive B cells the constant region of the IgH chain anchors the BCR in the B-cell membrane. Upon antigen encounter, however, the activated B cells differentiate into Ig-secreting plasma cells. The constant region of the Ig (also known as the Fc-tail), mediates its specific effector function. Nine different immunoglobulin isotypes exist in humans: IgM, IgD, IgG (IgG1, IgG2, IgG3, IgG4), IgA (IgA1, IgA2) and IgE (Figure 6).[29] In naive B cells the V(D)J exon is by default spliced towards the downstream constant region Cμ or by alternative splicing toward the Cδ, resulting in a BCR of the IgM or IgD isotype, respectively, both of which are expressed on naive B cells. During CSR, a switch region upstream of the Cμ exons recombines with a switch region upstream of one of the Cγ, Cα or Cε exons (Figure 6). This results in a new recombination product, replacing the IgM and IgD constant regions for either an IgG, IgA or IgE constant region (extensively reviewed in 45,46). Each of these Ig isotypes have their own predominant effector functions, as discussed in the following paragraph.

Immunoglobulin-producing plasma cells

Following proliferation, SHM and CSR, germinal center B cells differentiate either into plasma cells or memory B cells. Plasma cells are the effector B cells that, by producing Igs, directly fight the invading pathogen. As each Ig isotype has a distinct constant region; i.e. Fc-tail, each of them can be recognized by a distinct Fc receptor, subsequently inducing distinct downstream effector functions.[29] The nature of the pathogen and the combination of cytokines produced by the $CD4^+$ helper T cells, together define the selection of a certain isotype.[46,47] IgM is the first isotype to be expressed upon naive B-cell activation. Part of the IgM expressing cells does not undergo a germinal center response, but differentiates in extrafollicular niches, such as the marginal zone of the spleen. Consequently, IgM expressing cells can respond quickly to a pathogen trigger, but the IgM molecules contains low levels of SHM and consequently low affinity maturation. Although this low affinity can be (partially) overcome by forming large pentamer molecules, thereby

increasing binding efficiency, highly specific, long-lasting antibody responses will predominantly be of an Ig isotype different from IgM. Since B cells can undergo consecutive CSR steps (Figure 6), effector functions of the highly-specific Ig molecules can be adapted to specific needs.

IgG is the most predominant immunoglobulin in the circulation. It is mainly present as monomer and is easily transferred into local tissue, via binding to the neonatal Fc-Receptor, thereby providing protection throughout the human body.[48] In a similar process, IgG can also be transferred through the placenta and provide a fetus with maternal protection.[49] IgG coated pathogens can be recognized by Fcγ receptors, which either activate the complement system, stimulate phagocytosis, or induces antibody-dependent cellular cytotoxicity by NK cells and other cytotoxic cells. These actions result in the direct destruction of the pathogen.[50] IgA is predominantly localized at mucosal tissues lining the outside of the human body. Via binding of polymeric IgA to the polymeric Ig receptor on epithelial cells, it can be transported through the epithelial layer into mucus layers or excretions.[51] One of these IgA containing excretions is human breastmilk. IgA can thereby be passively transferred from a mother to her newborn's intestinal mucosa, providing early maternal protection to the child. In contrast to IgG, IgA is less capable of inducing antibody-dependent cytotoxicity. It, however, predominantly functions in blocking pathogen binding to host cells.[52] IgE predominantly functions by activating mast cells through the cross-linking of Fcε receptors on these cells, resulting in the release of granule content, including histamines and other inflammatory proteins. IgE antibodies function in response to parasitic infections, but are also known for their function in allergic responses.

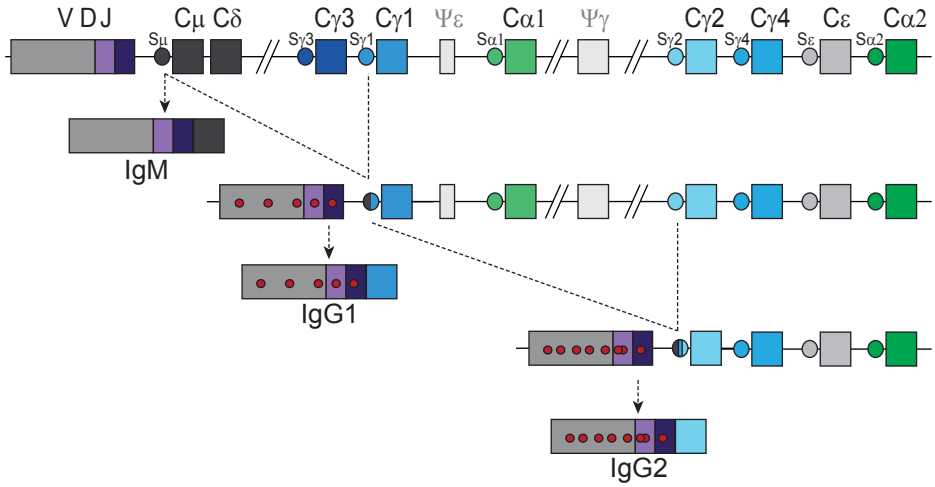


Figure 6. Class switch recombination and somatic hypermutation.
The human *IGH* locus contains 9 functional constant (C) regions. Apart from the Cδ region, each of the constant regions is preceded by a switch (S) region. RNA splicing of the V(D)J exon to the Cμ results in a B-cell receptor (BCR) of the IgM isotype. During class switch recombination, a switch region (in this figure Sμ) recombines with a downstream switch region (e.g Sγ1), which result in a new RNA splicing product and the production of a BCR of a different isotype (e.g. IgG1). During extensive proliferation, point mutations (somatic hypermutations; red dots) are introduced in the V(D)J exon to increase the affinity of the BCR. Consecutive switching to a downstream switch region (e.g. Sγ2), enables a further switching towards a more downstream BCR Ig isotype (e.g. IgG2).

Memory B cells

Memory B cells are able to respond fast upon subsequent encounter with the same antigen, providing long-lasting immunity to the host. Eight different memory B-cell populations can be defined (Figure 7). Five memory B-cell populations develop via classical T-cell dependent germinal center responses: IgM^+IgD^- (IgMonly), $CD27^-IgG^+$, $CD27^+IgE^+$, $CD27^+IgG^+$ and $CD27^+IgA^+$ memory B cells. All five have proliferated significantly and show high levels of SHM, indicative of affinity maturation. Still, $CD27^+IgG^+$ and $CD27^+IgA^+$ memory B cells underwent more proliferation and acquired more SHM than IgMonly, $CD27^-IgG^+$ and $CD27^+IgE^+$ memory B cells, suggesting that these cells underwent multiple cycles of affinity maturation.[53-55] Besides these classical germinal center-dependent memory B-cell populations, three other populations have been identified: IgM^+ memory B cells still co-expressing IgD (natural effector B cells) and $CD27^-IgA^+$ and $CD27^-IgE^+$ memory B cells. All three populations show low levels of proliferation and SHM, and can be detected in peripheral blood of immunodeficient patients who are unable to form germinal centers.[54-57] Together, this indicates a germinal center-independent (T-cell independent) origin of these populations. Natural effector B cells have been suggested to develop in the marginal zone of the spleen and are also referred to as marginal-zone B cells.[57-59] The $CD27^-IgA^+$ and $CD27^-IgE^+$ memory B-cell populations have been suggested to originate from mucosal tissues.[54,55]

Altogether, plasma cell formation, antibody production and memory development result from maturation of a selected set of naive B cells, thereby restricting the BCR repertoire, but increasing pathogen-specific responsiveness.

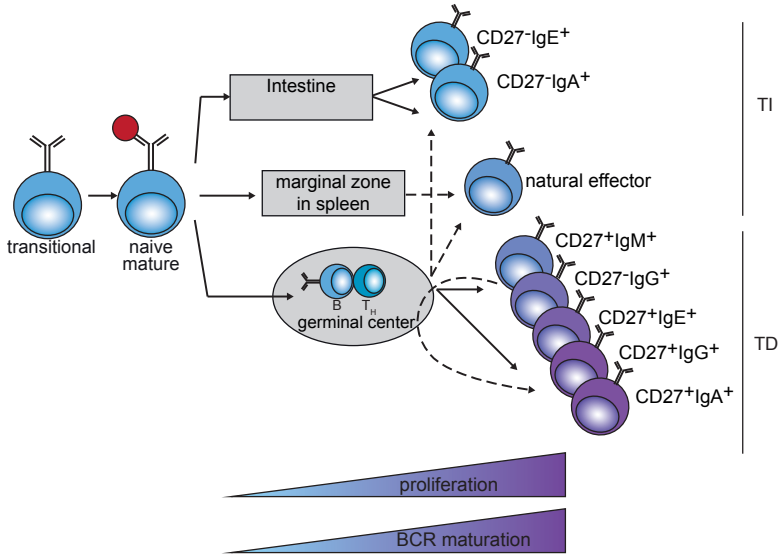


Figure 7. Memory B-cell formation.
Naive mature B cells can undergo T cell–independent (TI) or T cell–dependent (TD) immune responses. TI responses occur locally in the mucosal tissue, inducing the formation of $CD27^-IgE^+$ and $CD27^-IgA^+$ cells, or systemically in the splenic marginal zone, inducing natural effector B cells. TI responses are characterized by low proliferation and B-cell receptor (BCR) maturation levels as compared with TD responses. TD responses occur in germinal center structures. Primary germinal center responses induce the formation of $CD27^+IgM^+$, $CD27^-IgG^+$ and $CD27^+IgE^+$ memory B cells with intermediate proliferation and BCR maturation. Secondary TD germinal center responses induce the formation of $CD27^+IgG^+$ and $CD27^+IgA^+$ memory B cells that underwent even stronger proliferation and BCR maturation. Figure adapted from Berkowska *et al.*[54]

Antigen-dependent T-cell maturation

The majority of T cells produce a TCR using a TCRα and TCRβ chain, thereby forming an TCRαβ$^+$ T cell. Within these cells, around 65% comprises CD4$^+$ helper T cells, whereas the remaining 35% are CD8$^+$ cytotoxic T cells.[22,60]

CD4$^+$ TCRαβ$^+$ T-cell effector responses

When a naive CD4$^+$ T cell recognizes an antigenic peptide presented in HLA-class II proteins by an APC, further co-stimulation via CD40 expressed on the cell membrane of the APC, and CD40Ligand on the CD4$^+$ T cell, induces the activation and maturation of both cell types.[61] This induces the upregulated expression of co-stimulatory molecules on the APC, and the production and secretion of signaling molecules, i.e. cytokines, to the surroundings by both the cell types. The upregulated co-stimulatory molecules, such as CD80, CD86 or CD70, can stimulate and stabilize a subsequent interaction between an APC and an antigen-specific CD8$^+$ T cell.[62-65] The cytokines, including interferon γ, interleukin (IL)-2, IL-12 and IL-15,[22,63,66-68] furthermore stimulate proper activation of CD8$^+$ T cells (Figure 8A).

In addition, B cells are able to present antigenic peptides in HLA-class II molecules, thereby enabling a cognate interaction with an antigen-specific activated CD4$^+$ T cell in a germinal center. During this germinal center response (paragraph 'Antigen-dependent B-cell maturation'), the CD4$^+$ T cell stimulates B-cell maturation and differentiation via CD40 – CD40L interaction and the production of cytokines including IL21 and IL4 (Figure 8B).[41-43] An activated CD4$^+$ T cell can thereby provide help to both antigen-specific CD8$^+$ T cells and B cells.

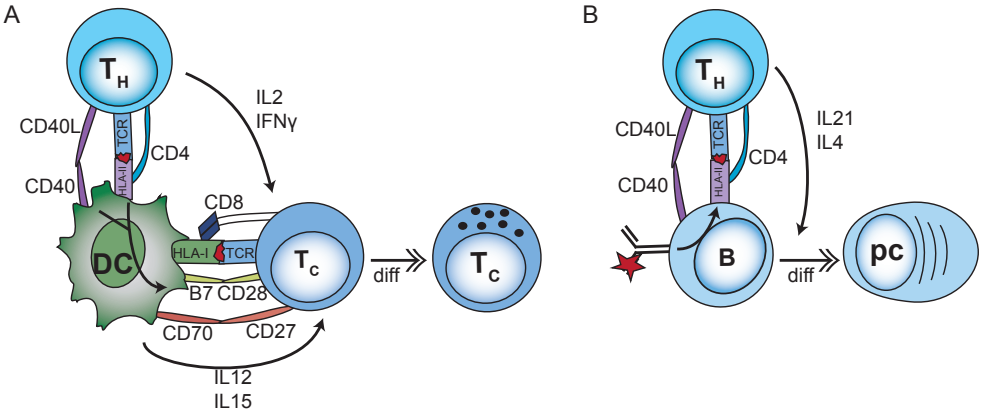


Figure 8. Helper T-cell functions.
A) An antigen-specific helper T (T_H) cell initiates a cognate interaction with a dendritic cell (DC) presenting antigenic peptides in HLA-class II molecules. This initiates the upregulation of co-stimulatory molecules, which stabilize a subsequent cognate interaction between the DC, presenting antigen in HLA-class I molecules, and an antigen-specific cytotoxic T (T_C) cell. Furthermore, both the T_H cell and DC produce cytokines to stimulate further T_C-cell differentiation. **B)** A B cell can present antigenic peptides in HLA-class II molecules to antigen-specific T_H cells. Subsequent signaling via co-stimulatory molecules and the production of cytokines by the T_H cell, stimulate the B cell towards plasma cell (pc) differentiation.

CD8$^+$ TCRαβ$^+$ T-cell effector responses

When a naive CD8$^+$ T cell recognizes an antigen presented in HLA-class I molecules, the additional co-stimulation provided by APCs, together with the cytokine production of both APCs and CD4$^+$ T cells, results in its full activation (as described above). Activated CD8$^+$ T cells then migrate towards the site of infection where they scan the cell membranes of cells in the surrounding tissue in search for cells that present their cognate antigen. Upon antigen encounter, the CD8$^+$ T cell induces the death of its infected target-cell, predominantly via the release of toxic granules containing perforins and granzymes, or FAS – FASLigand dependent cell death.[69-71]

TCRγδ$^+$ T-cell responses

TCRγδ$^+$ T cells are different from TCRαβ$^+$ T cells, not only in the TCR chains they use, but also with respect to their development and responses. Though details of their development and function still remain largely unknown, two major subsets of TCRγδ$^+$ T cells can be defined: Vδ1$^+$ and Vδ2$^+$ T cells. Vδ1$^+$ T cells predominate in the intestine and have been described to recognize stress-antigens (e.g. MHC-class I–related molecules MICA and MICB),[72,73] whereas Vδ2$^+$ T cells predominate in blood and recognize low molecular mass phospho-antigens.[72,74] Antigen recognition might be independent of presentation in HLA-class I or II molecules,[73] and does not require clonal expansion. Besides cytotoxic functions, TCRγδ$^+$ T cells have also been described to produce cytokines or chemokines and to perform a function in antigen presentation. Altogether, their quick, diverse, and stress-induced response make them important in stress-surveillance.[75,76]

Memory T cells

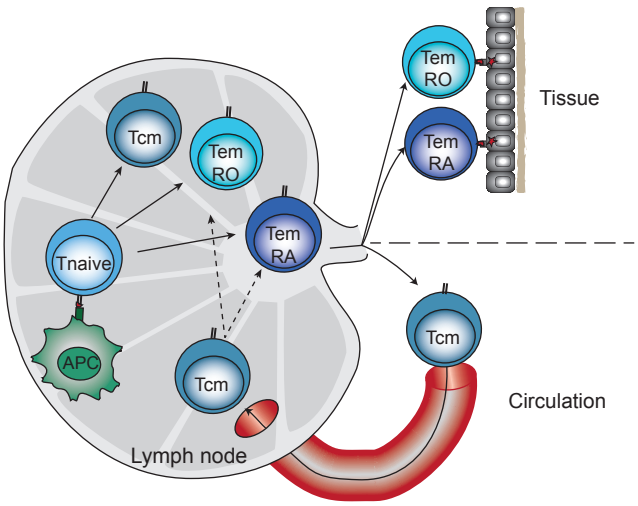
Upon pathogen clearance, the majority of effector T cells die by apoptosis to dampen the T-cell response and to prevent excessive effector T-cell accumulation after multiple pathogen encounters.[77,78] Instead, subsets of T cells remain present after pathogen clearance, in the form of memory T cells. The total pool of memory T cells is highly diverse, with the development of each memory T cell depending on the specific strength of TCR-triggering and/or cytokine stimulation,[79-81] and each possessing its own migration capacities, requirements for optimal stimulation and potential effector functions (Figure 9).[77,82-91]

One of the first proteins described to be differentially expressed in different T-cell subsets is the protein tyrosine phosphatase CD45. Naive T cells express CD45RA (an isotype of CD45), but upon antigen encounter and T-cell activation, the CD45RA switches isotype into CD45RO. Although the exact role of the CD45 isotypes is still largely unclear, this CD45-isotype-switch correlates with induced T-cell activation and proliferation.[92] Memory T cells can express either CD45RO, or re-express the CD45RA isotype, the latter being considered as having more effector cell properties.[93,94]

Similarly, depending on their function and predominant localization throughout the human body, T-cell subsets show differential expression of chemokine receptors (CCRs) and adhesion molecules.[86] Naive T cells express molecules important in lymph node homing, including CCR7 (also known as CD197) and L-selectin (also known as CD62L). Part of the CD45RO$^+$ memory T cells express similar

proteins, and have been designated "central memory" T cells (Tcm).[82,83] Due to the maintained expression of CCR7 and L-selectin, these Tcm cells circulate through the blood, lymphatics system and secondary lymphoid tissues. The remaining CD45RO$^+$ memory T cells and the CD45RA$^+$ memory T cells, however, lack the expression of both markers, but express other chemokine receptors, e.g. CCR5, CXCR1 or CX_3CR1, which enable their migration into local tissue, such as the skin or intestine.[77,84-87,91,94] Both of these memory populations are referred to as effector memory T cells (Tem), either CD45RO$^+$ (TemRO) or CD45RA$^+$ (TemRA)

A



B

		Tnaive	Tcm	Tem RO early	Tem RO interm	Tem RO late	Tem RA early	Tem RA interm	Tem RA late
co-stimulation cell survival regulation of activation	CD45RA								
	CD45RO								
	CD27								
	CD28								
	CD127 (IL-7R)								
	CD57								
	KLRG1								
	PD-1								
cell migration and adhesion	CCR7								
	CXCR4								
	CD62L								
	CCR4								
	CCR5								
	CXCR1								
	CX3CR1								
cytokine production and cytotoxic function	IL-2								
	IFNγ								
	TNFα								
	Perforin/Granzyme B								
	CD95L (FASL)								

Figure 9. CD8$^+$ T-cell responses.
A) Activated naive T cells differentiate into central memory T cells (Tcm) or effector memory T cells, the latter being either CD45RO$^+$ (TemRO) or CD45RO$^-$ (TemRA). Tcm cells circulate the lymphatics and blood circulation and lymphoid structures. TemRO and TemRA cells migrate into tissue and kill virus-infected cells. **B)** T-cell subsets expression levels of proteins involved in co-stimulation, cell survival and regulation of activation; cell migration and adhesion; and cytokine production and cytotoxic function. White, not expressed; light blue, intermediate expression; dark blue, strong expression.

The various memory T-cell subsets each possess distinct functional properties and different requirements for co-stimulation and survival. Naive T cells express high levels of survival factors, such as IL-7R (CD127), and different co-stimulatory molecules including CD27 and CD28, to enable their optimal activation upon antigen encounter.[84,85,87,94] Similarly, the systemic and lymph node localization of Tcm cells, will enable them to quickly recognize a subsequent infection with the same antigen, and to perform a central function in the induction of an adaptive immune response. In line with this function, Tcm cells maintain the expression of IL-7R, CD27 and CD28. Effector memory T cells gradually lose these survival factors and co-stimulatory molecules upon further differentiation (from early towards late differentiated TemRO or TemRA cells), but (especially cytotoxic T cells) show increased effector molecule production, including granzymes and perforin,[70,89] FASL (CD95L), and the cytokines IFNγ and TNFα, increasing their cytotoxic potential.[84,88,89] Increasing differentiation is, however, also correlated with an increased expression of inhibitory molecules, including killer cell lectin like receptor G1 (KLRG1), Programmed death 1 (PD-1 or CD279) and CD57,[95-97] and with increasing T-cell proliferative exhaustion.[81,84,95-97]

The exact differentiation pathway through which the various memory T-cell populations are generated remains debated.[91] Memory cells might develop from effector cells that escaped activation-induced cell death, or via a pathway independent from effector cell formation.[98] Furthermore, a consecutive differentiation pathway from naive T cells, via Tcm cells towards Tem cells has been described, in which Tcm cells might provide a renewing pool of cells continuously replenishing the Tem populations and in which effector memory T cells progressively lose co-stimulation, proliferative capacity and responsiveness.[79,80,84,89,99] Others, however, described a parallel differentiation,[100] or even a two-directional interchanging phenotype in which effector memory cells can regain expression of lymph node homing potential, self-renewal and functional properties comparable to central memory T cells.[101]

Still, irrespective of the differentiation pathway, the extensive characterization of the distinct memory T-cell subsets by markers described above (Figure 9) – which are still a selection of the available expression profiles in literature – enables detailed functional discrimination of memory T-cell subsets.

Maturation of the childhood immune system

During fetal development, a fetus resides in the protected environment of the uterus, shielded from the vast majority of external pathogens. Thus, the fetal immune system is largely immature and mainly composed of innate leukocytes and naive lymphocytes.[102-104] Maturation of the immune system largely occurs in the first few years of life, in response to environmental pathogens and other antigens. Important partial immune protection for the first few months of life, will be provided by the mother. Already during pregnancy maternal IgG molecules, can be transferred to the child through the placenta and can provide protection to the child.[49] Also after birth the mother can pass on immune protection to her child via breastfeeding. Breastfeeding contains various particles, including maternal IgA and IgG molecules and cytokines, which passively provide the child with immune protection for the time of breastfeeding duration.[105]

While receiving short-term passive protection via its mother, a child will have to develop its own protective immunity, mainly in the form of memory B and T cells. During childhood, the number of lymphocytes is relatively large, compared to adults.[102,104] With each naive lymphocyte having a unique antigen receptor, the combined BCR and TCR repertoires are enormous, with a high potential to mount responses to all distinct types of pathogens. As each pathogen induces a distinct functional response, associated with the expansions of distinct lymphocyte populations and the generation of distinct memory lymphocyte pools, the combination of pathogens we encounter in the environment eventually shapes the composition and antigen-receptor repertoire of our immune system.

With increasing age, the production of naive lymphocytes declines, together with a reduction in the size and functionality of the thymus. Consequently, the repertoire of new naive lymphocytes will become smaller in adults.[106] Adults, therefore, largely depend on their memory lymphocyte pools, produced as a result of the many pathogen-encounters during the first few years of life, to protect them from the recurrence of clinical illness.[107-113] The formation of a stable memory lymphocyte pool during childhood, and the maintenance hereof in adulthood, is therefore essential for long-term immune protection. A disbalance in the immune system can result in clinical complications, ranging from lack of protection, to the development of excessive immune responses, and can even lead to auto-immune diseases.

Persistent viral infections

Most of the encountered pathogens are eventually cleared by the immune system. Several viruses, however, escape from clearance by the immune system. Even though these can be seemingly well-controlled by the host, they can persist life-long, and cause complications in case of immunosuppression. Such persistent viruses include members of the family of herpesviruses and the human immunodeficiency virus (HIV).

Herpesviruses

Herpesviruses are double-stranded DNA viruses, and many are highly prevalent in the human population.[114] To date, 8 herpesviruses have been identified to infect humans. Four of these include the cytomegalovirus (CMV), Epstein Barr virus (EBV), herpes simplex virus (HSV) type 1 and varicella zoster virus (VZV). Seroprevalence for these viruses is high, varying between around 30 to >90% in adults.[115,116] Infection predominantly occurs during childhood and is mostly mild or even asymptomatic. However, infection in neonates and immunocompromised individuals might result in severe clinical complications. HSV-1 and VZV are two neurotropic viruses, meaning that they reside in sensory neurons.[117,118] HSV-1 can induce cold sores, recurring in situations of weakened immune surveillance. VZV induces chicken pox in children and can recur as shingles in most adults. CMV is able to infect a broad range of cell types including monocytes, epithelial cells, endothelial cell and to a lesser extend leukocytes.[119-121] CMV infection in endothelial cells also induces the attraction of neutrophils. CMV is transferred to these neutrophils, after which they recirculate and promote further dissemination of the virus.[122] EBV infects and persists in B cells.[123,124] EBV targets B cells through interactions with complement receptor 2 (CR2; CD21), HLA-class II co-receptor

surface molecules, or the Beta-1 integrin.[125-127] Even though all B cells express these molecules, EBV persists preferentially in memory cells.[123,128] Both CMV and EBV are able to induce infectious mononucleosis. CMV is, due to its tropism for endothelial cells, furthermore associated with vascular complications.[129,130] EBV is associated with increased development of malignant complications, including Burkitt lymphomas.[131]

The human immunodeficiency virus

HIV is a retrovirus responsible for inducing the acquired immunodeficiency syndrome (AIDS). HIV infects cells through binding to the CD4 proteins expressed on the membrane of the target cell, and therefore the predominant target cells are the $CD4^+$ T cells and monocytes/macrophages. HIV infection is characterized by $CD4^+$ T-cell depletion. HIV-infected adults in whom the $CD4^+$ T-cell numbers drop below 200 cells/µl of blood are eventually diagnosed with AIDS.

HIV uses co-receptors CCR5 and CXCR4, or integrin α4β7 for cell entry.[132-140] The integrin α4β7 is more recently described to form a complex with the CD4 protein on the cell membrane and $CD4^+α4β7^+$ T cells have been observed to be enriched for metabolically active cells expressing high levels of CCR5.[138] By targeting $α4β7^+$ cells, HIV thereby seems to increase its chance for effective infection. Furthermore, HIV is able to interact with DC-specific C-type lectin (DC-SIGN) on local DCs and CD21 on B cells, two cell types that are themselves not permissive for HIV infection, but that facilitate HIV virus transfer towards secondary lymphoid structures and into close proximity of $CD4^+$ T cells.[141,142] Moreover, HIV seems to preferentially infect HIV-specific $CD4^+$ T cells, most likely due to prolonged presence of both actively replicating HIV particles and HIV-specific $CD4^+$ T cells in lymphoid structures.[143] The latter might even further impair HIV clearance.

Immune evasion by persistent viral infections

Persistent viruses have developed various strategies to avoid their detection and destruction by the host's immune system. Although describing all the immunomodulatory effects of the viral proteins involved in immune evasion will be beyond the scope of this thesis (these have been extensively reviewed in 144-149), five major evasion strategies, used by herpesviruses and the HIV virus, will be addressed here (Table 1).

First, especially RNA viruses, including the HIV virus, have a high mutation rate of their viral genome, resulting in very high antigenic variability of the virus. Consequently, immune responses need to be continuously adapted to these mutating viruses. This provides the virus with a survival-advantage. DNA viruses, including the herpesviruses, seem to depend less on this evasion strategy as their DNA templates are more stable.

Second, CMV, EBV, HSV-1, VZV and HIV all inhibit antigen presentation by the infected host cell, masking them from the immune system. This is achieved through the low production of viral proteins during their state of latency. Furthermore, these viruses inhibit host HLA expression using mechanisms that can vary from decreasing HLA protein production, HLA stability, HLA transport to the cell membrane or HLA maintenance at the cell membrane.[150-155] Finally, viruses might limit the generation of presentable peptides by inhibiting host cell protease activity

or the TAP protein (transporter associated with antigen processing).[145,151,156-159]

Third, persistent viruses might directly inhibit the activation of immune cells; inhibiting cellular responses by avoiding NK cell and $CD8^+$ T cells activation,[160-163] or humoral responses by inhibiting antibodies from binding to their Fc-receptor and inducing antibody-dependent or complement-dependent cytotoxicity.[144]

Fourth, persistent viruses possess many mechanisms to affect cytokine and chemokine production or their subsequent signaling function, thereby regulating the balance between immune-activating molecules and immune-inhibiting molecules (reviewed in 144,148).

The last, important mechanism that can contribute to immune evasion is the selective tropism in certain host cell types. HSV-1 and VZV, for example, induces latency mainly in neuronal cells, which are protected from immune targeting, because of their importance and incapability to renew. EBV and HIV directly target lymphocyte populations. Killing the virus-infected cells, either by the virus itself, or as a result of an anti-viral immune response, therefore directly leads to destruction of the body's own immune system, eventually weakening the anti-viral immune responses.

Combined, the various evasion strategies, of which the ones described in this thesis are only a limited selection, enable the persistent viruses to escape from virus clearance and to induce long-term latency in the host.

Immunological consequences of persistent viral infection

As persistent viruses are continuously present, they also apply a constant pressure on the immune system. Consequently, important changes in the immune system have been correlated to the presence of a persistent viral infection. Some of these being common to persistent viruses, others being virus-specific (Figure 10).

Table 1. Evasion strategies used by different persistent viruses

Immune evasion strategy	Persistent virus				
	CMV	**EBV**	**HSV1**	**VZV**	**HIV**
1 High mutation rate and antigenic variability	±	±	±	±	+
2 Inhibiting virus peptide presentation					
reducing protein expression during latency	+	+	+	+	+
preventing HLA expression	+	+	+	+	+
inhibiting proteases and TAP function	+	+	+	?	?
3 Inhibiting immune cell activation					
inhibiting CD8+ T/ NK cell killing	+	+	+	?	+
inhibiting humoral immunity	+	+	+	+	+
4 Reducing cytokine/chemokine production	+	+	+	?	+
5 Predominant tropism	Broad range of cells	B cells	$CD4^+$ T cells, macro-phages	Neuronal cells	

TAP, transporter associated with antigen processing; +, virus possesses the evasion strategy; ±, virus can perform evasion strategy, but is less common; ?, evasion strategy not (yet) defined for the specific virus.

Direct destruction of the immune system

Some persistent viruses directly target the immune system and reside in immune cells. These viruses thereby directly affect the immune system and immune cell functioning (Figure 10a.). The two strongest examples hereof are the EBV and HIV viruses. EBV directly targets B cells, inducing persistence preferentially in memory B cells.[123,128] It is thought that this is the result of EBV infection in naive B cells that are driven into an immune response, after which the virus persists in the long-lived memory B-cell compartment.[123,164-166] Recent studies, however, indicate that both activated naive and memory B cells can be infected,[167] and that EBV can establish persistence in the absence of fully functional germinal center activity.[128,168] The true effect of EBV on B cells, is therefore still a matter of debate. Furthermore, although primary EBV infection induces a strong expansion of memory B cells that normalizes within 1 week after the appearance of clinical complications, long-term consequences of EBV persistence in memory B cells are still unknown.[169]

HIV directly targets $CD4^+$ T cells, focusing on activated, CCR5-expressing T cells,[132-140] and even HIV-specific $CD4^+$ T cells.[143] While HIV thereby directly impairs the $CD4^+$ T-cell functioning, it furthermore affects the function of $CD8^+$ T cells and B cells, both of which need $CD4^+$ T-cell help for optimal activation (as discussed earlier). B cells can furthermore be directly affected by HIV via direct binding and signaling of HIV to the B cell,[141] or via the transfer of HIV proteins, such as Nef (negative factor) onto B cells, reducing B-cell class switching towards IgG and IgA.[170] (extensively reviewed in 171-173) Both $CD4^+$ T-cell help-dependent and independent B-cell defects have been described, including hypergammaglobulinemia,[174,175] a reduction of $CD27^+$ class-switched memory B cells,[170,175,176] and an expansion of an aberrant, anergic B-cell population expressing low level of CD21 ($CD21^{low}$ B cells).[177,178]

Current combined antiretroviral therapy (cART) can nowadays effectively reduce HIV virus loads in peripheral blood and recover $CD4^+$ T-cell numbers to near-normal levels in both HIV-infected adults and children, suggesting that the treatment is effectively inhibiting viral replication and inducing immune recovery.[179-182] HIV thereby is changing from a lethal into a chronic disease. Still, full recovery of all immune defects is in adults not obtained, and long-term consequences of HIV infection and cART treatment in HIV-infected children remain largely unknown.

Overcrowding the immune system

The continuous presence of persistent viruses and the repeated cycles of activation and reactivation of virus production result in a continuous triggering of the immune system and a constant activation and stimulation of memory lymphocyte responses. Clinical consequences hereof have been best described for CMV and EBV infection. Memory lymphocyte pools directed against these viruses have been described to accumulate over time,[84,99] resulting in very large populations in especially the elderly. In these elderly they seem to occupy such a large part of the immune system that they overcrowd memory lymphocytes against different, unrelated pathogens, resulting in the loss of immunity (Figure 10b.)[183-187] Still, these effects are not consistently observed,[188-191] and despite high CMV and EBV seropositivity rates in elderly, only a minority develops clinical complications. Furthermore, childhood infection with these viruses is mostly asymptomatic and

might even be protective against the development of allergic or autoimmune diseases.[192,193] The reason for the different clinical outcome in children and adults, the discrepancy in literature on adult infection, as well as why only the minority of CMV- and/or EBV-infected elderly present clinical complications, remain largely unknown.

Immune exhaustion

Another important consequence of the continuous immune activation is immune exhaustion, also known as immunosenescence, a process that is also largely correlated to aging.[81,194,195] Immune exhaustion is a state in which cells show high replication history, reduced proliferative potential and reduced functional responsiveness (Figure 10c.). Exhaustion has been predominantly described for $CD8^+$ T cells and correlates with shortened telomere length, reduced expression of co-stimulatory molecules, e.g. CD27 and CD28, increased expression of inhibitory receptors, e.g. CD57, KLRG1 and PD-1, and reduced cytotoxic potential (see also Figure 9).[84,85,95-97]

Immune exhaustion has also been described for a B-cell population expressing low levels of CD21. Increased numbers of these $CD21^{low}$ B cells have been observed in various conditions of chronic immune activation, including immunodeficiencies,[196] autoimmune diseases,[197] and persistent viral infection with HIV.[198] In HIV-infected individuals, these $CD21^{low}$ B cells express increased levels of inhibitory receptors (including FcRL4), reduced replication potential, altered expression of homing receptors guiding them more towards inflamed tissue, and functional exhaustion.[198] They thereby share many characteristics with exhausted T cells. The presence of these $CD21^{low}$ B cells correlates with the presence of detectable HIV-viremia, suggesting that continuous viral exposure also induces exhaustion of B cells.[198] The ability of HIV to directly interact with B cells through binding of the CD21 protein, has been suggested to increase the chance of close contact between HIV particles and B cells, and enhance the induction of B-cell exhaustion.[141] This is even further underlined by the finding that $CD21^{low}$ B cells are enriched for HIV-specific cells, as recognition of HIV particles by BCRs could prolong the interaction period and subsequently enhance immunomodulatory effects and the induction of immune exhaustion.[198]

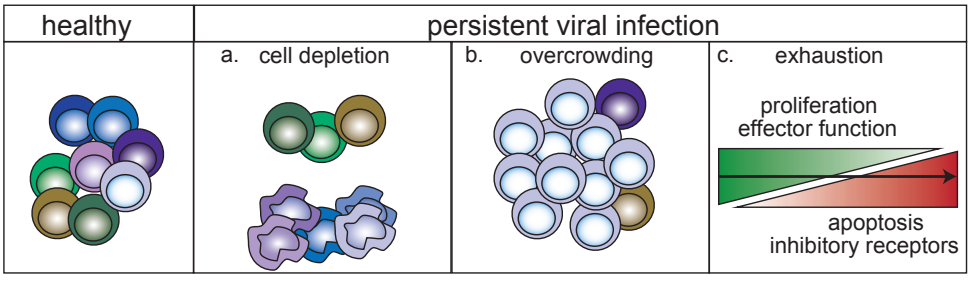


Figure 10. Immunological consequences of persistent viral infection.
Persistent viral infections can affect the immune system via three different mechanism: a. the targeted depletion of immune cells; b. the induction of extensive antiviral immune responses that overcrowd the pool of non-related immune cells; and c. extensive stimulation of the immune system which induces functional exhaustion, characterized by reduced proliferative potential and effector functions, but increased susceptibility to apoptosis and increased expression of inhibitory receptors.

Aims of this thesis

The importance of a well-functioning immune system can be appreciated in cases of either insufficient immune protection, such as immunodeficiency or immunosenescence, or excessive immune responses leading to clinical complications, such as autoimmune or allergic diseases. An optimal collaboration between the various cell types of the immune system is crucial for providing long-term protection of the host without eventually losing control. Understanding the mechanism behind this delicately balanced immune system will largely contribute to the field of immunology and might have implications for situations varying from vaccine strategies, the treatment of autoimmune or allergic diseases and even cancer therapy.

Most of the priming of the immune system is achieved during childhood, when the majority of the antigens encountered are still seen by the immune system for the first time. The research presented in this thesis aimed to further unravel the development and shaping of the immune system during childhood, focusing predominantly on the adaptive immune compartment. This in order to understand the mechanism by which children become optimally prepared for adulthood. **Chapter 2** focuses on the homeostasis during healthy immune development. In this chapter, we describe the different dynamics of the innate and adaptive immune compartments during their simultaneous development from birth until 6 years of age, and identify various environmental factors that associate to these dynamics. **Chapter 3** describes the effects of breastfeeding on the immune maturation at the age of 6 months. **Chapter 4** focuses on persistent herpesvirus infections, describing ethnic differences in herpesvirus seroprevalence. **Chapter 5** describes the presence of CMV- and EBV-associated T-cell expansions in 6-year-old children and their effect on the total T-cell compartment. **Chapter 6** focuses on the effect of EBV infection and persistence in memory B cells on the composition and functioning of the memory B-cell compartment in young children. In **Chapter 7**, we studied the effectiveness of current cART treatment in HIV-infected children and adolescents to enable B- and T-cell development.

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Chapter 2

EFFECTS OF EXTERNAL DETERMINANTS ON AGE-RELATED PATTERNS OF INNATE LEUKOCYTE AND NAIVE AND MEMORY B- AND T-LYMPHOCYTE NUMBERS IN EARLY CHILDHOOD: THE GENERATION R STUDY

Running title: Determinants of leukocyte subset kinetics in children

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ABSTRACT

Background. Blood leukocyte subsets in young children are highly dynamic, resulting in wide reference ranges. This is likely related to variable exposure to external antigens, and the generation of immunological memory following primary antigen responses. However, it is unclear which external determinants affect the dynamics of innate leukocytes, and naive and memory lymphocyte subsets.

Methods. Using 6-color flow cytometry and linear mixed effect modeling, we defined the dynamics of 62 leukocyte subsets from birth to 6 years of age in 1,182 children with one to five measurements per individual. Subsequently, we defined the impact of prenatal maternal lifestyle-related, prenatal maternal immune-mediated, birth characteristics and bacterial/viral exposure-related determinants on these leukocyte dynamics.

Results. Hierarchical clustering of leukocyte subset dynamics revealed four major age-related patterns that each contained functionally similar leukocyte populations. Innate leukocyte numbers were high at birth and were predominantly affected by maternal low education level. Naive lymphocytes peaked around 1 year, while most memory lymphocyte subsets more gradually increased during the first 1 to 4 years of life. Dynamics of $CD4^+$ T cells were predominantly associated with gender, birth characteristics, and persistent infections with cytomegalovirus (CMV) or Epstein Barr virus (EBV). $CD8^+$ T cells were predominantly associated with CMV and EBV infections. $TCR\gamma\delta^+$ T cells were associated with premature rupture of membranes and CMV infection. B-cell subsets were predominantly associated with gender, breastfeeding and Helicobacter Pylori carriership.

Conclusions. Our study identifies specific dynamic patterns of leukocyte subset numbers, as well as determinants that affect these patterns, thereby providing new insights into the shaping of the childhood immune system.

INTRODUCTION

The immune system in young children is highly dynamic in composition and cell numbers.[1-6] Innate cell numbers, such as neutrophils and NK cells, are higher in neonates than in children or adults,[1,5,7] and already display dynamic changes within the first few days after birth.[1,5,6] Furthermore, B and T cells are mostly naive in infants, while protective immunity is gradually built up in the form of increasing numbers of memory B and T cells during the first 5 years of life after which these numbers stabilize.[2-4,8-11]

Age-associated changes in leukocyte numbers have been extensively studied in the past two decades, and findings were quite consistent.[1-5,8-14] Both short-term follow-up of selected leukocyte subsets in infants in the first year of life,[5,6,12,13,15] and cross-sectional studies in children and adults,[3,4,6,10,11,14] have shown age-related differences in B- and T-cell numbers, whereas innate leukocyte subset numbers are quite stable. Still, it is difficult to make correlations between various leukocyte lineages, due to either the restricted numbers of analyzed subsets or the short-term follow-up. Furthermore, the determinants that drive the kinetics in immune development remain less well studied. Likely, various environmental determinants affect the development of certain leukocyte subsets during the first years of life, such as prenatal maternal life style and immune-mediated diseases, birth characteristics, and bacterial and viral exposure.[16-26] Studying the impact of environmental determinants on the dynamics of childhood immune development requires large cohorts of healthy young children, with multiple measurements per individual.

We investigated which external determinants, related to the prenatal maternal lifestyle, prenatal maternal immune-mediated diseases, birth characteristics or bacterial/viral exposure-related characteristics, influence the dynamics of blood leukocyte populations from birth until 6 years of age. This concerned a total of 62 leukocyte populations, including innate leukocyte subsets, naive and memory B-cell and T-cell subsets and TCRγδ^{+} T-cell subsets.

PATIENTS AND METHODS

Study subjects

This study was embedded in the Generation R Study, a prospective population-based cohort study from fetal life until young adulthood.[27,28] The current study focused on a subgroup of 1,182 two-generation Dutch children, born between August 2003 and August 2006. Peripheral blood was obtained at birth (cord blood; n=220), and median age of 6 months (5-9m; n=376), 14 months (13-17m; n=241), 25 months (22-30m; n=257) and 72 months (61-95m; n=916). Detailed immunophenotyping was performed at 1-5 time points per child, resulting in a total of 2,010 data points. Written informed consent was obtained from parents, according to the Medical Ethics Committee guidelines of Erasmus MC.

Immunophenotyping

Absolute numbers of leukocytes, NK cells, T cells and B cells were obtained with a diagnostic lyse-no-wash protocol, using a BD FACSCalibur. Six-color flow cytometry was performed to quantify 62 well-defined leukocyte populations

Table 1. Definition of leukocyte subsets

n= 62	n= 31	Name population	Phenotype definition
		Innate leukocytes	
1		**Granulocytes**	$SSC^{high}CD45^{+}$
2		CD15+ granulocytes	$SSC^{high}CD45^{+}CD15^{+}$
3	1′	Neutrophils	$SSC^{high}CD45^{+}CD16^{+}$
4	2′	Eosinophils	$SSC^{high}CD45^{+}CD16^{-}$
5		**Monocytes**	$FSC^{inter}SSC^{inter}CD45^{+}$
6	3′	classical monocytes	$FSC^{inter}SSC^{inter}CD45^{+}CD14^{+}CD16^{-}$
7	4′	intermediate monocytes	$FSC^{inter}SSC^{inter}CD45^{+}CD14^{+}CD16^{+}$
8	5′	Non-classical monocytes	$FSC^{inter}SSC^{inter}CD45^{+}CD14^{-}CD16^{+}$
9	6′	**NK cells**	$SSC^{low}CD45^{+}CD3^{-}CD16^{+}$or $CD56^{+}$
		Lymphocytes	
10		**T cells**	$SSC^{low}CD45^{+}CD3^{+}$
11		TCRαβ+ T cells	$SSC^{low}CD3^{+}TCR\gamma\delta^{-}TCR\alpha\beta^{+}$
12		∟CD4+ TCRαβ+ T cells	$SSC^{low}CD3^{+}TCR\gamma\delta^{-}TCR\alpha\beta^{+}CD8^{-}CD4^{+}$
13		∟CD8+ TCRαβ+ T cells	$SSC^{low}CD3^{+}TCR\gamma\delta^{-}TCR\alpha\beta^{+}CD8^{+}CD4^{-}$
14		TCRγδ+ T cells	$SSC^{low}CD3^{+}TCR\gamma\delta^{+}TCR\alpha\beta^{-}$
15		∟CD8+ TCRγδ+ T cells	$SSC^{low}CD3^{+}TCR\gamma\delta^{+}TCR\alpha\beta^{-}CD8^{+}CD4^{-}$
16		∟CD4+ TCRγδ+ T cells	$SSC^{low}CD3^{+}TCR\gamma\delta^{+}TCR\alpha\beta^{-}CD8^{-}CD4^{+}$
17		∟Vδ1-Vδ2- TCRαβ- T cells	$SSC^{low}CD3^{+}TCR\alpha\beta^{-}V\delta1^{-}V\delta2^{-}$
18		∟Vδ2+ T cells	$SSC^{low}CD3^{+}TCR\alpha\beta^{-}V\delta1^{-}V\delta2^{+}$
19	7′	∟Vδ1+ T cells	$SSC^{low}CD3^{+}TCR\alpha\beta^{-}V\delta1^{+}V\delta2^{-}$
20		∟Vγ9+ T cells	$FSC^{low}SSC^{low}CD3^{+}TCR\alpha\beta^{-}V\gamma9^{+}$
21	8′	∟Vδ2+Vγ9+ T cells	$FSC^{low}SSC^{low}CD3^{+}TCR\alpha\beta^{-}V\delta2^{+}V\gamma9^{+}$
22		CD4+ T cells	$SSC^{low}CD45^{+}CD3^{+}CD4^{+}CD8^{-}$
23	9′	∟CD4+ Tnaive	$FSC^{low}SSC^{low}CD3^{+}CD8^{-}CD197^{+}CD45RO^{-}CD27^{+}CD28^{+}$
24		∟CD4+ Tmem	$FSC^{low}SSC^{low}CD3^{+}CD8^{-}$ $CD197^{-}$ & $CD197^{+}CD45RO^{+}$
25	10′	∟CD4+ Tcm	$FSC^{low}SSC^{low}CD3^{+}CD8^{-}CD197^{+}CD45RO^{+}CD27^{+}CD28^{+}$
26		∟CD4+ TemRO	$FSC^{low}SSC^{low}CD3^{+}CD8^{-}CD197^{-}CD45RO^{+}$
27	11′	∟CD4+ early TemRO	$FSC^{low}SSC^{low}CD3^{+}CD8^{-}CD197^{-}CD45RO^{+}CD27^{+}CD28^{+}$
28	12′	∟ CD4+ interm TemRO	$FSC^{low}SSC^{low}CD3^{+}CD8^{-}CD197^{-}CD45RO^{+}CD27^{-}CD28^{+}$
29	13′	∟CD4+ late TemRO	$FSC^{low}SSC^{low}CD3^{+}CD8^{-}CD197^{-}CD45RO^{+}CD27^{-}CD28^{-}$
30		∟CD4+ TemRA	$FSC^{low}SSC^{low}CD3^{+}CD8^{-}CD197^{-}CD45RO^{-}$
31	14′	∟CD4+ early TemRA	$FSC^{low}SSC^{low}CD3^{+}CD8^{-}CD197^{-}CD45RO^{-}CD27^{+}CD28^{+}$
32	15′	∟ CD4+ interm TemRA	$FSC^{low}SSC^{low}CD3^{+}CD8^{-}CD197^{-}CD45RO^{-}CD27^{-}CD28^{+}$
33	16′	∟CD4+ late TemRA	$FSC^{low}SSC^{low}CD3^{+}CD8^{-}CD197^{-}CD45RO^{-}CD27^{-}CD28^{-}$
34		CD8+ T cells	$SSC^{low}CD45^{+}CD3^{+}CD4^{-}CD8^{+}$
35	17′	∟CD8+ Tnaive	$FSC^{low}SSC^{low}CD3^{+}CD8^{+}CD197^{+}CD45RO^{-}CD27^{+}CD28^{+}$
36		∟CD8+ Tmem	$FSC^{low}SSC^{low}CD3^{+}CD8^{+}$ $CD197^{-}$ & $CD197^{+}CD45RO^{+}$
37	18′	∟CD8+ Tcm	$FSC^{low}SSC^{low}CD3^{+}CD8^{+}CD197^{+}CD45RO^{+}CD27^{+}CD28^{+}$
38		∟CD8+ TemRO	$FSC^{low}SSC^{low}CD3^{+}CD8^{+}CD197^{-}CD45RO^{+}$
39	19′	∟CD8+ early TemRO	$FSC^{low}SSC^{low}CD3^{+}CD8^{+}CD197^{-}CD45RO^{+}CD27^{+}CD28^{+}$
40	20′	∟CD8+ interm TemRO	$FSC^{low}SSC^{low}CD3^{+}CD8^{+}CD197^{-}CD45RO^{+}CD27^{+}CD28^{-}$
41	21′	∟CD8+ late TemRO	$FSC^{low}SSC^{low}CD3^{+}CD8^{+}CD197^{-}CD45RO^{+}CD27^{-}CD28^{-}$
42		∟CD8+ TemRA	$FSC^{low}SSC^{low}CD3^{+}CD8^{+}CD197^{-}CD45RO^{-}$
43	22′	∟CD8+ early TemRA	$FSC^{low}SSC^{low}CD3^{+}CD8^{+}CD197^{-}CD45RO^{-}CD27^{+}CD28^{+}$
44	23′	∟CD8+ interm TemRA	$FSC^{low}SSC^{low}CD3^{+}CD8^{+}CD197^{-}CD45RO^{-}CD27^{+}CD28^{-}$
45	24′	∟CD8+ late TemRA	$FSC^{low}SSC^{low}CD3^{+}CD8^{+}CD197^{-}CD45RO^{-}CD27^{-}CD28^{-}$

Table 1 (continued)

n= 62	n= 31	Name population	Phenotype definition
46		**B cells**	$SSC^{low}CD45^{+}CD19^{+}$
47	25'	Bnaive	$SSC^{low}CD19^{+}CD27^{-}IgD^{+}$
48		Bmem	$SSC^{low}CD19^{+}$ IgD^{-} & $CD27^{+}IgD^{+}$
49		∟CD27- Bmem	$SSC^{low}CD19^{+}CD27^{-}IgD^{-}$
50		∟CD27+ Bmem	$SSC^{low}CD19^{+}CD27^{+}IgD^{-}$
51		∟IgM+ Bmem	$SSC^{low}CD19^{+}CD27^{+}IgM^{+}$
52	26'	∟Natural effector	$SSC^{low}CD19^{+}CD27^{+}IgM^{+}IgD^{+}$
53	27'	∟IgMonly	$SSC^{low}CD19^{+}CD27^{+}IgM^{+}IgD^{-}$
54		∟IgA+ Bmem	$SSC^{low}CD19^{+}IgA^{+}$
55	28'	∟CD27-IgA+	$SSC^{low}CD19^{+}CD27^{-}IgA^{+}$
56	29'	∟CD27+IgA+	$SSC^{low}CD19^{+}CD27^{+}IgA^{+}$
57		∟IgG+ Bmem	$SSC^{low}CD19^{+}IgG^{+}$
58	30'	∟CD27-IgG+	$SSC^{low}CD19^{+}CD27^{-}IgG^{+}$
59	31'	∟CD27+IgG+	$SSC^{low}CD19^{+}CD27^{+}IgG^{+}$
60		CD21low B cells	$SSC^{low}CD19^{+}CD38^{low}CD21^{low}$
61		Igλ+ B cells	$SSC^{low}CD19^{+}Igκ^{-}Igλ^{+}$
62		Igκ+ B cells	$SSC^{low}CD19^{+}Igκ^{+}Igλ^{-}$

(Table 1), which included NK-cell, monocyte, granulocyte, naive and memory B-cell, naive and memory $TCRαβ^{+}$ T-cell and $TCRγδ^{+}$ T-cell subsets (Supplemental Table 1). Flowcytometric analyses were performed on an LSRII (BD Biosciences) using standardized measurement settings.[29]

Determinants

Information on 26 dichotomized determinants was obtained (Table 2).[27,28] Six of these were related to prenatal maternal life style, and were evaluated using questionnaires in the first, second and third trimester of pregnancy:[28] maternal age (above 35y defined as a group with increased risks during pregnancy), education, net household income, smoking or alcohol use during pregnancy, and maternal body mass index before pregnancy (>25 defined as above healthy range). Three determinants were related to prenatal maternal immune-mediated diseases: maternal atopy defined by questionnaire data,[28] and serum anti-thyroid peroxidase IgG (anti-TPO) and anti-tissue transglutaminase IgA antibody (TG2A) levels.[30] Three birth-related determinants included gender, gestational age (preterm birth <37w) and birth weight (low birth weight <2,500g). Fourteen determinants related to bacterial or viral exposure included three perinatal determinants, i.e. premature rupture of membranes, birth season and mode of delivery; and eleven postnatal determinants, i.e. breastfeeding and breastfeeding duration, having siblings, antibiotics usage and presence of upper or lower respiratory tract infection in the first year of life (obtained using questionnaires at the child's age of 2, 3, 6 and 12 months);[28] Helicobacter pylori (H. pylori) carriership, and seropositivity (IgG) for cytomegalovirus (CMV), Epstein Barr virus (EBV), herpes simplex virus 1 (HSV-1) and varicella zoster virus (VZV) were defined at the age of 6 years.[26,31,32] We did not include the postnatal determinants of housing conditions, pets, day care, home environment or other potential sources for differences in antigenic or microbial pressure, because of incomplete data sets.

Statistical modeling

To model leukocyte dynamics between birth and the age of 6 years, linear mixed effect analyses were performed on the relationship between the age of the children and the size of individual leukocyte populations. By including random-effects in the model, this approach enabled modeling of cross-sectional data, with further improvement of the accuracy by incorporating longitudinal follow-up data from individual children. To capture the trend in the data more precisely, we included a natural spline with different knots (0-3 knots) into the models. Basically, the number of knots is inversely related to the smoothness of the curve. Positions of the knots in the 1-knot model was defined as the 50th percentile (25.5 months), the 2-knots model at 33rd and 66th percentiles (14.1 and 70 months); the knots in the 3-knot model were defined manually at 6, 14 and 24 months, focusing around the time points of data inclusion. Model selection was performed by likelihood ratio test.

Next, for each leukocyte population the effect of all 26 determinants on the models was assessed by first adding each determinant univariately into the model and analyzing fixed effect estimates. To correct for potential multiple testing errors, a correction for the four groups of determinants was performed, and consequently only determinants with an effect of $p<0.0125$ were defined as significant. Subsequently, for each leukocyte population, the determinants with a significant effect were combined in a multivariable model to correct for possible confounding effects. The p-value for significance for the multivariable analyses was $p<0.05$. Up to 3 determinants were added per model. Statistical analyses were performed in R (version R-3.2.1).[33]

Clustering analyses

To cluster patterns of leukocyte subsets kinetics, the modelled data of each population was normalized into zero mean and unit standard deviation (z-score), using the following calculation:

$$\text{z-score} = \frac{(\text{population size (at month x)} - \text{average population size (at months 0-72)})}{(\text{standard deviation of population size (at months 0-72)})}$$

The normalized leukocyte models were subsequently clustered using agglomerative ('bottom-up') Ward's hierarchical clustering, at each step clustering two clusters with minimum between-cluster distance, using the Euclidean distance measure.

RESULTS

Linear mixed effect modeling of leukocyte-subset cell numbers versus the child's age

To study the dynamics of different leukocyte subsets in young children, 2,010 measurements were performed in 1,182 children between birth and 72 months (6y) of age to quantify 11 leukocyte subsets: four major innate leukocyte subsets (monocytes, neutrophils, eosinophils and NK cells), naive B cells (Bnaive) and memory B cells (Bmem), naive and memory CD4$^+$ T cells (CD4$^+$ Tnaive; CD4$^+$ Tmem), naive and memory CD8$^+$ T cells (CD8$^+$ Tnaive; CD8$^+$ Tmem), and TCRγδ$^+$ T cells. All four innate leukocyte subsets followed a similar profile, starting with

high numbers at birth, which quickly declined within the first 6 months of age and subsequently remained stable (Figure 1A). In contrast, naive B-cell and T-cell numbers strongly increased after birth, peaked around 6-14 months of age and subsequently decreased to stable levels around the age of 2-6 years (Figure 1B). Memory B-cell and T-cell numbers slowly increased within the first 6-14 months of life, after which numbers declined marginally and stabilized from ~3 years onwards (Figure 1B). Total TCRγδ⁺ T-cell numbers increased until 6 months and remained quite stable at these levels (Figure 1C).

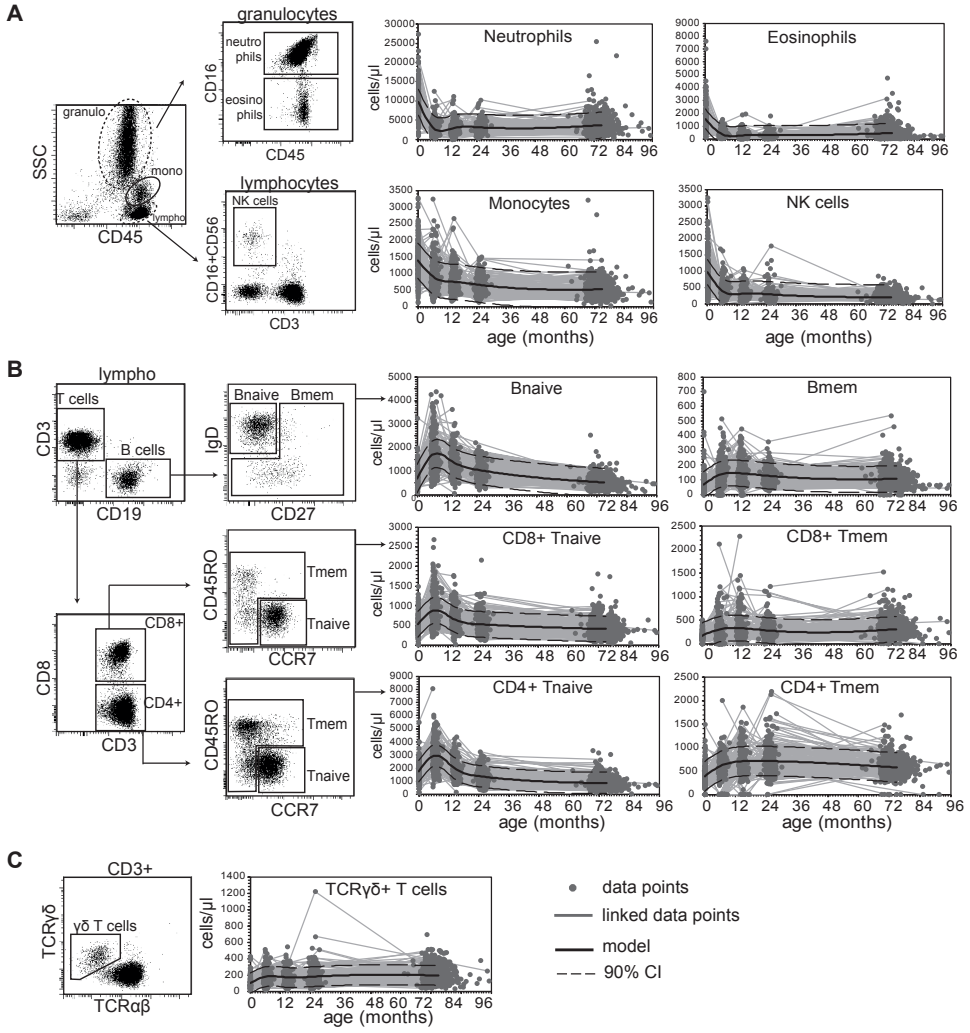


Figure 1. Dynamics of innate leukocyte and naive and memory lymphocyte populations in children between birth and 6 years of age.

A) Dynamics of monocytes, neutrophils, eosinophils and NK cells. **B)** Dynamics of naive and memory B-cell and T-cell subsets **C)** Dynamics of TCRγδ⁺ T cells. Flow cytometry plots depict population definitions in one representative 6-year-old individual. Graphs depict absolute numbers of cells in blood of 1,182 children with in total 2,010 measurements (gray dots). Linear mixed effect models were generated for each population (solid black line) and represented with the 90% confidence interval (CI) of the model (dashed black lines). For clarity, only direct follow-up time points within one individual were connected with gray lines; i.e. 0-6m, 6-14m, 14-25m or 25-72m.

Thus, innate leukocytes, naive lymphocytes and memory lymphocytes seem to display three distinct patterns in cell numbers versus the child's age, but patterns seemed common to subsets within each of these lineages.

Distinct dynamics of innate leukocytes, and naive and memory B and T cells

To evaluate whether functionally similar immune populations would follow similar dynamics with increasing age, we extended our analysis to 62 leukocyte subsets, including 31 phenotypically non-overlapping subsets, using a more detailed classification of innate cells, and naive and memory B and T cells (Table 1), in the same children. Linear mixed effect models were generated as described above and presented with the raw data points in Supplemental Figures 1-5.

Hierarchical clustering of the total 62 leukocyte populations resulted in 4 distinct clusters of leukocyte dynamics, in which functionally related populations, as well as populations with phenotypic overlapping definitions clustered together (Figure 2A). To test the effect of including populations with overlapping population definitions on the clustering, an additional clustering was performed on a selection of 31 non-overlapping populations (Table 1 and Supplemental Figure 6). The resulting clusters showed similar dynamics (Figure 2B and Supplemental Figure 6B) with only 3 populations being assigned to a different cluster, indicating that the overlapping populations did not overtly skew our clustering approach.

Cell numbers of all populations in the first cluster were high at birth, followed by a sharp decrease within the first 6 months of life, after which they stabilized (Figure 2B). This cluster exclusively contained innate leukocyte subsets, including the initially defined NK cells, monocytes, neutrophils and eosinophils (Figure 1A). All defined innate leukocyte subsets clustered within cluster 1, except for the intermediate monocytes, and except for the non-classical monocytes in the clustering of 31 non-overlapping populations (Supplemental Figure 6).

The populations in the second cluster strongly increased shortly after birth, peaked before 14 months of age, followed by a long-term gradual decrease. Populations in cluster 2 included the three major naive lymphocyte subsets defined in Figure 1B: Bnaive, $CD4^+$ Tnaive and $CD8^+$ Tnaive. Also the total B and T cells, the intermediate monocytes and the $V\delta1^+$ T cells clustered in cluster 2, as well as the non-classical monocytes in the clustering in Supplemental Figure 6. Furthermore, 5 memory populations also showed this pattern: $CD4^+$ central memory T cells (Tcm) and $CD8^+$ Tcm cells, early differentiated $CD8^+$ $CD45RO^-$ effector memory T cells ($CD8^+$ early TemRA), $CD27^-IgA^+$ memory B cells and natural effector memory B cells.

The populations in cluster 3 and 4 gradually increased in cell number and peaked either at 14 months (cluster 3) or after ~4 years (cluster 4). Clusters 3 and 4 contained only memory B-cell and T-cell subsets, and the $TCR\gamma\delta^+$ T-cell subsets. Cluster 3 contained the three major memory populations described in Figure 1B: Bmem, $CD4^+$ Tmem and $CD8^+$ Tmem. In particular, these memory populations included the early and intermediate $CD8^+$ TemRO subpopulations and the IgMonly, $CD27^+IgA^+$ and $CD27^-IgG^+$ memory B-cell subsets. Cluster 4 contained total $TCR\gamma\delta^+$ T cells and the $V\delta2^+$ and the $V\gamma9^+$ T-cell subsets, as well as the intermediate $CD8^+$ TemRO and TemRA cells, most of the $CD4^+$ effector memory T-cell subsets and the $CD27^+IgG^+$ memory B cells. $CD8^+$ late TemRO and TemRA cells clustered in cluster 3 in the analysis of all 62 populations (Figure 2) and in cluster 4 when only the 31 non-overlapping populations were included (Supplemental Figure 6).

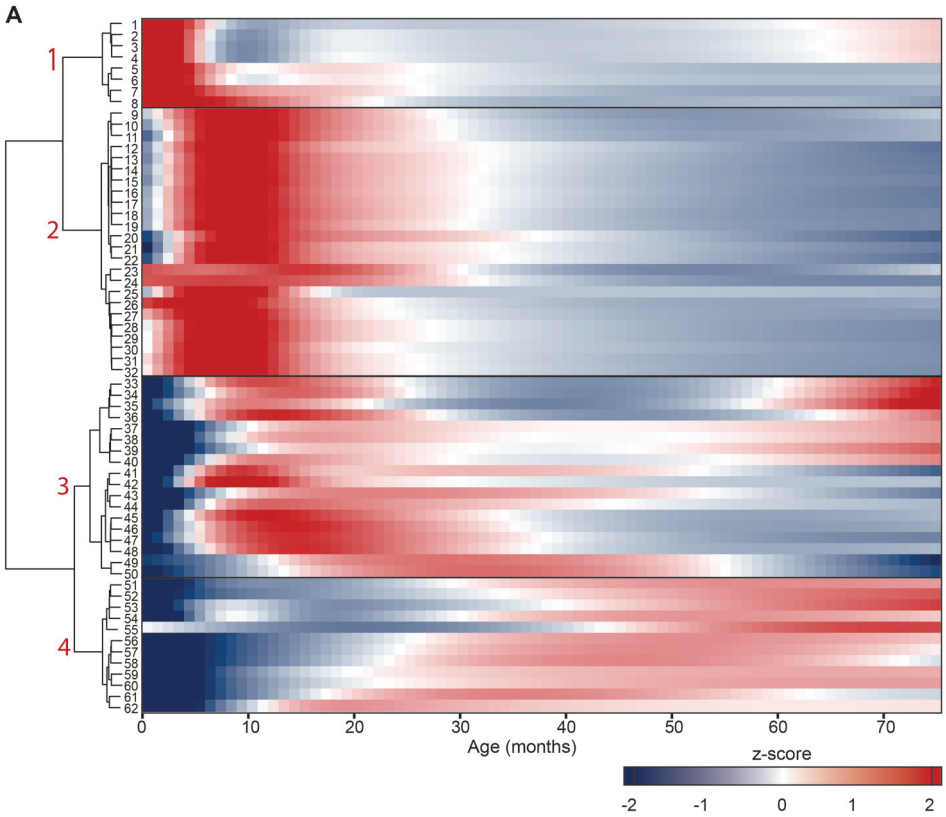


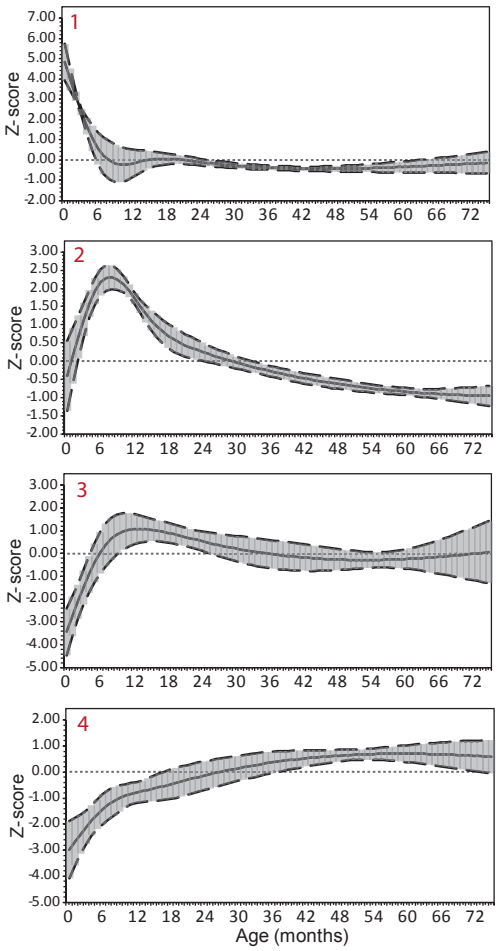
Figure 2. Hierarchical clustering of leukocyte subset dynamics in early childhood.
A) Absolute numbers of the 62 populations were calculated between 0-72 months of age using the linear mixed models. For each population, the data was converted to zero mean and unit standard deviation (z score). The resulting patterns of all 62 subsets were subjected to Ward's hierarchical clustering.
(Figure 2 continues on next page)

Associations between external determinants and leukocyte subset dynamics in the four clusters

To study which external determinants were associated with the four distinct age-related patterns of leukocyte numbers (Figure 2), we analyzed 6 prenatal maternal life style-related determinants, 3 prenatal maternal immune-mediated determinants, 3 birth characteristics, and 14 bacterial/viral exposure-related determinants (Table 2). We observed 1 prenatal maternal life style-related determinant, no prenatal maternal immune-mediated determinants, gender, and 9 bacterial/viral exposure-related determinants that showed a significant association with the dynamics of one or more of the 62 leukocyte populations after multivariable correction (Table 2). All of these 11 determinants also showed a significant association with the 31 non-overlapping leukocyte subsets.

Within cluster 1, 5 determinants were associated to changes in the kinetics of one or more of the leukocyte subsets. The strongest association was observed with maternal education level, both for the analysis of the total 62 populations (37.5%, Figure 3A,C) and the 31 non-overlapping populations (40%, Figure

B



1 Eosinophils
2 Granulocytes
3 CD15+ granuloytes
4 Neutrophils
5 NK cells
6 Classical monocytes
7 Monocytes
8 Non-classical monocytes

9 CD4+ Tcm
10 Natural effector B cells
11 IgM+ Bmem
12 CD27-IgA+
13 Igκ+ B cells
14 B cells
15 CD8+ T cells
16 Bnaive
17 Igλ+ B cells
18 CD21low B cells
19 CD8+ TCRαβ+ T cells
20 CD8+ TemRA early
21 Vδ1+ T cells
22 IgA+ Bmem
23 Intermediate monocytes
24 CD8+ Tcm
25 Vδ1-Vδ2- TCRαβ- T cells
26 CD4+ TCRγδ+ T cells
27 CD4+ Tnaive
28 T cells
29 TCRαβ+ T cells
30 CD8+ Tnaive
31 CD4+ T cells
32 CD4+ TCRαβ+ T cells

33 CD8+ TemRO interm
34 CD8+ TemRO
35 CD8+ TemRO early
36 CD8+ Tmem
37 CD8+ TemRO late
38 CD27+ Bmem
39 CD8+ TemRA late
40 CD8+ TemRA
41 CD8+ TCRγδ+ T cells
42 IgMonly
43 CD4+ Tmem
44 CD27+IgA+
45 Bmem
46 CD27-IgG+
47 CD27- Bmem
48 IgG+ Bmem
49 CD4+ TemRA early
50 CD4+ TemRA

51 Vδ2+ T cells
52 CD4+ TemRO interm
53 CD8+ TemRA interm
54 TCRγδ+ T cells
55 CD4+ TemRA interm
56 Vδ2+Vγ9+ T cells
57 CD4+ TemRA late
58 Vγ9+ T cells
59 CD4+ TemRO early
60 CD4+ TemRO
61 CD4+ TemRO late
62 CD27+IgG+

Figure 2. Hierarchical clustering of leukocyte subset dynamics in early childhood. *(continued)*
B) Average patterns +/- 1 standard deviation of the subsets in each of the 4 major clusters. A comparable hierarchical cluster analysis on 31 non-overlapping subsets is presented in Supplementary Figure 6.

3B,D). Specifically, low maternal education was associated with a reduction in the patterns of eosinophils and classical monocytes (Figure 4). Female gender was associated with a significant increase in the pattern of neutrophils, and the phenotypically overlapping $CD15^+$ granulocyte population. Having more than 1 sibling was associated with a reduction, and CMV infection with an increase, in NK cells. Antibiotics usage in the first year of life was associated with an increase in non-classical monocytes. Prenatal maternal immune-mediated determinants, and the bacterial/viral exposure-related determinants breastfeeding, respiratory tract infection or bacterial/ persistent viral infections did not affect any of the populations within cluster 1.

Bacterial/ viral exposure-related determinants were more frequently found to affect cell numbers in clusters 2, 3 and 4 than in cluster 1 (Figure 3A-B). None of the determinants included in our study affected the patterns of more than

Table 2. Characteristics of external determinants

	negative individuals (%)	positive individuals (%)	Missing (%)	Sign effect on cluster [a]	Sign effect confounder-corrected [b]
Prenatal maternal life style					
Maternal age >35yr	951 (80.5)	231 (19.5)	0 (0)	-	-
Low maternal educational level	400 (33.8)	760 (64.3)	22 (1.9)	1,2,3,4	1,2,3,4
Net household income per month >€ 2,200	242 (20.5)	847 (71.7)	93 (7.9)	-	-
Smoking during pregnancy	815 (69)	256 (21.7)	111 (9.4)	-	-
Alcohol use continued during pregnancy	299 (25.3)	750 (63.5)	133 (11.3)	-	-
Body Mass Index before pregnancy >25	761 (64.4)	240 (20.3)	181 (15.3)	-	-
Prenatal maternal immune-mediated diseases					
Maternal atopy (eczema, allergy HDM, hay-fever)	683 (57.8)	383 (32.4)	116 (9.8)	-	-
Anti-TPO >60 IU/ml (before 18 weeks of pregnancy)	849 (71.8)	55 (4.7)	278 (23.5)	-	-
Maternal TG2A during pregnancy >6 IU/ml	969 (82)	5 (0.4)	208 (17.6)	4	-
Birth characteristics					
Girl Gender	600 (50.8)	582 (49.2)	0 (0)	1,2,3,4	1,2,3,4
Preterm birth <37 weeks	76 (6.4)	1,106 (93.6)	0 (0)	-	-
Birth weight <2,500g	1,119 (94.7)	63 (5.3)	0 (0)	-	-
Bacterial/viral exposure-related characteristics					
Premature rupture of membranes	1,089 (92.1)	53 (4.5)	40 (3.4)	4	4
Caesarian section versus vaginal/ forceps/vacuum assisted	903 (76.4)	154 (13)	125 (10.6)	3,4	3,4
Birth season; born in fall/winter	682 (57.7)	500 (42.3)	0 (0)	-	-
Breastfeeding ever	109 (9.2)	969 (82)	104 (8.8)	-	-
Breastfeeding at 6 months of age	754 (63.8)	299 (25.3)	129 (10.9)	2,4	2,4
Siblings >1	687 (58.1)	480 (40.6)	15 (1.3)	1	1
Antibiotics/Penicillin use in 1st y	636 (53.8)	342 (28.9)	204 (17.3)	1,3	1,3
Upper respiratory tract infections in 1st y	547 (46.3)	475 (40.2)	160 (13.5)	-	-
Lower respiratory tract infections in 1st y (doctor attended)	819 (69.3)	131 (11.1)	232 (19.6)	-	-
Carrier of Helicobacter pylori within 6yrs	887 (75)	49 (4.1)	246 (20.8)	2,3	2,3
Seropositivity for Cyomegalovirus (CMV) at 6y	665 (56.3)	269 (22.8)	248 (21)	1,2,3,4	1,2,3,4
Seropositivity for Epstein Barr virus (EBV) at 6y	534 (45.2)	400 (33.8)	248 (21)	2,3,4	2,3,4
Seropositivity for Herpes simplex virus-1 (HSV-1) at 6y	810 (68.5)	124 (10.5)	248 (21)	2	2
Seropositivity for Varicella zoster virus (VZV) at 6y	73 (6.2)	861 (72.8)	248 (21)	-	-

[a] Effect in single variable analyses: each determinant was added to the linear mixed effect model univariately to test for a significant effect (Significance was defined as $p<0.0125$)
[b] Effect after multivariable correction: per leukocyte population, all determinants with a significant individual effect were added in a multivariable model to correct for confounding effects. Up to 3 determinants were added per multivariable model. Significance was defined as $p<0.05$. IU, international units

25% of the populations in cluster 2 (Figure 3C-D). However, female gender was associated with an increase in the patterns of $CD4^+$ naive and Tcm cells, and consequently in the phenotypically overlapping $CD4^+TCR\alpha\beta^+$ and total $CD4^+$ T cells. Whereas breastfeeding for more than 6 months was associated with a reduction in the pattern of total IgA^+ and $CD27^-IgA^+$ memory B cells, H. pylori carriership was associated with an increase in these populations. Furthermore, H. pylori carriership was associated with an increase in total $Ig\kappa^+$ B cells and $TCR\alpha\beta^+$ T cells. CMV and EBV were both associated with an increase in the strongly related total and $TCR\alpha\beta^+$ $CD8^+$ T cells, and CMV with an increase in $V\delta1^+$ T cells. HSV-1 seropositivity was associated with a decrease in the large population of naive B cells, and consequently with total B cells and $Ig\kappa^+$ and $Ig\lambda^+$ B-cell subsets.

In cluster 3, CMV and EBV seropositivity were most strongly associated with changes in lymphocyte dynamics (Figure 3C). Both viruses were associated with an increase in $CD8^+$ intermediate and late TemRO cells, and the phenotypically-related total $CD8^+$ TemRO and total $CD8^+$ Tmem populations. In addition, CMV was associated with an increase in $CD8^+TCR\gamma\delta^+$ T cells, and $CD8^+$ late TemRA cells, and the phenotypically-related total $CD8^+$ TemRA population. EBV was associated with an increase in $CD8^+$ early TemRO cells, and a decrease in total Bmem, and the $CD27^-$ and $CD27^+$ Bmem subsets. The effects on $CD8^+$ late TemRO and TemRA cells were still present in the analysis of 31 non-overlapping population, even though these were now included in cluster 4 (Figure 3D).

In cluster 4, gender, premature rupture of membranes, CMV and EBV were most strongly associated with changes in lymphocyte dynamics (Figure 3C-D). Female gender was associated with an increase in the patterns of $CD27^+IgG^+$ memory B cells, and $CD4^+$ early TemRO cells, and with a reduction in total $TCR\gamma\delta^+$ T cells and $CD4^+$ late TemRA populations. Premature rupture of membranes was associated with an increase in $CD4^+$ late TemRO and $V\delta2^+$ and $V\gamma9^+$ T-cell subsets. CMV and EBV seropositivity were both associated with an increase in total $CD4^+$ and $CD4^+$ late TemRO and TemRA cells, as well as $CD8^+$ intermediate TemRA cells. In addition, CMV was associated with increased $CD4^+$ intermediate TemRO cells, and EBV with an increase in $CD4^+$ early TemRO cells.

Altogether, we identified multiple determinants that had a significant effect on the dynamics of leukocyte subset numbers within one or more of the four distinct age-related patterns. Still, considerable variation of effects could be observed within individual clusters.

Effects of external determinants on leukocyte dynamics within distinct leukocyte lineages

As clusters 2, 3 and 4 each contained various B-cell and T-cell populations, each with distinct humoral or cellular functions, we next studied the effects of the 26 determinants on the patterns of individual subsets within the innate leukocytes, $TCR\gamma\delta^+$ T-cell, naive and memory B-cell, naive and memory $CD4^+$ T-cell and naive and memory $CD8^+$ T-cell and lineages (Figure 4).

Within the B-cell lineage, low maternal educational level was the only prenatal maternal life style-related factor that was associated with a change in B-cell dynamics, and was associated with a reduction in the patterns of total memory B cells. None of the prenatal maternal immune-mediated diseases were associated with leukocyte dynamics in any of the cell lineages. Of the birth characteristics,

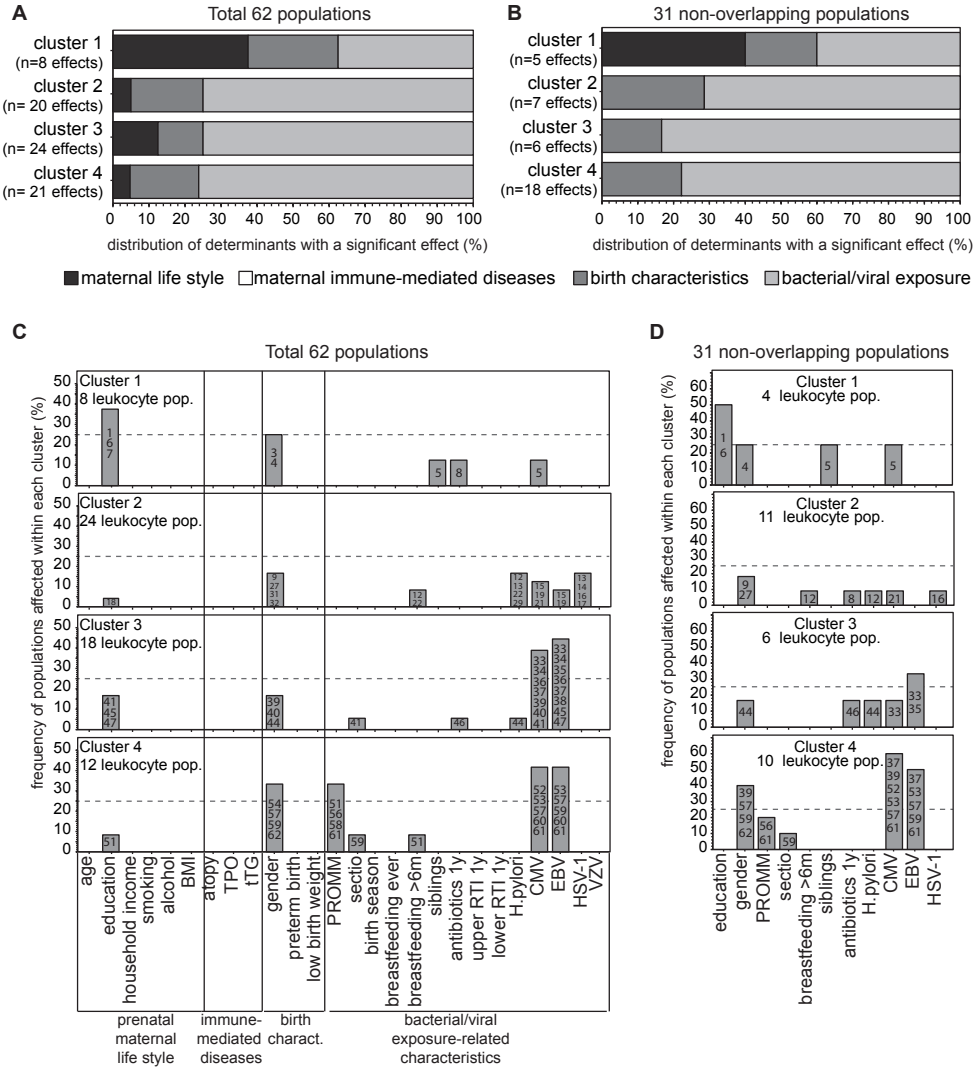


Figure 3. External determinants associated with leukocyte dynamics in the four clusters.
Twenty-six determinants were studied for having an independent effect on the linear mixed effect models of each of the 62 leukocyte populations (A and C) and each of the 31 non-overlapping leukocyte subsets (B and D). Subsequently, all determinants with a significant individual effect were added to a multivariable linear mixed effect analysis to correct for confounding effects. **A and B)** Relative distribution of significant effects by determinants within the groups: prenatal maternal life style, prenatal maternal immune-mediated diseases, birth characteristics or bacterial/ exposure-related determinants. Analyses were independently performed for all 62 defined subsets (**A**) and the subgroup of 31 non-overlapping subsets (**B**). **C and D)** Frequencies of populations within a cluster that were affected by individual determinants. In **C.** all 62 leukocyte populations and all 26 studied determinants are presented. In **D.**, the independent analysis on the 31 non-overlapping populations is depicted containing only the determinants that affected one or more cell types. Abbreviations: PROMM, premature rupture of membranes; RTI, respiratory tract infection. Numbers in each bar refer to the number of each population as indicated in Figure 2.

female gender was associated with an increase in the patterns of $CD27^+IgA^+$ and $CD27^+IgG^+$ memory B cells, though these effects were not reflected in the total memory B-cell populations. Breastfeeding for more than 6 months was associated with a selective reduction in the pattern of $CD27^-IgA^+$ memory B cells, antibiotics usage in the first year of life was associated with a selective increase in the pattern of $CD27^-IgG^+$ memory B cells, and H. pylori carriership was associated with an increase in the patterns of both $CD27^-IgA^+$ and $CD27^+IgA^+$ memory B cells. EBV infection was associated with a reduction in the pattern of total memory B cells, though not with a specific memory B-cell subset, HSV-1 infection was associated with a selective reduction in the pattern of naive B cells.

Within the $CD4^+$ and $CD8^+$ T-cell lineage, female gender was associated with an increase in the patterns of $CD4^+$ naive, Tcm and early TemRO cells, although these effects were not reflected in the total $CD4^+$ memory T-cell populations. In contrast, female gender was associated with a decrease in the patterns of $CD4^+$ and $CD8^+$ late TemRA cells. Whereas premature rupture of membranes was associated with an increase in $CD4^+$ late TemRO cells, child birth via caesarian section was associated with a reduction in the pattern of $CD4^+$ early TemRO cells. Breastfeeding, antibiotics usage and H. pylori carriership were not associated with $CD4^+$ or $CD8^+$ T-cell dynamics. Persistent viral infection with CMV and EBV strongly associated with $CD4^+$ and $CD8^+$ T-cell dynamics. CMV infection associated exclusively with an increase in the patterns of intermediate and late differentiated $CD4^+$ and $CD8^+$ TemRO and TemRA cells. Associations with EBV were slightly more variable, also including early differentiated $CD4^+$ and $CD8^+$ TemRO cells. In contrast to both CMV and EBV, HSV-1 and VZV did not associate with $CD4^+$ and $CD8^+$ T-cell dynamics.

Within the $TCR\gamma\delta^+$ T-cell lineage, CMV infection associated with an increase in the pattern of $V\delta1^+$ T cells; premature rupture of membranes was associated with an increase in $V\delta2^+V\gamma9^+$ T cells.

DISCUSSION

We here modelled the kinetics of 62 leukocyte subsets, including 31 non-overlapping subsets, and identified distinct patterns of cell numbers in the first 6 years of life for innate leukocytes, naive B and T cells, and memory B and T cells. Furthermore, we identified multiple determinants related to prenatal maternal life style, prenatal maternal immune-mediated diseases, birth characteristics or bacterial/viral exposure, that affected the dynamics of each of these leukocyte subsets between birth and 6 years of age.

Unsupervised clustering of all 62 leukocyte subsets, as well as of the set of 31 phenotypically non-overlapping populations, resulted in 4 clusters with the first cluster containing exclusively innate leukocyte subsets. These subsets followed highly similar patterns of kinetics with age: they were abundant at birth and quickly decreased before stabilizing at 6-14 months of age. Consistent with their early predominance, innate leukocyte kinetics was affected by maternal education level, which might already influence the fetus prenatally. Maternal education level is strongly related to life style with low education being an indicator for a less healthy life style, including differences in smoking and alcohol consumption, BMI, stress-levels, higher infection load and increased crowding.[34] These observations might suggests that maternal life style is especially important for shaping of innate

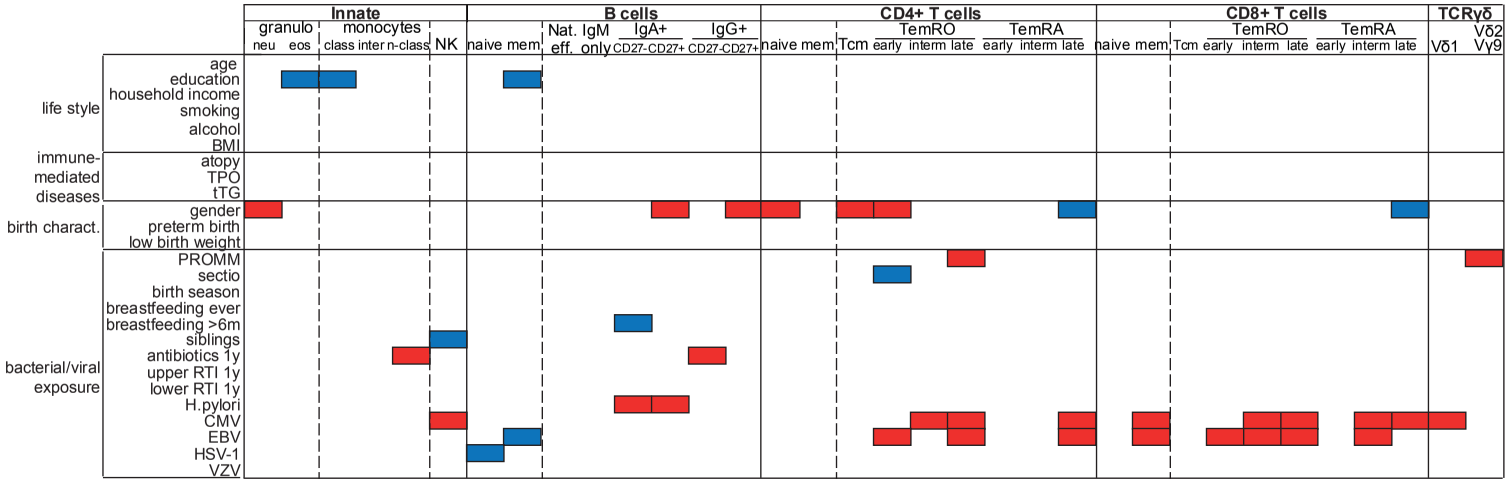


Figure 4. Effects of external determinants on the dynamics of innate leukocytes, and B-cell and T-cell subsets.
The effects are shown for all 26 determinants analyzed by multivariable linear mixed effect analysis. Depicted are the 31 leukocyte populations with non-overlapping phenotypic population definitions, and the total memory B-cell, CD4⁺ memory T-cell or CD8⁺ memory T-cell subsets (indicated as "mem" for each lineage). Significant associations with a subset are indicated with blue boxes representing a reduction, or red boxes representing an increase in the pattern of the indicated population. Details of each determinant are provided in Table 2. Additional abbreviations: PROMM, premature rupture of membranes; RTI, respiratory tract infection; neu, neutrophils; eos, eosinophils; class, classical monocytes; inter, intermediate monocytes; n-class, non-classical monocytes.

leukocyte populations, though the exact mediator of the effect will still have to be determined.

The non-classical and intermediate monocytes did not consistently cluster with the innate leukocyte subsets due to a later peak in numbers or a slower decline. This altered kinetics could be the result of these being derived from classical monocytes, and not directly from precursors in bone marrow.[35] Furthermore, the various subsets of monocytes show distinct expression levels of proteins involved in HLA-class II-dependent antigen presentation and CD40-CD40L co-stimulation,[35] and display distinct parasite pattern recognition abilities.[35-38] Thus, our data support the need to discriminate intermediate and non-classical monocytes from the dominant population of classical monocytes in kinetics studies.

All naive B-cell and T-cell populations clustered together in cluster 2 and their numbers increased in the first 14 months followed by a more gradual decline. After the first 1-2 years of life, B-lymphocyte production in bone marrow decreases,[39,40] and the thymus starts to involute.[41] These processes are likely causes of the decline we observed. The nature for decreased bone marrow output remains unclear. Recent studies, however, indicate a role for circulating long-lived memory B cells in this process.[42]

Memory B-cell subsets were found in several clusters. Natural effector and $CD27^{-}IgA^{+}$ memory B cells clustered together with naive B cells. Both memory B-cell populations are suggested to derive, at least in part, from germinal center-independent responses without T-cell help in the splenic marginal zone and the intestinal lamina propria.[43-45] The fast rise in cell numbers within the first 6 months of life contrasts the kinetics of the other, T-cell dependent, memory B-cell subsets in clusters 3 and 4. This supports the concept of these cells being generated in first and fast responses, in the absence of the extensive proliferation and selection processes of the germinal center.[45]

IgMonly, $CD27^{-}IgG^{+}$ and $CD27^{+}IgA^{+}$ memory B cells clustered in cluster 3, representing gradual memory formation. Only the $CD27^{+}IgG^{+}$ population clustered in cluster 4, and developed slightly slower than the other memory B-cell populations. In adults, this subset shows a higher level of affinity maturation, more extensive replication history, and more frequent development via consecutive class-switching, than $CD27^{-}IgG^{+}$ memory B cells.[45,46] It is perceivable that many of these $CD27^{+}IgG^{+}$ cells are generated from $CD27^{-}IgG^{+}$ memory B cells in secondary responses. This could explain the gradual decline in $CD27^{-}IgG^{+}$ B cell numbers after 14 months of age.

Several external determinants had a significant effect on the B-cell kinetics. First, we confirmed our previous observations regarding lower numbers of memory B cells in breastfed children.[21,23] However, in the current analysis we could not reproduce the previously reported association between breastfeeding duration and germinal center-dependent memory B-cell numbers at 6 months of age.[45] The difference is likely due to our current analysis in which duration of breastfeeding was not included and the overall pattern between birth and 6 years of age was studied.

IgA^{+} memory B cells were significantly increased in H. Pylori positive children. The colonization of the gastric mucus by H. Pylori has been found to correlate with an increase in total blood B-cell counts,[47] on top of the local expansion of antibody-secreting IgA^{+} cells,[48] which are important for the protection against H. Pylori

infection.[49] Our observed expansion of circulating IgA$^+$ memory B cells included both the CD27$^+$IgA$^+$ and mucosa-derived CD27$^-$IgA$^+$ subsets. This would suggest that the presence of H. pylori does not only result in a local expansion of plasma cells, but also a systemic expansion of memory B cells in otherwise asymptomatic carriers. It remains to be determined whether this expansion is beneficial for the host as the bacterial protein CagA inhibits B-cell apoptosis and thereby increases the risk for mucosa-associated B-cell malignancies.[50,51]

Memory T-cell subsets were found in clusters 2, 3 and 4. CD4$^+$ and CD8$^+$ Tcm (cluster 2) express lymph node homing markers and are the presumed precursors of effector memory T cells.[25,52,53] The early peak in Tcm numbers prior to those of effector memory T cells in young children would fit with this function. Similarly, CD8$^+$ early TemRA cells might be precursors for further differentiated TemRA subsets. Vδ1$^+$ T-cell numbers (cluster 2) peaked prior to the Vδ2$^+$ and Vγ9$^+$ subsets (cluster 4), thereby confirming the previously observed early shift from Vδ1$^+$ to Vδ2$^+$Vγ9$^+$ predominance in children,[54,55] Of note, Vδ1$^+$ T cells are abundant in adult intestine; their decline in blood might, at least in part, result from altered distribution towards mucosal tissue.

Both vaginal delivery and premature rupture of membranes were associated with an increase in the pattern of one of the CD4$^+$ TemRO subsets, in line with previous literature.[56] Stress upon child birth via vaginal delivery has been correlated with an increase in CD4$^+$ regulatory T cells.[57] As a large fraction of regulatory T cells has a memory phenotype, this could explain the observed increase of CD4$^+$ TemRO cells in children born by vaginal delivery. Since reduced numbers of regulatory T cells in cord blood are associated with the development of atopic dermatitis in the first year of life,[58] it will be of interest to study whether this effect is modified by the mode of delivery.

Both CD4$^+$ and CD8$^+$ memory T-cell dynamics were predominantly affected by CMV and/ or EBV seropositivity. This is consistent with previous observations in both children and elderly,[26,59-63] and in agreement with their function in cellular immunity. Moreover, these viruses displayed distinct effects with CMV being associated to increased late effector memory T-cell numbers and EBV with a slightly more variable effect, including also early effector memory T-cell subsets.[25,64] Furthermore, we confirmed that HSV-1 did not affect CD4$^+$ and CD8$^+$ T-cell populations.[26,63] In contrast, HSV-1 seropositivity was associated with a loss of naive B cells. This association has, to our knowledge, not been described before. EBV infection was associated with a reduction in memory B cells, likely due to the selective EBV persistence in these cells.[65,66] Our large-scale analysis allowed us to separate the herpesvirus-associated effects, with CMV and EBV being associated with memory T-cell expansions, and HSV-1 and EBV, with a decrease in naive or memory B-cell numbers, respectively.

Gender had a widespread effect on 13 subsets within multiple leukocyte lineages. Interestingly, female gender was associated with an increase in neutrophils, T-cell dependent IgA$^+$ and IgG$^+$ memory B-cell populations and CD4$^+$ naive, Tcm and early differentiated TemRO cells, while numbers of late differentiated CD4$^+$ and CD8$^+$ TemRA cells and TCRγδ$^+$ T cells were reduced. These combined effects result in a skewing of humoral and early differentiated CD4$^+$ T-cell responses over cellular cytotoxic responses in girls. These effects might be associated with differences in sex-hormone levels (testosterone, estradiol) in females versus males that are already detectable during early infancy, as well as with genetic

differences between females and males.[67] In adults, sex-hormones have been found to affect immune responses in both quantitative and qualitative aspects.[68,69] These insight can especially be important for dissection of auto-immune diseases, which are much more prevalent in females than in males, though predisposition for allergic diseases seems to be opposite in infancy.[67,70]

Methodological considerations

The strength of this study is its prospective longitudinal population-based design with >1,000 children and the possibility to study 26 external determinants with adjustments for major confounders. The linear mixed model approach enabled modeling of cross-sectional data, with further improvement of the accuracy by incorporating additionally available longitudinal follow-up data. Furthermore, we included only children with a two-generation Dutch ancestry, which prevented interference of our analyses by strong ethnic and cultural influences. However, extrapolation of our findings to different ethnic and cultural populations might be limited, and would require additional analysis of ethnically-different population cohorts.

The inclusion of 62 leukocyte populations allowed for the large-scale analysis of the effect of external determinants on both total cell lineages as well as on small subsets defined by extensive and detailed phenotypic definition. The overlap in some populations could have skewed the hierarchical clustering. However, our selection of 31 phenotypically non-overlapping populations resulted in clusters with similar patterns, indicating the robustness of the 4 major patterns of leukocyte dynamics and the observed effects of external determinants.

Our study was primarily explorative with a focus on the identification of determinants related to prenatal maternal life style, maternal immune-mediated diseases, birth characteristics or bacterial/viral exposure, that affected leukocyte dynamics between birth and 6 years of age. Although we defined whether the determinants had a positive or negative association with leukocyte numbers, we were unable to identify the exact nature of the effect, i.e. exactly when these effects presented and whether these effects will be transient, persistent or potentially even increasing over time. Consequently, more research into individual determinants will be needed to extend our observations by specifying these effects.

Finally, special consideration needs to be taken for serology of infectious agents that were measured at the age of 6 years. We cannot determine the exact timing of primary infections, and this can consequently be in the whole period preceding the age of 6 years. Still, most H. Pylori infections already occur in early childhood, and IgG seropositivity to herpesviruses only appears several weeks to 3 months after infection. Thus, it is likely that these determinants were present during or prior to the 6th year of life.

Conclusions

With our unbiased approach, we were, for the first time, able to classify leukocyte populations according to their dynamics between birth and 6 years of age, and to assess determinants that are associated with the dynamics of cell lineages or specific leukocyte subsets. We confirmed previously described effects of persistent CMV and EBV infections on memory T cells and identified new effects, including

the effects of HSV-1 on naive B cells. These newly identified determinants can provide new targets for studies on the molecular processes that regulate leukocyte development and immune responses, and that together underlie formation of long-lasting immunity without inducing destructive, excessive or insufficient immune responses.

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CONFLICT OF INTEREST

All authors declare that no competing interests exist.

J.J.M.v.D., E.G.v.L., L.E.B-J, H.H., W.A.D., and M.C.v.Z. designed and supervised the flowcytometric experiments; D.v.d.H., M.A.E.J. and H.B.-C. performed and analyzed most of the experiments and contributed to data analyses; V.W.V.J. designed the Generation R Study; H.A.M. designed the study, the collection and analyses of determinant data; K.N. contributed to the statistical modeling; D.v.d.H., M.C.v.Z., and M.A.E.J. wrote the manuscript; and all authors commented on the manuscript.

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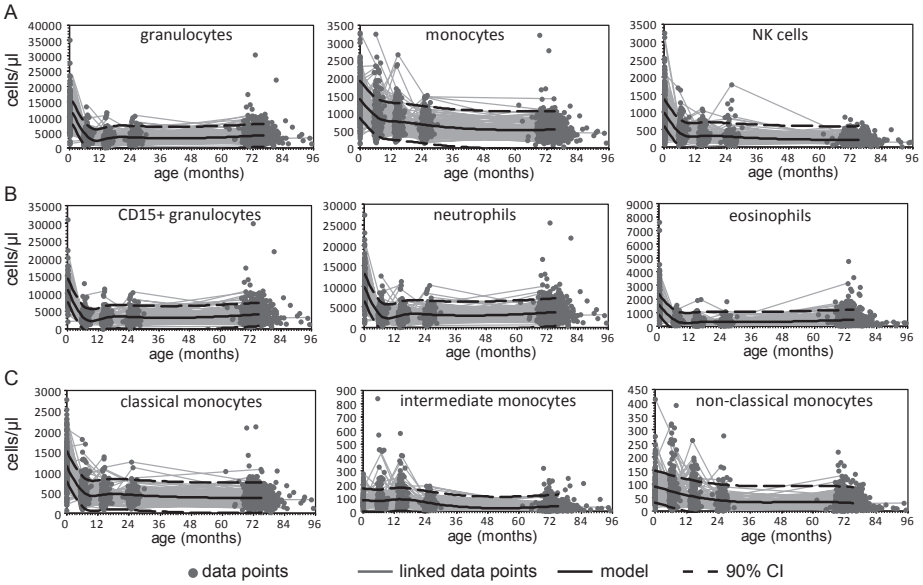
Supplemental Table 1. Antibody details

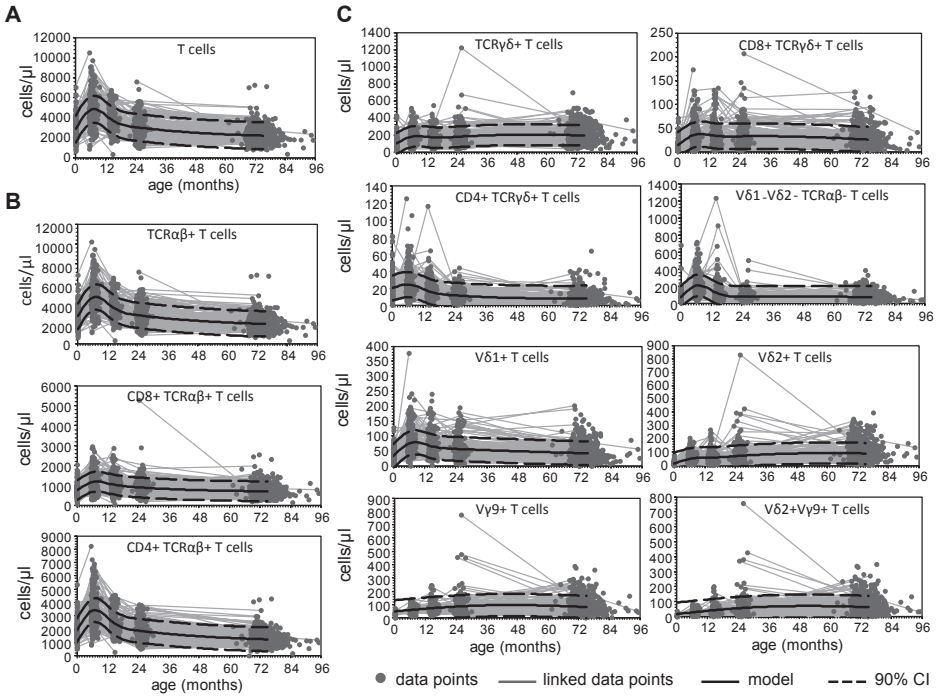
Tube		Fluorochrome					
		FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-Cy7
1	**Antibody**	**CD3**	**CD16+CD56**	**CD45**	**CD4**	**CD19**	**CD8**
	Clone	SK7	B73.1 + C5.9	2D1	SK3	SJ25C1	SK1
	Manufacturer	BD Biosciences	BD Biosciences+Dako	BD Biosciences	BD Biosciences	BD Biosciences	BD Biosciences
2	**Antibody**	**CD15**	**-**	**CD45**	**CD16**	**-**	**CD14**
	Clone	MMA	-	2D1	3G8	-	MO-P9
	Manufacturer	BD Biosciences	-	BD Biosciences	BD Biosciences	-	BD Biosciences
3	**Antibody**	**Igκ**	**Igλ**	**CD19**	**CD21**	**-**	**-**
	Clone	polyclonal	polyclonal	SJ25C1	B-ly-4	-	-
	Manufacturer	Dako	SBA	BD Biosciences	BD Biosciences	-	-
4	**Antibody**	**CD38**	**-**	**CD19**	**CD21**	**-**	**-**
	Clone	HB7	-	SJ25C1	B-ly-4	-	-
	Manufacturer	BD Biosciences	-	BD Biosciences	BD Biosciences	-	-
5	**Antibody**	**IgD**	**-**	**CD19**	**-**	**IgM**	**CD27**
	Clone	polyclonal	-	SJ25C1	-	polyclonal	L128
	Manufacturer	Southern Biotech	-	BD Biosciences	-	BD Biosciences	BD Biosciences
6	**Antibody**	**IgA**	**IgG**	**CD19**	**-**	**IgM**	**CD27**
	Clone	polyclonal	polyclonal	SJ25C1	-	polyclonal	L128
	Manufacturer	Kallestad	Southern Biotech	BD Biosciences	-	BD Biosciences	BD Biosciences
7	**Antibody**	**TCRαβ**	**TCRγδ**	**CD3**	**CD4**	**CD8**	**-**
	Clone	WT31	11F2	SK7	SK3	SK1	-
	Manufacturer	BD Biosciences	BD Biosciences	BD Biosciences	BD Biosciences	BD Biosciences	-
8	**Antibody**	**Vδ2**	**Vδ1 ***	**CD3**	**CD4**	**TCRαβ**	**CD8**
	Clone	B6.1	R9.12	SK7	SK3	IP26	SK1
	Manufacturer	BD Biosciences	Beckman Coulter	BD Biosciences	BD Biosciences	eBiosciences	BD Biosciences
9	**Antibody**	**CD28**	**CD197**	**CD3**	**CD8**	**CD45RO**	**CD27**
	Clone	CD28.2	3D13	SK7	SK1	UCHL-1	L128
	Manufacturer	BD Biosciences	eBiosciences	BD Biosciences	BD Biosciences	BD Biosciences	BD Biosciences
10	**Antibody**	**Vδ2**	**Vγ9**	**CD3**	**CD4**	**TCR**αβ	**CD8**
	Clone	B6.1	B3.1	SK7	SK3	IP26	SK1
	Manufacturer	BD Biosciences	BD Biosciences	BD Biosciences	BD Biosciences	eBiosciences	BD Biosciences

*, unconjugated antibody, detected with Goat anti-Mouse IgG PE (polyclonal; Invitrogen)



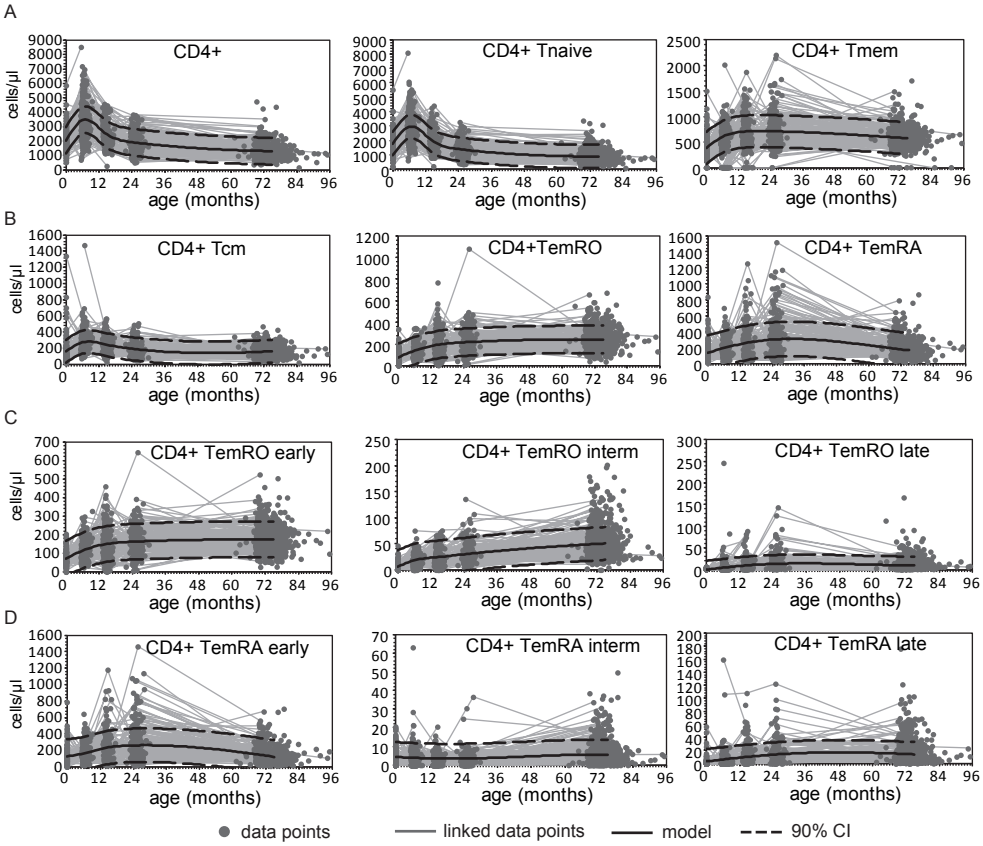
Supplemental Figure 1. Dynamics of innate leukocyte subsets in children between birth and 6 years of age.

Absolute numbers and modelled dynamics of granulocytes, monocytes and NK cells **(A)**, $CD15^{+}$, neutrophilic and eosinophilic granulocytes **(B)**, and classical, intermediate and non-classical monocyte subsets **(C),** from birth until 6 years of age. Linear mixed effect models were generated for each population (solid black line) and represented with the 90% confidence interval (CI) of the model (dashed black lines). For clarity of the graphs, gray lines connect only consecutive time points within one individual; i.e. 0-6m, 6-14m, 14-25m or 25-72m.

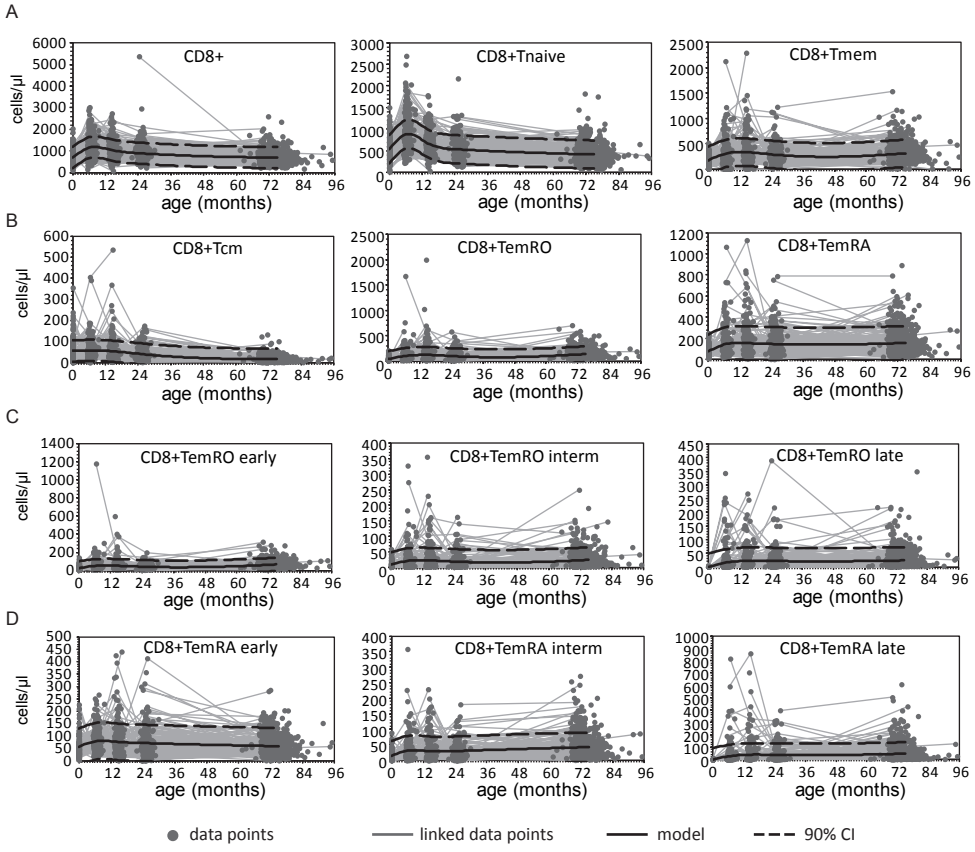


Supplemental Figure 2. Dynamics of TCRαβ⁺ T-cell and TCRγδ⁺ T-cell subsets in children between birth and 6 years of age.

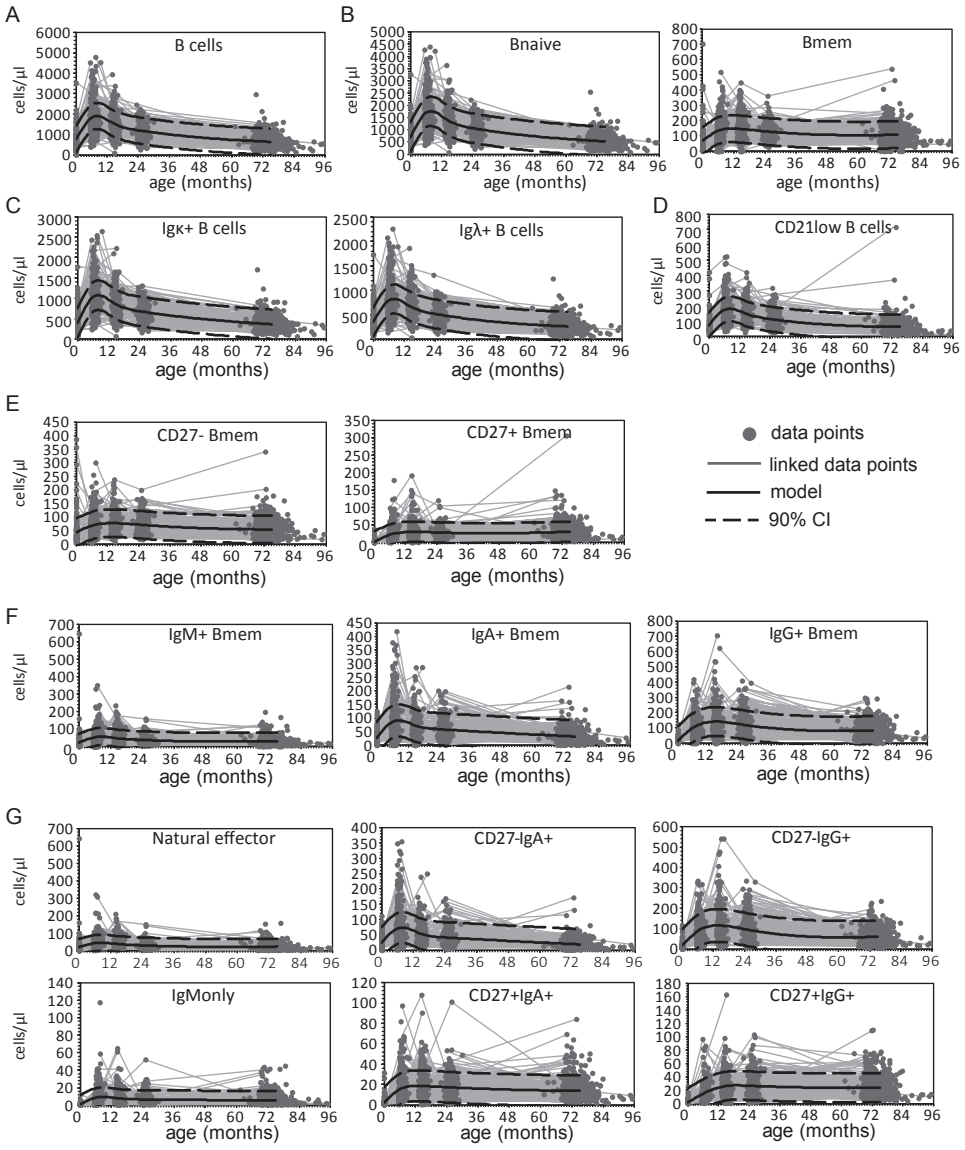
Absolute numbers and modelled dynamics of T cells **(A)**, TCRαβ⁺ T-cell populations **(B)**, and TCRγδ⁺ T-cell populations **(C),** from birth until 6 years of age. Linear mixed effect models were generated for each population (solid black line) and represented with the 90% confidence interval (CI) of the model (dashed black lines). For clarity of the graphs, gray lines connect only consecutive time points within one individual; i.e. 0-6m, 6-14m, 14-25m or 25-72m.



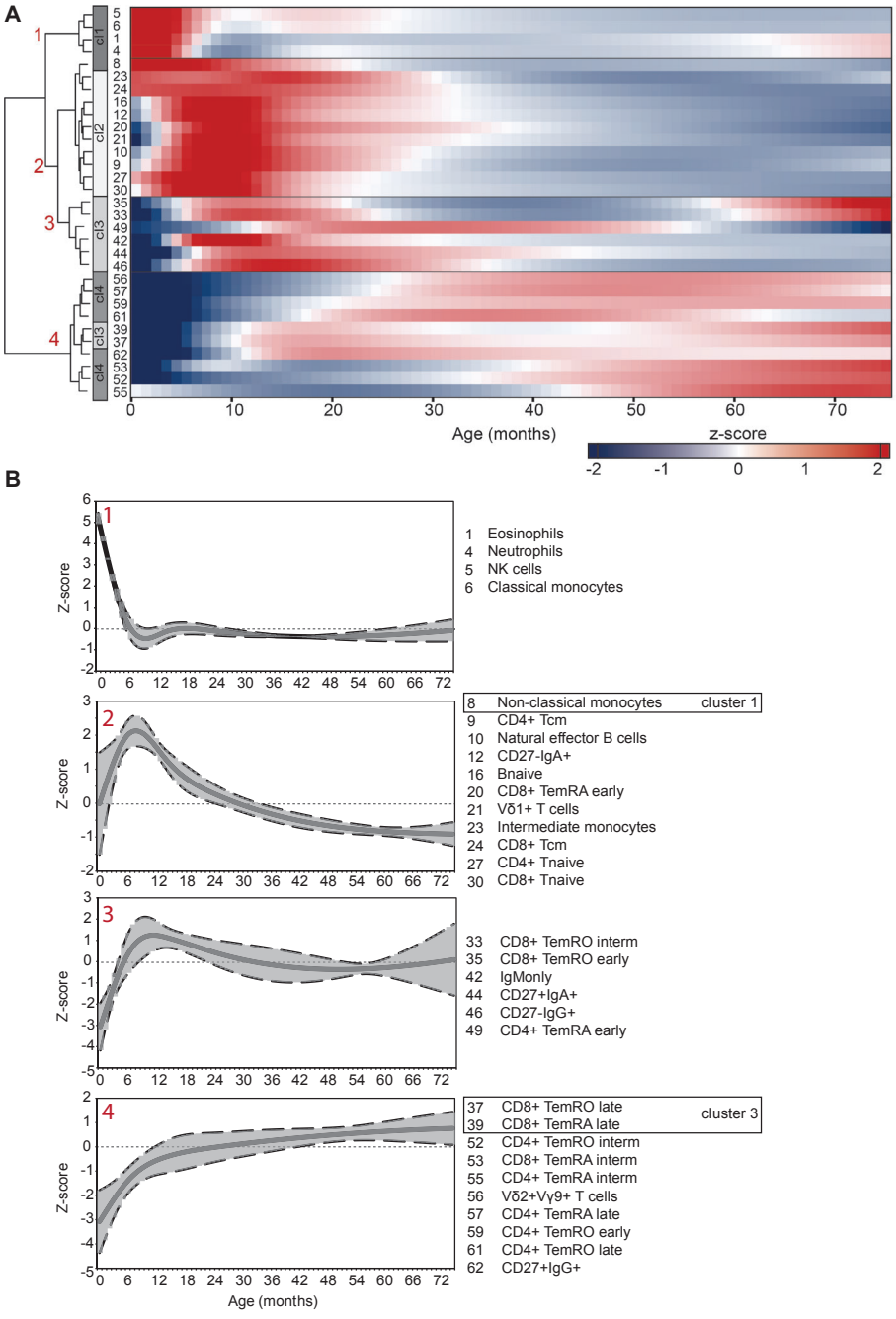
Supplemental Figure 3. Dynamics of CD4⁺ T-cell subsets in children between birth and 6 years of age. Absolute numbers and modelled dynamics of total, naive and memory CD4⁺ T cells **(A)**; CD4⁺ central memory T cells (Tcm), CD45RO⁺ effector memory (TemRO) and CD45RO⁻ effector memory (TemRA) T-cell subsets **(B)**; early, intermediate or late differentiated TemRO subsets **(C)**; or early, intermediate or late differentiated TemRA subsets **(D)**, between birth and 6 years of age. Linear mixed effect models were generated for each population (solid black line) and represented with the 90% confidence interval (CI) of the model (dashed black lines). For clarity of the graphs, gray lines connect only consecutive time points within one individual; i.e. 0-6m, 6-14m, 14-25m or 25-72m.



Supplemental Figure 4. Dynamics of CD8⁺ T-cell subsets in children between birth and 6 years of age. Absolute numbers and modelled dynamics of total, naive and memory CD8⁺ T cells **(A)**; CD8⁺ central memory T cells (Tcm), CD45RO⁺ effector memory (TemRO) and CD45RO⁻ effector memory (TemRA) T cell subsets **(B)**; early, intermediate or late differentiated TemRO subsets **(C)**; or early, intermediate or late differentiated TemRA subsets **(D)**, between birth and 6 years of age. Linear mixed effect models were generated for each population (solid black line) and represented with the 90% confidence interval (CI) of the model (dashed black lines). For clarity of the graphs, gray lines connect only consecutive time points within one individual; i.e. 0-6m, 6-14m, 14-25m or 25-72m.



Supplemental Figure 5. Dynamics of B-cell subsets in children between birth and 6 years of age. Absolute numbers and modelled dynamics of total B cells **(A)**; naive and total memory B cells **(B)**; total Igκ^{+} and Igλ^{+} B-cells populations **(C)**; and CD21^{low} B cells **(D)**; CD27^{-} and CD27^{+} memory B cells **(E)**; total IgM^{+}, IgA^{+} or IgG^{+} memory B cells **(F)**; IgM^{+}, IgA^{+} or IgG^{+} memory B-cell subsets **(G)** between birth and 6 years of age. Linear mixed effect models were generated for each population (solid black line) and represented with the 90% confidence interval (CI) of the model (dashed black lines). For clarity of the graphs, gray lines connect only consecutive time points within one individual; i.e. 0-6m, 6-14m, 14-25m or 25-72m.



Supplemental Figure 6. Hierarchical clustering of the dynamics of 31 non-overlapping leukocyte subsets in early childhood.
A) Ward's hierarchical clustering was performed as in Figure 2, including now only the 31 non-overlapping leukocyte subsets. Indicated in gray squares in front of the heat map is the cluster each population was assigned to in the analysis of the total 62 leukocyte subsets in Figure 2. **B)** Average patterns +/- 1 standard deviation of the subsets in each of the 4 major clusters. Indicated in black squares are 3 subsets that were assigned to different clusters than in Figure 2. *(Figure on next page)*

Chapter 3

DECREASED MEMORY B CELLS AND INCREASED CD8 MEMORY T CELLS IN BLOOD OF BREASTFED CHILDREN: THE GENERATION R STUDY

Running title: Impact of breastfeeding on adaptive immunity

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ABSTRACT

Background. Breastfeeding provides a protective effect against infectious diseases in infancy. Still, immunological evidence for enhanced adaptive immunity in breastfed children remains inconclusive.

Objective. To determine whether breastfeeding affects B- and T- cell memory in the first years of life.

Methods. We performed immunophenotypic analysis on blood samples within a population-based prospective cohort study. Participants included children at 6 months (n=258), 14 months (n=166), 25 months (n=112) and 6 years of age (n=332) with both data on breastfeeding and blood lymphocytes. Total B-cell and T-cell numbers and their memory subsets were determined with 6-color flow cytometry. Mothers completed questionnaires on breastfeeding when their children were aged 2, 6, and 12 months. Multiple linear regression models with adjustments for potential confounders were performed.

Results. Per month continuation of breastfeeding, a 3% (95% CI -6,-1) decrease in $CD27^{+}IgM^{+}$, a 2% (95 CI % -5,-1) decrease in $CD27^{+}IgA^{+}$ and a 2% (95% CI -4,-1) decrease in $CD27^{-}IgG^{+}$ memory B-cell numbers were observed at 6 months of age. $CD8^{+}$ T-cell numbers at 6 months of age were 20% (95% CI 3,37) higher in breastfed than in non-breastfed infants. This was mainly found for central memory $CD8^{+}$ T cells and associated with exposure to breast milk, rather than duration. The same trend was observed at 14 months, but associations disappeared at older ages.

Conclusions. Longer breastfeeding is associated with increased $CD8^{+}$ T-cell memory, but not B-cell memory numbers in the first 6 months of life. This transient skewing towards T-cell memory might contribute to the protective effect against infectious diseases in infancy.

INTRODUCTION

Breast milk contains factors that enhance nutrient absorption, stimulate growth and enhance the defense against pathogens.[1] Consequently, breastfeeding provides protection against infectious diseases during infancy.[2-4] The protective effect persists during childhood,[5,6] and modulates vaccination responses.[7-9] Thus, it is likely that breastfeeding not only provides passive immunization, but also enhances adaptive immunity.[10]

B and T lymphocytes comprise the cellular components of adaptive immunity, and are generated throughout life. B cells contribute to humoral immunity through the production of immunoglobulins (Ig), whereas $CD8^+$ cytotoxic T cells provide cellular immune responses. $CD4^+$ helper T cells support both humoral and cellular immune responses. Each B and T cell generates a unique antigen receptor during precursor differentiation in bone marrow or thymus, respectively. Only those cells that specifically recognize antigen with their receptor will undergo clonal proliferation and are involved in the antigen response. Cells generated from the clonal expansion will remain present in the body as long-lived memory cells and will initiate a fast and quantitatively stronger response upon secondary antigen encounter. In addition to $CD27^-$ naive B cells, six memory B-cell subsets can be identified.[11] Four of these express CD27 and are either positive for IgM, IgM and IgD, IgA or IgG. In addition, $CD27^-IgA^+$ and $CD27^-IgG^+$ memory B cells can be identified. Within both the $CD4^+$ and $CD8^+$ T-cell lineages, central memory ($CD45RO^+CCR7^+$), $CD45RO^+CCR7^-$ effector memory (TemRO) and $CD45RO^-CCR7^-$ (TemRA) can be distinguished from naive T cells ($CD45RO^-CCR7^+$).[12] Central memory T cells are most efficient in generating a new immune response by proliferating extensively in response to an antigen upon secondary antigen encounter.[13-15] The diversity and composition of the B-cell and T-cell compartments are highly dynamic in the first years of life; blood cell counts are especially high up to 2 years of age, following which they slowly decline to reach adult levels between 6 and 10 years.[16-20] At birth, nearly all B and T cells are naive, and memory cells are gradually built up in the first 6 years of life.[19]

Several studies have addressed the effects of breastfeeding on adaptive immunity. Breastfeeding was found to be associated with decreased frequencies of blood $CD4^+$ T cells.[21] This was mostly due to lower frequencies of naïve ($CD45RA^+$) T cells in breastfed children. Still, these observations were not consistently reproduced with some studies showing increased, and some decreased numbers of $CD4^+$ T cells.[21-24] Furthermore, long term breastfeeding was found to be associated with increasing numbers of $CD4^+$ and $CD8^+$ T cells.[24] Thus, although previous studies have addressed the influence of breastfeeding on blood lymphocyte populations,[21-24] the results remained inconclusive, mainly due to small samples sizes and limitations in the detection of memory cells.[21-24]

In the present study we used 6-color flow cytometric analysis of lymphocyte subsets in a population-based prospective cohort study to assess the impact of breastfeeding on build-up of memory B and T cells in infants and young children.

MATERIALS AND METHODS

Design and study population

This study was embedded in the Generation R Study, a prospective population-based cohort study that follows pregnant women and their children from fetal life onwards in the Netherlands.[25] The study has been approved by the Medical Ethics Committee of the Erasmus MC, University Medical Centre Rotterdam. Written informed consent was obtained from all parents of participants. We included 1,079 Dutch pregnant women and their children participating in a detailed subgroup study.[25] All children were born between February 2002 and August 2006. We excluded twins (n=27) in the present analysis to prevent bias due to correlation. Of these, data on both breastfeeding and immunophenotyping were available from 258 children at 6 months, 166 at 14 months, 112 at 25 months and 332 at 6 years of age. The main reasons for missing samples were due to non-consent of the parents (approximately 55% per visit) and technical or logistical failure (approximately 10% per visit).

Breastfeeding

Information regarding breastfeeding was obtained in postnatal questionnaires at the ages of 2, 6 and 12 months.[25] Mothers were asked whether they had ever breastfed their child (yes or no) and, if yes, at what age (months) they had stopped.[26,27] Breastfeeding duration was then categorized into four groups: never, $\leq$3 months, between 3 and 6 months and $\geq$6 months. An approximation of exclusive breastfeeding was performed according to whether the child received breastfeeding without any other bottle feeding, milk or solids.[27] Partial breastfeeding indicates infants receiving both breast-feeding, bottle feeding and/or solids in this period. Subsequently, the information on exclusiveness of breastfeeding was combined and categorized into the following breastfeeding categories: never; partial until 4 months and exclusive until 4 months.

Immunophenotyping of lymphocyte subsets

Flow cytometry was performed within 24 hours following sampling on fresh whole blood at the ages of 6 months (median 6.2; range 5.2; 8.2), 14 months (median 14.4; range 13.1-17.4), 25 months (median 25.2; range 23.3-29.8) and 6 years (median 5.9; range 5.1-7.2). Absolute counts of blood $CD3^+$ T cells, $CD16/56^+$ NK cells, and $CD19^+$ B cells were obtained with a diagnostic lyse-no-wash protocol. Lymphocytes were gated on the basis of CD45, FSC and SSC characteristics. Gates were set based on cells that are known to lack expression of the indicated marker. Additionally, 6-color flow cytometry was performed on an LSRII (BD Biosciences) to distinguish naive and memory B-cell and T-cell subsets as defined previously (Supplemental Table 3).[11,12] All flow cytometry acquisition was performed on whole blood after red blood cell lysis with ammonium chloride.

Covariates

The covariates that were assessed in this study were obtained from midwife and hospital registries at birth (birth weight, gestational age and gender) or through measurements at the research center (child anthropometrics). Additionally,

information on smoking and alcohol use during pregnancy and socio-economic status was obtained by prenatal questionnaires sent during the first, second and third trimesters of pregnancy.[28,29] Information on day-care attendance was obtained from parent-reported questionnaires at the ages of 6 and 12 months.

Statistical methods

Because the distribution of lymphocyte numbers in different age groups was skewed, these values were normalized by transformation to a natural log-scale. Differences in maternal and infant characteristics between breastfed versus never breastfed children were tested using independent samples t-tests and Chi-Square tests. Differences in baseline characteristics among the groups with different duration of breastfeeding were assessed using ANOVA tests and Chi-Square tests. Additionally, the associations of breastfeeding, breastfeeding duration (measured in groups, and measured continuously per month continuation of breastfeeding) and breastfeeding exclusivity with the change in lymphocyte numbers were assessed using multiple linear regression models with adjustment for potential confounders. For all ages, the category with no breastfeeding was the reference. First, associations between breastfeeding and total B, T, CD4 and CD8 counts were assessed. Subsequently, associations for B, $CD4^+$ and $CD8^+$ T-cell subpopulations were studied to assess the effect of breastfeeding on memory cells specifically. Multivariable regression models were created with stepwise adjustment for potential confounders, which were selected based on previous literature. Potential confounders included: maternal age, socio-economic status (SES), marital status, maternal BMI, maternal smoking and alcohol consumption during pregnancy, maternal reported autoimmune disease (including thyroid disease, multiple sclerosis, systemic lupus erythematosus, diabetes mellitus and arthritis), elevated anti-tTG level during pregnancy, maternal fever in the last trimester of pregnancy, family history of asthma or atopy (hay-fever, allergy, eczema), multiple parities, mode of delivery (caesarean section), gender, birth weight, gestational age, preterm birth, APGAR score, birth season, weight and age at focus visit, fever in the first 6 months (yes/no), frequency of upper and lower respiratory tract infections, and day-care attendance in the first year of life. Covariates were kept in the final multivariate model if the covariate resulted in an alteration in effect estimate of $\geq$ 10%,[30] or if the variables were associated with breastfeeding (determinant) and lymphocyte numbers (outcome) in our study. Because of the small numbers in the never breastfed group, final adjustment for potential confounders was restricted to those who attained the strongest alteration (%) in effect estimates. Because of the strong correlation between our outcomes (e.g. Pearson's correlation between total $CD8^+$ T cells and naive $CD8^+$ T cells r =0.82, and between total B cells and IgA r=0.57), and unweighted calculations only hold if the tests are independent,[31,32] we did not perform adjustments for multiple testing. All measures of associations are presented with their 95% confidence interval. All statistical analyses were performed using the Statistical Package for the Social Sciences version 20.0 for Windows (SPSS Inc, Chicago, IL, USA). P values <0.05 were considered to be statistically significant.

RESULTS

Population characteristics

No major differences in characteristics between children included at 6, 14, 25 months and 6 years of age were observed (Table 1, Supplemental Table 1) Overall, more than 86% of mothers started breastfeeding. Mother's educational level was significantly associated with the start of breastfeeding at 6 and 25 months, and 6

Table 1. Maternal and infant characteristics of the study population at 6 months

		Not breastfed (n=35)	Breastfed (n=223)
Maternal characteristics (n=258)			
Age (Mean ± SD; years)		32 (3.6)	32 (3.8)
Educational level (n; %)	Lower	24 (69%)	71 (32%)
	Higher	11 (31%)	152 (68%)*
Net household income per month (n; %)	< € 2400	2 (7%)	23 (11%)
	≥ € 2400	29 (93%)	178 (89%)
Smoking continued during pregnancy (n; %)		4 (14%)	21 (13%)
Alcohol use continued during pregnancy (n; %)		8 (29%)	55 (35%)
Body Mass Index before pregnancy (Mean ± SD; kg/m^2)		23 (3)	23 (4)
Fever in third trimester of pregnancy (n; %)		3 (9)	14 (6%)
Maternal atopy (eczema, allergy HDM, hay-fever)(n; %)		8 (25%)	72 (35%)
Paternal atopy (eczema, allergy HDM, hay-fever) (n; %)		8 (28%)	55 (28%)
Family history of asthma / atopy (n; %)		13 (37%)	107 (49%)
Any reported autoimmune disease (diabetes mellitus, SLE, arthritis, MS, thyroid disorder, or celiac disease) (n; %)		0 (0%)	5 (0.02%)
Mode of delivery (n; %)	Vaginal	21 (64%)	129 (61%)
	Forceps or vacuum assisted	5 (15%)	44 (20%)
	Caesarian section	7 (21%)	40 (19%)
Premature rupture of membranes (n; %)		3 (9%)	6 (3%)
Infant characteristics (n=258)			
Males (n; %)		19 (54%)	113 (51%)
Gestational age (Mean ± SD; weeks)		39.4 (2.4)	40.0 (1.7)
Preterm birth (<37 weeks) (n; %)		2 (6%)	11 (5%)
Birth weight (Mean ± SD; grams)		3439 (632)	3504 (524)
Apgar score at 5 min <7 (n; %)		0 (0%)	3 (1%)
Birth season (n; %)	Winter (dec-jan-feb)	5 (14%)	36 (16%)
	Spring (mar-apr-may)	10 (29%)	81 (36%)
	Summer (jun-jul-aug)	11 (31%)	62 (28%)
	Autumn (sept-oct-nov)	9 (26%)	44 (20%)
Siblings ≥1 (n; %)		4 (11%)	26 (12%)
Day-care >16 hours /week (n; %)		5 (33%)	77 (46%)
Fever in first 6 months (n; %)		12 (60%)	121 (62%)
Age at focus visit (Median ± range; months)		6.6 (6.2-8.2)	6.2 (5.2-7.9)*

Values are means (SD), absolute numbers (percentages) or #medians (90% range). *Significantly different between groups.
Data were missing on: Household income (n=26) Smoking during pregnancy (*n*=72), Alcohol during pregnancy (*n*=72) BMI before pregnancy (n=37), Fever in third trimester (n=9) Mode of delivery (n=12) Maternal atopy (n=22) Paternal atopy (n=29), Family history of asthma or atopy (n=3) Mode of delivery (n=12), maternal reported autoimmune disease (n=29), maternal reported any other chronic condition (n=31), premature rupture of membranes (n=5), Apgar (n=4), Day-care (n=42), weight at focus visit (n=1), fever in first 6 months (n=42). Any reported autoimmune disease included thyroid disease (n=4) and elevated anti-tTG level during pregnancy (n=1).

years of age. Moreover, mother's educational level was significantly associated with both the duration of breastfeeding and with B-cell memory subsets at 6 months of age [data not shown]. In addition, maternal alcohol use was related to both breastfeeding duration and total T, B, CD4$^+$ and CD8$^+$ cell numbers at 6 months [data not shown]. Both maternal educational level and alcohol use influenced the regression coefficients by more than 10%. Therefore, all subsequent analysis on breastfeeding duration and cell numbers were adjusted for both maternal education and maternal alcohol use during pregnancy.

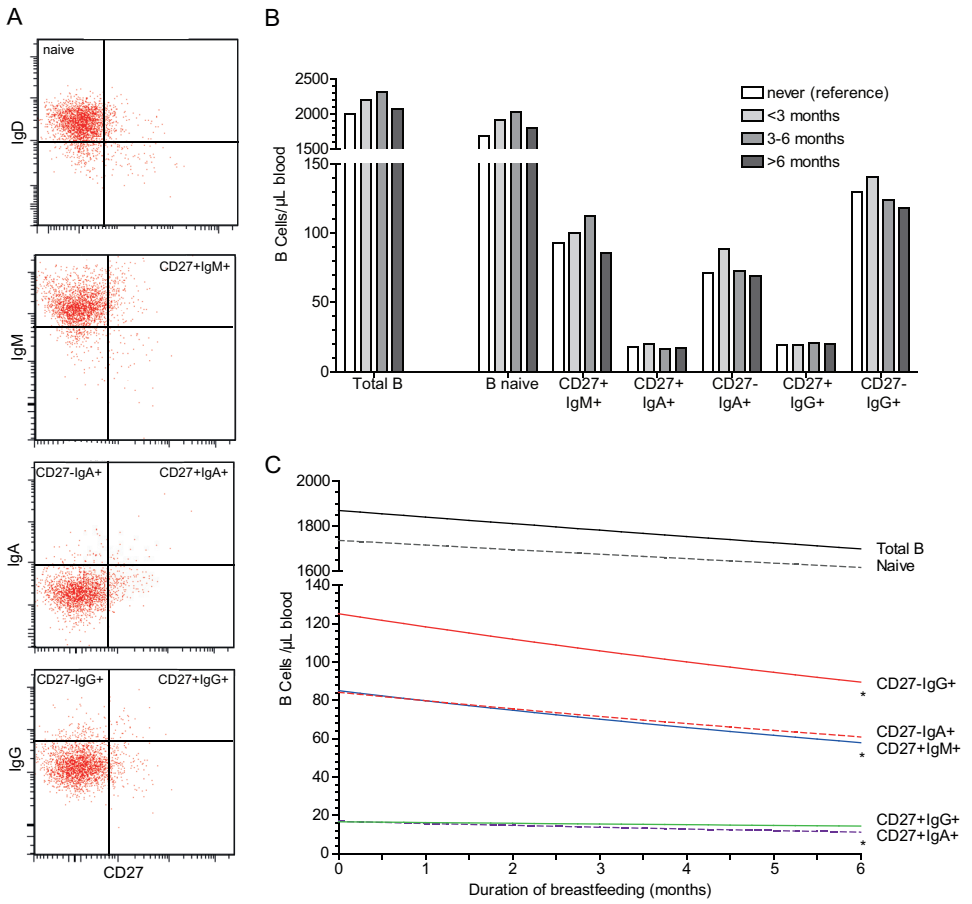

Figure 1. Impact of breastfeeding duration on B lymphocyte subsets at 6 months of age.
A) Gating strategy for dissection of CD19$^+$ B cells into 1 naive and 5 memory B-cell subsets by flow cytometry. The reference plots depict density plots of total lymphocytes and were used to set the gates accordingly. **B)** Backtransformed B-cell counts (cells/µL) at 6 months of age according to different breastfeeding duration categories (reference category is never). Categories of breastfed children contain both partial and exclusively breasted children. **C)** The estimated backtransformed regression line reflects B-cell counts (cells/µL) at 6 months of age, per month increase in breastfeeding duration.*, P<0.05.

Decrease in B-cell memory

Breastfeeding exposure (Figure 1B) and duration (Figure 1C) were not associated with total B-cell numbers at 6, 14, 25 months and 6 years of age (Table 2, Supplemental Table 2). Furthermore, no associations were observed between

breastfeeding exposure and duration and naive B cells, which constitute the majority of total B-cell numbers (Table 3, Figure 1B and 1C) However, a longer duration of breastfeeding, was associated with changes in the memory B-cell compartment. Per month longer breastfeeding, a 3% decrease in absolute numbers of $CD27^{+}IgM^{+}$, and a 2% decrease in both $CD27^{+}IgA^{+}$ and $CD27^{-}IgG^{+}$ memory B cells at 6 months of age were observed (Table 3, Figure 1C). $CD27^{+}IgG^{+}$ and $CD27^{-}IgA^{+}$ memory B cells at 6 months of age did not change with differences in breastfeeding duration. Stronger negative trends were observed for the associations between breastfeeding duration and frequencies of $CD27^{+}IgM^{+}$,

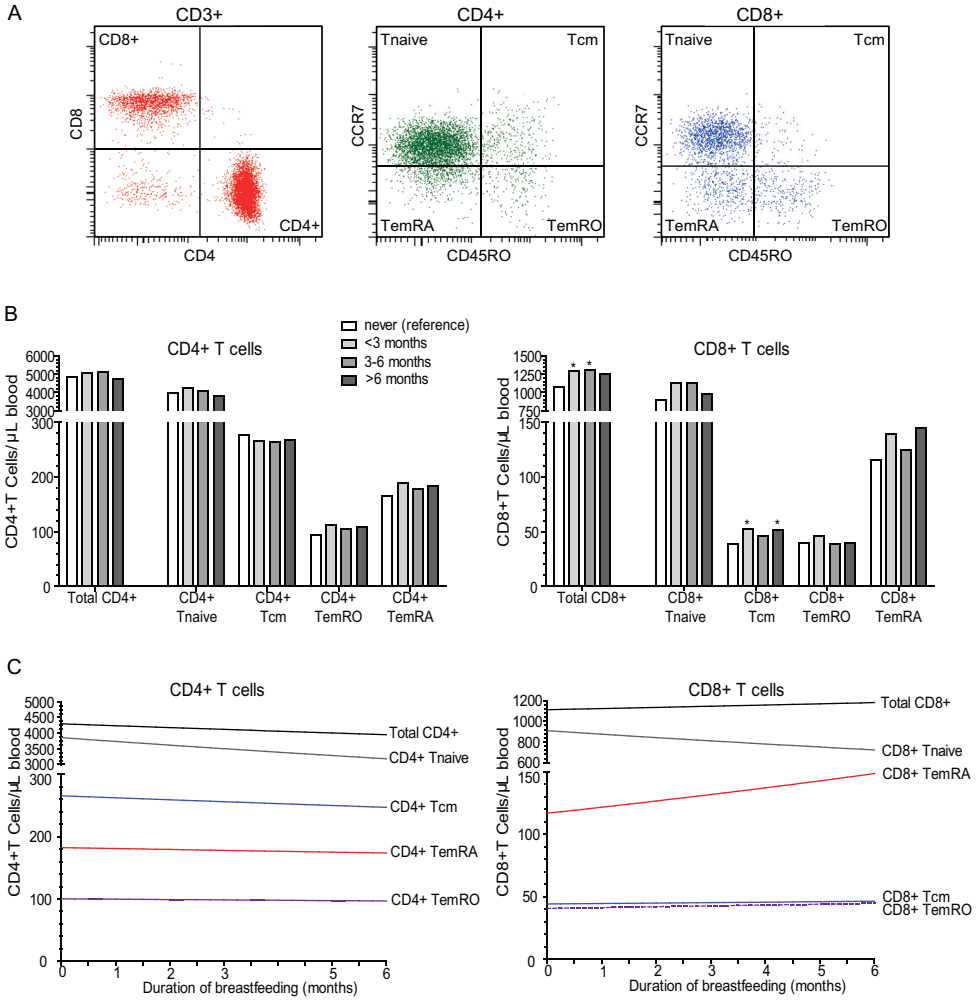

Figure 2. Impact of breastfeeding duration on T-lymphocyte subsets at 6 months of age.
A) Gating strategy for T-cell subset delineation. $CD4^{+}$ and $CD8^{+}$ T-cell subsets were defined within total $CD3^{+}$ T cells, and subsequently dissected using CD45RO and CCR7 into naive, central memory, $CD45RO^{+}$ effector memory (TemRO) and $CD45RO^{-}$ effector memory (TemRA) T cells. The reference depict density plots of total lymphocytes and were used to set the gates accordingly. **B)** Backtransformed CD4 and CD8 cell counts (cells/µL) at 6 months of age according to different breastfeeding duration categories (reference category is never). Categories of breastfed children contain both partial and exclusively breasted children. **C)** The estimated backtransformed regression line reflects CD4 and CD8 cell counts (cells/µL) at 6 months of age, per month increase in breastfeeding duration. *, $P<0.05$.

Table 2. Adjusted associations between breastfeeding and cell numbers at 6 and 14 months

	Regression coefficients for logtransformed cell numbers (*10e9/L)											
	6 months (n=258)						14 months (n=166)					
	n	B	NK	T	CD4	CD8	n	B	NK	T	CD4	CD8
DURATION												
Breastfeeding increase/month	223	-1 (-3,1)	0 (-2,2)	-1 (-2,1)	1 (-2,1)	1 (-1,2)	150	-2 (-4,0)	0 (-2,2)	0 (-2,1)	0 (-2,1)	0 (-2,2)
EXPOSURE												
Never	35	REF	REF	REF	REF	REF	16	REF	REF	REF	REF	REF
< 3 months	86	9 (-8,25)	4 (-16,24)	7 (-5,19)	6 (-7,19)	**19 (3,35)***	55	15 (10,40)	21 (-5,47)	16 (-3,35)	13 (-6,32)	19 (-5,44)
≥ 3 < 6 months	64	6 (-12,23)	0 (-21,22)	8 (-5,21)	7 (-7,22)	**20 (3,37)***	40	26 (0,52)	-2 (-28,25)	20 (0,39)	18 (-2,38)	25 (0,50)
≥ 6 months	73	0 (-18,18)	9 (-13,31)	2 (-11,16)	0 (-14,15)	16 (-2,33)	55	2 (-22,27)	9 (-16,34)	13 (-5,31)	13 (-6,31)	13 (-11,36)

Values are log transformed regression coefficients (95% confidence interval of cell numbers (*10^{e9}/L)) derived from linear regression models and reflect the increase or decrease (%) in log transformed cell numbers per month continuation of breastfeeding.(duration), and the increase or decrease (%) in log transformed cell numbers of groups exposed to breastfeeding relative to the reference group (never breastfeeding). Breastfed groups include both partially and exclusively breastfed children. *P-value <0.05.Adjusted for maternal education and maternal alcohol use during pregnancy.

Table 3. Adjusted associations between breastfeeding and memory B-cell and T-cell subsets at 6 months

	Regression coefficients for logtransformed cell numbers (*10e9/L)													
	B (n=258)						T CD4+ (n=258)				T CD8+ (n=258)			
	Naive B cells	CD27+ IgM+	CD27+ IgA+	CD27- IgA+	CD27+ IgG+	CD27- IgG+	CD4+ T naive	CD4+ T cm	CD4+ TemRO	CD4+ TemRA	CD8+ T naive	CD8+ T cm	CD8+ TemRO	CD8+ TemRA
DURATION														
Breastfeeding increase/ month (n=223)	-1 (-3,1)	**-3 (-6,-1)***	**-2 (-5,-1)***	-2 (-5,1)	1 (-3,2)	**-2 (-4,-1)***	-2 (-3,0)	-1 (-2,1)	-1 (-3,2)	0 (-3,2)	-2 (-4,1)	0 (-2,3)	0 (-3,3)	2 (-1,5)
EXPOSURE														
Never (n= 35)	REF	REF	REF	REF	REF	REF	REF	REF	REF	REF	REF	REF	REF	REF
< 3 months (n= 86)	12 (-6,31)	9 (-19,36)	1 (-28,30)	15 (-14,43)	-9 (-34,16)	-1 (-22,22)	7 (-10,24)	-3 (-20,14)	17 (-4,38)	14 (-8,37)	23 (0,46)	**30 (4,57)***	15 (-18,47)	20 (-8,47)
≥ 3 < 6 months (n= 64)	18 (-1,38)	18 (-11,48)	1 (-30,32)	14 (-17,44)	0 (-26,27)	1 (-22,25)	4 (-14,22)	-5 (-23,13)	9 (-14,31)	8 (-16,32)	23 (-2,448)	17 (-11,45)	-4 (-38,31)	9 (-20,39)
≥ 6 months (n= 73)	6 (-13,26)	-4 (-34,25)	-11 (-40,19)	-2 (-31,28)	-7 (-33,19)	-13 (-36,10)	-5 (-23,14)	-3 (-21,15)	13 (-10,35)	10 (-14,34)	10 (-15,34)	**29 (1,57)***	-5 (-39,30)	21 (-9,51)

Values are log transformed regression coefficients (95% confidence interval of cell numbers (*10^{e9}/L)) derived from linear regression models and reflect the increase or decrease (%) in log transformed cell numbers per month continuation of breastfeeding.(duration), and the increase or decrease (%) in log transformed cell numbers of groups exposed to breastfeeding relative to the reference group (never breastfeeding). Breastfed groups include both partially and exclusively breastfed children. *P-value <0.05.Adjusted for maternal education and maternal alcohol use during pregnancy.

$CD27^+IgA^+$ and $CD27^-IgG^+$ memory B cells within total B cells at 6 months of age, resp. -4% (95% CI -7,-1), -4% (95% CI -7, -1), and -3% (95% CI -5,-1) (data not shown). At 14 months of age, similar trends for breastfeeding duration and total B-cell numbers were observed, although not significant (Table 2). At older ages the effects disappeared. (Supplemental Table 2). Thus, a longer breastfeeding duration seems to negatively impact B-cell numbers in infants at least until the age of 6 months, at older ages these effects disappeared.

Table 4. Unadjusted associations between breastfeeding exclusivity and $CD8^+$ T cell numbers at 6 months

Regression coefficients for logtransformed cell numbers (*10e9/L); 6 months (n=258)					
	CD8 Total	**CD8 Tnaive**	**CD8 Tcm**	**CD8 TemRA**	**CD8 TemRO**
EXCLUSIVENESS					
Never (n= 35)	REF	REF	REF	REF	REF
Partial until 4 months (n=157)	13 (-3,29)	9 (-11,29)	17 (-7,41)	-5 (-30,21)	3 (-27,32)
Exclusive until 4 months (n=80)	14 (-3,31)	3 (-19,25)	**33 (7,59)***	12 (-16,40)	28 (-3,59)

Unadjusted values are log transformed regression coefficients (95% confidence interval of cell numbers ($*10^{e9}$/L)) derived from linear regression models and reflect the increase or decrease (%) in log transformed cell numbers of partial and exclusive breastfed groups relative to the reference group (never breastfeeding). Partial breastfeeding indicates infants receiving both breast-feeding, bottle feeding and/or solids in this period.*P-value <0.05.

Increase in T-cell memory

Breastfeeding exposure and duration were not associated with total T-cell and $CD4^+$ T-cell numbers at 6, 14, 25 months and 6 years of age (Table 2, Figures 2B and 2C, and Supplemental Table 2). However, the exposure to breastfeeding was associated with $CD8^+$ T cell numbers. $CD8^+$ T-cell numbers were 19% (95% CI 3, 35) higher in 6-month old infants who were breastfed for less than 3 months and remained 20% (95% CI 3, 37) higher in children who were breastfed until 6 months (Table 2, Figure 2B), than in children who were never breastfed. Comparable effect sizes were observed for exclusiveness of breastfeeding in relation to $CD8^+$ T cells (Table 4 and 5). Partial breastfeeding until 4 months was associated with a 20% increase in total CD8 T cells, and remained 21% higher in infants who were exclusively breastfed (Table 5). At 14 and 25 months of age, similar tendencies for breastfeeding exposure and $CD8^+$ T cell numbers were observed, however the associations were not significant. (Table 2, Supplemental Table 2).

Within the $CD8^+$ T-cell compartment at 6 months of age, central memory cell numbers ($CD45RO^+CCR7^+$) were 30% (95% CI 4, 57) higher in children who were breastfed until 3 months than in non-breastfed children (Table 3, Figure 2B). This effect was irrespective of duration; central memory cell numbers were still 29% (95% CI 1, 57) higher in infants who were breastfed for 6 months or longer than in infants who were never breastfed (Table 3, Figure 2B). In line with this, no significant associations were found between the duration of breastfeeding and $CD8^+$ central memory numbers (Table 3, Figure 2C).

Thus, $CD8^+$ central memory T cells were associated with breastfeeding exposure, but not with the duration of breastfeeding.

Table 5. Adjusted associations between breastfeeding exclusivity and CD8$^+$ T cell numbers at 6 months

Regression coefficients for logtransformed cell numbers (*10e9/L); 6 months (n=258)					
	CD8 Total	**CD8 Tnaive**	**CD8 Tcm**	**CD8 TemRA**	**CD8 TemRO**
EXCLUSIVENESS					
Never (n= 35)	REF	REF	REF	REF	REF
Partial until 4 months (n=143)	**20 (3,38)***	20 (-5,44)	12 (-17,40)	18 (-11,47)	4 (-31,39)
Exclusive until 4 months (n=80)	**21 (2,40)***	14 (-14,41)	16 (-15,48)	27 (-5,59)	12 (-28,51)

Values are log transformed regression coefficients (95% confidence interval of cell numbers (*10^{e9}/L)) derived from linear regression models and reflect the increase or decrease (%) in log transformed cell numbers of partial and exclusive breastfed groups relative to the reference group (never breastfeeding). Partial breastfeeding indicates infants receiving both breast-feeding, bottle feeding and/or solids in this period.*P-value <0.05. Adjusted for maternal education and maternal alcohol use during pregnancy.

DISCUSSION

Overall we found that breastfeeding was associated with a decrease in B-cell memory and an increase in CD8 T-cell memory at 6 months of age. The same trend was observed at 14 months of age, and disappeared at older ages. The decrease in B-cell memory was associated with the duration of breastfeeding, while the increase in total CD8$^+$ T cells and central memory CD8$^+$ T cells did not depend on the duration of breastfeeding. This suggests that breastfeeding enhances T-cell maturation, but not B-cell maturation.

Total B-cell numbers nor frequencies were significantly related with breastfeeding. This is in line with previous studies.[21,23] Only one study reported higher frequencies of total B cells in breastfed children at 6 months of age, however sample sizes were small (n=7 breastfed infants).[22] We found that the duration of breastfeeding was associated with decreased numbers of CD27$^+$IgA$^+$, CD27$^+$IgM$^+$ and CD27$^-$IgG$^+$ memory B cells, which are mostly derived from systemic T-cell dependent responses.[11] This suggests that continuous breastfeeding inhibits memory B-cell development. One of the candidate factors in breast milk that is likely to mediate this, is secretory IgA (sIgA).[33,34] Continuous breastfeeding will provide a constant supply of maternal sIgA onto the epithelial surface of the infant's gastrointestinal tract. This sIgA might prevent exposure of microorganisms to the infant's humoral immune system, and translocation of gut bacteria.[34,35] Thus, fewer naive B cells might be activated to differentiate into memory B cells (Supplemental Figure 1). Indeed, in suckling mice it has been suggested that maternal sIgA blocks mucosal B cell responses in the offspring.[36,37] Alternatively, the unchanged numbers of T-cell independent CD27$^-$IgA$^+$ B cells suggest that breastfeeding does not affect local IgA responses. It is therefore possible that maternal IgA helps to block translocation of intestinal bacteria, thereby only preventing systemic T-cell dependent B-cell memory formation. Other growth factors in breast milk that can reduce exposure of microbiota to B cells are epidermal growth factor, IGF-1, TGF-β, leptin and prolactin. These factors enhance maturation of the epithelial barrier,[33,38] and decrease uptake of foreign protein antigens. Factors such as lactoferrin, oligosaccharides and lipids may directly prevent attachment of the bacterial outer membrane to the mucosal surface.[33] Finally, the passive transfer of functional Ig-secreting plasma cells in breast milk may prevent bacterial or viral transmission.[39,40]

Total T-cell numbers nor CD4+ T-cell numbers were associated with breastfeeding exposure or breastfeeding duration. However, infants who were breastfed until 3 months had higher CD8+ T-cell numbers than infants who were never breastfed, and this change persisted when breastfeeding was prolonged until 6 months, suggesting an ongoing activation of CD8+ T cells by exposure to breast milk, but no accumulation over time. These results extend previous observations in small studies (n<40) of increased frequencies of CD8+ T cells or decreased CD4/CD8 ratios.[21,22,24] In addition, an increase in CD8+ T cells from 8 to 10 months of age was observed before,[24] suggesting a longer-lasting effect of breastfeeding. However, the children received breastfeeding until 8 months of age.[24] In addition, the number of children in the breastfeeding group was small (n=35), and no detailed analysis of CD8+ subsets was performed. Therefore, future studies will be needed to validate these findings.

Within total CD8+ T cells, the central memory subset was most significantly increased in breastfed children. This expansion will be the result of stimulation of mature T cells. Candidate immune stimulatory factors in breast milk are lactoferrin and exosomes. Lactoferrin has direct microbicidal properties, including binding of the bacterial cell wall and the concomitant release of lipopolysaccharide (LPS, endotoxin). Moreover, lactoferrin is known to enhance T-cell proliferation.[21,33] Exosomes are carrier vesicles of 50-100 nm that can bud from the membrane of eukaryotic cells.[41] Exosomes formed by B cells or dendritic cells contain MHC class I and class II, and have the potential to stimulate T cells.[42,43] Human breast milk has been found to contain immune modulatory exosomes that express MHC molecules, IL-2, IFNγ and TNFα. In line with our findings that breastfeeding did not affect memory CD4+ T cells, these vesicles did not appear to stimulate CD4+ T cells.[44] It is, however, likely that immunostimulatory compounds such as exosomes, do have CD8+ T-cell stimulating capacities and contribute to the increase in central memory T cells in breastfed infants (Supplemental Figure 1). In addition, it has been described that the infants take up live and functional maternal immune cells from the breast milk.[45-47] This might result in stimulation of CD8+ T cells. Thus, it remains unclear whether the increase in central memory CD8+ T cells can be attributed to immunostimulatory factors in breast milk, such as lactoferrin and exosomes, or reflects a role for maternal immune cells. Nevertheless, central memory T cells might mediate reactive immunity,[48] because they circulate between the spleen, blood and lymph nodes and proliferate extensively in response to a second encounter of an antigen.[13-15] Therefore, it could be hypothesized that an increase in central memory T cells is associated with increased reactive immunity.

Beneficial effects of breastfeeding on adaptive immune responses have previously been demonstrated in vaccination studies.[7-9] Breasted children showed increased interferon-γ production, and increased frequencies of CD8+ T cells after vaccination with measles, mumps and rubella.[9] Furthermore, breastfeeding had beneficial effects on virus-specific immune responses to poliovirus, diphtheria toxoid and tetanus toxoid,[49] whereas the responses to rotavirus are not clearly enhanced.[50,51] These studies included relatively few children, and confounding factors could not be taken into account. Thus, our results extend these previous observations from vaccination studies. Unfortunately, we were not able to study virus-specific memory cells and studies determining associations between central memory cell numbers and functional immunity are lacking. Thus, interpretations regarding cell-mediated immunity should be made with caution.

We did not observe associations between breastfeeding and naive T cells or effector memory T cells. In contrast, a previous study reported lower frequencies of naive $CD4^+$ T cells,[21] but no differences in memory T-cell frequencies. Therefore, it was suggested that the adaptive immune system develops slower in breastfed infants.[21] However, the study reported on a relatively small number of children in the breastfeeding group (n=34), and lacked data on absolute B-cell and T-cell counts.[21]

Our study was conducted in a large population-based prospective birth cohort. Previous studies that addressed the effects of breastfeeding on adaptive immunity, had smaller sample size and/or lacked follow-up.[21-24] Most of these retrospective studies were based on recall of infant feeding after several years, making recall bias of feeding habits an important concern.[52] Because of the prospective design of our study, detailed information on the duration of breastfeeding was collected at multiple time points shortly after breastfeeding was finished, thereby limiting potential recall bias.[52] Still, data on breastfeeding was collected retrospectively, and due to the use of questionnaires, misclassification may occur.[52] Because any misclassification would be independent of laboratory determined T-cell and B-cell numbers, it is unlikely that these affected the outcome. In addition, our study design provided information on a large number of potential confounders. We used an unbiased approach, investigating a broad panel of determinants on lymphocyte numbers and frequencies. Moreover, we performed measurements of lymphocytes at different ages, enabling us to study the effect of breastfeeding on adaptive immunity over a longer period of time. Finally, using detailed 6-color flow cytometry, we were able to discriminate multiple, functionally distinct B-cell and T-cell subsets.[11,12,53] Thus, we could evaluate the effects of breastfeeding on specific aspects of adaptive immunity.

At 14 months or age, we observed the same trends of decreasing B-cell numbers with longer breastfeeding duration, and higher CD8 T-cell numbers with breastfeeding exposure, as we did for 6 months, although effects were not significant. The sample size at 14 months was smaller than the sample size at 6 months. Hence, we cannot exclude that non-significant effects at 14 months are due to loss of statistical power, and some effects of breastfeeding remain at this age.

Our measurements were limited to peripheral blood. Thus, it is not possible to deduce whether B-cell and T-cell numbers in lymphoid tissue were affected. Theoretically, preferential homing to or away from tissue could result in changes in blood lymphocyte counts.[54] If breastfeeding affects this preferential homing, the effects will be lost once breastfeeding is discontinued. Nearly all children were no longer receiving breast milk and still showed lower memory B-cell numbers and higher $CD8^+$ T-cell numbers. Thus, we conclude that the effects of breastfeeding on preferential tissue homing was limited.

In conclusion, this prospective population-based cohort study among a large number of healthy children showed that $CD27^+IgA^+$, $CD27^+IgM^+$ and $CD27^-IgG^+$ memory B-cell numbers decreased with a longer breastfeeding duration. $CD8^+$ T cells, and especially $CD8^+$ central memory T-cell numbers, were higher in breastfed children up to 6 months of age. This suggests that breastfeeding enhances T-cell maturation in the first 6 months of life. On top of the protective effects of maternal IgA in breast milk, this might contribute to the protective effect against infectious diseases in infancy.

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Supplemental Table 1. Maternal and infant characteristics of the study population at 14, 25 months and 6 years

	14 months (n=166)		25 months (n=112)		6 years (n=332)	
	Not breastfed (n=16)	Breastfed (n=150)	Not breastfed (n=16)	Breastfed (n=96)	Not breastfed (n=37)	Breastfed (n=295)
Maternal characteristics						
Age (Mean ± SD; years)	31 (4.2)	32 (3.9)	31 (4.1)	32 (3.8)	31 (3.6)	32 (3.6)
Educational level (n; %)						
Lower	**7 (44%)**	**59 (40%)**	**12 (75%)**	**31 (33%)**	**22 (59%)**	**96 (33%)**
Higher	**9 (56%)**	**90 (60%)**	**4 (25%)**	**64 (67%)***	**15 (41%)**	**196 (67%)***
Net household income/month (n; %)						
< € 2400	0 (0%)	21 (15%)	1 (8%)	13 (15%)	4 (12%)	33 (13%)
≥ € 2400	13 (100%)	112 (84%)	12 (92%)	73 (85%)	30 (88%)	227 (87%)
Smoking during pregnancy (n; %)	2 (14%)	16 (15%)	1 (7%)	8 (11%)	6 (20%)	32 (15%)
Alcohol use during pregnancy (n; %)	6 (43%)	41 (38%)	4 (29%)	29 (39%)	11 (37%)	83 (37%)
Body Mass Index before pregnancy (Mean, SD)	25 (5)	24 (5)	25 (5)	23 (4)	24 (5)	24 (4)
Fever in third trimester of pregnancy (n; %)	1(6%)	9 (6%)	2 (13%)	5 (6%)	2 (6%)	19 (7%)
atopy (eczema, allergy HDM, hay-fever) (n; %)						
Maternal	3 (21%)	51 (36%)	6 (40%)	41 (46%)	9 (27%)	109 (40%)
Paternal	3 (23%)	41 (29%)	5 (39%)	24 (28%)	13 (39%)	77 (29%)
Family history of asthma / atopy (n; %)	5 (31%)	77 (52%)	9 (56%)	56 (59%)	20 (54%)	156 (54%)
Any reported autoimmune disease (diabetes mellitus, SLE, arthritis, MS, thyroid disorder, or celiac disease) (n; %)	0 (0%)	3 (2%)	0 (0%)	2 (2%)	2 (5%)	8 (3%)
Mode of delivery (n; %)						
Vaginal	9 (56%)	19 (64%)	7 (47%)	48 (55%)	20 (59%)	182 (65%)
Forceps or vacuum assisted	2 (13%)	30 (21%)	2 (13%)	21 (24%)	5 (15%)	59 (21%)
Caesarian section	5 (31%)	21 (15%)	6 (40%)	19 (22%)	9 (26%)	40 (14%)
Premature rupture of membranes (n; %)	1 (6%)	2 (1%)	1 (6%)	2 (2%)	3 (8%)	4 (2%)
Infant characteristics (n=258)						
Males (n; %)	9 (56%)	77 (51%)	7 (44%)	47 (49%)	20 (54%)	144 (49%)
Gestational age (Mean ± SD; weeks)	39.2 (2.2)	40.1 (1.9)	39.7 (1.9)	39.9(1.5)	39.7 (1.7)	40.1 (1.6)
Preterm birth (<37 weeks) (n; %)	1 (6%)	3 (2%)	1 (6%)	4 (4%)	2 (5%)	8 (3%)
Birth weight (Mean ± SD; grams)	3,455 (738)	3,505 (513)	3,516 (614)	3,542 (480)	3,543 (554)	3,531 (508)
Apgar score at 5 min <7 (n; %)	0 (0%)	3 (2%)	0 (0%)	0 (0%)	0 (0%)	3 (1%)
Birth season (n; %)						
Winter	2 (13%)	38 (25%)	3 (19%)	28 (29%)	6 (16%)	73 (25%)
Spring	5 (31%)	41 (27%)	3 (19%)	26 (27%)	6 (16%)	93 (31%)
Summer	3 (18%)	32 (21%)	4 (25%)	21 (22%)	11 (30%)	65 (22%)
Autumn	6 (38%)	39 (26)	6 (37%)	21 (22%)	14 (38%)	64 (22%)
Siblings ≥1 (n; %)	2 (12%)	17 (11%)	1 (6%)	8 (8%)	2 (5%)	26 (9%)
Day-care >16 hours /week (n; %)	3 (33%)	51 (47%)	4 (25%)	33 (34%)	5 (33%)	99 (47%)
Fever in first 6 months (n; %)	7 (78%)	80 (63%)	3 (60%)	51 (62%)	13 (57%)	175 (67%)
Age at focus visit (Median ± range; months/years)	14.4 (13.2-16.3)	14.4 (13.1-17.4)	25.3 (23.4-27.5)	25.2 (23.3-29.8)	6.0 (5.8-6.6)	5.8 (5.1-7.2)

Values are means (SD), absolute numbers (percentages) or #medians (90% range). * Significantly different between breastfeeding and no breastfeeding groups

Supplemental Table 2. Adjusted association between breastfeeding and cell numbers at 25 months and 6 years

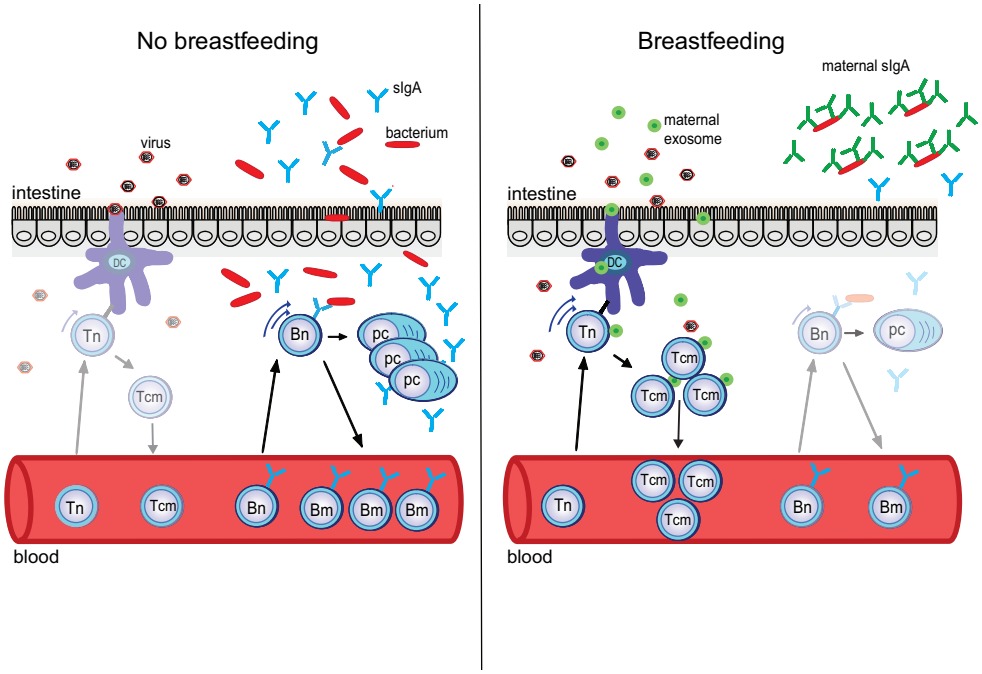
	Logtransformed cell numbers (*10e9/L)									
	25 months (n=112)						6 years (n=332)			
	n	B	NK	T	CD4	CD8	n	B	NK	T
DURATION										
Breastfeeding increase/month	96	-1 (-4,1)	0 (-3,3)	0 (-2,2)	1 (-2,3)	-2 (-4,1)	295	-1(-2,1)	0(-2,1)	1(-1,2)
EXPOSURE										
Never	16	REF	REF	REF	REF	REF	37	REF	REF	REF
< 3 months	29	14 (-10,38)	27 (-3, 58)	14 (-7,35)	11 (-10,32)	18 (-9,45)	112	-6(-19,8)	11(-5,26)	1(-10,12)
≥ 3 < 6 months	30	0 (-23,23)	7 (-23, 37)	-2 (-22,18)	-4 (-24,17)	1 (-25,27)	76	-5(-19,10)	7(-10,24)	2(-10,14)
≥ 6 months	37	11 (-14,35)	11 (-20,42)	12 (-9,33)	18 (-3,30)	-3 (-30, 24)	107	-12(-26,3)	-1(-17,16)	3(-8,15)
Never	15	REF	REF	REF	REF	REF	52	REF	REF	REF
≤ 6 months	50	-17 (-35, 2)	3 (-22,27)	-7 (-23,10)	-11 (-28,6)	3 (-18,25)	150	3(-8,13)	6(-6,18)	1(-8,10)
6-9 months	18	-4 (-29,21)	1 (-33,34)	-2 (-24,21)	0 (-23,23)	-2 (-31,28)	50	5(-10,20)	2(-15,19)	1(-11,13)
≥ 9 months	15	3 (-26, 32)	-17 (-55, 21)	9 (-17,35)	10 (-17,36)	8 (-26,41)	34	-3(-20,14	-6(-25,14)	1(-13,15)

Values are log transformed regression coefficients (95% confidence interval of cell numbers ($*10^{e9}$/L)) derived from linear regression models and reflect the increase or decrease (%) in log transformed cell numbers per months continuation of breastfeeding (duration), and the increase or decrease (%) in log transformed cell numbers of groups exposed to breastfeeding relative to the reference group (never breastfeeding). Missing in in categories 0-6-9 months (n=14 at 25 months, n=46 at 6 years) Breastfed groups include both partially and exclusively breastfed children. Adjusted for maternal education and maternal alcohol use during pregnancy. *P-value <0.05.

Supplemental Table 3. Antibody panel used for 6-color flow cytometry

Labeling	Conjugated monoclonal antibodies (clone)					
	FITC	**PE**	**PerCP-Cy5.5**	**PE-Cy7**	**APC**	**APC-Cy7**
1	CD3 (SK7)	CD16.56 (B73.1C5.9)	CD45 (2D1)	CD4 (SK3)	CD19 (SJ25C1)	CD8 (SK1)
2	IgD (poly)	CD23 (EBVCS5)	CD19 (SJ25C1)	CD21 (B-ly-4)	IgM (G20-127)	CD27 (M-T271)
3	IgA (poly)	IgG (poly)	CD19 (SJ25C1)	CD40 (5C3)	IgM (G20-127)	CD27 (M-T271)
4	CD28 (CD28.2)	CD197 (3D13)	CD3 (SK7)	CD8 (SK1)	CD45RO (UCHL-1)	CD27 (M-T271)

FITC= fluorescein isothiocyanate, PE=phycoerythrin, PerCPCy5.5= peridin chlorophyll protein, PE-Cy7= phycoerythrin-cyanin dye, APC=allophycocyanin and APC-Cy7= allophycocyanin-cyanin dye, poly = polyclonal antibody



Supplemental Figure 1. Summarizing mechanism of how breastfeeding might affect adaptive memory. In absence of breast milk, the infant's B and T cells respond to microorganisms in the intestine and generate long-lived memory cells and IgA (blue) that circulate through the body (left). Breast milk contains immune modulating components (right). Of these, maternal sIgA (green) is able to catch microorganisms and prevent recognition of these by B-cells. This might inhibit B-cell responses and B-cell memory formation. Other immunostimulatory components, such as exosomes, might stimulate naive T cells and increase T-cell memory formation. Abbreviations: Bn, naïve B cell; Bm, memory B cell; DC, dendritic cell; pc,plasma cell; Tn, naive T cell; Tm, memory T cell.

Chapter 4

CAN WE EXPLAIN ETHNIC DIFFERENCES IN CMV, EBV AND HSV-1 VIRUS SEROPREVALENCES IN CHILDHOOD? THE GENERATION R STUDY

Running title: Determinants of herpesviridae

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Submitted

ABSTRACT

Objective. To identify whether there are ethnic differences in CMV, EBV and HSV-1 seroprevalence rates in children at 6 years of age, and when present, to evaluate how these differences can be explained by socio-demographic and environmental factors.

Methods. This study was embedded within a multi-ethnic population-based prospective cohort study. Serum IgG levels against CMV, EBV and HSV-1 were measured by ELISA in 4,464 children (median age 6.0 years). Information on demographics and characteristics were assessed by questionnaires. Herpesvirus seroprevalences between Surinamese-Creole, Surinamese-Hindustani, Turkish, Moroccan, Cape Verdean and Antillean and Native Dutch children were compared.

Results. Non-western ethnicity was an independent risk factor for CMV (aOR, 2.16; 95% CI 1.81;2.57), EBV (aOR 1.76; 95% CI 1.48;2.09) and HSV-1 seropositivity (1.52; 1.39;1.66). Among the ethnic groups, CMV seroprevalences ranged between 29-65%, EBV between 43-69% and HSV-1 between 13-39%. Socio-economic position, crowding and lifestyle factors explained up to 48% of the ethnic differences in HSV-1 seroprevalences, and up to 39% of the ethnic differences in EBV seroprevalences. These factors did not explain ethnic differences in CMV seroprevalences.

Conclusion. Socio-economic position and factors related to lifestyle only explain a part of the large ethnic differences in EBV and HSV-1 seroprevalences, whereas they do not explain ethnic differences in CMV seroprevalences in childhood.

INTRODUCTION

Cytomegalovirus (CMV), Epstein Barr virus (EBV), and herpes simplex virus 1 (HSV-1) are ubiquitous herpesviruses, and often acquired in childhood. They are usually transmitted by contact with infected saliva (all three viruses), breastfeeding, placental transfer (CMV), {Longnecker, 2007 #3882;Boppana, 2007 #3880}or through contact with infectious skin lesions or secretions (HSV-1).[1,2] The viruses establish a lifelong latency after primary infection, and can periodically reactivate with shedding of the virus. Most primary infections are mild or asymptomatic in childhood, but they can cause serious complications in fetuses, immunocompromised individuals, or in elderly.[3-9] The incidence and severity of these herpesvirus associated diseases vary geographically, and many studies link age at primary infection to the pathogenesis of herpesvirus associated diseases.[10-14]

CMV seroprevalences vary between 40-60% in children aged 4-12 years,[8,15,16] with exclusion of African countries where almost all children have already been seroconverted by 3 years of age. Approximately 50% of children in the US and Europe are seropositive for EBV in the first years of life, but seroprevalences vary from 20-80% depending on age, race/ethnicity, geographic location and socio-economic development of the country.[9,12,17,18] HSV-1 is widespread during the first few years of life in developing countries, whereas in some European countries primary infection occurs relatively at older age.[19-22] Besides geographical differences, also ethnic differences have been described in population subgroups.[23] Ethnic and geographical differences in CMV, EBV and HSV-1 seroprevalences might be explained by differences in behavior that facilitate transmission, such as breastfeeding, daycare, family size and socio-economic position.[17,22,24,25] Because socio-economic position and ethnicity are strongly related, it remains unclear to what extent socio-economic factors explain ethnic differences in herpesvirus seroprevalences.[26] In addition, knowledge on other lifestyle related determinants is mainly based on prevalence studies from the late 90's,[27-30] but social conditions and lifestyle have changed over time.[31,32] Furthermore, the relative importance of each risk factor needs to be assessed,[26] and many studies lack the assessment of multiple risk factors. Previously, it has been suggested that early life exposures, in particular breastfeeding, are more important for CMV infection than adulthood exposures.[33] On the other hand, the ALADDIN birth cohort study found no strong influence of these early life risk factors.[34] Understanding transmission dynamics of herpesvirus infections within populations, and understanding how socio-economic and ethnic groups are differentially exposed to these infections, can improve existing preventive policies and interventions, including on the long run, vaccination programs.[35]

Therefore, the first aim is to assess whether there are ethnic differences in CMV, EBV and HSV-1 infection in children at 6 years of age. The second aim is to evaluate how ethnic differences in CMV, EBV and HSV-1 infections, when present, can be explained by socio-demographic and environmental factors.

METHODS

Study design

This study was embedded within the Generation R Study, a prospective population based cohort study from fetal life onwards.[36,37] All children were born between 2002 and 2006 in Rotterdam, the second largest city in the Netherlands. The study area covers more than half of the cities inhabitants, reflecting Rotterdam's multi-ethnic population.[36] The largest ethnic groups are of Dutch, Surinamese, Turkish, Moroccan, Dutch Antilles and Cape Verdean origin.[36] In total, n=8,305 children participated in the postnatal phase of the study, of whom 81% (n=6,690) visited the research centre (median age 6.0 years).[37] During this visit, blood samples were collected from 4,593 children. In total, n=4,464 children provided serological data on EBV, CMV, HSV-1 status (Supplemental Figure 1). The study was approved by the Medical Ethical Committee of Erasmus MC, University Medical Centre Rotterdam. Parents of the children gave written informed consent.

Herpesvirus serology

Venous blood plasma samples were analysed using enzyme immunoassays for IgG antibodies against CMV, EBV (capsid antigen), HSV-1 (glycoprotein C1; all from EUROIMMUN®, Lübeck, Germany). Results were evaluated semiquantitatively relative to a manufacturer-provided reference threshold sample. Per manufacturer's instructions, an optical density in the patient sample >10% above the provided threshold sample was defined as positive.

Ethnic background

Ethnicity of the child was determined by country of birth of the parents. The child was of non-native Dutch origin if one of the parents was born in another country than the Netherlands.[38] If both parents were born abroad, the country of birth of the mother prevailed. To study whether ethnic background was related to herpesvirus infections, we constructed a dichotomous variable 'Western/non-western' ethnicity. Next, to study to what extent socio-economic and lifestyle factors explained associations between ethnicity and herpesvirus infections, and to study the effect of cultural background of the mother (most often primary caregivers),[39] a distinction was made between mothers of Dutch, Turkish, Moroccan, Cape Verdean, Antillean, Surinamese Creole and Surinamese Hindustani ethnic background. Maternal ethnic background was based on country of birth of mother's parents and was assessed by questionnaires at enrollment. Mothers were considered non-native Dutch if one of her parents was born abroad.[40] If both parents were born abroad, country of birth of the mother's mother prevailed. Generational status of non-native Dutch mothers was based on their own country of birth. Foreign-born mothers were classified as 'first generation' and mothers born in the Netherlands were classified as 'second generation'.[39]

Other determinants

Other potential determinants of herpesvirus infections were selected based on existing literature.[2,12,24,32,33,41,42] These are: socio-economic position, family size/ parity, maternal lifestyle, breastfeeding, daycare and gender. Data on gender,

gestational age, birth weight, parity and mode of delivery were obtained from obstetric records from hospitals and mid-wife practices. Data on socio-demographic and lifestyle factors were obtained by a combination of pre- and postnatal questionnaires completed by both parents. Prenatal questionnaires included information on maternal lifestyle and socio-economic position. Information on socio-economic position was also determined by questionnaires obtained at 6 years of age. Information on breastfeeding and daycare was obtained by postnatal questionnaires. All questionnaires were available in three languages (Dutch, English and Turkish) and further support for verbal translation of questionnaires was available in Arabic, Portuguese and French.

Explanatory variables

Most effects of ethnicity on herpes virus infections are probably indirect ones, acting through more proximal determinants of CMV, EBV, and HSV-1. We considered the following factors to be potential explanatory variables: socio-economic position, breastfeeding, parity as indicator for siblings/family size, and day-care attendance.[2,12,24,32,33,41,42] Information on household income[43] and educational level,[44] both indicators of socio-economic position, were obtained using prenatal questionnaires. The Dutch Standard Classification of Education was used to categorize 4 subsequent levels of education: 1=high, 2= mid-high, 3=mid-low, and 4=low.[44] For additional analyses, income was subdivided into three categories: 1. Below modal <2,000 euro's per month, 2. Between 2,000 and 3,200 euro's per month, 3. > 3,200 euro's per month.[45,46] Child's age and gender were treated as potential confounders in the association between ethnicity and herpesvirus seroprevalences.

Statistical analysis

First, independent student's T-tests, and χ^2 tests were used to test whether characteristics were different between children who were seropositive for CMV, EBV and HSV-1, and who were not. To investigate which determinants were associated with CMV, EBV and HSV-1 seropositivity (y/n), logistic regression analyses were performed with herpesvirus seropositivity at the age of 6 years as dependent variables (CMV y/n, EBV y/n, and HSV-1 y/n). To study to which extent associations between ethnicity and CMV, EBV, and HSV-1 seropositivity were explained by factors associated with socio-economic position, crowding, breastfeeding behaviour and maternal lifestyle, we used Baron and Kenny's causal step approach.[47] Only those factors that were unequally distributed across the ethnicity groups (determinant) (Table 6; online) and were significant ($p<0.05$) associated with CMV, EBV and HSV-1 seropositivity (outcomes) (independent of ethnicity, gender and age); (Table 7; online) were added separately to the model.[47] To assess their mediating effects, the corresponding percentages of attenuation of effect estimates were calculated by comparing differences of model 1 with the adjusted ones (100 x (OR model 1 – OR model 2 with explanatory factor)/ (OR model 1)). Finally, a full model containing ethnicity and all the explanatory factors assessed the joint effects of explanatory factors. Interaction terms between ethnic background and the explanatory variables were tested for significance. If the test was significant ($p<0.05$), we also stratified the analysis by the variables. To reduce attrition bias, multiple imputation of the covariables was performed

Table 1. Maternal and infant characteristics of the study population (n=4,464)

	EBV		CMV		HSV-1	
	EBV - (n=2,185)	**EBV + (n=2,279)**	**CMV - (n=2,772)**	**CMV + (n=1,692)**	**HSV-1 - (n=3,592)**	**HSV-1 + (n=877)**
Maternal characteristics						
Age (Mean ± SD; years)	31.4 (4.9)	**30.1 (5.3)*** **	31.1 (5.0)	**30.1 (5.3)*** **	31.0 (5.0)	**29.5 (5.4)*** **
Educational level (n; %)						
Low	333 (16%)	535 (27%)	495 (19%)	373 (25%)	613 (19%)	255 (35%)
Mid low	604 (30%)	630 (31%)	794 (31%)	440 (29)	996 (30%)	238 (32%)
Mid high	504 (25%)	390 (19%)	582 (23%)	312 (21%)	768 (23%)	126 (17%)
Higher	595 (29%)	462 (23%)	689 (27%)	368 (25%)	936 (28%)	121 (16%)
Net household income per month (n; %)						
< € 2,200	594 (35%)	**799 (48%) *** **	802 (28%)	**591 (48%)*** **	1061 (38%)	**332 (57%)*** **
≥ € 2,200	1102 (65%)	864 (52%)	1320 (63%)	650 (52%)	1718 (62%)	252 (43%)
Smoking during pregnancy (n; %)	235 (14%)	**301 (17%)****	349 (16%)	187 (14%)	419 (15%)	117 (18%)
Alcohol use during pregnancy (n; %)	782 (45%)	**695 (39%)*** **	918 (44%)	**519 (39%)****	1262 (44%)	**215 (32%)*** **
Body Mass Index before pregnancy (Mean, SD)	23.3 (4.0)	**23.9 (4.3)*** **	23.7 (4.3)	23.5 (4.0)	23.4 (1.6)	**24.3 (4.9)****
Fever in 3rd trimester of pregnancy (n; %)	22 (7%)	17 (7%)	28 (7%)	11 (6%)	33 (7%)	6 (8%)
Maternal atopy (n; %)	670 (35%)	628 (33%)	845 (35%)	**453 (32%)***	1069 (34%)	229 (32%)
Family history of asthma atopy (n; %)	1018 (50%)	**937 (46%)***	1263 (49%)	**692 (46%)***	1611 (49%)	344 (45%)
Parity						
0	1198 (55%)	**1172 (54%)*** **	495 (19%)	**373 (25%)***	1947 (57%)	**423 (51%)***
1	661 (32%)	677 (31%)	794 (31%)	440 (30%)	1072 (31%)	266 (32%)
2	192 (9%)	257 (12%)	582 (23%)	312 (21%)	345 (10%)	104 (12%)
≥3	36 (2%)	76 (4%)	689 (27%)	368 (25%)	95 (2%)	51 (6%)
Mode of delivery (n; %)						
Vaginal	1342 (71%)	1468 (75%)	1746 (74%)	1066 (72%)	2240 (72%)	572 (76%)
Forceps or vacuum assisted	292 (16%)	240 12%)	305 (13%)	228 (15%)	449 (15%)	84 (11%)
Caesarian section	249 (13%)	255 (13%)	315 (13%)	190 (13%)	408 (13%)	97 (13%)
Premature rupture of membranes (n; %)	96 (5%)	85 (4%)	100 (4%)	81 (5%)	148 (4%)	33 (4%)

Table 1. continues on next page

Table 1. continued

	EBV		CMV		HSV-1	
	EBV - (n=2,185)	**EBV + (n=2,279)**	**CMV - (n=2,772)**	**CMV + (n=1,692)**	**HSV-1 - (n=3,592)**	**HSV-1 + (n=877)**
Infant characteristics (n=258)						
Males (n; %)	1121 (51%)	1183 (52%)	1469 (53%)	**836 (49%)***	1855 (52%)	450 (51%)
Western ethnicity (n;%)	1598 (74%)	**1266 (58%)*****	1991 (73%)	**877 (54%)*****	2426 (70%)	**407 (49%)*****
Gestational age (Mean ± SD; weeks)	39.7 (1.9)	39.8 (1.8)	39.8 (1.8)	39.8 (1.8)	39.8 (1.8)	**39.7 (1.8)***
Preterm birth (<37 weeks) (n; %)	159 (7%)	140 (6%)	181 (7%)	119 (7%)	246 (7%)	54 (6%)
Birth weight (Mean ± SD; grams)	3,427 (584)	3,401 (553)	3427 (576)	**3391 (555)***	3,424 (523)	**3,369(549)***
Day-care in first year (n; %)	940 (88%)	773 (88%)	1069 (86%)	**646 (92%)*****	1493 (89%)	**222 (84%)***
Breastfeeding (n; %)						
Never	140 (9%)	124 (9%)	211 (11%)	53 (5%)	215 (9%)	49 (10%)
< 3 months	518 (34%)	472 (34%)	650 (35%)	341 (33%)	812 (34%)	179 (36%)
3-6 months	305 (20%)	313 (23%)	395 (21%)	224 (22%)	524 (22%)	95 (19%)
>6 months	565 (37%)	479 (35%)	626 (33%)	421 (40%)	870 (36%)	177 (35%)
Infections first year (n; %)						
RTI	877 (58%)	808 (61%)	1054 (58%)	**631 (63%)****	1404 (60%)	281 (61%)
LRTI	190 (13%)	182 (15%)	241 (14%)	131 (14%)	314 (14%)	58 (14%)
URTI	810 (52%)	758 (54%)	975 (51%)	**593 (56%)****	1296 (52%)	272 (55%)
GI	530 (57%)	473 (61%)	623 (56%)	**380 (64%)****	833 (58%)	170 (61%)
Atopy first year (n; %)¶¶						
Eczema	440 (29%)	351 (26%)	486 (26%)	**305 (30%)***	678 (28%)	113 (24%)
Wheezing (≥ 1 episode)	545 (44%)	**520 (48%)***	680 (45%)	385 (48%)	884 (45%)	181 (48%)
Asthma diagnosis at age 6 (n; %)	133 (7%)	118 (7%)	168 (8%)	83 (7%)	209 (7%)	42 (7%)
Age (Median ± range; years)	6.1 (5.0-9.0)	**6.2 (4.9-9.1)*****	6.2 (5.0-9.0)	**6.2 (4.9-9.1)*****	6.2 (4.9-9.0)	**6.2 (5.0-9.1)*****
BMI for age (mean; sd; SDS)	0.21 (0.87)	**0.34 (0.94)*****	0.25 (0.90)	**0.32 (0.92)****	0.25 (0.89)	**0.41 (0.97)*****

Values are means (SD), absolute numbers (percentages) or #medians (range). Significantly different from not infected *(p<0.05), ** (p<0.01) *** (p<0.001)
Table is based on observed dataset.
¶ Western ethnicity includes children from Dutch, European, American Western, and Asian Western origin. Non-Western ethnicity included children from Turkish, Moroccan, Cape Verdean, Antillean, Surinamese-Creole and Surinamese-Hindustani origin.
¶¶ Atopy includes eczema, allergy HDM, hay-fever, any reported autoimmune disease (diabetes mellitus, SLE, arthritis, MS, thyroid disorder, or celiac disease).

(n=5 imputations).[48] Regression coefficients were pooled by taking the average of the coefficients of the 5 imputed datasets. The pooled results of the 5 imputed datasets were reported in this paper as Odds Ratios (ORs) and 95% confidence intervals (CI's). A 95% CI was calculated around the percentage attenuation using a bootstrap method with 1,000 resamplings per imputed dataset in the statistical program R.[49] All other statistical analyses were performed in SPSS 20.0 for Windows (SPSS Inc., Chicago, Il).

RESULTS

Maternal and child characteristics related to CMV, EBV and HSV-1 are shown in (Table 1). Within the total group of 4464 children (median age 6.0 years), n=1,692 (38%) were infected with CMV, n=2,279 (51%) were infected with EBV, and n=877 (20%) were infected with HSV-1. Thirty percent (n=1,319) were seronegative for CMV, EBV and HSV-1, 39% (n=1,735) were infected with one of these viruses, and 32% (n=1,410) were infected with two or more viruses. Of children with available data on herpesvirus serology, a total of 2,368 children were Native Dutch, 181 were Cape Verdean, 247 Moroccan, 109 Dutch-Antillean, 131 Suriname-Creole, and 136 children were of Surinamese-Hindustani origin (Figure 1).

Determinants of CMV, EBV and HSV-1 seropositivity

CMV seropositive children were more likely of non-Western ethnicity (aOR 2.16; 95% CI 1.81-2.57), to be girls (aOR 1.18, 95% CI 1.02, 1.36), and to be breastfed for 6 months or longer (aOR 1.96; 95% CI 1.25-3.07) than CMV seronegative children. Multiparity was inversely associated with CMV seropositivity (aOR 0.76; 95% CI 0.65-0.90).

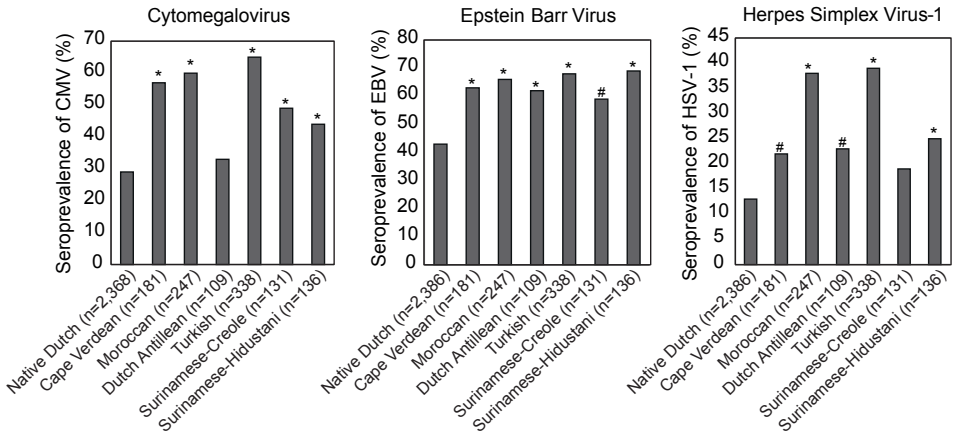

Figure 1. Seroprevalence of herpesvirus infections in childhood according to maternal ethnic background. Values are seroprevalences of CMV, EBV, and HSV-1 infections (%) per ethnicity category. CMV, EBV, and HSV-1 seroprevalences of Cape Verdean, Moroccan, Dutch Antillean, Turkish, Surinamese-Creole, and Surinamese-Hindustani children are compared to Dutch children. Footnotes: ¶ p-value <0.05, #P-value <0.01, *P-value <0.001 based on χ^2 test.

Similar to CMV, non-Western ethnicity was an independent risk factor for EBV (aOR 1.76; 95% CI 1.48-2.09) and HSV-1 (aOR 1.52; 95% CI 1.39-1.66). In contrast, EBV and HSV-1 seropositive children more often had 2 or more siblings (aOR EBV $_{\text{parity } \geq 2}$ 1.35; 95% CI 1.05-1.740, aOR HSV-1 $_{\text{parity } \geq 2}$ 1.31; 95% CI 1.14-1.50), than EBV and HSV-1 seronegative children (in contrast with the observations for CMV). Daycare and socio-economic position were only associated with HSV-1 seropositivity (aOR $_{\text{daycare}}$ 0.89, 95% CI 0.81-0.97). Mothers of HSV-1 seropositive children were lower educated (aOR 1.55, 95% CI 1.35, 1.79) and their family household income was lower (aOR 1.11 , 95% CI 1.01-1.23), than mothers of HSV-1 seronegative children (Table 5; online). Additional analyses between the seroprevalence of herpes virus infections and net household income more in detail strengthened the inverse associations between socio-economic position and herpesvirus seroprevalences (Table 8; online).

Ethnic background and CMV seropositivity

Children of Cape Verdean, Moroccan, Turkish, Surinamese-Creole and Surinamese Hindustani mothers were more often seropositive for CMV, than children of native Dutch mothers. CMV seroprevalences in the non-native Dutch groups ranged between 44-65%, compared to 29% in the native Dutch group (Figure 1). To explain the observed ethnic differences in CMV seroprevalences, the following explanatory variables were selected: maternal educational level, parity, daycare and breastfeeding duration,[47] (Table 6 and 7; online). However, after including all the explanatory factors in the model, differences in CMV seroprevalences compared to Dutch children increased with 55% (95% CI 24 to 124) for Surinamese-Hindustani children, 36% (95% CI 19 to 60) for Moroccan children, 34% (95% CI 14 to 66) for Surinamese-Creole children, 33% (95% CI 13 to 59) for Turkish children, and 23% (95% CI 5 to 47) for Cape Verdean children (Table 2).

Ethnic background and EBV seropositivity

Children in non-native Dutch groups were more often EBV seropositive than native Dutch children. EBV seroprevalences in the non-native Dutch minority groups ranged between 59-69%, compared to 43% in the native Dutch group (Figure 1). Maternal educational level, net household income and parity were selected to explain ethnic differences in EBV seroprevalences,[47] (Table 6 and 7; online). Socio-economic position was the most important contributor to the association between ethnicity and EBV seroprevalence. For example, net household income explained 22% (95% CI -41 to -6) of the differences in EBV seroprevalences between Cape Verdean and native Dutch children, followed by maternal educational level, which explained 18% (95% CI -31 to -7) (Table 3). Overall, 39% (95% CI -66 to -19) of the difference in EBV seroprevalences prevalence between Cape Verdean and Dutch children was explained by differences in socio-economic position and family size. These factors explained 36% (95% CI -55 to -19) of the differences in EBV seroprevalences between Moroccan and Dutch children, 33% (95% CI -48 to -17) of the differences in EBV seroprevalences between Turkish and Dutch children, and 25% (95% CI -60 to -3) of the differences in EBV seroprevalences between Antillean and Dutch children (Table 3).

Table 2. Attenuation of the OR of Cytomegalovirus seropositivity for different ethnicities after individual adjustment for the explanatory variables

	Surinamese-Creoles [a]		Surinamese-Hindustani [b]		Turkish [c]		Moroccan [d]		Cape Verdean [e]		Antillean [f]	
	OR	Att.	OR	Att.	OR	Att.	OR	Att.	OR	Att.	OR	Att.
CMV												
Model 1 (includes child sex and current age)	2.29 (1.61,3.28)		1.92 (1.35,2.72)		4.41 (3.47,5.62)		3.64 (2.77,4.79)		3.17 (2.33,4.32)		n.s.	NA
Socioeconomic status												
Model 1+ education	2.72 (1.87,3.88)	**+33% (17, 60) *****	2.26 (1.56,3.19)	**+37% (19, 80) *****	5.36 (4.01,6.76)	**+28% (14,44) *****	4.23 (3.17,5.61)	**+22% (11,36) *****	3.70 (2.69,5.12)	**+24% (12,40) *****		
Crowding												
Model 1 + parity	2.29 (1.60,3.28)	0% (-10,10)	1.89 (1.33,2.71)	-3% (-20,14)	4.49 (3.51,5.74)	+2% (-4,9)	3.94 (2.97,5.24)	**+11% (0.5,24) ***	3.14 (2.29,4.32)	-1% (-12,11)		
Model 1 + daycare	2.37 (1.62,3.32)	**+6% (1,14) ****	2.14 (1.49,3.07)	**+25% (8,58) ****	5.08 (3.84,6.42)	**+20% (7,36) ****	3.90 (3.00,5.40)	**+10% (3,19) *****	3.15 (2.28,4.24)	-1% (-3,2)		
Model 1 + breastfeeding	2.35 (1.63,3.33)	**+5% (0.4,12) ***	1.99 (1.37,2.79)	**+8% (2,9) ****	4.24 (3.33,5.43)	**-5% (-8,-2) ****	3.62 (2.70,4.69)	-1% (-4,1)	3.33 (2.45,4.58)	**+8% (2,15) ****		
Fully adjusted model												
	2.73 (1.81,3.83)	**+34% (14,66) *****	2.42 (1.62,3.42)	**+55% (24,124) *****	5.53 (3.98,6.97)	**+33% (13,59) ****	4.60 (3.36,6.31)	**+36% (19,60) *****	3.68, (2.6-,5.07)	**+23% (5,47) ****		

Abbreviations: OR, Odds Ratio; CI, confidence interval.

[a -f]The OR's represent the attenuated effect estimate and their 95% CIs relative to model 1 after individual adjustment for explanatory factors. Att. represent the attenuations of effect estimates and their 95% CIs for the different ethnicities relative to model 1 (includes confounders) after adjustment for explanatory variables ($100x (OR_{model\ 1} - OR_{model\ 1\ with\ explanatory\ factor}) / (OR_{model\ 1})$). Dutch is the reference group. No significant difference in CMV prevalence was observed for the Antillean group compared with the Dutch group, therefore attenuations in effect estimates for the Antillean group are not presented. * p<0.05, ** p<0.01, *** p<0.001

Table 3. Attenuation of the OR of Epstein Barr virus seropositivity for different ethnicities after individual adjustment for the explanatory variables

	Surinamese-Creoles [a]		Surinamese-Hindustani [b]		Turkish [c]		Moroccan [d]		Cape Verdean [e]		Antillean [f]	
	OR	Att.	OR	Att.	OR	Att.	OR	Att.	OR	Att.	OR	Att.
EBV												
Model 1 (includes child sex and current age)	1.77 (1.24,2.54)		2.85 (1.96,4.13)		2.69 (2.11,3.44)		2.40 (1.82,3.17)		2.18 (1.59,2.99)		1.98 (1.33,2.96)	
Socioeconomic status												
Model 1+ education	1.66 (1.15,2.40)	-15% (-43,0.2)	2.71 (1.85,3.95)	-7% (-17,3)	2.37 (1.84,3.08)	**-19% (-31,-7) ****	2.19 (1.63,2.91)	**-15% (-26,-6) ****	1.97 (1.41,2.70)	**-18% (-31,-7) ****	1.86 (1.24,2.80)	**-12% (-32,-0.6) ***
Model 1+ income	1.63 (1.13,2.36)	**-19% (-50,-5) ***	2.60 (1.80,3.86)	**-13% (-24,-4) ***	2.36 (1.86,3.16)	**-19% (-33,-5) ***	2.11 (1.60,2.91)	**-21% (-36,-6) ***	1.92 (1.41,2.74)	**-22% (-41,-6) ***	1.77 (1.19,2.73)	**-22% (-50,-6) ***
Crowding												
Model 1 + parity	1.80 (1.25,2.59)	+4% (-9,24)	2.86 (1.96,4.19)	1% (-10,16)	2.58 (2.02,3.31)	-6% (-13,0.7)	2.21 (1.66,2.94)	**-14% (-25,-4)***	2.03 (1.47,2.81)	-12% (-28,1)	1.97 (1.32,2.96)	-1% (-12,14)
Fully adjusted model												
	1.61 (1.12,2.37)	-21% (-56,3)	2.59 (1.77,3.87)	-14% (-30,5)	2.14 (1.67,2.88)	**-33% (-48,-17) *****	1.89 (1.42,2.61)	**-36% (-55,-19) *****	1.72 (1.23,2.44)	**-39% (-66,-19) *****	1.74 (1.17,2.69)	**-25% (-60,-3) ***

Abbreviations: OR, Odds Ratio; CI, confidence interval.

[a-f] The OR's represent the attenuated effect estimate and their 95% CIs relative to model 1 after individual adjustment for explanatory factors. Att. represent the attenuations of effect estimates and their 95% CIs for the different ethnicities relative to model 1 (includes confounders) after adjustment for explanatory variables ($100x (OR_{model\ 1} - OR_{model\ 1\ with\ explanatory\ factor}) / (OR_{model\ 1})$). Dutch is the reference group. *(p<0.05), ** (p<0.01) *** (p<0.001)

Ethnic background and HSV-1 seropositivity

All ethnic minority groups, except for Surinamese-Creole, were more often HSV-1 seropositive than native Dutch children. HSV-1 seroprevalences in the non-native Dutch groups ranged between 22-39%, compared to 13% in the native Dutch group (Figure 1). To explain ethnic differences in HSV-1 seroprevalences, maternal educational level, net household income and breastfeeding duration were selected as explanatory variables,[47] (Table 6 and 7; online). Maternal educational level and net household income were the most important factors to explain ethnic differences in HSV-1 seroprevalence. For example, 27% (95% CI -38 to -16) of this differences in HSV-1 seroprevalences between Turkish and Dutch children was explained by maternal educational level (Table 4). Socio-economic position and breastfeeding together explained 24% (95% CI -40 to -7) of the difference in HSV-1 seroprevalence between Turkish and Dutch children; they explained 48% (95% CI -150 to -18) of the differences in HSV-1 seroprevalences between Cape Verdean and Dutch children; 30% (95% CI -68 to -10) of the differences in HSV-1 prevalence between Surinamese-Hindustani and Dutch children and 22% (95% CI -36 to -5) of the differences in HSV-1 seroprevalence between Moroccan and Dutch children. Maternal educational level was the most important explanatory factor in the association between ethnicity and HSV-1 seroprevalence (Table 4).

Next, we observed that children who were seropositive for a single virus, were more likely to be infected with multiple herpesviruses. For example, the odds for CMV seropositivity was 1.69 times greater, and the odds for HSV-1 seropositivity was 2.17 times greater in EBV infected children than in EBV seronegative children. Also, the odds for both CMV and HSV-1 seropositivity was almost 3 times greater in EBV seropositive children than in EBV seronegative children (Table 9; online).

Also, CMV, EBV and HSV-1 seropositive children tended to have more frequently 'first' generation mothers, than seronegative children, although differences were not significant (Figure 2). Differences in characteristics between seronegative children and children with multiple herpes virus infections are described in Table 10; online. Mothers of seronegative children were more often higher educated, had a higher net household income, than mothers of children who had 1 or 2 herpes virus infections. Seronegative children were more often of Western origin, and had fewer siblings, than children who had 1 or 2 herpes virus infections (Table 10; online).

DISCUSSION

In this large and multi-ethnic population-based cohort, we found 1) large ethnic differences in seroprevalences of EBV (range 43-69%), CMV (range 29-65%) and HSV-1 (range 13-39%) among 6 year old children 2) the highest seroprevalence in non-Dutch children. Socio-economic factors and factors related to crowding early in life only partly explained ethnic differences in EBV and HSV-1 seroprevalence in childhood, but did not explain ethnic differences in CMV seroprevalence.

Our study design provided a unique opportunity to study determinants of these infections, to investigate variation in seroprevalence by ethnic group, and to study to which extent socio-economic and lifestyle factors explained these differences. Our results confirm and extend previous studies by observing that socio-economic position partly explains ethnic variation in EBV and HSV-1 seroprevalences, but not in CMV seroprevalences.[12,35,42] Although these three viruses are all members of the

Table 4. Attenuation of the OR of Herpes simplex virus 1 seroprevalence for different ethnicities after individual adjustment for the explanatory variables

	Surinamese-Creoles [a]		Surinamese-Hindustani [b]		Turkish [c]		Moroccan [d]		Cape Verdean [e]		Antillean [f]	
	OR	Att.	OR	Att.	OR	Att.	OR	Att.	OR	Att.	OR	Att.
HSV-1												
Model 1 (includes child sex and current age)	ns	NA	2.10 (1.40, 3.16)		4.08 (3.18,5.24)		3.73 (2.80,4.98)		1.75 (1.20,2.54)		1.70 (1.06,2.73)	
Socioeconomic status												
Model 1+ education			1.82 (1.19,2.73)	**-26% (-6,-10) ****	3.24 (2.49,4.26)	**-27% (-38,-16) *****	3.15 (2.28,4.17)	**-21% (-30,-12) *****	1.45 (0.95,2.07)	**-39% (-119,-19) ****	1.47 (0.91,2.36)	**-33% (-160,-7) ***
Model 1+ income			1.92 (1.24,2.87)	**-17% (-4,-0.1) ***	3.58 (2.66,4.68)	**-16% (-30,-1) ***	3.29 (2.35,4.42)	**-16% (-30,-0.5) ***	1.54 (1.01,2.24)	-28% (-92,1)	1.52 (0.91,2.43)	-26% (-115,11)
Crowding												
Model 1 + breastfeeding			2.04 (1.40,3.18)	**-6% (-14,-1) ***	4.29 (3.29,5.50)	**+7% (2,14) ****	3.80 (2.89,5.16)	+2% (0,6)	1.67 (1.16,2.47)	**-10% (-32,-2) ***	1.65 (1.02,2.65)	-8% (-43,2)
Fully adjusted model												
			1.78 (1.17,2.75)	**-30% (-68,-10) ****	3.33 (2.43,4.43)	**-24% (-40,-7) ****	3.14 (2.21,4.26)	**-22% (-36,-5) ***	1.39 (0.90,2.02)	**-48% (-150,-18) ***	1.42 (0.85,2.30)	-40% (-213,9)

Abbreviations: OR, Odds Ratio; CI, confidence interval.

[a-f] The OR's represent the attenuated effect estimate and their 95% CIs relative to model 1 after individual adjustment for explanatory factors. Att. represent the attenuations of effect estimates and their 95% CIs for the different ethnicities relative to model 1 (includes confounders) after adjustment for explanatory variables (100x ($OR_{model\ 1}$ - $OR_{model\ 1\ with\ explanatory\ factor}$) / ($OR_{model\ 1}$)). Dutch is the reference group.

No significant difference in HSV-1 prevalence was observed for the Surinamese-Creole group compared with the Dutch group, therefore attenuations in effect estimates for the Surinamese-Creole group are not presented. * p<0.05, ** p<0.01, *** p<0.001

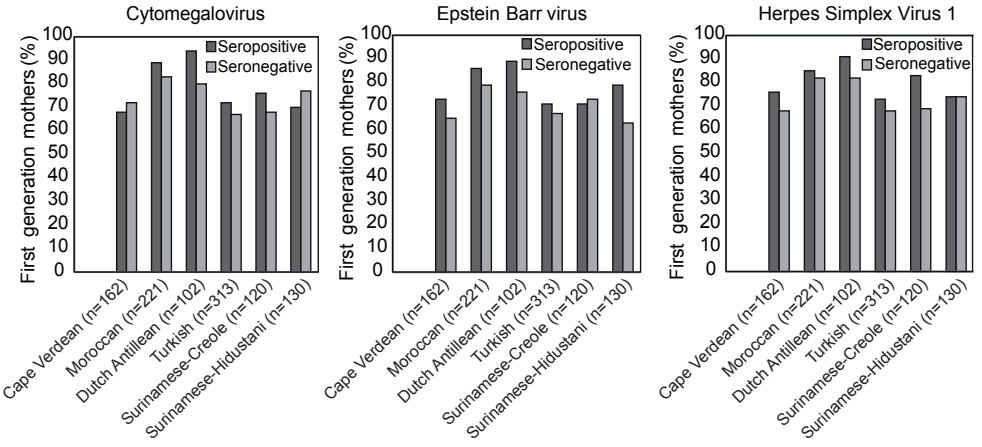

Figure 2. Percentages of first generation mothers according to CMV, EBV or HSV-1 seropositivity in childhood.
Values are percentages of 'first' generation mothers according to CMV, EBV or HSV-1 seroprevalences in childhood. Generational status of non-native Dutch mothers was based on their country of birth. Foreign-born mothers were classified as 'first generation' and mothers born in the Netherlands were classified as 'second generation'.

herpesvirus family, and thus share significant biological properties, transmission dynamics between these viruses seem to be different. Our results suggest that ethnic differences in CMV seroprevalences in childhood are mainly determined by factors that facilitate vertical transmission (e.g. pregnant women that transmit CMV to their child), which is in line with the idea that the most important source for CMV infection in childhood is breastmilk.[50-52] In contrast, ethnic differences in EBV and HSV-1 seroprevalences are partly determined by factors that facilitate horizontal transmission, such as family size and daycare, which are strongly related to socio-economic position. In fact, we were able to explain up to 39% of the ethnic differences in EBV seroprevalences, of which socio-economic position was the most important contributor, which is in line with a recent study.[53] However, in contrast with our study, the authors suggested that ethnic differences could not be explained by crowding. However, it could be speculated that socio-economic position reflects more proximal determinants (e.g. factors that facilitate horizontal transmission, such as family size, bed sharing and childcare arrangements). Indeed, the results from our multivariable model confirm that family size is important in estimating the risk of EBV infection.[18] In fact, we observed a dose-response relationship between parity and the risk of EBV and HSV-1 seropositivity.

In contrast with the results on EBV and HSV-1, ethnic differences in CMV seroprevalences could not be explained by socio-economic position, nor by other 'explanatory variables' such as breastfeeding, daycare and parity. In fact, these factors rather strengthened than weakened the association between ethnicity and CMV. We expected breastfeeding to partly mediate this association, because breastfeeding can transmit CMV, and breastfeeding behavior is influenced by ethnic background.[54] Nevertheless, vertical transmission from mother to child is probably more important in explaining ethnic differences in CMV seroprevalences than factors that facilitate horizontal transmission. Given the economic burden of CMV associated diseases, it has been suggested that vaccination in the general population would be the most straightforward way to control for all CMV

associated diseases.[3] Some recommend to include 12-year old children, whereas others suggested to include vaccination of toddlers.[33 55] However, thus far, safe and effective vaccines have not been developed. Nevertheless, on the basis of our results, ethnic and socio-economic differences should be taken into account when vaccination is contemplated.

HSV-1 prevalence was highest in Turkish and Moroccan children, which is in line with a previous Dutch study in adolescents.[42] Our results confirm that differences in HSV-1 seroprevalences between European countries could be partly explained by socio-economic factors.[22] It has been shown that lower socio-economic position indeed increases the risk for HSV-1.[42] All factors together, including socio-economic position, breastfeeding and crowding, explained up to 48% of ethnic differences in HSV-1 seroprevalence, of which socio-economic position was the most important contributor, suggesting an important role for factors that facilitate horizontal transmission.

Methodological considerations

An important strength of this study is that we had a large multi-ethnic cohort study population drawn from the general population of Rotterdam. Because immigration is common in Western countries, our findings may be more broadly applicable. In addition, our study design enabled us to assess multiple risk factors, and to study the relative importance of each using multivariate models. Moreover, an explanation of ethnic differences in herpesvirus infections using a mediator approach has never been performed before. Besides socio-economic position, numerous other factors vary between countries, and adjustment for these factors might decrease ethnic/racial or geographical variation.[12] Because our study was performed within a population based study of a region within a single country, we were able to adjust for all of these variables. A limitation of this study is that we did not have data on maternal CMV, EBV, HSV-1 seroprevalences, preventing analysis of mother-to-child transmission. A second limitation was lack of data on EBV, CMV and HSV-1 seroprevalences in fathers and siblings, precluding examination of their roles in horizontal transmission.

Conclusion

In this multiethnic population-based cohort, we found large ethnic differences in seroprevalences of CMV, EBV and HSV-1 among 6 year old children, with higher seroprevalences in non-Dutch children. Socio-economic factors and factors related to crowding early in life partly explained ethnic differences in EBV and HSV-1 seroprevalence, but did not explain ethnic differences in CMV seroprevalence. When immunization against these viruses is considered, ethnic and socio-economic differences should be taken into account.

CONTRIBUTORS' STATEMENT

Michelle A.E. Jansen contributed to the design of the study, completed the background literature research, data collection, performed the analyses, wrote the first draft of the manuscript, and collated comments from other authors. Diana van den Heuvel contributed to the design of the study, data collection,

immunological data analyses, data interpretation and writing of the manuscript. Selma H. Bouthoorn contributed to the data collection, statistical analyses, data interpretation and writing of the manuscript. Vincent W.V. Jaddoe conceptualized and designed the study and contributed to the writing of the manuscript. Herbert Hooijkaas contributed to the design of the study, interpretation of data analysis, and writing of the manuscript. Hein Raat contributed to the design of the study, interpretation of data analysis, and writing of the manuscript. Pieter L.A. Fraaij contributed to the design of the study, interpretation of data, and writing of the manuscript Menno C. van Zelm contributed to the design of the study, immunological data analyses, data interpretation, and writing of the manuscript. Henriette A. Moll obtained funds, designed the study, supervised the project, contributed to data interpretation and writing of the manuscript. All authors have access to all of the data, take responsibility for the integrity of the data, the accuracy of the data analysis and approved the final version of the manuscript. MAEJ and HAM are guarantors of the study and accept full responsibility for the work and the conduct of the study. All authors confirm that neither this manuscript nor any part of it has been published or is being considered for publication elsewhere.

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Table 5 (online). Determinants of herpesvirus seropositivity in children at 6 years of age

n=4,464	**Cytomegalovirus**	**Epstein-Barr Virus**	**Herpes simplex virus 1**
Determinants	(aOR 95% CI)	(aOR 95% CI)	(aOR 95% CI)
Gender			
Male	1.0	1.0	1.0
Female	**1.18 (1.02, 1.36)***	0.99 (0.87, 1.15)	1.05 (0.97, 1.13)
Ethnicity			
Western	1.0	1.0	1.0
Non western	**2.16 (1.81, 2.57)*****	**1.76 (1.48, 2.09)*****	**1.52 (1.39, 1.66)***
Income			
Low (<2200)	1.18 (0.98, 1.42)	1.09 (0.91, 1.30)	**1.11 (1.01, 1.23)***
High (≥2200)	1.0	1.0	1.0
Maternal educational level			
Low	0.95 (0.73, 1.23)	1.09 (0.85, 1.41)	**1.55 (1.35, 1.79)*****
Mid-low	0.82 (0.66, 1.01)	0.93 (0.76, 1.15)	**1.19 (1.05, 1.35)****
Mid-high	0.96 (0.77, 1.19)	0.94 (0.77, 1.15)	**1.16 (1.03, 1.31)***
High	1.0	1.0	1.0
Daycare	1.20 (1.00, 1.43)	1.06 (0.86, 1.31)	**0.89 (0.81, 0.97)***
Breastfeeding duration			
Never	1.0	1.0	1.0
<3 months	**1.53 (1.04, 2.23)***	0.98 (0.73, 1.33)	1.02 (0.89, 1.16)
3-6 months	**1.77 (1.21, 2.60)****	1.20 (0.85, 1.69)	1.08 (0.93, 1.26)
>6 months	**1.96 (1.25, 3.07)****	1.08 (0.78, 1.85)	1.06 (0.92, 1.22)
Parity			
0	1.0	1.0	1.0
1	**0.76 (0.65, 0.90)****	1.11 (0.95, 1.30)	**1.18 (1.08, 1.29)*****
2	0.90 (0.69, 1.18)	**1.35 (1.05, 1.74)***	**1.31 (1.14, 1.50)*****
≥3	0.85 (0.54, 1.33)	**1.58 (1.01, 2.48)***	**1.60 (1.29, 1.99)*****

Abbreviations: aOR, adjusted Odds Ratio; CI, confidence interval. Values reflect aOR's (95% CI). Table is based on imputed data set. *P<0.05, ** P<0.01, *** P<0.001. Models were adjusted for age, gender, socio-economic position (ethnicity, income, maternal educational level), crowding (daycare, breastfeeding, parity) and maternal lifestyle (maternal age, smoking and alcohol use during pregnancy, maternal BMI).

Table 6 (online). Associations between ethnic background and potential confounders and mediators (N=3528)

Ethnic background	Dutch (n=2386)	Surinamese-creoles (n=131)	Surinamese-hindustani (n=136)	Turkish (n=338)	Moroccan (n=247)	Cape Verdean (n=181)	Antillean (n=109)	P-value[a]
Potential confounders[b]								
Age (years)	6.1 (0.4)	6.4 (0.7)	6.3 (0.6)	6.3 (0.6)	6.4 (0.7)	6.3 (0.7)	6.6 (0.9)	<0.001
Parity (%)								
Nulliparous	60.3	54.3	53.8	43.7	37.9	47.7	51.9	<0.001
Potential mediators								
Maternal educational level (%)								
Low	12.7	32.8	31.5	53.3	45.1	50.7	36.0	<0.001
Mid-low	25.7	50.4	49.6	33.0	37.9	39.5	49.0	
Mid-high	26.7	14.3	15.0	10.0	14.6	8.6	9.0	
High	34.9	2.5	3.9	3.7	2.4	1.3	6.0	
Net household income (%)								
≤ 2200 euro	25.1	70.5	70.2	87.1	88.2	91.6	83.3	<0.001
> 2200 euro	74.9	29.5	29.8	12.9	11.8	8.4	16.7	
Daycare (%yes)	89.5	83.3	70.4	57.1	65.4	92.1	82.6	<0.001
Breastfeeding duration (%)								
never	10.1	8.3	4.2	1.2	3.7	11.7	13.0	<0.001
<3 months	31.8	41.7	56.9	35.5	41.1	51.9	52.2	
3-6 months	22.7	18.1	20.8	18.7	18.7	18.2	8.7	
>6 months	35.4	31.9	18.1	44.6	36.4	18.2	26.1	
Maternal smoking (% continued)	14.0	27.2	15.2	32.3	4.9	17.8	20.4	<0.001
Maternal alcohol use (% continued)	53.4	40.4	18.6	6.8	3.0	28.5	36.3	<0.001
BMI child (kg/m^2)	15.9 (1.4)	16.9 (2.7)	15.8 (2.1)	17.2 (2.3)	16.9 (2.0)	17.0 (2.3)	17.2 (2.5)	<0.001
Maternal BMI (kg/m^2)	23.2 (3.9)	24.9 (4.9)	23.8 (4.5)	24.9 (4.8)	25.2 (4.3)	23.7 (3.6)	25.5 (5.6)	<0.001

Values are percentages or means (SD).

[a] P-values are calculated with the Chi-square test for categorical variables and ANOVA for continuous variables.

[b] Data was missing for maternal educational level (9.2%), net household income (24.8%), daycare (56.3%), breastfeeding duration (34.7%), maternal smoking (13.3%), maternal alcohol use (21.0%), BMI child (0.2%) and maternal BMI (26.1%)

Table 7 (online). Associations between potential mediators and herpes virus seropositivity

	Cytomegalovirus		Epstein-Barr virus		Herpes Simplex virus 1	
	OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
Potential mediators						
Parity	**0.89 (0.82,0.98)**	0.012	1.08 (0.99,1.17)	0.096	1.07 (0.96,1.18)	0.22
Maternal educational level						
Low	**0.67 (0.53,0.85)**	0.001	**1.34 (1.07,1.69)**	0.011	**1.88 (1.41,2.51)**	<0.001
Mid-low	**0.66 (0.54,0.82)**	<0.001	0.99 (0.81,1.21)	0.93	**1.37 (1.03,1.83)**	0.03
Mid-high	0.89 (0.72,1.11)	0.31	0.92 (0.75,1.13)	0.43	1.14 (0.80,1.63)	0.46
High	Ref		Ref		Ref	
Net household income						
≤ 2200 euro	0.87 (0.72, 1.06)	0.16	1.20 (1.00, 1.44)	0.057	**1.29 (1.02,1.64)**	0.036
> 2200 euro	Ref		Ref		Ref	
Daycare						
No	Ref		Ref		Ref	
Yes	**1.38 (1.09, 1.76)**	0.009	0.97 (0.76,1.23)	0.77	0.86 (0.68, 1.09)	0.20
Breastfeeding duration						
never	Ref		Ref		Ref	
<3 months	1.34 (0.94,1.90)	0.10	0.89 (0.66,1.20)	0.44	0.74 (0.51,1.08)	0.12
3-6 months	**1.48 (1.07,2.06)**	0.019	1.01 (0.72,1.42)	0.95	0.67 (0.43,1.03)	0.067
>6 months	**1.63 (1.13,2.36)**	0.012	0.83 (0.60,1.14)	0.23	0.67 (0.44,1.02)	0.061

OR=odds ratio, CI=confidence interval. Values are OR's (95% CI)
Table is based on imputed dataset (potential confounders and mediators are multiple imputed, determinant observed).
Values are derived from logistic regression models and represent effect estimates (95% confidence intervals), adjusted for gender, age and ethnic background.

Table 8 (online). Odds Ratios for Infections and Coinfections According to Socio Economic Position

	CMV		EBV		HSV-1		CMV and HSV-1		EBV and HSV-1		EBV and CMV	
	Odds Ratio (95% CI)	*P* Value	Odds Ratio (95% CI)	*P* Value	Odds Ratio (95% CI)	*P* Value	Odds Ratio (95% CI)	*P* Value	Odds Ratio (95% CI)	*P* Value	Odds Ratio (95% CI)	*P* Value
Income												
<2000	**1.70 (1.45, 2.01)**	<0.0001	**1.92 (1.63, 2.27)**	<0.0001	**2.56 (2.09, 3.12)**	<0.0001	**3.43 (2.60, 4.54)**	<0.0001	**3.10 (2.44, 3.93)**	<0.0001	**2.19 (1.81, 2.64)**	<0.0001
2000-3200	0.93 (0.79, 1.10)	0.41	**1.32 (1.13, 1.55)**	0.001	**1.52 (1.23, 1.88)**	<0.0001	**1.66 (1.21, 2.28)**	0.002	**1.58 (1.21, 2.06)**	0.001	1.14 (0.93, 1.40)	0.22
>3200	1.00 (ref)		1.00 (ref)		1.00 (ref)		1.00 (ref)		1.00 (ref)		1.00 (ref)	
Education												
Low	**1.36 (1.08, 1.72)**	0.011	**1.92 (1.59, 2.33)**	<0.0001	**2.63 (2.11, 3.27)**	<0.0001	**3.21 (2.37, 4.35)**	<0.0001	**2.93 (2.21, 3.87)**	<0.0001	**2.01 (1.50, 2.68)**	<0.0001
Mid Low	0.97 (0.80, 1.19)	0.79	**1.23 (1.04, 1.46)**	0.02	**1.47 (1.17, 1.86)**	0.001	**1.40 (1.02, 1.94)**	0.04	**1.62 (1.22, 2.15)**	0.001	1.21 (0.95, 1.54)	0.13
Mid High	0.95 (0.79, 1.14)	0.58	0.94 (0.77, 1.14)	0.52	1.07 (0.77, 1.94)	0.67	1.02 (0.69, 1.51)	0.93	1.05 (0.71; 1.57)	0.79	1.12 (0.88, 1.42)	0.37
High	1.00 (ref)		1.00 (ref)		1.00 (ref)		1.00 (ref)		1.00 (ref)		1.00 (ref)	

Abbreviations: CI, confidence interval; CMV, cytomegalovirus, EBV, Epstein-Barr virus; HSV-1, herpes simplex virus type-1.
Data on income < 2000 n=870, 2000-3200 n=915, >3200 n=1827, Data on maternal educational level low n=1064, mid low n=1258, mid high n=940, high n=1202

Table 9 (online). Odds Ratios for Coinfections According to EBV Seropositivity Status

	CMV		HSV-1		CMV and HSV-1	
	Odds Ratio (95% CI)	*P* Value	Odds Ratio (95% CI)	*P* Value	Odds Ratio (95% CI)	*P* Value
EBV Positive	**1.69 (1.50; 1.91)**	<0.0001	**2.17 (1.86; 2.53)**	<0.0001	**2.95 (2.02; 3.11)**	<0.0001
EBV Negative	1.00 (reference)		1.00 (reference)		1.00 (reference)	

Abbreviations: CI, confidence interval; CMV, cytomegalovirus, EBV, Epstein-Barr virus; HSV-1, herpes somplex virus type-1.

Table 10 (online). Maternal and infant characteristics of the study population (n=4,464)

	EBV, CMV or HSV-1		
	0 infections (n=1,319)	**1 Infection (n=1,735)**	**≥2 infections (n=1,410)**
Maternal characteristics			
Age (Mean ± SD; years)	31.7 (4.7)	**30.8 (5.0)*** **	**29.7 (5.4)*** **
Educational level (n; %)			
Low	180 (14.4%)	**322 (20.3%)*** **	**366 (30.1%)*** **
Mid-Low	376 (30.0%)	476 (30.0%)	382 (31.5%)
Mid-High	329 (26.3%)	336 (21.2%)	229 (18.9%)
High	368 (29.3%)	452 (28.5%)	237 (19.5%)
Net household income per month (n; %)			
< € 2,000	177 (16%)	325 (23%)	**368 (35%)*** **
€ 2,000-3,200	291 (22%)	370 (26%)	254 (24%)
> € 3,200	668 (59%)	716 (51%)	443 (42%)
Smoking continued during pregnancy (n;%)	138 (13%)	**232 (17%)***	166 (15%)
Alcohol use continued during pregnancy (n;%)	487 (46%)	595 (43.4)	**395 (35.7%)*** **
BMI before pregnancy (Mean ± SD; kg/m^2)	23.4 (3.9)	**23.6 (1.7)**	**23.9 (4.3)***
Maternal atopy (n; %)	396 (33.8%)	548 (36.9%)	354 (30.2%)
Family history of asthma / atopy (n; %)	605 (48.7%)	809 (51.1%)	**541 (43.7%)***
Parity			
0	717 (57%)	**917 (55%)***	**736 (54%)*** **
1	404 (32%)	533 (32%)	401 (29%)
2	118 (9%)	162 (10%)	169 (12%)
≥3	23 (2%)	61 (3%)	62 (5%)
Mode of delivery (n; %)			
Vaginal	825 (73.4%)	1071 (72%)	914 (74.6%)
Forceps or vacuum assisted	166 (14.8%)	203 (13.6%)	163 (13.3%)
Caesarian section	133 (11.8%)	214 (14.4%)	148 (12.1%)
Premature rupture of membranes (n; %)	51 (4.1%)	72 (4.4%)	58 (4.3%)
Infant characteristics (n=258)			
Males (n; %)	683 (51.8%)	922 (53.1%)	699 (49.6%)
Western ethnicity (n;%)	1047 (80%)	**1162 (69%)*** **	**655 (49%)*** **
Gestational age (Mean ± SD; weeks)	39.8 (1.9)	39.8 (1.7)	39.8 (1.8)
Preterm birth (<37 weeks) (n; %)	76 (6.0%)	104 (6.0%)	79 (5.6%)
Birth weight (Mean ± SD; grams)	3442 (599)	3413 (557)	**3386 (552)**
Low birth weight (<2500 gr)	75 (5.7%)	98 (5.7%)	67 (4.8%)
Day-care in first year (n; %)	567 (85%)	**699 (89%)***	447 (88%)
Breastfeeding (n; %)			
Never	98 (10.2%)	114 (10.0%)	**52 (6.3%)***
< 3 months	340 (35.5%)	364 (32.0%)	286 (34.8%)
3-6 months	195 (20.4%)	237 (20.8%)	186 (22.7%)
>6 months	324 (33.9%)	423 (37.2%)	297 (36.2%)
Infections first year (n; %)			
RTI	538 (57.2%)	652 (59.4%)	**495 (63.3%)***
LRTI	125 (13.8%)	140 (13.5%)	107 (15.0%)
URTI	490 (49.8%)	607 (52.6%)	**471 (56.1%)***
GI	329 (55.7%)	378 (58.0%)	**296 (64.1%)***
Atopy first year			
Eczema	264 (27.4%)	312 (28.2%)	215 (27.2%)
Wheezing (≥ 1 epsiode)	338 (42.8%)	412 (45.5%)	**315 (49.5%)***
Nasopharyngeal bacterial carriership 6 years (n; %)			
No pathogen	606 (47.3%)	794 (46.8%)	606 (44.2%)
S Aureus	338 (26.4%)	459 (27.0%)	408 (29.8%)
S. Pneumoniae	255 (19.9%)	296 (17.4%)	246 (17.9%)
H. Influenza	125 (9.8%)	197 (11.6%)	152 (11.1%)
M. Catarrhalis	117 (9.1%)	156 (9.2%)	156 (11.4%)

Table 10. (continued)

	0 infections (n=1,319)	EBV, CMV or HSV-1 1 Infection (n=1,735)	≥2 infections (n=1,410)
Antibiotic use (n; %)			
year 1	386 (47.6%)	**504 (54.1%)****	340 (52.7%)
year 2	413 (41.6%)	520 (44.9%)	**374 (47.3%)***
year 3	313 (32.4%)	396 (36.4%)	**281 (37.5%)***
year 4	260 (27.6%)	331 (30.1%)	**247 (32.2%)***
year 6	227 (18.9%)	**339 (23.0%)***	**266 (23.9%)****
ever (at age 6 years)	830 (85.0%)	**1059 (89.4%)****	**774 (90.0%)****
Age at focus visit (Median ± range; years)	6.0 (5.0-8.8)	**6.0 (5.0-9.0)*****	**6.1 (4.9-9.1)*****
BMI for age (mean; sd; SDS)	0.20 (0.85)	0.25 (0.92)	**0.39 (0.95)*****

Values are means (SD), absolute numbers (percentages) or #medians (90% range). Based on observed dataset. Significantly different from 0 infections *(p<0.05), ** (p<0.01) *** (p<0.001). Maternal atopy: eczema, allergy HDM, hay-fever. Any reported autoimmune disease: diabetes mellitus, SLE, arthritis, MS, thyroid disorder, or celiac disease

Chapter 5

CMV- AND EBV-INDUCED T-CELL EXPANSIONS IN YOUNG CHILDREN DO NOT IMPAIR NAIVE T-CELL POPULATIONS OR VACCINATION RESPONSES: THE GENERATION R STUDY

Running title: CMV and EBV T-cell expansion in children

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ABSTRACT

Background. Cytomegalovirus (CMV) and Epstein Barr virus (EBV) induce effector memory T-cell expansion, which are variable and potentially depend on the age at primary exposure and co-infections. We evaluated the T-cell compartment and herpesvirus infections in 6-year-old children.

Methods. T-cell subsets and IgG seropositivity for CMV, EBV, herpes simplex virus-1 (HSV-1) and varicella zoster virus (VZV) were studied in 1,079 6-year-old children. A random subgroup of 225 children was evaluated for CMV and EBV seropositivity before 2y of age and vaccination responses against measles and tetanus.

Results. CMV and EBV infections were associated with significant expansions of $CD27^-$ and $CD27^+$ effector memory T cells, respectively. These expansions were enhanced in CMV+EBV+ children and were independent of VZV or HSV-1 co-infection. Naive and central memory T-cell numbers were not affected, nor were anti-tetanus and anti-measles IgG levels. Children infected before 2y of age showed smaller effector memory T-cell expansions than children infected between 2-6y.

Conclusions. CMV- and EBV-related T-cell expansions do not impair naive T-cell numbers nor maintenance of protective responses against non-related pathogens. Duration of infection was not directly related to larger expansions of effector memory T cells in children, suggesting that other mechanisms affect these expansions at later age.

INTRODUCTION

Cytomegalovirus (CMV) and Epstein Barr Virus (EBV) are ubiquitous in the human population and persist with presumed viral latency.[1,2] Infection occurs mainly in childhood, reaching ~50% seropositivity around the age of 6y and 80-90% in adults within the Western world.[3,4] CMV and EBV are associated with changes in immunological memory. Infected adults display persistent expansions of virus-specific effector memory T cells in both the $CD8^+$ and the $CD4^+$ lineages.[5,6] CMV-specific $CD8^+$ T cells are predominantly $CCR7^-CD45RO^+CD27^{+/-}$ in young adults, while more clonal $CCR7^-CD45RA^+CD27^-$ populations are found in elderly.[5,7-14] EBV-specific T cells are mainly $CCR7^-CD45RO^+CD27^+$.[5,8,15,16] The majorities of these populations have an extended lifespan, but a poor response to T-cell mitogens.[7-9,16-18]

Co-infection with CMV and EBV can have both synergistic and antagonistic effects: CMV co-infection can restore defective vaccination responses in EBV-infected children, while EBV co-infection can boost CMV-induced NK-cell differentiation, and together they affect the chance for developing allergic complications.[19-21] In contrast, infections with herpes simplex virus 1 (HSV-1) do not result in persistent effector T-cell expansions.[15] Less is known for co-infection with varicella zoster virus (VZV), because this already reaches 90-100% seropositivity in young adulthood in the Western world.

Accumulations of CMV- and EBV-induced effector memory T-cells have been suggested to overcrowd T cells with other specificities,[5,22] and to negatively impact immune responses to other infections and/or vaccinations.[9,17,23-25] Moreover, CMV and EBV persistence are associated with cardiovascular disease, infectious complications and with increased mortality rates, especially in immunosuppressed individuals or the very elderly.[23,26-31] Still, these effects are not consistently observed,[32-35] and despite high CMV and EBV seropositivity rates in elderly, only a minority develops clinical complications. An explanation could be the variation in T-cell responses: virus-specific T-cell numbers vary from barely detectable to >30% of total memory T-cells in peripheral blood. This diversity might be affected by the infectious dose,[36] and with long-term infection, and thus especially develop in individuals who had been infected early in life. However, in contrast to infection after puberty, primary infections with CMV or EBV in early childhood are mostly asymptomatic and might even be protective against the development of Celiac disease and allergies.[37,38]

To study whether the developing immune system in childhood provides more effective control of persistent viruses,[39] we studied 1,079 6-year old children in the Generation R cohort. The combined analysis of CMV, EBV, HSV-1 and VZV infection in this large cohort allowed us to study virus-specific and modifying effects on the T-cell compartment and their relation with vaccination responses to tetanus and measles.

MATERIALS AND METHODS

Study subjects

This study was conducted in the context of the Generation R Study, a prospective population-based cohort study from fetal life until young adulthood.[40] We included 1,079 6-year-old children (range 5.0-7.9y) from whom CMV, EBV, HSV-1 and VZV IgG serology, and detailed immunophenotyping of blood T cells was performed. From a random selection of 225 of these children, additional virus serology and immunophenotyping was performed on blood samples obtained in the second year of life (age range 13.1-29.9 months). Written informed consent was obtained from all parents of participating children. Ethical approval for the study was obtained from the Medical Ethical Committee of the Erasmus MC.

Serology

Blood plasma samples were subjected to enzyme-linked immunoassays for IgG antibodies against CMV, EBV capsid antigen (EBV-VCA), HSV-1 glycoprotein C1, VZV, Tetanus toxoid or Measles antigen (EUROIMMUN®, Lübeck, Germany). Results were evaluated relative to a manufacturer-provided reference threshold sample. Seropositivity, and assumed virus persistence, was defined by a sample/threshold ratio above 0.6 (CMV), 0.8 (EBV-VCA) 1.6 (HSV-1) and 1.0 (VZV). Anti-measles and anti-tetanus IgG levels were analyzed in international units/ml (IU/ml) as a continuous variable and with plasma levels above 275 IU/ml (measles) or 0.5 IU/ml (tetanus) being defined as protective vaccination responses.

Immunophenotyping

Absolute numbers of $CD3^+$ T cells were obtained with a routine diagnostic lyse-no-wash protocol. Detailed analysis of T-cell subsets was performed with 6-color flowcytometry (Supplemental Table 1 and Supplemental Figure 1).[22,41]

Antigen-specific T-cells were detected in thawed post-Ficoll PBMC of 15 CMV+ and 14 EBV+ children who carried the HLA-A*0201 allele, defined by SNPtags rs2844821(G) and rs762324(C) in previously generated SNP arrays.[40,42] Virus-specific $CD8^+$ T cells were detected with HLA-A*0201 tetramers loaded with CMV peptides NLVPMVATV (from pp65; APC-labeled) or VLEETSVML (from IE-1; PE-labeled) proteins and EBV peptides GLCTLVAML (from BMLF-1; APC-labeled) or YVLDHLIVV (BRLF-1; PE-labeled). Flowcytometric data were acquired on a FACSCalibur or LSRII (BD Biosciences) and analyzed using FACSDiva software (BD Biosciences; version 6.2) and Infinicyte (Cytognos; version 1.7) analysis software.[43]

Statistical analyses

Differences in infection prevalence were assessed using Chi-square tests. Differences in lymphocyte numbers (or frequencies) between uninfected controls and virus-infected groups were assessed with a Kruskal-Wallis test, followed by post-hoc Dunn's tests. Differences in longitudinal frequencies of lymphocyte populations were assessed using paired t-tests. P-values <0.05 were considered statistically significant.

RESULTS

Herpesvirus seropositivity

Among the 1,079 children included in the study, 36.2% was IgG-seropositive for CMV, 47.1% for EBV, 14.0% for HSV-1 and 92.1% for VZV. Because mainly CMV and EBV are reported to result in chronic T-cell expansions, we divided the 1,079 children into 4 groups: CMV-EBV- (uninfected; n=399); CMV+EBV- (CMV+; n=172); CMV-EBV+ (EBV+; n=289); CMV+EBV+ (n=219) (Table 1).

Co-infection with VZV was >90% in all groups. HSV-1 infection was significantly more frequent in EBV+ (p=0.02) or CMV+EBV+ (p<0.001) children (Table 1). Therefore, especially HSV-1 infection needs to be considered for possible confounding effects in our analyses on EBV.

CMV- and EBV-specific effector memory T-cells

To analyze whether 6-year-old children carried virus-specific T-cell expansions, we phenotyped virus-specific $CD8^+$ T cells in 15 CMV+ and 14 EBV+ children using CD27 and CD45RA and HLA-A*0201 tetramers loaded with CMV-specific peptides of pp65 and IE-1 and EBV-specific peptides of BMLF-1 and BRLF-1 (Figure 1). Virus-specific $CD8^+$ T cells were detected in blood at frequencies of 0.01-0.52% (pp65-NLV) and 0.01-2.32% (IE-1-VLE) for CMV peptides and 0.02-0.64% (BMLF-1-GLC) and 0.02-2.6% (BRLF-1-YVL) for EBV peptides. The CMV- and EBV-specific $CD8^+$ T-cells were phenotypically diverse and predominantly consisted of $CD45RA^-CD27^{+/-}$ and $CD45RA^+CD27^-$ memory T-cells (Figure 1). Thus, 6-year old children already display expansions of effector memory T cells directed against CMV and EBV antigens.

CMV and EBV associated memory T-cell expansions

To study the effects of CMV and EBV on the T-cell compartment, we immunophenotyped $CD8^+$ and $CD8^-$ ($CD4^+$) T cells in 18-20 randomly-selected children being either uninfected, CMV+, EBV+ or CMV+EBV+. T-cell data from each group of children were merged and subjected to automatic population separation (APS) with Infinicyte software,[43] based on the expression of CD3, CD8, CCR7, CD45RO, CD27 and CD28. Within both $CD8^+$ and $CD4^+$ T cells, four populations were distinguished in 2D plots of principle component (PC)1 vs PC2 (Figure 2). CMV was associated with a relative increase of two $CD8^+$ populations: population 2 ($CCR7^-CD45RO^-$; 9.2% uninfected vs 15.1% CMV+) and population

Table 1. HSV-1 or VZV co-infection in correlation to CMV- and EBV-IgG seropositivity at 6 years of age.

	n	%	HSV+ n (% of group)	VZV+ n (% of group)	HSV+VZV+ n (% of group)
Uninfected	399	37.0	39 (9.8%)ref[a]	367 (92%)ref[a]	35 (8.8%)ref[a]
CMV+	172	15.9	18 (10.5%)	165 (95.9%)	18 (10.5%)
EBV+	289	26.8	**46 (15.9%)***	260 (90%)	**42 (14.5%)***
CMV+EBV+	219	20.3	**48 (21.9%)******	202 (92.2%)	**40 (18.3%)*****
total	1,079	100	151 (14%)	994 (92.1%)	135 (12.5%)

[a] Significance of co-infection in the CMV+, EBV+ or CMV+EBV+ group is tested relative to the CMV-EBV- uninfected controls, using the Chi-square test; *, p<0.05; ***, p<0.001; ****, p<0.0001.

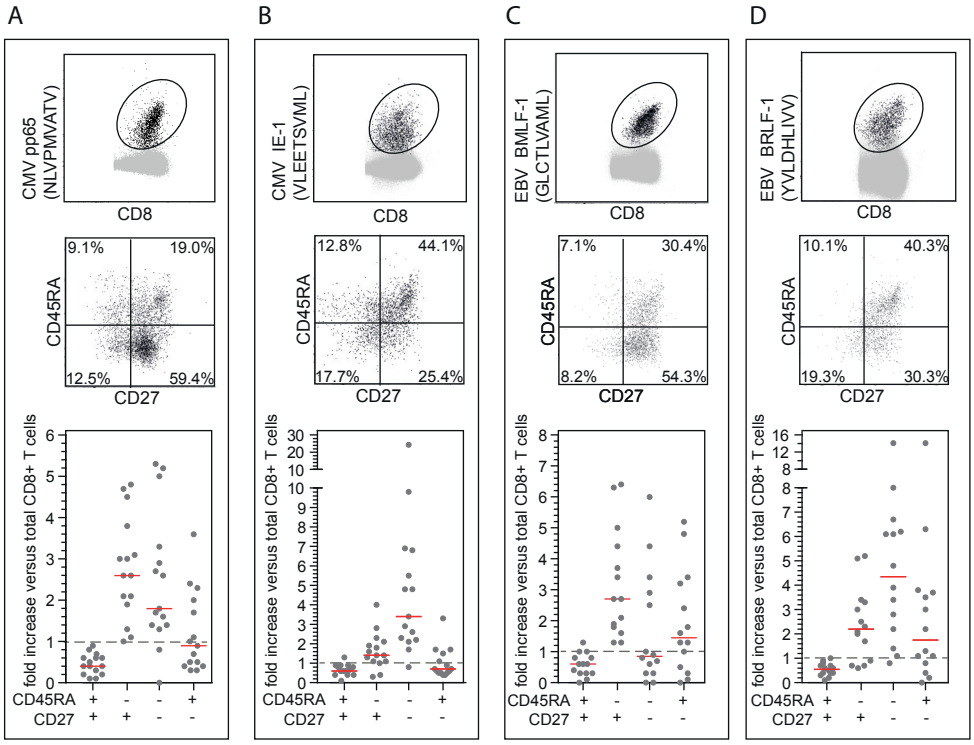
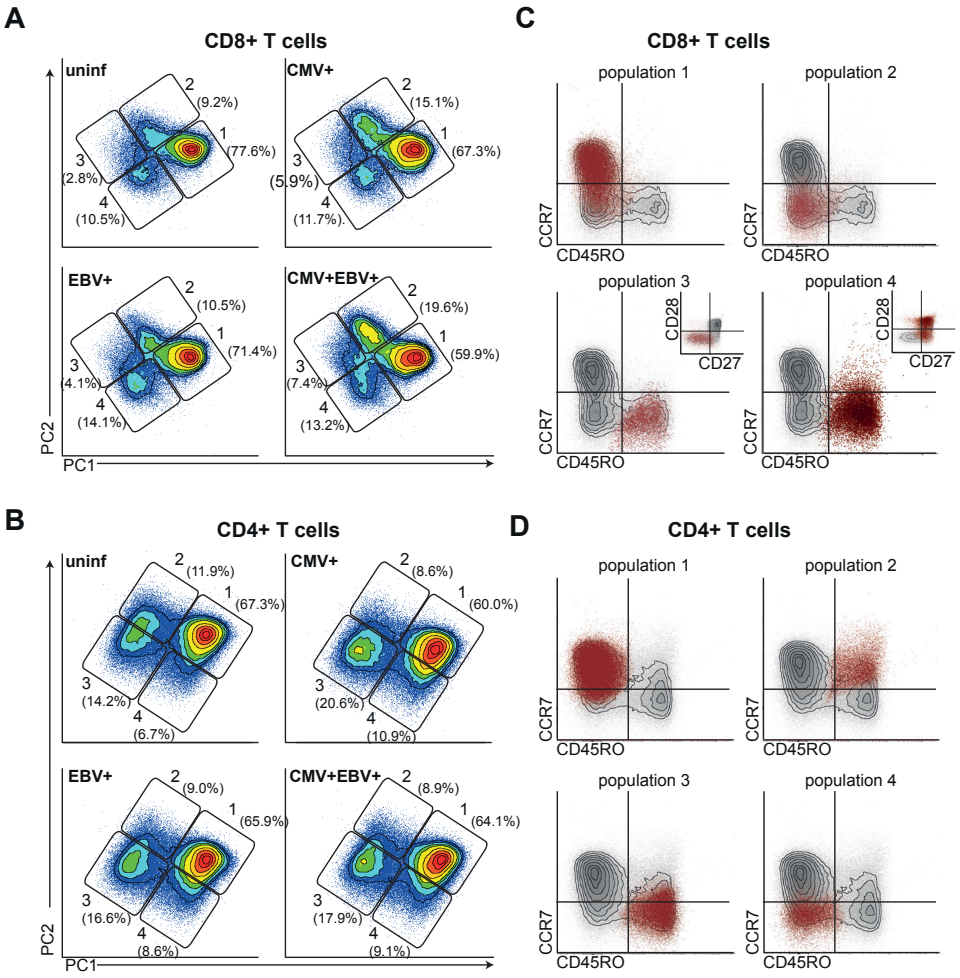


Figure 1. Frequencies and phenotypes of CMV- and EBV-specific CD8⁺ T cells in 6-year-old children. CD8⁺ T cells recognizing CMV- (**A-B**) or EBV-specific peptides (**C-B**) were detected with multicolor flowcytometry and further analyzed for CD27 and CD45RA expression. Flowcytometry plots consist of merged data files of 14 representative HLA-A*0201+ CMV+ (A-B) or EBV+ (C-D) children. The bottom graphs depict for each individual the relative distributions of tetramer-positive cells as fold increase compared to the relative distribution of total CD8⁺ T-cells.

3 (CCR7⁻CD45RO⁺; 2.8% uninfected vs 5.9% CMV+). EBV-infected children showed an increase in CCR7⁻CD45RO⁺ populations, both CD27⁻ (population 3; 2.8% uninfected vs 4.1% EBV+) and CD27⁺ (population 4; and 10.5% uninfected vs 14.1% EBV+, respectively). CMV- and EBV-infections were also associated with CD4⁺ T-cell memory expansions: CCR7⁻CD45RO⁺ (population 3; 14.2% uninfected; 20.6% CMV+; 16.6% EBV+) and CCR7⁻CD45RO⁻ (population 4; 6.7% uninfected; 10.9% CMV+; 8.6% EBV+) (Figure 2B/D). Thus, CMV and EBV infection in young children are associated with relative expansions of memory T cells.

Normal numbers of naive and central memory T cells in CMV- or EBV-infected children

To quantify whether the relative memory T cell expansions in CMV- and/or EBV-carriers also affected absolute T-cell numbers, we next evaluated the CD4⁺ and CD8⁺ T-cell lineages in 1,079 6-year-old children. CCR7⁺CD45RO⁻CD27⁺CD28⁺ naive T cells were distinguished from CCR7⁺CD45RO⁺CD27⁺CD28⁺ central memory T cells (Tcm), CCR7⁻CD45RO⁺ effector memory (TemRO) and CCR7⁻CD45RO⁻ effector memory (TemRA) (Supplemental Figure 1A).[22,41] TemRO and



CD8+ T cells

	PC1	PC2
CCR7	**32.7**	3.8
CD45RO	**23.1**	**35.5**
CD27	18.0	19.3
CD28	13.7	**25.8**

CD4+ T cells

	PC1	PC2
CCR7	**20.5**	**36.9**
CD45RO	**48.2**	15.8
CD27	10.6	12.4
CD28	4.4	**20.4**

Figure 2. Relative change in the T-cell phenotype in CMV- or EBV-infected children.
Unbiased flowcytometric analyses of $CD8^+$ T cells (**A**) and $CD8^-$ T cells (**B**) in uninfected (uninf), CMV+, EBV+ and CMV+EBV+ children. Data from 18-20 children in each group were merged into one file, and subjected to automatic population separation (APS) based on the expression of CD3, CD8, CCR7, CD45RO, CD27 and CD28. 2D projections of principle component (PC)1 versus PC2 revealed four populations. The relative contributions of CCR7, CD45RO, CD27 and CD28 to PC1 or PC2 are indicated. **C and D)** CD45RO vs CCR7 expression of the populations defined by APS. Populations 3 and 4 in panel C were both CCR7-CD45RO+, but differed in CD27 and CD28 expression (small inset).

TemRA CD8$^+$ T cells were significantly increased in children with CMV or EBV infection compared to uninfected controls (Figure 3B). For CMV, these specifically concerned a 4.4 fold increase of CD27$^-$CD28$^-$ late differentiated TemRA cells and 1.3-3.3 fold increase of CD27$^{-/+}$CD28$^-$ intermediate to late-differentiated TemRO cells over uninfected controls (Figure 3C-D). EBV-infected children showed a 1.2-1.3 fold increase in CD27$^{-/+}$CD28$^-$ intermediate to late-differentiated TemRA cells over uninfected controls and a 1.4-1.8 fold increase in all TemRO subsets (Figure 3C-D). Naive CD8$^+$ T cells and CD8$^+$ Tcm cells were present in normal numbers. Thus, the expansions of effector memory cells resulted in a significant 1.1 fold increase in the total number of CD8$^+$ T cells (Figure 3A). Combined CMV and EBV infections resulted in a further increase in total CD8$^+$ T-cell numbers (Figure 3A), with seemingly additive effects of the two viruses in double-infected children versus the single-infected children (Figure 3). Thus, in these 6-year-old children, infections with CMV and EBV were both associated with T-cell memory expansions. The phenotypes of the expanded populations differed for each of the viruses, did not affect each other in CMV+EBV+ double infection, and did not result in loss of naive CD8$^+$ T cells.

Total CD4$^+$ T-cell numbers were not affected by CMV and/or EBV infection (Supplemental Figure 1B). Still, EBV-infected children had 1.5 fold more CD27$^-$CD28$^-$ late-differentiated TemRO cells than uninfected controls. Furthermore, CMV-infected children showed a 1.3-2 fold increase in CD27$^-$ intermediate and late-differentiated TemRO cells, and a 1.9 fold increase in CD27$^-$CD28$^-$ late-differentiated TemRA cells over uninfected controls (Supplemental Figure 1B). Similar to CD8$^+$ T cells, CMV and EBV infection independently resulted in effector memory CD4$^+$ T-cell expansions, which did not affect naive T-cell numbers.

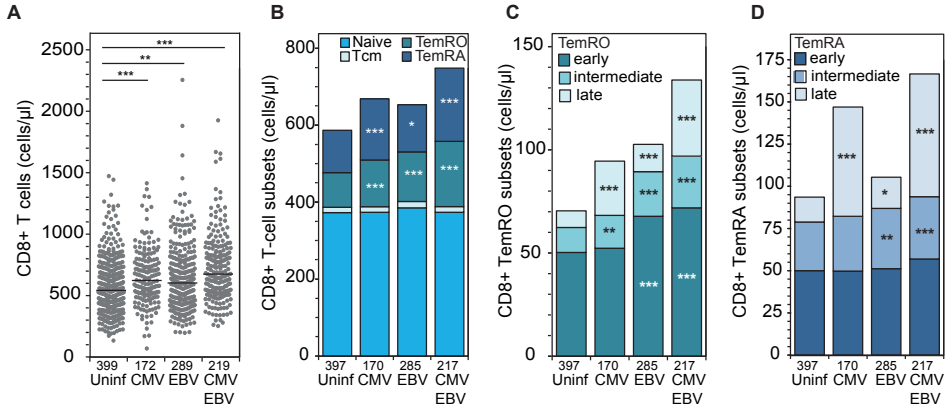


Figure 3. Absolute numbers of CD8$^+$ T-cell subsets in CMV- or EBV-infected children.
A) Absolute numbers of total CD8$^+$ T-cells in children uninfected with CMV or EBV (uninf), infected with only CMV, only EBV or CMV and EBV. **B)** Similar as in A, for 4 main CD8$^+$ T-cell subsets: CCR7$^+$CD45RO$^-$CD27$^+$CD28$^+$ Naive, CCR7$^+$CD45RO$^+$CD27$^+$CD28$^+$ Central memory (Tcm), CCR7$^-$CD45RO$^+$ Effector memory (TemRO) and CCR7$^-$CD45RO$^-$ Effector memory (TemRA) cells [41]. **C-D)** Similar as in A, for CD27$^+$CD28$^+$ early, CD27$^+$CD28$^-$ intermediate and CD27$^-$CD28$^-$ late TemRO (C) and TemRA (D) populations [22]. Bars depict stacked median values per T-cell population. The number of individuals per category is indicated underneath each plot. Significance was tested first by a Kruskal-Wallis test per T-cell population relative to the uninfected controls, and in case of significance ($p<0.05$) followed by a Dunn's test of individual patient groups. Significance for the Dunn's test is indicated in the plots: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

Effect of HSV-1 and VZV on T-cell subset numbers

Through combined analysis of children with single CMV, or single EBV, and double CMV/EBV infection, we could distinguish distinct effects of these viruses on the T-cell compartment in young children. Still, the observed effects could be influenced by infection with other viruses, such as HSV-1 or VZV, especially considering the increased HSV-1 positivity in EBV+ children (Table 1). Therefore, we separated the groups of uninfected controls, CMV+, EBV+ and CMV+EBV+ children further into VZV-HSV-1-, VZV+HSV-1 and VZV+HSV-1+ subgroups. Because VZV prevalence was >90% in our cohort, the effect of HSV-1, without VZV, could not be determined. Though the TemRO and TemRA populations were significantly different between all virus-infected groups (Kruskal Wallis; $p<0.0001$), these effects were only caused by CMV- or EBV-associated expansions. The presence of VZV and HSV-1 within the CMV+ or CMV+EBV+ group was only associated with a slight, but not significant increase of TemRA cells (Figure 4). Thus, co-infection with VZV and HSV-1 did not significantly affect CMV- or EBV-associated effector memory T-cell expansion.

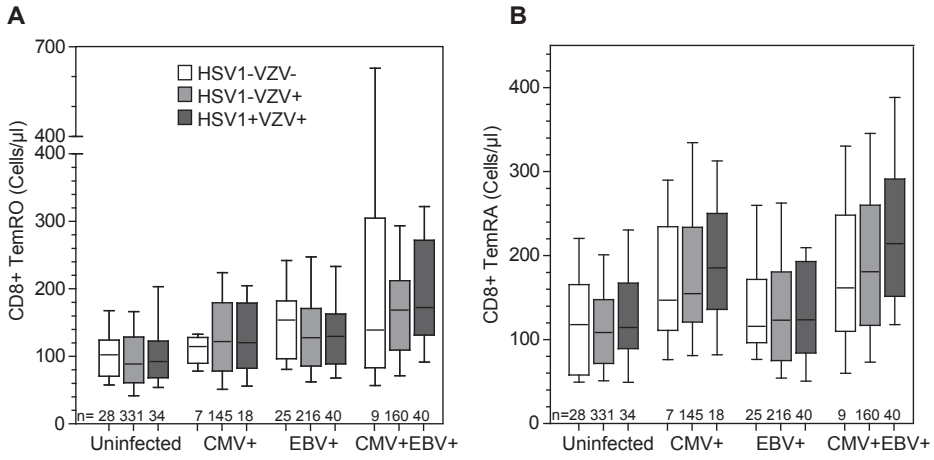


Figure 4. Effect of HSV-1 and VZV on blood CD8⁺ TemRO and TemRA cell numbers.
Uninfected, CMV+, EBV+ and CMV+EBV+ children were further subdivided in children without HSV-1 and VZV (white), co-infected with only VZV (light gray) or with both HSV-1 and VZV (dark gray). Absolute numbers of CD8⁺ TemRO **(A)** and TemRA **(B)** cells in all population groups depicted as 10-90% box-whisker-plots. The number of individuals per category is indicated underneath each plot. Significance was tested first by a Kruskal-Wallis test per T-cell population, and in case of significance ($p<0.05$) followed by a Dunn's test of individual patient groups, comparing HSV-1 and/or VZV subgroups within each category of uninfected, CMV, EBV or CMV+EBV+ children.

Normal vaccination responses in CMV- or EBV-infected children

The effector memory T-cell expansions in CMV- or EBV-infected children did not result in a reduction of naive and Tcm numbers (Figures 2 and 3). To study if immunological memory to other pathogens was normally present in these 6-year-old children, we tested their responses to previous vaccinations with tetanus at 2, 3, 4, 11 months and 4 years, and measles at 14 months according to the Dutch national vaccination protocol.[44] We defined vaccination responses in a randomly selected subgroup of 225 6-year-old children that showed similar seroprevalence for CMV and EBV (Supplemental Table 2), and similar effector memory T-cell

expansions (data not shown) as the total cohort of 1,079 children. Furthermore, the seroprevalence of co-infection with HSV-1 and VZV was not significantly different between the CMV+ or EBV+ groups in this selected cohort (Supplemental Table 3). In total, 81.2% of the children had IgG antibodies against measles, and 63.2% against tetanus. These frequencies were not significantly different in children with CMV and/or EBV infection (Figure 5). Furthermore, median titers of anti-measles and anti-tetanus IgG did not differ between the four groups. Thus, EBV and CMV seropositivity did not impair immunity to measles and tetanus vaccination in 6-year-old children.

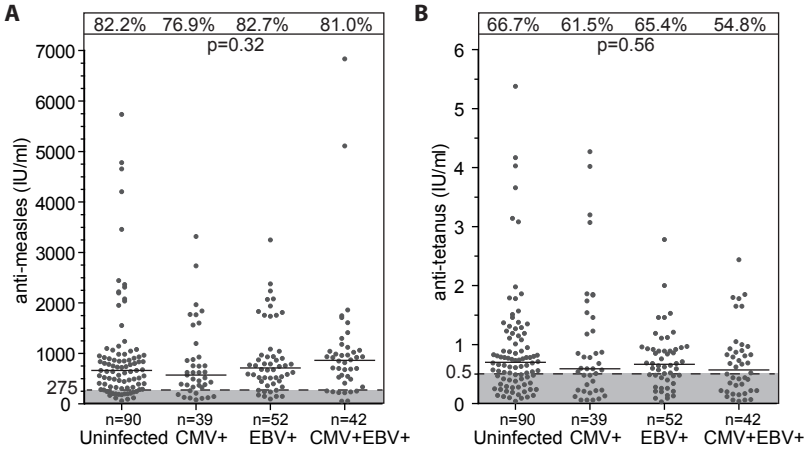


Figure 5. Vaccination responses in 6-year-old CMV or EBV infected children.
Anti-measles **(A)** and anti-tetanus **(B)** IgG levels in uninfected, CMV+, EBV+ or CMV+EBV+ children. Specific IgG levels were not significantly different between the virus positive groups as compared with uninfected controls as determined with the Kruskal-Wallis test; p-values are indicated at the top right of the plot. Percentages at the top represent the number of children with a protective anti-measles IgG response >275 international units/ml (IU/ml) (A) or anti-tetanus IgG response >0.5 IU/ml (B). Percentages were not significantly different as tested by Chi-square test.

No association between T-cell expansions and duration of CMV or EBV infection

Our results indicate that the EBV- and CMV-specific T-cell expansions do not affect naive T-cell numbers or vaccination responses to tetanus and measles in 6-year old children. To study whether infection early in childhood has different immunological consequences than infection later in childhood, we determined CMV and EBV seropositivity around 2 years of age in a subgroup of 225 children. We observed that of the CMV+EBV+ children, 80% already carried anti-CMV IgG before 2 years of age, and 46.8% anti-EBV IgG.

We subdivided the EBV+ and CMV+ children into early-infected (before 2y) and late-infected subgroups (2-6y), and compared their T-cell compartments at 6 years of age. Early nor late infection affected naive and Tcm cell numbers (not shown), whereas $CD4^+$ and $CD8^+$ effector memory T cells were significantly increased (Figure 6 and Supplemental Figure 1C). CMV+EBV+ early-infected children had significantly smaller $CD8^+$ TemRO and TemRA T-cell populations than late-infected children, with numbers in early-infected children being similar to uninfected controls (Figure 6A). Moreover, retrospective longitudinal analysis of early-infected children indicated that $CD8^+$ effector memory T-cell numbers

were stable between the age of 2y and 6y (Figure 6B). CD4+ effector memory T-cell numbers did not significantly differ between early-infected and late-infected children. Still, some subsets in late-infected children were significantly higher than in uninfected controls, whereas these were not increased in early-infected children (Supplemental Figure 1C). Furthermore, CD4+ effector memory T-cell numbers increased between the ages of 2y to 6y in early-infected children (Supplemental Figure 1D). Together, these results suggest that CD8+ effector memory T-cell numbers are not directly related to the time post infection. Rather, the early infections were associated with smaller expansions of effector memory T cells at 6 years.

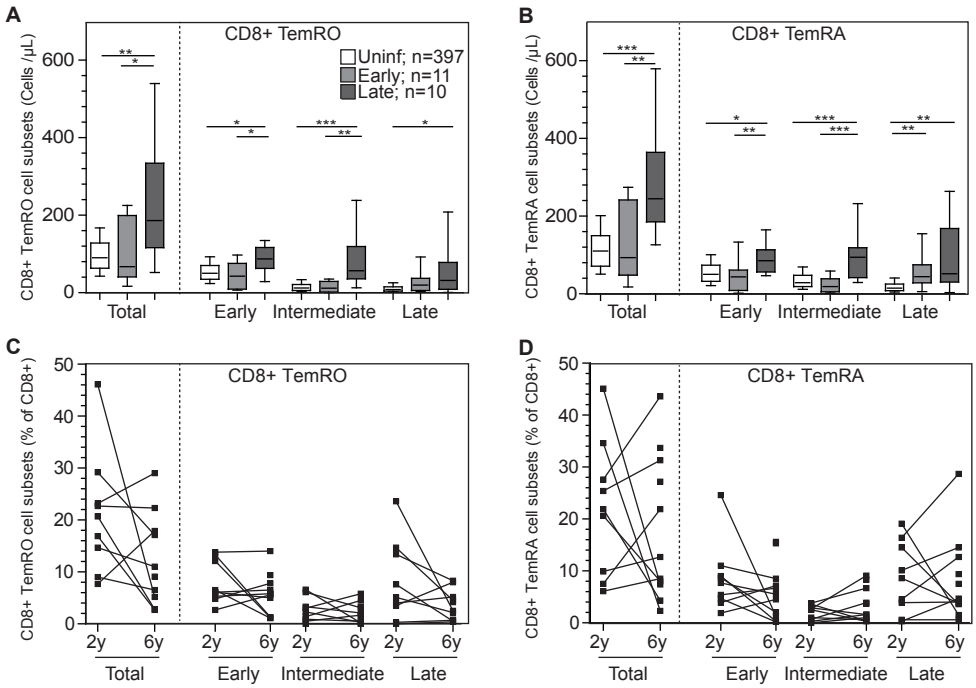


Figure 6. Limited effector memory T-cell expansions in children infected with EBV and CMV <2y. 6-Year old CMV+EBV+ children were subgrouped based on infection <2y of age (early; light gray; n=11) and >2y of age (late; dark gray; n=10), and compared to CMV-EBV- uninfected controls (white; n=397). **A-B)** Absolute number of CD8+ TemRO (A) and TemRA (B) cell subsets. Plots depict 10-90% box-whisker plots. Significance was tested first by a Kruskal-Wallis test per T-cell population, and in case of significance ($p<0.05$) followed by a Dunn's test of individual patient groups. Significance for the Dunn's test is indicated in the plots: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. **C-D)** Longitudinal follow-up of frequencies of CD8+ TemRO (C) and TemRA (D) subsets at the age of 2y and 6y in early infected children. No significant differences between 2y and 6y could be observed by paired T-test ($p>0.12$).

DISCUSSION

We studied the effects of single or combined infection with CMV, EBV, HSV-1 and/or VZV on naive and memory T cells in a large cohort of more than 1,000 6-year-old children. CMV and EBV infections each resulted in distinct effector memory T-cell expansions, which were additive in case of co-infection. HSV-1 and VZV infections did not significantly affect the T-cell compartment, but might slightly

enhance CMV- and EBV-associated T-cell expansions. In contrast to adults, $CD8^+$ Tem-cell expansions (either TemRO or TemRA) in CMV- or EBV-infected children did neither result in overcrowding of naive and Tcm compartments, nor in loss of vaccination responses to tetanus or measles. Notably, children infected <2y showed fewer Tem cells at 6-years than children infected between 2-6y. Thus, depending on the age at the time of infection, CMV and EBV infections seem to be controlled differently.

We, here, detected sizeable CMV- and EBV-specific $CD8^+$ memory T-cell expansions in 6-year-old children. Unfortunately, the real extent of virus-specific T-cell expansions is difficult to assess due to the large number of viral epitopes and the diverse HLA backgrounds of the children in our study. However, since 1) the phenotype of the antigen-specific T cells is largely similar to the phenotype of the expanded effector memory T-cell populations, both relatively and absolute, within the total $CD8^+$ T-cell pool in our cohort of children, and 2) the antigen-specific phenotypes are in accordance with previous literature on CMV and EBV infections in adults,[45] we are convinced that phenotyping the total $CD8^+$ T-cell compartment generates an reliable representation of virus-associated changes of the immune compartment.

Our observation that CMV and EBV were associated with an increase of distinct $CD8^+$ Tem subsets is consistent with previous literature in adults and children.[5,7-12,15,46,47] Importantly, our large cohort allowed us to further analyze the possible antagonistic, synergistic or independent impact of CMV and EBV on the immune compartment. Importantly, in the group of CMV and EBV co-infected children, an additive effect was seen for the Tem expansions compared to that seen in single-infected patients. Furthermore, the size of the CMV-associated TemRA late population was not (negatively) affected by the size of the EBV-associated TemRO populations in individual patients (data not shown). Thus, the distinct effects of CMV and EBV on $CD8^+$ Tem expansions, appear to be independent of each other.

In addition to $CD8^+$ Tem expansions, we found that $CD4^+$ Tem-cell numbers were increased in both CMV and EBV infections, which is similar to observations in adults.[6] Although these expansions were smaller and did not result in a significant increase in total $CD4^+$ T-cell numbers, they were distinct between both viruses. Furthermore, the phenotypes of the $CD4^+$ Tem expansions were remarkably similar to their $CD8^+$ counterparts and were additive in CMV and EBV double positive children. Despite these seemingly minor expansions, $CD4^+$ T cells have an important role in controlling primary CMV infection.[48] Apparently, the formation of sizeable Tem populations is necessary to successfully suppress the virus. Importantly, these expansions did not result in overcrowding of more immature T-cell subsets, something that for CMV infection in elderly has been associated with poor $CD4^+$ memory T-cell responses to influenza proteins.[24]

Our large cohort allowed us to study the effects of HSV-1 and VZV in EBV and CMV double negative children, as well as any modifying effects on CMV and/or EBV infections. Because virtually all adults are infected with VZV, little is known on the effects on T-cell memory[15] and we could for the first time conclude that VZV does not modify T-cell memory alone or in combination with CMV and/or EBV. Co-infection with HSV-1 was more frequent in EBV+ and CMV+EBV+ children and might therefore be an important contributor to the immune modulation that is currently assumed to be EBV-associated. Still, HSV-1 did not affect Tem in EBV+

children, suggesting that these expansions are EBV-specific and not due to HSV-1. The difference in T-cell modulation between CMV and EBV on the one hand and VZV and HSV-1 on the other hand is most likely due to tropism and anatomical localization of the viruses. In fact, it is well-possible that HSV-1 and VZV induce T-cell expansions, but these cells are thought to reside mostly in the human skin or locally around virus-infected cells.[49]

Despite the expansions of $CD8^+$, and $CD4^+$, Tem in children infected with CMV and/or EBV, these did not result in decreased naive or Tcm cell numbers. In fact, infected children had significantly more total $CD8^+$ T cells. Especially in immunosuppressed individuals, but also in CMV-infected elderly, effector memory T-cell expansions with a subsequent loss of naive T cells and loss of vaccination responses have been described to be hallmarks of CMV-associated immunosenescence.[9,17,24,25] However, literature is inconsistent,[32-35] and the loss of vaccination responsiveness in children, is not consistently observed.[19] In our cohort, IgG titers to previous measles and tetanus vaccinations were similar between uninfected and infected children. Combined, our data suggest that 6-year-old CMV- and/or EBV-infected children do not lose their immune responsiveness due to virus-related effects.

An important explanation for the discrepancy in the literature on the loss of naive T cells could be differences in analysis strategies. In fact, due to the Tem expansions, the relative proportions of naive and Tcm are decreased in infected children in our study. Moreover, the expansion of total $CD8^+$ T-cell numbers causes a shift in the CD4:CD8 T-cell ratio. These relative shifts are, however, the results of data analysis, rather than real defects, because the absolute naive $CD8^+$ and total $CD4^+$ T-cell numbers were normal. This is why we have for long time advocated for consistent analysis of absolute cell numbers with age-matched controls.[50]

Although 90% of elderly are infected with CMV and EBV, only a minority of these individuals shows loss of naive T-cell numbers, impaired vaccination responses and clinical complications. The reason why only a minority develops complications is still unclear. Our study at the age of 6y indicates that early infection before the age of 2y results in smaller Tem expansions than infection between 2-6y. Since the size of the expanded virus-specific Tem pool has been directly correlated to the extent of CMV-induced endothelial damage, the children that have been infected before 2y might have a reduced risk for developing cardiovascular complications.[31] CMV-related clinical complications might occur only in individuals infected later in life and who show the strongest CMV-associated T-cell expansions. An important factor influencing the variability in the response might be the infectious dose.[36] It would therefore be interesting to correlate the age at infection and the subsequent size of the Tem-cell expansions with the infectious dose and the development of clinical complications in children and adults, in a separate cohort or in longitudinal follow-up of the uninfected children in our cohort.

In conclusion, we here provide evidence that in young children, CMV and EBV infections lead to sizeable Tem expansions, but not to the associated immunosenescence. In fact, the Tem expansion result in increased total T-cell numbers, while naive and Tcm remain normally present, as are responses to previous vaccinations. Moreover, 6-year old children who were infected prior to 2 year of age maintained a stable control of these persistent viruses with only limited Tem expansions. These new insights into the immuno-modulatory effects of herpesviruses in young children are important to understand herpesvirus-associated immunosenescence in elderly.

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CONFLICT OF INTEREST

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D.v.d.H., W.A.D., J.J.M.v.D. and M.C.v.Z designed the experiments; D.v.d.H., M.A.E.J., H.B.-C., D.Z., K.A.M.v.K., M.A.W.S-t.N. and M.J.K.-C. performed and analyzed most of the experiments and contributed to data analyses; V.W.V.J. and H.A.M. designed the Generation R study and coordinated material collection. R.A. contributed to phenotyping virus-specific T-cell populations. D.v.d.H. and M.C.v.Z. wrote the manuscript; and all authors commented on the manuscript.

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Supplemental Table 1. Antibody details

Antibody	clone	manufacturer
CD45-PerCP	2D1	BD Biosciences
CD3-FITC	SK7	BD Biosciences
CD3-PerCP	SK7	BD Biosciences
CD4-PE-Cy7	SK3	BD Biosciences
CD8-APC-H7	SK1	BD Biosciences
CD8-PE-Cy7	SK1	BD Biosciences
CD45RO-APC	UCHL-1	BD Biosciences
CD28-FITC	CD28.2	BD Biosciences
CD27-APC-H7	M-T271	BD Biosciences
CD197(CCR7)-PE	3D13	e-Bioscience

Supplemental Table 2. CMV- and EBV- IgG seropositivities in the total and selected cohorts

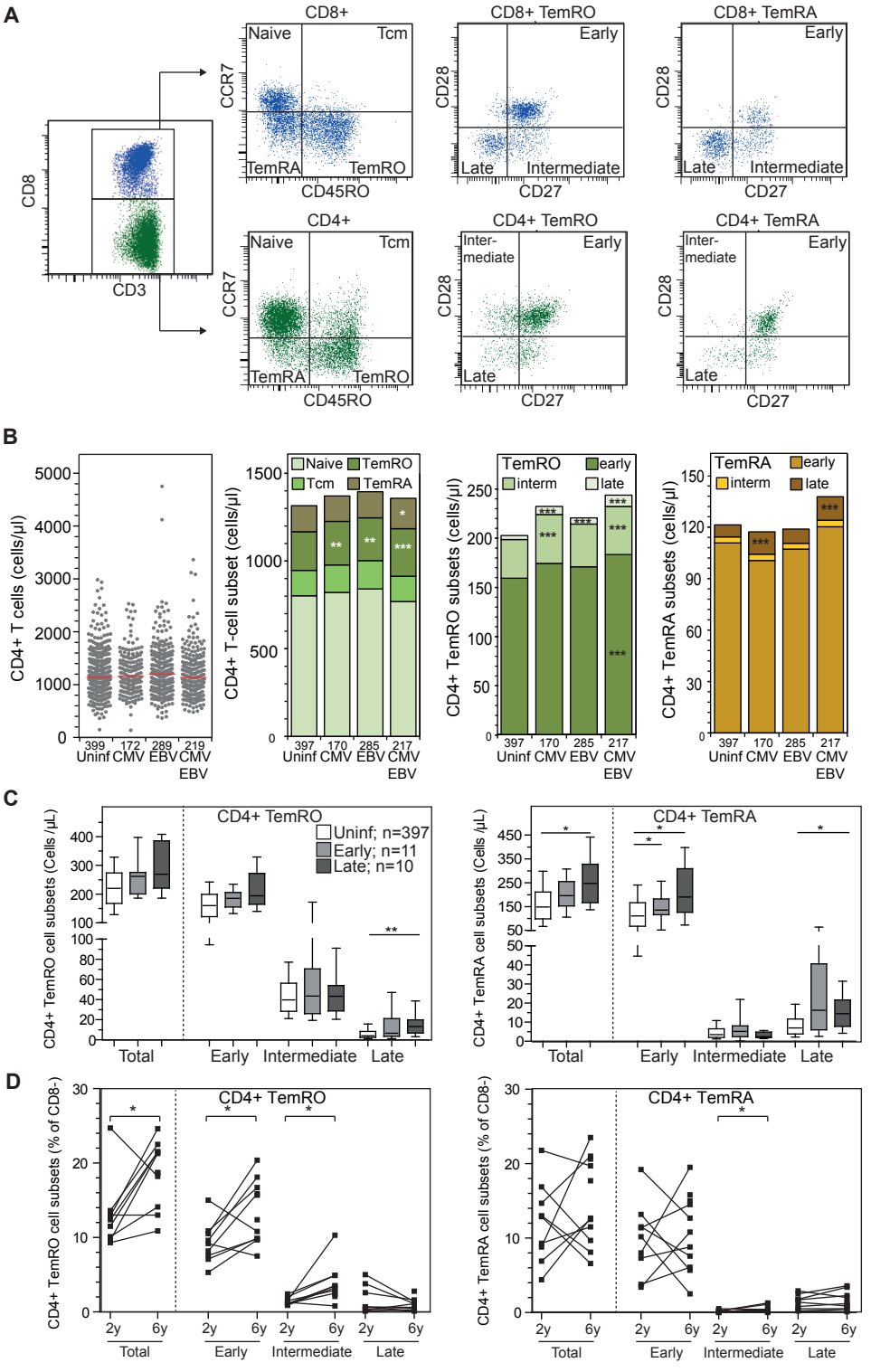
	Total cohort n(%)	Selected cohort n(%)
Uninfected	399 (37.0)	90 (40.0)
CMV+	172 (15.9)	41 (18.2)
EBV+	289 (26.8)	52 (23.1)
CMV+EBV+	219 (20.3)	42 (18.7)
total	1,079	225

No significant differences were observed in IgG seropositivity between the two cohorts using the Chi-square test.

Supplemental Table 3. HSV-1 or VZV co-infection in relation to CMV- and EBV-carriership.

	n	%	HSV+ n(% of group)	VZV+ n(% of group)	HSV+VZV+ n(% of group)
Uninfected	90	40.0	9 (10%)ref[a]	83 (92.2%)ref[a]	8 (8.9%)ref[a]
CMV+	41	18.2	5 (12.2%)	41 (100%)	5 (12.2%)
EBV+	52	23.1	10 (19.2%)	48 (92.3%)	9 (17.3%)
CMV+EBV+	42	18.7	6 (14.3%)	39 (92.9%)	6 (14.3%)
total	225	100.0	30 (13.3%)	211 (93.8%)	28 (12.4%)

[a] No significant differences were observed of co-infection in the CMV+, EBV+ or CMV+EBV+ groups relative to the CMV-EBV- uninfected controls using the Chi-square test.


A
CD8+
Naive
Tcm
TemRA
TemRO
CCR7
CD45RO
CD8
CD3
CD8+ TemRO
Early
Late
Intermediate
CD28
CD27
CD8+ TemRA
CD4+
CD4+ TemRO
Inter-
mediate
CD4+ TemRA
B
CD4+ T cells (cells/µl)
Uninf CMV EBV CMV
EBV
CD4+ T-cell subset (cells/µl)
TemRO
early
interm
late
CD4+ TemRO subsets (cells/µl)
TemRA
CD4+ TemRA subsets (cells/µl)
C
CD4+ TemRO cell subsets (Cells /µL)
Uninf; n=397
Early; n=11
Late; n=10
Total
CD4+ TemRA cell subsets (Cells /µL)
D
CD4+ TemRO cell subsets (% of CD8-)
2y
6y
CD4+ TemRA cell subsets (% of CD8-)

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Supplemental Figure 1. CD4$^+$ T cell subset analyses

A) Gating strategy for delineating T-cell subsets. Total CD3$^+$ T cells were subdivided into CD8$^+$ (blue) and CD8$^-$ (CD4$^+$; green) T cells. Both CD4$^+$ and CD8$^+$ T cells were further subdivided into CCR7$^+$CD45RO$^-$ Naive, CCR7$^+$CD45RO$^+$ central memory (Tcm), CCR7$^-$CD45RO$^+$ effector memory (TemRO) and CCR7$^-$CD45RO$^-$ effector memory (TemRA) cells [41]. TemRO and TemRA subsets were further subdivided into CD27$^+$CD28$^+$ early, CD27$^+$CD28$^-$ (CD8$^+$) or CD27$^-$CD28$^+$ (CD4$^+$) intermediate and CD27$^-$CD28$^-$ late populations [22]. **B)** Absolute numbers of total CD4$^+$ T-cells and CD4$^+$ T-cell subsets in children uninfected with CMV or EBV (uninf), infected with only CMV (CMV), only EBV (EBV) or both CMV and EBV (CMV EBV). Bars depict stacked median values per T-cell population. The number of individuals per category is indicated underneath each plot. **C)** Absolute numbers of CD8$^+$ TemRO (left) and TemRA (right) cell subsets in 6-year-old children who were either CMV-EBV-, became CMV+EBV+ before the age of 2y (early) or CMV+EBV+ between 2 and 6y of age (late). Plots depict 10-90% box-whisker plots. **B-C**) Significance was tested first by a Kruskal-Wallis test per lymphocyte population, and in case of significance ($p<0.05$) followed by a Dunn's test of individual patient groups. Significance for the Dunn's test is indicated in the plots: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. **D)** Longitudinal follow-up of frequencies of CD8$^-$ (CD4$^+$) TemRO (left) and TemRA (right) subsets at the age of 2y and 6y in 9 early infected children. Significance between 2y and 6y was tested by paired T-tests: *, $p<0.05$.

Chapter 6

TRANSIENT REDUCTION IN IGA$^+$ AND IGG$^+$ MEMORY B-CELL NUMBERS IN YOUNG CHILDREN PERSISTENTLY INFECTED WITH EBV: THE GENERATION R STUDY

Running title: EBV-associated depletion of memory B cells

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In preparation

ABSTRACT

The Epstein Barr virus (EBV) can persists both in IgM^+ and in Ig class-switched memory B cells. However, it remains unclear whether EBV infection results in depletion of these target cells. We here studied the presence of EBV genomes in 6 memory B-cells subset in adults, and we investigated how EBV infection during the first year of life affects the numbers of these memory B cells in young children at the ages of 14 months and 6 years. EBV genomes were equally numerous in $CD27^+IgG^+$, $CD27^+IgA^+$ and $CD27^-IgA^+$ memory B cells, and to a lesser extent in IgM-only, natural effector and $CD27^-IgG^+$ B cells. The blood counts of all memory B-cell subsets were highly dynamic in children in the first two years of life with peak levels around 14 months of age. IgM-only, $CD27^+IgG^+$, $CD27^+IgA^+$ and $CD27^-IgA^+$ memory B-cell counts at 14 months of age were significantly lower in EBV seropositive children than in uninfected controls. However, at 6 years, these counts were normalized, as were plasma IgG levels to previous primary measles and booster tetanus vaccinations. Thus, EBV persists mainly in Ig class-switched memory B-cells, even when derived from T-cell independent responses ($CD27^-IgA^+$), and results in transient depletion of these cells in young children. Combined, our studies demonstrate the impact of EBV infection on immunological memory in young children, as well as the plasticity of the immune system to overcome this.

INTRODUCTION

The Epstein Barr virus (EBV) is a gamma-herpesvirus that is ubiquitous in the human population. Primary infection occurs predominantly in childhood, after which EBV establishes life-long persistence by inducing viral latency in B cells.[1,2] EBV targets B cells through interactions with complement receptor 2 (CR2; CD21), MHC-II co-receptor surface molecules, or the Beta-1 integrin.[3-5] Even though all B cells express these molecules, EBV persists preferentially in memory B cells.[1,6] It is thought that this is the result of EBV infection in activated B cells that undergo an immune response after which the virus persists in the long-lived memory B-cell compartment.[1,7-9] Recent studies, however, indicate that both activated naive and memory B cells can be infected,[10] and that EBV can establish persistence in the absence of fully functional germinal center activity.[11]

Primary EBV infection induces a strong expansion of memory B cells that normalizes within 1 week after the appearance of clinical complications.[12] It is likely that infected cells are killed by the virus or anti-viral immune responses. However, there is limited information on how EBV infection and persistence affect the memory B-cell compartment.[9,12] Importantly, about 40% of EBV infections occur in the first 5 years of life,[13,14] during which the memory B-cell compartment is gradually build up.[15] Human memory B cells are phenotypically diverse. The majority originates from T-cell dependent germinal center responses: $CD27^+IgD^-IgM^+$ 'IgM-only', $CD27^-IgG^+$, $CD27^+IgG^+$ and $CD27^+IgA^+$ memory B cells.[16] In addition, T-cell independent responses in the splenic marginal zone and the intestinal lamina propria can generate $CD27^+IgD^+IgM^+$ 'natural effector' and $CD27^-IgA^+$ memory B cells, respectively.[16-18]

We here studied EBV persistence in all 6 memory B-cell subsets and investigated how EBV infection during the first year of life affects the memory B-cell counts in young children at the ages of 14 months and 6 years.

METHODS

Detection of EBV genomes in isolated memory B-cell subsets in EBV+ adults

Buffy coat material of 5 EBV-positive adults was obtained from Sanquin (Amsterdam, NL) and mononuclear cells were isolated by Ficoll-density gradient separation. Genomic DNA of 3.0 $x10^6$ mononuclear cells was isolated (Sigma-Aldrich) to determine the presence of EBV genomes with a quantitative PCR directed against specific for BALF5 (EBV DNA polymerase).[6,19]

The remaining post-Ficoll mononuclear cells were presorted using human $CD19^+$ MicroBeads (Miltenyi Biotech) on an autoMACS system and stored in liquid nitrogen. From EBV-positive donors, the $CD19^+$ B cells were thawed and B-cell subsets were purified after labeling with CD38-PE-Cy7 (clone HB7), CD24-APC-H7 (ML5), CD19-PerCP-Cy5.5 (SJ25C1), CD27-APC (L128), IgD-PO (IA6-2), IgM-PB (G20-127), IgG-PE (G18-145; all from BD Biosciences), and IgA-FITC (IS11-8E10; Miltenyi Biotech). Cell sorting was performed on a FACS Aria I (BD Biosciences) using standardized measurement settings.[20] Genomic DNA was isolated, and EBV genome copies per $1x10^6$ isolated cells were determined as described above.

The Generation R population cohort

Analysis of EBV infection in young children was conducted in the context of the Generation R study, which is a prospective population-based cohort study from fetal life until young adulthood.[21,22] In this study, a subgroup of 1,182 Dutch children were included, who were all born between August 2003 and August 2006. Ethical approval for the study was obtained from the Medical Ethical Committee of the Erasmus MC, University Medical Center Rotterdam. Written informed consent was obtained from all parents of participating children.

Flowcytometric Immunophenotyping

Peripheral blood was obtained at the mean ages of 0 (cord blood; n=220), 6 (n=376), 14 (n=241), 25 (n=257) or 72 (n=916) months for detailed immunophenotyping. With 1-5 measurements per child this resulted in a total of 2,010 data points. Absolute numbers of $CD19^+$ B cells were obtained with a routine diagnostic lyse-no-wash protocol and measured on a FACSCalibur (BD Biosciences). Six memory B-cell populations were defined,[16] using the following antibodies: CD19-PerCP (clone SJ25C1), CD27-APC-H7 (clone M-T27/L128), IgM-APC (polyclonal; all from BD Biosciences), IgD-FITC (polyclonal), IgG-PE (polyclonal; both Southern Biotech), and IgA-FITC (polyclonal; Kallestad). Flowcytometry was performed on a BD LSRII (BD Biosciences) using standardized measurement settings.[20]

EBV and measles serology

IgG antibody levels against the EBV viral capsid antigen (EBV-VCA) were determined in plasma samples from age 14 months and age 6 years of a subset of 219 children with enzyme-linked immunoassays (EUROIMMUN®). EBV positivity was determined based on a ratio of 0.8 of the sample over a manufacturer-provided reference threshold sample. In the same 219 children, anti-measles and anti-tetanus IgG levels were determined at 6 years of age (EUROIMMUN®). The presence of more than 275 international units per ml (IU/ml) for measles or 0.5IU/ml for tetanus were defined as protective vaccination responses.

Statistical analysis

Statistical comparison of EBV- and EBV+ groups were performed using the Mann-Whitney U test, paired T-test or Fisher's exact test as indicated in figure legends. P-values <0.05 were considered statistically significant.

To model the memory B-cell dynamics between birth and the age of 6 years, linear mixed effect analyses were performed on the relationship between the age of the children and the size of individual memory B-cell populations. By including random-effects in the model, this approach enabled modeling of cross-sectional data, with further improvement of the accuracy by incorporating longitudinal follow-up data from individual children. To capture the trend in the data more precisely, we included a natural spline with different knots (0-3 knots) into the models. Basically, the number of knots is inversely related to the smoothness of the curve. Positions of the knots in the 1-knot model was defined as the 50^{th} percentile (25.5 months), the 2-knots model at 33^{rd} and 66^{th} percentiles (14.1 and 70 months); the knots in the 3-knot model were defined manually at 6, 14

and 24 months, focusing around the time points of data inclusion. Model selection was performed by likelihood ratio test. Statistical analyses were performed in R (version R-3.2.1).[23]

RESULTS

EBV persists in IgA$^+$ and IgG$^+$ class-switched memory B cells in adults

To test whether EBV preferentially persists in certain B-cell subsets, we purified 8 previously defined populations from human blood.[16] Naive B cells were separated into CD38$^+$CD24$^+$ transitional and CD38^{dim}CD24^{dim}CD27$^-$IgD$^+$IgM$^+$ naive mature B cells. In addition 6 CD38^{dim}CD24^{dim} memory B-cell subsets were studied: CD27$^+$IgM$^+$IgD$^+$ 'natural effector', CD27$^+$IgM$^+$IgD$^-$ 'IgM-only', CD27$^+$IgA$^+$, CD27$^-$IgA$^+$, CD27$^+$IgG$^+$ and CD27$^-$IgG$^+$ memory B cells (Figure 1A). The numbers of EBV genomes per million cells were determined in all subsets from 5 EBV+ adults and represented relative to the number of EBV genome copies in CD27$^+$IgG$^+$ memory B cells (Figure 1B). EBV genomes were readily detectable in CD27$^+$ Ig-class switched memory B cells, but hardly detected in transitional, naive mature and IgM-only B cells (containing <8% of the number of copies detected in CD27$^+$IgG$^+$ memory B cells).[1,6,11] While low numbers of CD27$^+$IgM$^+$IgD$^+$ and CD27$^-$IgG$^+$ memory B cells carried EBV (25% and 39% compared to CD27$^+$IgG$^+$ memory B cells,

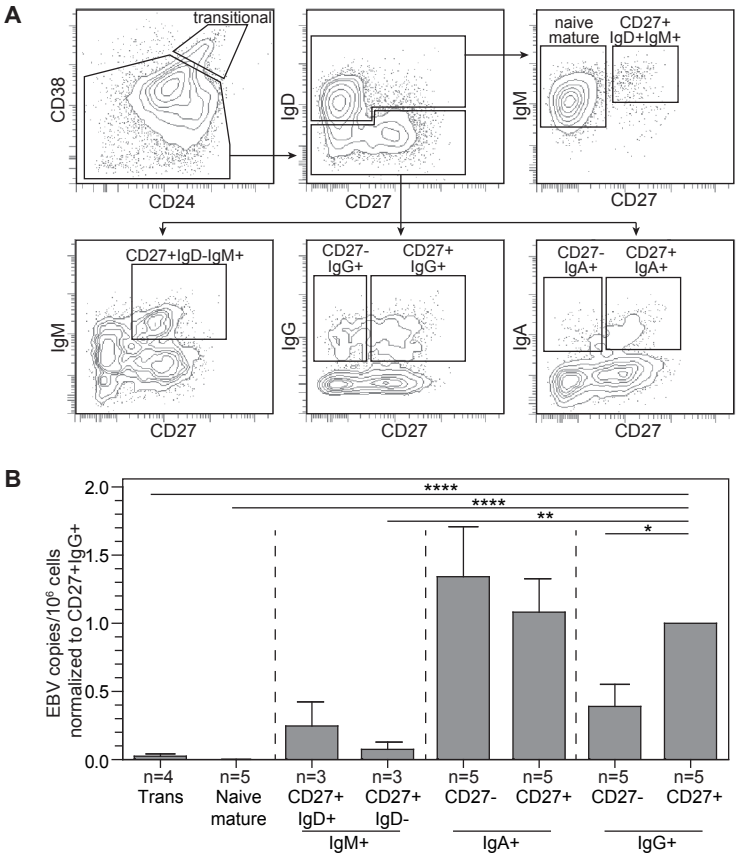

Figure 1.
EBV persistence in isolated memory B-cell subsets in adults.
A) Definition of transitional B cells, naive mature B cells and IgM$^+$, IgG$^+$ or IgA$^+$ memory B-cell subsets. **B)** Relative EBV load in the populations defined as in A in peripheral blood of 5 EBV+ adults. Data is depicted as mean (+ SEM) EBV load relative to EBV copies/10^6 cells in CD27$^+$IgG$^+$ memory B cells. Number of donors tested in each individual populations is indicated under the plot. Significance was tested between all subsets and CD27$^+$IgG$^+$ memory B cells with paired T-tests. *, p<0.05; **, p<0.01; ****, p<0.0001.

respectively), EBV was as frequent in CD27$^-$IgA$^+$ memory B cells as in the CD27$^+$ Ig-class switched memory B cells. Thus, EBV can persist in all 6 memory B-cell populations, but is most abundant in the T-cell dependent CD27$^+$IgG$^+$, CD27$^+$IgA$^+$ subsets and the T-cell independent CD27$^-$IgA$^+$ subset.

Dynamics of memory B cells in the first two years of life

In previous transversal studies, it has been found that memory B cells are gradually build-up in the first years of life.[15,24-27] To study the longitudinal dynamics of memory B cells in the first years of life, we performed repeated flowcytometric immunophenotyping measurements between birth and 6 years of age in 1,182 children in the context of the Generation R study. We performed linear mixed effect modeling of memory B-cell populations to define the immune dynamics while taking into account the information from longitudinal measurements within individuals (Figure 2). All memory B-cell populations showed the largest dynamics during the first two years of life. Blood B-cell counts strongly increased within the first 14 months of age, followed by a gradual decline and finally a stabilization of cell counts between 4-6 years. Thus, the build-up of the various memory B-cell subsets occurs most strongly in the first year of life.

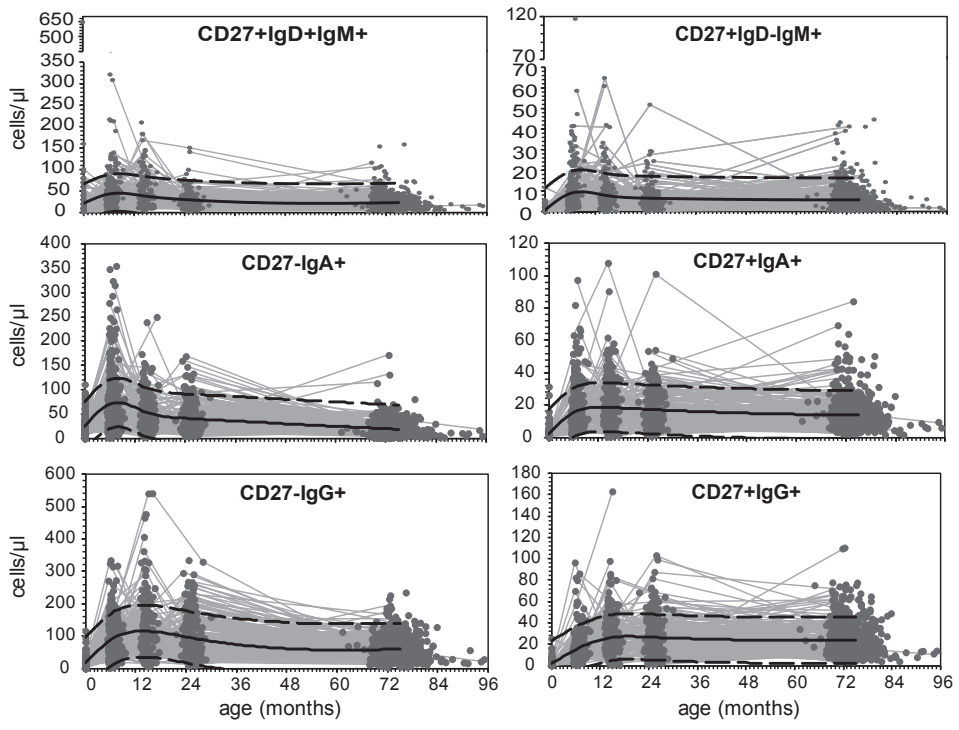

Figure 2. Dynamics of memory B-cell populations from birth until 6 years of age.
Included were 1,182 individuals with 1-5 measurements per individual, resulting in a total of 2,010 data points. Individual data is depicted in gray dots; directly consequetive timepoints within one individual are connected by gray lines (0-6m, 6-14m, 14-25m, 25-72m). Longitudinal linear mixed effect models describe the overall dynamics between birth and 6 years of age. Solid black lines represent the modelled data, dashed lines are 90% confidence intervals.

Transient decline in memory B cells of EBV positive children

To study whether EBV infection in the first year of life affects the dynamic development of memory B cells, we studied the naive and memory B-cell counts in blood of 149 14-month-old children, consisting of 13 EBV carriers and 136 EBV-negative children, as assessed by anti-EBV-VCA IgG serology. While total, as well as naive, natural effector and $CD27^-IgG^+$ B cells were similar between EBV+ and EBV- children, four memory B-cell populations were significantly reduced in EBV-infected children: $CD27^+IgD^-IgM^+$ 'IgM-only', $CD27^-IgA^+$, $CD27^+IgA^+$ and $CD27^+IgG^+$ (40.6-60.6% reduction in median values of EBV+ vs EBV- children) (Figure 3A).

The long-term effects of this decline were studied by follow-up analysis of the blood B cells of the same children at 6 years of age. At this age, 81 children were still EBV negative and 53 became seropositive between 14 months and 6 years of age. Regardless of early (<14 months) or late (>14 months) EBV infection, nearly all memory B-cell counts were comparable to those in uninfected controls (Figure 3B). Only the numbers of IgM-only memory B cells were significantly lower in late EBV+ than in EBV- children (31% decrease) (Figure 3B). Thus, the decline in memory B cells at 14 months seems to be transient and the numbers are normalized at 6 years of age.

Effects of B-cell depletion on vaccination responses

To study whether the low memory B-cell counts at 14 months of age were associated with abnormal antibody responses, we measured IgG levels in plasma samples of 6-year-old children to previous primary vaccination against measles and booster vaccination against tetanus. Both vaccinations are performed in the context of the Dutch national vaccination protocol.[28] Primary measles vaccination is performed at the age of 14 months when EBV+ children show a decline in memory B cells. Tetanus vaccination is performed at the ages of 2, 3, 4 and 11 months during the

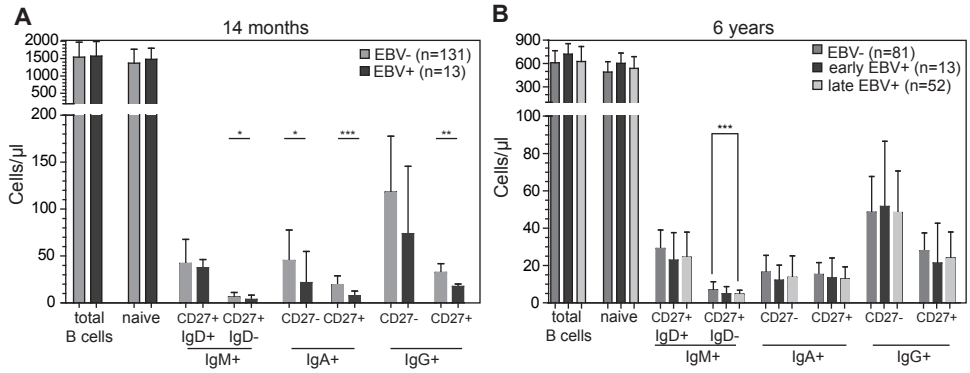

Figure 3. Effects of EBV persistence on circulating B cells in children.
A) Absolute numbers of total B cells and naive, IgM^+ memory, IgA^+ memory or IgG^+ memory B-cell subsets in EBV- (n=131) and EBV+ (n=13) children at the age of 14 months. **B)** Absolute numbers of total B cells and naive, IgM^+ memory, IgA^+ memory or IgG^+ memory B-cell subsets at the age of 6 years in EBV- children (n=81), children infected with EBV between 1-6 years of age (late EBV+; n=52) and children infected with EBV within the first 14 months of age (early EBV+; n=13). Bars depict median and interquartile range. Significance was tested by Mann-Whitney U test relative to EBV- individuals. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

first year of life, followed by a booster at 4 years (Figure 4A). Thus, in plasma of children at 6 years of age, we could evaluate build-up and/or maintenance of protective primary responses against measles, as well as memory responses against tetanus. Both the anti-measles (Figure 4B) and the anti-tetanus (Figure 4C) IgG levels at 6 years of age were similar between early EBV+, late EBV+ and EBV- children. Furthermore, the percentage of children with a protective response was similar between early EBV+, late EBV+ and EBV- children, with >80% (measles) and >67% (tetanus) of children having a protective titer at 6 years. Thus, both primary responses against measles and memory responses against tetanus were not affected by EBV infection and/or memory B-cell depletion in the first year of life.

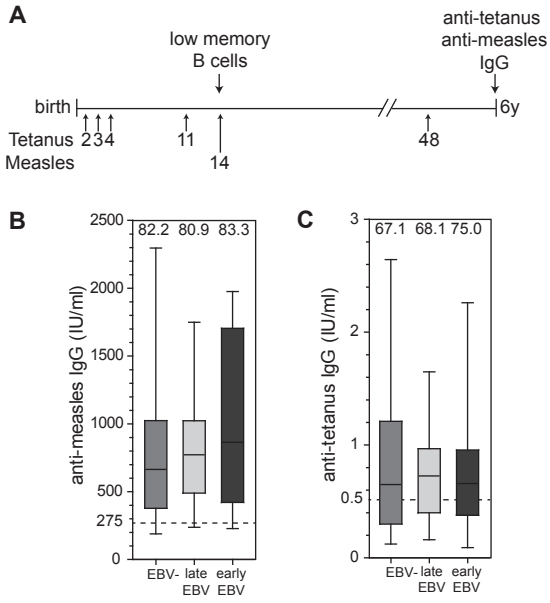

Figure 4. Vaccination responses in EBV-infected children.
A) Schematic representation of the measles and tetanus vaccination protocol in the Netherlands [28]. Tetanus vaccinations were performed at the age of 2,3,4,11 months and 4 years, and measles vaccination at 14 months of age. EBV+ children carried low numbers of memory B cells at 14 months of age, and IgG titers to previous vaccinations were determined at 6 years of age. **B,C)** Anti-measles IgG titers (B) and anti-tetanus IgG titers (C) in international units per ml (IU/ml) at 6-years of age in EBV- (n=73), late EBV+ (n=47) or early EBV+ children (n=12). The threshold for a protective vaccination response (anti-measles, 275 IU/ml; anti-tetanus, 0.5 IU/ml) is indicated by the dashed line. The frequencies of individuals with vaccination responses above this threshold are indicated at the top of the plots. Significance was tested by Mann-Whitney U test or Fisher's exact test relative to EBV- individuals. No significant differences were observed; all p values >0.46.

DISCUSSION

We here studied the persistence of EBV in memory B-cell populations and its effect on blood memory B-cell counts. EBV was able to persist in all class-switched memory B-cell populations. Memory B-cell populations were highly dynamic in size in the first 2 years of life, peaking around 14 months of age. Early EBV infection associated with a transient decline of memory B-cell numbers at 14 months of age, which normalized before 6 years and which did not affect vaccination responses against measles and tetanus.

In line with previous observations,[1,6,11] we hardly detected any EBV genomes in naive B cells, and only to a limited extent in IgM-only memory B cells and natural effector B cells.[1,6,11] Interestingly, EBV genome copies were equally numerous in $CD27^{-}IgA^{+}$ memory B cells as in the classical $CD27^{+}$ IgA^{+} and IgG^{+} subsets. $CD27^{-}IgA^{+}$ memory B cells appear to originate through germinal center-independent maturation pathways in local tissue, as they have been observed in patients with a lack of germinal centers due to a mutation in the CD40L gene and show strong similarities with IgA^{+} memory B cells in the intestinal lamina

propria.[16] The observation that these mucosa-derived $CD27^{-}IgA^{+}$ memory B cells contained the highest number of EBV copies, therefore, fits with the oral pathway of EBV infection. Previously, EBV was also detected in natural effector B cells that originate from T-cell independent responses in the splenic marginal zone.[11,18,29,30] Our observations therefore strengthen the concept that EBV is able to infect cells outside of the germinal center, not only in the spleen, but also in mucosal tissue.

We here observed decreased numbers of memory B cells in blood of 14-month-old EBV-infected children. These children were unlikely to be in the phase of acute infection, as they had a positive IgG serology against the viral capsid antigen (VCA) of EBV. Since these antibodies typically appear a week after acute infection, and remain positive for life,[31] these children most likely were infected during their first year of life. Even though the impact of EBV on T-cell populations has been extensively described,[13,32] effects of EBV on the B-cell compartment are less well described. Primary EBV infection was shown to result in a large relative expansion of EBV+ B cells, which subsequently contracted resulting in low frequencies of virus-carrying cells 1 week after appearance of clinical symptoms.[12] This depletion of virus-expressing cells is most likely the result of virus-induced or immune-mediated cell death. Our observations extend these observation with long-term effects on memory-B-cell numbers in young children. Importantly, we did not observe effects on memory B cell numbers in older children who were infected >14 month of age. During the first 5 years of life, memory B cells show dynamic changes in cell number with peaks around 14 month of age. It is therefore conceivable that EBV infection has the greatest effect at this age.

Despite a reduction in memory B-cell numbers at 14 months of age, early EBV-infected children had normal memory B-cells counts at 6 years of age, and carried normal IgG titers to previous measles and tetanus vaccinations. Since antigen-specific memory B-cell numbers correlate well with anti-measles IgG titers in the first years after vaccination,[33,34] it appears that these were normally formed in our EBV+ children at 14 months. Moreover, anti-tetanus IgG titers were normal in 6-year-old EBV-infected children. These children had received a booster vaccination at 4 years after initial vaccinations during the first year of life. This booster is expected to trigger long-lived memory B cells. Thus, despite a depletion in memory B cell, the remaining cells were sufficient to mount an antigen-specific response with protective IgG levels. Thus, the B-cell response shows large plasticity in immunological memory in these young children. Still, the outcomes we measured are of low-resolution. We cannot exclude that the antigen-specific memory B cells have a more restricted Ig gene repertoire following EBV-associated memory B cell depletion.

It is unlikely that natural exposure to measles or tetanus had a large effect on our observations, because of the >86% vaccination coverage in the Netherlands.[35] The last reported national measles outbreak was in 1999/2000, at least two years before the children in our study were born.[36] The vast moajority of infected children were not previously vaccinated, and a large-scale outbreak could be prevented due to the high overall vaccination coverage in the Netherlands.[35] Tetanus infection is rare in the Netherlands, with 5 or less reported cases of tetanus infection per year.[37] It will, therefore, be unlikely that natural exposure increased the anti-measles or anti-tetanus IgG titers and thereby interfered with our analyses.

Altogether, our data indicates that EBV is able to persist in all class-switched memory B-cell populations in blood, including $CD27^{-}IgA^{+}$ and $CD27^{-}IgG^{+}$ memory

B cells, and that although early EBV infection associated with a significantly smaller memory B-cell expansion in the first year of life, long-term effect on the composition of the blood memory B-cell compartment or its function in vaccination responses seem to be limited.

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CONFLICT OF INTEREST

All authors declare that no competing interests exist.

D.v.d.H., A.B., A.B.R., M.C.v.Z., and J.J.M.v.D. designed the experiments; D.v.d.H., M.A.E.J. performed experiments and most of the data analyses; A.B. and A.B.R. contributed to determining EBV persistence in memory B-cell subsets. V.W.V.J. and H.A.M. designed the Generation R study and coordinated material collection. D.v.d.H. and M.C.v.Z wrote the manuscript; and all authors commented on the manuscript.

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Chapter 7

PERSISTENT SUBCLINICAL IMMUNE DEFECTS IN HIV-1 INFECTED CHILDREN TREATED WITH ANTIRETROVIRAL THERAPY

Running title: Immune defects in HIV+ children on ART

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ABSTRACT

Objectives. With the introduction of combined antiretroviral treatment (cART), HIV-infected children can reach adulthood with minimal clinical complications. However, long-term HIV and cART in adults is associated with immunosenescence and end-organ damage. Long-term consequences of HIV and cART in children are currently unknown.

Design and method. We studied 69 HIV-infected children and adolescents under cART (0-23y) for the occurrence of subclinical immunological aberrations in blood B and T cells, using detailed flow cytometric immunophenotyping and molecular analyses.

Results. Children with undetectable plasma HIV viral loads for >1 year showed near-normal to normal $CD4^+$ T-cell numbers and near-normal numbers of most class-switched memory B cells. Furthermore, expansions of aberrant $CD21^{low}$ B cells contracted in patients with virus suppression. In contrast, $CD8^+$ effector T cells were increased, and $CD4^+$ memory T cells, $V\gamma9^+V\delta2^+$ T cells and $CD27^-$ IgA^+ memory B cells were decreased and did not normalize under ART. Moreover, $V\gamma9^+V\delta2^+$ T cells showed defects in their T-cell receptor repertoire selection.

Conclusion. Our results show the effectiveness of current cART to enable the build-up of phenotypically diverse B-cell and T-cell memory in HIV-infected children. However, several subclinical immune abnormalities were detected, which were partially caused by defective immune maturation. These persistent abnormalities were most severe in adolescents and therefore warrant long-term follow-up of HIV-infected children. Early identification of such immune defects might provide targets for monitoring future treatment optimization.

INTRODUCTION

Current combined antiretroviral therapy (cART) protocols have been successful in the treatment of human immunodefiency virus (HIV)-infected children, who can now reach adulthood with minimal clinical complications.[1] cART effectively induces viral suppression and in most patients plasma HIV RNA levels are below the detection limit within 1 year after start of treatment.[2] Furthermore, treatment restores total $CD4^+$ T cell numbers to levels within the normal range,[3] suggesting that the treatment is effectively inhibiting viral replication and inducing immune recovery.[4,5] Signs of immune activation in HIV-infected adults and children, i.e. the $CD8^+$ T-cell expansions and hypergammaglobulinemia, resolve after the initiation of cART.[1] Thus, cART changes HIV-infection from a lethal disease into a chronic, treatable disease.

However, long-term HIV infection has in adults been associated with clinical complications, such as cardiovascular complications, neurological diseases and malignancies.[6-8] Both HIV-associated immune aberrations and immunosenescence, as well as long-term cART are important contributors to the development of these diseases.[6-8] Immune aberrations in HIV-infected adults include an expansion of $CD8^+$ T cells with an effector memory phenotype: $CD45RA^-CCR7^-$. These HIV-specific cells display reduced cytolytic potential and are more prone to apoptosis,[9-11] mechanisms that could contribute to impaired clearing of HIV.[12,13] Furthermore, the Vδ2-expressing subset of $TCR\gamma\delta^+$ T cells is contracted both in mucosal tissues and in blood. This defect is associated with increased microbial translocation in the intestine.[14-16] Finally, HIV-infection affects B-cell responses leading to hypergammaglobulinemia,[17,18] an expansion of the aberrant, anergic $CD21^{low}$ B-cell population,[19-22] and a reduction of $CD27^+$ class-switched memory B cells,[18,23] of which especially the latter persists despite ART.[18,24-26]

Long-term effects of HIV infection and cART in children currently remain unclear. The first few years of life are crucial for the proper human immune maturation, and thereby to prepare them for adulthood.[27] Even though cART successfully suppressed HIV, it is unclear whether the dynamic adaptive immune maturation is normal in HIV-infected children. Early detection of immune aberrations could be predictive of future complications. Some persistent aberrancies in T-cell and B-cell memory have been described in HIV-infected children,[28-30] and their nature seems to depend on the child's age at the start of ART.[30-37] Still, an in-depth study into immune aberrations that might have long-term pathogenic effects has, to our knowledge, not been performed.

We, therefore, studied the blood B- and T-cell compartments in a cohort of 69 perinatally HIV-infected children and adolescents (0-23 years). The vast majority of these individuals responded well to ART with HIV levels below the detection limit and total $CD4^+$ T-cell numbers within the normal range. However, several persistent subclinical immune abnormalities could be identified in both B and T cells that warrant long-term follow-up of these perinatally HIV-infected children and might be predictive for future complications.

METHODS

Study subjects

Blood was obtained from 69 HIV-positive children between 0 and 23 years of age during routine outpatient clinic visits (Supplemental Table 1).[1,5,38] HIV-infected individuals were stringently treated and monitored every 3-6 months at Erasmus MC-Sophia Children's hospital and part of The Dutch vertically HIV-infected pediatric population cohort as registered by the Dutch HIV Monitoring Foundation.[1,5,39] Except for 1 child that did not give consent, all children of the cohort that were below 23 year of age and visited the outpatient clinic between December 2009 and December 2012 were included in the study. 148 age- and sex-matched HIV-negative healthy individuals were recruited in the same hospital.[1,5,38] Included were children undergoing orthopedic, ophthalmic, urologic or other non-infectious surgical procedures in otherwise healthy condition. Excluded were children having fever, burns or using antibiotics at the moment of sampling, and children having a known immunodeficiency, cardiovascular disease, coagulopathy, renal or hepatic impairment, and children undergoing organ transplantation. Patients and controls were included following informed consent from the parents according to the guidelines of the Medical Ethics Committee of Erasmus MC.

Flow cytometric immunophenotyping and isolation of B- and T-cell subsets from peripheral blood

Absolute counts of blood $CD4^+$ and $CD8^+$ T cells were obtained with a diagnostic lyse-no-wash protocol. Detailed flow cytometric immunophenotyping was performed on fresh blood samples after red blood cell lysis with ammonium chloride. $CD4^+$ T cells, $CD8^+$ T cells, $TCR\gamma\delta^+$ T cells and B cells were characterized using the antibodies described in Supplemental Table 2. Flowcytometric analyses were performed on an LSRII or CantoII (both from BD Biosciences) using standardized measurement settings.[40]

Post-Ficoll PBMCs were stored in liquid nitrogen and later thawed for sorting of $CD24^{dim}CD38^{dim}CD27^-CD21^{low}$ ($CD21^{low}$) B cells and three $CD24^{dim}CD38^{dim}CD21^+$ subsets: $CD27^-IgD^+$ naive mature, $CD27^+IgD^+$ natural effector and $CD27^+IgD^-$ memory B cells on a FACS Aria I (BD Biosciences).

Sequence analysis of rearranged *TCR* and *IGH* transcripts

The presence of the invariant-T nucleotide within the Vδ2-Jδ1 junctional region [41] was determined in rearranged transcripts amplified from PBMC cDNA, using the BIOMED-2 Vδ2 forward and Jδ1 reverse primers.[42] Rearranged Vγ9-Jγ1.2 transcripts were amplified with the BIOMED-2 Vγ9 forward primer and the fluorescently labeled BIOMED-1 Jγ1.2 reverse primer.[42,43] Amplified products were subjected to GeneScan analyses using an ABI PRISM 3130XL fluorescent sequencer (Applied Biosystems) to determine the frequencies of canonical rearrangements.[41,44]

IgM and IgG transcripts were amplified and cloned from cDNA of sorted B-cell subsets, using *IGHV3* and *IGHV4/6* leader forward primers and *IGHM* and *IGHG* reverse primers.[45,46] Sequences were generated on an ABI PRISM 3130XL and analyzed with the IMGT (http://imgt.org/), and BASELINe (http://selection.med.yale.edu/baseline/) software.[47,48]

B-cell replication history using the KREC assay

DNA was isolated from sorted B-cell subsets with the GenElute Mammalian Total DNA Miniprep Kit (Sigma-Aldrich) to determine the replication history with the Kappa-deleting Recombination Excision Circles (KREC) assay as described previously on a StepOnePlus Real-Time PCR system (Applied Biosystems).[49]

Statistical analyses

Statistical analyses were performed using the Mann-Whitney U test, unpaired T-test, Fisher exact test or Chi-square test. P-values <0.05 were considered statistically significant.

RESULTS

Patient characteristics

In this study, we included 69 children and adolescents who were infected with HIV in early childhood (Supplemental Table 1). Of these, 4 patients had plasma HIV viral loads (VL) >15,000 copies/ml at the moment of sampling (HIVhigh). Of the remaining 65 patients (HIVlow), 26 had detectable VL <900 copies/ml at the time of first sampling or detectable VL at least once in the preceding year (suboptimal viral suppression; HIVdetect) and 39 patients had undetectable VL for >1y (stable viral suppression; HIVund) (Supplemental Table 1).

All HIVlow patients received ART treatment for an average of 7.3y (HIVund) and 5.1y (HIVdetect). Two of the four HIVhigh patients received ART for 9.9 and 12.3y; the other two patients were clinically well and treatment was postponed according to guidelines at that time. Disease severity scores at diagnosis, according to Center for Disease Control and Prevention (CDC) guidelines,[50] varied between N1 and C3. The nadir $CD4^+$ T-cell counts were not significantly different between the three patient groups, as was the age at which the patients reached their nadir CD4. During the study follow-up, patients were clinically well; only 8 children needed a short-term hospitalization within the cumulative cohort follow-up. Total $CD4^+$ T-cell numbers at the moment of inclusion were normal in 81.5% of HIVlow patients (87.2% HIVund; 73.1% HIVdetect) and in 50% of HIVhigh patients (1 treated, 1 untreated).[27] Serum IgA, IgG and/or IgM levels above the normal range were detected in only 17.9% of HIVund patients, but were significantly more frequent in HIVdetect (53.8%) and HIVhigh (75%) patients.[51] Vaccinations against Hepatitis A and B induced a protective response (>100 International units/L (IU/L)) in 29/29 vaccinated HIVlow children and 1/2 vaccinated HIVhigh patients. Within the HIVlow group, increasing age of the patients correlated to significantly lower nadir CD4 counts, which were furthermore reached at significantly later age. Also treatment was initiated later in older patients and children of 16-23y more frequently showed reduced $CD4^+$ T-cell counts at the moment of sampling, suggesting persistent $CD4^+$ T-cell defects. Clinical severity score, high serum Ig and plasma HIV RNA levels were, however, independent of the age of the child at inclusion or treatment duration.

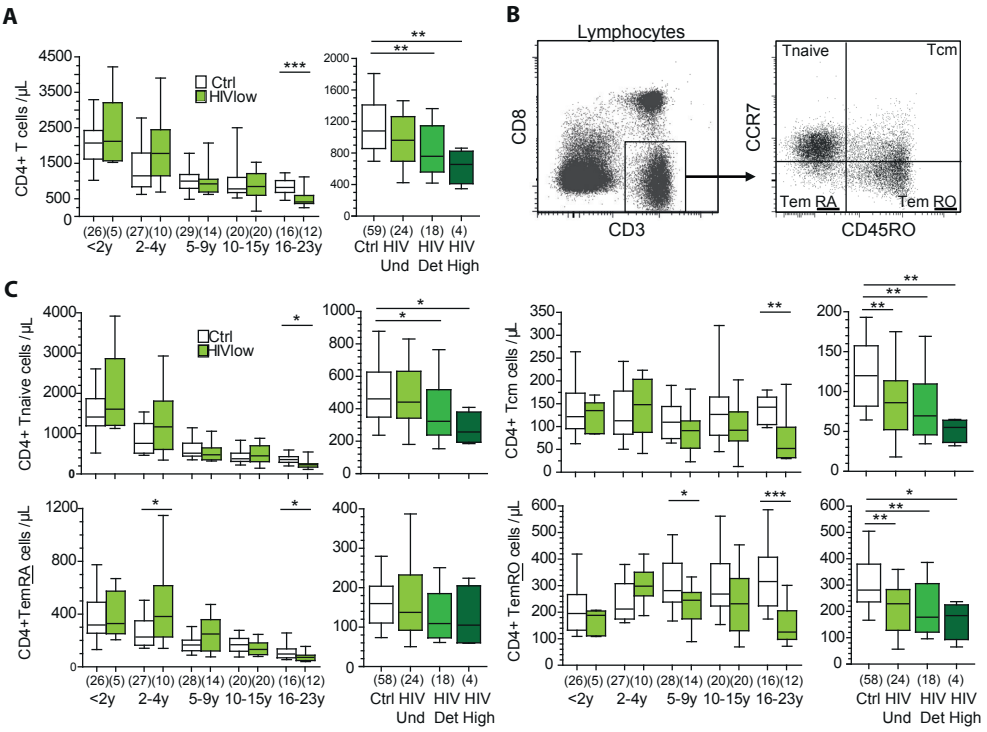
Altogether, cART appeared effective in most patients as evidenced by the limited clinical complications, the normal response to vaccinations and the increase in $CD4^+$ T-cell counts.

Low CD4+ memory T-cell numbers in cART-responsive adolescents

CD4+ T-cell numbers were within the normal range in most children <16y, but were reduced in many older children and adolescents (Supplemental Table 1). When divided over 5 age-groups (<2y, 2-4y, 5-9y, 10-15y, 16-23y), CD4+ T-cell numbers in HIVlow patients were comparable to age-matched controls, except for a significant reduction in adolescents of 16-23y (Figure 1A).

To address whether CD4+ T-cell numbers were related to plasma HIV RNA levels, children of 5-20y were separated into HIVund, HIVdetect and HIVhigh (Supplemental Table 1). CD4+ T-cell numbers were only slightly, but not significantly, reduced in HIVund patients, and more severely reduced in HIVdetect and HIVhigh patients (Figure 1A).

To study the nature of the CD4+ T-cell reduction, we dissected total CD4+ T cells into CD45RO-CCR7+CD27+CD28+ naive, CD45RO+CCR7+CD27+CD28+ central memory (Tcm), CD45RO+CCR7- effector memory (TemRO) and CD45RO-CCR7- effector memory (TemRA) (Figure 1B). These four subsets were all significantly reduced in 16-23y HIVlow patients (Figure 1C). In younger children, these subsets were normally present, except for significantly increased TemRA in 2-4y and decreased TemRO in 5-9y. Within TemRO, the early differentiated CD27+CD28+ subset was not only significantly reduced in the 16-23y, but all age groups >5y (Supplemental Figure 1A). The reductions in Tcm and TemRO numbers were similar in HIVund or HIVdetect patients, but slightly stronger in patients with high viral load (Figure 1C and Supplemental Figure 1A). Thus, viral suppression seems to restore the CD4+ T-cell compartment to a large extent in HIVlow patients, but especially adolescents showed reduced Tcm and TemRO numbers that are typically seen in viremic patients.



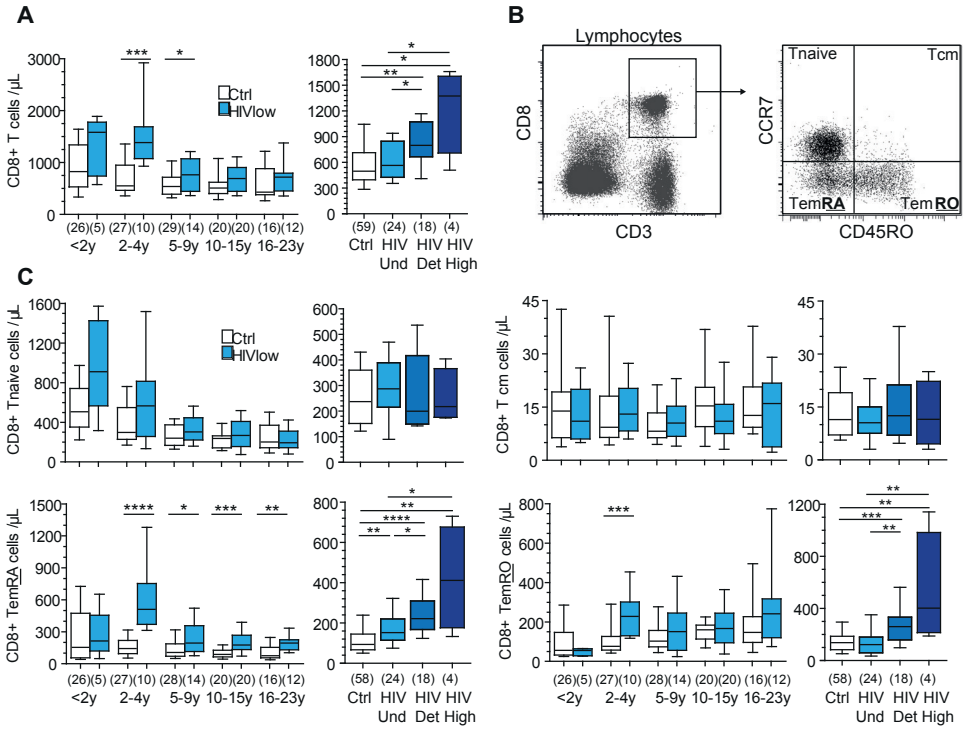


Figure 2. The CD8⁺ T-cell compartment in HIV-infected children and adolescents.
A) CD8⁺ T-cell counts in controls and HIVlow patients under ART (left) and in uninfected controls (ctrl), HIVund, HIVdetect or HIVhigh patients of 5-23y old (right). The number of individuals per group are indicated in parentheses. Plots depict 25-75th percentiles (box) and 10-90th percentile (whiskers). **B)** Definition of naive (Tnaive), central memory (Tcm), CD45RO⁺ effector memory (TemRO) and CD45RO⁻ effector memory (TemRA) subsets within CD3⁺CD8⁺ T cells. **C)** Absolute numbers of CD8⁺ Tnaive, Tcm, TemRO and TemRA subsets presented similarly as in panel A. Statistical significance was determined using the Mann-Whitney U test; *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001

CD8⁺ effector memory T-cell expansions in young children

Of the 65 HIVlow patients, only 4 showed the increase in total CD8⁺ T-cell numbers that is typically associated with HIV infection. Still, direct comparison of patients groups with age-matched controls showed significantly increased numbers of CD8⁺ T cells in children of 2-4 and 5-9y. (Figure 2A). Subset analysis revealed that this increase was mainly due to significantly high numbers of TemRA and to a lesser extent of TemRO (Figure 2B-C). Within these effector subsets, especially CD27⁻CD28⁻ late effectors were significantly increased in HIV-infected children (Supplemental Figure 1B-C). In line with this, more CD8⁺ T cells expressed CD57 and HLA-DR (not shown).[52-54] The expansions of total and TemRO CD8⁺ effector T

Figure 1. The CD4⁺ T-cell compartment in HIV-infected children and adolescents.
A) CD4⁺ T-cell counts in controls and HIVlow patients under ART (left) and in uninfected controls (ctrl), HIVund, HIVdetect or HIVhigh patients of 5-23y old (right). The number of individuals per group are indicated in parentheses. Plots depict 25-75th percentiles (box) and 10-90th percentile (whiskers). **B)** Definition of naive (Tnaive), central memory (Tcm), CD45RO⁺ effector memory (TemRO) and CD45RO⁻ effector memory (TemRA) subsets within CD3⁺CD8⁻ T cells. **C)** Absolute numbers of CD4⁺ Tnaive, Tcm, TemRO and TemRA subsets presented similarly as in panel A. Statistical significance was determined using the Mann-Whitney U test; *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.

cells significantly related to the presence of virus, whereas TemRA T-cell expansions were found in all three VL-groups (Figure 2C and Supplemental Figure 1B-C). Thus, good control of viremia seems to inhibit the $CD8^+$ effector memory T-cell expansions, but abnormalities can still be found, especially in young children.

Cellular and molecular TCRγδ$^+$ T-cell repertoire changed in HIV patients

TCRγδ$^+$ T-cell numbers were low to normal in our patients with the typically increased ratio of Vδ1$^+$ over Vδ2$^+$ T cells (Figure 3A).[15,16] In young children, this was mainly due to significantly increased numbers of Vδ1$^+$ T cells, whereas older children and adolescents had significantly reduced Vδ2$^+$ T-cell numbers compared to controls (Figure 3B-C). Moreover, Vγ9$^+$ T-cell numbers were slightly reduced in children of 5-9y and 16-23y, suggestive of a reduced number of Vγ9$^+$Vδ2$^+$ T cells, the dominant population in healthy adults (Supplemental Figure 2A-B).[44]

To study the nature of the Vγ9$^+$Vδ2$^+$ T-cell defects, we analyzed two typical selection determinants in these cells: 1) selection for the invariant-T nucleotide in the Vδ2-Jδ1 junctional regions, normally occurring within the first year of life and present in 90% of healthy adults, and 2) the canonical Vγ9-Jγ1.2 rearrangement, formed through homology-mediated repair and yielding a canonical Complementarity Determining Region 3 (CDR3) of 14 amino acids.[41,44] In 15 patients, we did not find the invariant-T (Figure 3D). These mainly concerned young children <2y, but their frequency was also significantly reduced in the patients of 2-23y. Furthermore, patients showed a trend towards reduced usage of the canonical Vγ9-Jγ1.2 rearrangements, even despite effective virus suppression in HIVund patients (Supplementary figure 2C). Thus, in adolescents Vγ9$^+$Vδ2$^+$ T-cells were impaired in numbers and in their TCR maturation and repertoire selection, irrespective of ART.

Reductions in IgA$^+$ B-cell memory despite ART

Total B-cell numbers, including the transitional and naive mature B-cell subsets, were normal in HIV patients of all age-groups (Figure 4A and data not shown). Within the memory B-cell compartment, all subsets were decreased in number, except for a slight increase in IgM-only B cells (Figure 4BC). Partial normalization of $CD27^-IgG^+$, $CD27^+IgG^+$ and $CD27^+IgA^+$ memory B-cell numbers was related to viral suppression, whereas the IgM^+ populations were equally affected in HIVund, HIVdetect and HIVhigh patients. The strongest effect was observed in the $CD27^-IgA^+$ subset that is thought to originate from T-cell independent responses in the gut.[46] This population was consistently reduced in all age categories and to the same extent in HIVhigh, as well as HIVdetect or HIVund children (Figure 4C). Thus, even though ART seems to partially restore class-switched memory B-cell defects, increased IgM-only and decreased $CD27^-IgA^+$ memory B-cell numbers persist during ART in HIV-infected children.

CD21^{low} B cells are associated with high HIV loads

HIV-infected adults show expansions of atypical CD21^{low} B cells with molecular signs of antigen-maturation and reactivity to HIV.[55] They are functionally impaired due to reduced replication history and somatic hypermutation (SHM) levels,

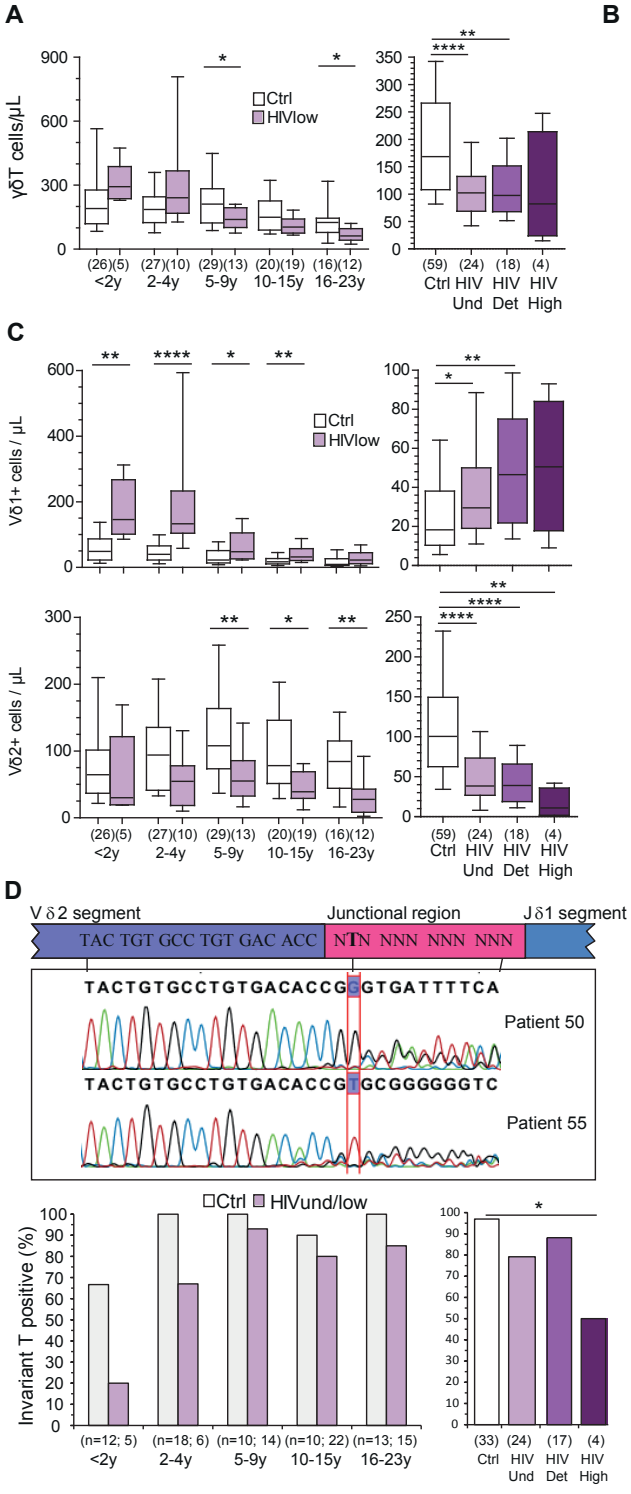


Figure 3. Cellular and molecular defects in TCRγδ⁺ T cells of HIV-infected children and adolescents

A) TCRγδ⁺ T-cell counts in controls and HIVlow patients under ART (left) and in uninfected controls (ctrl), HIVund, HIVdetect or HIVhigh patients of 5-23y old (right). The number of individuals per group are indicated in parentheses. Plots depict 25-75th percentiles (box) and 10-90th percentile (whiskers). **B)** Definition of Vδ1- and Vδ2-expressing T-cell subsets. **C)** Absolute numbers of Vδ1- and Vδ2-expressing T-cell subsets presented as in panel A. D) Molecular analysis of TCRDV2-TCRDJ1 rearrangements for detection of the invariant-T nucleotide in the first codon of the complementary determining region (CDR)3. A/C) Plots depict 25-75th percentile (box) and 10-90th percentile (whiskers). Statistical significance was determined, using the Mann-Whitney U test; *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001. D) Statistical significance was determined, using the Chi-square test.

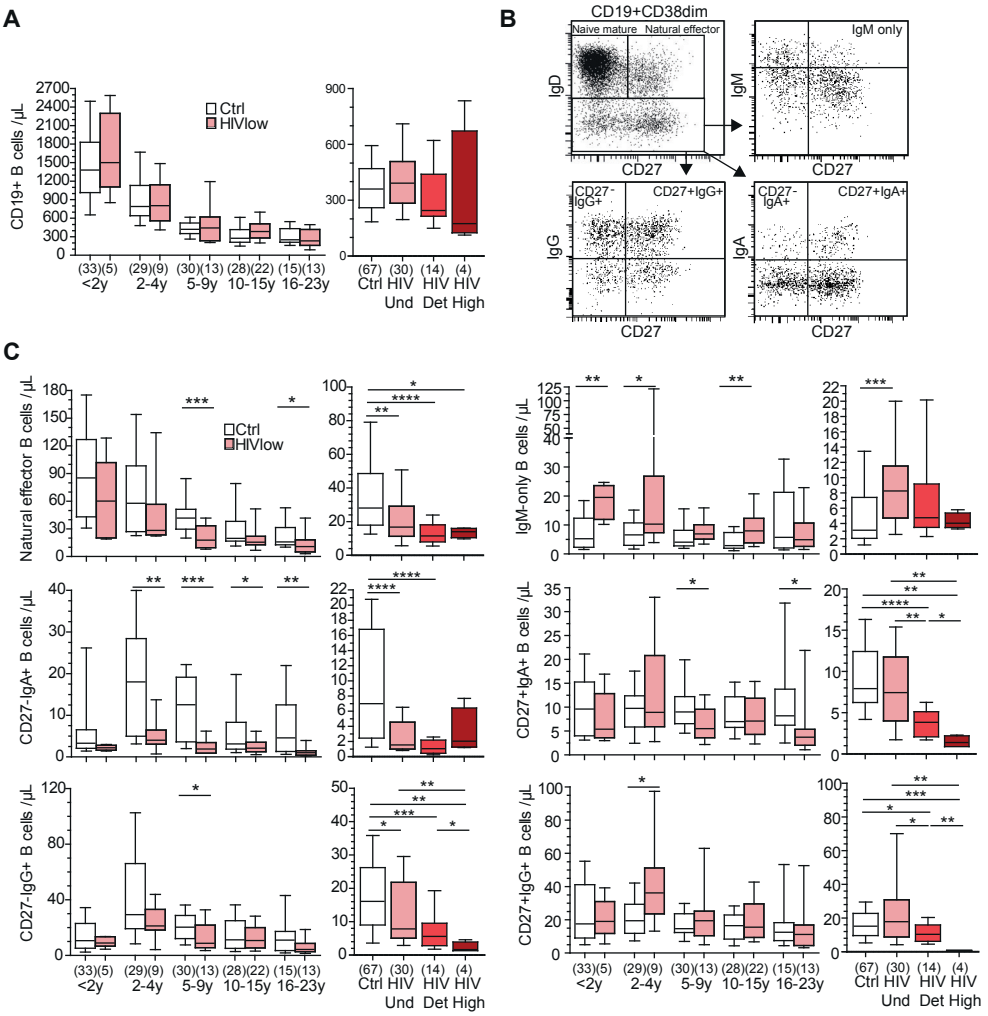


Figure 4. The memory B-cell compartment in HIV-infected children and adolescents.
A) CD19⁺ B-cell counts in controls and HIVlow patients under ART (left) and in uninfected controls (ctrl), HIVund, HIVdetect or HIVhigh patients of 5-23y old (right). The number of individuals per group are indicated in parentheses. Plots depict 25-75th percentiles (box) and 10-90th percentile (whiskers). **B)** Definition of 6 memory B-cell subsets: CD27⁺IgD⁻IgM⁺ (IgM-only), CD27⁺IgD⁺IgM⁺ (natural effector), CD27⁻IgA⁺, CD27⁺IgA⁺, CD27⁻IgG⁺ and CD27⁺IgG⁺.[46] **C)** Absolute numbers of 6 memory B-cell subsets in HIV-infected children presented similarly as in panel A. Statistical significance was determined, using the Mann-Whitney U test; *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.

and reduced responsiveness to stimulation.[55] Our HIVhigh children also carried significant expansions of CD38^{dim}CD27^{-}CD21^{low} B cells (Figure 5AB). Importantly, numbers of these CD21^{low} B cells were near-normal in HIVlow patients in all age groups (Figure 5A-C). To study whether CD21^{low} B-cell numbers were directly related to HIV viral loads, we performed longitudinal analysis (1.7-2.9 year) of 14 patients (Figure 5D and Supplemental Figure 3). In all four patients with high viral loads, we observed increased CD21^{low} B-cell numbers. In 2 patients, the CD21^{low} B-cell numbers increased within 0.5 year following the rise of plasma HIV RNA

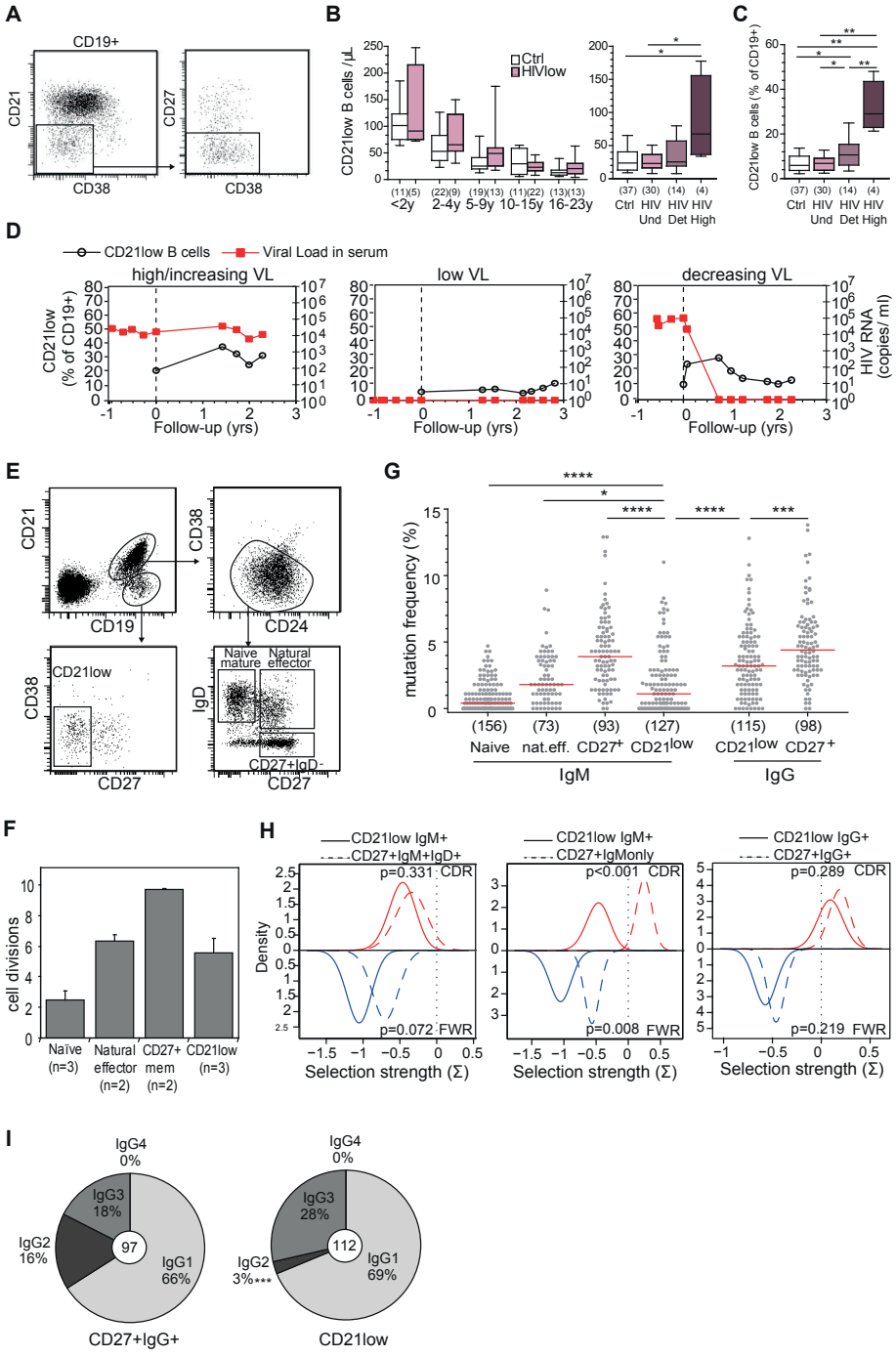


Figure 5. legend on the next page

Figure 5. Kinetics and molecular diversity of CD21^{low} B cells of HIV-infected children.
A) Definition of CD19^{+}CD38^{dim}CD21^{low}CD27^{-} "CD21^{low} B cells". **B)** CD21^{low} B-cell counts in controls and HIVlow patients under ART (left) and in uninfected controls (ctrl), HIVund, HIVdetect or HIVhigh patients of 5-23y old (right). The number of individuals per group are indicated in parentheses. Plots depict 25-75th percentiles (box) and 10-90th percentile (whiskers). **C)** CD21^{low} B-cell frequencies within total CD19^{+} B cells presented similarly as panel B. **D)** Longitudinal follow up of HIV plasma loads and CD21^{low} B-cell frequencies in one representative patients with high-VL (left column), low-VL (middle) and decreasing VL (right). **E)** Sorting strategy and definition of CD21^{low}, naive mature, natural effector and CD27^{+}IgD^{-} memory B cells. **F)** Replication history of purified B-cell subsets of 3 HIV-infected children (patients 28 29 and 34) as determined with the KREC assay.[49] **G)** Mutation frequencies in *IGHV* regions of IgM and IgG transcripts in 5 HIV-infected children (patients 52, 56, 59, 67 and 68). The number of transcripts analyzed in each category is indicated in parentheses. **H)** BASELINe analysis of IgM and IgG transcripts to determine the selection strength for replacement mutations.[47,48] **I)** IgG subclass distributions of sequenced IgG transcripts from CD21^{low} and CD27^{+}IgD^{-} B cells. The number of sequences included is depicted in the center of the chart. Statistical significance was determined using the Mann-Whitney U test (B/C/G) or Chi-Square test (I); *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.

levels. Patients with consistently low plasma HIV RNA levels carried a persistently small CD21^{low} population of <20% of total B cells. Finally, patients who successfully started ART showed a concomitant decrease in CD21^{low} B-cell numbers within 0.5 year of decreased plasma HIV RNA levels (Figure 5D and Supplemental Figure 3). Thus, CD21^{low} B-cell numbers seem directly related to active HIV infection and can be normalized by ART in children.

CD21^{low} B-cells in HIV patients are a mixture of naive and antigen-experienced B cells

To study the nature of these CD21^{low} B cells, we purified these, as well as the CD21-expressing CD27^{-}IgD^{+} naive B cells, CD27^{+}IgD^{+} natural effector and CD27^{+}IgD^{-} memory B cells from 5 HIV-infected children over 13y for comparative analysis of replication history and SHM levels (Figure 5E). Similar to previous observations in adults,[55] CD21^{low} B cells had a replication history of ~6 cell divisions, which was comparable with natural effector B cells, but clearly less than CD27^{+}IgD^{-} memory B cells (Figure 5F). The CD21^{low} population consisted mainly of IgM^{+} (with or without IgD) and IgG^{+} B cells (data not shown). To distinguish between these subsets for SHM analysis, we sequenced *IGHV* genes from separately amplified *IGHM* and *IGHG* transcripts. IgG transcripts of CD21^{low} B cells were nearly all mutated, although the SHM loads were slightly lower than in IgG transcripts from classical CD27^{+}IgD^{-} memory B cells (Figure 5G). Still, the transcripts of CD21^{low}IgG^{+} and CD27^{+}IgD^{-} cells showed similar selection for replacement mutations in CDRs (Figure 5H). Furthermore, both subsets showed similar IgG subclass distributions with the IgM-proximal IgG1 and IgG3 mostly used (Figure 5I). In contrast, about half of the IgM transcripts in CD21^{low} B cells were unmutated. The range of SHM levels and selection for replacement mutations of the mutated clones were more similar to natural effector B cells, but clearly lower than CD27^{+}IgD^{-} memory B cells (Figure 5G-H). Thus, our molecular analyses indicate that the CD21^{low} population in HIV patients is actually a mixture of naive and memory B cells, and that the molecular signs of antigen maturation in the IgG^{+} subset are more similar to classical CD27^{+}IgG^{+} memory B cells than might have been previously appreciated.

DISCUSSION

We here performed an in-depth study on the effect of perinatal HIV infection and cART treatment on the immune compartment of children and adolescents. Our results show the effectiveness of current cART to enable the build-up of phenotypically diverse B- and T-cell memory in HIV-infected children. This included,

at least in part, the normalization of $CD4^+$ T-cell numbers in the youngest children, normalization of class-switched memory B-cell numbers, and the reduction of $CD21^{low}$ B-cell expansions. However, persistent expansions of $CD8^+$ effector T-cells, and the reductions in $V\gamma9^+V\delta2^+$ T-cells and $CD27^-IgA^+$ memory B cells were not restored by cART.

Nearly all children in our cohort responded well to ART; they hardly had clinical complaints, mounted protective vaccination responses, showed low to undetectable HIV counts in plasma, and carried near-normal blood $CD4^+$ T-cell counts.[4,5] Adolescents more often showed a persistent reduction in their $CD4^+$ T-cell numbers. Various reasons might underlie this age-associated effect, including a lack of medication adherence, waning of the T-cell compartment over time due to prolonged HIV infection,[56] or the change in treatment protocols over the last 10 years (Supplemental Table 1).[57] The later start of ART treatment in children >10y of age was associated with significantly lower nadir $CD4^+$ T-cell counts than in children <10y, suggesting a more disrupted immune compartment in the adolescents. The near-normal $CD4^+$ T-cell compartment in the young children might therefore highlight a strong improvement in ART regimens in recent years and might further emphasize the importance of an early start of ART treatment. Still, future longitudinal studies will be needed to address whether the waning of $CD4^+$ T cells over time is stably inhibited in these young children.

While the depletion of $CD4^+$ T cells in adolescent patients concerned all naive, memory and effector subsets, Tcm and early TemRO cells were most severely affected. Even in adolescents with undetectable HIV for >1y $CD4^+$ Tcm and TemRO cells were lower than in uninfected controls, indicating that, once lost, these populations might be impossible to fully restore by ART. Loss of Tcm cells was reported to correlate with rapid disease progression,[58] whereas a small HIV reservoir in Tcm cells and stable Tcm functionality were observed in long-term non-progressors and natural HIV-controllers.[59,60] The higher numbers of Tcm cells in young HIVlow patients in our study might, therefore, again indicate an important immunological improvement due to ART, suggesting that early diagnosis and treatment might be important to prevent the initial loss of these populations.

All patients >2y had increased numbers of effector memory $CD8^+$ T cells, which might reflect ongoing response caused by residual HIV replication or might potentially be an early sign of immunesenescence. It will be important to longitudinally follow-up these $CD8^+$ TemRA expansions, to address whether they indeed correlate to chronic immune activation or possibly indicate early signs of immunosenescence.

The HIV-infected children in our cohort had increased numbers of $V\delta1^+$ T cells and decreased $V\delta2^+$ T cells, extending previous $TCR\gamma\delta^+$ T-cell aberrations found in blood and the intestinal mucosa of HIV-infected adults.[15,16,61] The increase in $V\delta1^+$ T cells did not result from clonal expansion (data not shown), but could still be the result of chronic stimulation.[15] The low $V\delta2^+$ T cell numbers were mainly due to a reduction in the $V\gamma9^+V\delta2^+$ subset. On top of the depletion that has previously been described in adults,[62] we here showed that in children $V\gamma9^+V\delta2^+$ T cells showed defective repertoire selection. Because the defects in $V\gamma9^+V\delta2^+$ T cells were not restored by ART, their levels could be good biomarkers to monitor future treatment optimization. Still, longitudinal follow-up of our patients will be important to define potential clinical complications that are associated with defects in $V\gamma9^+V\delta2^+$ T cells.

In our cohort, we observed severely reduced numbers of blood $CD27^-IgA^+$ memory B cells, which are derived from T-cell independent responses in the intestinal lamina propria.[46] Because these responses do not critically depend on $CD4^+$ T-cell help, this defect is most likely independent of any impaired $CD4^+$ T-cell responses, but likely depend on other, possibly HIV-targeted, mechanisms.[63,64] In line with the increased monocyte activation and intestinal microbial translocation observed by others,[37] the persistent loss of $CD27^-IgA^+$ B cells might result from persistent intestinal complications and ongoing HIV replication. However, we cannot exclude a possible effect of the oral intake of antivirals, which might affect B-cell responses in the intestine. Studies in HIV-negative individuals receiving post-exposure prophylaxis treatment might help to unravel the effect of treatment on the intestinal immune responses.

$CD27^-IgG^+$, $CD27^+IgG^+$ and $CD27^+IgA^+$ memory B-cell numbers were near-normal in children under ART. Ig serum levels and memory responses to common vaccination antigens, such as measles, pneumococci, influenza and tetanus antigens are reported to be reduced in HIV-infected patients, and it is debated whether ART is able to restore these.[65-68] The normalization of these T-cell dependent memory B-cell subsets in our patients, coinciding with a normalization of $CD4^+$ memory T cells and protective hepatitis A/B vaccination responses suggests maintenance or recovery of T-cell dependent humoral responses in our cohort.

We found that $CD21^{low}$ B-cell expansions correlated with plasma HIV RNA levels. We, moreover, showed that this subset was composed of both naive and memory IgM^+ B cells and IgG^+ B cells. This could explain the low replication history of the total subset. The $CD21^{low}IgG^+$ subset contained SHM and IgG1 and IgG3 usage that was more similar to $CD27^-IgG^+$ than to $CD27^+IgG^+$ memory B cells.[46,69] Considering that $CD21^{low}$ B cells display poor response to antigen and contain high frequencies of HIV-reactive B cells,[55] downregulation of CD21 could be a mechanism for HIV to impair the host's protective Ig responses, which, we here showed for the first time, could affect both naive and memory B-cell responsiveness. The direct and stable contraction in $CD21^{low}$ B-cell numbers following declines in plasma HIV RNA levels after cART treatment, therefore, indicates an important beneficial effect of cART treatment and makes it a good candidate marker for successful cART.

Altogether, our study showed the effectiveness of current ART to enable the build-up of phenotypically diverse B- and T-cell memory in HIV-infected children, especially in the younger children in our study, who receive the most recent treatment protocols and in whom treatment was started significantly earlier than in adolescents. However, even with undetectable viral loads, subclinical defects in $CD4^+$ TemRO and Tcm, $CD8^+$ TemRA, $V\delta2^+$ T cells and $CD27^-IgA^+$ memory B cells persisted, which were partially caused by defective immune maturation. Careful prospective monitoring of these persistent defects will be important for the early detection of clinical complications, ongoing virus replication or immunosenescence that might arise from these defects when these children grow older.[37,56]

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CONFLICT OF INTEREST

All authors declare that no competing interests exist.

D.v.d.H., M.A.B., J.J.M.v.D. and M.C.v.Z. designed the experiments; G.J.A.D., M.A.B., M.v.d.B., A.W.L., N.G.H., A.M.C.v.R. and P.L.A.F. provided conceptual advice; D.v.d.H., D.Z. and H.C. performed and analyzed most of the experiments and contributed to data analyses; G.J.A.D., N.G.H., A.M.C.v.R. and P.L.A.F. provided material necessary for performing experiments; D.v.d.H. and M.C.v.Z. wrote the manuscript; and all authors commented on the manuscript.

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Supplemental Table 1A. Characteristics of HIV patient groups

	HIVlow	HIVlow 0-1y	HIVlow 2-4y	HIVlow 5-9y	HIVlow 10-15y	HIVlow 16-23y
n	65	6	9	15	20	15
Plasma HIV RNA copies/ml at inclusion (average (range)) [a]	25.3 (0-900)	192 (0-900)	27.0 (0-123)	6.8 (0-102)	0 (0-0)	9.7 (0-145)
Age at inclusion in years (average (range))	10.6 (0.4-20.6)	1.2 (0.4-1.9)	3.5 (2.3-4.9)	7.6 (5.1-9.9)	13.0 (10.2-15.9)	18.4 (16.2-20.6)
ART treatment (n (%))	65 (100)	6 (100)	9 (100)	15 (100)	20 (100)	15 (100)
CDC score (at diagnosis) [b]	N1-C3	A1-C3	A1-C1	N1-C3	N1-C3	N1-C3
Age at start ART in years (average (range))	4 (0-17)	0.3 (0-0.6)	1.2 (0.1-3.2)	2.5 (0-7.3)	3.7 (0.3-14.7)	9.1 (5.4-17)
ART duration in years (average (range))	6.4 (0.2-13.3)	0.7 (0.2-1.6)	2.3 (1-4.7)	5.1 (0.5-8.8)	9.1 (1.2-13.3)	9.3 (0.9-12.7)
Nadir CD4 in % of age-matched reference value (average (range)) [c]	61.6 (0-301.5)	86 (29.6-112.7)	110.5 (36.8-301.5)	60.6 (1.4-215.0)	51.1 (0-118.8)	37.5 (1.0-91.4)
Age at nadir CD4 in years (average (range))	6.4 (0.2-19.4)	1.0 (0.2-1.9)	3.1 (1.6-5.8)	3.8 (0.3-8.4)	6.8 (0.3-15.0)	12.6 (5.4-19.4)
Reduced $CD4^+$ T-cell numbers at inclusion (n (%)) [c]	12 (18.5)	0 (0)	1 (11.1)	0 (0)	2 (10)	9 (60)
Hyperglobulinemia (n (%)) [d]	21 (32.3)	4 (66.7)	2 (22.2)	5 (33.3)	3 (15)	7 (46.7)
Protective Hepatitis A/ B response (n[protected]/n[tested] (%))	29/29 (100)	3/3 (100)	3/3 (100)	11/11 (100)	11/11 (100)	1/1 (100)

[a] Undetectable virus load was depicted as 0
[b] CDC= Center for Disease Control and Prevention; N/A/B/C, increasing clinical severity; 1/2/3, increasing CD4 depletion [50]
[c] CD4+ T-cell numbers below age-matched reference values [27,50]
[d] Ig serum levels above age-matched reference values [51]

Supplemental Table 1B. Characteristics of HIV patient groups

	HIVlow		
	HIVund	**HIVdetect**	**HIVhigh**
n	39	26	4
Plasma HIV RNA copies/ml at inclusion (average (range)) [a]	0 (0-0)	63.2 (0-900)	111,275 (16,600-328,000)
Age at inclusion in years (average (range))	10.4 (1.0-18.9)	10.9 (0.4-20.6)	15.6 (12.2-19.0)
ART treatment (n (%))	39 (100)	26 (100)	2 (50)
CDC score (at diagnosis) [b]	N1-C3	N1-C3	N1-A1
Age at start ART in years (average (range))	2.6 (0-11.1)	6.2 (0-17.0)	4.4 (1.1-7.8)
ART duration in years (average (range))	7.3 (0.8-13.3)	5.1 (0.2-12.8)	11.1 (9.9-12.3)
Nadir CD4 in % of age-matched reference value (average (range)) [c]	67.4 (6.9-301.5)	53.1 (0-160)	43.5 (21-64.3)
Age at nadir CD4 in years (average (range))	6.1 (0.3-19.4)	6.9 (0.2-17.0)	9.9 (1.6-16.8)
Reduced $CD4^+$ T-cell numbers at inclusion (n (%)) [c]	5 (12.8)	7 (26.9)	2 (50)
Hyperglobulinemia (n (%)) [d]	7 (17.9)	14 (53.8)	3 (75)
Protective Hepatitis A/ B response ($n_{protected}/n_{tested}$ (%))	16/16 (100)	13/13 (100)	1/2 (50)

[a] Undetectable virus load was depicted as 0

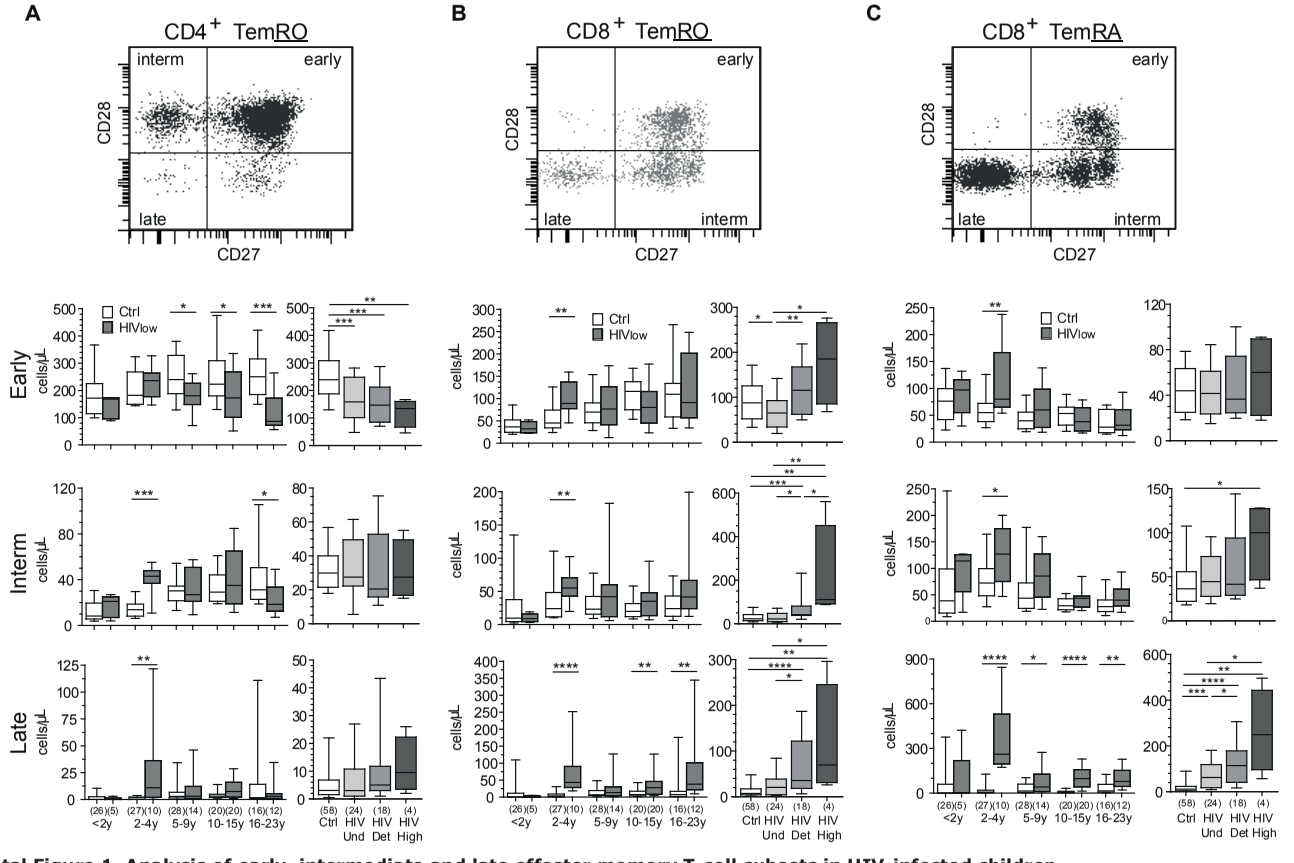
[b] CDC= Center for Disease Control and Prevention; N/A/B/C, increasing clinical severity; 1/2/3, increasing CD4 depletion [50]

[c] CD4+ T-cell numbers below age-matched reference values [27,50]

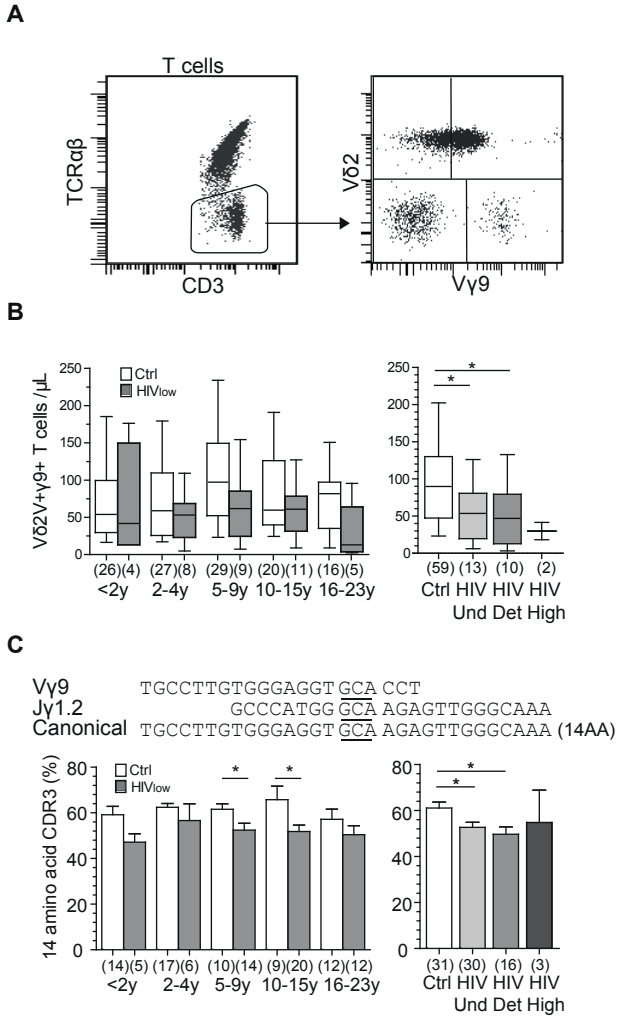
[d] Ig serum levels above age-matched reference values [51]

Supplemental Table 2. Antibodies used for flow cytometry

	Antibody	Fluorochrome	Clone	Manufacturer
T cells	CD3	PerCP-Cy5.5	SK7	BD Biosciences
	CD4	PB	RPA-T4	BD Biosciences
	CD8	APC-H7	SK1	BD Biosciences
	CCR7	PE	3D13	eBiosciences
	CD45RO	FITC	UCHL1	Zebra/Dako
	CD27	APC	L128	BD Biosciences
	CD28	PE-Cy7	CD28.2	eBiosciences
	TCRαβ	APC	IP26	eBiosciences
	TCRγδ	PE-Cy7	11F2	BD Biosciences
	Vδ1	FITC	TS8.2	Thermo Scientific
	Vδ2	FITC	B6.1	BD Biosciences
	Vδ2	PE	B6	BD Biosciences
	Vγ9	PE	B3.1	BD Biosciences
	CD57	FITC	HNK-1	BD Biosciences
	HLADR	PE-Cy7	L243	BD Biosciences
	CD45	PO	HI30	Invitogen
B cells	CD19	PerCP-Cy5.5	SJ25C1	BD Biosciences
	CD21	PE-Cy7	B-ly4	BD Biosciences
	CD24	PB	SN3	Exbio
	CD27	APC	L128	BD Biosciences
	CD38	APC-H7	HB7	BD Biosciences
	IgA	FITC	IS11-8E10	Miltenyi Biotech
	IgA	PE	IS11-8E10	Miltenyi Biotech
	IgA	FITC	polyclonal	Kallestad
	IgD	bio	IA6-2	BD Biosciences
	IgD	FITC	polyclonal	SBA
	IgG	PE	G18-145	BD Biosciences
	IgG	PE	polyclonal	SBA
	IgM	PB	G20-127	BD Biosciences
	IgM	PE	polyclonal	SBA

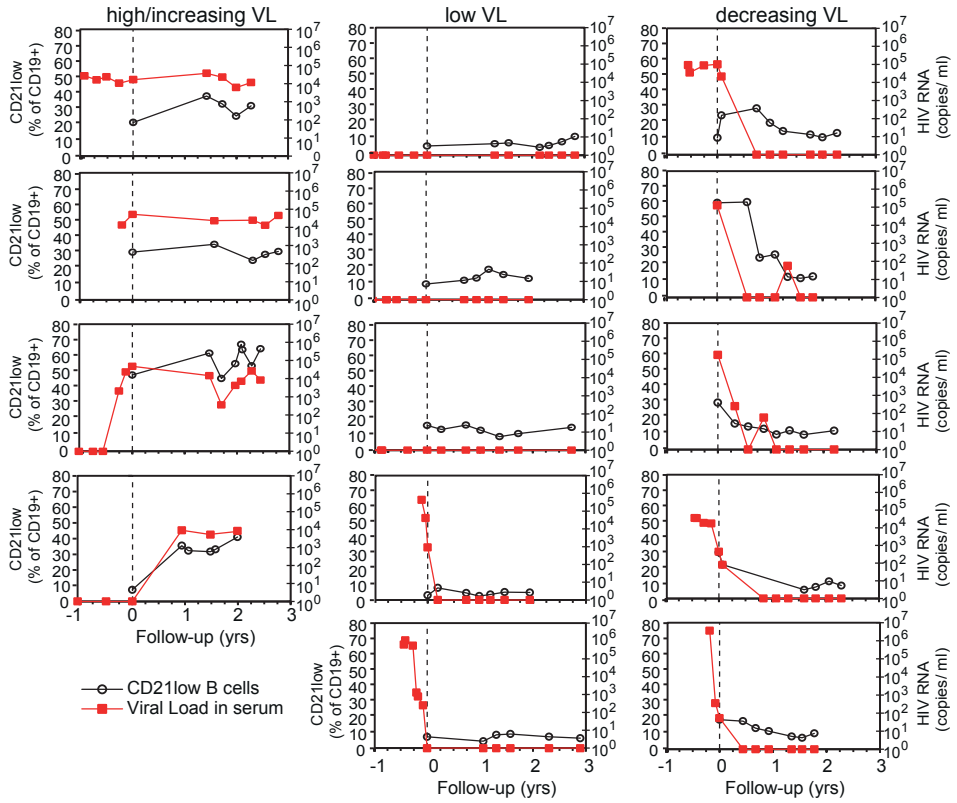


Supplemental Figure 1. Analysis of early, intermediate and late effector memory T-cell subsets in HIV-infected children.
CD4+ TemRO **(panel A)**, CD8+ TemRO **(panel B)** and CD8+ TemRA **(panel C)** were dissected into early, intermediate (interm) and late subsets based on differential expression of CD27 and CD28.[50] Left plot depicts the absolute numbers of each cell type in controls and HIVlow patients under ART. Right plot depicts the subset of 5-23y-old children in either uninfected controls (ctrl), HIVund, HIVdetect or HIVhigh patients. The number of individuals in each category is represented in parentheses underneath the plots. Plots depict 25-75th percentile (box) and 10-90th percentile (whiskers) of data. Statistical significance was determined, using the Mann-Whitney U test; *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.



Supplemental Figure 2. Cellular and molecular defects in Vγ9⁺Vδ2⁺ T cells of HIV-infected children and adolescents

A) Gating strategy for Vγ9⁺Vδ2⁺ T cells. **B)** Left plot depicts the absolute numbers of Vγ9⁺Vδ2⁺ T cells in controls and HIVlow patients under ART. Right plot depicts the subset of 5-23y-old children in either uninfected controls (ctrl), HIVund, HIVdetect or HIVhigh patients. The number of individuals in each category is represented in parentheses underneath the plots. Plots depict 25-75th percentile (box) and 10-90th percentile (whiskers) of data. Statistical significance was determined, using the Mann-Whitney U test; *, $p<0.05$. **C)** Frequency of Vγ9-Jγ1.2 rearrangement of 14 amino acids in size, representing the canonical rearrangement. Patients are represented as in panel B. Statistical significance was determined, using the unpaired T-test; *, $p<0.05$.



Supplemental Figure 3: Longitudinal follow-up of CD21^{low} B-cell frequencies and HIV VL in HIV+ children.

Longitudinal follow up of plasma HIV RNA levels and CD21^{low} B-cell frequencies in 4 patients with high-VL (left column), 5 patients with low-VL (middle) and 5 patients with decreasing VL (right). The 3 top panels are the same as shown in Figure 5D in the main paper.

Chapter 8

GENERAL DISCUSSION

During the first few years of life, newborns mount immune responses to diverse pathogenic threats and generate immunological memory post infection. This will prevent clinical illness during subsequent encounters with the same pathogen and thereby provide functional immunity. Maturation of the largely immature neonatal immune system is necessary to provide a young child with an immune system that supports long-term survival without the development of clinical complications due to either insufficient responses (immunodeficiency or immunosenescence) or unwanted responses (autoimmunity or allergies). In adults and elderly, persistent viral infections have been shown to skew T-cell memory. This mostly concerns the cytomegalovirus (CMV), Epstein Barr virus (EBV) and human immunodeficiency virus (HIV).[1-3] Little is known about the factors that affect leukocyte subset numbers and formation of immunological memory in young children.

In this thesis, we aimed to identify the effects of various external determinants during pregnancy, around child birth and during the first years of life on the leukocyte populations in young children. In **Chapter 2**, we studied leukocyte population dynamics between birth and 6 years of age in relation to a large number of external determinants. The association between breastfeeding and reduced B-cell memory was further studied in more detail in **Chapter 3**. Various aspects of persistent viral infections were studied: i.e. factors that affect the prevalence of herpesvirus infections (**Chapter 4**), the CMV- and EBV-associated changes in T-cell memory (**Chapter 5**), the EBV-associated reduction of memory B cells (**Chapter 6**), and the effect of perinatal HIV infection on the B-cell and T-cell compartments of HIV-infected children receiving combined antiretroviral (cART) treatment (**Chapter 7**). Together, the studies in this thesis illustrate the dynamics of childhood immune maturation and the plasticity of the childhood immune system to cope with persistent viral infections.

BREASTFEEDING PROVIDES STEPWISE MATURATION OF CELLULAR AND HUMORAL IMMUNITY

In **Chapter 2** we observed a selective negative effect of breastfeeding on the pattern of $CD27^{-}IgA^{+}$ memory B-cell dynamics, but not the germinal center-dependent memory B-cell populations, between birth and the age of 6 years. Breastfeeding is known to provide passive protection to the newborn during the first few months of life, in the form of maternal immunoglobulins or anti-pathogenic molecules, growth factors, lactoferrin and exosomes.[4-6] Maternal IgA is important to reduce bacterial adhesion to epithelial cells and intestinal translocation in the neonate and thereby to prevent bacterial exposure to the newborn's humoral immune system.[4] The $CD27^{-}IgA^{+}$ memory B-cell population is generated from local intestinal IgA responses,[7] which is the direct target organ of breastfeeding. This would suggest that breastfeeding reduces especially the formation of local humoral responses, whereas systemic germinal center-dependent responses are less or not affected. However, in **Chapter 3**, we observed that breastfeeding duration was associated with smaller T-cell dependent memory B-cell populations, an effect that resolved after discontinuation of breastfeeding. Growth factor proteins (e.g. epidermal growth factor and insulin-like growth factor) and hormones (e.g. erythropoietin) in breast milk were observed to reduce the intestinal permeability and subsequent microbial translocation.[6,8-10] This would be in line with our observation of a reducing effect of breastfeeding on systemic memory B-cell populations during breastfeeding duration. The longitudinal approach in **Chapter 2** focused on

the impact of breastfeeding on the overall pattern between birth and 6 years of age without including information on breastfeeding duration, which might in part explain why we did not observe the reduced numbers of systemic T-cell dependent memory B-cell populations in breastfed children in this study. Though speculative, breastfeeding might transiently delay the induction of systemic B-cell responses during the period of breastfeeding, whereas the effect of breastfeeding on the intestinal microbiota and subsequent intestinal $CD27^{-}IgA^{+}$ memory B-cell responses might be more long-lasting and therefore detectable on the overall pattern of $CD27^{-}IgA^{+}$ memory B-cell development. Detailed follow-up analyses of $CD27^{-}IgA^{+}$ memory B cells in association with breastfeeding or formula feeding will be important to understand the long-term consequences of breastfeeding on this population.

In contrast to inhibiting effects on B-cell memory, we observed expansions of central memory T (Tcm) cells in the presence of breastfeeding on. Expansion of the Tcm subset depended on breastfeeding exposure, but not on breastfeeding duration. This might suggest that it primes the newborn's T-cell compartment, and would be in line with the more effective vaccination responses observed in breastfed children.[11] Currently no direct $CD8^{+}$ T-cell stimulating component has been identified in breastfeeding. Potential factors in breast milk that might stimulate $CD8^{+}$ T-cell maturation include vitamins, cytokines, exosomes, and potentially viral or bacterial structures.[4-6] Cytokines, such as interleukin (IL)-15, can have $CD8^{+}$ T-cell stimulatory potential,[12] and IL-15 in breast milk has been associated to protection from mother-to-child HIV-transmission.[13] Exosomes in breast milk contain major histocompatibility complex (MHC)-class I and MHC-class II proteins, as well as various co-stimulatory molecules, and functional micro-RNAs, all of which might provide them with immunomodulatory potential.[14-16] Exosomes might be involved in presentation of maternal viral or bacterial antigens, and thereby stimulate T-cell maturation, either directly, or by bringing together $CD4^{+}$ and $CD8^{+}$ T cells.[15] This is in line with the described increase in $CD4^{+}$ regulatory T-cell differentiation under the control of exosomes in breast milk.[14] Based on the complex membrane protein composition of exosome pools and the extensive content of these vesicles, I am convinced that more regulatory properties are likely to be identified in the future, which might provide an explanation for the $CD8^{+}$ T-cell stimulation that we observed in breastfed children.

One might speculate that breastfeeding reduces the need for humoral immune responses, while T-cell responses are primed, thereby boosting cellular immunity. It will be important to study the receptor diversity and reactivity of this expanded $CD8^{+}$ Tcm pool, to define whether they are the result of antigen-independent or antigen-driven differentiation and proliferation, the first of which might provide a broadly reactive Tcm pool, whereas the latter would likely skew the repertoire of these cells. Future research into the immune stimulatory properties of exosomes, cytokines, and other components of breast milk, will be important to provide insights into the broad or targeted effect of breastfeeding-associated $CD8^{+}$ T-cell expansions.

PERSISTENT VIRAL INFECTIONS INDUCE DISTINCT $CD8^{+}$ EFFECTOR MEMORY T-CELL EXPANSIONS IN YOUNG CHILDREN

We observed that in 6-year-old children, CMV and EBV (**Chapter 5**), and HIV (**Chapter 7**), induces distinct $CD8^{+}$ effector memory T-cell expansions: EBV was

predominantly associated with expansions of early CD45RO$^+$ effector memory; HIV predominantly with intermediate to late CD45RO$^+$ or CD45RA$^+$ effector memory; and CMV predominantly with late CD45RA$^+$ effector memory (Figure 1). Why infections with these viruses result in CD8$^+$ T-cell expansions with distinct immunophenotypes is currently still under debate.

An important aspect will be the distinct functional properties of early differentiated T cells (high proliferative potential, but low cytotoxicity) and terminally differentiated T cells (low proliferative potential, but high cytotoxicity) in relation to the optimal anti-viral response required to clear each distinct virus.[12,17-19] Poly-functionality, measured as simultaneous production of IL-2, TNFα and IFNγ, is assumed to be essential for long term viral control.[20-22] However, these poly-functional T cells might have a more intermediate differentiation phenotype, in both CMV and HIV infection,[23,24] whereas the predominantly late-differentiated CMV-specific cells might be enriched for low avidity cells that are less responsive to antigenic stimulation.[25] Apparently, these functional properties alone do not explain the phenotypic differences in anti-viral responses.

CMV-specific T cells in children (**Chapter 5**) were mostly CD45RA$^-$ rather than CD45RA$^+$. This was in line with previous data on children,[25] but contrasted observation in adults of expansions of CMV-specific CD45RA$^+$ effector memory

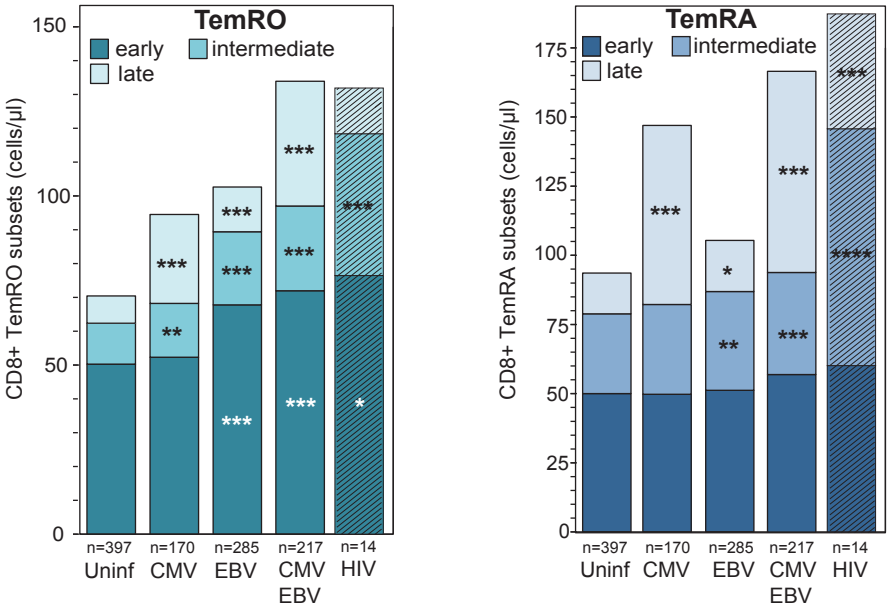


Figure 1. CD8$^+$ effector memory T-cell expansions in children infected with CMV, EBV or HIV. Absolute numbers of early, intermediate are late differentiated CD45RO$^+$ (TemRO; left) or CD45RO$^-$ (CD45RA$^+$ TemRA; right) CD8$^+$ effector memory T-cell populations in 5-9 year old children of the Generation R birth cohort (Uninfected, CMV+ and/or EBV+) and the HIV-cohort (HIV+). Included HIV-infected children showed undetectable viral load for more than 1 year, or detectable viral load in the preceding year, but below 900 copies/ml at the time of inclusion. Data in HIV infected children was not corrected for co-infection with CMV or EBV. Bars depict stacked median values per T-cell population. The number of individuals per category is indicated underneath each plot. Significance in CMV- and EBV-infected children was tested first by a Kruskal-Wallis test per lymphocyte population, and in case of significance ($p<0.05$) followed by a Dunn's test of individual patient groups relative to the uninfected controls. Significance between uninfected controls and HIV-infected children was subsequently tested by Mann Whitney U test. Significance for the Dunn's test or Mann Whitney U test is indicated in the plots: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

T cells. This might mean that terminally differentiated CMV-specific CD45RA$^+$ effector memory T cells accumulate with increasing age as a result from repetitive antigenic stimulation.[18,21,26] As these terminally-differentiated CD45RA$^+$ effector memory T cells show signs of replicative senescence,[27,28] this might result in the eventual loss of this response. In line with this hypothesis, especially T-cell clones with high HIV-antigen binding sensitivity that was associated with high initial anti-viral functionality and anti-viral protection, showed CD57 expression and frequent loss of dominant clones.[21] This might also explain the need for large accumulations of terminally differentiated T cells to maintain viral control in CMV-infected elderly.[25] It will be of interest to longitudinally follow-up the phenotype of CMV-specific cells in the children studied in **Chapter 5**, as this will show whether the phenotype is stable or changes over time into a terminally-differentiated phenotype. Furthermore, the effect of high antigenic stimulation on the phenotype of these T cells might be tested by comparing early treated HIV-infected children that show virus-control prior to the onset of high viremia, with children treated after the development of high HIV viremia.

Multiple findings in literature contrast the hypothesis of replicative senescence upon repetitive stimulation. The terminally differentiated CMV-specific T cells that predominate in elderly retain the potential to function upon re-stimulation.[29] Furthermore, poly-functional T cells accumulate in CMV-infected elderly,[22] and a low HIV viral setpoint and high CD4$^+$ T-cell count correlated with the presence of T cells with a terminally-differentiated phenotype, instead of the intermediate phenotype related to poly-functionality.[23,24,30,31] Furthermore, initiation of cART treatment, and subsequent reduction in HIV loads in serum, in HIV infected patients, might not change the phenotype of the initially developed CD8$^+$ T-cell populations. This latter observation might suggest that the initial anti-viral response defines the long-term phenotypic (and potentially functional) characteristics of anti-viral CD8$^+$ T-cell populations.

In **Chapter 5**, we observed higher CMV than EBV prevalence in 2-year-olds, suggesting younger infection with CMV than EBV. Furthermore, we observed in **Chapter 4** that CMV infection might depend more on vertical transmission, such as via breastfeeding, whereas EBV and HSV-1 depended more on horizontal transmission as they were associated with having siblings or attending daycare. As childhood immune maturation is associated with strong changes in the inflammatory milieu,[32-34] the inflammatory milieu at the time of infection might be an additional factor adding to the complexity of determining the T-cell phenotype. Antigen-independent cytokine stimulation (e.g. by IL-15) has been described to induce a terminally-differentiated phenotype in otherwise functional CMV- and EBV-specific T cells in elderly.[12,17,24,25,35,36] Their increased resistance to apoptosis might subsequently contribute to their accumulation. A similar increase in survival and effector functions has also been described upon IL-15 treatment of HIV-specific effector memory T cells, although this did not directly induce their terminal differentiation.[37] In the studies presented in this thesis, we were, unfortunately, unable to correlate the age of infection with the phenotype of the virus-specific CD8$^+$ T cells and the inflammatory state of the immune system. Correlating this information will be essential to further test the importance of the inflammatory milieu on the priming of anti-viral responses.

The potentially distinct routes of infection for CMV (vertical transmission, such as via breastfeeding), compared to EBV and HSV-1 (more horizontal transmission, via contact between children) (**Chapter 4**), might be another important factor

influencing the primary anti-viral response as it can affect virus load or virus-stability. Cytokines and growth factors in breast milk have been suggested to perform protective functions either by enhancing activation of $CD8^+$ T cells in breast milk, via the reduction of intestinal permeability, or via the activation of the infant's intestinal epithelial intestinal tissue to prime the infant against intestinal infection.[13,38] Although this was only shown for HIV infection, a similar situation might apply for infectious CMV in breast milk.[12] While inducing a more terminally differentiated phenotype of $CD8^+$ effector memory T cells, [12,24] it might induce early and strong control of infection, and could explain the relatively small $CD8^+$ T-cell expansions that we observed in **Chapter 5**. This hypothesis could be tested by comparing the phenotype and size of virus-specific or virus-associate $CD8^+$ T-cell populations in children fed with breast milk versus formula milk. Additional insights into the effect of maternal cytokines on direct immune priming in the breastfed infant, instead of on the virus within the breast milk, could be obtained by studying EBV- and HSV-1-infected children. These two viruses are less frequently acquired via breastfeeding (**Chapter 4**). Differences between EBV- or HSV-1-infected children fed with breast milk or formula milk would therefore indicate a direct effect of breastfeeding on the infant's immune system.

INHIBITION OF IMMUNE RESPONSES BY PERSISTENT VIRAL INFECTIONS IN YOUNG CHILDREN

Besides the strong induction of anti-viral $CD8^+$ effector memory T-cell expansions, we also observed two negative associations of these viruses with B-cell and T-cell numbers. First, EBV infection was found to associate with a transient reduction in memory B-cell numbers, likely resulting from direct infection and persistence of the virus in these cells (**Chapters 2** and **6**). Second, HSV-1 infection did not induce $CD8^+$ T-cell expansions, but instead was associated with reduced naive B-cell numbers (**Chapter 2**). Naive B cells themselves are not permissive for HSV-1 infection, suggesting indirect effects. The reduction in naive B cells might involve increased differentiation into memory or effector cells, which would suggest the involvement of more humoral responses against HSV-1 instead of cytotoxic T-cell responses. However, we did not observe an increase in memory B cells in our analyses and were unable to study plasma cell responses. It will be important to define whether the effects are persistent or transient in nature. The effect of EBV seemed mainly due to a reduced expansion of memory B cells within the first year of life. Although these cell numbers have normalized after their initial decline at 14 months (**Chapter 6**), it remains unclear whether these are as diverse as in uninfected children, or children infected after 14 months of age. Specifically the clonal diversity and replication history could reveal insights into whether memory B-cell numbers normalized through replenishment from naive cells or through enhanced compensatory proliferation of the few remaining memory cells. When the normalization of cell numbers would be due to increased bone marrow output, it could potentially restore this diversity, whereas we might speculate that normalization by increased homeostatic proliferation of circulating memory B cells could induce permanent reduced diversity of the memory B-cell compartment, and potentially reduced humoral immunity, in these EBV-infected children.

PERSISTENT SUBCLINICAL IMMUNE DEFECTS IN HIV-INFECTED CHILDREN

In **Chapter 7**, we found that current cART protocols restored (or prevented the loss of) many aspects of memory B and T cells in perinatally HIV-infected children and adolescents. However, we also identified a selected set of persistent defects that were not prevented nor restored under cART treatment. These especially concerned: expansions of $CD8^+$ effector memory T cells, and reductions in $CD4^+$ central memory and $CD45RO^+$ effector memory T cells, $V\delta2^+V\gamma9^+$ T cells and $CD27^-$ IgA^+ memory B cells. Even though these defects were currently still subclinical, complete normalization of these populations might be essential for an efficient immune system and for long-term protection of HIV-infected individuals.[39-42] Current cART protocols provide HIV-infected children with a relatively good prognosis, but the challenge will be to have these children to age with minimal clinical complications. A detailed understanding of the immunological conditions of these children and the identification of potential early signs of clinical complications in the future, will be highly important for future health care and potential further treatment optimization. Whereas follow-up of the normalized memory B-cell and T-cell populations will be important to define the stability of the beneficial effects of cART, the identified persistent defects might indicate potential pitfalls in the current treatment that could in the long term lead to clinical complications. Therefore, follow-up of these subclinical defective lymphocyte populations is highly important.

Combined, the persistent, but still subclinical, defects identified in **Chapter 7**, might be signs of incomplete HIV control. $CD4^+$ central memory and $CD45RO^+$ effector memory T-cell populations might be preferentially infected and provide important HIV reservoirs that can be established in a little as 10 days after the onset of clinical symptoms.[43-47] Though clearly speculative, the observed subclinical defects might suggest that $CD4^+$ memory T cells are persistently depleted and that latent reservoirs in $CD4^+$ memory T cells might not be fully eliminated by cART.[48] Such a persistent reservoir and low-level ongoing virus replication, might subsequently trigger persistent $CD8^+$ T-cell expansions, including the highly HIV-related $CD8^+$ intermediate differentiated effector memory T cells (Figure 1).[1,2] The intestine has been described to be an important reservoir for HIV infection.[43,49-51] In line with this, we observed a severe decrease of intestinal-derived $CD27^-$ IgA^+ memory B-cell numbers in blood. Whether this population is reduced due to increased retention, lack of generation, or depletion of these cells in the intestine will require additional studies. Moreover, it will be highly interesting to study whether this population is affected as a direct result of ongoing local HIV-replication, of increased intestinal bacterial translocation after HIV-induced intestinal damage,[49,52] or of the effect of the predominantly oral treatment on the integrity of the intestinal tissue. The latter might be tested in HIV-exposed, but HIV-uninfected individuals receiving post-exposure prophylaxis. Finally, the reduction of $V\delta2^+V\gamma9^+$ T-cell numbers in HIV-infected children, which was partially due to impaired maturation of these cells in response to phospho-antigens,[53-56] might be an early predictor for potential mycobacterial complications and impaired anti-HIV responses.[42,57] Altogether, detailed monitoring of these children will be needed to address the clinical relevance of these, still subclinical, defects at the long-term, but the populations identified here might provide important targets for longitudinal follow-up.

PLASTICITY IN THE CHILDHOOD IMMUNE SYSTEM UPON VIRUS ENCOUNTER

In **Chapters 5** and **7**, we observed that the CMV-, EBV- and HIV-associated $CD8^+$ effector memory T-cell expansions in 6-year-old children did not affect the naive and central memory T-cell compartments. Furthermore, in CMV and EBV infection it, at the same age, did not result in the loss of vaccination responses against non-related measles or tetanus infection. Moreover, infection before the age of 2 years induced smaller T-cell expansions than infection between 2-6y. In **Chapter 6**, we observed that EBV infection within the first two years of life resulted in an only transient decline of memory B-cell numbers around 24m that normalized before the age of 6y. Combined with the mostly asymptomatic nature of CMV and EBV infection in early childhood, and the potential protective effect of these viruses on the development of celiac disease (Jansen et al submitted) and allergies,[58-60] this might suggest only mild changes in immune homeostasis after childhood infection. However, it is important to realize that the studies in this thesis focused predominantly on quantitative measures, and less on functional responsiveness or the development of a broad repertoire of lymphocyte receptors. Even though our data in **Chapter 5** suggests that effector memory T-cell populations did not increase in early CMV- and EBV-infected children between 2 and 6 years of age, long-term follow-up of these children will be essential to confirm the stability of this population and the associated mild changes in the composition of the immune system, without excessive accumulation of $CD8^+$ effector memory T cells, the loss of naive cells or immunity against non-related pathogens. Furthermore, the reduction of memory B-cell numbers upon EBV-infection normalized before the age of 6 years and vaccination responses against measles and tetanus seemed normal. However, it needs to be investigated whether also the repertoire and responsiveness of this memory B-cell population fully normalized compared to EBV-uninfected children. When cell numbers normalize due to homeostatic proliferation, this might reduce the diversity and impair subsequent responsiveness of the memory B-cell population in EBV-infected children.

The importance of a proper immune maturation might be even more clearly visible in perinatally HIV-infected children. In **Chapter 7**, we observed that the HIV-infected children below 10y were treated at significantly younger age and showed less immune dysbalance than children >10y. Though current cART treatment is able to reduce the virus levels in serum to below the detection limit, and to recover a large part of the immune system, the extent of immune recovery was described to be inversely related to the patient's age at the start of cART treatment.[61,62] (Reviewed in [63]) Whereas the start of treatment early during immune maturation has been described to prevent the loss of humoral vaccination responsiveness, this responsiveness could not be restored by treatment started later during childhood.[64] Based on these beneficial effects of early treatment, current cART protocols changed in the last decade; whereas according to the 2006 guidelines cART had to be initiated in <11-months-old HIV-infected infants with clinical complications, $CD4^+$ T-cell frequencies below 25% of the age-related normal frequency, $CD4^+$ T-cell count <1,500 cells/ul, or a total lymphocyte counts below 4,000 cells/µl,[65] these recommendations have been updated, suggesting to initiate treatment in all HIV-infected children below 24 months of age (2010),[66] or even in all HIV-infected children below 5 years of age (2013).[67] Long-term follow-up of these early treated HIV-infected children will be essential to confirm whether the beneficial effects of early treatment might wane over time or persist into adulthood.

THE CLINICAL RELEVANCE OF UNDERSTANDING HERPESVIRUS IMMUNITY

In **Chapter 4**, we describe the large variation in herpesvirus seropositivity within populations with different ethnicities; the incidence of herpesvirus infection was significantly lower in the Western world compared to non-Western populations. Infection with CMV, EBV, HSV-1 and VZV are common, and might reach >50 to even 100% seropositivity in the adult population in the Netherlands.[68-73] Genetic variation between different ethnic groups likely contributes to infection susceptibility. However, we described in **Chapter 4**, that a significant part of the difference in infection prevalence between ethnic groups could also be explained by differences in external determinants associated with (likely cultural) behavior. Lower socioeconomic situation and larger family size (more siblings) in certain ethnic population were associated to the increased prevalence of EBV and HSV-1 infection, whereas the prevalence of CMV infection might be increased by more frequent breastfeeding over bottle feeding of infants. As a consequence, the smaller family size and use of bottle feeding in Western populations might delay or fully prevent infection. As especially infections with CMV and EBV have been associated with severe clinical complication in neonates, immunosuppressed individuals and elderly,[74-83] the reduced prevalence of herpesvirus infection in the Western world might seem beneficial.

However, we observed that a lack of CMV infection or seropositivity for less than 2 herpesviruses was associate with an increased risk for the development of celiac disease autoimmunity (Jansen et al submitted), suggesting a protective effect of herpesvirus infection. A similar effect of herpesviruses has previously also been described for allergic diseases.[58-60] Importantly, this protective effect might be limited to infection before the second year of life,[60,84] and might require wild type infection and not vaccination.[59] These observations are in line with the relatively limited immune disturbance that we observed upon early CMV and EBV infection in both the T-cell compartment (**Chapter 5**) and the B-cell compartment (**Chapter 6**), and are in line with the hygiene hypothesis. The increasing hygiene, smaller family size and increasing use of bottle feeding in the Western world, and the concomitant change in our microbiome and infection pressure in the Western population, is negatively correlated with the prevalence of allergic or autoimmune diseases.[33,34]

As a result of the diverse range of above-described clinical aspects to be considered, opinions on whether or not herpesvirus infection should be prevented by universal immunization are varying. Our data might argue against herpesvirus vaccination in young childhood (e.g. below 2 years of age) as herpesvirus infection at young age might be beneficial in priming immune maturation. However, as delayed infection poses important clinical risks, especially upon primary CMV infection during transplantation or pregnancy,[74] delayed herpesvirus infection might be disadvantageous and should preferably be prevented. Therefore, immunization against especially CMV and EBV might prove beneficial in children that were not naturally infected during the first few years of life.

THE UNIQUE OPPORTUNITIES PROVIDED BY LARGE COHORT STUDIES

The studies described in this thesis were performed in two unique population cohorts: the Generation R cohort and the Dutch vertically HIV-infected pediatric population cohort (HIV-cohort).[85-87] Both cohorts are prospective population-based cohort studies involving long-term follow-up of young children and include a detailed clinical or environmental characterization of the included individuals.

The large size of the Generation R cohort enabled studies on clinically minor patient groups, which would otherwise be too small to be studied in detail. One of those opportunities was the inclusion of children with slightly delayed or lack of VZV infection. As VZV infection reaches nearly 100% in adults, and already >90% in 6-year-old children in the Netherlands,[73] effects of VZV infection on the composition of the immune compartment could never be studied in detail before.[88] Moreover, in such studies, the effects of other persistent viruses, such as CMV and EBV, would need to be excluded. By studying this large Generation R population cohort, and focusing on 6-year-old children, we could identify 69 children being negative for HSV-1 and VZV. This allowed us, for the first time, to study the effect of VZV on the immune system (**Chapters 5**), while correcting our analyses for confounding effects that would otherwise interfere with proper interpretation of the data, including potential interference by CMV- and or EBV-associated effects. Thus, we showed that VZV did not affect memory T cells, in contrast to CMV or EBV. Consequently, VZV infection might be either relatively mild in terms of T-cell responses, or persistent T-cell expansions are only present locally and are not detectable in blood.

Our studies on the HIV-cohort, including 69 children, enabled a detailed analysis of blood B and T cells (**Chapter 7)**. This number of children, with different age between 0-23 years, made it possible to compare the effect of treatment in different age-groups. Furthermore, the detailed and longitudinal characterization of these children further enabled the discrimination between children with stable HIV suppression for >1y, children with suboptimal HIV suppression presenting low virus loads at the moment of sampling or detectable virus loads within the preceding year, and children that lacked virus suppression. Thereby we were able to correlate our findings to the presence of HIV in serum, and to define the relevance of optimal virus control. The detailed 8-color flow cytometry analyses of this large HIV-cohort enabled the identification of persistent immune defects, despite current cART protocols, that might provide important new targets for future studies (see Section "Persistent subclinical immune defects in HIV-infected children")

CONCLUDING REMARKS

A properly build immune system during the first few years of life is essential for long-term survival without the development of clinical complications. Persistent viral infections are known to have a strong impact on this immune maturation. The studies presented in this thesis combine both large-scale analyses on the complexity of immune maturation, and detailed immunological analyses on the origin, maturation or responsiveness of selected lymphocyte populations. Where large-scale cohort studies provide the unique possibility to analyze isolated determinants while excluding interference by the large variety of confounding

factors, only the addition of detailed cellular and molecular analyses will allow the study of immune maturation in its complete depth and complexity.

Combined, the studies presented in this thesis stress the plasticity of the childhood immune system and its capability to control viral infection without large-scale immune dysfunction. This information will be important, not only for our basic understanding of healthy immune maturation, but might also contribute to our understanding of immune dysfunction during chronic immune stimulation. Furthermore, it might add to the ongoing debate of herpesvirus vaccination. In addition, the identification of persistent subclinical defects in selected lymphocyte populations in blood of cART treated HIV-infected children, might directly contribute to targeted long-term clinical follow-up of these children.

It will be essential to continue combined epidemiological and detailed molecular immunological studies on the composition of the immune system in children under the influence of different inflammatory conditions or microbial colonization, as well as in children carrying different viral infections and being infected at different age. These studies will even further improve our understanding of what drives the maturation of the immune compartment during childhood and what defines its homeostasis.

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Addenda

Addendum

SUMMARY
SAMENVATTING

SUMMARY

During pregnancy, a fetus is protected from a large part of the pathogens of the environment. As a result, a newborn's immune system is immature and unexperienced, and mainly composed of innate leukocytes and naive lymphocytes. Memory lymphocytes, and concomitant functional immunity, needs to be formed in response to various pathogen encounters. Most of the immune maturation occurs during the first few years of childhood, during which the child comes into contact with a large variety of pathogens. Though most of the pathogens are cleared, after which life-long immunity is generated, some viruses evade virus clearance and induce a state of latency and viral persistence. Due to their continuous presence and continuous pressure on the immune system, persistent viral infections are known to have a strong impact on the immune system of especially elderly. The research described in this thesis was set out to characterize immune maturation during early childhood, in response to various environmental determinants with a specific focus on persistent viral infections.

In **Chapter 2**, we studied the dynamics of leukocyte subsets within the innate leukocyte, naive and memory B-cell and naive and memory T-cell lineages, and investigated which external determinants were associated with childhood leukocyte dynamics. We demonstrated that the dynamics of leukocyte subsets could be divided in four main age-related patterns, with functionally similar leukocyte populations following similar kinetics. Furthermore, the effect on immune dynamics by various external determinants related to prenatal maternal life style, maternal immune-mediated diseases, birth characteristics or bacterial/viral exposure, were described. Our data confirms previously described effects of persistent Cytomegalovirus (CMV) and Epstein Barr virus (EBV) infections on memory B-cell and T-cell dynamics, and identified new effects, including the association between Herpes Simplex virus 1 (HSV-1) seropositivity and reduced naive B-cell numbers. Our large-scale analysis of all leukocyte subsets and various external determinants allowed for the identification of new determinants that can provide targets for studies on the molecular processes that regulate leukocyte development and immune responses.

In **Chapter 3**, the effect of breastfeeding was studied in more detail in a cross-sectional study on breastfed versus bottle-fed children at the age of 6, 14, 25 and 72 months. We demonstrated a negative association between longer breastfeeding duration and the numbers of T-cell dependent $CD27^{-}IgG^{+}$, $CD27^{+}IgM^{+}$ and $CD27^{+}IgA^{+}$ memory B-cell populations at the age of 6 months. These effects disappeared after breastfeeding discontinuation. Furthermore, we demonstrated that breastfeeding exposure, independent of breastfeeding duration, had a positive effect on the size of the cytotoxic $CD8^{+}$ T-cell compartment, potentially suggesting a lasting priming of $CD8^{+}$ T-cell immunity via breastfeeding. Combined these observations might suggest that breastfeeding reduces the need for humoral immune responses, while priming cellular responses, thereby enabling step-wise immune maturation.

In **Chapter 4**, we demonstrated the differences in infection prevalence for CMV, EBV and HSV-1 in populations with different ethnicities. The ethnic differences in EBV and HSV-1 prevalence could in part (up to 39%) be ascribed to socioeconomic position and factors related to life style. This might suggest an important involvement of horizontal transmission for EBV and HSV-1 infection, which would be related to factors such as family size or daycare attendance. Ethnic differences

in the prevalence of CMV infection were independent of socioeconomic position and factors related to life style. In contrast CMV infection seemed to be positively associated to breastfeeding, suggesting the involvement of vertical transmission from mother to child.

CMV and EBV are associated with changes in immunological memory. Infected adults display persistent expansions of virus-specific effector memory T cells in both the $CD8^+$ and the $CD4^+$ T-cell lineages. In **Chapter 5**, we studied the memory T-cell compartment in detail in 6-year-old children with or without infection with CMV and/or EBV. We demonstrated similar virus-associated T-cell expansions as those described in adults. Furthermore, we demonstrated that these effects were independent of HSV-1 and Varicella Zoster virus (VZV) infection. However, in contrast to suggestions in elderly, CMV- and/or EBV-associated effector memory T-cell expansions in 6-year-old children did not result in a loss of the naive or central memory T cells, nor in a loss of vaccination responses against non-related Measles or Tetanus vaccination. Moreover, in 6-year-old children infected before age 2 years, stable control of these persistent viruses was maintained with only limited effector memory T-cell expansions. This might suggest an only mild change in the $CD8^+$ T-cell compartment upon childhood infection with CMV or EBV. These new insights into the immunomodulatory effects of herpesviruses in young children are important for our understanding of herpesvirus-associated immunosenescence in the elderly.

EBV has been shown to persist in memory B cells. Still, it is unclear whether persistent EBV infection results in altered B-cell memory. Therefore, in **Chapter 6**, we studied persistence of EBV in the various memory B-cell subsets and the memory-B-cell compartments in children persistently infected with EBV. We demonstrated that, in adults, EBV was able to persist in all class-switched memory B-cell populations, independent of their germinal center dependent or independent origin. In addition, through extensive flowcytometric immunophenotyping in EBV-infected children, we were able to demonstrate that EBV infection in the first year of life resulted in a reduction in the memory B-cell expansion that normally occurs at the age of 14 months. However, this reduction seemed transient as it normalized before the age of 6 years. Moreover, it did not seem to have long-term consequences on vaccination responses against tetanus and measles vaccination. Combined, this study demonstrates the impact of EBV infection on immunological memory in young children, as well as the plasticity of the immune system to overcome this.

With the introduction of combined antiretroviral therapy (cART), Human Immunodeficiency virus (HIV) infection is becoming a chronic instead of lethal disease, and perinatally HIV-infected children can reach adulthood with minimal clinical complications. However, long-term effects of persistent HIV infection and cART in children currently remain unclear. In **Chapter 7** we studied a cohort HIV-infected children and adolescents receiving cART. We demonstrated that cART is able to effectively reduce virus load in serum and restore total $CD4^+$ T-cell numbers and many of the other HIV-related immune defects. Still, a selection of subclinical immunological defects was identified in blood of cART treated HIV-infected children. These involved the $CD4^+$ T-cell, $CD8^+$ T-cell, $TCR\gamma\delta^+$ T-cell and memory B-cell lineages, and were shown to be in part caused by defective immune maturation. A detailed understanding of the immunological conditions of HIV-infected children and the identification of potential early signs of clinical complications in the future, will be highly important for future health care and

potential further treatment optimization in these HIV-infected children. Though still subclinical, the identified defects might provide important targets for targeted clinical follow-up of the HIV-infected children.

Combined, the studies presented in this thesis underline the complexity of the childhood immune dynamics and identify various external factors associated with immune maturation, with a focus on breastfeeding and persistent viral infections. Moreover, these studies stress the plasticity of the childhood immune system upon viral infection with herpesviruses and the HIV virus. Children seem to control persistent herpesvirus infections without the negative effects that have been observed in elderly. This information will be important, not only for our basic understanding of healthy immune maturation, but might also contribute to our understanding of immune dysfunction during chronic immune stimulation. Knowledge on immune dynamics and the external determinants that shape the childhood immune system will provide more understanding of the processes that underlie the formation of long-lasting immunity without inducing destructive, excessive or insufficient immune responses.

SAMENVATTING

Het afweersysteem van de mens bestaat uit een complex geheel van cellen en moleculen. Het kan worden onderverdeeld in aangeboren afweer en verworven afweer. Aangeboren afweercellen functioneren direct na hun aanmaak en deze eigenschappen veranderen niet tijdens of na een afweerreactie. In tegenstelling, wordt de verworven afweer grotendeels gevormd als gevolg van contact met ziekteverwekkers. De verworven afweer bestaat uit B en T cellen. B en T cellen starten hun ontwikkeling als naïeve cellen die nog niet in contact zijn gekomen met een ziekteverwekker. Pas na contact met een ziekteverwekker zal een naïeve B of T cel gaan delen en uitrijpen tot effectorcellen die de ziekteverwekker bestrijden, of tot geheugencellen. Deze geheugencellen blijven lange tijd in het lichaam aanwezig en kunnen zich bij een volgend contact met dezelfde ziekteverwekker snel ontwikkelen tot een effectorcel. Daardoor kan de ziekteverwekker bij een volgend contact meestal snel uit het lichaam worden verwijderd voordat er ziekteverschijnselen ontstaan. In dit geval spreken we van immuniteit tegen deze ziekteverwekker. Het verworven afweersysteem past zich op deze manier als het ware aan aan de omgeving.

Tijdens de zwangerschap wordt een foetus in de baarmoeder beschermd tegen een groot deel van de ziekteverwekkers uit de omgeving. Daarom bestaat het afweersysteem in pasgeborenen voornamelijk uit aangeboren afweercellen en naïeve B en T cellen. Geheugencellen en bijbehorende immuniteit, zullen grotendeels gevormd worden na de geboorte als gevolg van contact met de vele verschillende ziekteverwekkers in de omgeving. De veelheid aan ziekteverwekkers die een kind vooral in de eerste levensjaren tegenkomt bepalen dus grotendeels de vorming van zijn afweersysteem.

Hoewel de meeste virussen door middel van een afweerreactie worden verwijderd uit het lichaam, zijn sommige virussen in staat het afweersysteem te ontwijken en zich blijvend in het lichaam te 'verschuilen'. Dit zijn zogeheten persistente virussen. Een aantal bekende persistente virussen zijn het humaan immunodeficiëntie virus (HIV), en leden van de herpesvirusfamilie, waaronder het cytomegalovirus (CMV), Epstein Barr virus (EBV), herpes simplex virus-1 (HSV-1) en varicella zoster virus (VZV). Doordat deze virussen continu in het lichaam aanwezig zijn, oefenen ze een sterke druk uit op het afweersysteem. Bij een deel van de ouderen beïnvloedt dragerschap van deze virussen het afweersysteem sterk, waardoor ze minder goed kunnen reageren op andere ziekteverwekkers, waaronder het influenza (griep) virus. Het is echter niet duidelijk of deze effecten al op jonge leeftijd ontstaan, of dat het afweersysteem bij jongeren anders reageert op deze virussen dan bij ouderen. Daarom was het doel van het onderzoek beschreven in dit proefschrift het bestuderen van de vorming van het afweersysteem in jonge kinderen en hoe infecties met persistente virussen deze vorming beïnvloeden.

In **Hoofdstuk 2** hebben we bestudeerd hoe de aantallen van de diverse typen afweercellen in bloed van kinderen veranderen tijdens de eerste 6 jaren van hun leven. Deze metingen zijn verricht binnen de Generation R Study waarin 10.000 kinderen vanaf het eerste trimester van de zwangerschap worden gevolgd. Hierdoor zijn van veel kinderen gegevens van meerdere tijdspunten beschikbaar evenals vele sociale en biologische gegevens. Bij deze kinderen hebben we aangetoond dat de afweercellen in het bloed kunnen worden onderverdeeld in 4 groepen op basis van de dynamiek in de cel aantallen. Hierbij volgden functioneel vergelijkbare afweercelpopulaties hetzelfde patroon. Deze grote Generation R

Study maakte het daarnaast mogelijk om te bestuderen welke omgevingsfactoren geassocieerd zijn met de dynamiek van afweercellen. We hebben hierbij gefocust op omgevingsfactoren die gerelateerd zijn aan 1) de levensstijl van de moeder tijdens de zwangerschap (bijvoorbeeld roken en alcoholgebruik), 2) de aanwezigheid van auto-immuunziektes of allergieën bij de moeder, 3) karakteristieken van de geboorte (bijvoorbeeld of een kind is geboren via een keizersnede of vaginale bevalling, of informatie over het geboortegewicht), of 4) het contact met bacteriën/virussen. Allereerst bevestigen onze resultaten eerdere bevindingen uit de literatuur over het effect van CMV en EBV op de verandering in de aantallen B en T geheugencellen. Daarnaast hebben we nieuwe effecten geïdentificeerd, zoals een verminderd aantal naïeve B cellen in bloed van HSV-1-geïnfecteerde kinderen. Onze grootschalige analyses van dit grote aantal leukocytenpopulaties en de vele omgevingsfactoren hebben het mogelijk gemaakt om nieuwe factoren te identificeren die kunnen worden gebruikt in vervolgstudies naar de processen die de vorming van het afweersysteem reguleren.

In **Hoofstuk 3** hebben we op de leeftijden van 6, 14, 25 of 72 maanden de effecten van borstvoeding en flesvoeding op het afweersysteem bestudeerd. Op de leeftijd van 6 maanden vonden we dat naar mate een kind langer borstvoeding kreeg, verschillende populaties binnen de B geheugencellen kleiner werden. Deze effecten waren niet meer detecteerbaar nadat de borstvoeding werd gestopt. Daarnaast hebben we gevonden dat kinderen die borstvoeding kregen, een vergrootte populatie van T geheugencellen hadden, namelijk de $CD8^+$ centrale T geheugencellen. Dit effect was onafhankelijk van de hoe lang een kind borstvoeding kreeg. Samen, suggereren deze bevindingen dat een kind dankzij de beschermende werking van borstvoeding tijdelijk minder B geheugencellen hoeft te vormen, terwijl borstvoeding de vorming van T geheugencellen juist stimuleert. Mogelijk faseert het geven van borstvoeding de vorming van het afweersysteem door eerst de vorming van T geheugencellen te laten plaatsvinden en pas later de B geheugencellen.

In **Hoofdstuk 4** hebben we laten zien dat de frequentie van CMV, EBV en HSV-1 infectie binnen een bevolking sterk afhangt van de etniciteit van de geteste groep mensen. Deze variatie tussen verschillende etnische groepen kon in het geval van EBV en HSV-1 infectie voor ≤39% worden verklaard door verschillen tussen de etnische groepen in de levensstijl en socio-economische status van de moeder. Dit hangt mogelijk samen met factoren zoals familiegrootte of het gebruik van kinderopvang en sluit aan bij eerdere bevindingen dat HSV-1 en EBV veelal horizontaal worden overgedragen door contact tussen familieleden of tussen kinderen op kinderdagverblijven. De verschillen in de frequentie van CMV infectie tussen verschillende etnische groepen waren niet afhankelijk van de socio-economische status of levensstijl van de moeder; CMV infectie kwam juist frequenter voor in bevolkingsgroepen die vaker borstvoeding gaven dan flesvoeding. Dit suggereert een rol voor de zogeheten 'verticale overdracht' van CMV van moeder naar kind.

Een selectie van CMV- en EBV-geïnfecteerde ouderen hebben sterk toegenomen aantallen CMV- en EBV-specifieke T geheugencellen en een verminderde afweerrespons op vaccinaties tegen bijvoorbeeld influenza. Om te bepalen of CMV en EBV ook op jonge leeftijd zulke effecten geven, hebben we, in **Hoofdstuk 5**, T geheugencellen in detail bestudeerd bij 6-jaar-oude kinderen met of zonder CMV en/of EBV infectie. We hebben aangetoond dat CMV en EBV infectie elk resulteerden in een toename van een ander type T geheugencel, en dat beide

toenames aanwezig waren in dubbel-geïnfecteerde kinderen. Deze toenames waren onafhankelijk van infectie met twee andere herpesvirussen: HSV-1 en VZV. In tegenstelling tot ouderen, leidden deze vergrote populaties van T geheugencellen bij kinderen niet tot verminderde aantallen naïeve T cellen of andere T geheugencellen, of tot het verlies van vaccinatieresponsen tegen de niet-gerelateerde ziekteverwekkers mazelen en tetanus. Het toegenomen aantal T geheugencellen was op 6 jarige leeftijd kleiner bij kinderen die al voor het 2^{e} levensjaar waren geïnfecteerd met CMV of EBV, dan bij kinderen die op latere leeftijd waren geïnfecteerd. Onze resultaten suggereren dat er slechts een milde verschuiving ontstaat in de T-celpopulaties na infectie met CMV of EBV in de vroege kindertijd. Deze bevindingen zijn in tegenstelling met eerder beschreven sterke effecten van deze virussen op de T cellen in ouderen. Deze nieuwe inzichten in de effecten van herpesvirussen op het afweersysteem van jonge kinderen kunnen een belangrijk bijdrage leveren aan onze kennis over de herpesvirus-geassocieerde veroudering van ons afweersysteem bij ouderen.

In de literatuur is beschreven dat het EBV persisteert in B geheugencellen, maar het is niet bekend wat het effect daarvan is op de grootte en de verdeling van de verschillende B geheugencelpopulaties. Daarom hebben wij in **Hoofdstuk 6** bestudeerd in welke B geheugencelpopulaties EBV persisteert in het bloed van volwassenen. Daarnaast hebben we bestudeerd wat het effect is van persistente EBV infectie op de aantallen B geheugencellen in bloed van kinderen. We hebben aangetoond dat EBV in alle 6 typen B geheugencellen kan persisteren, maar dat de frequentie het hoogst was in de IgA^{+} en IgG^{+} subsets. Op de leeftijd van 14 maanden waren de aantallen van deze B geheugencellen significant lager in het bloed van EBV-geïnfecteerde kinderen, in vergelijking met EBV-negatieve kinderen. Dit effect was kortstondig en op 6-jarige leeftijd waren de aantallen B geheugencellen in deze EBV-geïnfecteerde kinderen vergelijkbaar met die in EBV-negatieve kinderen en kinderen die pas tussen 14 maanden en 6 jarige leeftijd waren geïnfecteerd met EBV. Bovendien hadden de EBV infectie en de bijbehorende tijdelijke verlaging van B geheugencellen geen langdurig effect op vaccinatieresponsen tegen tetanus en mazelen. Hiermee hebben we voor het eerst een negatief effect gezien van EBV persistentie op B geheugencellen en hebben we vooral het vermogen aangetoond van het afweersysteem om deze gevolgen te herstellen.

Infectie met HIV heeft een enorme invloed op het afweersysteem, maar dankzij de invoering van gecombineerde antiretrovirale therapie (cART) zijn de effecten veel kleiner dan voorheen. Veelal kunnen HIV-geïnfecteerde kinderen tegenwoordig de volwassen leeftijd bereiken zonder veel klinische problemen. Toch is het lange-termijn effect van HIV infectie en de bijbehorende cART behandeling nog onvoldoende bekend. In **Hoofdstuk 7** hebben we het verworven afweersysteem bestudeerd in een groep met HIV-geïnfecteerde kinderen en jongvolwassenen die onder behandeling waren van cART. We hebben gevonden dat cART in staat was de hoeveelheid virus in het bloed te verlagen tot beneden de detectiegrens van de meettechniek. Als gevolg werden CD4^{+} T cellen niet massaal geïnfecteerd en waren deze aantallen vrijwel normaal. Daarnaast zorgde cART behandeling ervoor dat de aantallen B en T geheugencellen, en de meeste andere effecten van het virus op het afweersysteem, bijna volledig normaliseerden. Desalniettemin konden we een aantal subklinische defecten identificeren die niet verdwenen door de behandeling. Dit betrof onder andere een blijvend verlies van CD4^{+} T cellen, TCRγδ^{+} T cellen en B geheugencellen, en een blijvend toegenomen CD8^{+} T

celpopulatie. Een gedetailleerd beeld van de conditie van het afweersysteem van HIV-geïnfecteerde kinderen, en het herkennen van signalen die mogelijk wijzen op toekomstige klinische problemen, is uiterst belangrijk voor de gezondheidszorg en eventuele verdere optimalisatie van behandeling. Hoewel de geïdentificeerde defecten (nog) subklinisch zijn, kunnen de geïdentificeerde populaties mogelijke belangrijke targetpopulaties zijn voor immunologische monitoring van HIV-geïnfecteerde kinderen.

Gezamenlijk illustreren de studies in dit proefschrift de complexe dynamiek in het afweersysteem van jonge kinderen en het effect van persistente virussen hierop. Onze bevindingen benadrukken het vermogen van het afweersysteem van kinderen om een infectie met een persistent virus zoals de herpesvirussen of HIV onder controle te houden zonder de negatieve effecten die beschreven zijn bij ouderen. Deze informatie kan niet alleen bijdragen aan onze basale kennis over de vorming van het gezonde afweersysteem, maar kan mogelijk ook een belangrijke bijdrage leveren aan onze kennis over de disfunctie van het afweersysteem tijdens chronische (virus-gerelateerde) stimulatie. Bovenal zal deze informatie bruikbaar zijn bij het verder ontrafelen van de processen die de vorming van levenslange immuniteit reguleren zonder de vorming van ontoereikende, excessieve of zelfs destructieve responsen.

Addendum

LIST OF ABBREVIATIONS

AID	Activation-induced cytidine deaminase
AIDS	Acquired immunodeficiency syndrome
anti-TPO	anti-thyroid peroxidase IgG
anti-tTG	Anti-tissue transglutaminase IgA antibody
APC	Antigen presenting cell
BCR	B-cell receptor
BMI	Body mass index
cART	Combined antiretroviral therapy
CCR	Chemokine receptor
CD	Cluster of differentiation
CI	Confidence interval
CMV	Cytomegalovirus
CR	Complement receptor
CSR	Class switch recombination
D	Diversity
DC	Dendritic cell
EBV	Epstein Barr virus
FSC	Forward scatter
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
HSV-1	Herpes simplex virus-1
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
IgL	Immunoglobulin light chain
Igκ	Immunoglobulin kappa
Igλ	Immunoglobulin lambda
IL	Interleukin
INF	Interferron
J	Joining
KLRG1	Killer cell lectin like receptor G1
MHC	Major histocompatibility complex
NK cell	Natural killer cell
OR	Odds ratio
PD-1	Programmed death
SD	Standard deviation
SES	Socio-economic status
SHM	Somatic hypermutation
SSC	Side scatter
TI	T cell-independent
TD	T cell-dependent

Tc	Cytotoxic T cell; CD8+ T cell
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
TG2A	Anti-tissue transglutaminase IgA antibody
Th	Helper T cell; CD4+ T cell
TNF	Tumor necrosis factor
Treg	Regulatory T cell
V	Variable
VCA	Viral capsid antigen
VZV	Varicella zoster virus

Addendum

CURRICULUM VITAE
PHD PORTFOLIO
LIST OF PUBLICATIONS

CURRICULUM VITAE

Personal details:

Name	Diana van den Heuvel
Date of birth	12 July 1987
Place of birth	Rotterdam; The Netherlands
Nationality	Dutch

Education:

2010-2015 **PhD study: Department of Immunology of the Erasmus MC**
The Immune System out of Shape? Shaping of adaptive immunity by persistent viral infections in young children
Promotor: Prof.dr. J.J.M. van Dongen
Co-promotor: Dr. M.C. van Zelm

2008 - 2010 (cum laude) **Master of Science: Biomolecular Sciences**
Vrije Universiteit Amsterdam; Specialization Cell Biology

2005 - 2008 **Bachelor of Applied Science: Biology and Medical Laboratory research;**
Hogeschool Rotterdam; Differentiation: research/ biochemistry; extra subjects: histology, gene cloning and signal transduction

1999 - 2005 **VWO** (pre-university education)
Comenius College, Capelle aan den IJssel.

Research experience:

2010 **Literature study:**
An overprotective immune system: The origin, development and reactivity of IgE responses in immune protection and allergy
Erasmus MC, Department of Immunology, Rotterdam
Supervisors Dr. M.C. van Zelm and Drs. M.A. Berkowska

2010 **2nd Master internship:**
Characterization of B-cell defects in HIV-infected children
Erasmus MC, Department of Immunology, Rotterdam
Supervisors Dr. M.C. van Zelm and Drs. M.A. Berkowska

2009 **1st Master internship:**
DC-exosome binding to T-cells: Retention at the plasma membrane and functional consequences
University Utrecht, Department of Biochemistry and Cell biology, Utrecht
Supervisors Dr. M. Wauben Dr. E. Nolte-'t Hoen

2008 **Graduation internship bachelor:**
Determining the mutation rate and mutation characteristics of 18 Y-chromosomal Short Tandem Repeat Markers (Y-STRs)
Erasmus MC, Department of Forensic Molecular Biology, Rotterdam
Supervisors Prof.dr. M. Kayser and M. Goedbloed

2007	**1st Bachelor internship:** *Tuberous Sclerosis complex: Characterization of TSC2 and TSC1 variants* Erasmus MC, Department of Clinical Genetics, Rotterdam Supervisor Dr. M. Nellist

Courses (additional to the PhD-portfolio):

2005 (certificate)	Coaching of first year bachelor students

Work history:

2008	**Research Technician** Erasmus MC, Department of Forensic Molecular Biology Head of the group: Prof.dr. M. Kayser
2006 - 2008	**Student coach** at the Hogeschool Rotterdam

PHD PORTFOLIO

Name PhD student	Diana van den Heuvel
Erasmus MC Department	Immunology
PhD period	September 2010 – February 2015
Promotor:	Prof.dr. J.J.M. van Dongen MD
Supervisor:	Dr. M.C. van Zelm
Research School:	Molmed

1. PhD Courses	Year
General courses	
- Teach the teacher I	2014
- Management voor promovendi en postdocs (NIBI)	2013
- Biomedical English Writing and Communication (15 weeks)	2012
- The Basic Introduction Course on SPSS	2011
Specific courses	
- Basic flow training (advanced)	2013
- Regulatory B-cell symposium	2012
- The 4th Symposium & master classes on Mucosal Immunology 'Adaptive immune response in the mucosa: B Cells and Beyond'	2011
- The Advanced Course 'Molecular Immunology' at the Molecular Medicine postgraduate school within the Erasmus MC	2011
- Molmed course (introduction to the topics of the 15th Molecular Medicine day 2011)	2011
- Mucosal Immunology symposium + masterclass; Molecular Medicine postgraduate school of the Erasmus MC; Rotterdam	2011
- Biomedical research techniques at the Molecular Medicine postgraduate school within the Erasmus MC	2010
- Lectures and refreshment classes of The Advanced Course 'Molecular Immunology' at the Molecular Medicine postgraduate school within the Erasmus MC	2009

2. Congresses, seminars, presentations	Year
Seminars and workshops	
- Annual Molmed Day (4x)	2010-2014
- Medische Immunologie, Rotterdam	2010
Presentations	
- Mucosal Immunology symposium + masterclass; Molecular Medicine postgraduate school of the Erasmus MC; Rotterdam *(presentation in the masterclass)*	2011
- 29th annual meeting of the European Society for Paediatric Infectious Diseases (ESPID) *(ePoster presentation)*	2011

(Inter)national conferences	
- Keystone: 'HIV Vaccines' combined with 'B cell development and function'	2013
- The 5th Netherlands Conference on HIV Pathogenesis, Prevention and Treatment (NCHIV 2011)	2011
- 29th annual meeting of the European Society for Paediatric Infectious Diseases (ESPID)	2011
- Nederlandse Vereniging Voor Immunologie (NVVI) Lunteren symposium 2011; Time for high T: Features and functions of T-cells in health and disease	2011
- Nederlandse Vereniging Voor Immunologie (NVVI) annual meeting; Noordwijkerhout (5x)	2010-2014
Other	
- Journal club department of Immunology	2009-2013
- Seminars and minisymposia	2009-2014

3. Teaching	**Year**
- Vaardigheidsonderwijs immunologie 2nd year Medical curriculum	2011-2014
- Interviews with students, 2nd year Keuzeonderwijs "Paradise or Evil Empire"	2012-2013
- Supervising 3 Bachelor theses / 1 Master thesis	2011-2014

PUBLICATIONS

Accepted publications

D. van den Heuvel, G.J.A. Driessen, M.A. Berkowska, M. van der Burg, A.W. Langerak, D. Zhao, H. Charif, N.G. Hartwig, A.M.C. van Rossum, P.L.A. Fraaij, J.J.M. van Dongen and M.C. van Zelm; **Persistent subclinical immune defects in HIV-1 infected children treated with antiretroviral therapy.**
AIDS (2015), in press

D. van den Heuvel, M.A.E. Jansen, W.A. Dik, H. Bouallouch-Charif, D. Zhao, K.A.M. van Kester, M.A.W. Smits-te Nijenhuis, M.J. Kolijn-Couwenberg, V.W.V. Jaddoe, R. Arens, J.J.M. van Dongen, H.A. Moll and M.C. van Zelm; **CMV- and EBV-induced T-cell expansions in young children do not impair naive T-cell populations or vaccination responses: The Generation R Study.**
Journal of Infectious Diseases (2015), in press

M.A.E. Jansen, D. van den Heuvel, M.C. van Zelm, V.W.V. Jaddoe, A. Hofman, J.C. de Jongste, H. Hooijkaas and H.A. Moll; **Decreased memory B cells and increased CD8 memory T cells in blood of breastfed children: The Generation R study.**
PLoS One. 2015; 10(5): e0126019.

Submitted publications

M.A.E. Jansen, D. van den Heuvel, S.H. Bouthoorn, V.W.V. Jaddoe, H. Hooijkaas, H. Raat, P.L.A. Fraaij, M.C. van Zelm and H.A. Moll; **Can we explain ethnic differences in CMV, EBV and HSV-1 virus seroprevalences in childhood? The Generation R Study.**
Submitted

M.A.E. Jansen, D. van den Heuvel, K.V.M. van der Zwet, V.W.V. Jaddoe, A. Hofman, J.C. Escher, P.L.A. Fraaij, H. Hooijkaas, M.C. van Zelm and H.A. Moll; **Herpesvirus infections and transglutaminase type 2 antibody positivity in childhood: The Generation R Study.**
Submitted

Manuscripts in preparation

D. van den Heuvel, M.A.E. Jansen, A. Bell, A.B. Rickinson, V.W.V. Jaddoe, J.J.M. van Dongen, H.A. Moll and M.C. van Zelm; **Transient reduction in IgA$^+$ and IgG$^+$ memory B-cell numbers in young children persistently infected with EBV: The Generation R study.**
Manuscript in preparation

D. van den Heuvel, M.A.E. Jansen, K. Nasserinejad, W.A. Dik, E.G. van Lochem, L.E. Bakker-Jonges, H. Bouallouch-Charif, V.W.V. Jaddoe, H. Hooijkaas, J.J.M. van Dongen, H.A. Moll and M.C. van Zelm; **Effects of external determinants on age-related patterns of innate leukocyte and naive and memory B- and T-lymphocyte numbers in early childhood: The Generation R Study.**
Manuscript in preparation

Addendum

ACKNOWLEDGEMENTS/DANKWOORD

Hier zit ik dan, relaxed en languit op de bank mijn dankwoord te schrijven. Ik had een jaar, of zelfs een maand geleden niet gedacht dat dit moment ooit nog ging komen. Dit proefschrift was nooit tot stand gekomen zonder de hulp van heel veel mensen. Ik ga mijn best doen jullie allemaal te bedanken, dus ga er maar even goed voor zitten…

Allereerst wil ik mijn promotor Jacques van Dongen en copromotor Menno van Zelm bedanken voor alle hulp en begeleiding tijdens mijn uiteindelijk bijna 5-jarige promotieonderzoek. Ik heb veel van jullie geleerd. Jullie beiden zijn zeer enthousiast over het doen van onderzoek en stonden altijd klaar met nieuwe plannen en ideeën. Menno, ik begon al eerder in jouw groep als een master student. Ook al had ik officieel gekozen voor een project naar de reactiviteit van IgA^+ B cellen, was mijn project na twee dagen al compleet veranderd en bestudeerde ik opeens de B en T cellen in HIV-geïnfecteerde kinderen. Deze switch was even wennen, maar uiteindelijk heb ik er toch maar mooi een hele interessante promotiestudie aan overgehouden. Niet alleen het onderwerp, maar ook zeker jouw tempo en niveau van denken en werken was even wennen. Zeker in het begin was het niet altijd even gemakkelijk om je bij te benen, maar ik heb hier wel heel veel van geleerd. Nu ben je zelf aan een nieuw avontuur begonnen in Australië. Heel veel succes daar. Je gaat het er vast fantastisch doen!

Vervolgens wil ik graag mijn commissieleden bedanken. Prof.dr. H.A. Moll, Prof.dr R.A.W. van Lier en Dr. A.M.C. van Rossum van de kleine commissie, en ook Prof. dr. R. de Groot en Prof.dr. G.M. Verjans: bedankt dat jullie plaats wilden nemen in mijn promotiecommissie en dat jullie de tijd wilden nemen om mijn stukken te beoordelen. Speciale dank gaat uit naar Henriette. Bedankt dat je niet alleen secretaris van de commissie wilde zijn, maar me ook altijd nuttige feedback hebt gegeven en me hebt geholpen bij alle epidemiologische analyses die we samen hebben gedaan. Hoewel we in het begin soms twee verschillende talen leken te spreken, heb je me geholpen om de 'epidemiologische taal' genoeg onder de knie te krijgen om mijn promotieonderzoek mooi te kunnen afronden. Daarnaast ben je een belangrijke buffer geweest tussen de verschillende expertises die allemaal meespeelden tijdens het doen van onderzoek op het grensgebied van de epidemiologie en immunologie, bedankt daarvoor.

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Next, I would also like to thank the people that introduced me into the immunology department and that guided me through my master internship and into my PhD-project. Magda (Berkowska), you taught me very patiently everything I had to know during my internship, and after that we have been roommates for a while during my PhD-project. You have set the bar for us PhD-students in the group of Menno, which I am trying to reach. Thanks a lot for your help and guidance and I hope that we can meet once in a while to catch up. Maar ook Edwin, je bent ondertussen niet meer op het Erasmus, maar je hebt me tijdens mijn stage onder je hoede genomen wat betreft de flowcytometrie van HIV-geïnfecteerde kinderen. Ik kon altijd op je rekenen. Bedankt voor je hulp.

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but you were always very good in everything you did. Plates full of bacteria, plates full of ELISA tests, or loads of flowcytometry analyses, nothing was too much. You decided (unfortunately) to continue a master outside the field of science, but you are of course also doing very well at that. Good luck with all your future plans. Rachid, je kreeg een technische uitdaging om de tetrameerkleuringen te starten en dat heeft zo zijn hobbels gekend, maar het is gelukt. Je bent inmiddels bezig met de I&I master. Heel veel succes! Ingrid (Snijdewind), ook al was je niet mijn student, je hebt me wel geholpen met de flowcytometrie van de HIV-studie. Jij bent zo'n bezige bij die alles tegelijk doet en de ballen in de lucht houdt. Je master, je coschappen, je promotieonderzoek en dan ook nog zwanger. Je bent altijd druk, maar nooit te beroerd om iemand anders te helpen. Succes met alles afronden (gaat zeker goed komen) en geniet van je gezinnetje!

Dan zijn er nog mijn kamergenootjes. Hanna, in de oudbouw hebben wij lange tijd een kamer gedeeld. Je was altijd de vraagbaak voor mij als startende PhD-student. Ondertussen zie ik je eigenlijk als 'één van ons BCDers' en zit je ook op de BCD-kamer (al mogen we hem niet zo noemen ;-P). Nu nog loop ik bij je langs zodra ik ben vastgelopen in mijn Excel analyses en lossen we het op. Maar ook naast de praktische dingen weet ik je te vinden voor hulp en mental support. Ondertussen als postdoc aan de gang en net terug van je zwangerschapsverlof ben je al weer druk met alle studies. Maar hoe druk ook, jij lijkt altijd alles onder controle te hebben, daar ben ik stiekem jaloers op. Anna, wij waren in onze tijd samen op de kamer allebei aan het stoeien met flowcytometrie analyses en daardoor elkaars mental support. Ondertussen ben je gepromoveerd en druk in de kliniek. Succes met alles. Martine ("lekker ding") en Marjolein, het is altijd een drukke boel op de kamer. Met veel gekakel en koffietjes of theetjes. Altijd gezellig. Jullie zijn allebei ook al lekker op weg in jullie promotieonderzoek. Succes met afronden. Jullie kunnen het!

En dan is er natuurlijk nog de hele (ex-)BCD groep. Magda B., Magda R., Christina, Britt, Jorn, Marieke, Edwin, Benjamin, Halima en Katharina. Sommigen heb ik hierboven al bedankt. Magda R., we started together on the FRET project, are now defending on two consecutive days, and even party together. Both of us struggled through the process of writing and finishing our thesis together. When you were stressed out, I would try to listen, but what happened most of the time is that I would freak out and you would listen and tell me that the world is not going to fall apart. With your complex project and your ability to focus and organize your projects, I always considered you as the most 'true scientist' in our group. It was a tough start being alone in The Netherlands and running a project in which almost no-one could help. But you nailed it! Jorn, jij bent altijd de rust zelve en hebt alles onder controle. Je gaat bijna naar Australië en daarna je promotieonderzoek afronden. Veel stukken op de planning, maar ik heb er vertrouwen in dat je ook dat allemaal onder controle hebt. Marieke, hoewel de start van je aanstelling niet even soepel liep, heb je je helemaal in je project gegooid en ben je ook nu hard op weg. Ik hoop dat je stukken snel geaccepteerd worden. Je kunt wel een positieve review gebruiken. Benjamin, wij hebben heel wat gesprekken gehad over onderzoek en daarbuiten. Nu je op de 11e zit spreek ik je helaas minder. Ik hoop dat we contact houden. Succes met je nieuwe vaste contract! Katharina, it was only for a relatively short time, but thanks for working together. You just started your new job. Good luck back in Germany!

Uiteraard wil ik ook alle andere collega's en coauteurs bedanken voor alle hulp en gezelligheid tijdens deze PhD-studie. Ik kan jullie in dit dankwoord helaas niet allemaal bij naam noemen, en zou ook alleen maar mensen vergeten als ik het toch ga proberen. Daarom aan jullie allemaal: dankzij jullie heb ik een hele fijne tijd gehad binnen de afdeling Immunologie.

En dan komen we uiteraard bij de mensen die misschien nog wel het allerbelangrijkst zijn geweest in de afgelopen jaren. Pa, Ma, Linda en Anita, wie had gedacht dat ik nog eens ontspannen en languit op mijn eigen bank mijn dankwoord zou schrijven. Bedankt voor jullie hulp. Vooral de laatste twee jaar hebben ook van jullie veel gevraagd, en dan heb ik het niet alleen over de promotietijd… Deze periode is zwaarder geweest dan ik me van tevoren kon bedenken, maar dankzij jullie ben ik toch op dit punt terecht gekomen en heb ik alles weer op de rit. Jullie hebben me er doorheen getrokken ook al was het voor jullie ook onwijs zwaar, daar kan ik jullie niet genoeg voor bedanken. Ik heb meer geleerd in deze periode dan tijdens de volledige rest van mijn promotieonderzoek. Het belangrijkste wat ik heb geleerd is om te genieten van wat je doet. Laat niemand oordelen over wie je bent. Geniet van de weg naar je doel, want die is soms belangrijker dan de uitkomst zelf. En pieker niet, want piekeren is als een schommelstoel; het houdt je bezig maar brengt je nergens.

Maar boven alles: Het leven is als een vlinder; ook al zou de wereld zonder ze misschien precies hetzelfde functioneren, hij zou minder mooi zijn.

Iedereen bedankt; Thank you all!

Diana