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Immune maturation in early childhood and the influence of herpesvirus infections

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Den här avhandlingen handlar om utvecklingen av barns immunförsvar och hur den kan påverkas av herpesvirus-infektioner. Vid födseln är immunsystemet inte färdigutvecklat och därför är nyfödda barn särskilt mottagliga för infektioner. Några bakterier och virus kan dock hjälpa till att stimulera mognaden av immunceller i barnet. Herpesvirusen Epstein-Barr-virus (EBV) och cytomegalovirus (CMV) har utvecklats sida vid sida med människan under vår evolution. Idag är mellan 70-90% av alla vuxna människor infekterade med EBV och/eller CMV. Man får ofta de här virusen i barndomen och som med många andra infektioner blir man inte så sjuk som man skulle blivit om man fått sjukdomen när man är äldre. När man väl fått infektionen stannar virusen kvar i kroppen i en vilande (latent) form. Då och då aktiveras de igen, men man märker oftast ingenting eftersom immunsystemet minns infektionen och snabbt kan slå ner den. Om man däremot har ett försvagat immunförsvar, som t.ex. vid HIV/AIDS, kan svåra sjukdomar utvecklas som följd av EBV- och CMV-infektioner. Det faktum att vi sällan besväras av dessa livslånga passagerare tyder dock på att vi hittat en balans under vår gemensamma evolution. Trots detta interagerar anti-virala och infekterade celler ständigt även under en latent infektion. Vår hypotes var att den här interaktionen och de substanser som immuncellerna släpper ifrån sig påverkar deras mognadsnivå och har betydelse för barnets immunförsvar. I vår forskning har vi använt blodprover från barn och renat fram immunceller från dem. Vi har gjort experiment där vi tillsatt bakteriefragment eller virus. Med hjälp av biokemiska analysmetoder har vi mätt hur mycket försvarssubstanser som cellerna tillverkar, och har även kunnat titta på vilka molekyler olika cellpopulationer har på cellytan genom en fluorescensbaserad analysmetod som kallas flödescytometri.

I studie I undersökte vi monocyter, en celltyp som är viktig för infektionsförsvar inte minst hos nyfödda barn. Här studerade vi inte påverkan av herpesvirus men konstaterade att monocyter hos nyfödda var lika bra på att producera anti-bakteriella substanser som monocyter hos vuxna. Däremot såg vi att de kanske inte mognade lika mycket efter stimuleringen som vuxna monocyter. I studie II jämförde vi immunsvar hos 2-åriga barn med eller utan latent EBV- och CMV-infektion. Vi undersökte interaktionen mellan monocyter och naturliga mördarceller (NK-celler), som också har en extra viktig roll hos nyfödda. Vi såg att NK-celler hos barn med latent herpesinfektion producerade mindre av ett anti-viralt ämne (interferon-gamma/IFN-γ). De lägsta svaren återfanns i barn som var infekterade med båda virusen. I studie III jämförde vi immunsvar hos 5-åriga barn med eller utan latent EBV- och CMV-infektion. Vi kunde konstatera att barn med latent CMV hade förhöjda populationer av mogna NK- och T-celler. Även här fanns det en additiv effekt av att ha båda virusen latent, i alla fall vad gällde NK-cellerna. I studie IV EBV-infekterade vi celler från EBV-negativa barn (nyfödda, 2- eller 5-åringar) med eller utan latent CMV. Vi tittade på hur B-cellerna påverkades och på immunsvar från NK- och T-celler. Vi visade att infektionsgraden berodde på hur stora mängder anti-viral IFN-γ som cellerna tillverkade. Vi kunde även visa att B-cellerna hos CMV-positiva barn inte följde samma infektionsförlopp. Detta föreföll vara kopplat till de stora populationer av mogna T-celler som finns i CMV-positiva barn.

Av dessa fynd framgår det att de virus som vi bär med oss hela livet kan bidra till en ökad mognadsgrad hos barnets immunceller. Detta kan i sin tur ha betydelse för reaktioner på andra infektioner och/eller harmlösa stimuli som allergener, och till och med ha en skyddande effekt. Vidare forskning kring hur immunsystemet utvecklas, mognar och bemöter infektioner gör att vi bättre kan förstå oss på sjukdomsförlopp för att kunna utveckla nya behandlingsformer och vaccin.

SCIENTIFIC SUMMARY

The quality of immune responses develops gradually from birth into adulthood and in the context of the host microbial environment. The aim of the work presented in this thesis was to study immune maturation during childhood, and how this process can be affected by the commonly acquired herpesviruses; Epstein-Barr virus (EBV) and cytomegalovirus (CMV).

In newborns, the innate immune system is relatively more important as adaptive functions are slower to mature. The capacity of cells from the newborn to produce cytokines has been extensively studied, but never the prevalence or functions of the two major innate monocyte subsets. **In paper I** we found that neonatal monocyte subsets had a similar phenotype and existed in similar frequencies as in adults. They had a potent capacity in their production of pro-inflammatory cytokines following stimulation with a bacterial ligand. The $CD14^+CD16^+$ monocyte subset remained at a higher degree in neonatal cell cultures post-stimulation, which could reflect a failure to further differentiate. These results show that the neonatal monocyte compartment is not inherently immature and we suggest that impairment in the neonatal immune response may lay predominately in the continued differentiation of monocytes to e.g. DCs.

Infections with EBV and CMV are commonly asymptomatic, occur during childhood and are followed by life-long persistence. Mouse studies have shown that latent infections with these herpesviruses confer increased functionality of natural killer (NK) cells that are important effectors against viral infection. **In paper II** we investigated whether latency with EBV and CMV in 2-year old children could have similar effects and examined the collaboration between monocytes and NK cells. We found that monocyte-induced NK-cell production of IFN-γ was in fact decreased in seropositive (EBV and/or CMV positive) children as compared to seronegative. IFN-γ levels were lower also in the corresponding plasmas and this was most pronounced in children co-infected with both viruses. Also, seropositive children tended to have a lower proportion of $CD14^+CD16^+$ monocytes that were potent inducers of NK-cell IFN-γ. This indicates that herpesvirus latency in childhood can affect functionality of NK cells, and we suggest that in this system it could be via decreased stimulation from $CD14^+CD16^+$ monocytes.

As we found that herpesvirus latency, and particularly co-infection with EBV and CMV, affected the functional capacity of NK cells, and since recent findings indicated a role for CMV in the differentiation of NK- and T cells, we broadened our approach **in paper III** to include both NK- and T cells and followed up on our findings in 5-year old children. We found that EBV and CMV co-infection was associated with the highest levels of differentiated $NKG2C^+$ NK cells. Further, CMV^+ children had higher plasma IFN-γ and IL-15 levels and higher NK-cell cytotoxic capacity. We found high frequencies of $NKG2C^+$ NK cells upon *in vitro* EBV infection of PBMC from EBV-naïve CMV^+ children and in co-cultures with adult PBMC and an EBV-positive cell line and IL-15. Together this indicates an *in vivo* imprint of co-infection by these two viruses, possibly dependent on the induced cytokine environment and interactions between NK- and infected B cells.

Previous studies from our group showed that the time-point of EBV contraction during childhood affected the extent of immune-modulation by viral latency. Very little is known regarding the infection process of EBV during childhood due to the asymptomatic presentation. **In paper IV** we used the *in vitro* EBV infection model to infect PBMC from children of different ages, and with a positive or negative serostatus to CMV. We found that age and subsequent capacity for production of anti-viral cytokines affected infection rate in terms of B-cell proliferation and B-cell acquisition of memory phenotype. CMV seropositive children had lower EBV-induced accumulation of memory B cells, which was correlated to a high prevalence of $CD57^+CD8^+$ T cells and IFN-γ production. These findings demonstrate that herpesvirus seropositivity could be connected to altered responses to secondary infections through maturation of highly differentiated T cells.

Taken together, the work in this thesis shows that monocyte subsets at birth can give potent functional responses, and that latency with the common herpesviruses EBV and CMV have significant effects on the maturation process and functional capacity of anti-viral effector cells during childhood. This in turn could affect responses to related or unrelated infections or even to non-invasive antigens such as allergens.

LIST OF PAPERS

This thesis is based on the original papers listed below, which will be referred to by their roman numerals in the text:

I. **Sohlberg E,** Saghafian-Hedengren S, Bremme K, Sverremark-Ekström E. Cord blood monocyte subsets are similar to adult and show potent peptidoglycan-stimulated cytokine responses. Immunology. 2011 May;133(1):41-50.

II. Saghafian-Hedengren S[1], Sundström Y[1], **Sohlberg E,** Nilsson C, Linde A, Troye Blomberg M, Berg L, Sverremark-Ekström E. [1]Shared first authorship. Herpesvirus seropositivity in childhood associates with decreased monocyte-induced NK-cell IFN-gamma production. J Immunol. 2009 Feb 15;182(4):2511-7.

III. Saghafian-Hedengren S[1], **Sohlberg E[1],** Theorell J, Carvalho-Queiroz C, Nagy N, Persson JO, Nilsson C, Bryceson YT and Sverremark-Ekström E. [1]Shared first authorship.
Epstein-Barr virus co-infection in children boosts cytomegalovirus-related differentiation of Natural Killer cells. *Submitted manuscript.*

IV. **Sohlberg E,** Saghafian-Hedengren S, Rasul E, Marchini G, Nilsson C, Klein E, Nagy N and Sverremark-Ekström E.
CMV Seropositive Children Show Inhibition of *In Vitro* EBV-induced B-cell Transformation That is Associated with $CD8^+CD57^+$ T-cell Enrichment and IFN-γ. *Revised and resubmitted.*

LIST OF PAPERS (not included in thesis)

The following original articles are relevant but not included in this thesis. The papers will be cited by their roman numerals:

V. Béziat V, Liu L, Malmberg JA, Ivarsson MA, **Sohlberg E,** Björklund AT, Retière C, Sverremark-Ekström E, Traherne J, Ljungman P, Schaffer M, Price D, Trowsdale J, Michaëlsson J, Ljunggren HG, Malmberg KJ.
NK Cell Responses to Cytomegalovirus Infection Lead to Stable Imprints in the Human KIR Repertoire and Involve Activating KIRs. Blood. 2013 Apr 4;121(14):2678-88.

VI. Rasul AE, Nagy N, **Sohlberg E,** Adori M, Claesson HE, Klein G, Klein E. J Simultaneous detection of the two main proliferation driving EBV encoded proteins, EBNA-2 and LMP-1 in single B cells. Immunol Methods. 2012 Nov 30;385(1-2):60-70.

The following original articles are included in the authors' works but do not concern the main topic of this thesis.

VII. Wennstedt S, **Sohlberg E,** Hamad RR, Bremme K, Sverremark-Ekström E, Mincheva-Nilsson L and Holmlund U. "Alterations in the NKG2D/NKG2D-ligand system in preeclamptic pregnancies". *Manuscript in preparation*.

VIII. **Sohlberg E,** Saghafian-Hedengren S, Bachmayer N, Hamad RR, Bremme K and Holmlund U. Preeclampsia affects cord blood NK-cell expression of activation receptors and serum cytokine levels but not CB monocyte characteristics. *Accepted.*

IX. Bachmayer N, **Sohlberg E,** Sundström Y, Hamad RR, Berg L, Bremme K, Sverremark-Ekström E.
Women with pre-eclampsia have an altered NKG2A and NKG2C receptor expression on peripheral blood natural killer cells. Am J Reprod Immunol. 2009 Sep;62(3):147-57.

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ABBREVIATIONS

AID	activation-induced deaminase
AICL	activation-induced C-type lectin
APC	antigen-presenting cell
BCR	B-cell receptor
BL	Burkitts lymphoma
CBMC	cord blood mononuclear cells
CMV	Cytomegalovirus
CSR	class-switch recombination
CTL	cytotoxic T lymphocyte
DC	dendritic cell
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
GC	germinal center
HLA	human leukocyte antigen
IFN	interferon
IL	interleukin
ILC	innate-lymphoid cell
IM	Infectious mononucleosis
KIR	killer-cell immunoglobulin-like receptor
LCL	lymphoblastoid cell line
LMP	latent membrane protein
LPD	lymhoproliferative disease
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
MICA/B	MHC class I chain-related A/B
NK cell	natural killer cell
NCR	NK-cell receptor
PBMC	peripheral blood mononuclear cells
PGN	peptidoglycan
PRR	pattern recognition receptor
ROS	reactive oxygen species
SHM	somatic hypermutation
TCR	T-cell receptor
Tfh	T-follicular helper cell
Th1	T helper type 1
Th2	T helper type 2
Th17	T helper type 17
TLR	toll-like receptor

TNF	tumor necrosis factor
Treg	regulatory T cell
VCA	viral capsid antigen

FOREWORD

From the point of birth, our immune system develops in concert with the external and internal microbial world. Newborns are vulnerable to infections as their immune system is not yet fully mature. Maturation of immune cells and responses occurs progressively with age and is essential to cope with the pressure from infectious organisms that face us in our day-to-day life. One family of microbes that is commonly encountered in childhood is the herpesviruses, such as Epstein-Barr (EBV) and cytomegalovirus (CMV). They have co-evolved with humans for millions of years and are carried as lifelong latent infections once they are acquired. Latent viruses are continuously monitored by our immune system and ongoing responses to these viruses could uniquely affect differentiation of effector cells and unrelated immune responses. The overall aim of this thesis was to understand how the functionality of the immune system develops during childhood and if and how latently carried viruses can modulate this process.

INTRODUCTION

BASIC OVERVIEW OF THE HUMAN IMMUNE SYSTEM

The field of immunology strives to encompass all aspects of the immune system in living organisms. During evolution, some microbes developed the ability to invade other organisms and cause disease, and thus the immune system evolved based on the need for recognition of tissue damage and danger. Even the simplest of organisms such as single-cell eukaryotes and plants have evolved mechanisms of immunity to preserve integrity of the host system. In fact, many of the features of this system are shared going from plants to higher vertebrates. These include the ability to recognize and distinguish between infectious agents and the host, the use of germ-line encoded receptors that recognize molecular structures of microorganisms and, upon such recognition, the induction of genes that encode antimicrobial peptides.[1] In humans, physical barriers, specialized immune cells and immune organs act in concert with antimicrobial compounds to battle infectious agents (Figure 1). Immune cells are generated from precursor cells in the bone marrow through a process known as hematopoiesis. Pluripotent stem cells develop into common lymphoid or myeloid progenitors that further develop into distinct lineages of immune cells. Upon completion of this stage, immune cells exit the bone marrow and may develop further in lymphoid or non-lymphoid organs. Immune

cells are present throughout the body in circulating blood, lymph and lymphatic tissues and in specialized immune compartments such as the thymus, spleen and lymph nodes.

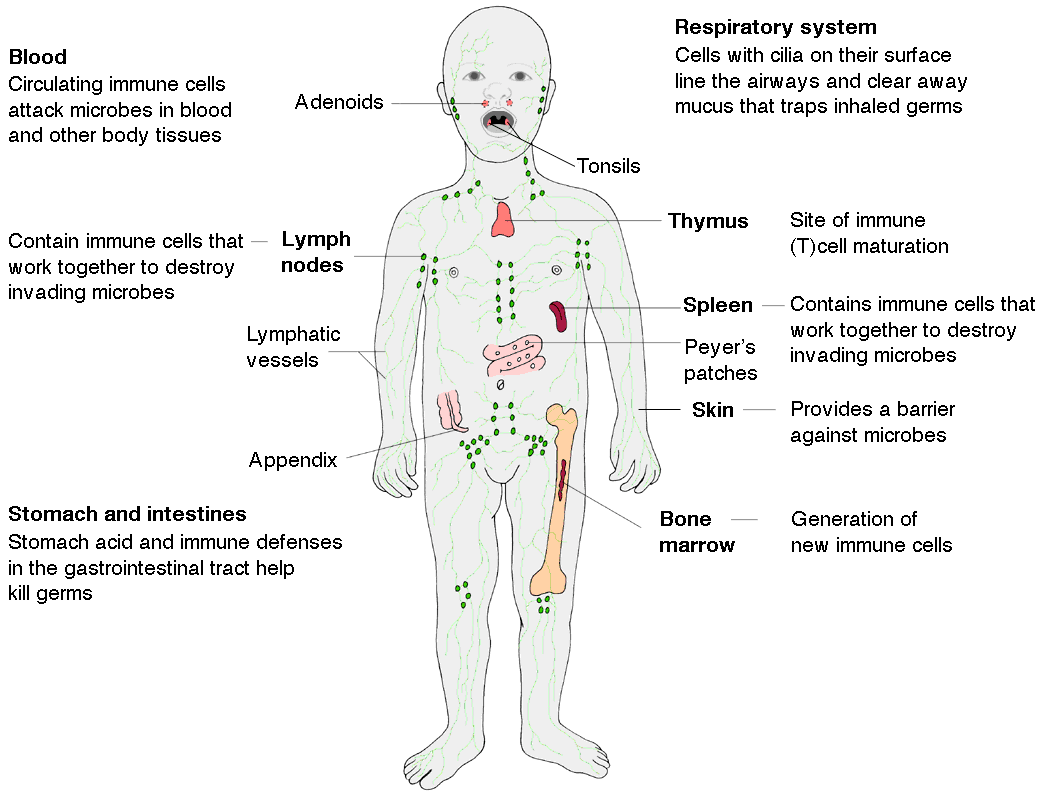

Figure 1. Simplified view of the bodys' defense mechanisms. Adapted from www.childrenshospital.org and www.humanillnesses.com.

Besides fighting infections, the immune system has many other roles in the body including protection against altered/malignant cells, preservation of tissue integrity upon sterile inflammation and in the reproductive system. Detection of an ongoing pregnancy by the immune system is recognized to be important for successfully maintaining gestation. The maternal immune system changes both locally in the intrauterine environment, in the peripheral circulation and in the implantation site which is filled with immune cells that among other functions contribute to vascularization.[2] Throughout pregnancy there is a continuous exchange of maternal and fetal factors and exciting new research shows that the conditions we face in the womb may influence future health and disease.[3,4]

Traditionally, the immune system has been divided into two branches that cooperate to eliminate threats to the host, the innate and adaptive immune system. They differ in their speed of initiation, their specificity and their ability to 'remember' infectious events. They also employ different modes of recognition of danger and have different effector mechanisms. Nonetheless their actions are highly interrelated and the initial quality of the innate response will direct subsequent adaptive functions.[5]

Constitutive innate defenses ensure rapid limitation of microbial invasion through preexisting molecules, such as a diverse range of complement factors and acute phase proteins, and phagocytic cells such as neutrophils and macrophages. Innate cells use pattern-recognition receptors (PRRs) to detect microbes. The PRRs are encoded in the germ-line and are therefore similar in all humans. They are called PRRs because they can recognize particular molecular patterns associated with microbes. These patterns or structures are often carbohydrates found in bacterial cell walls, yeasts and protozoa, and are essential for the organisms' virulence and survival. Upon recognition by the innate immune cell, microbes can be engulfed by the formation of a phagosome. Intracellular vesicles that contain enzymes and reactive oxygen species (ROS) fuse with this phagosome and the microbe can be digested. Alternatively, the content of intracellular vesicles is released to the outside and affects microbial membrane integrity.

Innate defenses may be sufficient to clear invading pathogens, but if not they can limit infection until cells of the adaptive immune branch have expanded enough to ensure elimination of the threat. Adaptive immune cells also recognize microbe-specific molecules or 'antigens', which refers to any molecule that binds specifically to a B- or a T-cell receptor. B cells can recognize antigen directly while T cells recognize the antigen when it is presented in association with another molecule on host cells, the major histocompatibility complex (MHC). This function is carried out by antigen-presenting cells (APCs). Activated B cells will start to produce antibodies that can neutralize the pathogen and/or target it for elimination by other cells, while activated T cells kill infected cells directly by the release of cytotoxic granules or by the induction of programmed cell death – apoptosis. T cells can also produce a number of substances that act on other immune cells and provide help to cells participating in an immune response. B- and T cells undergo somatic re-arrangement of the genes encoding their receptors, a process that can generate almost unlimited ability to recognize different microbes. They also form long-lived memory cells after resolution of an immune response and these memory cells are poised for immediate attack upon re-encounter with the original

pathogen. Therefore the repertoire of memory cells is individual depending on which infections a person has encountered during his or her lifetime.

In order to coordinate immune responses cells need to communicate. For this purpose, the production and secretion of soluble proteins called cytokines is of major importance. Cytokines bind to a variety of receptors and induce cellular responses regulating the intensity and duration of an immune response. Specialized cytokines called chemokines can induce movement of immune cells to a site of infection. This regulated and coordinated development of the immune response is extremely important in order to avoid inappropriate and damaging inflammatory responses after the pathogen is eliminated. The different functions of cytokines, and the cells of the human immune system will be further discussed in separate chapters.

INNATE IMMUNITY

Innate cells

The principal innate effector cells are neutrophils, NK cells, monocytes, macrophages and dendritic cells (DCs). Other innate cells include γδ T cells, basophils, eosinophils, mast cells, but also epithelial and endothelial cells can carry out some innate functions. Neutrophils are the most numerous among leukocytes representing 50-70% of circulating immune cells. They are highly efficient phagocytes that engulf and eliminate microbes by production of reactive oxygen species (ROS) and inducible nitric oxide synthase (iNOS). Another mechanism whereby neutrophils can kill bacteria is by the release of neutrophil extracellular traps (NETs). NETs are fibrous structures that are made up of de-condensed chromatin and contain anti-microbial proteins that are thrown into the extracellular space when the neutrophil undergoes active cell death. Neutrophils can also produce chemokines, like IL-8, that can recruit and instruct other immune cells at the site of infection.[6] NK cells constitute approximately 5-20% of the blood lymphocyte population. They contribute to host defense through cytolysis-mediated killing of stressed or infected cells, and are an important source of cytokines and chemokines that contribute to inflammation and polarization of T-cell responses.[7] Monocytes constitute 5-10% of the leukocyte pool, are important initiators of immune responses and a source of early cytokines following infection.[8] Monocytes are also precursors of macrophages and dendritic cells (DCs) that are central in antigen presentation and belong to the so-called professional APCs. Macrophages are highly adaptable, found in all tissues and show great functional diversity. They are effective phagocytes that are

specialized in the uptake, digestion and presentation of antigens to adaptive cells. They also have roles in development, homeostasis and tissue repair.[9] DCs are the most effective APCs, they are present in peripheral tissues where they sample antigen to later migrate to lymph nodes to prime naïve T cells and activate B- and NK cells. DCs are a very heterogeneous group of cells with varying origin and have specialized functions in different locations.[10] The main lineages are the plasmacytoid (pDCs) and myeloid (mDCs) that recognize microbes through expression of distinct PRR repertoires and that can differentially regulate adaptive immune function.[11]

The activity of, and collaboration between, monocytes and NK cells was explored within the papers in this thesis and these cells will be further described in the section "Related background'.

Pattern recognition

As previously mentioned recognition of microbes by innate cells is mediated through PRRs that can distinguish between different classes of pathogens by detection of structures such as components of the bacterial cell wall or viral nucleic acids.[12,13] PRRs are found both on the cell surface and either free or membrane-bound in the cytosol, which enables detection of microbes that employ different modes of infection. Their importance for host defense is underlined by the many microbial interference strategies targeted at PRRs[14] and that their deficient expression or function has been associated with increased susceptibility to infectious and persistent inflammatory diseases.[15-17] These types of receptors can also respond to endogenous danger signals such as nucleic acids and heat shock proteins that are released by necrotic cells. In an evolution of the now famous danger model by Matzinger, PRRs are suggested to respond to this type of cell damage induced in necrotic cells rather than to microbial presence per se, and the type of immune response initiated will depend on the tissue in which it is instigated.[18]

There are many types and families of PRRs including the RIG-I like receptors, C-type lectin receptors and NOD-like receptors. The most well known family of PRRs is the transmembrane Toll-like receptors (TLRs). These are found on the cell surface or on intracellular vesicles (Figure 2). So far 10 functional TLRs have been identified in the mammalian genome that together display a wide specificity. For example, TLR2 recognizes bacterial lipoproteins but also lipoteichoic acid and peptidoglycan (PGN) together with NOD1/NOD2. TLR4 recognizes lipopolysaccharide (LPS), TLR5 bacterial flagellin, TLR7

and TLR8 single stranded RNA and TLR9 unmethylated CpG motifs in DNA.[12] TLR activation initiates intracellular signaling cascades, which result in transcription of genes encoding inflammatory cytokines and chemokines, co-stimulatory molecules and antimicrobial compounds. TLRs are composed of extracellular ligand-binding and intracellular signaling domains. Upon ligation, the TLRs dimerize and associate with adaptor proteins such as MyD88, IRAK and TAK1. Downstream of TAK1, mitogen-activated protein kinases (MAPKs) such as the extracellular signal-regulated protein kinases (ERK) and p38-MAPK are activated through phosphorylation.[19] MAPKs in turn phosphorylate the transcription factor AP-1 that switches on genes encoding the inflammatory cytokines,[12] (Figure 2.)

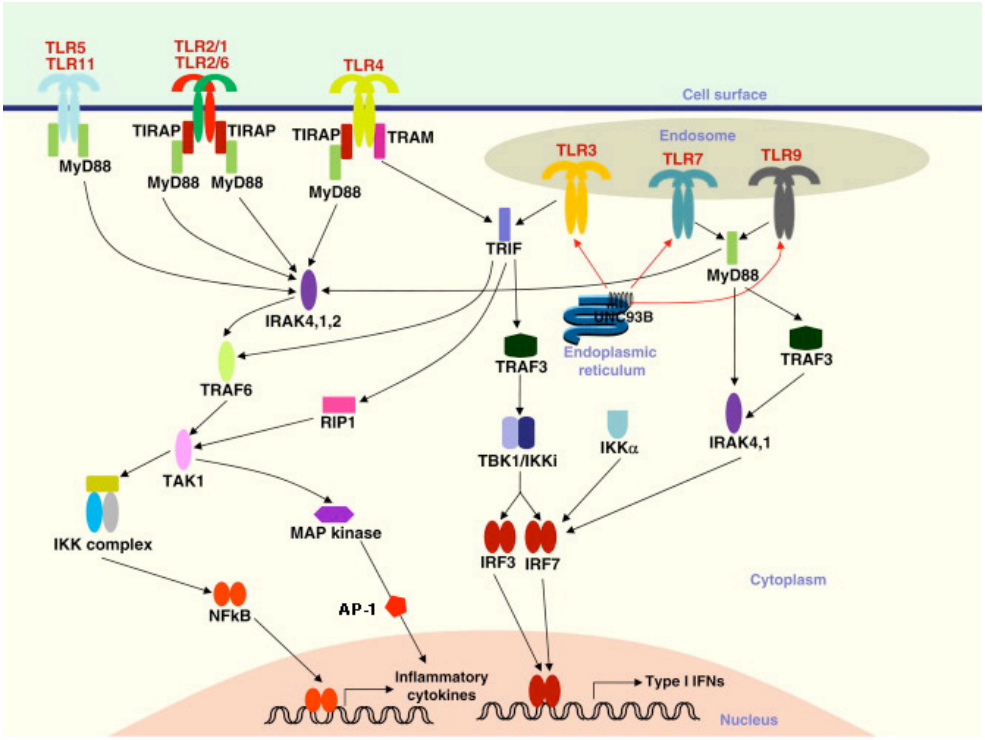

Figure 2. TLR signaling elicits production of inflammatory cytokines and type I interferons. Adapted from Kumar, Kawai & Akira.[20] Reprinted with the permission of Elsevier.

ADAPTIVE IMMUNITY

The adaptive branch of immunity is highly specific to previously encountered antigens. Responses can be categorized into cell-mediated immunity involving T cells and humoral immunity involving B cells.

T LYMPHOCYTES

The prefix T is derived from the thymus where progenitor T cells from the bone marrow undergo further maturation. T cells recognize antigen via the T-cell receptor (TCR) consisting of the CD3 molecule and associated signaling proteins. The TCR is expressed as a heterodimer consisting of a α- and a β chain in the case of classical ($\alpha\beta$ T cells) and a γ- and a δ chain in case of non-classical ($\gamma\delta$ T cells). For $\alpha\beta$ T cells to recognize antigens they need to be presented to them by cells bearing MHC class I or II molecules which interact with the CD8 and CD4 co-receptor, respectively.

During maturation, immature T cells undergo genetic rearrangement of the TCR genes (VDJ recombination), which generates their specificity. They also start to express both CD4 and CD8. The T cells will then interact with MHC-peptide complexes displayed in the thymus. T cells that are able to recognize self-MHC molecules survive and receive signals to only express CD4 or CD8. Those T cells that interact too strongly with MHC molecules presenting self-peptides, or those that do not regonize any MHC-peptide complex, undergo apoptosis. In this way naïve $CD3^+CD4^+$ T helper (Th) cells and $CD3^+CD8^+$ T cytotoxic (CTL) cells are generated and pass out of the thymus into the bloodstream or via the lymphatic system.

The MHC complex and antigen-presenting cells

Presentation of antigen in the periphery can be carried out by professional APCs expressing MHC II (macrophages, DCs and B cells) and non-professional APCs expressing MHC I (all nucleated cells). Non-professional APCs can process cytosolic proteins derived from the cell itself or from intracellular bacteria, into peptides that are transported to the surface in complex with MHC I molecules. Naïve $CD3^+CD8^+$ T cells can engage MHC I but additional signals from co-stimulatory molecules and cytokines are needed for generation of effector T cells. The professional APCs can endocytose extracellular particles and process these into peptides which can couple with MHC II molecules and be presented on the cell surface. Naïve $CD3^+CD4^+$ T cells can engage MHC II and after additional co-stimulatory signals be fully activated. Upon activation, clonal proliferation of T cells generates highly specific effector

cells but also long-lasting memory cells. Memory T cells persist long after an infection has resolved and can quickly expand to large numbers of effector cells upon re-exposure to their cognate antigen.

$CD3^+CD8^+$ cytotoxic T cells

Naïve $CD8^+$ T cells develop into effector cytotoxic T cells (CTLs) and proliferate under the influence of IL-2. CTLs got their name from their ability to induce apoptosis in virus infected- or tumor cells. Killing of target cells is achieved through a multi-step process that involves adherence and TCR-dependent recognition between the CTL and a target cell. This leads to formation of an immunological synapse and polarized release of granules that contain cytotoxic proteins like perforin. Perforin forms pores in the target membrane and co-secreted granzymes activate a cascade of apoptosis-inducing proteases. Killing can also be induced by interaction with the membrane bound Fas ligand on CTLs with the death receptor Fas on the target cell. Cross-linked Fas rapidly induces the apoptosis-related cellular machinery. CTLs are also important producers of cytokines, like TNF and IFN-γ, that can be directionally or broadly secreted.[21,22]

$CD3^+CD4^+$ T-helper cells

T-helper (Th) cells provide help to cells participating in an immune response through the production of cytokines. There is a vast array of Th-cell subsets that display different functionalities, these include but are not limited to Th1, Th2, Th9, Th17, Th22, T follicular helper cells (Tfh) and T regulatory (Treg) cells. Differentiation of $CD4^+$ T cells into these distinct Th-subsets is essential for proper host defense and normal immune-regulation.[23] Their respective differentiation depends on the signals that naïve $CD4^+$ T cells receive during their activation and will be tailored to suit the ongoing immune response in that particular tissue.[18]

Th-cell subset functions

Th1, Th2, and Th17 cells seem to cooperate with a different branch of the granulocyte-monocyte axis (Figure 3). Th1 cells support cell-mediated immunity and the eradication of intracellular pathogens through their production of important cytokines such IL-2, TNF and IFN-γ. IFN-γ is a potent activator of monocytes/macrophages and promotes cytotoxic effector functions of NK cells. IFN-γ also contributes to class-switch recombination of B cells to IgG isotypes that are effective in opsonization of pathogens. Th2 cells, on the other hand, support humoral immunity and enhance elimination of parasitic infections (e.g., helminths), through

production of IL-4, IL-5 and IL-13. These cytokines act on basophils, eosinophils and mast cells and promote antibody class switch recombination (CSR) of B cells to antibody types (IgE and IgG1) that enhance functions in cells that fight parasitic infections.[23,24] Th17 cells got their name from their production of IL-17, and are closely connected to enhancement of neutrophil function. IL-17 promotes cytokine production in innate and epithelial cells that in turn induce neutrophil production and recruitment. Besides IL-17, Th17 cells also produce other cytokines like IL-22, IL-26, granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor (TNF) that act on epithelial cells of barrier tissues, to enhance antimicrobial defense and epithelial barrier integrity. This can have an importance in the protection against extracellular bacteria and fungi.

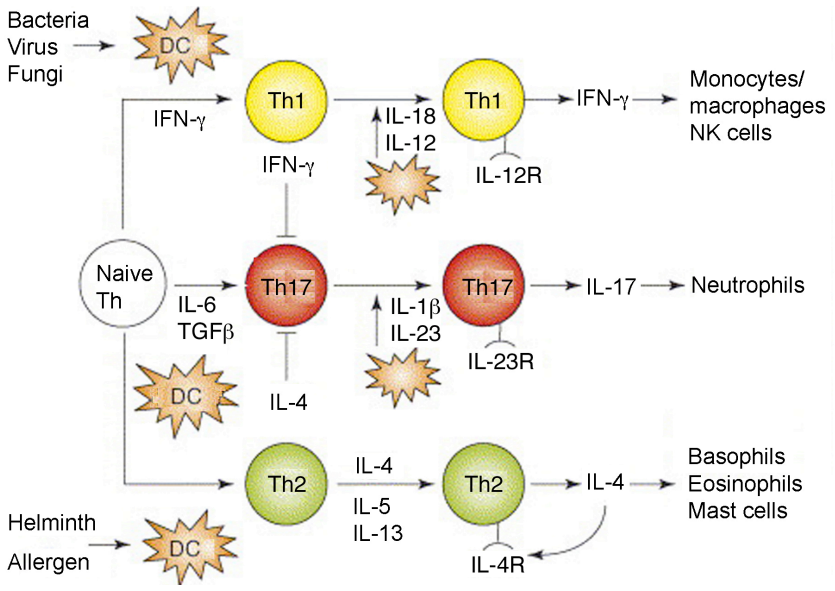

Figure 3. Cross-talk between Th1, Th2 and Th17 cell subsets and distinct innate cells. Adapted from Reinhardt *et al*.[25] Reprinted with the permission of Elsevier.

The phenotype of Th cell populations is defined by expression of signature cytokines and 'master regulator' transcription factors.[26,27] For instance, the phenotype of Th1 and Th2 is set by epigenetic modifications that during differentiation activate one set of genes (e.g. IFN-γ or IL-4) and silence the other.[28] Thus, it is perhaps not surprising that rather than representing distinct lineages, differentiated Th cells display plasticity and can changeover between the different subsets.[27] Perhaps the most dramatic example is that Th2 cells can be reprogrammed

to IFN-γ producing Th1 cells under the influence of type I interferons (IFN) in viral infections.[29]

Tfh cells can be distinguished from the other $CD4^+$ T cell lineages in that they express very low levels of the cytokines and transcription factors that distinguish eg Th1, Th2 and Th17. Tfh cells are important in the formation of germinal centres (GC) where they reside in close proximity to B cells. Tfh cells have a key role in promoting B-cell function through production of IL-21 and in the promotion of antibody class switch recombination (CSR). They are important for the generation of long-lived serological memory.[30]

Treg cells are vital to keep immunological unresponsiveness to self-antigens and suppress excessive deleterious immune responses. They can dampen the activity of effector cells through the production of IL-10 and TGF-β, by metabolic disruption of activated cells and via targeting of DCs thus preventing further activation of T cells.[31] There are two different sources of Treg cells, those derived from the thymus (natural or nTreg) and those that develop in the periphery and acquire a Treg phenotype and function in the periphery (induced or iTreg).[32]

Memory T cell subsets

When T cells are activated they will form effector and/or memory cells. The generation of memory ensures that we have a pool of antigen-experienced T cells, and these cells will accumulate over the lifetime of all individuals. Memory T cells can be broadly divided into central memory (T_{CM}), effector memory (T_{EM}) and terminally differentiated effector memory T cells (T_{EMRA}) subsets. They have distinct homing patterns, differ in their ability to proliferate in response to antigen or cytokines and in their effector functions. T_{CM} is predominant in the CD4 compartment whereas T_{EM} are more common in the CD8 compartment in the blood. Within tissues, T_{CM} are enriched in lymph nodes and tonsils, whereas lung, liver, and gut contain greater proportions of T_{EM}.[33] T_{CM} cells are $CD45RO^+$ memory cells that express the chemokine receptors CCR7 and CD62L that facilitate migration to T cell areas of secondary lymphoid organs. Due to efficient upregulation of CD40L they are believed to provide effective stimulatory feedback to DCs and B cells. After activation and proliferation, T_{CM} produce IL-2, IFN-γ or IL-4. The T_{EM} cells are memory cells that have lost expression of the lymphoid homing receptor CCR7 but display chemokine receptors that allow migration to inflamed tissues. Once in these tissues they can display direct effector functions. $CD8^+$ T_{EM} cells express large amounts of perforin and both $CD4^+$ and $CD8^+$ T_{EM}

can rapidly produce IFN-γ, IL-4 and IL-5. The T_{EMRA} cells are $CD45RA^+$ and are considered to be the most differentiated subset. T_{EMRA} cells are characterized by the highest expression levels of perforin and the most efficient effector functions. The relationship between these memory subsets is not entirely clear, but they have been suggested to develop from T_{CM} to T_{EM} and T_{EMRA}.[34]

B LYMPHOCYTES

B cells originate in the bone marrow and fully mature in secondary lymphoid organs like lymph nodes or spleen. One role for B cells is in the production of immunoglobulins (Igs). Igs are expressed on the B-cell surface as a part of the B-cell receptor (BCR) together with CD79 and co-receptors like CD19 and CD21. Igs can also be secreted in a soluble form, which is then referred to as antibodies. Unlike T cells that recognize antigens only in the context of MHC molecules, B cells can recognize free antigen. For example, carbohydrate structures on microbial surfaces can cross-link Igs on the B-cell surface thereby inducing activation. B cells can also be activated in a T-cell dependent fashion where Th cells are activated via professional APCs that promote Th-B cell interaction.[35] B cells themselves can act as the APCs and present MHC II restricted antigens to T cells. Recognition of antigen in this manner prompts binding between CD40 on the B-cell and CD40-ligand on the Th cell that will strengthen their interaction. B cells also express co-stimulatory molecules like CD80/86 that can interact with CD28 (activating) or CTLA-4 (inhibitory) on the Th cell. B cells also produce a wide variety of cytokines that can affect T-cell differentiation. Furthermore, B cells also express TLRs and signals can be integrated with those from the BCR to fine-tune functional B cell responses.[36]

Antibodies and affinity maturation

Antibodies recognize antigen and have three major roles, neutralization of a pathogen, activation of the complement cascade and enhancement of phagocytosis. Antibodies can bind to the surface of any cell that expresses Fc receptors. For instance, binding between antigen-antibody complexes and Fc receptors on macrophages or neutrophils induces phagocytosis. In NK cells, Fc binding of antibodies that are attached to the surface of a target cell initiates a process termed antibody-dependent cellular cytotoxicity (ADCC), leading to lysis of the target cell.[37]

Antibody molecules have antigen-binding and constant regions. The antigen-binding portion of the antibody is highly variable which is achieved through VDJ recombination early in B-cell development. The constant region is used to distinguish 5 different classes or isotypes of antibody; IgD, IgM, IgG, IgA and IgE. These classes have distinct effector functions. IgD mainly acts as a BCR. IgM is the first antibody produced upon antigen recognition and is efficient in activation of complement due to its pentameric shape and many antigen-binding sites. IgA is found in many bodily fluids and secretory IgA can cross-link large antigens and prevent the attachment of pathogens to the epithelium. IgE is involved in the protection against parasitic infection and in the allergic reaction. IgG is the major circulating antibody and can aid in neutralization of toxins, Fc-mediated phagocytosis and in activation of complement.

Naïve B cells express membrane-bound IgD and IgM. In order to produce IgG, IgA or IgE, class-switching is needed. Every naïve B cell has the potential to switch to any of these classes however isotype switching largely depends on the nature of the event prompting the switch, T cell and DC activity and the cytokines that are present in the local microenvironment.[38-40] When B cells are activated they can either generate short-lived antibody secreting plasmablasts or form GCs in nearby lymph node follicles. Short-lived antibody-producing B cells (i.e. plasma cells) provide an initial burst of low affinity antibodies that can limit infection until higher affinity antibody producing cells emerge from the GC.[41] In the GC, affinity maturation and isotype switching of B cells occurs through the process of somatic hypermutation (SHM) and CSR (Figure 4). SHM is initiated by the activity of the enzyme activation-induced deaminase (AID) that contributes to the deposition of point mutations into the variable antigen-binding region of the Ig gene to generate higher (or lower) affinity variants.[42] B cells with improved affinity will recognize antigen presented by follicular DCs. Then CSR will be initiated which exchanges the initial constant (Ig)M region for one of the downstream constant regions (IgG, E or A) through deletional recombination, allowing for switch between different Ig isotypes. When the B cell has gone through both these processes successfully it expresses highly specific isotype-switched antibodies. The B cell can then differentiate into either a long-lived antibody-secreting cell that can produce copious amounts of antibodies or a long-lived memory cell.

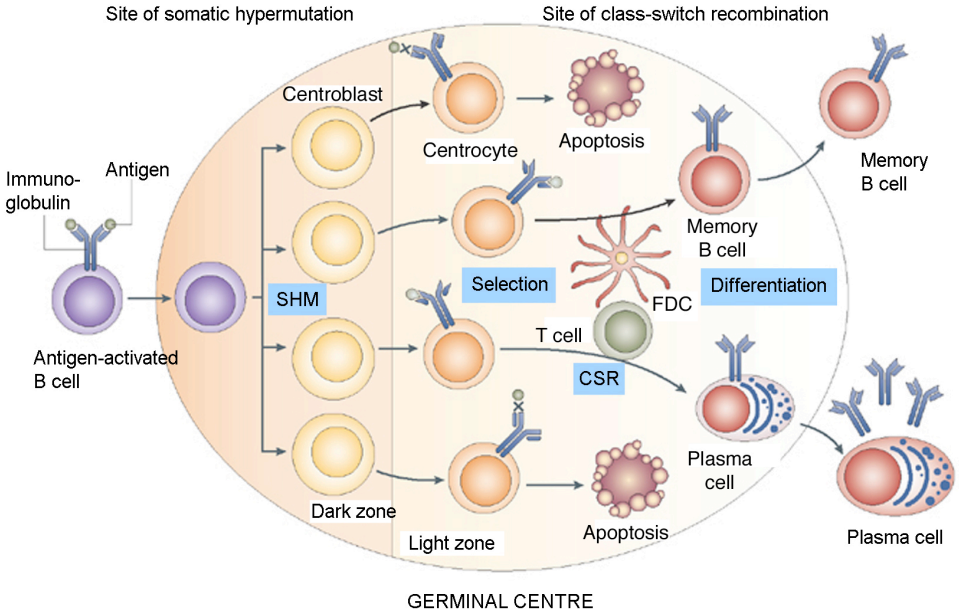

Figure 4. Fates of activated B cells. Adapted from Klein & Dalla-Favera.[35] Reprinted with the permission of Nature publishing.

B-cell subsets in blood

Mature B cells in the blood can be divided into subsets that separate naïve from memory B cells. The division between the subsets is based on expression of IgD and the TNF-receptor superfamily member CD27, following findings that in addition to mutated Ig genes, memory B cells express CD27.[43]

Antigen-inexperienced naïve B cells are described as $IgD^{+}CD27^{-}$. The antigen-selected B cells that have undergone clonal expansion along with isotype switching are referred to as $IgD^{-}CD27^{+}$ switched memory B cells. There are also $IgD^{+}CD27^{+}$ non-switched memory cells whose origins are unclear. Although the $IgD^{+}CD27^{+}$ B cells can be found in various anatomical locations they share a phenotype with marginal zone B cells and are commonly referred to as that. There is also a small subset of $IgD^{-}CD27^{-}$ cells that express IgM only. These cells may be unable to carry out somatic hypermutation or class-switch recombination.[44] Although CD27 is commonly used as a marker for memory, one study found that some Ig switched cells do not express CD27.[45]

BRANCHING OUT – NEW PLAYERS IN THE IMMUNE SYSTEM

Over the past decade many cells that do not fit the traditional division into the innate or adaptive branch have been discovered. A rapidly evolving field describes cells whose development, functions and activation mechanisms overlap the two branches. Some of these cells will be briefly mentioned below but are outside the scope of this thesis.

γδ T cells are prime examples of cells able to perform both conventional adaptive features, through the recognition of specific antigens by their unconventional TCR, and rapid innate-like responses with cytokine production and direct lysis of stressed or infected cells. Recognition of antigens by γδ T cells does not rely on MHC or MHC related molecules such as CD1, although this type of activation is also possible. They can instead themselves present antigen to conventional αβ T cells, provide help to B cells and participate in DC maturation.[46]

Natural killer T (NKT) cells are a subset of T cells that express NK-cell surface markers and participate in the defense against viral infection.[47] A subset of these cells is called invariant NKT (iNKT) cells. The T-cell receptor of iNKT cells recognizes self and foreign lipid antigens presented by CD1d in a conserved manner, and activation triggers a variety of polarized immune responses.[48]

Mucosal-associated invariant T cells (MAIT) display a semi-invariant TCR and are restricted by the MHC-related molecule MR1. They are present in the intestine, liver and draining lymph nodes, which implies a close relationship with the microflora. MAIT cells have been suggested to have dual roles; control of the commensal flora during homeostasis and effector functions via IFN-γ and TNF production after TCR-MR1 interactions during bacterial infection.[49]

Innate lymphoid cells (ILCs) are various developmentally related cells that have roles in mediating immune responses and regulating tissue homeostasis and inflammation. ILCs are defined by their lymphoid morphology, lack of recombined antigen receptors and absence of myeloid or DC phenotypes. Cells that fall under this denomination include lymphoid tissue-inducer (LTi), NK22, natural helper cells and NK cells that produce many of the Th-cell associated cytokines following stimulation. This year a nomenclature for ILCs was proposed based on the cytokines that these cells produce and the transcription factors that regulate their development and function.[50] Some ILC subsets are prevalent at mucosal surfaces and can rapidly secrete immunoregulatory cytokines, suggesting that they contribute to infection defense.[51]

CYTOKINES – soluble messengers

The importance of cytokines in the direction of immune responses and in cell-cell communication cannot be underestimated. There are different families of cytokines based on function; the prototype cytokines are called interleukins (ILs) since they act between leukocytes. Among other functions, ILs are involved in perpetuation of inflammation together with the TNF family. IFNs are key molecules in the early defense against viruses and induce an antiviral state in cells. Chemokines influence migratory behavior of leukocytes. A given cytokine can have different actions depending on its antagonistic or synergistic effects with other cytokines and whether the target cell has prior antigen priming. Below will follow a brief description of cytokines relevant for this thesis.

IL-1β and TNF

Some of the earliest cytokines initiated during inflammation are IL-1β and TNF that are produced primarily by activated monocytes, macrophages and DCs. In addition, NK-, B- and T cells also produce TNF. Both IL-1β and TNF are very potent pro-inflammatory cytokines and are therefore not normally found in high levels in circulation in healthy individuals. The two cytokines synergize to mediate the systemic acute phase response that follows local inflammation including induction of fever, production of acute phase proteins, such as C-reactive protein by the liver, and activation of the vascular endothelium.[52,53]

IL-6

Many of the systemic acute phase effects are mediated by IL-1β and TNF in combination with IL-6. Mainly monocytes, macrophages, DCs, endothelial- and B cells produce IL-6 in response to PRR and/or IL-1β/TNF stimulation. In the initial phase of infection or tissue injury, IL-6 induces acute-phase proteins, activation of macrophages and mediates lymphocyte arrival to the site of inflammation. In the resolution phase of inflammation IL-6 favors the onset of acquired responses by e.g. inducing neutrophil apoptosis whilst acting as a lymphocyte stimulatory factor. Also, IL-6 can block further pro-inflammatory cytokine and chemokine production thus affecting leukocyte recruitment.[54,55]

IL-8

IL-8 (CXCL8) is a chemokine produced during inflammation by a range of cells like monocytes, granulocytes, lymphocytes, fibroblasts and endothelial cells. Neutrophils are the primary producers of, and targets for, IL-8 and respond by chemotaxis and subsequent

upregulation of surface adhesion molecules. IL-8 also enhances generation of ROS and delays neutrophil apoptosis.[56]

IL-10

IL-10 is a regulatory cytokine and acts a negative feedback switch when the cells involved in an immune reaction also start to produce IL-10. IL-10 producing cells include monocytes, DCs, B-, NK- and the majority of T-cell subsets, which underscores its role as a critical negative regulator. IL-10 has diverse effects on most hematopoietic cell types such as growth and/or differentiation of T cells, B cells and NK cells. IL-10 dampens the inflammatory response through down-regulation of genes encoding inflammatory mediators, co-stimulatory molecules and by enhancing expression of molecules that antagonize the inflammatory response.[57] IL-10 also enhances differentiation of IL-10 secreting Tregs, which contribute to control of immune responses and tolerance *in vivo*.[58]

IL-12p70

IL-12p70 is a pro-inflammatory cytokine that is closely involved in innate and adaptive immune responses. It is composed of two subunits, p40 and p35, that together form the biologically active cytokine. IL-12 is predominantly produced by monocytes, macrophages, DCs and neutrophils in response to activation of PRRs and/or co-stimulatory signals and cytokines from activated T cells and NK cells. IL-12 is a potent inducer of IFN-γ and an activator of NK cells. IL-12 has a crucial role for establishing antigen-specific Th1 responses, and enhancing activation of B cells and the production of Th1-related antibody isotypes, which are essential to control infections with many microbial pathogens. Further IL-12 could be involved in inducing clonal expansion and fixing the phenotype of already committed IFN-γ secreting Th1 cells.[59]

IL-2 and IL-15

IL-2 and IL-15 are closely related cytokines that share structural and signaling components. They have roles in maintenance and proliferation of $CD8^+$ T cells, memory $CD8^+$ T cells and NK cells, and in enhancing cytotoxic activity in these cell types.[60] IL-2 is normally produced by T cells (predominately $CD4^+$), upon engagement of the TCR. IL-2 is necessary for the development of T-cell memory and has a unique role in supporting the development of Treg.[61] IL-15 is produced by myeloid cells in response to TLR stimuli and type I IFNs. For NK cells, in contrast to IL-2, IL-15 is required for optimal NK-cell development and

maturation.[62,63] Further, IL-15 induces NK-cell surface expression of LFA-1 (an integrin), which enables formation of a conjugate between NK cells and target cells, thereby directly contributing to the induction of cytotoxicity.[64]

IL-18

IL-18 is produced by myeloid cells through activation of a molecular complex called the inflammasome, by microbial products.[65] IL-18 was originally described as an IFN-γ inducing factor, and it was later clarified that this action is in concert with IL-12. IL-18 can also induce other immunostimulatory cytokines and direct differentiation of NK cells into a distinct NK-cell 'helper' pathway characterized by expression of several mature DC-associated surface markers.[66]

IFN-α

IFN-α belongs to the type I IFNs and is one of many in this group of cytokines that have been found to interfere with the viral infection process.[67] pDCs are strong producers of IFN-α but also other lymphocytes and infected cells can release IFN-α. Early production of IFN-α plays a crucial role in anti-viral defense and acts on many other cell types that carry IFN-α receptors. Binding/recognition will result in production of other cytokines, proliferation of memory T cells,[68] regulation of NK-cell homeostasis, activation and function.[69,70]

IFN-γ

IFN-γ is a type II IFN that is essential for control of viral infection, intracellular bacteria and tumor malignancies. The main IFN-γ producers are NK cells, NKT cells and T cells. IFN-γ directly promotes antiviral mechanisms by induction of antiviral enzymes but its main function is in immunomodulation. Over 300 genes are estimated to be upregulated by IFN signaling including genes coding for molecules involved in adhesion, apoptosis, host defense, cell-signaling and transcription.[71] IFN-γ distinguishes Th1 type of immune responses and IFN-γ signaling is fundamental in the maintenance of these responses.[72] Its production is induced by mitogenic/antigenic stimuli and cytokines that are secreted by PRR-activated myeloid cells. IFN-γ is also a major activator of APCs and enhances responses to microbial inducers such as TLR ligands and antigen processing and presentation capabilities.[73]

RELATED BACKGROUND

In this section there will be more detailed information regarding the cells and viral infections that were studied and that constitute the topic of this thesis.

MONOCYTES

Monocytes ($CD14^+$ cells) are important initiators of immune responses, supply cytokines early following infection and are precursors of macrophages and DCs.[8] They play a crucial role not only in innate responses but also in priming and maintenance of adaptive responses. After differentiation in the bone marrow, monocytes enter the blood stream. When pathogen invasion occurs, monocytes can act as efficient effector cells and recognize danger through their expression of PRRs. In particular, monocytes express high levels of NOD1/NOD2 and TLR2 and TLR4.[74,75] Monocytes can migrate from the blood into inflamed tissues under the influence of chemoattractants such as monocyte chemotactic protein-1 MCP-1/CCL2, and adhesion molecules such as integrins and selectins. At the inflamed site monocytes can differentiate into recruited macrophages and/or DCs. Furthermore, circulating monocytes can act as precursors of tissue-resident macrophages and DCs during homeostasis, although it is unclear to what extent this occurs.[8,76]

Heterogeneity of the monocyte population

The blood monocyte population is heterogeneous and the subpopulations most likely play different roles in antimicrobial responses and during homeostasis (Figure 5). Based on expression of CD16 (FCγ receptor III) and CD14 (LPS co-receptor) two major subsets were initially described; the $CD14^{++}CD16^-$ and $CD14^+CD16^+$.[77] $CD14^+CD16^+$ cells are now further subdivided into $CD14^{++}CD16^+$ and $CD14^{dim}CD16^+$ cells and the 3 types of monocytes are termed classical ($CD14^{++}CD16^-$), intermediate ($CD14^{hi}CD16^+$), and non-classical monocytes ($CD14^{dim}CD16^{++}$).[78]

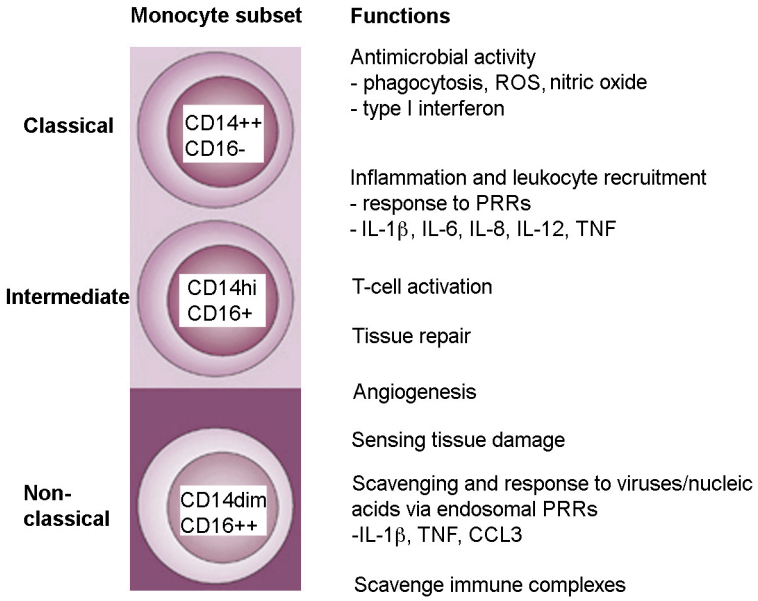

Figure 5. Different roles of monocyte subsets. Adapted from Saha and Geissmann.[79] Reprinted with the permission of Nature publishing.

Recently two studies of genome wide analyses of all three monocyte subsets were reported.[80,81] Although they described contradictory results regarding their developmental relationship, the intermediate and non-classical subsets seem to be more closely related.[80-82] Previously, classical and non-classical subsets were suggested to represent sequential stages of monocyte differentiation.[83,84] Support for this theory came from transcriptome analysis[83] and studies where classical monocytes acquired CD16 and the chemokine receptor CXC3R1 under the influence of TGF-β, IL-10, M-CSF and CCL2.[85,86] Further, TLR2/1-induced differentiation of monocytes into $CD1b^+$ DCs was restricted to non-classical monocytes, while differentiation into DC-$SIGN^+$ cells was enhanced in the classical subset.[87] Thus, the classical subset could give rise to more mature macrophage and DC-like non-classical monocytes.[86,88] Most functional studies so far also concern the classical versus non-classical monocytes, where the intermediate cells are most often included in the non-classical subset. Non-classical monocytes display the highest levels of TLRs[89,90] and were initially described as pro-inflammatory with regards to their capacity for cytokine production.[89] In a study by Cros et al. non-classical monocytes were shown to respond primarily to viral stimuli[82] whereas other studies showed potent TNF responses also to LPS.[80,89] LPS stimulation further induced high levels of IL-6, IL-8, CCL2 and CCL3 in classical monocytes whereas intermediate monocytes produce IL-1β, TNF, IL-6 and CCL3. All three subsets produce IL-10.[82,89,91]

Classical and non-classical monocytes were early on described to differ in their chemokine receptor expression (CCR2 and CD62L vs CX3CR1) and trafficking behavior.[92-95] The non-classical subset has now been suggested to act as 'patrolling' monocytes due to their crawling behavior on endothelium after adoptive transfer into mice.[82] Non-classical monocytes are very motile[86] and express genes associated with cytoskeleton mobility,[80,81] indicating that this subset may have the capacity to migrate and respond quickly to damage on inflamed endothelium.

The intermediate monocyte subset may act as efficient APCs. They express high levels of MHC II processing and presentation genes and surface levels of CD40 and CD54 that are important in APC-T-cell interactions, and can potently induce staphylococcal enterotoxin B mediated T-cell proliferation.[80,81] However, their ability to capture and process antigen, and therefore the *in vivo* relevance, is so far unknown.

Expansion of the intermediate and non-classical subset has been described in inflammatory conditions such as asthma[96] and in bacterial infections such as sepsis and tuberculosis.[97,98] Viral infections (hepatitis B and C infection,[99] HIV[100] and Dengue fever[101]) can also cause expansion of these subsets but the cause for this, and if they contribute to infection defense or may worsen the disease state through cytokine production, is currently unclear.

NK CELLS

NK cells ($CD3^-CD56^+$ cells) contribute to host defense by their involvement in the control of pathogens including virus, bacteria, fungi and helminths.[102] They are also implicated in the killing of tumor cells (reviewed in[103]). The cytolytic function of NK cells is accomplished by release of pre-formed cytoplasmic granules that contain lytic proteins like granzyme and perforin, and/or by induction of apoptosis through interactions of FAS and TNF-related apoptosis-inducing ligand (TRAIL) on NK cells with their respective ligands. NK cells are an important source of cytokines and chemokines, like IFN-γ, TNF and MIP-1β, that contribute to inflammation and polarization of T-cell responses and enhances recruitment of immune cells to sites of inflammation.

NK cells are widely distributed in lymphoid and non-lymphoid tissues.[7] There is a vast heterogeneity in the NK-cell compartment but there are two major subsets with functional differences and distribution between anatomical sites. The predominant subset in blood is the $CD56^{dim}CD16^+$ cytolytic NK cells that express homing receptors for inflamed sites. The

minor subset is the $CD56^{bright}CD16^{-}$ NK cell subset that displays low cytolytic activity but high production of IFN-γ and express homing receptors for secondary lymphoid tissues.[104,105]

NK-cell activation

The NK-cell response is regulated in a complex fashion by integration of inhibitory and activating signals provided by NK-cell receptors, in combination with signals received from the surrounding cytokine environment. For direct NK-cell activation the presence or absence of MHC I on the cell surface forms the basis for how NK-cells can recognize aberrant cells ("missing-self" recognition).[106] Interactions between inhibitory receptors on NK cells and MHC class I on healthy cells prevent NK-cell activation and protect from cytolysis. Stressed, transformed or infected cells can lack normal MHC expression and fail to provide the inhibitory signal which renders them susceptible to lysis. In addition, NK-cell activation receptors react to changes in the expression levels of their ligands that are induced by cellular stress, which seals the fate of the target cell.

There is also an indirect pathway to NK-cell activation. This involves activation of accessory cells such as DCs by pathogen recognition and subsequent upregulation of co-stimulatory molecules and cytokine release that lead to the recruitment and activation of NK cells.[107,108] This type of indirect NK-cell activation over-rides inhibitory signals provided by MHC I-expressing competent cells and is augmented by antigen-specific T cells through the production of IL-2.[109] Overall, accessory cells are required to provide soluble and contact-dependent activation signals for proper pathogen mediated NK-cell activation.[110] NK cells are dependent on cytokines such as monocyte-secreted IL-12, IL-15 and IL-18 that induce proliferation, enhance cytotoxicity and IFN-γ secretion in NK cells. Further, IFN-γ stimulation of myeloid cells amplifies production of IL-12/IL-18, creating a feed-forward loop.[72]

NK-cell receptors

As already stated, the integration of signals derived from a multitude of NK-cell receptors form the basis for NK-cell activation. The various combinations of NK-cell receptors that induce different types of NK-cell effector functions have been described in detail in Bryceson *et al.* 2006.[111] Below will be a brief description of some of the major inhibitory and activating receptors.

Killer-cell Immunoglobulin-like receptors (KIRs) are specific for MHC class I molecules including human leukocyte antigen (HLA)-A, -B, -C and –G, and have mainly inhibitory functions. The KIR genes are highly variable and different NK-cell clones express a random combination of KIRs. However, each individual has a determined repertoire of KIRs that is stable over time.[112] The KIR family also has members with activating or dual functions.[113,114] The function of activating KIRs is largely elusive but recent findings suggest that they participate in the response against herpesvirus infection, described in co-publication V in this thesis and by Stewart *et al*. in.[115]

CD94 and NKG2 are two receptors that form heterodimers. Depending on the version of the NKG2 polypeptide, inhibition (NKG2A) or activation (NKG2C) is mediated. Both NKG2A and -C recognize a non-classical version of MHC I - HLA-E in complex with leader sequence peptides of classical MHC class I molecules. NKG2C has lower binding affinity to HLA-E, which may contribute to the regulation between inhibition and activation although these receptors seem to be expressed by different subset of NK cells depending on differentiation status.[116-118]

The main activating receptors constitutively found on all NK cells in peripheral blood are NKG2D and the natural cytotoxic receptors (NCRs) NKp30 and NKp46.[119] NKp46 has been suggested as an alternative marker instead of CD56 to define NK cells in order to facilitate cross-species comparisons.[120] The ligands for the NCRs are largely unknown but NKp30 can recognize the tumor related protein B7-H6,[121] influenza hemagglutinin is recognized by NKp46[122] and poxvirus hemagglutinins are ligands for both.[123] NKG2D recognizes the MHC class I chain-related proteins A and B (MICA/MICB) and the UL-16 binding proteins (ULBP) that are induced by DNA damage, oxidative stress and inflammation.[124] Other activation receptors include CD16, 2B4, DNAM-1 and NKp80. CD16 is the low affinity receptor for IgG. Binding of IgG, either on target cells coated with antibody or immune complexes, to CD16 on NK cells induces lysis through antibody-dependent cellular cytotoxicity[37] and production of several cytokines.[125]

NK-cell development and differentiation

The NK-cell developmental pathway is not fully clear but commitment to the NK-cell lineage, education of NK cells towards self-markers and establishment of functional competence occurs in the bone marrow.[126] NK cells may also develop in peripheral sites such as secondary lymphoid organs.[127]

Differentiation of NK cells is sequential and driven by cytokines and/or via interactions between cell-surface receptors.[128-130] NK-cell differentiation is associated with multiple phenotypic and functional changes, including low expression of cytokine- and chemokine-receptors, a gradual decline in proliferative capacity and responsiveness to cytokines, and increased ability to perform cytotoxic responses. When NK cells differentiate they loose expression of some NK-cell receptors such as NKG2A and in parallel gain NKG2C and KIRs along with CD57.[116-118] CD57 is a carbohydrate epitope that can be found on subsets of NK- and T cells. CD57 expression on NK cells has recently been described to denote a mature NK-cell subset. These cells have a distinct functional response profile characterized by low responsiveness to IL-12+IL-18 stimulation but potent activity when stimulated via CD16.[118]
Recently several papers have described increased NK-cell responses during secondary exposure to antigen and their features have been compared to those of cytotoxic T lymphocytes.[131,132] The concept of NK-cell 'memory' has generated a lot of interest and has been described in detail for the mouse model of CMV infection.[132] Murine and human NK cells that have been stimulated and subsequently re-stimulated with IL-12, IL-15 and IL-18 also exhibit enhanced functionality upon re-stimulation,[133] and these 'memory-like' NK cells were suggested to have potential in clinical settings.[134]

MONOCYTE AND NK-CELL COLLABORATION

Although NK cell-DC interactions have received the most attention, several studies have shown mutual activation between NK cells and monocytes/macrophages.[110,135-137] Besides the reciprocal effects of the prototype cytokines produced by monocytes and NK cells, this cross-talk is partially dependent on cell-cell contact,[137] and has been proposed to occur between NKp80 on NK cells and the transmembrane receptor activation-induced C-type lectin (AICL) on monocytes and macrophages.[135] In the presence of proinflammatory cytokines, NKp80-AICL engagement promotes cytokine release from both cell types suggesting that this interaction may be functionally relevant at sites of inflammation.
Interestingly, the monocyte subsets could influence NK-cell activation differently. Gene set enrichment analysis has shown that $CD14^{+}CD16^{+}$ display upregulation of transcripts related to NK-cell mediated cytotoxicity,[83] indicating a close relationship between these cells in battling infection. Further the $CD14^{hi}CD16^{+}$ intermediate monocyte subset expresses more AICL than the classical $CD14^{++}CD16^{-}$ subset and engagement of AICL augmented production of pro-inflammatory cytokines in co-cultures of monocytes and NK cells.[135]

IMMUNE FUNCTION EARLY IN LIFE

Newborns and young children typically have low immune responsiveness associated with heightened sensitivity to pathogens in early life.[138] The development of the immune system starts *in utero* in a normally microbe-free environment. The immune system of the newborn is therefore largely antigen inexperienced with very few memory cells. The increased susceptibility of newborns to infections is often attributed to a number of functional immaturities exhibited by the innate and particularly the adaptive branch of neonatal immunity. Antibody production is diminished in newborns with limited production of IgG to a range of T-cell dependent and –independent antigens. The microarchitecture of structures needed for proper antibody production, i.e. lymphoid follicles and GCs, is not fully developed at birth.[139] Differentiation of neonatal follicular DCs in response to signals from neonatal B cells is defective, leading to a delayed maturation of the follicular DC network.[140] Further, there seems to be a bias for the differentiation pathway to memory- instead of plasma B cells, which complicates early-life immunization.[141]

In the T-cell compartment, CTL can mount potent cytotoxic responses under certain circumstances, such as congenital CMV infection.[142,143] In terms of cytokine production however, early studies showed that newborns have defective T-cell production of IFN-γ and a Th2-biased immunity.[144,145] Lately this concept has been widened to incorporate a Th2/Th17-type of immunity over Th1-type of immunity,[146-148] (Figure 6). A Th2-skewed immunity at birth could be associated with intrinsic epigenetic modifications in Th cells that allow enhanced secretion of IL-4.[149] IL-4 production in turn may reinforce the Th2-bias via IL-4 driven apoptosis of primary Th1 cells.[150] Although Th1 responses appear to be down-regulated it is also clear that the neonatal response strongly depends on the stimulus used and the setting in which the cells are cultured,[146,148,151-154] and a global Th2-bias at birth is not evident.[155] During ontogeny this Th2-skew will be successively balanced with increasing Th1 maturation. An important factor for slow development of Th1 responses may be impaired DC support of Th1-cell differentiation connected to low production of IL-12 in response to a number of microbial stimuli.[156-159]

Functional Treg cells can be found in umbilical cord blood and in the thymus of newborns but appear to be less suppressive as compared to those from adults.[160,161] These Treg generally have a naive phenotype and express predominately homing receptors for the gut, which later in childhood switch to more adult-like extra-intestinal homing patterns.[162]

With regards to monocytes and NK cells, there are adult-level NK-cell percentages, including the $CD56^{dim}$ and $CD56^{bright}$ subpopulations at birth.[163] Cytotoxicity and IFN-γ production after IL-12+IL-18 stimulation are at adult levels.[164] However, neonatal NK cells have high expression of inhibitory NKG2A/CD94 receptors, and low expression of granzyme B that might contribute to low cytolytic activity of cord blood NK cells without prior cytokine stimulation.[165] Monocytes in the newborn have been extensively studied and have been described to give both immature and mature responses. Very little is known regarding the expression of PRRs or their signaling intermediates during childhood. TLR2 and TLR4 are expressed at an adult level on neonatal monocytes.[166,167] Expression of basal MyD88 have been shown to be either lower[168] or adult-level,[169] and ERK1/2 and p38-MAPK activation in response to LPS and/or R-848 (a TLR7/8 ligand) either adult-level[167-169] or deficient.[167,169] Maturation of TLR responses takes place over time as shown by a recent longitudinal study where cytokine production capacity to multiple TLR stimuli increased from newborns to the age of 5.[170] Furthermore, TLR-mediated cytokine production during the perinatal period could be influenced by the level of soluble factors in neonatal plasma. One study found that compared to adult plasma, the level of the purine metabolite adenosine in neonatal plasma is higher. Enhanced adenosine levels were connected to enhanced cAMP content in mononuclear cells, which conferred suppression of TLR-induced TNF production, whereas IL-6 production was not affected.[153]

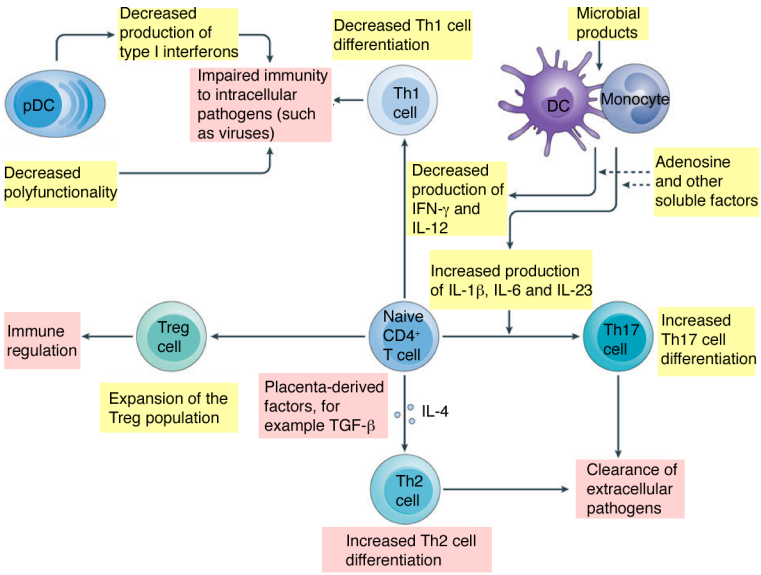

Figure 6. Immune characteristics early in life. Adapted from Prendergast, Klenerman & Goulder.[171] Reprinted with the permission of Nature publishing.

PERSISTENT VIRAL INFECTIONS

Viral infection leads to activation of innate immunity and generation of effector and memory T- and B cells. A successful immune response leads to viral clearance and resolution of the infection. An alternative route is the establishment of persistent infection where the virus remains within the host. During persistent infection the host immune response must adapt to viral presence and control viral replication without damage to virus-infected host tissues. The virus in turn must avoid complete elimination and too strong immune responses that might lead to the death of its host. This equilibrium is highly dynamic with continuous adaptation from both host and virus. Viruses have a multitude of evasion strategies to escape immune surveillance that may also help in establishing persistent infection. These include downregulation of molecules needed for T- and NK-cell recognition (such as MHC and NKG2D), production of viral homologues to immunoregulatory cytokines (such as viral IL-10) and inhibition of antigen presentation.[172] Viruses have also developed a wide diversity of tactics to avoid the type I IFN system via the disruption of initial detection of the virus by PRRs, by disarming or blocking host cell transcription of factors involved in IFN expression or by disturbing IFN signaling (reviewed in[173]).

HERPESVIRUSES

Herpesviruses are among the most successful viruses in the human population and are exceptionally efficient in establishing persistent infections. Herpesviruses are large double-stranded DNA viruses with viral particles in the range of 150-200 nm in diameter. There are three major subclasses of herpesvirus mainly based on the type of cells they infect and their ability to induce cell proliferation. The α-Herpesviruses, including Varicella Zoster and Herpes Simplex persist in neurons; the β-Herpesviruses, including cytomegalovirus (CMV) persist in lymphocytes and the γ-herpesviruses, including Epstein-Barr Virus (EBV) also persist in lymphocytes and are able to induce lymphoproliferation.

These viruses have evolved alongside their vertebrate hosts for millions of years,[174] which has enabled host and virus to adapt to one another. Primary infection is followed by a lifelong latent infection where the virus does not overtly affect the host, and host immunity in turn is able to control latently infected cells. Primary herpesvirus infection is usually asymptomatic or leads to an acute transient disease. However all of the herpesviruses have the capacity to induce severe disease upon infection and following the establishment of latency, especially in immunocompromised individuals.[175,176]

Herpesviruses often have one target cell in the host for initial replication and another one for the maintenance of the viral genomes. When the virus is encountered for the first time there is an initial lytic cycle of infection, in which large numbers of new virions are generated. This commonly takes place in epithelial cells at mucosal sites. Lytic infection is followed by the dormant or latent stage of infection where the virus persists within a different target cell, typically lymphocytes or neurons. The latent stage is characterized by very low-level viral replication and minimal expression of most viral genes.

Both innate and adaptive immunity play a role in controlling herpesviruses, including humoral and cell-mediated immune responses in both primary and latent infection. Infected cells within the latent cycle are not eradicated, although some T cells are specific for latent antigen.[177] Re-activation events frequently occur as a part of the viral life cycle but may also occur as a consequence of disruptions in host immunity. These reactivation events are usually controlled by host immunity.

EPSTEIN-BARR VIRUS

EBV was discovered in 1964 by electron microscopy of cells cultured from Burkitt's lymphoma (BL) tissue by Epstein, Achong, and Barr.[178] EBV is widely spread with around 95% prevalence in the adult population and primary infection often occurs during early childhood. EBV transmission commonly occurs via saliva where to EBV is continuously shed,[179] and children are often infected through contact with family members. When the infection is acquired in childhood there are usually no symptoms or only mild pathology. When individuals acquire EBV later in life, the infection often manifests as a self-limiting lymphoproliferative disease with clinical symptoms, referred to as infectious mononucleosis (IM). This disease is also called glandular fever or kissing disease as it is commonly seen in teenagers getting their first "taste" of adult life.

EBV targets and infects B cells. EBV infection in the absence of immune control is associated with uncontrolled B-cell proliferation and transformation, and immunocompromised individuals have an increased risk of EBV-associated lymphoproliferative disease (LPD), including Burkitt's lymphoma, Hodgkin's lymphoma, and nasopharyngeal carcinoma.[175,180]

EBV COLONIZATION

Primary infection

As stated above, EBV is a B-lymphotropic virus, however which cell EBV initially infects has been intensely investigated. Early studies of throat washings from IM patients suggested that viral replication takes place in the oropharyngeal epithelium during primary infection.[181,182] However, epithelial cells are very difficult to infect with cell-free virus alone[183,184] and therefore infection has been suggested to take place via surface transfer of virus from adjacent B cells.[185] B cells can be infected by cell-free virus at a vastly greater infection rate than epithelial cells, which points to the B cell as the primary target cell for EBV. This is supported by several studies including work performed by Thorley-Lawson *et al* on cultures of tonsillar epithelium.[184,186]

Upon infection, the EBV virion will attach to the B-cell surface through interactions between the viral proteins gp350 and gp42, with CD21 and HLA II on the B cell. This is followed by virus-cell fusion, the expression of growth-transforming genes and the outgrowth of EBV-positive B lymphoblastoid cells. The events following initial infection to persistence in the memory B cell population are still a matter of debate. Thorley-Lawson *et al.* put forward a popular theory in 2004.[187] They suggested that the virus uses a pattern of gene expression that mimics antigen-driven activation and differentiation of B cells. This is achieved through a GC reaction where the infected naïve B cell is driven to become a B cell blast and subsequently undergoes the process of SHM and CSR. In the end this process will give a latently infected memory B cell that carries the virus but that does not express any viral genes.[187] The competing theory argues against the need of GC transit to achieve differentiation of a naive B cell into memory and suggests that the B cell memory population may be infected directly[188-190] (both theories shown in Figure 7). Although this may indeed occur, EBV does not preferentially infect any particular subset of B cells in peripheral blood or tonsils, such as naïve $IgD^{+}CD27^{-}$ or $IgD^{-}CD27^{+}$ memory B cells.[191,192] Further, the proliferation rate of all B-cell subsets after *in vitro* EBV infection is similar as shown by Heath *et al*,[191] which indicates that no preferential expansion of a particular B-cell subset occurs after infection.

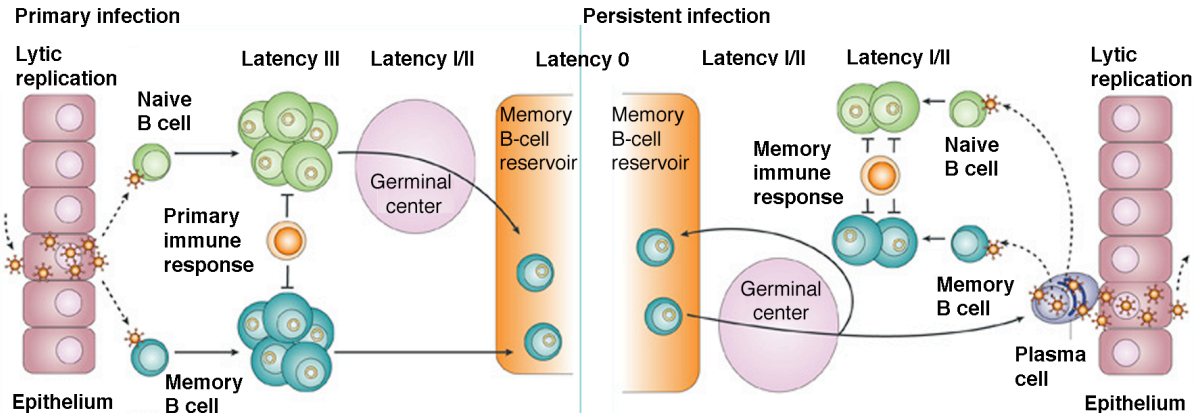

Figure 7. Putative *in vivo* interactions between EBV and host cells. Adapted from Young & Rickinson.[193] Reprinted with the permission of Nature publishing.

Latent EBV infection

In latently infected individuals the frequency of EBV-infected B cells is approximately 1-50 in 10^6 B cells and stays stable over time.[194] EBV can largely be found within the IgD^-CD27^+ switched memory population but also exists in the IgD^+CD27^+ non-switched memory population.[186,195-197] Once the virus has established latent infection, only a limited number of non-immunogenic viral genes are expressed.[198] Likely this facilitates evasion of cytotoxic T cells so that EBV can remain within the host. Different patterns of gene expression (latency programs) have been observed during latency. The latency programs comprise virally encoded nuclear and membrane associated proteins that can manipulate B-cell activation and differentiation (Table 1). Latency programs have been divided into four types: Latency 0, I, II and III. Latency III, also referred to as the 'growth program' is the classic gene expression program observed after *in vitro* infection.[199] Latency II and I programs have a more restricted gene expression pattern. Latency type I is typically found in BL tumor cells and latency II in various carcinomas and lymphomas. Latency type 0 represents a stage where all viral genes are silent (reviewed in[200]). The specific roles of some of the proteins in the latency programs will be briefly described below.

Latency program	Genes expressed	*In vivo*	Gene product	Function
0		Memory subset	**EBNA-1**	Maintains viral episome, regulates viral promoters, anti-apoptotic
I	EBNA-1	Germinal center, IM	**EBNA-2**	Upregulation of viral LMPs and cellular c-myc, CD21 and CD23
II	EBNA-1, LMP-1, LMP-2A, LMP-2B	Germinal center, IM	**LMP-1**	Survival and proliferation, mimics CD40 signaling
III	EBNA-1-6, LMP-1, LMP-2A, LMP-2B	IM	**LMP-2**	Survival and proliferation, blocks BCR signaling and lytic cycle

Table 1. Latency programs and gene products. Adapted from Klein, Klein and Kashuba.[200] Reprinted with the permission of Elsevier.

EBV gene products

Epstein-Barr nuclear antigen (EBNA)-1 plays an essential role in viral genome replication/maintenance and possibly also for B-cell transformation. Expression of EBNA-1 can also inhibit apoptosis in BL cells.[201] EBNA-2 is the first viral protein expressed after B-cell infection and has a key role in growth transformation. EBNA-2 expression induces upregulation of viral latent membrane proteins (LMPs) and various cellular genes including the oncogene c-myc, CD21 and CD23.[202,203] LMP-1 is a classical viral oncogene and its expression can be found in all EBV-positive tumors.[200,204] LMP-1 shows homology with CD40, and through the expression of LMP-1, EBV can mimic the CD40-CD40 ligand interaction and thereby provide the signals normally given by T-cell help. This is believed to be important in the transition of the infected naïve B cell into a latently infected memory B cell.[187] The LMP-2 complex is also essential for cell survival and proliferation[205] and LMP-2A and -2B can disrupt BCR signaling through recruitment of tyrosine kinases.[206] It is conceivable that LMP2A's capacity to imitate BCR signaling may act in conjunction with LMP1's surrogate T-cell help to drive infected cells through GC reactions during primary infection of B cells *in vivo*.

Reactivation and propagation

EBV lytic infection occurs initially during primary infection and intermittently during latent infection. Lytic infection in the oropharynx leads to the release of infectious virions into saliva that ensures transmission to new hosts and maintains infection of the current host.[179,207,208] Re-activation of the virus probably occurs when memory B cells undergo differentiation into plasma cells.[209] Most healthy EBV carriers will shed virus intermittently

as a result of viral replication within the oropharyngeal epithelium.[179,208,210] In fact, one study found that the level of virus is consistently high in saliva, suggesting continuous replication at this site following re-activation of B cells.[179] EBV can also display abortive reactivation that results in repetitive immune stimulation despite the absence of new virions.[209]

Immune responses to EBV

Innate responses

As primary EBV infection is mostly asymptomatic in childhood, data concerning the infectious process have largely come from studies of patients suffering from IM and from *in vitro* studies with EBV-transformed B-cell lines. For many viruses, including EBV, the first wave of cytokine/chemokine production is an important step for the outcome of the infection. *In vitro* studies indicate a marked EBV-induced innate immune activation with accessory cell release of cytokines including type I IFNs and IL-12.[211-213] IFN-α and –β production by DCs can be induced upon pattern recognition of genomic DNA[213] or of small non-coding RNAs encoded by EBV (EBERs).[212] Further, it was demonstrated that cross-presentation by DCs of EBV antigens derived from B cells was critical for the initiation of T-cell mediated antiviral responses and resistance to EBV transformation.[214]

NK cells have also been shown to participate in defense against EBV. Early *in vitro* studies demonstrated that NK cells can inhibit the EBV-induced transformation of resting B cells if added within a few days of infection, at least in part through the release of IFN-γ.[215,216] Tonsillar NK cells are particularly active IFN-γ producers and can act as inhibitors of *in vitro* transformation if challenged with EBV-infected B lymphocytes in the presence of DCs as a source of IL-12.[211] Although *in vitro* studies show activity of NK cells against EBV-infected cells, their role *in vivo* is less clear. In one study of IM patients the number of NK cells was elevated at the time of diagnosis, inversely correlated with viral load and NK cells showed an enhanced ability to kill MHC class I deficient EBV-infected cell lines, probably due to a priming cytokine environment.[217] Further, individuals who are NK-cell deficient can suffer from severe EBV infections.[218] It was recently shown that those harbouring deficiencies in the magnesium transporter MAGT1 have high levels of EBV and a predisposition to lymphoma which was connected to defective expression of NKG2D in NK- and $CD8^+$ T cells and impaired cytolytic responses against EBV.[219] However, recipients of T-cell depleted stem cell transplants can develop EBV-related LPD in the first 3–6 months post-transplant, by which time NK cell numbers have recovered but the patients are still T-cell deficient.[220]

Adaptive responses

Many types of antibodies are generated in response to EBV however to what extent they contribute to defense against EBV is unclear. Some gp350-specific IgG antibodies are neutralizing but tend to arise later on in primary infection.[175] In acute IM, IgM reactivity against viral capsid antigen (VCA) can be detected early whereas IgG against VCA and EBNA-2 arrives later in infection.[221] In addition IgA can also be detected, indicating polyclonal B-cell activation. ELISAs detecting anti-EBNA-1 and anti-VCA IgG and IgM antibodies are commonly used to determined EBV seropositivity, as they are detectable in serum during the latent phase in all healthy EBV carriers. The fact that VCA is a lytic cycle antigen indicates that EBV undergoes regular reactivation. With regards to cytokine production, there is evidence that B cells release cytokines like IL-5, IL-6 and IL-10 following infection that could potentially affect T-cell responses.[222-224] Further, expression of LMP-1 in latently infected B cells primes them for potent IFN-α production upon secondary viral infection.[225]

In IM, the most pronounced clinical feature is the massive proliferation of $CD8^+$ T cells. These cells are possibly the most important component in the immune response against EBV. The $CD8^+$ T cells that arise are directed mainly against lytic cycle antigen but a smaller percentage is specific for latent proteins.[177] The expanded EBV-specific $CD8^+$ T cells have high cytolytic capacity and levels of perforin, but vary in their cytokine production ability.[226] Although IFN-γ production is potent it only takes place in subpopulations of these cells as shown by *in vitro* studies with autologous LCL or EBV peptide stimulation.[226,227] Further, they have high susceptibility to apoptosis and require active stimulation to persist.[228] Following control of the infection, most EBV-specific T cells die, especially those specific for lytic antigens, but memory T cells for both lytic and latent antigen persist and form the basis for long-term control of latent EBV infection.

Although there is little expansion of the $CD4^+$ T-cell compartment in IM, EBV stimulates both cytolytic and helper $CD4^+$ T cells that can produce IFN-γ and aid in restriction of virus replication.[229-231] Activated $CD4^+$ T cells in IM were found to be specific for lytic cycle proteins or the latent protein EBNA-1.[232,233] Upon re-stimulation, memory $CD4^+$ T cells from seropositive individuals are even able to control B-cell outgrowth.[231,233,234] Therefore $CD4^+$ T cells could be directly killing infected B cells, secreting cytokines and thereby providing help for T- and B cells.

CYTOMEGALOVIRUS

CMV was identified as a virus in the 1950's after collaboration between Weller, Rowe and Smith that independently isolated the virus from patient samples (described in[235]). CMV typically infects cells of the myeloid lineage along with epithelial and endothelial cells. Like EBV, CMV is carried by a majority of adults (60-90%). A similar etiology is seen for CMV as for EBV where the infection most commonly occurs early on in life. The virus is spread by person-to-person contact and all bodily excretions are a possible source of infectious CMV. Most children are infected via breast milk[236] or by transmission from other children at day care or school.[237,238] CMV can also cause mononucleosis-like disease with fever and leukopenia, and is associated with severe immunopathology in immunosuppressed hosts.[176]

CMV COLONIZATION

Primary and latent infection

As stated above, CMV can infect cells of the myeloid lineage along with epithelial and endothelial cells.[176,239] CMV is carried latently in the same types of cells it infects along with hematopoietic progenitor cells.[240,241] During primary infection, infected cells can be found in cervix, salivary gland, breast and intestine. By establishing in these locations, the virus can spread to cells in underlying tissues and into bodily excretions.[242] CMV most likely spreads as free virus particles in the blood or by using cells as a means of transfer. Infected macrophages can be detected in tissues from people with CMV disease[243] and also upon *in vitro* infections.[244] CMV can replicate within myeloid cells but this is dependent upon the differentiation status of the cell.[245,246] Primary infection is often followed by prolonged periods of viral shedding, in adults up to several months after seroconversion.[247] Children may shed virus for years and having a child that sheds CMV is the strongest predictor for seroconversion in adults.[248,249] Reactivation of CMV from latency is associated with the release of pro-inflammatory cytokines[250] and seems to be largely controlled by $CD4^+$ and $CD8^+$ T cells along with NK cells.[251] Some CMV-specific T cells are directed against viral proteins that are expressed upon viral entry and can lyse cells without the requirement for viral gene expression, providing rapid elimination of reactivating cells.[252] In the latently infected host a huge part of the memory $CD8^+$ T cell compartment is dedicated to CMV, and this percentage increases with age.[253-255]

Immune response to CMV

Like for EBV, there have been difficulties in studying the primary infection, as it is often asymptomatic in childhood. Therefore re-activation events have been studied, such as those occurring after hematopoeitic stem cell transplantation of CMV seropositive donors, along with *in vitro* infections.[256,257] CMV infection leads to activation of PRRs on DCs by viral DNA products, which generates secretion of cytokines and chemokines such as type I IFNs, IL-12, IL-18, and IL-15.[258] In turn, these molecules will activate NK cells, thus promoting their cytotoxicity, IFN-γ production, and proliferation, leading to viral clearance.[259] CMV expresses immunodominant antigens and elicits strong humoral as well as cellular responses, although the activity of NK cells, $CD4^+$ T cells and $CD8^+$ T cells are thought to be the crucial factor for control of CMV replication.[176] Upon infection CMV will induce a rapid expansion of $CD8^+$ T cells. Expansions have also been shown following cases of *in utero* infection where $CD8^+$ T cells produce IFN-γ and contribute to lysis of virus-infected cells.[143] Also $CD4^+$ T cells respond to CMV antigens with the production of IFN-γ along with IL-2 and TNF. $CD4^+$ T cells can also directly control viral replication through cytotoxicity.[260-262] In addition, CMV-specific $CD4^+$ T cells may help CMV-specific $CD8^+$ T-cell responses.[263]

CMV-driven differentiation of NK- and T cells

A number of studies have put the spotlight on CMV as a driver of immune-cell maturation. In the mouse model of CMV infection a particular subset of $Ly49H^+$ NK cells expand and persist long after primary infection, which is dependent on IL-12 signaling.[264] These expanded NK cells show enhanced proliferation, degranulation and IFN-γ secretion months after primary infection, and were therefore termed 'memory' NK cells.[132] However, the slow contraction of NK cells following CMV infection appears to occur without real memory formation.[265] Pioneering work by Lopez-Botet and colleagues showed that in humans, latently CMV-infected individuals had expanded subsets of NK cells expressing the activation receptor NKG2C.[266] Further, CMV^+ children, and those born with congenital CMV infection also display preferential accumulation of the $NKG2C^+$ NK-cell subset,[267,268] and they are thus thought to be the equivalent of murine $Ly49H^+$ NK cells. Recent findings have associated the NKG2C phenotype with CD57 expression indicating that these cells are highly mature.[118,269] $NKG2C^+$ NK cells in CMV^+ individuals exhibit polyfunctional responses, including heightened ability to produce IFN-γ against HLA-E expressing targets and antibody-coated targets but not to IL-12+IL-18 stimulation.[270]

In CMV^+ donors, unrelated acute and chronic infections can drive expansion of $NKG2C^+$ cells.[270-273] How expansion occurs in primary infection or following secondary infections is not clear but could be a result of direct interaction between NKG2C on NK cells and HLA-E on CMV-infected target cells.[266,273] Thus for secondary infections, expansion of the $NKG2C^+$ NK-subset would include CMV reactivation and/or bystander activation. In co-publication V, Beziat *et al.* showed that the expanded $NKG2C^+$ NK cell populations predominately display inhibitory KIRs specific for self HLA-C, suggesting that their interaction modulates their CMV-driven differentiation. They further showed a unique contribution of activating KIRs, in addition to NKG2C, in the expansion indicating a role for both activating and inhibitory KIRs in immunity to CMV infection.

In the T-cell compartment, CMV is associated with elevated numbers of highly differentiated $CD8^+CD57^+$ cells.[274-276] CD57 expression on T cells correlates with higher expression of granzyme A, granzyme B and perforin[277] and heightened lytic activity.[278] Although CD57 has been associated with replicative senescence[279] these cells are able to divide when provided with distinct co-stimulatory signals including IL-2 and IL-15.[280,281] Populations of $CD8^+CD57^+$ cells typically accumulate with age, during persistent antigen stimulation and viral reactivation.[278,282,283]

CO-INFECTIONS – INTERPLAY BETWEEN EBV AND CMV

Only a few studies exist that investigate the interplay between EBV and CMV, but they indicate that harbouring both viruses latently is different from having a single infection with regards to many different immune parameters. In a study where mice were latently co-infected with the murine homologues of EBV and CMV, the immune response gene-expression profiles were distinct from those seen in infection with either herpesvirus alone.[284] Studies of aging humans show inflations of herpesvirus-specific T-cell responses. Interestingly, one study showed that the effect of age upon EBV-specific responses was dependent on CMV serostatus. CMV^+ donors displayed stable EBV-specific immune responses but in CMV^- donors, the response to EBV increased significantly with age, an effect that was not seen with influenza-specific $CD8^+$ T-cell immune response.[253] Additionally, our group has shown that co-infection with CMV further reduced the risk for IgE sensitization that EBV seropositivity conferred,[285] suggesting that the immune profile under the influence of herpesviruses may differ depending on single or co-existence in the host.

HERPESVIRUS LATENCY – BENEFITS OF CO-EVOLUTION?

There has been an increasing understanding of the importance of our microbiota in the development and shaping of immune cells and responses, from infancy and into adulthood. A lot of attention has been directed at the cross-talk between microbes, epithelial and immune cells that takes place in the gastrointestinal tract and that contribute to immune maturation. Disturbances in this process may lead to faulty differentiation of immune cells and subsequent disease development.[286] For instance, children who develop allergy during their first 5 years of life have an altered gut flora composition with fewer bacterial species,[287,288] which influences both mucosal and systemic immune responses during childhood.[288,289] Whereas the bacterial microbiota has generated immense interest, much less is known regarding the importance of the human virobiota and its associated genes - the "virome". Our virome constitutes all the viruses that we carry transiently or as persistent infections for life. The impact of the virome on host physiology could be negative as viruses can cause disease but may in fact also have mutualistic symbiotic effects and this is beginning to be acknowledged.[284,290,291] Surprisingly little is known regarding the potential benefits of herpesvirus latency in humans but some findings will be discussed below.

Herpesvirus and allergy

A delayed encounter with microorganisms, from early in childhood to later in life, has been hypothesized to relate to the increase in diseases such as allergy and asthma seen during the last decades.[292] This theory has been termed the hygiene hypothesis. Both EBV and CMV could be potential candidates for etiological agents of the hygiene hypothesis. Infections with EBV and CMV are becoming delayed in westernized societies, as compared to in the developing world, which may be connected to socioeconomic factors.[249,293] The connection between herpesvirus latency and allergy is relatively understudied field. Our group has shown that children who were EBV seropositive were less likely of being IgE-sensitized at the age of 2, an association that was further enhanced by CMV co-infection.[285] Studies by other groups also found that an early EBV infection can modulate the risk of atopy.[294] This ¨protective¨ effect might be dependent on what time-point during childhood the infection is contracted. Our group later found that 5-year old children who were EBV seropositive before the age of 2 were at a lower risk of being persistently IgE-sensitized, (IgE-sensitized at both 2- and 5-years of age) whereas children infected after the age of 2 were at a higher risk.[295] Children that were persistently IgE-sensitized had higher levels of allergen-specific IgE than those that

were late sensitized (after 2 years). This indicated that an early EBV infection might be beneficial not only regarding risk modulation but also regarding the levels of IgE. These data support the indication that a delayed primary EBV infection may contribute to a higher prevalence of allergies seen in westernized societies.

Herpesvirus and protection against secondary infections

Having a latent EBV or CMV infection could modulate steady-state immune reactivity through alterations of the cytokine environment and differentiation of effector cells. Together this imprint on the immune system could influence the course of secondary infections. Mouse studies have shown that TNF and IFN-γ production during latency with the murine analogues of EBV and CMV conferred protection against infection with *Listeria monocytogenes* and *Yersinia pestis*.[296,297] This was attributed mainly to activation of the innate arm of immunity, e.g. NK cells and macrophages. In the same study,[296] Barton *et al*. elegantly showed that the cross-protection was dependent on true latency, by infecting mice with a virus strain that was not able to establish latency. Other studies in mice have shown that latency with the murine analogue of EBV confers NK-cell 'arming' in terms of increased expression of cytolytic proteins, IFN-γ and subsequent enhanced cytotoxicity. NK cells that were armed by this mechanism protected the host against a lethal lymphoma challenge.[298] In one study of human infants, in contrast to the authors' beliefs, CMV seropositivity was associated with a robust response against measles and staphylococcus enterotoxin B vaccines, as measured by proliferation and IFN-γ production.[299] Thus, it seemed that in this case carriage of CMV could enhance the immune responses to this type of immune challenge in infancy.

Another phenomenon described for herpesvirus latency is the activation of herpesvirus-specific memory T cells during secondary infections that are able to mediate cross-protection, or heterologous immunity.[300] It is conceivable that these memory T cells are activated either via MHC cross-reactive peptides expressed by other pathogens or through antigen-independent bystander mechanisms. Activation by antigenic similarity has not been shown for herpesvirus-specific $CD8^+$ T cells but acute EBV infection triggers cross-reactive T cells specific for influenza A through this mechanism.[301,302] Bystander activation of these cells on the other hand occurs in infections with denguevirus, hantavirus, hepatitis B virus, adenovirus, influenzavirus and human immune deficiency virus (HIV).[282,303-305] In one interesting study Sandalova *et al.* showed that EBV- and CMV-specific T cells contributed to the expansion of the $CD8^+$ T-cell compartment in response to hepatitis B infection, and

showed potent IFN-γ production indicating enhanced effector T-cell function.[303] They found no evidence of herpesvirus DNA in serum of infected patients suggesting the mechanism responsible for activation of EBV- and CMV specific T cells is distinct from reactivation of the viruses themselves, something that was previously suggested.[304] However, lack of detection of DNA might no be exclusively associated with lack of reactivation.[209] Taken together this suggests that a herpesvirus-seropositive individual could respond to secondary infections differently than a seronegative person, and display an altered infection course and/or enhanced immune protection.

PRESENT STUDY

OBJECTIVES

The overall aim of this work was to examine the response capacity of the immune system in childhood, and how common herpesviruses can affect differentiation and activation status of immune cells in the healthy host.

Specific aims:

- To determine the phenotype and functionality of neonatal monocyte subsets in contrast to adult, with regards to expression of activation markers and cytokine responses to a common bacterial ligand (Paper I).

- To evaluate whether children with latent EBV or EBV/CMV co-infection had altered innate responsiveness focusing on monocyte-NK cell collaboration (Paper II).

- To investigate whether CMV^+ children with EBV co-infection had an altered CMV-related T- and NK-cell differentiation and if so, what a possible mechanism behind this could be (Paper III).

- To evaluate the course of *in vitro* EBV infection in children of different ages, and if EBV infection in CMV^+ children would proceed differently due to high levels of mature anti-viral cells (Paper IV).

METHODS

A general description of the cohort material and methods is provided below and in more detail in each paper.

Cohort material

Cell samples from 2-year and 5-year old children were obtained from a cohort of children recruited to the Sachs Children's Hospital in collaboration with the pediatrician Caroline Nilsson. In these children serostatus to a range of viruses was determined. Serostatus (positive or negative) describes whether an individual has been exposed to a virus, developed immune responses and/or acquired latent forms of infection. At the age of 2, serostatus to 13 viruses was investigated by assaying for antiviral antibodies in blood plasma: respiratory tract infections, including adenovirus, influenza (A/H1, A/H3, and influenza B), parainfluenza (types 1, 2, 3), and RSV; and herpesvirus, including CMV, EBV, herpes simplex virus (HSV), human herpesvirus 6 (HHV6), and varicella-zoster virus (VZV).[285] At the age of 5 serostatus against EBV and CMV was determined.[295] For EBV this was carried out using immunofluorescence assays,[306] where the presence of IgG against EBV capsid antigen (EBV VCA) and EBNA-1 was tested. For CMV, ELISA was utilized to assess the presence of IgG directed against CMV nuclear antigens.[307] IgG was assessed as the development of IgM antibodies in children from 0 to >4 years experiencing IM is highly variable and dependent on age, whereas IgG is more frequently occurring at the onset of infection. In asymptomatic children there is as yet no knowledge about the development of the immune response in relation to infection time.

Overall experimental procedure

Peripheral blood (PB) was collected from healthy adult volunteers, from 2-year or 5-year old children or from umbilical cord blood (CB) right after delivery. Serum was separated and mononuclear cells (MC) were isolated from blood by Ficoll-Paque gradient centrifugation and frozen until analysis of the relevant groups. Experiments were carried out and cell phenotype and function was analyzed by flow cytometry. Cytokine levels were assessed in serum and supernatants by ELISA or cytometric bead array.

Paper I

Monocyte subsets from CBMC or PBMC from adults were simultaneously analyzed for cell surface markers (CD11c, CD14, CD16, CD80/86, CD163, HLA-DR). Monocyte functional responses were tested by stimulation with the TLR2 ligand PGN. Upon PGN stimulation, intracellular IL-12p70 and TNF was analyzed as well as phosphorylation of the signaling intermediate p38-MAPK. Also, levels of TNF, IL-1β, IL-6, IL-8 and IL-10 in supernatants were assayed. Serum substitution experiments were carried out exchanging fetal calf serum (FCS) (the standard serum used for cell culture) with cord blood or adult human sera and the same cytokines as above were analyzed in these supernatants.

Paper II

PBMC from 2-year old children with known serostatus to CMV and EBV were stimulated with IL-15+PGN to give monocyte-induced NK-cell activation, and NK-cell intracellular IFN-γ production was assessed along with IFN-γ release into supernatants. Also, frequency and phenotype of monocyte subsets was analyzed (CD14, CD16, CD11c, CD80/86). Further, monocytes and monocyte subsets were isolated and co-cultured with NK cells to investigate their relative stimulatory capacity on NK-cell IFN-γ production upon IL-15+PGN stimulation. Finally blood plasma levels of IFN-γ in these children were determined.

Paper III

PBMC from 5-year old children with known serostatus to CMV and EBV were phenotyped *ex vivo* and the T- and NK-cell compartment were analyzed (CD3, CD4, CD8, CD16, CD56, CD57, CD69, NKG2A, NKG2C, NKG2D, NKp30, CD45RA, 2B4, DNAM-1). NK-cell functionality was assessed by stimulation with MHC-deficient K562 or Daudi (EBV-positive) target cells, CD16 stimulation or IL-15+PGN stimulation and expression of CD107a, intracellular IFN-γ, MIP-1β, TNF and CD69 was evaluated. Changes in frequency and activity of the $NKG2C^+$ NK cell population were evaluated in an *in vitro* infection model where PBMC from EBV-naïve CMV^+ 5-year old children were infected with EBV. This was achieved through short time exposure of PBMC to supernatant containing EBV particles followed by cell culture for 7 or 14 days and subsequent analysis. Further, a co-culture model was set up where T-cell depleted PBMC from adults with enriched $NKG2C^+$ NK cell populations were co-cultured under various cytokine conditions with EBV-positive lymphoblastoid cells (LCL) or EBV-negative (Ramos) cells, or only in medium spiked with supernatant from LCL or Ramos. Post co-culture these cells were subjected to a K562 assay

and CD107a, intracellular IFN-γ and ki-67 (proliferation) was assessed. HLA-E expression on LCL and Ramos was determined.

Paper IV

CBMC or PBMC from EBV-naïve 2-year or 5-year old children with known serostatus to CMV were phenotyped *ex vivo* and the NK-, T- and B cell population were analyzed (CD3, CD4, CD8, CD19, CD27, CD56, CD57, NKG2C, IgD). Cells were subjected to *in vitro* EBV infection as before and cultured for 3, 7 or 14 days. Post-infection, phenotype of the NK-, T- and B cell population was analyzed as above. The following parameters were also assessed: Levels of IL-2, IL-4, IL-5, IL-10, IL-21, TGF-β, IFN-α and IFN-γ in supernatants. Intracellular T- and NK-cell IFN-γ. B-cell phenotype after addition of recombinant human IFN-γ in different doses and intervals to infected cultures of CMV+ 2-year old children. T-cell phenotype (CD57 and CD69) and IFN-γ release into supernatants after incubation of PBMC from 2-year old children with EBV-positive LCL, or EBV-negative Ramos cells.

Methodological considerations

The type of studies that we carried out necessitated the use of frozen cell samples. This enabled collection of cells from children over long periods of time for later simultaneous analysis of experiment and control groups. We are aware of the considerations of the impact on cells upon freezing. To this end our group has carried out extensive assessments of cell viability, proportions of cell populations, expression of activation markers, cytokine responses and susceptibility to EBV of frozen versus freshly isolated PBMC. We have found that 1. Cell viability in our PBMC samples after thawing is typically around 90%. If cells are frozen at too low or too high cell concentration viability decreases. If cell samples had low viability they were not used for experiments. 2. No cell population decreases preferentially after freezing (monocytes, B-, T-, or NK cells). 3. Cytokine responses of fresh and frozen PBMC are of similar magnitude, for instance monocyte production of IL-6 upon PGN stimulation. 4. The phenotype of monocytes and NK cells regarding the markers we have assessed is stabilized after short time rest/cell culture, for example CD16 expression on monocytes 5. Freshly isolated CBMC and CBMC that have been frozen > 8 years (!) can be equally infected by EBV and survive over a 14 day period.

Thus the potential freezer-induced effect on PBMC was carefully considered but should not have affected the experimental parameters significantly.

RESULTS AND DISCUSSION

PAPER I

Newborns are vulnerable to infections due to an immature immune system. Responses from innate cells, such as the monocyte, are generally of greater importance in infants than that of adaptive cells that mature at a slower pace. In general, the newborn cytokine response to innate stimuli is impaired in comparison to the adult response, as shown by numerous studies.[146,148,151-154] Despite the different roles in antimicrobial responses of the two major monocyte subsets,[89,90] their occurrence and functional maturity in the newborn had not previously been investigated. In this paper, we compared human newborn and adult monocytes and their subsets, with focus on functionality and expression of activation markers. We hypothesized that altered ratios, and/or functional diversity of these cells, could contribute to the qualitative difference between neonatal and adult antimicrobial responses.

Firstly, CBMC and PBMC were phenotyped *ex vivo*. We found that the $CD14^{++}CD16^{-}$ and $CD14^{+}CD16^{+}$ neonatal monocyte subsets had similar phenotype and frequencies in neonatal samples as in adult. When we stimulated cells with the bacterial ligand PGN, which is highly activating for monocytes,[74,75,308] neonatal monocytes were not impaired in their functional response and even had a higher capacity for production of TNF and IL-12p70 than adult monocytes. This was linked to higher phosphorylation of p38-MAPK. Further, $CD14^{+}CD16^{+}$ monocytes were demonstrated to have higher IL-12p70 production capacity than $CD14^{++}CD16^{-}$ monocytes in both newborns and adults.

Extensive studies of cytokine responses of neonatal cells to different innate and adaptive ligands have revealed some common patterns pointing to a Th2-Th17 biased immunity over Th1.[146-148] However, results are often inconsistent between studies using whole blood or MCs as the source of cells, which could be due to adenosine levels in serum. Neonatal serum is reported to contain high levels of adenosine that can suppress cytokine responses of neonatal cells *in vivo*.[153] As we found that the intrinsic capacity (eg. without influence of serum factors) of neonatal monocytes for cytokine production was not impaired, we addressed the question of serum influence and substituted FCS in culture medium with either neonatal or adult human sera and assayed whether cytokine production from CBMC or PBMC was affected. We found selective effects depending on the cytokine rather than the cell origin where IL-6 and IL-8 levels were increased but IL-10 and TNF levels were decreased when FCS was replaced with human sera. This implies that the use of FCS in cell cultures might yield substantially different results than when cells are cultured in autologous sera. It does,

however, not preclude that the comparison made here of intrinsic ability for cytokine production between neonatal and adult monocytes is not valid.

Inherent production of TNF by newborns was particularly strong. *In utero*, it is easy to envision that tight regulation of TNF by serum factors would be desirable. TNF is a potent inflammatory mediator[52] that has been connected to pregnancy complications.[309] In fact, blockade of TNF has been suggested as a treatment for immune-mediated fertility problems.[310] If indeed regulatory factors act to suppress TNF responses, it is possible that their concentration wane after birth enabling the successive maturation of TNF responses observed in childhood,[152] although this is yet to be investigated.

Our findings were somewhat at odds with the general functional impairment described for neonatal cells. Since we found remaining high percentages of $CD14^{+}CD16^{+}$ monocytes after stimulation in neonatal cultures, we suggested that they might fail to undergo further differentiation into macrophage or DC effector cells upon bacterial encounter. Further we argued that the functional impairment of the neonatal innate arm might lie mainly in DC function, as opposed to the innate monocyte responses *per se*, as DC maturation in childhood is slow.[156-158] In conclusion, these data add to our knowledge regarding innate responses at birth and describes for the first time the frequencies, phenotype and functional capacity to antibacterial challenge of the neonatal monocyte subsets.

PAPER II

In paper I we investigated the neonatal antibacterial responses with focus on monocytes. Besides their role as direct effectors, monocytes are in close collaboration with NK cells and provide signals for NK-cell survival/proliferation and cytokine production. Primary infection with EBV and CMV is usually asymptomatic and occurs during early childhood and the viruses then persist in a latent form. We had previous indications that serostatus to EBV and CMV (but not to other acute or persistent viral infections) would affect the risk of becoming IgE-sensitized[285] and the response capacity of the T-cell compartment.[311] Further, a recent paper at the time showed that latency with EBV in mice primed NK cells for IFN-γ production through activation of macrophages.[296] In this study we further explored the collaboration between accessory cells and NK cells and investigated monocyte-NK-cell activation in relation to herpesvirus serostatus in early life. We hypothesized that the innate response capacity would be affected, especially given our previous findings on EBV latency as an immune-modulatory factor.

We used PBMC from a cohort of 2-year old children with known serostatus to EBV and CMV. We had two groups of children; those that did not carry either virus (CMV^-EBV^-, seronegative) and those single-positive for EBV or co-infected with both viruses ($CMV^{+/-}$ $EBV^{+/-}$, seropositive). We stimulated cells with IL-15+PGN to induce monocyte-induced NK-cell activation. In contrast to findings from mice, we found reduced NK-cell IFN-γ production following stimulation, both intracellular in NK cells and in supernatants, as well as lower plasma levels of IFN-γ in seropositive children.

We speculated on whether this was dependent on an intrinsic defect in IFN-γ production by NK cells or was due to reduced capacity of monocytes to provide ample stimulation. We found that $CD14^+CD16^+$ monocytes could more efficiently induce IFN-γ by NK cells through this stimulation pathway (IL-15+PGN) (probably due to potent IL-12 production see Paper I) and that they were slightly reduced in seropositive subjects thereby providing a possible explanation to poor monocyte-induced NK-cell IFN-γ responses.

Interestingly, when we subdivided the groups the lowest levels of IFN-γ were found in children co-infected with EBV and CMV. CMV resides in myeloid cells[242] and infection of monocyte-derived DCs with CMV inhibits their maturation process and release of pro-inflammatory cytokines.[312] Although there was no statistical difference we found that the release of IL-6 by monocytes appeared to be lowest in the co-infected group. This further implicates the functionality of the accessory cell in seropositive children rather than the NK cell in itself. However, this does not exclude that NK cells were not as efficient in receiving monocyte-derived signals. $CD57^+$ NK cells have reduced transcription of the IL-12 receptor-β2 and also reduced IL-12 induced responses.[118] As it has later been shown that CMV can drive the differentiation of NK cells into $CD57^+$ (e.g. $NKG2C^+CD57^+$)[269] cells, an increased ratio of these cells in those children that had seroconverted to CMV might confer overall lesser IFN-γ responses.

A previous study from our group had shown an overall increased capacity of the T cell compartment in children that were EBV/CMV seropositive, and particular in IFN-γ producing cells in CMV^+ children upon polyclonal activation. The $CD57^+$ T cells that are generated in persistent CMV infection are potent IFN-γ producers[277] and thus, the effect of herpesvirus latency on effector cells might differ depending on the mode of activation and differentiation status induced by CMV (directly or via the induced cytokine environment).

Because of the fact that viral exposure factors (e.g. day care and siblings) were similar between groups, a question that was raised was whether these children differed in their innate

capacity prior to acquiring herpesvirus, i.e. whether a weak innate capacity for IFN-γ production set the stage for, rather than was a consequence of, contraction of EBV and/or CMV. By this logic, children who remain seronegative would do so because of strong innate activation leading to blockage of productive viral infection. However, although NK cells can control infection in tonsils,[211] the importance of the innate response in control of EBV infection is unclear. Furthermore seropositive 2-year old children have strong polyclonal T-cell responses[311] indicating that primary infection should be able to be controlled by this arm of immunity. One factor that has been suggested to underlie differential predisposition for acquiring herpesvirus infections is polymorphism in the IL-10 gene promoter and serum IL-10 levels.[313] Here we could not find any differences in plasma IL-10 levels between seronegative and seropositive children although we could not rule out that differences might have been masked by viral IL-10 production. Therefore, and in the light of an increased understanding of how persistent viral infections can modulate host immunity, we believe that these findings represent an example of how herpesvirus latency affects innate responses in children and possibly subsequent adaptive responses induced by monocyte-NK cell cross-talk.

PAPER III

Here we followed up on findings from paper II on herpesvirus seropositivity and anti-viral responses in children in light of recent discoveries on CMV-driven immune differentiation. Several studies had shown an imprint on the phenotype and function of NK- and T cells by CMV infection as mentioned above. Along with ours and others results[284] on additive effects of EBV and CMV co-latency, and as infection with these two viruses can happen in a close spatial time frame during childhood, we asked whether EBV co-infection could influence the CMV-induced imprint on anti-viral effector cells. We investigated the T- and NK-cell compartment and hypothesized that immune characteristics in children harboring both viruses might differ from those with only CMV.

We utilized samples from the same cohort of children but now at the age of 5. We had two groups of children, those that carried CMV (CMV^+) and those that did not (CMV^-). These groups were further subdivided to investigate the influence of co-infection with EBV into; those children that did not carry either virus (CMV^-EBV^-), those with only CMV (CMV^+) or those co-infected with both viruses (CMV^+EBV^+). A few children carrying only EBV (EBV^+) were included as a reference.

Our first approach was to study the expression of various differentiation markers and activating receptors on the NK- and T cell population. Firstly, in accordance with previous studies,[266,269,275,276] we found that a positive serostatus for CMV, but not EBV, was associated with increased frequencies of $NKG2C^+$ and $NKG2C^+CD57^+$ NK cells, and $CD8^+CD57^+$ T cells. However, previous studies only addressed single infections or did not show the serostatus to other viruses. When we investigated the effect of EBV co-infection in CMV^+ children we found associations within the NK- (but not T-) cell population, where EBV latency further promoted CMV-expanded cells ($NKG2C^+$ and $CD57^+NKG2C^+$ NK cells). We tried to shed some light on how EBV was able to affect frequencies of $NKG2C^+$ cells in CMV^+ children. We employed an *in vitro* infection model where PBMC from EBV-naïve CMV^+ 5-year old children were infected with EBV. In EBV-infected cultures the proportions of $NKG2C^+$ NK cells were increased in 7 out of 8 subjects, sometimes by as much as double as compared to non-infected controls, although we were not able to establish whether this was due to proliferation or increased survival/resistance to apoptosis as compared to $NKG2C^-$ NK cells.

We also found that CMV^+ children in the cohort displayed higher *ex vivo* degranulation in response to K562 target cells and had higher IL-15 levels in plasma. Co-infected children appeared to have the highest plasma IL-12p70 and IL-15 levels. With regards to the known effects of these cytokines on NK-cell functionality, we wanted to more closely examine NK-cell interaction with EBV-positive B cells in the presence of IL-15 and IL-12p70. As the sample cell number and availability from 5-year old CMV^+ children was limited, we co-cultured PBMC from adults that had large populations of $NKG2C^+$ and $NKG2C^+CD57^+$ NK cells with an EBV-positive cell line (LCL) with or without IL-12p70 and/or IL-15. As a majority of adults are seropositive for EBV, we depleted T cells from the PBMC to avoid memory responses. Co-culture in the presence of IL-15 or IL-15+IL-12p70 modestly increased the frequency of $NKG2C^+$ NK cells, which was not seen with an EBV-negative cell line. We found that a fraction of the LCL expressed HLA-E, a ligand for NKG2C, providing a possible link to propagation/maintenance of the $NKG2C^+$ population.[266,314] LCLs are a very heterogeneous population of cells with subsets of cells in lytic or latent phase.[315] Cells undergoing lytic replication decrease their expression of HLA-E[316] indicating that the effect on the NKG2C population may have been more prominent if the LCL had been a more homogenous. Further, cell-cell contact (or the environment it induced) was required for re-distribution of the NK-cell compartment, as cultures of PBMC in supernatant derived from LCL cultures did not affect the percentage of $NKG2C^+$ NK cells.

The most important finding from this paper was that dual latency with CMV and EBV had an additive effect on the frequency of differentiated NK cell populations, and possibly the cytokine environment, as compared to latency with CMV alone. It is clear that co-infection with two herpesviruses can have unexpected effects on host gene expression. In the mouse model of EBV and CMV infection, the genes specifically upregulated in co-infection were those connected to heightened inflammation or enhanced lymphocyte responses.[284] In agreement with study II and our previous studies on cytokine production capacity of the T-cell compartment,[311] this study illustrated a differential effect on the T- and the NK-cell compartment of herpesvirus latency.

It is possible that the accumulation of late-differentiated NK cells in co-infected children occurs as a result of reactivation of CMV upon EBV infection, although there is as yet no evidence supporting that primary EBV infection in healthy carriers would induce CMV reactivation *in vivo*.[317,318] Other studies have found that acute and chronic viral infections can induce an increase in $NKG2C^+$ and $CD57^+NKG2C^+$ cells dependent on a positive CMV serostatus.[270-273] In one report the percentage of $NKG2C^+$ NK cells tended to be increased in children actively secreting CMV,[267] and CMV can reactivate following inflammatory signals.[242] Serum IFN-γ levels were high in CMV^+ and co-infected children which could indicate reactivation as indicated by high levels of IFN-γ transcripts seen in transplant patients undergoing CMV reactivation.[319] Although an increased frequency of subclinical CMV re-activation may underlie high proportions of differentiated NK cells in co-infected children, CMV effector cells could also react to EBV-infected cells upon reactivation of EBV. As CMV latency confers increased levels of NK-cell promoting IL-15, the presence of HLA-E expressing EBV^+ cells could contribute to the expansion of the $NKG2C^+$ population upon interactions with NK cells. In connection to this, antigen-specific IL-2 secretion from T cells has been suggested to synergize with a potential enhanced direct activation of 'memory' NK cells and to lead to even more potent NK-cell responses during secondary infections.[109]

To our knowledge this is the first study breeching the subject of co-infection with these two viruses and our findings illustrate that the modulatory effects of different persistent herpesviruses may synergize in maturation of the immune system early in childhood.

PAPER IV

In paper II and III we found that herpesvirus latency affected the frequencies of NK- and T-cell subsets and NK-cell responses in children, and that co-infection with CMV and EBV differed from having either virus alone in terms of immune-modulation. Although EBV is normally encountered in early childhood, knowledge regarding the immune response to EBV has largely originated from studies of IM in adults, as clinical symptoms are seldom seen in infants. In study IV we set out to define the course of EBV infection in children of different ages, and whether a positive serostatus to CMV, and thus enriched T- and NK-cell subsets with mature phenotype, could alter the immune response to EBV. In connection to this, our group has demonstrated that the time-point of primary EBV infection during childhood could be of importance in modulating the risk of developing IgE sensitization,[295] further underscoring the importance of defining the immune response to EBV at different time-points during childhood.

PBMC samples originated from the same cohort of children as described in study II and III. We selected subjects that were EBV-naïve and $CMV^{-/+}$ in the age groups 2-years and 5-years of age and further we included unrelated CBMC samples (newborns). We utilized the *in vitro* infection model and monitored changes in the B-, T- and NK cell populations along with soluble factors released in to supernatants of infected cultures, to give insights in to both the fates of infected cells as well as those of anti-viral effectors.

Initially we established that the EBV infection model was functional by examining the target cell population, i.e. B cells, in terms of morphological changes (blasting), increase in frequency (proliferation), and acquisition of CD23 and CD27 along with expression of EBNA-1 and LMP-1 in CBMC cultures upon *in vitro* EBV infection. Upon establishment of successful EBV infection we compared infection of cells from different age groups and found differences in B-cell activation and release of interferons into supernatants. CBMC had low interferon responses and high accumulation of $IgD^{+}CD27^{+}$ non-switched memory B cells whereas older children had higher interferon responses and accumulation of $IgD^{-}CD27^{+}$ switched memory B cells in infected cultures. It is well known that the capacity to mount anti-viral responses increases with age as there is a slow development of accessory cell responses, such as production of IFN-α and DC production of IL-12.[154,156,158] Decreased activation of accessory cells will in turn affect NK- and subsequent T-cell responses such as IFN-γ production.

We showed that the B-cell subset distribution differed between age groups with higher proportions of memory cells in older children as expected. That this might have influenced the accumulation of memory IgD^-CD27^+ B cells in infected cultures of 2- and 5-year old children was considered. There is an ongoing discussion whether EBV preferentially infects memory or naïve B cells.[188,320] This discussion centers on the fact that EBV resides in memory B cells during latent infection and thus has to either infect them directly or drive a non-memory B cell through differentiation into memory phenotype. If both naïve and memory B cells are targeted it has been suggested that the latter cell might possess a growth advantage following infection.[188] There are now studies showing that EBV can equally infect all B-cell subsets and that they proliferate at an equal rate following infection.[191,192] EBV infection can induce differentiation factors involved in the SHM and CSR processes[191,321,322] and when the appropriate T-cell signals are present (CD40, IL-4 and IL-21) EBV is able to induce upregulation of IgG and IgA and concurrent downregulation of IgD in B cells.[191] Therefore we believe that the populations of IgD^-CD27^+ B cells seen in cultures of older children may have arisen through downregulation of IgD and/or class-switching of infected naïve B cells, rather than just expansion of pre-existing memory subsets. IL-21 and TGF-β were found in all supernatants and may have contributed to switching, although inherent B-cell factors (other than subset distribution) probably played a role in generating the difference between age groups.

Interestingly 2-year old CMV^+ children did not display the same EBV-driven accumulation of IgD^-CD27^+ switched memory B cells as those that did not have latent CMV. Further they had greater amounts of $CD8^+CD57^+$ T cells and higher levels of IFN-γ in supernatants and these factors correlated strongly. When recombinant IFN-γ was added to cultures of PBMC from CMV^- children formation of switched memory B cells was restricted. The central role for IFN-γ in mediating restriction of EBV transformation has been demonstrated earlier,[211,216] but not in connection to mature T cells in CMV^+ individuals. In this setting CMV-associated T cells with high IFN-γ production capacity may be activated through bystander mechanisms (cytokine environment), reactivation of CMV and/or through cross-recognition of related epitopes and we suggest that they may be involved in the immune response against EBV, directly or indirectly. Although the strongest connection to IFN-γ production on day 14 was to highly mature $CD57^+$ $CD8^+$ T cells this does not rule out that NK cells contributed to the secretion of IFN-γ in the early stages of the infection. In murine CMV infection NK-cell

cytotoxicity is maintained into the later stages of infection whereas IFN-γ production is impaired due to reduced availability of IL-12.[323,324]

Herpesvirus-specific memory T cells can confer enhanced immunity against a secondary infection through so-called heterologous immunity.[300] Studies with a panel of viruses have shown that prior immunity to one virus can confer enhanced clearance of a second unrelated virus. This was related to high production of T-cell IFN-γ for which the second virus was highly susceptible.[325] Although probably transient,[297] this type of unrelated or secondary activation may well be of clinical relevance. Barton *et al*. speculate on this topic in a series of papers stating that in the case of EBV, heterologous immunity may play a role in the variable presentation of IM at different ages.[284] IM develops more frequently in older individuals which could possibly be due to massive expansion of cross-reacting T cells, which would not occur in the infant.[300] In connection to this, we did not study regression, i.e. the complete reversal of infection, and have no reasons to assume that the infection is blocked. When the cohort material was assessed for connections between carriage of CMV at 2-years of age and the likelihood of EBV seropositivity at the age of 5, no significant relation was discovered (unpublished observation). However, we speculate that the infection course may be prolonged in CMV^+ children. If indeed it is essential for EBV to transform a naïve B cell into a memory phenotype to establish latency and thus be 'invisible' to the immune system,[320] the rapid transition into memory should be desirable. As the transition of EBV infected cells into the switched memory subset is inhibited in CMV^+ children, one could wonder whether these children may suffer a prolonged infection course, stronger T-cell activation due to more cells in lytic cycle and possibly be overrepresented among children developing IM, although this remains to be proven.

In conclusion, we studied EBV infection in children who are the most common target group for EBV and for whom very little is known regarding primary infection. During the time period between birth and 5 years of age the immune system goes through rapid maturation and could be uniquely susceptible to differentiating factors such as herpesvirus latency. Herein we were able to show both age- and CMV-related effects on the course of EBV infection. The data provided here may aid in future understanding of why EBV infection follows differential courses in childhood, and further invites for speculation that the order in which herpesvirus infections are acquired in early life could make a difference for the outcome of infection.

PRELIMINARY DATA AND EXTENDED DISCUSSION

Go back to Start - Herpesvirus and allergies

Although my studies came to focus on the general effect of herpesvirus latency on differentiation and activation status of immune cells, the starting point was the finding that EBV infection decreased the risk for IgE-sensitization in children, an effect enhanced by CMV co-infection.[285] No connections were found for the other acute or persistent viral infections investigated (see Cohort Material). EBV and CMV might be unique in that they persist in immune cells and that such a large part of the memory compartment is dedicated to control over these viruses. Of course this does not rule out that other viruses may influence immune-maturation, but was out of the scope of this thesis.

We were initially interested in the relation between herpesvirus and Th1/Th2 polarization that is important in the development of allergic responses.[292] With the setup and cohort material from 5-year old children used in study III we assessed frequencies and cytokine profiles of Th subsets. Firstly, there were similar bulk frequencies of $CD4^+$ T cells in CMV^- and CMV^+ children (study III). We then stimulated PBMC with PMA and ionomycin (potent polyclonal activator) and measured the intracellular production of IFN-γ, IL-4 and IL-17 in $CD4^+$ Th cells. Elevated frequencies of IFN-γ^+ Th cells seemed to be connected to CMV carriage although only significantly so in co-infected children (Fig. 8). This result was consistent with plasma IFN-γ levels (study III) and our previous reports of high IFN-γ production following polyclonal activation of T cells in 2-year old CMV^+ children.[311] Together with high frequencies of $CD57^+CD4^+$ T cells (unpublished result), also the $CD4^+$ T-cell compartment seems to be affected by latent herpesvirus infection, with increased Th1-skewed functionality as the result.

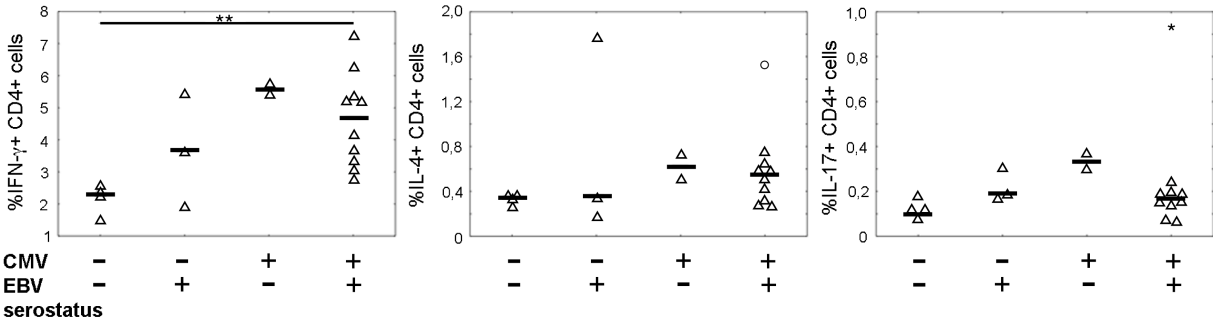

Figure 8. IFN-γ skewed cytokine profile of $CD4^+CD3^+$ cells from CMV and EBV co-infected 5-year old children.

Although the Th1/Th2 balance has a fundamental role in the development of allergies, Treg cells have now come into focus because of their ability to dampen effector cell responses and modulate immune reactivity and inflammation.[31] We assessed Treg cells in our cohort of 5-year old children but found similar frequencies regardless of serostatus to EBV and CMV (Fig. 9). There appeared to be no connection between Th-cell cytokine profiles or Treg frequencies to IgE sensitization in these children (unpublished observation).

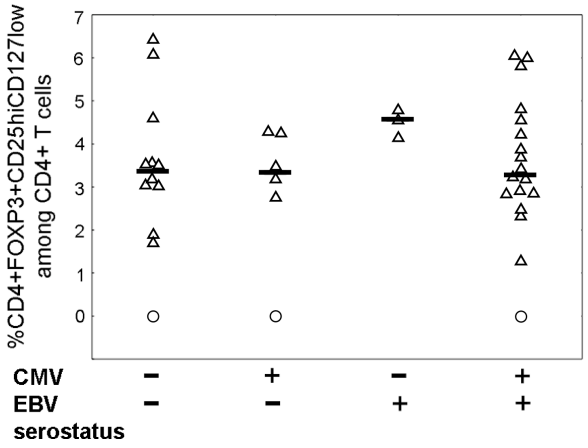

Figure 9. Similar *ex vivo* frequencies of Treg cells in 5-year old children regardless of EBV and CMV serostatus.

As of yet it is not clear how our findings on herpesvirus-mediated immune differentiation of the anti-viral effector cell compartment relate to a protective effect against the development of IgE-sensitization. Altered T- and/or NK-cell cytokine production capacity may have consequences for subsequent B-cell responses in the form of altered cytokine environment or other as of yet unidentified co-stimulatory pathways. Additional effects of CMV co-infection on a decreased risk of IgE sensitization[285] may be due to elevated IFN-γ levels, which can block IL-4 and IgE synthesis. It is also conceivable that the connection to allergy might lie closer to heart, namely in EBVs target cell - the B cell. We have performed an extensive phenotyping of the B-cell compartment in the 5-year old children of our cohort and are in the process of analyzing results.

Production of IgE by plasma B cells is central to the allergic response. There are some interesting connections between EBV and the production of IgE. IgE synthesis is regulated in a complex manner that involves IL-4 and CD23/CD21. CD23 exists in a membrane bound (mCD23) and a soluble form (sCD23). sCD23 is induced in *in vitro* and *in vivo* EBV infection,[326-328] can act as a low affinity IgE receptor,[329] and is involved in the regulation of

IgE production.[330] Furthermore CD40 plays a crucial role in Ig switching and CD40/CD40L pairing + IL-4 and IL-13 leads to IgE production. EBV induced expression of LMP-1 can completely substitute CD40 signaling in B cells, leading to normal B-cell development, activation, and immune responses including CSR, GC formation, and SHM. Interestingly, LMP1-signaling can induce CSR to IgG1 independent of cytokines.[331]

Time-point for primary herpesvirus infection during childhood – does it matter?

We had indications that age at the time of herpesvirus contraction was important for immune-modulation since EBV infection before the age of 2 years conferred a decreased risk of IgE sensitization whereas infection after 2 years increased the risk.[295] In connection to this, we speculated that EBVs polyclonal effects on B cells might propagate proliferation of already IgE-committed memory B cells in older children whereas this would not occur in younger antigen inexperienced children (Saghafian-Hedengren personal communication). We assessed plasma levels of IL-2, IL-4 and IL-5 for the CMV^+ and co-infected children of paper III. There were no differences between CMV seronegative and seropositive children overall. Interestingly however, when the children were divided into groups depending on the time of contraction of EBV and/or CMV, IL-2 and IL-4 levels were increased in the co-infected group that had contracted EBV after the age of 2 (late L) (Fig. 10), i.e. the group of children with increased risk of late-onset IgE sensitization.[295]

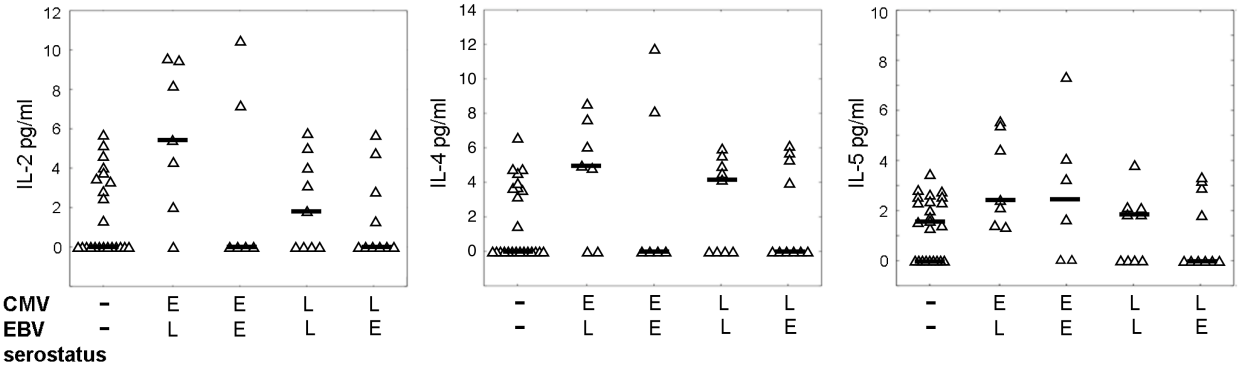

Figure 10. Raised plasma levels of IL-2 and IL-4 in 5-year old co-infected children that have contracted EBV after 2-years of age (late L). Before 2-years of age (early E).

The discrepancies in plasma levels of IFN-γ seen between study II and study III (e.g. low versus high levels in seropositive subjects) suggests that the shaping of immune cells by herpesvirus latency could differ depending on age (2y versus 5y). When we divided the co-infected group of 5-year olds from study III into those that had contracted CMV early and then either contracted EBV early or late we found that expansions of mature T- and NK cell populations were most pronounced in those children infected with CMV and EBV before the age of 2. This group also had higher IFN-γ levels in plasma (Fig. 11).

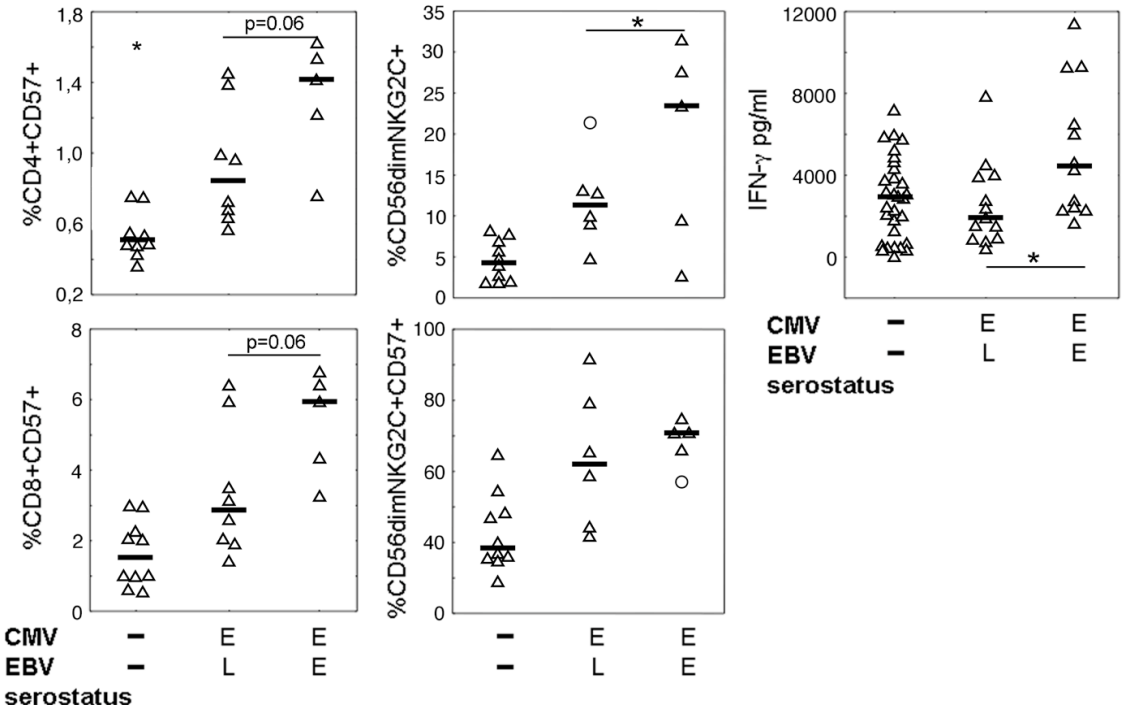

Figure 11. Time-point of primary EBV co-infection, before the age of 2 (early E) or after the age of 2 (late L) affects levels of differentiated T- and NK cells and plasma IFN-γ levels in CMV-seropositive children.

Dynamics of herpesvirus-related immune maturation during childhood – a model

Herpesvirus-induced differentiation of immune cells and of subsequent immune responses appears to be dependent on dynamic factors. During childhood and adolescence we are in a constant stage of development and maturation and it is conceivable that there are ”windows of opportunity” when we are susceptible to extrinsic influences that will interact with our genetic background and shape our immune system.

Young infants (around and below 2) are peak shedders of CMV[248] and have detectable CMV viremia for a prolonged period of time following primary infection. We believe that a pre-existing CMV-induced redistribution of the NK-cell compartment can be amplified by an (early) EBV co-infection (paper III). NK cells preferentially target EBV-infected B cells in the lytic cycle,[316] which primarily occurs in secondary lymphoid tissues. NK cells can be recruited to draining lymph nodes following viral infection,[332] and have been shown to control EBV in tonsillar sites.[211] It is possible that interactions between HLA-E^+ target cells and $NKG2C^+$ NK cells occur upon primary EBV infection. Target cells could include both EBV-infected and/or reactivating CMV-infected cells. The anti-viral cytokine environment generated in response to EBV could promote CMV reactivation[250] and boost NK-cell function and the proliferation of bystander $NKG2C^+$ NK cells.[333] The persistence of $NKG2C^+$ NK cells may be dependent on presence of CMV antigen,[319] and reactivation of CMV may be subclinical but sufficient to prime newly emerging NK cells.[334] Reactivation of latent viral infections are probably a common phenomenon in infancy due to the many other infections contracted during this time. In connection to this, the level of IFN-γ in plasma at the age of 5 is correlated to the number of common respiratory tract infections contracted during the first year of life (Maria Johansson unpublished). One could envision that the $NKG2C^+$ NK-cell compartment may be boosted whenever the anti-viral T-cell mediated responses fails to keep CMV at bay and it is possible that CMV is not as well controlled in those infants who simultaneously harbor EBV.

One interpretation of how early contraction of EBV would differ from late contraction is that the duration of time that EBV has existed within the host system has enabled more re-activations of EBV and/or CMV and thus accumulation of late-differentiated cells. Furthermore, the maturational state of the immune system when contracting the infection will be of importance. We showed that for *in vitro* EBV infection, age was significantly related to the proliferation of EBV-infected cells and the redistribution of B-cell subsets into memory

phenotypes (paper IV). Recent studies have found that children infected early in life, and those with active co-infections carried higher EBV viral load.[335,336] The possibility for successful reactivation of a persistent virus is increased if the virus initially establishes a high latent virus genome load as shown by studies of human alpha herpesviruses, and this in turn is reliant on the level of virus replication achieved during primary infection of the naive host.[337-339] In fact, the magnitude of the CMV imprint on the NK-cell compartment is quite variable. Studies of CMV^+ adults (such as in co-publication V and Muntasell *et al*.[333]) have found that not all individuals show these expansions of $NKG2C^+$ NK cells with differentiated phenotype, indicating the influence of many factors on their generation and persistence. However their frequency in adults is remarkably stable over a period of years whereas children display a more dynamic behavior with a decline in some of the expanded NK-cell phenotype between primary contraction and 5-years of age (co-publication V). When we simultaneously assessed CMV^+ 5-year olds with the cohort described in paper IV the frequency of their $NKG2C^+$ NK cells and $CD8^+CD57^+$ T cells were similar to non-infected 2-year olds but still higher than non-infected 5-year olds suggesting stabilization in the period following infancy. This type of expansion followed by contraction is also noted in acute CMV infection.[269,319]

In conclusion, herpesvirus-induced differentiation of anti-viral effector cell populations is dynamic and it is possible that the proximity to primary infection, re-activation events, other acute or persistent infections and age at time of primary herpesvirus infection determine the frequencies and activation status of these populations. This in turn might affect the level of immune reactivity towards other infections or immune stimuli (Figure 12 overleaf).

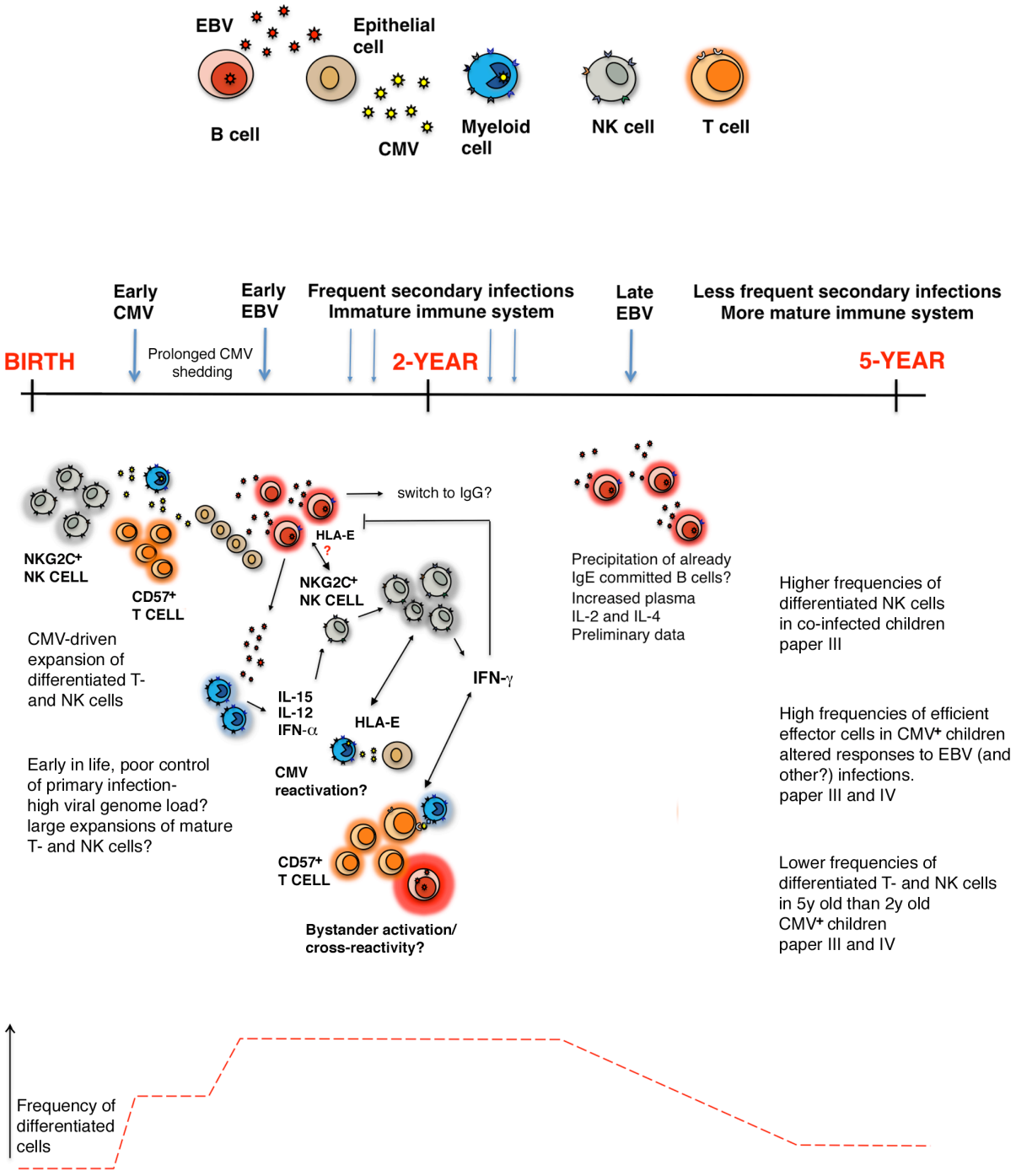

Figure 12. Hypothetical scenario of influence of herpesvirus on immune cells and responses in childhood based on data presented in this thesis and previous studies from independent research groups, all references within the main body of text.

FUTURE PERSPECTIVES

Our studies have shown herpesvirus-induced differentiation of immune cells in children, altered functional responses of viral effector cells in seropositive children and additive effects of CMV and EBV co-infection on these parameters. In relation to this the following would be interesting to study in children of different ages and/or with different serostatus to CMV and EBV:

- The effector functions and interactions with NK cells of the third subset of intermediate $CD14^{dim}CD16^{+}$ monocytes, which been connected to viral infections.

- Whether any interaction involving HLA-E occurs between NK cells and EBV-infected B cells leading to subsequent propagation of $NKG2C^{+}$ NK cells.

- The occurrence of differentiated T- and NK cells in the oropharynx, where the natural EBV infection occurs, and their expression of homing receptors enabling migration to these sites.

- Detailed effects on the B cell population upon *in vitro* EBV infection and whether expression of viral products and latency programs differs depending on the maturational state of the B cell. IgE switch/production and expression of molecules and transcription factors related to control of IgE synthesis. IgA levels in saliva.

- The underlying mechanism and contribution of IFN-γ in the alteration of B-cell subset composition after *in vitro* EBV infection in CMV^{+} children.

- The nature of activation of differentiated $CD8^{+}$ T cells in EBV-infected cultures of CMV^{+} children in terms of bystander activation versus cross-recognition of related epitopes.

- Possible effect of large populations of differentiated T- and NK cells in CMV+ children on other bacterial or viral infections besides EBV.

- On the larger scale, clinical investigations of a possible connection between CMV latency, cross-reactivity and IM.

GENERAL CONCLUSIONS

Our reliance upon the microbial world for proper maturation of the immune system in childhood is now becoming fully appreciated. We as humans are in fact not alone but composed of many communities of species whose interplay has a great impact on the survival success of the overall "holobiont".[340] The co-evolution of herpesviruses and their hosts have been taking place over eons of time and have enabled both parties to adapt to one another. Therefore, although these viruses can act as causative agents of disease, it is not unconceivable that they may also drive differentiation of immune cells that might be beneficial to their host species. By studying a pediatric cohort, and due to late EBV and CMV infection in Sweden, we had a unique possibility to study contraction/infection and modulation of immune responses by these viruses in childhood. Through this series of papers, and related work from our group, we build the argument that herpesvirus latency can and does modulate the immune system in an asymptomatic host and that this may have effects on immune responses to both related and unrelated infections and allergens. Therefore every person's microbiome, including the intestinal microbiota and the virome, may have a great impact on individual immune reactions.

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I was born by the river in a little tent
Oh and just like the river I've been running ever since
It's been a long, a long time coming
But I know a change gonna come, oh yes it will.

Sam Cooke

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