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The Effect of Human Cytomegalovirus on Innate Immune Responses – Immune Activation and Evasion

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Till min älskade familj

ABSTRACT

Human cytomegalovirus (HCMV) is a wide-spread virus infecting about 60-90 % of the healthy human population. After a primary infection, the virus establishes life-long latency in its host. Although HCMV seldom give any symptoms in immunocompetent individuals, it may cause severe disease in persons with a deficient immune system, such as AIDS patients, transplant recipients and fetuses. The infection is controlled mainly by virus-specific T cells and NK cells, but to co-exist with its host, HCMV has developed several mechanisms to influence the host immune response. Reactivation of HCMV is mediated by inflammation with activated immune cells and secretion of strong pro-inflammatory cytokines, and HCMV has been linked to several inflammatory diseases such as inflammatory bowel disease (IBD), systemic lupus erythematosus (SLE) and cardiovascular disease. In addition, HCMV has lately been detected in different tumors and is suggested to play a role in cancers such as prostate cancer, colon cancer and glioblastome multiforme (GBM). HCMV is believed to act as an onco-modulatoty virus, contributing to tumor cell growth and survival.

In this thesis I aim to further understand the mechanisms behind HCMV immune modulation and how these effects may contribute to the development of cancer. I describe that;
(I) HCMV is able to partially stimulate PDC maturation and to induce a strong IFN-α secretion. HCMV activated PDCs stimulate B cell activation and proliferation but conversely depress T cell proliferation. This may enhance virally mediated immunosuppression and help HCMV escape immune recognition.
(II) HCMV influence the PDC mediated activation of NK cells. HCMV infected PDCs reduce NK cell cytotoxicity, and instead facilitate the development of non-cytotoxic cytokine producing NK cells. NK cells are vital in the anti-viral immune response, and this may contribute to viral immune evasion as well as cytokine induced viral reactivation and replication.
(III) Virally induced soluble molecules from *in vitro* infected cells are able to downregulate NK cell killing of target cells. Interestingly, HCMV positive glioblastoma cells demonstrated the same inhibition of NK cell cytotoxicity. This observation describes a mechanism by which HCMV hampers a vital NK cell function, which may led to reduced anti-viral and anti-tumor immune responses.
(IV) Peripheral neutrophil activation correlates to tumor progression in patients with GBM and levels of several pro-and anti-inflammatry cytokines are altered compared to healthy individuals. HCMV is known to activate neutrophils and enhance secretion of cytokines important in inflammation, and HCMV may thereby contribute to the immune dysfunction often seen in these patients.

In conclusion, the ability of HCMV to alter the host immune system is vital for virus latency and reactivation. Here I describe novel mechanisms that may contribute to HCMV immune evasion and to the development of cancer.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to by their roman numerals:

I. Varani S, **Cederarv M**, Feld S, Tammik C, Frascaroli G, Landini MP, Söderberg-Nauclér C. Human cytomegalovirus differentially controls B cell and T cell responses through effects on plasmacytoid dendritic cells. *J Immunol. 2007;179(11):7767-76.*

II. **Cederarv M**, Odeberg J*, Söderberg-Nauclér C*. HCMV infection of PDCs deviates the NK cell response into cytokine producing cells unable to perform cytotoxicity. *Immunobiology. 2009;214(5):331-41.*

III. **Cederarv M**, Fritz N, Wolmer-Solberg N, Uhlen P, Odeberg J*, Söderberg-Nauclér C* Specific suppression of NK cell cytotoxicity by primary glioblastoma cells is mediated by Human Cytomegalovirus. *Manuscript.*

IV. **Cederarv M,** Rahbar M, Wolmer-Solberg N, Stragliotto, G, Peredo, I, Orrego A, Skarman P, Xu, X, Dzabic M, Taher C, Söderberg-Nauclér C. Immunological patterns in HCMV infected glioblastoma patients; induced PMNL activity is associated with tumor progression. *Manuscript.*

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LIST OF ABBREVIATIONS

ADCC	antibody-dependent cell mediated cytotoxicity
BCR	B cell receptor
CVD	cardiovascular disease
DC	dendritic cell
E	early
EBV	Epstein-Barr virus
EC	endothelial cell
EGFR	epidermal growth factor receptor
FasL	Fas ligand
GM-CSF	granulocyte-macrophage colony-stimulating factor
gp	glycoprotein
GrB	Granzyme B
HCMV	Human Cytomegalovirus
HHV	Human Herpesvirus
HSPG	heparin sulphate proteoglycans
HSV	Herpes Simplex virus
IBD	inflammatory bowel disease
IE	immediately early
IFN	interferon
IR	internal repeat
IRL	internal repetitive sequence long
IRS	internal repetitive sequence short
KIR	immunoglobulin-like receptor
L	late
MCMV	murine cytomegalovirus
MCP	major capsid protein
MCP-1	monocyte chemotactic protein-1
MDC	myeloid dendritic cell
MHC	major histocompability complex
MIC	MHC class I-related chain
MIP	macrophage inflammatory protein
MoDC	monocyte-derived dendritic cell
ORF	open reading frame
PBMC	peripheral blood mononuclear cell
PDC	plasmacytoid dendritic cell
PDGFR	platelet-derived growth factor receptor
pp	phosphoprotein
SCT	stem cell transplantation
SLE	systemic lupus erythematosus
SMC	smooth muscle cells
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper
TNF	tumor necrosis factor

TR	terminal repeat
TRL	terminal repetitive sequence long
TRS	terminal repetitive sequence short
TTP	time to tumor progression
UL	unique long
ULBP	UL16 binding protein
US	unique short
VZV	Varicella Zoster virus

AIMS OF THE STUDY

Human cytomegalovirus (HCMV) is known to establish life-long latency in its host and may cause severe morbidity in immunocompromized individuals. In order to accomplish latnecy, HCMV has developed several strong immune regulatory functions that are vital for the virus to avoid recognition and elimination by the immune system and to establish latency. Exerting powerful effects on the immune system have consequences on other aspects of the host's morbidity, and recent data imply a high frequency of HCMV positive cells in several cancer forms, suggesting a role of HCMV in tumor development.

The aims of my thesis projects were;

1. **To further characterize the effect of HCMV on the immune system, in particular the innate immune response**. I wanted to investigate how a HCMV infection of immune cells that are important in the host anti-viral defense influences their maturation, differentiation, effector functions and their ability to communicate with other cells.
2. **To investigate the effect of HCMV on the immune system in patients with glioblastoma multiformae (GBM), a very malignant brain tumor with a high degree of HCMV positive cells.** These patients have a highly dysfunctional immune response, and the effect of HCMV on the function of immune cells could possibly contribute to their impaired immune system. Despite aggressive anti-tumor treatment, patients have a very short life expectancy after diagnosis. Since a functional immune system is vital in the fight against cancer, a better understanding of the mechanisms behind the immunosuppression in these patients may aid in the development of more powerful treatments.

BIOLOGY OF HUMAN HERPESVIRUSES

HCMV is a member of the herpesvirus family. In humans, eight herpesviruses are described so far; herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2), varicella zoster virus (VZV), Epstein-Barr virus (EBV), human cytomegalovirus (HCMV) and human herpesviruses 6 (A and B variant), 7 and 8 (HHV-7 and HHV-8). Viruses belonging to the herpesvirus family share a common virion architecture, which is the inclusion criteria for this virus family. All herpesviruses consist of an icosadeltahedral capsid of approximately 100 nm in diameter containing a core of double stranded viral DNA. The capsid is covered by the tegument that consists of amorphous asymmetric protein material of several HCMV proteins. In the mature virion, the tegumented capsid is enclosed by a lipid bilayer envelope containing viral glycoprotein complexes on its surface [1]. Herpesviruses are widely spread throughout nature and in the world. There are over 130 different herpesviruses known today and most animals have at least one herpesvirus, but one virus strain rarely infect more than one species.

Herpesviruses share four significant biological properties;
1) Following a primary infection, the virus establishes life-long latency in its host, and may be reactivated later in life. The viral genome persists as closed circular molecules in the cell harboring the latent virus and only express a small number of viral genes.
2) They encode a large array of enzymes involved in nucleic acid metabolism, DNA synthesis and processing of proteins such as enzymes important in virus replication.
3) DNA synthesis takes place in the nucleus of the infected cells and matures on its way to the cell surface; viral DNA synthesis and capsid assembly occurs in the nucleus, and capsid particles are enveloped as they transit through the nuclear membrane. The virus particle matures in the cytoplasm and intracellular compartments before leaving the cell.
4) Production of infectious virus particles is most often accompanied by destruction of the infected cell.

With respect to differences in their biological properties, such as host range and reproductive cycle, herpesviruses are further divided into 3 subfamilies; alphaherpesviridae, betaherpesviridae and gammaherpesviridae. Members of the alphaherpesviridae subfamily have a short reproductive cycle, establish latency by infecting mainly sensory ganglia and spread rapidly in culture, and include HSV-1, HSV-2 and VZV. Betaherpesviruses have a restricted host range with longer replication cycles; HCMV, HHV-6 and -7. HCMV is believed to establish latency in monocytes, HHV-6 in macrophages and $CD4^+$ T cells, and HHV-7 in $CD4^+$ T cells. The gammaherpesvirus family consist of EBV and HHV-8 which also have a restricted host range, and establish latency mainly in B cells [2].

HUMAN CYTOMEGALOVIRUS

Human cytomegalovirus (HCMV) is a widely spread human betaherpesvirus carried by a large majority of the healthy adult population. The virus usually spreads at an early age and establishes life-long latency after the primary infection. Infection is most often subclinical in immunocompetent individuals but during the past decades the interest and importance of HCMV as a pathogen has increased significantly [3, 4]. HCMV is a major cause of morbidity in AIDS patients and transplant patients [5] and is believed to be of importance in several clinical conditions such as inflammatory bowel disease (IBD) [6], systemic lupus erythematosus (SLE) [7], myositis [8], cardiovascular disease (CVD) [9] and psoriasis [10]. In addition, HCMV has been described to be able to contribute to tumor cell survival and growth and has been detected in different tumors, and is therefore also regarded as an oncomodulatory virus [11-15].

HCMV BIOLOGY

HCMV GENOME AND STUCTURE

The HCMV genome consists of a linear, double stranded DNA molecule which is the largest genome among the herpesviruses with its 230 kilobase pairs, based on studies of the laboratory HCMV strain AD169. Clinical isolates have been shown to have even larger and more complex genomes [4, 16, 17]. The genome is composed of two unique domains; the unique long (UL) and the unique short (US) domain. On each sides of these domains are internal and terminal repetitive sequences (IRS, IRL and TRS, TRL respectively) [4]. Open reading frames (OFRs) are named using prefixes UL, US, IR or TR depending on their location in the genome. The HCMV genome encodes about 200-250 ORFs which are divided into immediate early (IE), early (E) and late (L) depending on at what time points after infection their mRNA is detectable in the cytoplasm of an infected cell [4, 18]. However, only around 50 of these ORFs encode for proteins believed to be essential in virus replication and the production of new virus particles, which means that the majority of HCMV proteins might have functions important for virus modulation of cellular and immunological responses of the host.

The HCMV genome is enclosed in an icosahedral shaped capsid comprising of at least five different proteins. The major capsid protein (MCP) makes up around 90% of the total protein content and is encoded by UL86. Other proteins forming the capsid are the smallest capsid protein UL48-49, the minor capsid protein UL85, the minor capsid binding protein UL46 and fragments of the assembly protein UL80 [16, 17]. Surrounding the capsid is the tegument layer, an amorphous layer containing least 25 different proteins. Many of these proteins have an unclear function, but some are believed to be important in maintaining the structure of the virus particle and regulating viral gene expression and host cell responses to the infection. Yet some are important in the process of viral particle assembly and egress. Most of these proteins are phosphorylated and are therefore given the prefix pp. The most abundant proteins are pp150 (ppUL32) and pp65 (ppUL83) which are both highly immunogenic. pp65 is associated with dense body production and is also used as target in anti-genemia assays

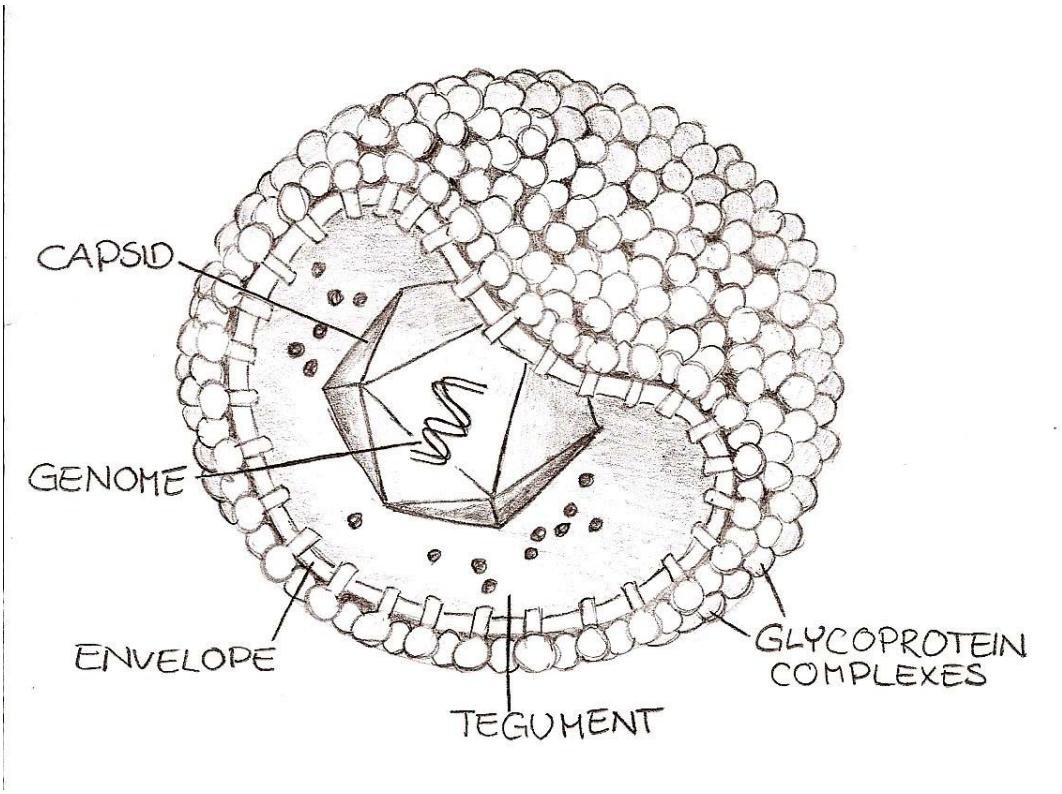

Figure 1. An illustration of the HCMV structure.

for diagnosis of clinical HCMV infection. Although pp65 depleted viruses grown *in vitro* are growth modified, pp65 is not needed for an efficient viral replication in culture. pp150 makes up 20% of the virion mass and is a broadly recognized viral antigen recognized in sera from the vast majority of HCMV positive individuals.

The outer surface of the virus particle is the envelope which consists of viral glycoproteins and host lipids and proteins derived from intracellular membranes. Viral envelope proteins are important targets of the host antibody response to the infection. Approximately 8 viral glycoprotein species are believed to be included in the envelope, the highly conserved gB (gUL55), and gM (gpUL100)/gN (gpUL73) being the most abundant. gB can always be found on the surface of infected cells and is involved in cell-to-cell transmission, and is together with gH (gpUL75) important in virus binding to and fusion with target cells. Also gM/gN and gH are important glycoproteins building the envelope and are together with gB essential in the production of infectious virus particles. Several additional virally encoded glycoproteins, such as gpUL4 and gpUL132 are believed to be minor constituents of the envelope. The major host proteins incorporated in the virus envelope are β_2-microglobulin [19], actin [17], CD13 [20], topoisomerase II [21], CD55, DC59 [22] and annexin II [23].

HCMV ENTRY AND TISSUE SPECIFICITY

HCMV entry into host cells is initiated by the binding of HCMV glycoproteins to host cell heparin sulphate proteoglycans (HSPG), integrins [24] and cell surface receptors.

After attachment, the virus envelope fuses with the plasma membrane in a rapid, pH dependent or independent fashion [25]. Several different host cell glycoproteins have been suggested to act as receptors facilitating HCMV entry; β2 microglobulin [19], CD13 [26], epithelial growth factor receptor [27], epidermal growth factor receptor (EGFR) [27], platelet-derived growth factor receptor-α (PDGFR-α) [28], DC-SIGN [29] and annexin II [23, 30]. The site of entry is most often the epithelial cells in mucosal surfaces of the upper alimentary tract, genitourinary tract or respiratory tract although cells of almost all body organs can get infected [31]. In addition to epithelial cells, infection can occur in monocytes, macrophages, dendritic cells (DC), endothelial cells (EC), smooth muscle cells (SMC), fibroblasts, stromal cells, neuronal cells and hepatocytes. There is a difference between different cells with regard to susceptibility, permissiveness and establishment of latency. Virus replication is not allowed in all cell types, and is less efficient and sometimes not permissive in ECs, monocytes and macrophages, compared to infection in fibroblasts, the cell type often preferred for *in vitro* growth of the virus due to the high infection rate and permissive infection. During non permissive infection, which often occurs in monocytes, only HCMV IE and E genes are expressed and no virus particles are formed [32]. When fully differentiated to macrophages, also late genes are transcribed, and macrophages are together with fibroblasts, EC, SMC and epithelial cells, the only cell types that allow a fully permissive virus replication resulting in infectious virus particles.

HCMV LATENCY AND VIRUS REACTIVATON

After the primary infection, HCMV establishes a life-long latency in its host. Although HCMV can infect virtually all human organs, the major site of HCMV persistence is believed to be monocytes/macrophages together with ECs, that have been implicated to harbor latent HCMV as well. HCMV is thought to facilitate the interaction between these two cell types in order to enhance virus spread in the host [33, 34]. Both the type of endothelial cell and the HCMV strain is believed to be of importance in the infection [35]. Fibroblasts are commonly used for *in vitro* production of HCMV with high virus titers. Clinical HCMV isolates have the ability to replicate in both ECs and fibroblasts, but several studies have shown that clinical HCMV strains cultured *in vitro* in fibroblasts lose their endothelial tropism. This is thought to result from genetic alterations in the UL150-128 region which leads to a loss of genes essential for endothelial tropism [36, 37]. $CD14^+$ monocytes are believed to be the major cell type responsible for harboring latent HCMV *in vivo* [38-40], where the virus genome is maintained at a low copy number of approximately 10 copies/cell [41]. However, the virus is not able to replicate efficiently in monocytes and the infection is non-permissive. Also, monocytes are non-replicating, relatively short-lived cells. On the other hand, reactivation and a complete replication cycle have been described in differentiated macrophages, and monocyte differentiation into macrophages is thought to activate latent HCMV [39, 40]. Still, one could expect to find latent HCMV stored in a more long-lived cell type. Indeed, HCMV DNA has been detected in $CD34^+$ bone marrow cells, and HCMV has been shown *in vitro* to infect $CD33^+$ myeloid progenitor cells [42-44]. After differentiation to $CD14^+$ macrophages or DCs, $CD34^+$ supported production of infectious HCMV particles. These progenitor cell types are together with $CD14^+$ cells also considered to harbor latent HCMV [42, 45].

In the process of activating latent HCMV during macrophage differentiation from monocytes, the presence of $CD8^+$ T cells and the secretion of the anti-viral inflammatory cytokines IFN-γ and TNF-α is believed to be vital [46]. Still, studies have described an enhanced HCMV replication in the presence of TNF-α [47]. *In vivo*, elevated levels of these cytokines have been detected in sera of patients with an active HCMV infection and HCMV activation is often a consequence of allogenic organ or bone marrow transplantation, or bacterial infections [48, 49]. Taken together, immune activation with cytokine production is believed to be necessary for HCMV reactivation and virus production from monocytes/macrophages. In addition to $CD8^+$ T cells, NK cells have the ability to secrete TNF-α and IFN-γ and also PDCs are strong producers of TNF-α. As these cells are believed to be able to enhance each other's activation and cytokine secretion [50-53], we analyzed the secretion of these cytokines in PDC-NK cell co-cultures and how the cytokine release from these cells is affected by the presence of HCMV (**paper II**). We found that a HCMV infection of PDCs prior to co-culture with NK cells resulted in a significantly increased secretion of both IFN-γ and TNF-α. The secretion of these cytokines was increased in uninfected PDC-NK cell coculture as well, but not significantly, compared to PDCs and NK cells cultured alone. Also, this increased secretion was dependent of an active virus replication since infection of PDCs with a UV-inactivated virus prior to coculture resulted in cytokine levels comparable to uninfected cocultures. Thus, HCMV infection of PDCs might affect their cross-talk with NK cells and aid in producing an environment conducive for viral reactivation and replication.

Stimulating monocytes with IFN-γ and TNF-α produces HCMV-permissive macrophages, which describes the importance of these cytokines in a productive HCMV infection [46]. Also, TNF-α induces HCMV IE promoter activity, thereby enhancing virus replication [54]. Both cytokines are strong inflammatory mediators and are produced by activated NK cells and T cells, and HCMV reactivation is often seen in patients with inflammatory conditions. Also, HCMV is known to induce the expression of Cox-2, 5-lipooxygenas (5-LO) and leukotriens in infected cells [55, 56] and since 5-LO expression in HCMV infected areas of the bowel in patients with IBD correlates to leukocyte infiltration, HCMV may enhance inflammation and cytokine secretion in these patients. This may describe the importance of HCMV in the development of inflammatory diseases.

HCMV REPLICATION CYCLE AND VIRUS ASSEMBLY

HCMV replication cycle takes approximately 48-72 hrs, which is long compared to other herpes viruses. After virus envelope fusion with the host cell membrane, the virus capsid with surrounding tegument proteins is transported to the cell nucleus and viral DNA is delivered inside the nucleus. Viral genes are expressed in three structured phases based on appearance of viral mRNA and proteins in the infected cell; IE, E and L, where expression of IE genes starts immediately after entry into the nucleus. IE expression is independent of the expression of other viral genes, but depends on cellular transcription factors and possibly virus tegument proteins for activation. IE genes encode mainly non-structural proteins important in regulating the expression of E and L genes and possibly also regulate host cell gene expression. E genes are believed to mainly encode for non-structural proteins needed for virus DNA replication; for

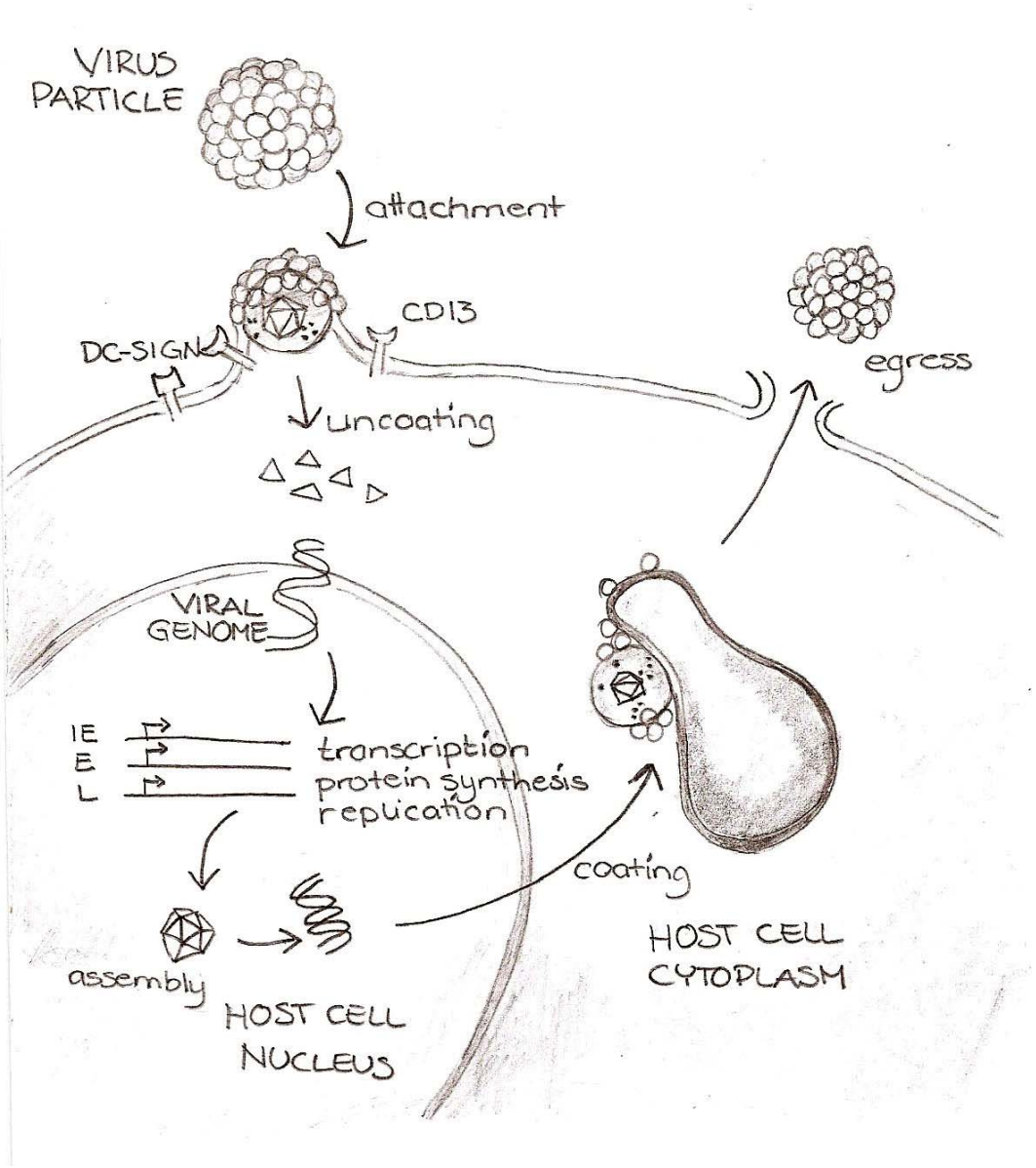

Figure 2. HCMV entry, replication and egress from a host cell.

example HCMV-DNA polymerase and DNA binding proteins. L genes are primarily believed to encode for virus structural genes such as viral capsid proteins and envelope proteins important in virus particle assembly. Both E and L genes are dependent of functional transcribed EI gene products. Cells are not immediately killed by the virus infection, the importance of this is still unclear. A full virus replication takes approximately 72 hrs and the release of virus particles reaches high levels at about 5-7 days of virus particle accumulation. After this there is a continuous high virus production for days until cell lysis [4, 17].

The process of viral particle formation and transportation out of the host cell is still not well understood. Viral capsids containing newly synthesized viral DNA are thought to be formed in the cell nucleus. Envelopment is believed to occur in steps. Gold electron microscopy images of infected cells have identified different sites; when passing

through the nuclear membrane, in cytoplasmatic Golgi-derived vesicles and at the plasma membrane. Virions are released to the extracellular matrix by fusion of the virus containing vacuoles with the plasma membrane [4, 57].

HCMV EPIDEMIOLOGY, VIRUS TRANSMISSION AND REACTIVATION

HCMV is believed to be universally spread among human populations but the percentage of infected individuals varies between 60% and 100% among different populations. However, since HCMV infections in immunocompetent individuals most often are subclinical and unnoticed, the exact percentage is difficult to assess [58]. The infection rate is depending on geographical location and the socioeconomic situation, where the highest rates are seen in groups with low socioeconomic status in developing countries [59]. Among Swedish blood donors more than 70% have been shown to be HCMV seropositive [60]. HCMV is transmitted from one person to the other via direct contact of body fluids. After primary infection, infectious HCMV particles are present in urine, saliva, tears, semen and cervical secretions up to years after infection [59]. High rates of HCMV infected individuals are seen where close contact with body fluids are common, such as between sex partners, in day care centers and in schools. Indeed, HCMV is most often acquired early in childhood through close contact with other children or through breast-feeding and the percentage of infected individuals increase with age [61]. Also, blood products [62], solid organs [63] and bone marrow transplants [64] can transmit active as well as latent HCMV.

HCMV AND THE IMMUNE SYSTEM

INTRODUCTION TO THE IMMUNE SYSTEM

The immune system is a complex, adaptable and specific system that protects the host from invading pathogens and cancer transformed cells. It consists of a large variety of cells and molecules specialized on recognizing and eliminating what is considered foreign. The immune system is commonly divided into two functional components; the innate and the adaptive immune system that differ in specificity. The innate immunity is the first, early line of defense against infections and is considered less specific with a broad reactivity. It consists of cells and molecules that are not pathogen-specific, but rather recognize classes of highly conserved molecules commonly expressed by pathogens, infected host cells or tumor cells. Many components of the innate immune system are present before the infection or tumor formation and thus constitute a rapid, but unspecific, host immune response. In general, it comprises of four defense barriers; anatomic, physiologic, phagocytic and inflammatory. In contrast to the innate immune system, the adaptive immune system requires some time for activation and is activated after infection. It is distinguished both by a high degree of specificity to a wide range of pathogen molecules, by memory and by being highly adaptable, in contrast to the innate immunity which is mainly pre-determined. The adaptive immune response usually peaks 7-10 days after activation, but a second encounter to the same pathogen will result in a much more rapid response due to immunological memory. This response is also often much stronger and efficient than the initial response. The major effectors are immune cells, antibodies and other molecules produced by the cells.

Innate and adaptive immunity are not independent of each other but work in a cooperative way. The innate immunity produces signals and molecules that stimulate, activate and direct the subsequent adaptive response [65, 66]. Together, the innate and the adaptive immune responses serve as a strong defense system protecting the host against pathogens and tumor transformed cells.

Both cell-mediated and humoral immunity are believed to be important in controlling HCMV infection in the host [67-69]. The virus can reactivate in immunocompetent HCMV positive individuals, but the infection is controlled by the immune system of the host with a strong immune response against the virus. Both $CD4^+$ T helper cells and cytotoxic $CD8^+$ T cells take part in the cell mediated HCMV immunity [70]. $CD8^+$ T cells are important in lysis of HCMV infected cells. In the clinical situation, lack of a proper T cell response may lead to increased risk of and a delayed recovery from HCMV associated disease [71, 72]. Also, patients having a poor NK cell response have been described to suffer from HCMV associated pneumonia, suggesting a role of NK cells in controlling HCMV infection [73]. In addition, a proper humoral immune response is important in HCMV immunity. Upon acute infection, most often after the primary infection but to a lesser extent also after a recurrent infection, the host responds by a transient IgM production. This IgM secretion is followed by sustained IgG levels, and anti-HCMV antibodies are thought to contribute to control of virus infection and virus dissemination [74, 75].

HCMV is a highly host specific virus, but shares several features with murine cytomegalovirus (MCMV) including a similar immune response. This makes the mouse model suitable for studying CMV, and several studies on mice have increased our knowledge on CMV and the immune system. As in humans, immunocompromized mice with an active MCMV infection suffer from severe morbidity and mortality [76]. These mice have high levels of virus in blood and MCMV infection has been linked to severe pneumonia [76], myocarditis [77] and an increase of immune cells in cardiovascular lesions [78]. MCMV infection in mice gives rise to a strong, specific T cell response, and T cells together with NK cells and dendritic cells (DC) are important in controlling the infection [79-81]. The importance of murine NK cells in the anti-MCMV immune response have been shown by depleting NK cells and thereby increasing the susceptibility to MCMV [82].

INTRODUCTION TO HCMV IMMUNE EVASION

A functional immune system is crucial to combat virus infections. In order to establish a life-long infection and to co-exist with its host, HCMV must be able to avoid elimination by the immune system. HCMV has developed several immune evasion strategies that interfere with both the early innate and the following adaptive immune responses. Still, the mechanisms behind HCMV induced immunosuppression are not fully understood.

All these mechanisms employed by HCMV to evade the host immune system also cause a general immunosuppression both against HCMV itself but also against

unrelated pathogens, which is most pronounced during primary infection and after transfusion-mediated infection [83, 84]. Patients with an acute HCMV infection have been described to have a higher risk of suffering from other infections, in particular bacterial and fungal infections [85]. Peripheral blood mononuclear cells (PBMCs) from patients with an active HCMV infection have a reduced proliferation in response to proliferative stimuli and herpes virus antigens.

The immune system and HCMV immune evasion mechanisms will be further discussed in the following sections.

THE INNATE IMMUNE SYSTEM

The innate immune system main function is to eliminate invading microbes as well as activating and steering the following adaptive immune response. Cells of the innate immune system mainly recognizes pathogens by binding of so called pattern recognition receptors (PRRs) to preserved structures commonly expressed on most microbes. PRRs are specific to certain patterns shared by large groups of organisms and unique to microbes. Toll-like receptors (TLRs), one type of PRRs, are triggered by bacterial toxins, stress proteins and bacterial and viral nucleic acids. Phagocytosing cells such as monocytes/macrophages, DCs and neutrophiles are an important part of the innate immunity. These cells recognize and engulf pathogens; macrophages promote inflammation and steer the following immune response. DCs are professional APCs and also direct other immune cells by presenting pathogen epitopes on the surface together with costimulatory molecules as well as secrete cytokines and chemokines. Neutrophils on the other hand take part in the host defense by engulfing and destroying pathogens. Also, NK cells are important in the immune response mainly against viruses due to their ability to rapidly kill an infected cell, thereby eliminating the virus. Alongside the cellular immunity, the complement system is a complex system with activating and inhibitory proteolytic enzymes. The complement system can be activated by antibodies covering a microbe, through binding of mannan binding lectin to microbes or by a direct recognition of and binding to pathogens. Regardless of activation pathway, complement activation lead to cell lysis, pathogen opsonization and induction of inflammation.

CYTOKINES AND CHEMOKINES

Pro- and anti-inflammatory cytokines are important messenger molecules of the immune system, secreted by both innate and adaptive immune cells and can stimulate cell activation, growth and differentiation. They are often produced in response to cell stimuli such as antibody binding or pathogen recognition, and regulate the subsequent innate and adaptive immune reactions. Some cytokines, such as TNF-α, IL-12 and IFN-α are produced by phagocytic cells in an early response to infection and are strong inducers of a cellular adaptive immune response as well as further enhances the cellular innate immunity. Also cytokines produced later in the immune reaction to an infection by cells of the adaptive immune response regulate the immune system. Th1 T cells secrete IFN-γ and TNF-α in the response to intracellular bacteria and viruses which will activate macrophages and B cells. Parasite infection will on the other hand induce a

Th2 response with the production of IL-4, IL-5 and IL-13. Cytokines of the different Th profiles have a downregulating effect on each other [86].

Cytokines can be strong activators of immune responses and an overproduction after an infection may lead to sepsis; a systemic inflammatory state, also called systemic inflammatory response syndrome. HCMV have been described to prolong the disease course of septic patients, possibly due to the array of cytokines induced during HCMV infection [87].

Chemokines are important in regulating migration of leukocytes and recruiting them to sites of infection and inflammation (pro-inflammatory chemokines) but also to direct cells through normal tissue (homeostatic chemokines). Pro-inflammatory chemokines are often secreted in response to an infection. Secreted chemokines influence inflammatory cells migrate to the site of inflammation, where chemokines also induce leukocyte cell adhesion and tissue infiltration. Thus, chemokines are essential regulators of the immune system and important in mounting a proper anti-microbial defense. Chemokines are usually divided in two subgroups; the CC chemokines and the CXC chemokines. Examples of chemokines belonging to the CC subfamily are monocyte chemotactic protein (MCP)-1-3, MIP (macrophage inflammatory protein)-1α/β and RANTES. MCP-1 (or CCL2) is a strong pro-inflammatory chemokine that induce monocyte, neutrophil, T cell and DC chemotaxis, and is expressed by a large array of cells such as peripheral leukocytes, SMC and endothelial cells in response to infection. The MIP proteins MIP-1α and MIP-1β (or CCL-3 and CCL-4 respectively) are secreted by activated macrophages upon bacterial encounter, and are important in activation of neutrophils and macrophages and the induction of cytokine release. RANTES (or CCL-5) is considered a strong regulator of T cell migration [88, 89].

HCMV IMMUNE EVASION BY AN ALTERED CYTOKINE AND CHEMOKINE PATTERN

As mentioned, cytokines and chemokines have vital roles in the development of a proper innate and adaptive immune response to an infection. Upon infection, HCMV have been shown to induce the secretion of several different cytokines and chemokines important in steering the immune response; IL-1, IL-6, IL-8, IL-10, IFN-β, TGF-β, MCP-1, MIP-1α/β and RANTES [9] and **paper I and II**. Since HCMV reactivation from latent state is thought to be induced by cytokine production, this "cytokine storm" seen during the acute phase might facilitate HCMV reactivation and replication and enhance viral spread.

The importance of the anti-viral cytokines IFN-γ and TNF-α in controlling HCMV infection has been well described in mice. These cytokines reduce viral replication and induce a strong anti-viral $CD4^+$ and $CD8^+$ T cell response [90]. However, although known to be anti-viral cytokines also in humans, TNF-α and IFN-γ work synergistically to induce the formation of HCMV-permissive monocyte-derived macrophages and TNF-α induce HCMV IE promoter activity thus enhancing virus replication [38, 46, 54]. We found that a HCMV infection of PDCs and a subsequent co-culture with NK cells induce both TNF-α and IFN-γ induction (**paper II**). In the light of previous data, this implies that by interfering with the important PDC-NK cross-talk, HCMV may

further enhance its replication and persistence. Both TNF-α and IFN-γ are strong inducers of an anti-viral immunity, and the enhanced secretion of these cytokines illustrates two sides of the role of cytokines in the immune response against HCMV; they both activate immune cells important in a proper anti-HCMV immune response, and they induce viral replication and proliferation of HCMV susceptible cells.

Also IL-8 and TGF-β have been shown to induce HCMV replication *in vitro* and might aid viral spread by recruitment of leukocytes [91]. In addition, HCMV is known to encode for its own viral IL-10 homologue expressed in infected cells. This viral IL-10 is able to downregulate MDC maturation and cytokine release, as well as inhibit virally induced IFN-α secretion from PDCs [92, 93].

HCMV is known to encode for two CXC chemokine homologues; UL146 and UL147 [94]. UL146 are believed to have dual actions in the same manner as HCMV induction of cytokines by recruiting neutrophils to the site of infection. Neutrophils are among the first immune cells to respond to an infection and are important in phagocytosing microorganisms and secreting cytokines thereby activating and steering the following immune response (the action of neutrophils are further discussed below). But neutrophils are susceptible to infection by HCM, and a release of the UL146 encoded chemokines homologue will recruit neutrophils to the site of infection and thereby enhance viral dissemination. Also MCMV encodes a chemokine homologue; the CC homologue m131/129, which has been shown to increase inflammation, viral production and viral spread [95].

NK CELLS AND THEIR RECEPTORS

NK cells were first described in the 1970s on a functional basis as cells able to kill tumors cells without prior sensitization, and were considered to be 'non-specific' lymphocytes [96, 97]. Since then, a large number of studies have demonstrated their efficiency in killing many types of tumor cells as well as infected cells. NK cells, constituting 5-10% of total peripheral lymphocytes, were originally considered to be non-specific due to their lack of specific surface receptors such as the T cell receptor or immunoglobulin (Ig) molecules for pathogen recognition [98]. Instead NK cells activation is dependent on a balance between activating and inhibitory receptors on the cell surface, but the exact mechanisms and receptors involved are still mainly unknown. Activation can occur either by a decrease in inhibitor signals or an increase in activating signals.

NK cell activation is mainly regulated by the so called 'missing self' hypothesis. NK cells express a wide range of inhibitory receptors specific for MHC class I molecules on target cells. These molecules are abundantly expressed on healthy cells and protect cells from NK cell killing by providing inhibitory signals. Infected cells and tumor cells on the other hand often down-regulate these molecules in order to protect them from T cell recognition. Without a proper MHC class I expression (termed "missing self") no inhibitory signal is delivered to the NK cell and it can be activated. Inhibitory receptors are mainly members of the killer immunoglobulin-like receptor (KIR) family, the leucocyte Ig-like receptors (LIRs) and the C-type lectin-like family comprising of

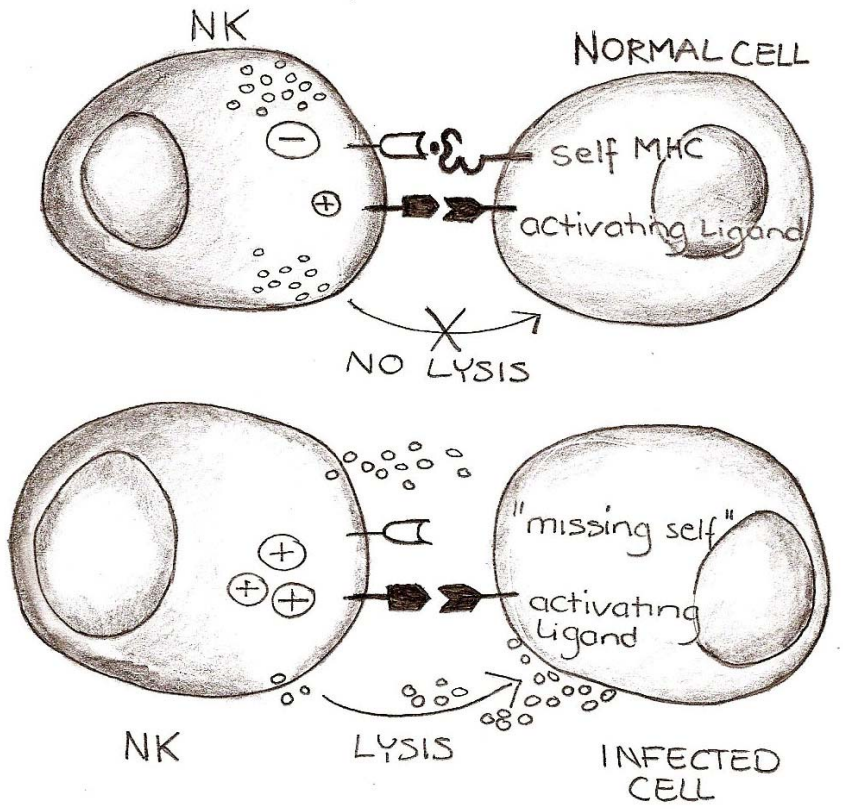

Figure 3. Illustration of the "missing-self hypothesis" important in NK cell activation.

CD94/NKG2A and CD94/NKG2C heterodimers [99, 100], all having great affinity for MHC class I molecules [101]. The CD94/NKG2 heterodimers recognize the non-classical MHC class I molecule HLA-E, which is also seen in mice where CD94/NKG2A, -C or E interacts with the non-classical MVH class I molecule Qa-1 [102]. But in contrast to humans, instead of KIRs, mice express Ly49 C-type receptors that recognize MCH class I molecules, thereby having similar functions to human KIRs [99]. NK cells also express a large variety of activating receptors that after recognizing target cell molecules stimulate NK cell killing and cytokine production [103, 104]. Among the activating receptors are the 'natural cytotoxicity receptors' NKp30, NKp44, NKp46 that together with NKG2D are considered to be the major NK cell activating receptors [105]. Also ligation of surface CD2 receptors, CD16, activatory KIRs and TLRs will deliver activating signals to the cell [105, 106]. The ligands for the natural cytotoxicity receptors are mainly unknown but NKG2D is believed to bind the stress-induced membrane-bound proteins MHC class I-related chain A and B (MICA and MICB respectively) as well as UL16 binding protein (ULBP) 1-4. In contrast, soluble forms of these proteins have been shown to bind to and inhibit NK cell activating receptors resulting in decreased NK cell cytotoxicity [107]. Soluble MICB described in cancer patients is believed to contribute to tumor cell survival by downregulating NK cell activity [108, 109]. In addition, NK cells can be activated when binding to the Fc region of antibodies bound to tumor cells or infected cells, termed antibody-dependent cell mediated cytotoxicity (ADCC) [110].

NK cells are vital in the host tumor rejection. Tumor cell may induce NK cell anti-tumor reactivity by down-regulating their MHC class I expression, thereby activating NK cell by the missing self hypothesis. Also, through binding of activating receptors, NK cells recognize structures that are upregulated on transformed cells, such as MICA, MICB and ULBP1-3 [111, 112]. The efficiency of NKG2D-mediated killing by NK cells have been shown to correlate to the surface expression of its ligands on target cells [113]. However, MIC-expressing tumors have been described to progress, which implies that NK cell response against these tumors is hampered. Soluble NKG2D ligands have been shown to be released from tumors in patients with colon carcinoma, leukemia and neuroblastoma. This was followed by a downregulation of activating receptors on NK cells, thereby inhibiting a proper NK cell response [108, 114, 115].

HCMV INTERFENCE WITH NK CELL RECEPTOR LIGATION AS IMMUNE EVASION MECHANISMS

HCMV is known to downregulate the expression of MHC class I in order to avoid recognition by other immune cells [116-118]. A decreased expression of MHC class I would make the infected cells vulnerable to NK cell lysis, since NK cells become activated in the absence of proper MHC class I expression (missing self-hypothesis). In mice, several mechanisms that interfere with NK cell killing of infected cells have been suggested. The stimulatory NK cell receptor Ly49H is believed to be central in the NK cell response against MCMV. It binds to the virally encoded m157 gene product that is a structural homologue of the MHC class I molecule [119, 120]. However, due to alterations in the m157 gene, MCMV strains that are resistant to Ly49H mediated NK cell killing have emerged [121]. Also, several MCMV encoded proteins have been reported to interfere with NKG2D mediated activation of NK cells, mainly by decreasing surface expression of its activating ligands. NKG2D is as mentioned a strong activating NK cell receptor and murine ligands include RAE-1, MULT-1 and H60 [122]. The m152, m155 and m145 genes all encode proteins that downregulate expression of NKG2D ligands [123-125].

Also HCMV infected cells are less sensitive to NK cell killing [126, 127], and different mechanisms are postulated. The virus affects expression of both triggering and inhibitory molecules on infected cells regulating NK cell activation, by downregulating activating receptors and enhancing surface expression of inhibitory molecules [128, 129]. The HCMV encoded MHC class I homologues UL18 and UL142 have been suggested to mediate protection against immune activation due to recognition of missing self. UL18 is reported to mediate resistance against NK cell lysis by binding to the inhibitory receptor LIR-1 [129]. In addition to NK cells, LIR-1 is expressed on T cells, DCs, monocytes and B cells and UL18 may contribute to HCMV immune evasion by interfering with the activation of these cells, and it was recently shown that UL18 can inhibit MDC migration and MDC mediated T cell activation [130, 131]. However, the importance of UL18 in protecting infected cells against NK cell lysis is debated and the role of UL18 in HCMV immune evasion is unclear [127]. The recently discovered HCMV protein UL142 mediates protection from NK cell lysis of HCMV infected fibroblasts, possibly by a downregulation of the NKG2D ligand MICA [132]. Also NKG2D ligand MICB has been shown to be downregulated by the recently identified HCMV microRNA-UL112 [133]. In addition, HCMV UL40 mediate

resistance to NK cell lysis by enhancing the expression of inhibitory HLA-E and its binding to NK cell inhibitory receptor CD94/NKG2A [128, 134].

The expression of NK cell receptors, both inhibitory and activating, and the function of NK cells have been described to be altered in HIV-infected viremic individuals [135, 136]. Several studies describe a NK repertoire in these patients with increased surface expression of inhibitory NK cell receptors in combination with a low expression of activating receptors [135, 137, 138]. Also, soluble HCMV protein pp65 have been reported to downregulate NKp30, having an impact on late NK cell cytotoxicity [139]. This shows that a soluble HCMV protein can cause a general suppression of NK cell activity by specifically binding to an NK cell activating receptor. We investigated how HCMV influence the expression of NK cell receptors (**paper III**). We found that soluble protein(s), virally or host cell encoded, released from HCMV infected cells were able to significantly downregulate NK cell activating receptor NKG2D after 24 hrs of coculture. No downregulation of the other major NK cell activating receptors were detected. The downregulation of NKG2D was observed on both classical large $CD56^{+}/Cd16^{+}/CD3^{-}$ NK cells as well as on $CD56^{bright}/CD16^{-}$ NK cells (see section on NK cell subtypes). This decreased surface receptor expression was accompanied by a reduction of NK cell killing. These observations suggest that HCMV have the ability to hamper NK cell activity by mediating a downregulation of an important activating receptor. This could suggest another mechanism employed by HCMV to evade the elimination by NK cells.

NK CELL CYTOTXICITY

Cytotoxicity is considered as the main effector function of NK cells, targeting tumor cells and virally infected cells. This effect is mainly carried out by the extracellular release of cytolytic proteins stored in cytoplasmatic granules in NK cells. [140, 141]. When NK cells have recognized a target cell, these granules migrate toward the contact site, fuse with the NK cell membrane and the granule content diffuse toward the target cell [142].

The key lytic proteins released by NK cells are believed to be the pore-forming protein perforin and granzymes, mainly Granzyme B (GrB) [143]. When binding to and entering the target cell, caspase-dependent and independent apoptosis is induced, but the mechanism by which these proteins exert their function is still unknown, although both proteins need to be present in order to induce apoptosis [144, 145]. Previously, the main understanding was that perforin proteins were inserted in the cell membrane of the target cell in a pore-shaped formation [146, 147]. This pore was thought to induce cell death by puncturing the membrane, thereby inducing cell death. However, this effect has only been observed *in vitro* with high concentrations of pure perforin added to cells, and is not believed to take place *in vivo*. Alternatively, soluble granzymes may enter cells via pinocytosis or diffusion [148]. In addition, several studies have shown that GrB can induce target cell apoptosis in the presence of perforin concentrations so low that not induction of cell membrane damage occurred [149, 150]. Indeed, subsequent studies have described induction of granule-mediated apoptosis in the absence of detectable plasma membrane perforin pores. Instead, perforin and GrB are suggested to be delivered to the target cell in multimeric complexes together with the

proteoglycan serglycin (SG), also present in the cytolytic granules of NK cells. Perforin-SG complexes were shown to mediate cytosolic delivery of GrB-SG macromolecules withour forming membrane pores [149, 151]. Granzyme-mediated apoptosis is carried out in two main ways; one is by the cleavage and activation of several different caspases, including caspase-3, -6, -7, -8, -9 and -10 with subsequent DNA fragmentation, but GrB is also able to induce BID-mediated mitochondrial permeabilization [152]. Granzymes may also enter target cells independently of perforin, via receptor-mediated endocytosis or macropinocytosis. However, perforin is still required to induce apoptosis since granzymes are retained inside endocytic vesicles, only in the presence of perforin are granzymes transported out of these vesicles and into the cell nucleus where they can initiate apoptosis [153, 154].

A continuous expression and storage of perforin and GrB in cytoplasmatic granules in NK cells vouches for a rapid response when encountering a target cell, but mRNA production and accumulation can also increase upon activation [155]. The HCMV protein UL16 has been shown to protect infected cells against the action of NK cell cytolytic proteins perforin and Granzyme B and also block surface expression of UL16 binding proteins (ULBP) 1 and 2 and thereby inhibiting their binding to the NK cell activating receptor NKG2D [126, 156].

The importance of perforin in NK cell cytotoxicity has been shown in mice both *in vivo* by perforin knock-out mice and *in vitro* with a mutation in the perforin gene, where NK cell function is significantly reduced [157, 158]. These mice were unable to clear an active lymphocytic choriomeningitis virus infection and had a reduced anti-tumor response *in vivo*. Also, NK cell killing of target cells *in vitro* was hampered. In addition, humans suffering from familiar hemophagocytic lymphohistiocytosis (FHL) have a mutation in the gene encoding for perforin. These patients suffer from an uncontrolled hyperinflammation with prolonged fever, hepatosplenomegaly and cytopenia. They have severely impaired NK cell cytotoxicity due to an absolute deficiency of perforin, again pointing to the importance of perforin in NK cell function [159, 160].

One may wonder why the NK cell itself is not killed by its own lytic proteins after release. Although the exact mechanisms remains to be determined, NK cells seem to be resistant to lytic proteins after degranulation [161]. One possible explanation is the insertion of cathepsin B into NK cell membrane upon degranulation, thereby providing self-protection [144]. Also the intracellular protein proteinase inhibitor 9 (PI-9) is thought to inhibit apoptosis induction by GrB [162].

The Fas-Fas ligand (FasL) pathway, or ”the kiss of death” is a mechanism important in the control of cytotoxic T cell homeostasis and promotion of tolerance to self-antigens [163]. NK cells together with effector T cells express FasL (CD178) molecules on their surface which are able to bind Fas (CD95) on target cells susceptible to apoptosis [164]. This binding initiates intracellular events activating pro-caspase 8 or 10 which lead to apoptosis.

In addition to cytotoxicity, activated NK cells are able to produce large amount of cytokines that are important both in the anti-viral defense and in steering the subsequent

immune response. For example, IFN-γ production by NK cell has been shown to be vital in clearing an infection [165]. In addition, NK cells have the ability to secrete IL-2, granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor (TNF)-α and transforming growth factor (TGF)-β [141, 166]. TNF-α has been described to induce cell apoptosis by enhancing ligand binding affinity of receptors mediating cell death, inflammatory processes, cell activation and differentiation [167].

Although NK cells by definition don't need to be activated to perform its effector functions, NK cell activation is regulated by the release of cytokines and chemokines by surrounding cells. IL-2, IL-12 and IFN-α induce NK cell cytotoxicity, and are believed to be important in promoting a strong anti-viral response during infection. *In vitro,* the efficiency of NK cell killing of tumor cells is enhanced by stimulation of NK cells with IL-2 and IL-12. Also, IFN-α has been described to induce NK cell cytotoxicity [168]. This increased killing is thought to result from an enhanced binding of perforin to target cell membranes [169]. IL-12 is also believed to drive NK cells to produce IFN-γ [165]. IL-2 is commonly used for NK cell activation *in vitro*. IL-15 produced *in vivo* by DCs is important in NK cell activation, differentiation and for cell survival [170]. TNF-α, IL-18, IL-1 all enhance the effects of IL-12, IL-15 and IFN-α and promote NK cell cytokine secretion, proliferation and cytotoxicity [171-173]. NK cell chemotaxis in culture has been induced by the chemokines MIP-1α/β, MCP-1 and RANTES [174]. In contrast, TGF-β and IL-10 downregulate NK cell activity by inhibiting cytokine production, proliferation and cytotoxicity.

NK CELL SUBTYPES

NK cells were originally defined as large $CD56^{+}/CD16^{+}/CD3^{-}$ lymphocytes, but today $CD56^{-}/CD16^{+}/CD3^{-}$ subsets of NK cells have also been identified; these cells differ from 'classical' $CD56^{+}/CD16^{+}/CD3^{-}$ NK cells in regard to surface markers, cytokine production and killing abilities. $CD56^{-}$ cells both have a reduced ability to release lytic proteins, possibly due to low perforin content, and to produce cytokines, and as described above; have an abnormal expression of inhibitory and activating receptors. A shift in NK cell subpopulations towards cells less able to secrete cytokines and lytic proteins have for example been described in HIV-infected individuals with high levels of viral replication, and is thought to contribute to the impaired NK cell function and poor immunological response in these patients [136, 138]. Also chronic HCV/HIV-1 co-infected individuals have been described with elevated numbers of $CD56^{-}/CD16^{+}/CD3^{-}$ cells, which is not seen in HCV mono-infected patients [175], again with a possible contribution to the poor viral immune response in these patients.

Another recently identified NK cell subset are the $CD56^{bright}/CD16^{-}/CD3^{-}$ NK cells. These cells display no KIR surface molecules, but respond to cytokine stimulation, mainly IL-2 due to a constant expression of IL-2 surface receptors. $CD56^{bright}/CD16^{-}/CD3^{-}$ NK cells are potent producers of cytokines, but have a weak ability to kill target cells, compared to classical $CD56^{+}/Cd16^{+}/CD3^{-}$ NK cells, which have strong cytotoxic activity. However, cytotoxicity and proliferation can be enhanced by IL-2 and IL-12 stimulation [176]. $CD56^{bright}$ cells can produce high amounts of TNF-α, IFN-γ, GM-CSF, IL-10 and IL-13, and are believed to have important functional roles in regulating

the immune response [177]. These NK cell subtypes comprise approximately 10% of the total NK cell count.

DENDRITIC CELLS

Dendritic cells (DC) represent a vital part of the immune system, critical in the induction of the innate immunity, in the regulation of the following adaptive immune response and in maintaining self-tolerance. Immature DCs act as sentinels of the immune system; they are constantly searching the blood, peripheral tissues, lymph nodes and secondary lymphoid organs for invading microorganisms. DCs are the most potent antigen-presenting cells (APC). When encountering an inflammation or infection, resting DCs engulf and process antigens to display them in the form of MHC-peptide complexes. The DCs then undergo maturation, leading to cells which are less efficient in antigen uptake but with a high cytokine secretion and expression of lymphocyte co-stimulatory molecules on the cell surface. Mature DCs are critical for activating and directing subsequent innate and adaptive immune responses [178]. After maturation, DCs migrate to draining lymph nodes where they prime following immune responses.

DCs are further divided into different subsets on the basis of phenotypic markers and functional properties and these DC subsets induce different subsequent immune responses; myeloid DCs (MDCs) and plasmacytoid DCs (PDCs).

MYELOID DENDRITIC CELLS

MDCs can be further divided into Langerhans cells residing in epidermis, interstitial DCs and circulating DC, that all have the capacity to engulf pathogens and display antigens on their surface and thereby they trigger subsequent immune reactions. They are thought to differentiate from myeloid precursor cells. MDCs express the myeloid marker CD11b and toll-like receptors TLR3 and TLR4 that mediate DC activation upon stimulation with LPS and poly(I:C) [179]. MDCs are important in regulating the adaptive immune response, by having the unique capacity to prime naïve CD4+ T cells [180] and skewing the T helper (Th) response towards Th1 or Th2 depending on cytokine secretion [181]. MDCs capable of secreting high amounts of IL-12, IL-18 and RANTES direct the following T cell response towards a Th1 profile. MDCs with a weaker ability to secrete IL-12, but instead secreting IL-6 and IL-10 skew the response into a Th2 phenotype (see section T cells below). MDCs are also important in stimulating differentiation of naïve B cells into Ig-secreting plasma cells by secretion of IL-12 and IL-6 in response to IL-2.

PLASMACYTOID DENDRITIC CELLS

PDCs represent 0.2% - 0.8% of peripheral blood mononuclear cells and have a plasma cell like morphology. The origin of PDCs is unclear, but they are believed to either be derived from a myeloid or lymphoid progenitor [182]. In contrast to MDCs, PDCs lack myeloid markers such as CD11c and CD33 as well as T cell receptors, CD3 chains or B cell markers, but do express the cell surface molecules CD4, BDCA 2 and 4, CD123 and high levels of MHC class II molecules. PDCs are activated upon stimulation of

TLR7 and TLR9 by nucleic acids and CpG DNA. PDCs are believed to be an important part of the innate immune response to in particular viruses by having the capacity to secrete large amounts of cytokines. IFN-α is secreted at early stages of infection and is thought to be vital in initiating an effective anti-viral response by stimulating NK cells and macrophages [183]. In addition to IFN-α, PDCs have the ability to rapidly secrete the pro-inflammatory cytokines TNF-α and IL-6 upon viral infection. TNF-α can drive the maturation process of PDCs into APCs, while IL-6 together with IFN-α induce B cell differentiation into Ig-producing plasma cell [184, 185]

After antigen recognition, PDCs mature and differentiate; they upregulate the expression of costimulatory molecules, MHC class I and MHC class II molecules on the surface, while the capacity to produce INF-α is reduced. PDCs are thought to modulate the NK cell response by presenting antigens as well as releasing cytokines and chemokines (see section on PDC – NK interaction below). Like MDCs, mature PDCs present antigens, and are able to induce expansion of T cell populations, although less efficient than MDC. Depending on maturation state, concentration of the antigen and other circumstances, PDCs can prime different T cell responses, preferable inducing a Th1 differentiation [186]. Mature PDCs present antigens like others APC and can trigger subsequent T cell responses by providing co-stimulatory signals. PDCs are also believed to prime naïve $CD4^+$ T cells and produce IL-12.

Furthermore, PDCs have been shown to be important in the development of humoral immune responses to virus infections by affecting the production of virus specific antibodies and polyclonal Ig secretion. PDCs activated though CpG DNA stimulation activate B cells to differentiate into plasma cells secreting Ig, after B cells stimulation through B cell receptor (BCR). This was independent of T cells. Also, in response to virus infections, PDCs can amplify the adaptive immunity of other antigen-presenting cells such as B cells, MDCs and monocytes by the release of IFN-α, TNF-α and/or IL-6 [184, 187].

The importance of PDCs in immune responses to tumors is debated. As described, PDCs have the ability to regulate the following immune response by activating important anti-tumor immune cells, such as NK cells and T cells. Also, PDCs have a unique ability to secrete high amounts of the pro-inflammatory cytokine INF-α upon stimulation. In mice, PDCs have been shown to be able to induce an antigen-specific CD8+ T cell response and were able to protect mice from tumor challenge [188]. This was also shown in humans, after immunization with a combination of mature PDCs and MDCs, but not with PDCs alone [189], suggesting that therapeutic vaccination with activated PDCs may have limited results on reducing tumor growth. Still, PDCs may be used to enhance the anti-tumor response.

HCMV EVASION OF DC RESPONSES

HCMV is known to interfere with multiple cellular functions of MDCs, mechanisms of great advantage for the virus due to the important role of MDCs in regulating proper immune activation and regulation. Previously presented data from our group shows that HCMV may block the cytokine-activated differentiation of monocytes into functionally active MDCs *in vitro*, and instead induced cells with greatly depressed immunological

functions [190]. They had a reduced endocytosis and a reduced migrating capability. In a similar manner, HCMV also inhibit macrophage migration, phagocytosis and differentiation from monocytes [191]. Although HCMV infected MDCs had an increased expression of the costimulatory molecule CD86 compared to uninfected cells, they were unable to induce a proper T cell response [190]. To subvert recognition by antigen by DCs and other APCs, HCMV infection of MDCs lead to a decrease of both MHC class I and II surface expression, CD40 and CD80 costimulatory molecule expression, as well as a hampered cytokine release, that are all important mechanisms in activating a strong B cell and T cell response [192, 193].

Many studies have implied that PDCs are activated during viral infections. PDCs express CD4, CXCR4 and CCR5, target receptors for human immunodeficient virus (HIV)-1, and are thus highly susceptible to infection by HIV-1, and can transfer virus to antigen-specific $CD4^+$ T cells [194, 195]. *In vivo*, HIV-1 is able to alter and impair the PDC phenotype, IFN-α release and reduce the total number of PDCs [196, 197]. These alterations are correlated to a fall in $CD4^+$ T cell count and the occurrence of opportunistic infections and Kaposi's sarcoma. As with a HIV-1 infection, PDC numbers and capacity to produce IFN-α is greatly reduced upon an acute hepatit C virus (HCV) which is thought to contribute to viral persistence and chronic liver disease [198]. The same clinical findings are observed in patients suffering from chronic hepatitis B infection [199].

We have shown that an HCMV infection of PDCs lead to their partial maturation with an intact ability to produce IFN-α together with TNF-α, IL-6 and IL-10, but with reduced capabilities to properly stimulate and activate T cells and B cells **(paper I)**. PDCs treated with soluble factors released from HCMV infected cells, trigger B cell activation and proliferation in the presence of B cell receptor stimulation, in a T cell independent manner. Co-culture of B cells, anti-Ig, PDCs and HCMV lead to B cell activation as shown by upregulation of CD86 and the release of IL-6 and IL-8. This activation of B cells was mediated through the infection of PDCs since HCMV treated B cells in the absence of PDCs did not induce CD86. Activation of B cells through the infection of PDCs seems to be HCMV specific, since influenza infected PDCs did result in an upregulation of CD86 expression. In contrast, HCMV infected PDCs did not induce plasma cell differentiation, nor antibody production. This mechanism may contribute to an unspecific B cell activation and enhanced humoral response. This is often observed in patients with active HCMV infection, who may suffer from hypergammaglobulinemia, cryoglobulinemia and autoantibody production [200-203]. In opposite, in **paper I** we describe that HCMV infection of PDCs hampers their T cell stimulatory ability, and HCMV infection instead leads to a decreased proliferation of both $CD4^+$ and $CD8^+$ T cells. This may be a result from an insufficient expression of costimulatory molecules on HCMV infected PDCs. These data are in contrast to previous data on PDCs infected with HSV, HIV or influenza virus, where virus infected PDCs were able to expand naïve T cells [186, 204, 205], suggesting that the hampering effect on T cells is HCMV specific. As mentioned, T cells are important players in controlling the HCMV infection and this downregulation of T cell proliferation may enhance viral spread.

NK CELLS AND DENDRITIC CELLS

In recent years, several publications have focused on the improrta bi-directional cross-talk between DCs and NK cells both in humans and in mice. Depending of the maturation state and NK:DC ratio, activated NK cells are able to either induce DC lysis or DC maturation. A high NK:DC ratio will lead to killing of autologous DC derived *in vitro* from monocytes (MoDC), particularly immature MoDC. Mature MoDC seem to be protected against NK cell lysis, and this is thought to be mediated by upregulation of MHC class I molecules on the surface of mature DCs and by engagement of the NK cell surface receptor NKp30 [206-208]. Although lysis of DCs by activated NK cells have been shown *in vitro,* the importance of this function *in vivo* is still not fully understood. Killing of DCs have been shown to result in a hampered anti-tumor response, but NK cell mediated lysis of DCs have also been shown to be important in preventing graft-versus-host disease following bone marrow transplantation [209, 210]. With low NK:DC ratios, NK cells can induce MoDC maturation and IL-12 secretion, a process dependent on both cytokines such as TNF-α and IFN-γ, and on cell-cell contact, again with engagement of NKp30 [179, 211]. This could aid in enhancing DC responses when DCs are not fully activated by infections or tumors.

Both MoDCs and MDCs have been shown to stimulate NK cell functions, such as NK cell cytotoxicity, CD69 expression and cytokine secretion, both via cell-cell contact and cytokine secretion [179]. PDCs activated via TLR9 are also able to activate autologous NK cells. The increase in CD69 expression and cytotoxicity seem to be dependent on secretion of soluble factors, mostly TNF-α but to a minor extent also IFN-α, working in an additive fashion. In addition, IL-2 secretion by activated MDCs have also been shown to enhance NK cell IFN-γ production [212]. NK cells cultured with activated PDC secreted only low amounts of IFN-γ and no IL-10 [50, 52, 53]. The interaction between NK cells and DCs are believed to take place mainly in lymph nodes [213]. PDCs produce chemokines, e.g: CCL2, CCL3, CCL5, CXCL10 and IL-8 that are likely to recruit NK cells in the periphery to sites of inflammation [214]. *In vivo* this may contribute to an effective anti-viral and anti-tumor response.

HCMV AND PDC-NK CELL CROSS-TALK

It is likely that the cross-talk between these cells has a large impact on the host defense against invading pathogens and greatly influences and steer the following adaptive immune response. Because of this and since HCMV has several mechanisms to avoid the immune system, we hypothesized that HCMV could influence the interaction of these cells in order to escape recognition and elimination. In **paper II**, we show that a HCMV infection of PDCs prior to coculture with NK cells *in vitro* lead to NK cells less able to kill target cells. Despite of an increased CD69 surface expression and induced migration, these NK cells had a significantly reduced cytotoxicity when incubated with NK cell sensitive target cells K562. This decrease in killing ability was seen in parallel with a reduction of intracellular perforin levels. These data implicate that HCMV have the ability to interfere with PDC activation of NK cells, rendering NK cells less able to kill target cells, and possibly with a reduced anti-viral function. One could speculate that this might have a great impact on the immune response in patients with an active HCMV infection. NK cells are as mentioned vital in the host response against viral

infections and tumors, and PDCs can strongly enhance their function by both cell-cell contact and cytokine release. Thus, patients that are co-infected with HCMV and an additional virus, or HCMV positive cancer patients, may suffer from a decreased anti-viral immune response due to the hampering effects of HCMV on PDCs and NK cells. This could contribute to a worsening of symptoms and a poorer outcome.

NEUTROPHILS

Neutrophils are one of the first immune cell types arriving at the site of an inflammation and are therefore important in the first line of defense against bacterial and fungal infections. They are attracted to inflammatory sites by complement components and cytokines released from other activated immune cells, mainly T helper cells and macrophages. Neutrophils are phagocytosing cells and engulf encountered pathogens coated with either complement or Ig particles, much in the same way as macrophages does. Phagocytosed microorganisms are then eliminated by fusion with intracellular vesicles containing lytic antibacterial proteins or oxygen and nitrogen radicals [110]. Neutrophils are also capable of secreting inflammatory cytokines such as IL-1, IL-6, IL-8, TNF-α, TGF-β and GM-CSF. Since these cells have a high potential of releasing toxic substances, it is important that cell homeostasis is tightly regulated to minimize the risk of tissue damage. Therefore, neutrophils have a short life span in peripheral blood, 6-9 hours, after which they undergo apoptosis and are engulfed by macrophages, thereby limiting the leakage of toxic compounds [215]. However, lytic enzymes and oxygen radicals released by neutrophils have been shown to be involved in tissue destruction in different chronic inflammatory disorders such as rheumatoid arthritis, asthma and inflammatory bowel disease [216].

Although neutrophils don't harbor latent HCMV, the virus has been shown to infect neutrophils early during a primary or reactivating infection. This makes neutrophils a suitable target to use for HCMV diagnosis. The commonly used HCMV antigenemia test is based on detection of HCMV early protein pp65 in neutrophils using immunocytochemistry, which correlate well with HCMV disease [217]. HCMV infection of neutrophils may lead to clinical complications in patients with an active HCMV infection due to HCMV ability to affect the function of these cells. Our group has previously described that HCMV infected neutrophils have upregulated effector functions and are less sensitive to apoptosis. In individuals with an active HCMV infection, this might lead to long-lived neutrophils that have an enhanced ability to secrete their toxic content into surrounding tissues. HCMV is believed to be of importance in several inflammatory disorders such as IBD, a disease characterized by infiltration of inflammatory cells and tissue damage [218]. Through the infection of neutrophils, HCMV may enhance the damaging effect of neutrophils by rendering over-reactive, long-lived cells which could remain in tissues for prolonged time periods. This may contribute to worsening of the disease progression in inflammatory disorders such as IBD.

Also, an increase of circulating neutrophils has been reported in patients with GBM, and an increase of neutrophil infiltration in the margins of the tumor is thought to correlate to GBM malignancy [219]. Upon degranulation, neutrophils release the tissue disrupting enzyme elastase, and the secretion of this enzyme from neutrophils is

believed to contribute to GBM progression and aid tumor invasiveness [220]. Since HCMV infection of neutrophils has been shown to increase cell survival, we were interested to investigate the correlation between circulating neutrophils, HCMV infection and the progression of GBM. We characterized neutrophil activation in GBM patients included in a randomized double-blind study aiming to evaluate the effect of Valganciclovir, a commonly used anti-viral drug, as additional therapy for these patients. In **paper IV**, we found that the activation of circulating neutrophils is correlated to the progression of GBM. We analyzed neutrophil with regard to their activation status at start of anti-tumor treatment and at 3, 12 and 24 weeks after diagnosis and treatment onset. Patients with a high neutrophil activity early after diagnosis (before 12 weeks and at 24 weeks) had a significantly shorter time to tumor progression (TTP) compared to patients with a decreasing or unchanged neutrophil activation. This suggests that the activation of circulating neutrophils may contribute to a worsening of the disease in these patients, and could possibly be used as markers for disease progression. However, when dividing patients depending on whether they received anti-HCMV treatment or placebo, we found no correlation between HCMV treatment and neutrophil activity. On the other hand, patients with HCMV treatment had a significantly decrease in MCP-1 serum concentrations. MCP-1 is a pro-inflammatory cytokine important in enhancing neutrophil tissue infiltration as well as recruiting monocytes, T cells and DCs to the site of infection. Our data suggest that HCMV induces systemic MCP-1 secretion which may enhance neutrophil infiltration. Since neutrophil tumor infiltration in patients with GBM is associated with a worsening of disease, HCMV may contribute to the tumor progression in these patients.

THE ADAPTIVE IMMUNE SYSTEM

The adaptive, or acquired, immune system is mainly constituted by T and B cells, and cytokines and antibodies secreted from those cells. The adaptive immune system is, in contrast to the innate immunity, characterized by a large repertoire of very specific antigen receptors which gives a pathogen-specific response. The response takes some time to develop, and the peak response is usually acquired several days post infection. After infection, microbial antigens are presented together with costimulatory molecules on the surface of APCs, which home to the lymph nodes after antigen uptake. There, T and B cells specific for this particular antigen recognize the presented molecule and become activated which will cause them to develop and expand. In this way the adaptive immune response is directed to this precise pathogen and the efficiency of the immune response improves over time. T and B cells are also influenced by cytokines secreted earlier in the immune response to the infection by cells of the innate immunity. Depending on the kind of cytokines secreted and what type of infection, the adaptive immunity will develop in different directions, either to a Th1 or a Th2 response. A Th1 response mainly involves cell-mediated immune responses, with activated macrophages and cytotoxic $CD8^+$ T cells and an increased secretion of pro-inflammatory cytokines IFN-γ and TNF-α. Th2 on the other hand activates a humoral immune response with B cells that proliferate and differentiate into plasma cells and secrete neutralizing antibodies. Main cytokines produced are IL-4, -5, -6, -10 and -13. After the infection is cleared the adaptive immune system returns to its basal state.

T CELLS

T cells can be divided into two groups depending on their function and their surface markers, T helper (Th) cells and cytotoxic T cells. Cytotoxic T cells are $CD8^+$ and primarily MHC class I restricted, with the main function to kill infected cells and tumor cells. The effector functions of $CD8^+$ T cells are the same as those of NK cells; they secrete cytotoxic granules containing lytic proteins such as perforin and granzymes. Th cells are $CD4^+$ and reacts mainly to MHC class II presentation of antigens by secreting cytokines stimulating other cells in the immune response. They help increase macrophage phagocytosis and differentiate B cells into antibody-producing plasma cells [221].

The type and function of the T cell response is entirely dependent on interactions between naïve T cells and other cells of the immune system, either by close contact or by secreted immunostimulatory molecules such as cytokines. Pathogen antigens bound to either MHC class I or MHC class II molecules presented on the surface of infected cells or APCs will bind to the antigen-specific T cell receptor (TCR) which initiates a cascade of intracellular signals leading to clonal expansion and differentiation of naïve T cells into effector T cells [222]. The interaction between the TCR and the MHC bound peptide is not enough for T cell activation, but other molecules distinct from the antigen recognition, such as adhesion and co-stimulatory molecules are essential to mount a proper T cell response. CD28 expressed on T cells deliver the second signal required for cell activation when binding to B7 molecules (CD80 or CD86) on APCs. Also, the binding between CD40L on T cells and CD40 on APCs contributes to efficient T cell activation [223]. The lack of proper co-stimulatory signals can lead to T cell anergy, where the T cell is unable to respond to MHC signaling. The type of T cell response is also dependent on the array of cytokines produced by other immune cells, mainly APCs but also NK cells. Immature Th cells can mature to either Th1 cells or Th2 cells. Cytokines such as IL-12 and IFN-γ will induce a Th1 response, leading to Th cells secreting IL-2, IFN-γ and TNF-α in their turn. This cytokine burst from Th1 cells will lead to stimulation of a cell mediated immune response with activated $CD8^+$ T cells, NK cells and macrophages. On the other hand, IL-4 will cause naïve Th cells to develop into Th2 cells that will trigger a humoral immune response by their secretion of IL-4, IL-5 and IL-10. B cells are stimulated to proliferate and differentiate into antibody-producing plasma cells [224, 225].

The initial frequency of T cells specific for a particular antigen is quite low. After activation the specific T cell clone is expanded in order to increase the number of cells capable to react with this specific antigen and thereby mounting an adequate immune response. T cells have the ability of memory, and some T cells will develop into memory T cells; long-lived cells able of a rapid and enhanced immune response to antigen previously encountered [226]. Memory T cells are mainly distinguished by the expression of surface markers CD45RA and CD45RO [227].

HCMV AND T CELLS

The cellular immunity against HCMV is believed to play a central role in controlling the infection. During the acute infection, there is a large increase in HCMV specific T

cells, in particular $CD8^+$ T cells about one week post infection. T cells become activated when recognizing virus antigens bound to MHC class I molecules together with stimulation from cytokines produced from other activated immune cells. T cells have been shown to be directed against a broad range of viral epitopes, but the tegument protein pp65 and the major IE protein IE1 are believed to immunodominant [228, 229]. These T cells become cytotoxic, killing infected cells, and start producing large quantities of cytokines, such as IFN-γ and TNF-α. As mentioned above, TNF-α induce the HCMV IE promoter activity and thereby enhance virus replication, and TNF-α together with IFN-γ are important in the reactivation and replication of HCMV. Thus, in contrast to having anti-viral functions, activation of T cells in response to infection may further enhance viral replication and spread. When viral load is reduced, the T cell pool returns to basal state. As mentioned above, we investigated the effect of HCMV infection of PDCs on T cell activation (**paper I**). Viruses such as HSV, HIV and influenza are described to activate T cells through infection of PDCs [186, 204], but on the contrary, we found that HCMV infection of PDCs rather hamper their ability to act as APC and stimulate T cells. Both $CD4^+$ and $CD8^+$ T cells proliferation was significantly reduced. Also, an enhanced TNF-α production from HCMV *in vitro* have been reported to increase release of arachidonic acids and prostaglandin E_2 (PGE_2) causing inhibition of T cell activity [230]. We describe an increased TNF-α secretion in coculture with HCMV infected PDCs and NK cells, which may be another way for HCMV to increase PGE_2 release and T cell suppression (**paper II**). Since a proper T cell response is an important part of the host anti-HCMV response, inhibition of T cells may be of great benefit for the virus in order to evade recognition and elimination by the immune system. This might also contribute to the general immunosuppression often seen in individuals with an active HCMV infection with an increased risk of suffering from other co-infections.

HCMV has been described to down-regulate the expression of MHC class I and II on infected cells, in order to escape immune recognition of CD8+ and CD4+ T cells. HCMV has multiple virally encoded proteins that interfere with antigen presentation. At least five different HCMV proteins interfere with antigen presentation on the cell surface in form of MHC class I complexes, important in activating $CD8^+$ CTL elimination of infected cells. gpUS2 and gpUS11 accelerate MHC class I degradation in the cytoplasm, gpUS3 and gpUS10 hamper MHC class I transport from the endoplasmatic reticulum (ER) to the cell surface and gpUS6 inhibit binding of antigen peptides to the MHC class I molecule [231, 232]. Also, HCMV interferes with MHC class II surface expression, important in activating $CD4^+$ T cells which have a vital role in activating and directing other immune cells. HCMV gpUS2 and gpUS3, in addition to having an effect on MCH class I expression, have been shown to decrease MHC class II surface expression and HCMV protein pp65 mediate intracellular degradation of HLA-DR molecules [116, 117]. HCMV inhibit the IFN-γ induced MHC class II surface expression by disrupting the Jak/Stat pathway thereby reducing Jak-1 levels and STAT 2 protein levels, although the mechanisms behind this are still unclear [233]. Also, the HCMV encoded IL-10 homologue mentioned above have the ability to inhibit both MHC class I and II expression, in addition to reducing DC activity and cytokine secretion [92, 93, 118]. In addition to downregulating MHC molecules, HCMV inhibit T cell proliferation by inducing soluble CD83 from infected MDCs [234].

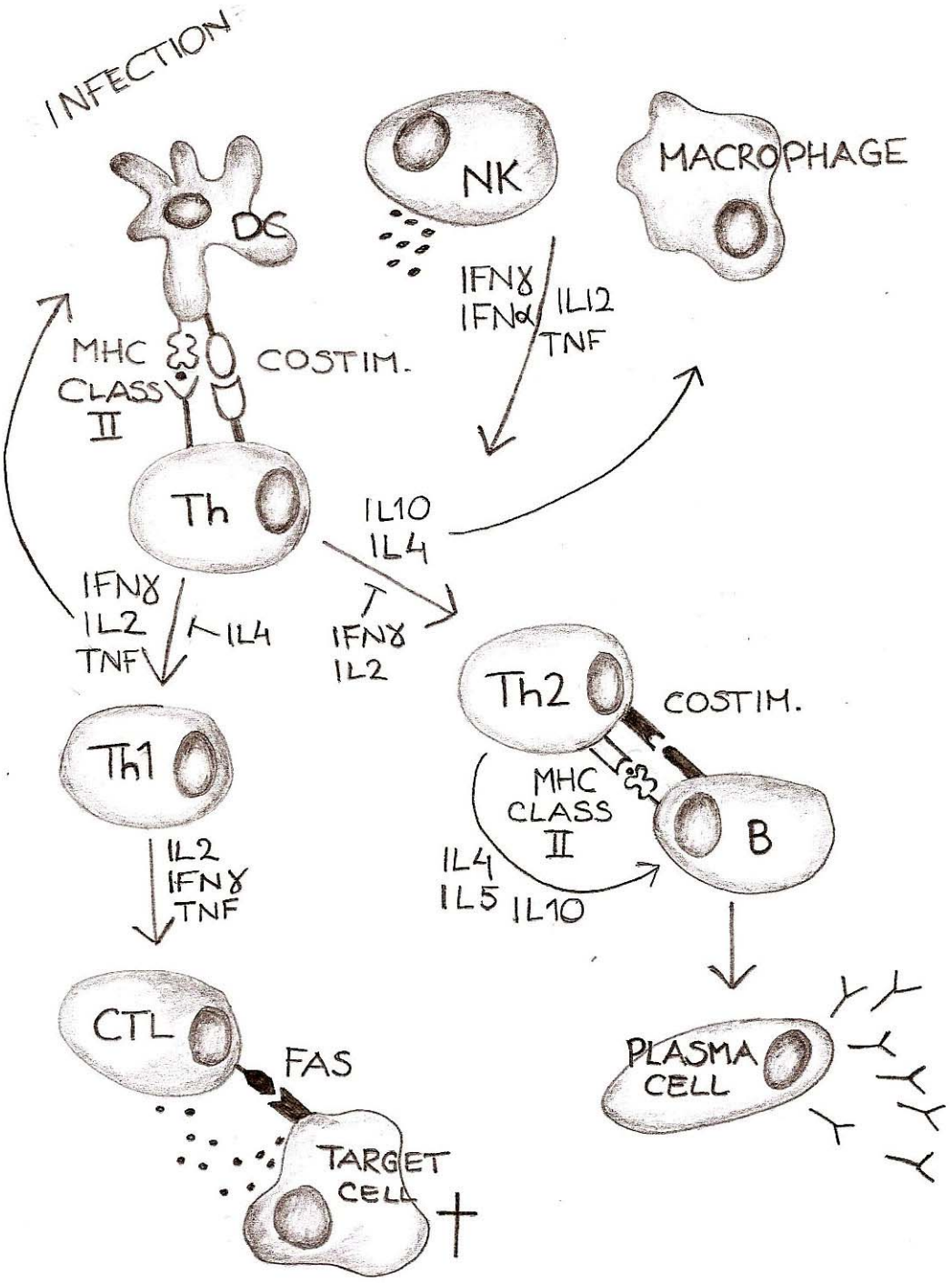

Figure 4. A simplified illustration of the immune response to an infection.

As in humans, T cells are vital in the mouse immune response against MCMV infection and three genes are described that aim to hamper a proper MHC class I expression and the presentation of antigens [76]. gp40, mentioned above to be important in MCMV NK cell evasion, is also believed to inhibit MHC class I expression by binding to and inhibiting its transport to the cell surface [235]. gp48, encoded by the MCMV m06 gene, also inhibit MCH class I surface expression but by retaining the molecule in the ER and target it for degradation in lysosomes [236]. The m04 gene encodes for gp34 which has been described to bind to MHC class I molecules in the ER [237]. This

complex is expressed together on the cell surface and gp34 is thereby believed to inhibit T cell recognition. However, this is debated, recent data suggest that gp34 rather enhances MHC class I expression and antigen presentation [238].

B CELLS

The humoral immune response is comprised of B cells and antibodies produced when these cells are activated and differentiated into effector cells, or plasma cells. Naïve B cells express antibodies bound to the cell surface, which are part of the B cell receptor (BCR). These surface-bound antibodies allow the B cell to detect and bnd to pathogens with a very high affinity leading to B cell activation. B cells then differentiate into effector cells and start to produce and secrete soluble antibodies, and it is only these soluble antibodies that mediate the effect. Binding of antibodies specific to certain pathogens and toxins serve to neutralize them by sterical hindrance, which prevents their interaction with other cells. Also, antibody covered particles can be recognized by phagocytosing cells, such as macrophages and neutrophils which targets them for elimination by these cells. In addition, phagocytosis of antigen coated particles, together with enhancement of humoral responses and lysis of the microbe, can also activate the complement. Furthermore, NK cells have the ability to recognize antibodies and this trigger their effector functions and ADCC.

The initial antibody response is the secretion of IgM and IgD, but in response to cytokines and CD40L stimulation from T cells, plasma cells may switch to produce IgG, IgA and IgE. The IgM response is quite rapid and is mainly aimed to activate complement, while IgG is important in ADCC. IgE is secreted in response to parasites and also mediate allergic responses. Cytokines produced during a Th2 response will drive B cells into producing mainly IgE and IgG1 while a Th1 response induce IgG2 production. Antigens bound to the BCR can also be internalized and presented on the B cell surface in the form of MHC class II complexes, which activate other immune cells. Antigen binding also induces upregulation of B7 co-simulatory molecules, important in the activation of T cells [239].

As with T cells, B cells also have the ability to form memory cells after activation, characterized by the expression of CD27 [240]. These cells are very long-lived and vouch for a rapid, specific response when encountering the same antigen again. Upon exposure to a pathogen, they rapidly proliferate and differentiate into antibody-producing plasma cells.

HCMV EVASION OF HUMORAL RESPONSES

The humoral immune response against HCMV is thought to be important, although it does not offer a complete protection against infection. HCMV specific antibodies cannot neutralize the virus, thus neither eliminate the virus nor protect against reinfections. Rather it reduces the severity of HCMV mediated morbidity and might also limit the generalized spread of the virus within the infected host. In humans, antibodies specific for at least 15 different HCMV epitopes, both from envelope and tengument proteins, have been detected. These antibodies are mainly of IgM, IgA and IgG type [74, 241]. To counteract this, HCMV encodes two Fc receptor homologues;

UL119-118 and TRL11/IRL11, which mediate binding of unspecific antibodies to the infected cell in order to hide viral antigens displayed on the cell surface as well as inhibit complement binding and activation. [242, 243]. In addition, HCMV induce host-cell expression of CD35, CD46 and CD55 regulating complement activation on infected cells. CD35 and CD46 mediate cleavage of C3b and C4b thereby inhibiting opsonization by phagocytosing cells and CD55 protect against complement-mediated cell lysis [244, 245].

HCMV activated PDCs secrete cytokines, such as IL-6 and IFN-α, that are important in the activation of B cells [184]. As mentioned, **in paper I** we investigated the ability of HCMV infected PDCs to activate B cells. After incubation with soluble factors released from infected PDCs, B cells showed signs of activation, and together with BCR stimulation also proliferated and differentiated to plasma cells although no antibody production was detected. But when adding T cells to cocultures with BCR stimulated B cells and HCMV infected PDCs, there was a significant increase in antibody production. Thus, HCMV seems to have the ability, through infection of PDCs, to activate B cells and to stimulate their proliferation, differentiation and production of antibodies. HCMV has previously been described to induce non-specific B cell activation and antibody production, which may contribute to the hypergammaglobulinemia often seen in HCMV viremic individuals [200, 201, 203, 246]. The HCMV mediated activation of B cells described here might further explain the mechanism behind these serological abnormalities.

HCMV INHIBITS APOPTOSIS TO AVOID THE IMMUNE SYSTEM

Induction of apoptosis is a very common and important mechanism used to lyse virally infected cells as part of the host's anti-viral defense, but also to eliminate tumor cells. Infection of a cell can both trigger 'intrinsic' host cell apoptosis as well as make the host cell susceptible for 'extrinsic' triggered apoptosis by activated cytotoxic effector cells of the immune system. Therefore many viruses have developed mechanisms to block cell apoptosis. HCMV has multiple proteins that are involved in inhibiting cell apoptosis. HCMV proteins IE1 and IE2 have each been described to block apoptosis of infected fibroblast cells induced by TNF-α [247]. The mechanisms behind this are mainly undefined, but IE2 was described to bind to p53 and inhibit its activation, thereby protecting against p53 mediated apoptosis [248]. The HCMV gene UL36 encodes for another cell-death suppressor called vICA, which bind to the inactive form of caspase-8 and thereby prevent its activation and initiation of apoptosis. It is thought to be important mainly in avoiding elimination by Fas-FasL binding and clearance by cytotoxic $CD8^+$ T cells and NK cells [249]. HCMV UL37 encodes for the viral protein vMIA that, like vICA inhibits Fas-mediated apoptosis in fibroblasts but acts downstream to caspase-8. vMIA is localized to the mitochondria and act by altering the fusion process of mitochondrial networks believed to induce apoptosis [250, 251]. vICA is thought to be dispensable for virus growth *in vitro*, but in opposite vMIA is required for efficient virus replication [249, 252]. In addition, HCMV UL144 encodes a structural homologue to the herpesvirus mediator, a member of the TNF receptor superfamily, and is believed to be able to block apoptosis in a yet undefined way [253].

Resistance to apoptosis is a mechanism commonly employed by cancer cells in order to enhance tumor survival and growth. HCMV has been suggested to protect neutroblastoma tumor cells against cell apoptosis [254] and increasing number of reports describe the presence of HCMV in tumor cells. This implies that the anti-apoptosis mechanisms induced by HCMV may be beneficial for tumor cells, however the clinical relevance of HCMV in tumor development needs to be further described. The correlation between HCMV and cancer is further discussed below.

CLINICAL ASPECTS OF HCMV INFECTION

HCMV INFECTION IN THE IMMUNOCOMPETENT HOST

HCMV infection in individuals with a functional immune system is usually subclinical, but can sometimes result in disease symptoms, and accounts for about 8% of all mononucleosis cases [255]. An active HCMV infection may cause symptoms for weeks, including fatigue, rash, fever, myalgia, headache, cervical lymphadenopathy and splenomegaly. More unusual symptoms are pneumonia, myocarditis, anemia, retinitis, gastrointestinal ulceration, hepatitis, peripheral neuropathy and Guillain-Barré syndrome [256]. Since an acute HCMV infection often is associated with a sustained immunosuppression, these individuals also have an increased risk of suffering from reactivation of other latent infections. After infection, the virus may persist up to a year in blood and patients with clinical HCMV symptoms are at risk of transferring HCMV to other persons compared to HCMV infected with subclinical symptoms [257].

HCMV INFECTION IN THE IMMUNOCOMPROMISED HOST

Although HCMV seldom give rise to clinical symptoms in an immunocompetent individual, HCMV is a common cause of morbidity in immunocompromized individuals and may be caused by a primary infection, reactivation of a latent infection or a reinfection [256]. Patients at risk of severe HCMV disease are AIDS patients, patients receiving solid organ or bone marrow transplants, cancer patients undergoing chemotherapy and fetuses and newborns with a congenital infection.

HCMV AND OPPORTUNISTIC INFECTIONS

Opportunistic infections, with HCMV being one of the most important, have long been a cause of severe disease in patients with HIV infection, although highly active anti retroviral treatment (HAART) has dramatically decreased the incidence of opportunistic diseases [258, 259]. Before HAART, about 40% of HIV infected patients who had developed AIDS suffered from HCMV related manifestations, but the numbers have now decreased significantly [260-263]. Although the risk of HCMV related disease is decreased with HAART, during the initial months of treatment before $CD4^+$ T cell count is reconstituted the high risk of HCMV manifestations is still high, which also points to the importance of HAART treatment in avoiding HCMV disease

[264, 265]. Also, a majority of HIV infected individuals in developing countries do not have the possibility to get HAART treatment. In this patient group, retinitis is the most common HCMV mediated manifestation. Proper HIV treatment in the developed countries has reduced the occurrence of retinal disease, but in developing countries HCMV retinitis is still the most common cause of visual loss [259]. The cause of HCMV related disease in HIV patients is thought to be the decreased cellular immunity, in particular the low $CD4^+$ T cell count but also a low $CD8^+$ T cell count, often seen in HIV patients [266]. Although HAART has significantly increased quality of life for HIV infected individuals, HCMV and other latent infections have been shown to cause inflammatory complications, collectively called 'immune reconstitution inflammatory syndrome' due to a regenerating immune system [267].

HCMV IN TRANSPLANT PATIENTS

HCMV infection in transplant patients used to be a major problem and a common cause of HCMV disease symptoms such as fever, malaise, myalgia and headache. End-organ disease, primarily affecting the transplanted organ but is also seen in other organs, affected 10-30% of patients with a substantial risk of mortality [268]. The risk is highest for HCMV seronegative recipients receiving a HCMV positive transplant [269]. The use of anti-viral prophylactic treatment of all transplant patients has led to a dramatic decline in HCMV disease during immunosuppressive treatment following transplantation [270, 271]. Beside prophylactic treatment, pre-emptive therapy is often used, where patients are treated with anti-virals when viremia is detected, as in opposite to standard care where anti-HCMV treatment is used for treating clinically symptoms of HCMV. Pre-emptive treatment is believed to reduce drug toxicity and enhance the efficiency of treatment in patients at risk of HCMV disease. This method relies on a rapid and sensitive HCMV viremia detection in order to predict HCMV disease [272]. However, the risk of late-onset HCMV disease has recently been described, and is thought to be a considerable risk of morbidity and mortality after prophylactic treatment [273, 274]. HCMV disease is much more difficult to treat in stem cell transplantations (SCT) although a lot of progress has been done using pre-emptive treatment with anti-virals the last years [275]. HCMV associated pneumonia and gastrointestinal involvement are still causes of mortality among BMT recipients but other clinical manifestations are common such as HCMV-associated graft failure, hepatitis, retinitis, high fever and encephalitis [276]. As with solid-organ transplantations, the serological status of both donor and recipient are important for the outcome of the transplantation [277]. Pre-emptive treatment and donor matching is believed to reduce the risk of HCMV-associated disease and death [275].

CONGENITAL HCMV INFECTIONS

HCMV is the most common cause of congenital infections and is known to be the reason of many different diseases in the fetus and the newborn. Approximately 1% of all newborns are congenitally infected with HCMV, and can be a result from both a primary or a recurrent infection in the mother. However the rate of infection of the fetus is much higher if the mother has a primary infection [278]. Infection of the fetus can occur during all three trimesters. The risk of a more severe outcome is considered larger if the fetus is infected during the first half of pregnancy, but if HCMV is

transferred to the fetus during the third trimester the risk of hearing loss is the highest [279]. A primary infection during the first trimester is associated with a higher risk of abortion and fetal loss than women with a recurrent infection or infection at later time points [280]. Around 5-10% of infected newborns show symptoms at birth but up to 15% asymptomatic children may develop HCMV associated manifestations later in life. In addition to an intrauterine infection route, HCMV may also be transmitted from mother to child at birth or during breast-feeding. At one year of age 30-40% of all children are HCMV positive and children with a congenital HCMV infection may secrete virus in urine for up to 14 years of age. Symptoms in newborns include smallness, microcephaly, sensorineural hearing loss and other neurological abnormalities, intracranial calcifications, heptaomegaly, splenomegaly, anemia, jaundice and trombocytopenia [279]. Children with pure somatic symptoms are less likely to develop intellectual and neurodevelopmental disabilities at later age. The mechanisms behind the neuronal disorders are not fully understood, but may involve a hampered differentiation of progenitor cells into neurons and astrocytes [281, 282]. Treatment of symptomatic newborns with anti-virals is possible and may decrease the risk of hearing loss and neurological symptoms, but is often not given as a routine treatment to infants with HCMV, although it is becoming more common. Since the majority of HCMV infections are asymptomatic, preventive treatment during pregnancy is difficult, and the development of a HCMV vaccine is thought to have the greatest impact on congenital HCMV disease.

HCMV AND AUTOIMMUNITY

Autoimmunity is characterized by a sustained immune response towards self antigens. Immune cells react against non-foreign antigens and since these cannot be eliminated, the immune response leads to a chronic inflammation and injury to tissues. It is believed that autoimmune diseases arise both from genetic factors, mainly HLA genotype, and environmental and life style aspects like smoking and infections. Viruses are thought to play a significant role, since they may induce autoimmunity through several mechanisms; upregulation of key molecules on APCs, molecular mimicry, presentation of cross-reactive epitopes and the induction of auto-antibodies [283, 284]. HCMV has been linked to different autoimmune diseases, possibly through induction of anti-CD13 autoantibodies [285, 286], although several other HCMV associated auto-antibodies have been detected; anti-nuclear antibodies, anti-SMC antibodies and anti-EC antibodies. The self-antigen CD13 was shown to become associated with the HCMV particle during virus maturation and become immunogenic during active infection [20, 203]. CD13 can be found on several cell types, like ECs, SMCs, granulocytes and macrophages.

Active HCMV infection and the presence of CD13 specific auto-antibodies has been linked to the chronic inflammation seen in patients with ulcerative colitis and Crohn's disease [6, 286]. HCMV is thought to contribute to the ongoing inflammatory process and the induction of anti-CD13 autoantibodies which may mediate cytotoxic effects when binding to epithelial cells, SMCs and ECs in the intestine. The systemic autoimmune disease SLE involves multiple organs throughout the body, including skin, kidneys and the central nervous system. It is a disease characterized by autoreactive T and B cells, high titers of autoaintbodies against DNA and nucleosomes. A correlation

between HCMV infection and the disease course of SLE have been made. Anti-HCMV antibodies have been shown to be significantly higher in SLE patients, also clinical symptoms and disease severity was increased in HCMV-SLE patients, where all patients included had an active infection [7, 287]. SLE is believed to be driven by altered DC function and high IFN-α secretion [288]. HCMV has several known mechanisms by which it alters the function of different DC subtypes (see section on HCMV immune evasion). In **paper I** and **paper II** we show that an infection of PDCs with HCMV result in a large IFN-α secretion, which could partly explain the correlation between an active HCMV infection and worsening of SLE symptoms. In addition, the cytotoxic abilities of NK cells have been shown to be greatly reduced in SLE patients, possibly due to a reduction of perforin and granzymes, and this is thought to contribute to the dysregulation of the immune system seen in these patients [289]. The effect of HCMV in NK cells described in **paper II** and **paper III**, along with previously described NK cells inhibitor actions by HCMV (see section on HCMV immune evasion), might describe additional mechanisms behind these hampered NK cells in HCMV positive SLE patients.

HCMV AS AN ONCOMODULATORY VIRUS

Several different viruses, such as human papilloma virus, hepatitis B and C virus, EBV and SV40, are thought to be involved in the development of different tumors. Due to the many mechanisms employed by HCMV to alter cell fuctions, it is believed to function as an oncomodulatory virus, meaning that the virus has the ability to catalyze the oncogenic process, although the clinical importance of HCMV is debated. HCMV is known to influence cell apoptosis, differentiation, proliferation and DNA repair mechanisms, all which may contribute to tumor formation and growth [14]. Also, HCMV immune evasion strategies and the induction of immunosuppression might help infected tumor cells to avoid recognition and elimination by the immune system. Several studies have described a high presence of HCMV in tumor cells of different malignancies, such as colon cancer [11], prostate cancer [12], cervical cancer [290] and glioblastoma multiformae [13]. Still, the role of HCMV in the development of cancer needs to be further clarified.

As mentioned above, the HCMV expressed proteins vICA and vMIA, encoded by UL36 and UL37, are known to suppress cell death in infected cells by inhibiting caspase activation by Fas engagement and by inhibiting mitochondrial-mediated apoptosis, respectively. These mechanisms might be important in avoiding immune clearance by cytotoxic $CD8^+$ T cells and NK cells, cells vital in the elimination of tumor cells. If HCMV is able to protect infected tumor cells against the cytotoxic mechanisms employed by these cells, it would be of great advantage for tumor growth. We report that HCMV infected PDCs hamper their stimulatory ability, with a decreased proliferation and of both $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells, which might describe yet another mechanism by which HCMV avoids T cell mediated clearance (**paper I)**. We also describe additional mechanisms by which HCMV avoid elimination by NK cells (**paper II** and **paper III)**. HCMV infection of PDCs reduce the cytotoxic abilities of NK cells, generating cells possibly less able to perform their key function of killing virally infected cells and tumor cells. In addition, there is a direct effect of HCMV on the function of NK cells. NK cells did not become infected by HCMV.

Rather, soluble protein(s) produced by infected cells, virally or host cell encoded, had the ability to reduce the cytotoxic ability of NK cells, accompanied by a reduction of cell surface expression of the NK cell activating receptor NKG2D. In addition, we show that supernatants from HCMV positive, but not HCMV negative, GBM cells in culture have the same ability to downregulate NK cell cytotoxicity. Although the brain is an immunologically privileged site with little infiltration of immune cells, with a growing brain tumor the protective blood-brain barrier is weakened and cells from the blood can infiltrate the brain. Still the ability of NK cells to reach the site of a brain tumor needs to be further studied. Previous studies have shown that glioblastoma cells are less sensitive to NK cells by a strong overexpression of HLA-E, a ligand for the NK cell inhibitory receptor CD94/NKG2A [291]. Blocking this binding enabled a NKG2D-mediated NK cell killing of the tumor cells. NK cells have a very important role in the control and elimination of tumor cells, these mechanisms described in **paper II** and **III** might further explain the contributing role of HCMV in tumor immune evasion and growth.

For a tumor to be able to grow it needs to develop new blood vessels, which is referred to as angiogenesis. HCMV has the ability to downregulate inhibitors of angiogenesis, such as trombopondin-1 and -2, in gliblastoma cells, thus promoting the formation of new blood vessels and supporting tumor growth [14]. In addition, several studies have shown induction of TNF-α by HCMV, a cytokine known for its abilities to stimulate angiogenetic factors. HCMV has also been reported to increase migration and adhesion of SMCs, neuroblastoma cells and neuronal cells in mice [15]. Increased cell motility as well as increased adhesion to endothelium are important mechanisms in the development of metastases. The increased adhesion and disruption of endothelial monolayer integrity was thought to be mediated by an upregulation of β1α5 integrin and proteases by the virus. This might describe mechanisms by which HCMV is able to increase tumor invasiveness. Recently, our group reported that HCMV infection leads to the activation of telomerase by expression of telomerase reverse transcriptase (hTERT), in both normal human fibroblasts and malignant glioblastoma cell lines [292]. This could describe an important mechanism for HCMV in cell immortalization and tumor progression.

CONCLUSIONS

The work included in my thesis has focused on further characterizing the effects of HCMV on the immune system. Evading the host immune response to the infection is crucial for the virus' to survive, replicate and establish latency, and several HCMV immune evasion mechanisms have already been described in the literature. Beside enhancing the virus continued existence and spread, these strong effects on immune cells have additional consequences for the host. HCMV viremic individuals may suffer from severe morbidity including additional opportunistic infections, other inflammatory conditions and cancer.

In paper I we show the effect of HCMV on PDCs, with consequences on subsequent T and B cell activation. PDCs are important immune cells acting as sentinels of the blood that initiate and direct both the innate and the adaptive immune response, so that a proper anti-viral response is mounted upon infection. Affecting these cells would be of great benefit for the virus in its effort to evade elimination of the immune system. HCMV infection of PDCs results in their partial activation. Although a minority of cells becomes infected, these cells have a hampered ability to stimulate T cell proliferation. Thus, by infecting PDCs, HCMV obstructs the function of two cell types central in the host immune response to a viral infection and this may contribute to the overall immunosuppression often seen in individuals with an active HCMV infection. In contrast, HCMV infected cells were able to stimulate B cell activation and proliferation, a mechanism possibly leading to the abnormal humoral response in HCMV viremic persons.

Lately, the cross-talk between PDCs and NK cells has received a lot of attention. Both cell types are vital in the anti-viral immune response. NK cells are rapid responders to an infection, and are also important in eliminating tumor cells. Paper II describes the effect of HCMV on PDC activation of NK cells. We show that HCMV infected PDCs induce the production of TNF-α and IFN-γ, strong anti-viral cytokines inhibiting both viral replication directly as well as activating and directing the surrounding immune cells. Interestingly, in the case of HCMV, TNF-α and IFN-γ have contradictory effects. They have been shown to induce HCMV reactivation and replication, thus HCMV would greatly profit from this increased secretion. However, NK cells activated by HCMV infected PDCs had a reduced ability to kill target cells. This hampering of such a vital anti-viral mechanism may significantly increase the virus ability to escape elimination by the immune system. In addition, as NK cells are important players in the host anti-tumor response, one might speculate that this can have consequences for tumor cell survival enhancing tumor immune evasion.

NK cells do not become infected by HCMV themselves. However, in paper III, soluble factor(s) released by HCMV infected cells are able to hamper NK cell cytotoxicity. Interestingly, this was also observed when treating NK cells with supernatant from HCMV positive glioblastoma cells. GBM tumors are highly malignant and patients diagnosed with this tumor have a very short life expectancy, despite aggressive treatment. The immune system in general and NK cells in particular are vital in controlling the growth of cancer transformed cells. Since these patients often suffer from a general systemic immunosuppression, one may hypothesize that this hampered

immune function contributes to the malignancy of this tumor. By inhibiting cytotoxicity, the primary anti-tumor cell function of NK cells, HCMV may contribute to this immunosuppression and poor immune clearance of tumor cells.

In paper IV, we characterize the cytokine pattern and neutrophil activation status in patients included in a study aiming to evaluate the effect of anti-viral therapy in parallel to anti-tumor treatment. We found that the activation of peripheral neutrophils correlates to the progression of tumors in these patients, where activated neutrophils were associated to a shorter time to tumor progression. Peripheral neutrophils have previously been shown be increased in patients with GBM, and this correlation between neutrophil activation and time to tumor progression suggests that following the activation of these cells in patients may be useful in monitoring the disease progression. In addition, we describe that these patients have altered expression patterns of important pro- and anti-inflammatory cytokines. Several of these have previously been shown to be affected by a HCMV infection. This skewed cytokine secretion may contribute to the immune abnormalities often seen in these patients, and it is possible that it adds to the reduced ability of the immune system to fight these tumors.

In summary, I believe that HCMV have strong affects on the host's immune system with consequences both for healthy individuals and for patients suffering from either HCMV associated complications or other diseases.

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