GENERAL INTRODUCTION AND SCOPE OF THE THESIS
INTRODUCTION

The family Herpesviridae

Human cytomegalovirus (HCMV) is the fifth member of the family of human herpesviruses. This family is composed of eight members and includes herpes simplex virus 1 and 2 (HSV-1, 2), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), CMV, and the human herpesviruses 6, 7 and 8 (HHV-6, 7, 8) [1,2]. The typical herpesvirus architecture comprises a core containing linear double-stranded DNA, within an icosahedral symmetric capsid. This capsid is surrounded by proteinaceous tegument. In turn, the viral envelope with spiked glycoprotein clusters surrounds the tegument and capsid. This is called a virion. Four significant biologic properties are shared within the herpes family; namely 1) the viruses carry an extensive array of enzymes necessary for their replication. 2) Viral synthesis and capsid assembly takes place in the nucleus of the host cell. 3) Production of infectious viral particles results in destruction of the host cell. 4) After initial infection the virus remains lifelong in the host in a latent state. During latency the virus has a closed circular genome and expresses only a few proteins [1].

Human cytomegalovirus:

Genome

Within the herpes family, CMV belongs to the betaherpes viruses. These are characterized by salivary gland tropism, slow growth kinetics and high species specificity [3]. A whole array of hosts, such as humans but also other primates, domestic animals and rodents can be infected by a species specific CMV [4].

Of all known viruses, HCMV has one of the largest genomes. It is about 235 kilobase pairs (kbp) long and codes for more than 200 putative open reading frames (ORFs) [5]. The linear HCMV genome is composed of a unique long domain (UL) and a unique short (US) domain. Both ends of these domains are flanked by a repeat sequence, an internal and terminal long repeat sequence and an internal and terminal short repeat sequence, IR_L, TR_L and IR_S, TR_S respectively. The UL domain and US domain can be arranged head to tail or head to head and in the order UL-US or US-UL. Thus, four different isomers of the HCMV genome can be formed [6,7].

As with many viruses, small parts of DNA, not directly necessary for replication in vitro can be lost after serial propagation [8]. For example, the laboratory CMV strain AD169 as well as the low-passage clinical isolate Toledo had lost a part of the CMV genome compared to clinical isolates; AD169 misses a region of about 15 kbp representing at least 19 genes and Toledo misses a region of about 13 kbp [9]. Both regions are located in the same part of the viral genome.

Structure of the virus

CMV infection and replication in permissive cells is caused by the virions (Fig. 1B). CMV inoculum, for instance of the laboratory strain AD169 produced in fibroblasts, contains two other types of particles having the capability to enter the cell [10]. These are the non-
infectious enveloped particles (NIEP) and dense bodies (DB) [11,12]. NIEP are enveloped capsids with a translucent core that lack viral DNA and thus have the same size as a capsid (Fig. 1B). DB have no nucleocapsid at all and consist of a viral envelope filled with the massively packed phosphoprotein pp65 (Fig. 1A) [13]. The size of DB vary considerably and can become very large. Sometimes, DB are seen in the cytoplasm of infected cells by light microscopy. NIEP and DB are presumably products of defective viral replication and, together with complete virions, they can be released from progressively infected cells [11]. The fraction of NIEP in the virus inoculum can accumulate to 60%, and is mainly composed of DB (50% of all particles) [12]. Infected cells in the host produce NIEP and DB together with the virion as well, although the ratio between the different particles is unknown [14].

Figure 1
Electron microscopy graph of a CMV infected endothelial cell. Fig. 1B represents a close up of the indicated region in Fig. 1A. Dense bodies (Fig. 1A) and virions (Fig. 1B) are indicated by arrows. A NIEP is shown by an arrow head (Fig. 1B)

Major structural proteins
The complete virion contains a limited set of proteins out of its total genomic expression potential. The inner element of the virion, the nucleocapsid, expresses four viral proteins: major capsid protein (MCP) (pUL86), minor capsid protein (pUL85), minor capsid binding protein (pUL46) and smaller capsid protein (pUL48/49). The major capsid protein is the main component of the pentamers and hexamers, which gives the capsid its icosahedral form.

The tegument, that surrounds the capsid, is constituted of about 20 proteins with pp65 (ppUL83) and pp150 (ppUL32) as major representatives.
Pp65, or the lower matrix protein, is one of the most extensively studied proteins. It is of special importance in the diagnostic setting. During active infection pp65 is detectable in a fraction of the nuclei of polymorphonuclear cells [15,16]. The development of the CMV-pp65 antigenemia method enabled the early and rapid diagnosis of CMV infection [17]. In vitro, pp65 is abundantly produced in infected fibroblasts. DB are almost totally composed of pp65 [18]. The expression level of pp65 in infected cells is lower if low-passage clinical strains are used [19]. Furthermore, infection by a low multiplicity of the virus results in less production of pp65 [13]. In infected cells, pp65 is the major phosphate acceptor [20,21]. The protein has kinase activity [22], and auto-phosphorylation capacity [22]. After viral entry pp65 is translocated to the nucleus immediately [15]. In the nucleus pp65 can bind to metaphase chromosomes, but pp65 has no DNA binding capacity [23]. During viral replication pp65 is located in specific nuclear compartments and a direct interaction with lamins is demonstrated [24]. However, pp65 is not required for viral replication in vitro [22], but in its absence replication is markedly reduced.

Pp150 or basic phosphoprotein is another phosphate acceptor in the tegument and composed about 20% of the virion mass. Acquisition of pp150 starts in the nucleus [25]. Experiments with antisense pp150 mRNA reveals an essential role for this protein in late stage virus maturation [26].

The outer element of the virion, the viral membrane, includes proteins essential for viral entry. Three major complexes of glycoproteins can be identified: glycoprotein complex I (gCI), gCII and gCIII.

The gCI is composed of glycoprotein B (gB), a glycoprotein early expressed in the replication cycle and existing as a heterodimer of glycoprotein (gp)115 and gp55, that remain complexed in the virion envelope. Up to 50% of the protein mass of the viral envelope is represented by gB. Prior to infection, gB is involved in both attachment to and fusion with the host cell membrane [27]. The gCII complex includes several different highly glycosylated proteins in the range of 47 kD to 200kD. One of these is glycoprotein M (gM), a 100 kD protein that may be responsible for the heparin-binding capacity of gCII [28]. The third complex gCIII is composed of a heterotrimeric complex of glycoprotein H-glycoprotein L-glycoprotein O [29,30]. Aside of gB, gCIII is most abundantly expressed in the viral membrane.

Penetration and replication

The entry of CMV into a target cell is described in a model for CMV entry in fibroblasts [31], which is based upon in vitro studies with cell free laboratory strains. Because the infectivity, the rate and kinetic of infection with CMV differ among various cell types, the entry route of other target cells such as endothelial cells may be different. Nevertheless, in fibroblasts, entry of CMV starts with a low affinity interaction of the viral components gC-II or gB [32,33] with cell surface heparan sulphate proteoglycans. This is rapidly followed by a phase of stable attachment, which may involve gB too, together with as yet unknown viral proteins. The next step is a direct pH-independent fusion of the viral membrane with the plasma membrane [34].
Besides gB [35], gHgLgO also has a role in fusion and penetration. A number of molecules on target cells are reported to involve in the process of virus entry. CD13 is candidate for the stable attachment phase [31,36] but is not expressed on all target cell types. A 30-34kD cell surface protein, annexin II [37,38] can bind gB [39] and a 92.5 kD protein on fibroblasts binds and is phosphorylated by gH [40-42]. Furthermore HLA class I molecules are reported to have a role in viral entry [43]. It seems likely that the virus utilizes different receptors on each of the cell types permissive for CMV.

After the virion has entered the cell, it is uncoated in the cytoplasm [10]. The capsid moves to the nucleus in a way comparable as has been described for HSV-1 [44,45] and delivers the viral DNA to the nucleus via the nuclear pores. The process of viral transcription and translation is strictly regulated, whereby a time-scheme of separate phases (with some overlap) can be identified. Replication starts with transcription of immediate early (IE) genes and IE proteins can be detected within 30 minutes after exposure to viral inoculum [46]. The immediate early phase lasts about four hours, followed by the early phase up to approximately 48 hours after infection. The IE proteins, together with cellular factors, transactivate a set of viral and host promoters resulting in transcription of the viral early genes. Both IE and early transcripts are necessary for late gene transcription. During the late stage of replication structural virus proteins are produced and particles are assembled. In vitro, the late phase ends after approximately 72 hours [6]. However, in the late phase, the immediate early proteins are maximally produced.

After initial infection with CMV (primary infection) active replication is started. In a normal healthy host this induces an immune response that serves to eliminate the virus. The interference of the immunological defense with viral replication and dissemination triggers the virus to enter a silent state without replication of the viral genome (viral latency). Viral replication is shut off and just a limited set of viral transcripts is produced [47]. It is still a matter of debate whether complete latency is induced or (also) low-grade viral replication (persistent infection) occurs. The life-long maintenance of antibody and T cell responses give evidence of at least periods of persistent infection in the host.

**Latency in the host**

Transplantation of a seropositive organ in a seronegative recipient almost always evokes a primary infection [48], thus the source of the transmitted virus has to be the transplanted organ or blood products that were given during the surgery. Studies in mice using PCR in situ hybridization demonstrate that endothelial cells of small vessels in multiple organs and tissues [49] are positive for viral DNA. In the lungs viral DNA positive cells are identified as macrophages, whereas in bone marrow the viral DNA positive cells are not identified [49]. These findings indicate that multiple tissues can serve as a site of viral latency. In humans many organs harbour CMV DNA [50,51]. The cell types containing the virus are often organ specific such as red pulp splenocytes, hepatocytes, tubular and glomerular cells [52]. Haematogenous cells also are a source of latent or persistent CMV. In peripheral blood of seropositive healthy individuals, monocytes but not lymphocytes or polymorphonuclear cells contain CMV DNA [53,54]. Allogeneic stimulation can induce viral replication in
monocytes of almost all seropositive donors [55]. In monocytes, CMV DNA is detected in a closed circular conformation, typical for the latent virus [56]. Reactivation of the virus is only possible when cells undergo differentiation to macrophages [57]. However, monocytes have a rapid turnover. To maintain latency, the monocytes have to obtain their virus from a resident pool of persistent and latent virus. In healthy individuals, in CD34+ bone marrow progenitors CMV DNA can be detected without IE-expression [58]. Furthermore, granulocyte-macrophage progenitors (CD33+CD34+) of healthy, CMV seropositive individuals contained latency-associated transcripts [59,60], supporting the hypothesis that in bone marrow especially the myelo-monocytic lineage is involved in viral latency [61].

Clinical aspects
CMV is widely distributed among the population. Almost 60% to 90% of the human population, depending on the population density and the social economic circumstances [62], have been infected with CMV. People can acquire the virus during several stages of human life. Already before birth, congenital infections may occur [62]. Babies can become infected by breast milk containing infectious virus from the mother. During childhood many children acquire CMV, in particularly when they are in day care centres. Furthermore, people in close contact with these children have a high risk as well. The virus can be acquired via sexual contact [62]. Another well-known cause for CMV infection is transplantation of an organ from a seropositive donor in a seronegative recipient.

Most healthy individuals remain asymptomatic after infection with CMV, but some of them will develop a mononucleosis-like syndrome. In contrast, congenital infection with CMV may cause severe birth defects. Immunocompromised patients are a risk group for CMV disease, such as the transplant recipients, AIDS patients or patients treated with chemotherapy. They may develop CMV disease with complications of high spiking fever, painful joints, malaise, elevated liver enzymes, thrombocytopenia and/or leukopenia [63]. A small number of immunocompromised patients develop more serious complications during infection. Multiple manifestation of gastrointestinal symptoms during CMV infection were reported as well as vasculitis and neurological complications. CMV pneumonia is a potentially lethal complication after bone marrow transplantation. AIDS patients frequently develop retinitis that can result in blindness. In AIDS patients as well as in the transplant recipients, almost all organs systems can be involved in CMV infection [64].

About 60% to 90% of the transplant recipients develop an active CMV infection, either primary or secondary. Primary infections, starting with virus that originates from the transplanted organ or from infected blood products, result in the most severe infections. Secondary infections are caused by reactivation of virus in the host itself or by superinfection of a different strain from the donor organ [65]. Despite immunosuppression, the patients with secondary infections have benefit of the pre-existing immune memory and are thus able to clear the infection more efficiently than the patients with a primary infection. The course and severity of CMV infection among the different types of organ transplantation vary considerably. This can be explained by different regimens of immunosuppression [66-68].
The broad range of symptoms that can be developed during CMV disease indicate a generalized infection whereby multiple organs are more or less affected. This was demonstrated for lungs and the gastrointestinal tract. Lungs (decrease in CO diffusion [69]) as well as the intestines (increased permeability to lactulose [70]) have a decrease in organ function during active CMV infection. Even in CMV patients without clinical symptomatology, subtle decreases in functioning of the lungs can be observed [69].

CMV and transplant rejection
In transplant recipients, CMV infection may directly cause complications during active infection. Indirectly, CMV infections are associated with development of acute or chronic transplant dysfunction. [71-74]. Potent immunosuppression to treat acute rejection, such as anti-thymocyte globulins, is accompanied by an increased risk for CMV infection. A second indication for the development of CMV infection after acute rejection comes from in vitro experiments. It is supposed that release of cytokines during acute rejection, especially TNFα, has a role in reactivation of CMV. In vitro studies show that binding of TNFα via activated NFκB stimulates the CMV IE promoter/enhancer region in reporter gene assays [75]. The NFκB can bind to the CMV IE promoter/enhancer region [76]. The same group showed a correlation between high plasma levels of TNFα and high CMV antigenemia values in transplant recipients [77].
The inversed order of CMV infection followed by acute rejection is associated with a higher risk to develop acute rejection has been reported as well [78]. Alongside with the development of an effective immune response against CMV, alloreactivity might be induced against the graft [79].
In the long run, chronic transplant dysfunction is a serious threat for the transplanted graft. CMV infection is one of the risk factors for chronic transplant dysfunction that might enforce its development [80,81]. The endothelium is considered to have a central role in the development of chronic transplant dysfunction, therefore it will be discussed afterwards.

CMV and endothelium
The endothelium constitutes the inner lining of the blood vessels and thus it is distributed throughout the body. Endothelial cells form the interface between tissues and the blood stream. Infection of endothelial cells gives CMV access to the bloodstream as well as to tissues. In vivo studies showed infected endothelial cells in the capillaries [62,82,83]. Moreover, the appearance of CMV infected cytomegalic endothelial cells (CEC) in peripheral blood of patients demonstrates vascular involvement. This is also related to organ involvement [84,85]. In vitro experiments with CMV infected endothelial cells demonstrate several effects of CMV on the function of endothelial cells. Particularly, the role of endothelial cells in inflammatory reactions is negatively affected by CMV infection. Besides the immediate effects of CMV infection on endothelial cells, a relation is suggested between CMV infection and the development of chronic transplant dysfunction and atherosclerosis [80,86].
**Introduction**

**Infection of endothelial cells in vivo**

In specimen derived from organs such as brain, lung, gastrointestinal tract and heart, infected endothelial cells are observed in capillary vessels or venules [83,87-89]. Doublestaining with tissue specific markers and CMV specific markers show infected endothelial cells early in the replication stage with a ‘normal’ morphology as well as late stage infected cells with the cytomegalic appearance. In some samples, the cytomegalic cells seem to detach from the basal membrane [83].

More evidence of infected endothelial cells comes from peripheral blood samples of patients with active CMV infection. During severe infection, cytomegalic endothelial cells (CEC) are detected in peripheral blood [85,90]. Although in general the occurrence of CEC is low [84,85], higher frequencies are observed in patients with a stronger regimen of immunosuppression [84,85,91,92]. The CEC have a diameter of 35-45 µm, a condensed nucleus with nuclear inclusion bodies and morphologically they show the characteristic owl’s eye appearance. Furthermore, CMV proteins of all replication stages can be detected; thus the CEC are late-stage infected [84,85].

The extraordinary large size of these CEC as compared to uninfected EC is remarkable. The diameters of small capillaries in organs is about 5-10 µm. Therefore, it is postulated that CEC become trapped in capillary vessels and thus will hinder the blood flow. Plugging of the cytomegalic endothelial cells in capillary vessels is held responsible for the aforementioned CMV associated decrease in organ function of lungs or intestines [69,70]. As an alternative to plugging, CEC may become damaged or malformed. However, apart from these circumstances, CEC also can disseminate virus and induce new sites of infection. Until now, it is not known from which types of vessels the CEC originate. Histopathological studies have demonstrated that infected sites are mainly located in capillary vessels or venules of organs and tissues [83,87]. Moreover, CEC numbers are increased in venous blood obtained of the forearm after venous stasis and rubbing of the forearm. Obviously, these CEC originate from capillaries or venules from the same forearm [84].

**Table 1: CMV endothelial cell tropic strains**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Isolation on fibroblasts (no.passages)</th>
<th>Cell associated (C) or supernatant (S)</th>
<th>Max. % of infection</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>VHL-E</td>
<td>+</td>
<td>C</td>
<td>95</td>
<td>[100]</td>
</tr>
<tr>
<td>C3</td>
<td>+ (5)</td>
<td>S</td>
<td>30</td>
<td>[102,103]</td>
</tr>
<tr>
<td>C1FE, a.o.</td>
<td>+</td>
<td>S</td>
<td>60-100</td>
<td>[97,104]</td>
</tr>
<tr>
<td>TB40E, TB42E, a.o.</td>
<td></td>
<td>C</td>
<td>95</td>
<td>[101]</td>
</tr>
<tr>
<td>VR#6110/28E, a.o.</td>
<td>+ (3)</td>
<td>C</td>
<td>80</td>
<td>[105]</td>
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</tbody>
</table>
Infection of endothelial cells in vitro

In contrast to fibroblasts, *in vitro* cultured endothelial cells are not easily infected with the ubiquitously used laboratory strains [93-95]. Infection of human umbilical vein endothelial cells (HUVEC) with cell-free AD169 maximally results in 2%-10% infection [95,96]. A number of strategies are employed to improve infection of endothelial cells with CMV labstrains, like treatment with sodium butyrate [96,97], electroporation [98] or protein kinase C activation [99]. These methods can increase the percentage of infection to 30%. One report mentions 80% infection [97].

Waldman et al. published a paper that described passaging of a clinical CMV isolate separately in either fibroblasts or endothelial cells. After 20 passages in fibroblasts, HUVEC are infected with a very low efficiency, whereas the infectivity of fibroblasts is not altered. In contrast, 20 passages of the virus in HUVEC results in similar infection kinetics of HUVEC whereas fibroblasts are infected with slightly lower rates [100]. Obviously, after multiple passages in fibroblasts the virus is altered in its behaviour to infect different host cells.

Five groups have described one or more clinical isolates that have an improved infectability for endothelial cells (Table 1). Even between different clinical isolates considerable differences exist in the capability to infect endothelial cells [97,101], thus indicating that CMV strains have a different tropism for certain cell types. Moreover, this is also reflected in infection conditions. Some of the virus strains efficiently infect endothelial cells via cell-cell contact whereas other strains infect endothelial cells as free virus (Table 1).

**Endothelial cell tropism**

The factors that determine endothelial cell tropism are probably due to differences at the level of viral entry, transport to the nucleus or further on during viral replication. The reports of two groups conflict concerning the putative entry mechanism of CMV in HUVEC: namely endocytosis and fusion with the cell membrane [106](C. Sinzger, the 7th Cytomegalovirus workshop, 1999, Brighton). A study of AD169 entry into fibroblasts or HUVEC demonstrated that the viral genome can be detected in the cytoplasm of both cell types in similar frequencies. However, only in a small fraction of the HUVEC the viral genome is observed in the nucleus and several hours later compared to fibroblasts, which argued of a hampered transport of AD169 DNA to the nucleus of endothelial cells [107].

The differences in CMV tropism for endothelial cells are supposed be determined by the viral DNA. A study reports large-scale deletions of the viral genome in laboratory strains compared to recent clinical isolates [9].

Furthermore, the viral genomes of endotheliotropic and non-endotheliotropic strains are compared and regions that were different between both strains are identified. The involved proteins are mainly glycoproteins [108]; thus a role at the level of viral entry was suggested. The studies of infection of endothelial cells are *in vitro* experiments. Still, these results might be a better reflection of the *in vivo* situation than the high rates of infection obtained in fibroblasts. Histopathological studies on tissue specimen of patients with CMV infection...
show that infected fibroblasts are rarely detected, in contrast to epithelial cells, endothelial cells, parenchymal cells, smooth muscle cells or tissue specific cell types [87,109].

**Diversity of endothelial cells**

Although endothelial cells are distributed throughout the body, arterial derived endothelial cells have different characteristics than endothelial cells from high endothelial venules or capillaries. Most studies, including the majority of experiments with CMV infected endothelial cells are performed with HUVEC, an fetal large vein derived cell. Comparative studies have shown that they are a good representative of adult venous and capillary endothelial cells [110-112]. Nevertheless, CMV infection of arterial, microvascular or venous endothelial cells may reveal differences with respect to infection with CMV.

**Table 2: Endothelial effector functions altered by CMV infection**

<table>
<thead>
<tr>
<th>Function</th>
<th>Ref.</th>
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<tbody>
<tr>
<td>MHC class I</td>
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<tr>
<td>MHC class II</td>
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<tr>
<td>Cytokines</td>
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<tr>
<td>IL-1α,</td>
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<tr>
<td>IL-1β</td>
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<td>IL-6</td>
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<td>IFN-β</td>
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<tr>
<td>Cytokines</td>
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<tr>
<td>IL-8</td>
<td></td>
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<tr>
<td>GRO-α</td>
<td></td>
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<tr>
<td>Growth factors</td>
<td></td>
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<tr>
<td>G-CSF</td>
<td></td>
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<tr>
<td>GM-CSF</td>
<td></td>
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<tr>
<td>PDGF and bFGF</td>
<td></td>
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<tr>
<td>Adhesion molecules</td>
<td></td>
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<tr>
<td>ICAM-1</td>
<td></td>
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<tr>
<td>VCAM-1</td>
<td></td>
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<tr>
<td>E-selectin</td>
<td></td>
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<tr>
<td>ICAM-2</td>
<td></td>
</tr>
<tr>
<td>Viral Fc-γ receptor</td>
<td></td>
</tr>
<tr>
<td>Coagulation</td>
<td></td>
</tr>
<tr>
<td>Plasminogen activator inhibitor-1 (PAI-1) +</td>
<td>167</td>
</tr>
<tr>
<td>Procoagulant activity</td>
<td></td>
</tr>
</tbody>
</table>

+ upregulated, 0 not affected, n.i. not inducable, - downregulated
Two studies indicate a higher susceptibility of brain microvascular endothelial for labstrains (infection percentage of 50%) compared to HUVEC [113,114]. CMV infection of arterial cells has produced more conflicting results. In contrast to infected HUVEC or microvascular cells, the infection is not lytic for arterial cells [115]. Two other groups were not able to reproduce these findings and reported similar infection percentages of arterial, microvascular or venous endothelial cells [116] (M. Kahl, the 7th Cytomegalovirus workshop, 1999, Brighton). The variation in infectivity of the different endothelial cell types can reveal more about the viral pathogenesis in the body, especially sites that are at risk to develop CMV mediated vascular damage.

**Endothelial function after CMV infection**

Endothelium has an essential role in many physiological processes. It forms the barrier between blood and tissue, thus the transport of molecules in and out of tissues is very important. It has a key function in wound healing and angiogenesis, regulation of the influx of inflammatory cells and the regulation of (anti)coagulation. Morphologically, endothelium is composed of a thin closed layer of cells with apical a smooth surface to prevent spontaneous coagulation of blood. Infection with CMV, especially lytic infection, interferes with normal endothelial function. Already after one day of infection the infected cells enlarge and round up. This is observed in vitro as well as in vivo in rats. The infected cells progress to cytomegaly and finally detach from the basal membrane [83,95,117]. In vitro studies have shown that these morphological changes are accompanied by alterations in the expression of integrins on infected EC [103,118], the attachment sites to the extracellular matrix. Furthermore, the production of matrix molecules, like fibronectin, is reduced [119], together with an increased release of matrix degrading collagenases [114]. Recently, a study in fibroblasts has shown that the synthesis of a series of molecules involved in cell shape and attachment is inhibited during CMV infection [120].

Besides effects of CMV infection on individual cells, in vitro experiments reveals that CMV infection affects endothelial interactions with the inflammatory response as well. Table 2 shows a summary of events that are known to be modulated by CMV infection of endothelial cells. Early in infection, CMV infection of endothelial cells results in a reduced production of the cytokines IL-1α and IL-1β [102], but increased production of IL-6 [121]. Another event early in infection is the upregulation of adhesion molecules at the cell surface [122-126], which in turn causes an increased adherence of polymorphonuclear cells and monocytes [127]. Transmigration across CMV infected endothelial cells of polymorphonuclear cells, monocytes or T cells is enhanced as well [128,129]. A higher production of the chemokines IL-8 and GRO-α will attract increasing numbers of inflammatory cells [129], in vivo this may amplify the immune response. Intriguingly, the virus itself also encodes chemokines and chemokine receptors that interfere with this process. CMV produces an α-chemokine, that attracts PMN in particularly [130]. The intimate contact between monocytes or polymorphonuclear cells and endothelial cells during adhesion or transmigration is one of the routes that CMV may use for viral dissemination (Fig. 2). The virus can be transmitted from CMV infected endothelial cells to monocytes.
Expression of chemokine receptors may serve as an evasion strategy. The CMV open reading frames UL33 [132], US27 and US 28 [133,134] encode proteins homologous to the seven membrane span chemokine receptors. Of these, US28 is a functional CC chemokine receptor and can sequestrate extracellularly derived chemokines, such as RANTES, in the cytoplasm of infected cells [135,136]. RANTES is one of the CC chemokines, involved in attraction of cytotoxic T lymphocytes. Thus elimination of this molecule may hinder the immunological response against CMV (Fig. 2). Besides this, US 28 enhances cell-cell fusion [137], and binds membrane-bound fractalkine [138]. Maybe, US 28 has a role in viral entry as well.

The modulatory effects of CMV infection have both pro- and anti-inflammatory consequences. Sometimes, effects of CMV infection on endothelial cells are counteracted by a different mechanism of the EC. This is particularly illustrated in the activation of CMV specific cytotoxic T lymphocytes by infected endothelial cells. At first, immediate early proteins of CMV are responsible for the upregulation of ICAM-1 expression on the cell surface [123,139], and thus the adherence of T cells will be increased. Then, HLA class II expression on endothelial cells, normally induced by an inflammatory stimulus, is downregulated after infection with CMV [140]. For this, the virus directly interferes with the Jak/Stat pathway involved in the induction of MHC class II expression [141]. Despite the lack of adequate HLA class II expression, CMV infected endothelial cells are still able to induce an activating response in autologous T cells. Blocking antibodies against (upregulated) ICAM-1 cause an inhibition of T cell activation [142]. Apparently, the CMV-induced ICAM-1 is involved in the T cell activation independent of HLA class II and thus counteracts the effects of its own downmodulation of MHC class II.

Role of CMV in atherosclerosis

Apart of the acute effects caused by CMV infection, the virus is also associated with long term processes like atherosclerosis. A pathophysiological relationship of CMV has been described with transplant associated atherosclerosis (chronic transplant dysfunction) [80,143], atherosclerosis [86,144] or restenosis after angioplasty [145,146]. Although these three phenomena are induced in very different situations, the underlying process resulting in occlusion of vessels has much in common. The development of atherosclerosis is described by Ross in his response-to-injury model [147,148]. Basically, an initial and/or continuing insult to the endothelial surface (Ox-LDL, mechanical, immunological, viruses etc,) may result in the process of atherosclerosis. Several stages are identified, to begin with endothelial activation resulting in expression of adhesion molecules. This attracts monocytes and lymphocytes, which transmigrate into the subendothelial space. Here, these cells accumulate lipids (fatty streak) and produce growth factors. In response to these factors, smooth muscle cells start to proliferate into the intima and form a fibrous plaque that narrowed the lumen.
Figure 2
Schematic presentation of the sequence of events in CMV infected vascular endothelial cells. The adherent cells with grey nuclei represent CMV infected endothelial cells. During CMV infection inflammatory cells, such as T helper cells (Th), produce cytokines that induce activation of the endothelial cells (A). This results in upregulation of adhesion molecules and MHC class II expression. The activated cells start to secrete chemokines and become more thrombogenic (A). CMV infection of endothelial cells results in an enhanced release of chemokines as well (B), which can attract inflammatory cells, such as PMN and cytotoxic T cells (Tc). CMV specific cytotoxic T cells can recognize infected cells resulting in reactive oxygen species (ROS) that damage and lyse infected cells (C). CMV infection and damage of endothelial cells also results in the release of soluble factors (vWF, sVCAM-1) of vascular damage (D). Infection with CMV downmodulates the expression of MHC class I, MHC class II as well as of adhesion molecules. Furthermore the infected cells can sequestrate chemokines inside the cytoplasm (E). PMN can acquire CMV protein pp65 resulting in positive CMV antigenemia (F). The late stage CMV infected endothelial cells become cytomegalic and can release from the basement membrane (G).
Most studies to elucidate the role of CMV infection in the development of chronic transplant dysfunction are performed in rats infected with rat cytomegalovirus (RCMV) and transplanted with solid organs [149-152]. Conform the process described for atherosclerosis, end-stage chronic transplant dysfunction is histologically characterized by occluded vessels with extensive proliferation of smooth muscle cells into the intima. Large numbers of macrophages and activated T cells are enclosed in the intimal mass. In contrast to atherosclerosis, the time scale to chronic transplant dysfunction is much shorter. Furthermore, the insults to the endothelial surface are mainly caused by inflammatory reactions, therefore no extensive fat accumulation is observed. CMV infections influence many steps in the cascade to chronic transplant dysfunction. It induces endothelial activation with expression of adhesion molecules and MHC class II and influx of inflammatory cells. Furthermore, the production of growth factors and proliferation of smooth muscle cells is enhanced as well after infection with RCMV [152-155].

Several studies suggest a relation between CMV infection and atherosclerosis. In patients with atherosclerosis, higher levels of CMV specific antibody levels are observed that in healthy controls [156]. Furthermore, latent CMV DNA can be observed in atherosclerotic lesions [157]. In addition, epidemiological studies of healthy individuals show that higher titers of CMV antibodies are associated with a slight intimal-medial thickening. The higher levels of lipoproteins and fibrinogen with higher titers of CMV antibodies indicate weak procoagulant properties, which are in turn associated with an increased risk for atherosclerosis. [86,158].

CMV infection in relation to the development of restenosis has been studied in vivo as well in vitro. A study in patients that underwent coronary angioplasty reveals a striking role for CMV protein IE84. Smooth muscle cells of restenotic lesions have accumulated p53, a nuclear transcription factor that negatively regulates cell growth and tumour formation. IE84 interacts with the p53 resulting in proliferation of smooth muscle cells [159]. Other steps influenced by CMV infection are the expression of scavenger receptors, which bind oxidized lipids. The uptake of oxidized lipids is increased by CMV[160]. The intracellular lipid metabolism is associated with the production of free radicals and oxidative stress. Furthermore, oxidative stress has been described to have a stimulatory effect on the CMV IE promoter [161,162]. However, these experiments are all performed in vitro, the effects of local reactivation of CMV at sites of atherogenesis in vivo still have to be investigated. In vivo, other groups had difficulties to confirm the relation between CMV and restenosis [163-165].

In summary, many factors are involved in the development of chronic transplant dysfunction, atherosclerosis or restenosis. In some of these local CMV may interfere, however the extent and the significance of the contribution of CMV is unknown and awaits further investigation.
References


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Scope of the thesis
Grefte et al. were the first to describe the occurrence of cytomegalic endothelial cells (CEC) in peripheral blood of CMV patients. The occurrence of CEC in blood indicated the involvement of vascular endothelium during CMV infection. We hypothesized that infection of endothelial cells could have an important role in the pathophysiology of CMV infection. CMV infection of endothelial cells may cause cytopathogenic lesions in the vascular endothelium. Late stage infected endothelial cells may detach from the basal membrane and enter the blood stream. Because of their large size, these CEC may plug in capillary vessels and thus play a role in CMV mediated organ dysfunction. Moreover, because CEC are fully infected, they may disseminate the virus throughout the body.

In the present thesis we investigated the clinical significance of CEC in the pathophysiology of CMV infection. For this, the occurrence of CEC during CMV infection was studied in a cohort of kidney transplant recipients and related to CMV induced clinical symptoms, CMV related organ dysfunction as well as soluble parameters of endothelial damage. We employed a series of in vitro experiments to examine the role of CMV infected endothelial in viral dissemination and we studied the effects of CMV infection on endothelial cells.

Chapter 1 gives an overview of the literature related to our topic: the role of endothelium in CMV infection.
To test the hypothesis, the study was started with the development of a quantitative method to isolate CEC from blood samples of CMV patients. Cells were sorted onto adhesion slides, using fluorescent activated cell sorting (FACS) sorting of the mononuclear cell (MNC) fraction after staining with an EC-specific antibody. The sorted cells were stained with CMV specific specific markers allowing the identification of the cells as genuine CEC (Chapter 2). In a longitudinal study of kidney transplant recipients (Chapter 3) we studied the occurrence and concentration of CEC and endothelial cells in different stages of infection in the course of CMV infection. Beside CEC, which are late-stage CMV infected endothelial cells, we also observed uninfected endothelial cells in blood of patients. However, endothelial cells in intermediate stages of CMV infection were never found. The relationship of CEC and EC with the severity of infection and clinical symptoms was studied. The occurrence of CEC and EC in blood appeared to be strongly related to acute rejection episodes that happened before the CMV infection. In the same cohort of patients we have measured soluble parameters of endothelial involvement during CMV infection: von Willebrand Factor (vWF), soluble vascular cellular adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1) as well as soluble E-selectin (sE-sel) respectively. In parallel to the CEC study, we investigated the relationship of these parameters with the severity of infection, clinical symptoms, preceding acute rejection episodes and we studied the correlation of vWF and sVCAM-1 levels with occurrence of CEC (Chapter 4).

The role of CEC in CMV-mediated organ dysfunction was not only related to clinical symptoms, but also investigated by functional tests of patients with an active CMV infection. These patients have a decrease in their pulmonary diffusion capacity for CO. By
determination of components specific for the capillary flow (Vcap) or the diffusion across the membrane between alveolus and capillary (Dm) the contribution of CEC to the decrease in pulmonary could be assessed (Chapter 5.1). In a number of patients, the pulmonary diffusion capacity was determined and blood samples were analysed for the occurrence of CEC at the same day during the course of CMV infection. In Chapter 5.2 we report the relation between detectable CEC in blood, the severity of CMV antigenemia and the pulmonary diffusion capacity for CO with its components: Dm and Vcap.

Chapters 6 and 7 represent the in vitro experiments performed to study the interaction of CMV infected endothelial cells and polymorphonuclear cells. We describe the in vitro generation of pp65 positive granulocytes using human umbilical vein endothelial cells (HUVEC) infected with the endothelial-adapted CMV strain TB42E as a model for CMV antigenemia. In Chapter 6 the role of CMV specific antibodies in the generation of pp65 positive granulocytes was analyzed by using patient sera as well as purified IgG from these patient sera. A series of experiments was performed to provide insight in the route of the viral protein pp65 from entering the polymorphonuclear cell (PMN) to become detectable in the nucleus (Chapter 7). In the last chapter the increased expression of ecto-ATPase (CD39) and ecto-5’nucleotidase (CD73) is described, which are two enzymes with a key role in anticoagulation. Furthermore, CD73 has an anti-inflammatory effect by production of adenosine (Chapter 8).