Cytomegalovirus Infection in Immunocompetent and Renal Transplant Patients

Clinical Aspects and T-cell Specific Immunity

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Dissertation presented at Uppsala University to be publicly examined in Rudbecksalen, Rudbecklaboratoriet, Dag Hammarskjölds väg 20, Uppsala, Saturday, November 29, 2008 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in Swedish.

Abstract

Cytomegalovirus (CMV) is a β-herpesvirus that, after primary infection, establishes a life-long persistence in the human host. Up to 90% of humans are infected with CMV, that is kept under control by CMV-specific CD8+ and CD4+ T cells. In patients with an impaired cellular immunity, however, CMV infections can be life-threatening. Thus, it is vital to identify risk factors and target high-risk patients. In this thesis we have evaluated low-dose valacyclovir prophylaxis in renal transplant patients and studied CMV-specific T cell immunity in healthy and renal transplant patients.

In renal transplant patients, the CMV serostatus of both the recipient (R) and the donor (D) has a major impact on the risk of developing CMV disease. In the high-risk D+/R- population, >50% are likely to develop CMV disease in the absence of prophylaxis and/or pre-emptive therapy. We have used low-dose valacyclovir prophylaxis for high-risk renal transplant patients, and graft and patient survival up to 5 years after transplantation was comparable to data reported for other prophylactic protocols. The incidence of CMV disease and graft rejection during the first year after transplantation was also comparable to that achieved with other protocols, and without the adverse effects reported for other therapies.

In the D+/R+ population, with a 15-35% risk of developing CMV disease, it is important to identify those individuals that are subject to a higher risk because of risk factors other than CMV serostatus. We therefore measured several immunologic parameters in renal transplant patients and in immunocompetent individuals with latent and primary CMV infection. In patients with a primary symptomatic CMV infection, CMV-specific CD8+ T cells peaked within a month after onset of symptoms but declined rapidly. In renal transplant patients, we found that the reduction in IFNγ-producing CMV-specific CD4+ T cells at 2 months post-transplantation may predict high-grade CMV DNAemia.

Keywords: Cytomegalovirus, renal transplantation, cellular immunity, valacyclovir, mismatched, prophylaxis, tetramer, CD4 T cells, CD8 T cells

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ISSN 1651-6206
urn:nbn:se:uu:diva-9324 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-9324)
To my family
This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:


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Abbreviations

aa amino acids
ACV acyclovir
CD cluster of differentiation
CD4/Th T helper cell, Th
CD8 T killer cell, Tk
CFC cytokine flow cytometry
CMV cytomegalovirus
CTL cytotoxic lymphocytes
D+/− donor CMV serostatus
EBV Epstein-Barr virus
FITC fluorescein isothiocyanate
gB/H/M/N glycoprotein B/H/M/N
GCV ganciclovir
HHV human herpes virus
HIV human immunodeficiency virus
HLA human leukocyte antigen
HSV herpes simplex virus
IE immediate-early
IgG/M immunoglobulin G/M
IFN interferon
IFNγCD4/8 interferon-gamma-producing CD4/8 T cell
kbp kilobase pair
MHC major histocompatibility complex
NK natural killer cell
ORF open reading frame
pp phosphoproteins
PE phycoerythrin
PTLD post transplant lymphoproliferative disease
R+/− recipient CMV serostatus
SCT stem cell transplantation
SOT solid organ transplantation
TetraCD8 tetramer+CD8 T cell
UL unique long domain
VACV valacyclovir
VGCV valganciclovir
VZV varicella-zoster virus
INTRODUCTION

Cytomegalovirus (CMV) is a member of the human herpesvirus family that consists of 8 viruses: herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), CMV, human herpes virus 6 A and B (HHV-6A and B), human herpes virus 7 (HHV-7), and human herpes virus 8 (HHV-8). Herpesviruses can be further divided into subfamilies: α-herpesviruses (HSV-1, HSV-2 and VZV), β-herpesviruses (CMV, HHV-6A/B and HHV-7) and γ-herpesviruses (EBV and HHV-8). Herpesviruses are enveloped viruses with an icosahedral capsid that encloses a double-stranded DNA genome (Fig.1). CMV is the largest member of the human herpesvirus family, with a genome of 236 kbp and more than 200 open reading frames (ORFs) [1] encoding more than 80 viral proteins, including glycoproteins (e.g., gB), phosphoproteins (e.g., pp65), and other transcription/replication proteins.

![Figure 1. A schematic picture of the CMV structure](http://www.biografix.de/)


Figure 1. A schematic picture of the CMV structure
(Reproduced with the permission of Dr. Marko Reschke, http://www.biografix.de/)
Genome analysis has indicated that mammalian CMV have co-speciated with their respective hosts over the last 80 million years [2]. This prolonged period of co-evolution has resulted in a high level of co-adaptation between the virus and its host and, like other herpesviruses, CMV establishes latency after primary infection. CMV has been shown to persist in monocytes, bone marrow progenitor populations and endothelial cells [3-9]. During latency, CMV cannot be eliminated by host defenses but the immune system keeps the virus under close surveillance, giving it little chance to reactivate and cause symptomatic disease.

The latency and reactivation phases have developed through evolution and are maintained by a number of genes that allow the manipulation of different cellular processes involved in cell-cycle control. These processes ensure for example that cellular precursors could be used for viral replication. If the host’s immune system is impaired for any reason, CMV can reactivate and cause transient episodes of viremia that are generally asymptomatic in immunocompetent individuals [10]. In immunocompromised subjects, such as transplant recipients and HIV/AIDS patients, however, the consequences of a reactivation may be severe and even fatal.

**Epidemiology**

CMV is one of the most successful of human pathogens, since it can be transmitted both vertically and horizontally, usually with little effect on the host. CMV is shed in body fluids (e.g., saliva, urine, semen, breast milk). Transmission usually occurs from person to person, including through the intrauterine route, but it can also be spread during transfusion or organ transplantation. Globally, between 60 and 90% of the general population is infected with CMV [11], with generally higher rates in developing countries. The high seroprevalence rate in Sweden (70-80%) is believed to be related to the high rate of breastfeeding and the widespread use of daycare facilities for children [12]. CMV is usually acquired during early childhood, with 40% of all individuals being seropositive at 4 years of age [13]; the prevalence of infection increases with age after infancy and ongoing seroconversion is seen throughout life [14].
CMV replication

When CMV enters the human body, it begins to rapidly and effectively penetrate virtually all types of cells, including monocytes/macrophages, neutrophils, endothelial cells, epithelial cells, smooth muscle cells, fibroblasts, stromal cells, neurons and hepatocytes [9]. This penetration is accomplished by a pH-independent fusion in the case of most cells, such as fibroblasts, but in epithelial and endothelial cells it occurs through endocytosis and fusion at low-pH [15]. It is important, however, to note that only certain cells, such as fibroblasts and fully differentiated macrophages, allow a fully permissive replication cycle that results in the production of infectious virus. Other cells such as monocytes are non-permissive, meaning that the CMV infection is restricted to early events of gene expression and does not result in the production of complete virus. Reactivation of latent CMV in vivo is also restricted to certain cell types and has thus far been shown only in fully differentiated monocytes/macrophages [16]. After the virus penetrates the cell, it replicates and matures in the nucleus, and, as for other herpes-viruses, this process occurs in three relatively distinct phases:

Immediate-early (IE) phase
This phase begins with the transcription of the IE (alpha) genes during the first 4 hours after viral entry. The transcription event is dependent only on cellular factors and does not require de novo viral protein synthesis. Non-structural proteins appear in the nucleus within 4 hours after infection. These proteins are essential for the regulation of the expression of the early- and late-phase genes, and also for manipulating various cellular processes.

Early (E) phase
The transcription of the E (beta) genes is dependent on the expression of functional IE gene products. In this phase a variety of essential proteins involved in viral replication are produced, including DNA polymerase and helicase-primase.

Late (L) phase
The transcription of L (gamma) genes occurs approximately 24 hours after infection and includes structural and maturation proteins. This synthesis is highly dependent on viral DNA replication and can be blocked by inhibitors of viral DNA polymerase such as ganciclovir. The viral products are then assembled and packaged in the nucleus whereas the final maturation and budding of the virus take place in a Golgi-derived vacuole [17] from which the virus is released. CMV replication in vivo is a very dynamic process with
doubling times of 12-24 hours [18] and a maximum virus release at 72 to 96 hours post-infection. The replication continues for several days until cell lysis occurs.

Immune response to CMV infection

CMV infection triggers a forceful immune reaction in the human body, including both antibody- and T-cell-mediated responses. Because of its effective immune evasion strategies, CMV is able to establish latency despite the strong immune response by the host. The primary infection usually resolves without complications, leaving the immune system to maintain an effective balance and avoid reactivation of the virus.

B-cell immune responses

Primary CMV infection elicits a transient IgM response within 1-3 weeks that is followed by the development of persistent IgG antibodies. These antibodies play a minor role in clearing the infection but are believed to play an important role in reducing the severity of CMV disease in adults and protecting the fetus from congenital infection. The antibodies are directed against at least 15 different proteins; the most immunogenic is pp150, against which nearly 100% of the CMV-seropositive individuals have antibodies [19]. Another important immunogenic protein is pp65; the antibody response against this protein is very high during the acute phase of the infection. The best-characterized protein, however, is glycoprotein B (gB), and up to 50-70% of the host’s neutralizing antibody response is accounted for by the response to his protein [19].

T cell immune responses

Cellular immunity against CMV is multifaceted, and both CD8 (T killer/CTL) and CD4 (T helper) T cells, as well as natural killer (NK) cells and γδ T cells are involved. A substantial proportion of the T-cell immune system, in both the CD8 and CD4 T-cell populations, is dedicated to controlling CMV replication, and it has been demonstrated that the majority of the CMV proteome is targeted by the T-cell arm of the immune system [20].

CD8 T-cell responses

After primary infection, the virus-infected cells display viral peptides together with MHC I, and these combinations are recognized by the T-cell receptors of the CD8+ CMV-specific T cells. Together with cytokines produced by CMV-specific CD4 T cells, the CD8 T cells become activated and start to produce cytokines (e.g., IFNγ and TNFα) and effector proteins (e.g.,
granzyme and perforin) to destroy the infected cell. The main targets of the CD8 T cells are pp65, IE1, and gB, and it is possible that the responses to pp65 and IE1 vary from one phase of the infection to another. The pp65 response may be of importance during the initial phase in newly infected cells before IE1 antigen is expressed, whereas IE1 responses may be expanded during reactivation from latency, since the IE1 response appears before the pp65 response in the event of a reactivation [21]. During the replicative phase of the infection, CMV-specific CD8 T cells display a uniform phenotype with high proliferation and expression of CD45RO and CD27. When the infection resolves into the latent phase, effector populations contract by apoptosis and CMV-specific T cells differentiate into resting primed memory cells (e.g., CD45RA+CD27-), but there is a large variation in the different subsets in the CD8+ population [22]. This central role for CD8 T cells in protection from disease has been substantiated by the inverse correlation observed between CMV disease and CD8 T-cell reconstitution after stem cell transplantation [23].

**CD4 T-cell responses**

In recent years, the role and importance of CD4 T cells in controlling the CMV infection has been recognized. Their involvement was first demonstrated in bone marrow transplant patients who received adoptive transfer of CMV-specific CD8 T cells, with patients deficient in virus-specific CD4 T cells displaying a lower level of CD8 anti-CMV T-cell activity [24]. This situation has also been observed in other populations with reduced capacity to mount a CMV-specific CD4 T-cell response. Deficiencies in CD4 T cells in children appear to correlate with prolonged viral shedding [25], whereas CD8 T-cell deficiencies do not [26]. In HIV patients, significant reductions in CMV-specific CD4 T cells are associated with increased CMV replication despite the presence of CD8 T cells, as well as a diminished ability of the CD8 T cells to secrete IFNγ after peptide stimulation [27]. A similar pattern has been demonstrated in the case of organ transplantation and will be discussed in more detail below.

**NK cell responses**

NK cells are members of the innate immune system and recognize pathogen-infected cells and tumors. These cells are vital in the event of a viral infection, and if NK cells are depleted, host resistance against CMV infection is markedly reduced [28].
Immune evasion

The capacity of CMV to establish latency and to co-exist with the host may be the result of multiple immune evasion strategies, and increasing evidence exists that both the innate and adaptive immune responses are affected. CMV has been shown to downregulate both MHC class I and II expression in infected cells and to interfere with MHC class II expression in several ways [29-31]. The balance between host defenses and virus is maintained through an intricate series of pathways; for example, although downregulation of MHC class I antigens would make the cells more vulnerable to NK cell-mediated lysis, other viral products (UL 16, UL 18) are responsible for helping the virus resist NK cells [32]. One potential clinical relevance of CMV’s immune evasion has been proposed by Humar et al.: CMV immune evasion gene expression levels in patients with CMV infection decline exponentially with antiviral therapy, and can be independently correlated with clinical outcome [33].

Immunological methods

The functional status of T cells in organ transplant recipients is of great importance, and the development of techniques such as ELISPOT, cytokine flow cytometry (CFC), and major histocompatibility complex (MHC) tetramers has facilitated the exploration of CMV-specific immunity [34].

- **ELISPOT** is based on the detection of a cytokine produced by single cells after stimulation with antigens [35]. The secreted cytokine is then detected by specific monoclonal antibodies that generate spots reflecting the number of cytokine-secreting cells. This method therefore measures both function and frequency, but one potential disadvantage is subjectivity.

- **CFC** is based on the detection of secreted cytokines from lymphocytes. One approach to detection is to create an artificial affinity matrix with antibodies that cover the cell surface and captures the secreted cytokines (upon stimulation), making it possible to label and measure the number of activated cells [36]. An alternative approach involves the intracellular staining of cytokines and requires the use of inhibitors of intracellular transport such as brefeldin [37]. This method allows for categorization of T-cell subsets, such as Th1 or Th2. The advantages of this method are that there is no need for HLA-specific epitopes, and the total capacity to respond to CMV antigen is measured. The drawbacks are that the method is time-consuming, and the samples need urgent attention.
- MHC tetramers consist of four MHC peptide complexes linked together by biotin and avidin with an attached fluorochrome [38]. The tetramerization increases the avidity of the peptides and produces a consistent binding to the T cells that facilitates the identification of the CMV-specific T cells by flow cytometry. The advantages of this method are its very high sensitivity and specificity and the fact that it requires very little blood; however, the obvious disadvantage is that HLA-specific epitopes are required.

CMV diagnosis

Historically, diagnosis of CMV disease has been made by histopathology, with large "protozoan-like" cells (cytomegaly [39]), with intranuclear inclusions being identified in biopsy material (Fig.2). This method is still used in conjunction with immunochemistry for the diagnosis of tissue-invasive CMV infection, but it has been superseded by serological and virological assays for determining the level of CMV replication and serostatus prior to transplantation.

![Figure 2. Typical owl-eye inclusions in CMV disease](Reproduced with the permission of Dr. Dan Wiedbrauk)

Serological assays

Serological assays involving the detection of antibodies (IgM and IgG) against CMV are highly specific and sensitive in immunocompetent individuals; the presence of anti-CMV antibodies indicates previous infection but does not indicate the level of immunity. The most commonly available assay is ELISA [40], but other methods such as latex agglutination, radio-immunoassay, complement fixation, and immunofluorescence tests are also available. One area in which serological analysis is of particular interest is in pregnant women, for whom the introduction of IgG avidity assays has improved the accuracy of diagnosis [41]. On the other hand, serological assays
cannot be recommended for monitoring CMV infection in immunocompromised individuals, since the results can be unreliable because of the reduced level of antibody production in these individuals. In transplant populations, serological assays can be used before transplantation in both the donor and recipient to identify subgroups with high or low risk of contracting CMV disease.

Virological assays

Virological assays have been developed for use in immunocompromised patients, in whom both rapid diagnosis and the ability to monitor CMV infection and the effects of antiviral therapy are vital for optimal patient management.

Isolation of CMV and immunohistochemistry

Viral culture has been considered the "gold standard" for many years. The endpoint is the characteristic cytopathic effect produced by productive replication of CMV in cell cultures such as human fibroblasts and routinely takes 2-4 weeks to reach. In the 1980s, a rapid cultured-based assay was developed that combined the inoculation of fibroblasts with the clinical sample followed by a 16- to 24-hour incubation followed by immunohistochemical detection of CMV proteins [42]. The disadvantage of this assay is that it is not possible to quantify the viral load; thus, the capacity to monitor the effects of treatment is greatly decreased [43]. The use of culture-based assays is still of importance, however, in epidemiological studies and in resistance testing.

Antigenemia assays

Antigenemia assays are based on the use of monoclonal antibodies against a viral protein, usually pp65, that is present in peripheral blood leukocytes during active CMV infection [44]. The antigenemia assay is highly specific and sensitive in diagnosing CMV infection, and has superior prognostic value when compared to cell culture-based methods. Other advantages are its reliability and rapidity, together with the fact that the viral load can be estimated from the number of pp65-positive leukocytes. It does, however, have some disadvantages when compared to PCR (see below): The major disadvantage is the need for a sufficient number of leukocytes (the absolute neutrophil count must be >0.2x10^9 cells/ml) [45], a requirement that limits the use of antigenemia assays in neutropenic populations. Other limitations are the need for rapid analysis within 6-8 hours of veni-puncture [46], and its difficulties to process large numbers of samples.
DNAemia assays
Since the early 1990s, DNAemia assays have made use of PCR or other hybridization techniques for both qualitative and quantitative detection of viral DNA. CMV DNA assays can be used on all body fluids, including blood components, and since CMV is a strictly cell-associated virus, the most sensitive blood compartment for detecting DNA is whole blood rather than plasma or leukocytes [47-50]. CMV DNA testing of whole blood reflects both the content of CMV in leukocytes and the cell-free virus; this testing of whole blood generally gives higher values than plasma does [43, 49, 51]. Quantitative DNAemia has been shown to correlate with virus replication and clinical symptoms [52, 53] and is used in the clinical setting to identifying patients at risk of developing CMV disease and to monitor the response to antiviral therapy. CMV DNA assays can also be performed on dried blood spots on Guthrie cards as a means of diagnosing congenital infection retrospectively [54].

RNAemia assays
RNAemia assays are used to detect a viral product, the pp67 mRNA transcript, which is more directly related to virus replication than is DNA. Nucleic acid sequence-based amplification (NASBA) is used to amplify the viral mRNA. The low sensitivity of this method, however, limits its usefulness in guiding of pre-emptive therapy [55].

Clinical symptoms of CMV infection – direct effects
Immunocompetent individuals
In immunocompetent persons of all ages, primary CMV infection is usually subclinical or associated with mild clinical symptoms such as fever, headache, and fatigue. Some healthy individuals, most often adolescents, may experience a mononucleosis-like syndrome, often called CMV syndrome, with prolonged fever, myalgia, splenomegaly, and a mild hepatitis with elevated liver transaminases that may continue for several weeks [10]. The infection is usually benign, without sequelae, and needs no treatment; however, on rare occasions, CMV can cause meningoencephalitis, Guillan-Barré syndrome, pneumonitis, or myocarditis. Reactivation of CMV in immunocompetent individuals is rather uncommon, and in two studies of healthy blood donors, the prevalence of DNA in the blood and plasma has been found to be <0.5% [56, 57].
Neonates
CMV is the most common congenital infection and is an important cause of severe congenital disease, including premature birth and death. The proportion of congenitally infected individuals is between 0.1 and 2.4% [58]. Congenital CMV infection can follow both primary and recurrent maternal infection but severe consequences of the disease are mainly related to primary infection [58]. Primary infection of a seronegative mother is associated with a 30-40% risk of intrauterine transmission, as compared to approximately 1% for seropositive mothers with a reactivated CMV infection. If the virus is transmitted, approximately 10% of the babies display symptoms of CMV disease at birth [58, 59]. These symptoms include microcephaly, hepatosplenomegaly, petechiae, hearing loss, and intracranial calcifications, and they are associated with a poor prognosis [60] and risk of long-term sequelae.

Immunocompromised individuals
In patients with a reduced T-cell capacity, CMV infection and disease are likely to cause more severe clinical consequences than in immunocompetent individuals. This population, which include solid organ transplant (SOT) recipients, stem cell transplant (SCT) recipients, HIV-infected patients, and other patients with heavy immunosuppression such as rheumatologic patients, is at high risk of developing invasive disease, with high morbidity and mortality. The risk of symptomatic disease in these individuals is variable and depends on many factors, including the immunosuppressive protocol used, the type of transplant, various host factors (e.g., age, neutropenia, comorbidity), and the serostatus of both the donor and recipient. Among the different SOT types, heart-lung transplants are associated with the highest risk of symptomatic disease (39-41%), followed by pancreas, liver, and kidney transplants (8-32%) [61].

In renal transplant populations, the CMV serostatus of both the recipient (R) and donor (D) has a major impact on the risk of developing CMV disease. The risk ranges from 5 to 10% in the D-/R- population, provided that these patients are given CMV-negative or leukocyte-depleted blood products [62]. The risk is intermediate (15-35%) in the D+/R+ and D-/R+ groups, and highest in the high-risk D+/R- population who lack both cellular and humoral immunity: Greater than 50% of these individuals are likely to develop CMV disease in the absence of prophylaxis and/or pre-emptive therapy [63].

Another high-risk population is those patients who receive depleting antibodies such as ATG and OKT-3 for the treatment of organ rejection. With such treatment protocols, CMV disease can develop in up to 65% of the treated individuals, as compared to 15-25% when these antibodies are used for induction therapy [64]. CMV disease in immunocompromised patients
can range from the previously described CMV syndrome (together with CMV DNAemia) to end-organ diseases such as pneumonia, colitis, hepatitis, encephalitis, myelitis, and retinitis.

Clinical symptoms of CMV infection – indirect effects

In addition to the direct effects of invasive CMV infection, this virus is also associated with a number of indirect effects that result in part from the influence on the host immune response. These indirect effects have been suggested to be a result of persistent low-level CMV infection [65] and have been associated with transplant-associated vasculopathy and atherosclerosis, posttransplantation diabetes, an increased risk of opportunistic infections (mainly bacterial and fungal), development of cancer, and decreased graft and patient survival [52, 53, 65-69]. An association between continuous dysregulation of the immune system and increasing age has also been proposed. This could be due to the persistence of CMV as a chronic antigenic stressor and would mainly affect the shrinking T-cell antigen repertoire and potentially contribute to the increased incidence of infections in the elderly [70, 71], and perhaps even depression and anxiety [72].

Prevention and treatment of CMV disease

Strategies for the prevention and treatment of CMV infection and disease in the transplant community include:

- Prophylaxis
- Pre-emptive therapy
- Deferred therapy; treatment for established CMV disease

Prior to the availability of therapy with foscarnet and ganciclovir (developed in the 1980s), CMV disease was often fatal, with up to 80% succumbing to SCT-associated CMV pneumonitis [73]. During the past few decades, however, the options for treating CMV disease have expanded, with the various antivirals, immunoglobulins and combinations thereof making CMV-associated mortality rare. CMV morbidity, on the other hand, is still a source of great concern, and the question of prevention versus pre-emptive or deferred therapy is a matter of debate. The approaches vary widely among different transplant programs as a result of the lack of large multicenter randomized trials and the difficulties involved in comparing the existing studies.
**Prophylaxis**

Antiviral prophylaxis is used in patients at risk of CMV infection/disease before any sign of an active CMV infection can be detected. Prophylaxis is administered over a defined period, usually correlated with the time during which the patient is most intensely immunosuppressed (commonly 100 days following transplantation), but longer periods (up to 6-12 months) are being investigated in certain high-risk groups such as D+/R- recipients [74]. The advantages of prophylaxis are a reduced morbidity and mortality related to the direct effects of the CMV infection, as well as a reduction in the indirect effects of CMV infection [75-79]. It has also been shown in renal transplant populations that a prophylactic strategy using ganciclovir is less costly than pre-emptive therapy [80, 81].
The disadvantages of prophylaxis may include a prolonged antiviral drug exposure that can be associated with toxicity and the emergence of resistance, the development of late-onset CMV disease (CMV disease occurring after cessation of the prophylaxis), and higher drug costs. The over-treatment associated with a prophylactic strategy can be substantial and in SCT, up to 65% of the patients have been reported to receive unnecessary antiviral therapy [82]. It is therefore important to consider the necessity for prophylaxis in low-risk populations. The risks and symptoms of late-onset disease are also a matter of discussion, and there is still a need for a strategy to manage this aspect of CMV disease.

The drugs most commonly used for antiviral prophylaxis are:

- **Acyclovir (ACV)** – a cyclic nucleoside homologue of guanosine that is phosphorylated by viral thymidine kinase (UL97) and cellular kinases to a triphosphate form that inhibits the viral DNA polymerase and act as a chain terminator of CMV DNA synthesis. ACV was first discovered in 1974 and became available for clinical use in 1982. Despite its rather poor in vitro activity, it has been found effective compared to placebo for prophylaxis in SCT patients [83], renal transplant patients [84], and AIDS patients [85].

- **Valacyclovir (VACV)** – a valine ester of ACV with increased bioavailability that has been in use since the early 1990s. VACV has been shown to reduce both CMV disease and acute rejection in renal transplant recipients when administered in a high (8g daily) or low (3g daily) dose regimen [86, 87]. It is also more effective than ACV as a prophylactic agent in SCT patients [88]. However, neurotoxic adverse effects such as confusion and hallucinations have been seen with high-dose VACV[86, 89].

- **Ganciclovir (GCV)** – a cyclic nucleoside homologue of guanosine that also is phosphorylated by viral thymidine kinase (UL97) and cellular kinases to a triphosphate form that inhibits viral DNA polymerase and act as a chain terminator of CMV DNA synthesis. Used since the 1980s, its effect on CMV is superior compared to that of ACV, and despite the poor bioavailability (3-5%) of the oral form of GCV, studies have confirmed its benefits when used as a prophylactic agent in liver transplant [90] and high-risk kidney transplant recipients [91]. In populations with the highest risk of CMV disease, such as those receiving lung or heart-lung transplants, intravenous (iv) GCV is used initially. The most common side effects are renal toxicity and neutropenia, which often cause problem in SCT patients.
• **Valganciclovir (VGCV)** – a valine ester of GCV with improved bioavailability that has been used since 2000. The efficacy of VGCV as a prophylactic agent in various transplant populations is comparable to that of GCV [92]. VGCV prophylaxis is as economical as sequential GCV prophylaxis in high-risk D+/R− SOT patients [93]. The spectrum of side effects is the same as GCV.

• **CMV immunoglobulin (CMVIG)** – a pool of anti-CMV IgG antibodies that has been used since two trials demonstrated a decrease in ”severe” CMV-associated disease in kidney and liver transplant recipients [94, 95]. The trials did not, however, demonstrate any reduction in the overall risk of CMV disease, and CMVIG is mostly used in combination with GCV in high-risk heart or lung transplant recipients in some centers.

**Pre-emptive therapy**

The alternative approach to minimizing CMV disease is pre-emptive therapy, which is initiated when a laboratory marker (e.g., antigenemia, DNAemia) indicative of a high-level of replication and/or disease becomes positive. The aim is to reduce the viral burden and to prevent progression from asymptomatic infection to CMV disease. A successful strategy is built on several factors, including:

• Selection of the appropriate patient population
• The use of rapid, sensitive and reliable diagnostic methods such as PCR or pp65Ag assays
• Regular monitoring, with excellent patient and doctor compliance
• Initiation of treatment quickly after detection of viral replication
• Choosing the appropriate type, dose, and duration of an antiviral agent

If these factors cannot be achieved, it is likely that the prophylactic strategy might be the more suitable option. The advantages of pre-emptive therapy include a reduced drug exposure and decreased risk of toxicity and resistance. It is also thought that pre-emptive therapy enables limited replication of CMV to occur, thus facilitating immune priming, which may be of importance for future control of CMV replication [96]. The disadvantages of this strategy include the logistic demands of laboratory testing and uncertainty about the impact on the indirect effects of CMV disease. It has also been reported that up to 33% of patients receiving pre-emptive treatment later develop CMV DNAemia that requires treatment.
The drugs most commonly used for antiviral pre-emptive therapy are:

- **Ganciclovir**
- **Valganciclovir**
- **Foscarnet** – an inorganic pyrophosphate analogue that directly inhibits the CMV DNA polymerase. Foscarnet has been used since the 1980s, most often in the event of GCV resistance or in cases of SCT-associated neutropenia. It can only be administered iv, and the most common side effects are renal toxicity and hypocalcemia.

To summarize the preventive strategies: There is currently more or less agreement that both prophylactic and pre-emptive strategies reduce the impact of CMV, and it is likely that a mixed approach is needed to optimize the well-being of patients. Evidence-based guidelines from the International Herpes Management Forum (IHMF) [43] and the Canadian Society of Transplantation (CST) [97] recommend universal prophylaxis for patients at the highest risk for CMV disease whereas preemptive therapy may be most appropriate for those at a moderate or low risk of CMV. The choice of antiviral drug regimens used for universal prophylaxis depends on the type of organ transplanted and the CMV serostatus of the donor and recipient. The optimal pre-emptive drug regimen and laboratory monitoring strategy are still unknown.

**Treatment**

Despite the use of preventive strategies, some patients ultimately develop CMV disease, and the treatment options available for these patients are similar to those used in the pre-emptive strategies. In recent years there has been a gradual shift from iv GCV toward the use of VGCV [98], and in the case of neutropenia and/or GCV resistance, foscarnet is used/added. Treatment with GCV in neonates has been proven to be effective in reducing hearing loss [99]. Additional drugs used in treating established CMV disease are:

- **Cidofovir** – a nucleotide monophosphate that is triphosphorylated by cellular kinases to triphosphate that directly inhibits CMV DNA polymerase. It has a long half-life that allows once-weekly therapy. The most common side effect is a significant renal toxicity.
- **Maribavir** – a novel benzimidazole drug in ongoing phase 3 trials that directly inhibits the UL97 kinase and CMV DNA synthesis. It has also been shown to be effective against GCV-resistant CMV strains. It is not associated with nephrotoxicity or hematologic toxicities.
• **Leflunomide** – A pyrimidine synthesis inhibitor that interferes with virion assembly. It has a good bioavailability (80%) and is already approved for autoimmune conditions because of its immunosuppressive abilities. It has been used in SOT recipients with good effect, whereas the results in SCT recipients have been less positive [100, 101].

Adoptive immunotherapy

Since the early 1990s [24], infusing CMV-specific CD8 and CD4 T cells into patients to stop viral replication has been a subject of research. Together with the progress associated with new immunological methods such as MHC class I tetramers, the introduction of this approach has enabled several groups to use this method as a treatment option in SCT patients who are unresponsive to GCV [102, 103]. This method is not yet in routine use, but it could be used as a final option in patients in whom all other treatment options have been explored.

CMV resistance

The emergence of resistance to anti-CMV drugs has become an increasing problem in recent decades. Already in the late 1980s, shortly after the introduction of GCV, sporadic cases of GCV-resistant CMV were reported in severely immunocompromised patients [104], and during the AIDS epidemic, the GCV resistance increased. In recent years, the widespread use of more intensive immunosuppressive strategies and prophylaxis with GCV in the SOT population has resulted in an increasing level of GCV resistance. Risk factors for the development of GCV resistance are: D+/R- serological status, prolonged exposure to GCV, potent immunosuppression, suboptimal GCV levels, and high viral load [105-107]. The highest incidence of GCV-resistance (20-30%) is seen in the D+/R- lung transplant and kidney-pancreas transplant populations, whereas the incidence in D+/R- kidney transplant patients ranges from 2 to 5%. In the R+ transplant population, however, the incidence is virtually zero, except for lung transplant patients, resulting in an overall incidence of GCV resistance of 0-13% [108].

The main mechanisms of GCV resistance involves mutations in UL97 (viral phosphotransferase), UL54 (DNA polymerase), or both [109-111]. Mutations in UL97 ultimately result in decreased levels of active GCV, whereas mutations in UL54 result in a virus that is less susceptible to GCV, with cross-resistance to cidofovir and foscarnet. Mutations in UL97 are far more common, and a majority of the mutations occur in one specific region. It has been demonstrated that the mutation in UL97 can be detected at up to 1% in the original CMV population in patients, and this resistant virus is
selected by GCV treatment [18]. The frequency of GCV-resistant virus has been found to increase from 8% after 3 months of GCV treatment to 64% after 15 months [112, 113]. In patients who develop CMV disease because of drug-resistant virus, the clinical outcome is generally poor, with a mortality rate of up to 20% [108, 114, 115]. The reason for this high rate may be that the second-line therapy options are limited; thus, the need for additional drugs with good anti-CMV effect is evident.

Vaccine development

Because of the high risk in neonates and the immunocompromised individuals of developing CMV infection and disease, intensive research has been carried out during the past 30 years to develop a vaccine against CMV infection. Despite these efforts, no CMV vaccine has yet been licensed. The development of a vaccine to prevent CMV infection or disease has therefore been assigned the highest priority by medical authorities such as the U.S. Institute of Medicine, but there are still many problems to be solved.

What viral proteins should be included?

In order to be effective, viral vaccines must induce antibodies that neutralize viral entry into the host cells. Earlier studies of laboratory CMV strains have focused on viral entry into fibroblasts and have revealed that the major neutralizing epitopes reside in glycoproteins gB, gH, and gM/gN [116-118]. It has also been suggested that epitopes within gB can represent up to half the neutralizing activity of human immune sera [116]. Recent findings, however, have indicated that CMV enters different cell types via various distinct mechanisms. Fibroblast entry occurs at the cell membrane and is pH-independent, while entry into endothelial and epithelial cells requires a complex of gH and gL, along with three additional viral proteins that are unnecessary for fibroblast entry, UL128, UL130, and UL131 [119, 120]. This discovery suggests that previously unrecognized neutralizing epitopes may exist. Recent findings also imply that in naturally infected individuals, the titers of neutralizing antibodies to these epitopes far exceed those seen in fibroblast-neutralizing responses [121].

At present, two vaccines are in phase II studies, one based on the gB complex and one based on a live attenuated Towne strain. The gB vaccine has been shown to induce high levels of monoclonal antibodies that neutralize both wild-type virus and laboratory strains. It is safe and immunogenic and is probably the most likely candidate for a subunit vaccine. While the gB vaccine would not be expected to elicit antibodies to UL128–131, antibodies to certain epitopes within gB could selectively inhibit epithelial or endothelial cell entry. The Towne vaccine has been used extensively in clinical trials; it is safe and stimulates neutralizing antibodies. This vaccine, however, contains a mutation in UL 130 but
could potentially elicit antibodies to epitopes within UL128 or UL131. These antibodies can specifically neutralize epithelial or endothelial cell entry [120], but the levels of antibody produced have been demonstrated to be much lower than those found in natural sera [122]. These findings may indicate that the endocytic entry mediators UL128, UL130, and UL131 are strong potential candidates for antigens containing epithelial neutralizing epitopes. It is therefore possible that the induction of epithelial-neutralizing antibodies comparable to those induced by natural infection may be necessary for an effective CMV vaccine.

What population should a clinical vaccine trial focus on?

Another problem is how to design a vaccine trial: Should it be aimed at immunocompromised patients or at mothers who are at risk for transmitting congenital CMV? If women who are not yet pregnant are the target population, one problem would be the great number of subjects needed to demonstrate efficacy. Given an estimated efficacy of the vaccine of between 50 and 80% with congenital infection as an endpoint, somewhere between 3,000-10,000 mothers and their newborns would have to be enrolled [123].

A vaccine would have the greatest impact if it were given to infants before the age of two. In this population, much smaller number of subjects would be needed (around 300). In children however, it has been demonstrated that those who shed CMV for a prolonged period have greatly diminished CMV-specific CD4 responses [25]. Therefore, it may be necessary to develop a vaccine that induces CMV-specific CD4-responses in order for it to be effective.

Renal transplantation and allograft rejection

Since the onset of transplantation procedures in the 1950s, patient and graft survival has improved, mostly because of the availability of better immunosuppressive drugs, but also as a result of better surgical techniques and infection control. Patient and graft survival after 1 year is now approaching 100% in living donor transplants (98% patient and 95% graft survival) and >90% in deceased donor renal transplants (95% patient and 89% graft survival). Five-year survival, however, is less encouraging with 90% (patient) and 80% (graft) survival in living donors and 80% (patient) and 67% (graft) survival in deceased donor transplants [124]. Today, the major causes of late allograft loss are death with a functioning graft and chronic renal allograft nephropathy (CAN, see below), each of which accounts for about 50% of all allograft loss. The major identified causes of mortality among renal transplant recipients are cardiovascular disease (22%), infection (16%), malignancy (7%), cerebrovascular disease (6%), and graft failure (1%) (Fig.4).

Rejection

There are four major clinical types of rejection in human renal allografts:

- Hyperacute
- Humoral/antibody-mediated
- Acute cellular/vascular
- Chronic

Hyperacute rejection

Hyperacute rejection occurs very rapidly upon reperfusion of the graft: within minutes to 24 hours post-transplantation. It requires pre-formed antibodies of either the HLA or ABO type against the donor tissue. These antibodies are produced by plasma cells and have been induced by blood transfusions, previous transplantation, or pregnancies. The antibodies attack the endothelium and cause activation of both the coagulation and complement cascades, which in turn leads to vascular coagulation and immediate necrosis and graft failure. These rejections can usually be avoided by a thorough HLA matching of the donor and recipient to avoid an HLA mismatch that increases the risk.
Humoral/antibody-mediated rejection

Humoral- or antibody-mediated rejection often occurs within 30 to 90 days after transplantation. Clinical signs of rejection are a rapid increase in serum creatinine, hypertension, and reduced urine output. Neutrophilic infiltration and C4d deposition in the glomeruli can be seen on biopsy. The pathogenesis is based on the post-transplantation development of anti-HLA antibodies. This condition requires intensive treatment with monoclonal or polyclonal antibodies, possible plasmapheresis and/or immune globulin, and a change in the maintenance immunosuppressive therapy.

Acute rejection

In recent years, the incidence of acute rejection has decreased dramatically, from >50% in the pre-cyclosporine era to about 10% with the newer immunosuppressive protocols. Acute rejection occurs mainly within the first 12 months after transplantation. Clinically, an increase in the serum creatinine and blood pressure are typically noted. The acute rejection, further divided into cellular and vascular rejection, is characterized by an infiltration of both CD4+ and CD8+ T cells, together with histiocytes, macrophages, and granulocytes. In cellular rejection, the infiltration is seen in the renal tubules, and in the event of a vascular rejection in the intima of small arteries. Acute rejection is still classified according to the BANFF criteria [125, 126], which combines the topographical categorization of the immune injury and the severity of the injury. These scores are then summarized into a scheme with different severity grades. Primary treatment of the acute rejection is based on intravenous methylprednisolone and/or a change in the maintenance immunosuppressive therapy.

Chronic rejection

Chronic rejection can occur months to years after transplantation, with clinical signs of a gradual increase in serum creatinine, proteinuria and hypertension. The pathology of this condition is diverse and features interstitial fibrosis, tubular atrophy, and glomerulosclerosis. The pathogenesis involves factors such as HLA-antibody formation, inadequate immunosuppression, drug toxicity, and previous rejections and infections. Another condition that resembles chronic rejection is the multi-etiological, non-specific and chronically progressive parenchymal scarring previously [127] known as chronic allograft nephropathy (CAN), the commonest cause of graft failure in the first decade after transplantation. This allograft dysfunction may have an unpredictable onset but occurs after the first 3 months post-transplantation, with a slow deterioration of renal function. Biopsies display a nonspecific histology with tubular atrophy and thickening of arteries. Depending on the cause (which is often unknown), therapy may be beneficial, but in most cases there is no cure.
Immunosuppressive treatment in renal transplantation

The central issue in organ transplantation remains suppression of allograft rejection. Immunosuppressive drugs are used for induction (intense immunosuppression during the initial days after transplantation), maintenance therapy, and reversal of established rejection. Until recently, the strategy was quite simple: After induction with depleting antibodies, maintenance therapy consisted of three drugs (a calcineurin inhibitor, azathioprin, and corticosteroids) that were continuously reduced. Rejection was treated with high-dose steroid or depleting antibodies. Today, hundreds of combinations exist, a situation that has improved both graft outcome and patient morbidity and mortality, and the combinations are now being more and more individualized, partly as a result of our increasing knowledge in the field of pharmacogenomics [128, 129].

Immunosuppression can be achieved by depleting lymphocytes, diverting lymphocyte traffic, or blocking lymphocyte response pathways (Fig. 5). As a result of the immunosuppression, rejection is suppressed, but this situation also leads to undesired consequences such as infection, cancer, post-transplant lymphoproliferative disease (PTLD), and nonimmune toxicity. Immunosuppressive drugs can be classified into the following categories:

- Small-molecule drugs (e.g., cyclosporine)
- Depleting protein drugs (e.g., ATG)
- Nondepleting protein drugs (e.g., basiliximab)
- Fusion proteins (e.g., belatacept)
- Glucocorticoids
- Intravenous immune globulin

Small-molecule drugs

Most small-molecule immunosuppressive agents are derived from microbial products and target proteins that have been highly conserved in evolution. These drugs probably do not saturate their targets at clinically tolerated concentrations, and without target saturation, the drug's effects are proportional to the concentration of the drug, which makes dosing and monitoring critical.

Azathioprine was the first immunosuppressive drug widely used in organ transplantation, but it is now considered a second-line drug because of the introduction of cyclosporine. Its effect is mainly on DNA synthesis, with serious side effects that include infections, neoplasias, and hematologic condition (leukopenia and/or anemia).
**Calcineurin inhibitors**
Calcineurin inhibitors can be considered the cornerstone of immunosuppression and can be used as a single drug, even though they are mainly used together with other immunosuppressive drugs.

*Cyclosporine*, the most widely used immunosuppressive drug, is a fungal peptide composed of 11 amino acids (aa). It is a prodrug that forms a complex with cyclophilin, an intracellular protein that inhibits calcineurin and reduces the transcription of IL-2, IL-4, and IFN$\gamma$. The side effects of cyclosporine are related to its concentration and, in addition to an elevated risk of opportunistic and other infections, include nephrotoxicity, hypertension, hyperlipidemia, gingival hyperplasia, hirsutism, and tremor. This drug is also associated with the hemolytic-uremic syndrome and post-transplantation diabetes mellitus. Drug concentrations must be monitored, but there is no consensus on the optimal time for sampling [130].

**Tacrolimus (FK506)**, a macrolide antibiotic licensed since 1994, uses another intracellular protein, FKBP12, to create a complex similar to that involving cyclosporine but with much greater potency. The effect on rejection frequency is superior to that of cyclosporine [131]. The choice between cyclosporine and tacrolimus is based on the differences in their side effects: Tacrolimus use poses the same risk of renal toxicity and the hemolytic-uremic syndrome, but it is less likely to cause hypertension, hyperlipidemia, gingival hyperplasia, or hirsutism. On the other hand, an elevated risk of developing post-transplantation diabetes [132] and BK-virus-related nephropathy has been demonstrated with this drug [133]. The concentration needs to be monitored, and trough levels are generally measured.

**Purine synthesis inhibitors**
*Mycophenolic acid (Myfortic)* and the prodrug *mycophenolate mofetil (MMF)* block purine synthesis and prevent the proliferation of T and B cells. They are widely used in combination with calcineurin inhibitors and have replaced azathioprine because of their superior effect on graft rejection. Addition of MMF in the event of rejection could improve graft outcome. Other advantages are that there is no absolute need for concentration monitoring, and the risk of cardiovascular events and nephrotoxicity is low. The drug has been demonstrated to inhibit pneumocystic activity in vitro, but the risk of CMV disease may be elevated [134].

**Target-of-Rapamycin inhibitors**
*Sirolimus (rapamycin)* and *everolimus* are other macrolide antibiotics that form a complex together with FKBP12, but their effect is inhibition of the rapamycin target instead of calcineurin. This inhibition prevents cytokine
(IL-2) receptors from activating the cell cycle. These drugs are not nephrotoxic by themselves, but they increase the nephrotoxicity of calcineurin inhibitors [135]. Other important side effects are hyperlipidemia, pneumonitis, and thrombocytopenia, and recently an association between sirolimus and new-onset diabetes has been demonstrated [136]. Sirolimus has also been demonstrated to have both antineoplastic and arterial protective effects [137, 138], and the available evidence indicates that with respect to tumor growth, rapamycin’s anti-cancer activity outweighs its immunosuppressant effects [139]. Its arterial protective effects could be due to an impaired wound healing and/or an antiangiogenetic effect and have led to the development of drug-eluting stents used in coronary arteries.

Figure 5. Immunosuppressive drugs and sites of action. CsA-cyclosporine. MPA-mycophenolic acid. See text for details. (Figure reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology 3, 831-838, copyright 2003)
Depleting antibodies

Depleting protein immunosuppressive agents are antibodies targeted against T cells, B cells, or both. When T-cell depletion treatment is initiated, large amounts of cytokines are released, causing severe systemic symptoms. Depleting antibodies are used to reduce early rejection but also increase the risk of infection and PTLD. When the immune system recovers, after months to years, late rejection may occur. The depletion of antibody-producing cells is better tolerated than is T-cell depletion because of the absence of cytokine release and the maintenance of immunoglobulin levels.

*Antithymocyte globulin (ATG)* is produced by immunizing horses or rabbits (more potent) with human lymphoid cells; IgG without toxic antibodies is then harvested. ATG blocks T-cell membrane proteins and causes a profound and durable T-cell functional depletion as a result of both altered function and lysis. Polyclonal ATG can be used as an induction agent for 3 to 10 days or in the event of steroid-resistant rejection. Unwanted side effects are, most notably, the cytokine release syndrome (fever, chills, and hypotension), neutropenia, thrombocytopenia, and serum sickness.

*Muromonab-CD3 (OKT3)* is a mouse monoclonal antibody against CD3 that is used to treat rejection and for induction. It binds to T-cell receptor-associated CD3-complexes and triggers a massive cytokine-release syndrome before depleting T cells and altering T-cell function. Because of the severe immunosuppression and elevated risk for CMV disease that are associated with this drug, prophylaxis against CMV is given when muromonab-CD3 is used as rejection treatment. Unfortunately, humans can make neutralizing antibodies to the drug, limiting its reuse; also, prolonged use increases the risk of PTLD. Its other main adverse effects are gastrointestinal and neurotoxic. In general, the use of OKT-3 has decreased since the incidence of rejection has declined.

*Alemtuzumab* is a humanized monoclonal antibody against CD52 that massively depletes lymphocyte populations. It was originally approved for treating refractory B-cell chronic lymphocytic leukemia but has recently been used as an induction agent in renal transplantation. The risks of immunodeficiency complications with alemtuzumab, such as infections and cancer, are being investigated, but some studies have demonstrated a similar or reduced incidence of infectious complications [140, 141]. Toxicity is generally related to neutropenia, anemia, and a mild cytokine-release syndrome.
Rituximab is a monoclonal antibody that targets anti-CD20, eliminating B cells for 6-9 months; it is considered a very safe drug. It was originally approved for treating refractory non-Hodgkin's B-cell lymphomas and PTLD in organ-transplant recipients, but has recently been used in both autoimmune disorders and transplant settings. It is used to diminish the levels of alloreactive antibodies in highly sensitized patients [142], to manage ABO-incompatible transplants [143], and to treat rejection associated with B cells and antibodies [144, 145]. The use of rituximab in the transplant setting needs further studies in randomized controlled trials before it can be recommended as a standard treatment.

Non-depleting antibodies

Non-depleting protein drugs are monoclonal antibodies or fusion proteins (still in clinical trials) that target proteins expressed only in activated immune cells; this strategy effectively reduces the immune response without depleting entire lymphocyte populations. The somewhat limited efficacy of non-depleting antibodies is balanced by an absence of immunodeficiency complications and low levels of non-immune toxicity and cytokine release.

Basiliximab and daclizumab are anti-CD25 monoclonal antibodies that block the IL-2 receptor α-chain. They are widely used for induction, with a low-to-moderate risk of rejection, but they require T-cell activation and therefore only deplete activated T-cells. They are considered moderately effective, since they only reduce rejection by about one-third in combination with calcineurin inhibitors. On the other hand, they have minimal toxic effects and are associated with a reduced risk of CMV infection [146].

Fusion proteins

Belatacept (LEA29Y) is a second-generation cytotoxic T lymphocyte–associated antigen 4 (CTLA-4) immune globulin. It is a fusion protein that combines CTLA-4 (which engages CD80 and CD86) with the Fc portion of IgG, thereby inhibiting a T-cell costimulatory pathway. Belatacept, now in phase III trials, is used as a long-term iv-administered drug to reduce reliance on toxic small-molecule immunosuppressive drugs and has been demonstrated to have a superior effect on graft function when compared to treatment with cyclosporine.
Glucocorticoids

Glucocorticoids have played a critical role in the evolution of organ transplantation. Their mechanism of action is yet not fully understood, but they are known to affect T-cell activation both directly and indirectly and display different effects in low- and high-dose regimens. Higher doses are used during the first days after transplantation or in the case of rejection to reduce non-specific inflammation; the lower doses used during maintenance therapy act via receptor-mediated effects to target transcription factors. Long-term glucocorticoid use is associated with numerous adverse effects, including an increased risk of infection, new-onset diabetes, hyperlipidemia, hypertension, and accelerated bone loss. An early steroid withdrawal in renal transplant recipients would reduce many of these steroid-related side effects, and it has recently been demonstrated that this can be accomplished, at 2 to 6 days post-transplantation, without compromising graft function [147, 148].

Intravenous immune globulin

The mode of action of this therapy depends largely on antigen binding and modification of effector functions. Antigen binding is mediated via immune antibodies and a wide spectrum of autoantibodies. The effector functions include modulation of expression and function of Fc receptors, complement activation, complement binding, anti-inflammatory effects due to interference with the cytokine network, and modulation of T- and B-cell activation. Organ transplantation is an important field for this treatment, primarily for patients with high titers of anti-HLA antibodies.
AIMS

General aim
CMV infections are still a source of great concern, especially in transplant populations. A better understanding of the immunologic response in primary and reactivated CMV infection as well as better diagnostic methods and optimal strategies for preventing and treating CMV disease in transplant populations are still needed. In these papers, we wanted to evaluate a modified prevention strategy in high-risk renal transplant patients, and to characterize the dynamics of the CMV-specific immune response in both renal transplant patients and healthy controls.

Specific aims
Paper I
The aim of this retrospective study of CMV-mismatched (D+/R-) renal transplant patients was to determine whether a comparatively low dose of prophylactic valacyclovir (3g/day) given for 90 days post-transplantation could prevent CMV disease with fewer CNS adverse effects than were reported by participants in an earlier study in which 8g/day was used. We also wanted to compare the incidence of rejection to that of historical controls.

Paper II
The aim of this study was to characterize the CMV-specific CD8+ immune response in healthy individuals with primary or latent CMV infection. We wanted to see whether, in latently infected individuals, the frequencies of different CMV epitopes within the same donor were of equal magnitude or whether any of the epitopes displayed immunodominance. The frequencies of the CMV-specific T cells were then related to the function of the CMV-specific T cells.
Paper III
The aim of this longitudinal, prospective study of CMV-seropositive renal transplant recipients was to evaluate the dynamics of CMV-specific T cells, in terms of both function and absolute number, as well as the viral load up to 1 year after transplantation. We also wanted to assess possible correlations between CMV-specific T-cell activity and clinical symptoms of CMV infection.

Paper IV
The aim of this retrospective study of CMV-mismatched (D+/R-) renal transplant patients given low-dose valacyclovir (3g/day) for 90 days post-transplantation was to evaluate patient and graft survival up to 5 years post-transplantation and to compare these survival rates to international data from the CTS database. We also wanted to analyze the incidence of CMV disease, CMV resistance, biopsy-proven acute graft rejection, and major neurotoxic adverse effects up to 1 year after transplantation.
METHODS

Ethics
The ethics committees at Uppsala University Hospital (papers I-IV, when applicable) and Rikshospitalet, Oslo (paper III) approved the studies. When applicable, information about the study was provided to the participants by research nurses and physicians, and informed consent was given by all participants. To protect the integrity and privacy of the participating individuals, all participants were assigned study codes, when applicable, which were used throughout our analyses and in correspondence.

Subject inclusion (I-IV)
Renal transplant patients (I, III, IV)
The patients in the study described in papers I and IV were studied retrospectively on the basis of medical charts and nursing notes. All CMV-seronegative patients at Uppsala University Hospital who consecutively received a renal transplant from a CMV-seropositive donor between September 1998 and November 2000 (I) or between September 1998 and June 2007 (IV) and also received low-dose prophylaxis with valacyclovir 3g/day for 90 days post-transplantation were included.

In paper I, 25 patients (D+/R-) were followed for 6 months after transplantation. Nine patients who received basiliximab induction therapy were analyzed as a subgroup.

In paper III, all consecutive CMV-seropositive renal transplant recipients (n=17) with haplotype HLA A*0201 and/or B*0702, from two transplant centers, Uppsala University Hospital (n=13) and Rikshospitalet – Radiumhospitalet Medical Centre, Oslo (n=4), between March 2002 and September 2003 were recruited. All donors were CMV-seropositive, resulting in a homogenous D+/R+ group.

In paper IV, 102 patients (D+/R-) were followed for up to 5 years after transplantation. The results from these patients were compared to analogous results from approximately 4,000 patients (see paper IV for details) from the international Collaborative Transplant Study (CTS) database, to which Uppsala is one of the almost 300 contributing renal transplant centers.
Patients with primary CMV disease (II)

Five otherwise healthy persons with a symptomatic primary CMV infection diagnosed at the Department of Infectious Diseases, Uppsala University Hospital were included. They displayed classic symptoms of CMV infection in combination with positive CMV-specific IgM and low IgG levels. They had to express HLA allele A1 or A2, since these tetramers were the only ones available at the start of the study.

Healthy controls with latent CMV infection (II, III)

CMV-seropositive and serologically HLA typed blood donors (II) and staff from the Departments of Clinical Immunology (II, III) and Infectious Diseases (III) were included in these studies. In paper II, 53 subjects (30 male and 23 female) aged 27-80 were recruited, and in paper III, 11 subjects (1 male and 10 female) aged 29-55 were studied. In paper II, all subjects had to express at least two of the following HLA alleles: HLA A1, A2, A3, A24, B7, B8, and B35; in paper III, they had to express the HLA A*0201 and/or B0702 haplotypes (corresponding to the only tetramers available at the start of the study). All of the subjects stated that, as far as they knew, they were perfectly healthy and had not experienced any signs of infection during the past 2 weeks during the sampling period.

Blood sampling (II, III)

In paper II, symptomatic patients were sampled as soon as possible after diagnosis, and subsequent samples were then obtained on weeks 4, 6, and 8, and then every month up to 6 months after the onset of symptoms. Of the 53 latently infected subjects, 41 were tested twice, the second occasion occurring at a median of 58 days (range 14-269) after the first sample.

The patients in paper III were repeatedly tested on day 0 (the day of transplantation) and days 14 and 28, then at least once a month up to 1 year after transplantation. Blood samples were collected for routine laboratory tests and for quantification of CMV DNA, tetraCD8, IFNγCD8, and IFNγCD4. Samples were drawn by nurses at hospitals and other health care facilities and sent to the Department of Clinical Immunology for analysis within 6 hours (functional assays) or 24 hours (tetramer assays) of venipuncture. Samples for CMV PCR analysis were frozen for storage and analyzed later (III). The latently infected subjects were repeatedly tested at the same intervals as the patients.
Determination of CMV load (III, IV)

In paper III, CMV-DNA was quantified by a real-time PCR assay (TaqMan), and the sequences of the PCR primers and probes were selected from the UL65 gene encoding pp67 [149]. In paper IV, the commercial COBAS AmpliCor CMV Monitor (Roche) was used from 1998 to 2000. From 2001 to 2008, we used an in-house PCR from our local laboratory [150].

MHC I tetramer analysis (II, III)

The numbers of CMV-specific CD8+ T cells were determined using MHC I CMV tetramers (II,III) as previously described [38]. Titrated amounts of phycoerythrin (PE)-labeled HLA-matched MHC I tetramers (Table 1) and an antibody mixture containing αCD8-FITC and αCD3-APC were added to EDTA-blood and incubated for 30 min at 4°C. FACS Lysing Buffer was added, and the cells were repeatedly washed with PBS containing 1% FCS and 0.1% sodium azide before FACS analysis was performed. In patients with ongoing rejection and/or CMV disease, excessive nonspecific binding was sometimes seen. In these cases, the experiment was repeated, with the order of the lysing and staining reversed. A total of 20,000 to 30,000 CD3+ lymphocyte events were collected, and CMV-specific cells were expressed as the percentage of CD8+ cells and considered positive if the value was >0.10%. The number of CMV-specific cells/μl was recalculated by using the absolute number of CD8+ cells, which was simultaneously determined.

Figure 6. A schematic drawing of a CD8 T cell binding a MHC I tetramer. To the right, a FACS plot showing tetramer-binding cells in the upper right quadrant. (Reproduced with the permission of Anna-Karin Lidehäll.)
Table 1. MHC I tetramers used in the studies

<table>
<thead>
<tr>
<th>HLA A or B allele restriction element</th>
<th>Peptide sequence</th>
<th>Peptide origin</th>
<th>Paper #</th>
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</thead>
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<td>YSEHPTFTSQY</td>
<td>pp65</td>
<td>II</td>
</tr>
<tr>
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<td>NLVPMVATV</td>
<td>pp65</td>
<td>II,III</td>
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<td>IPSINVHHY</td>
<td>pp65</td>
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CMV-specific T-cell stimulation and intracellular cytokine staining (II, III)

The function of CMV-specific CD4+ and CD8+ T cells was measured by assessing their ability to secrete IFNγ in response to specific stimulation; no cytotoxic assays or proliferation assays were used. After blood sampling, T cells were stimulated by adding CMV antigen (see below) to the sodium-heparinized whole blood and incubating the samples at 37°C for 6 hours.

Stimulation of CD4+ T cells

CMV-specific CD4+T cells were stimulated by adding CMV-infected lysed fibroblasts. Viral proteins were processed and presented by antigen-presenting cells in the blood. The CD4+ T cells with the right specificity became activated upon recognition of the combination of MHC II and viral peptides [151].

Stimulation of CD8+ T cells

CMV-specific CD8+ T cells were stimulated by two different methods: In paper II, a pool of overlapping peptides spanning the CMV protein pp65 was used. The peptide pool consisted of 15 amino acid-long peptides covering the whole protein and overlapping by 9 amino acids. Peptide pools mainly activate CD8+ T cells but also activate CD4+ T cells to some extent [152]. In paper III, stimulation was achieved by adding the pp65-derived immunodominant peptides NLVPMVATV (A*0201) or TPRVTGGGAM (B*0702).
Intracellular cytokine staining

After the first 2 hours of a 6-hour of incubation with CMV antigen, brefeldin A was added to inhibit the Golgi apparatus, thus containing the cytokines intracellularly. For practical reasons, the last 4 hours of incubation took place in a programmable waterbath that automatically cooled the samples after 4 hours and kept the tubes cold overnight. On day 2, the erythrocytes in the sample were lysed (with FACS Lysing Solution) and the T cells were then permeabilized (FACS Permeabilizing Solution 2). Antibody staining was performed with the addition of αCD3-APC, αCD8-PerCP, αCD38-, or 69-PE and αIFNγ-FITC antibodies. The tubes were then incubated for 30 minutes at 4°C and then washed once with PBS containing 1% BSA before FACS analysis was performed.

Figure 7. Intracellular cytokine staining after stimulation with CMV antigen.
(a) and (b) - gating of CD3+ lymphocytes in FACS plots.
(c) - IFNγ-production in an unstimulated sample.
(d) - IFNγ-production in CD8 T cells after in vitro stimulation with a mixture of overlapping peptides covering the entire CMV protein pp65.
(e) - IFNγ-production in CD4 T cells after in vitro stimulation with a CMV-infected and lysed fibroblast cell line.
(Reproduced with the permission of Anna-Karin Lidehäll)
Absolute counting of lymphocyte subsets (II, III)

Enumeration of CMV-specific CD8+ and CD4+ T cells
The numbers of CMV-specific CD8+ T cells were determined using MHC I CMV tetramers. IFNγ-production was examined by intracellular cytokine staining after stimulation with a peptide pool spanning the entire pp65 (II) or with a HLA-specific peptide (III). Because of the greater difficulty experienced in synthesizing MHC class II tetramers, we used cytokine responses against CMV lysate (III) to determine the number of CMV-specific CD4+ T cells.

Flow cytometry and FACS analysis
Flow cytometry-based absolute cell counting was performed essentially as described by Gratama et al. [153]. An antibody mixture containing titrated amounts of αCD45-FITC, αCD8-PE, αCD4-PerCP, and αCD3-APC was incubated with exactly 100 μl of EDTA-anticoagulated blood. After lysis of the erythrocytes, 100 μl of Flow-Count Beads was added. To add exact amounts of blood and beads, a reverse pipetting technique was used. Gating and data analysis were performed as described by Gratama et al. [153]. All samples were analyzed in duplicate, and mean values were calculated.

For all flow cytometric analyses, a four-color FACSCalibur instrument with CellQuest or CellQuest Pro Software was used. FACS analyses were performed within a few hours after staining. A total of 20,000 to 30,000 CD3+ lymphocytes were collected, and the percentage of IFNγ-producing CD8+ cells, as determined by cell counting, was calculated and expressed as IFNγ-producing CD8+ cells/μl. To correct for background cytokine release, the IFNγ result from a control tube without antigen was subtracted from the experimental results (Fig. 7c).

Statistics
In paper I, the differences in the incidence of CMV disease/graft rejection between the various groups with different immunosuppressive protocols were analyzed with Fisher’s exact test.

In paper II and III, paired data obtained at various time points in the same subject were compared using the Wilcoxon signed rank test. Differences between groups were assessed by Mann-Whitney U-test and correlations were calculated using Spearman’s nonparametric correlation test. Median (minimum-maximum) values were used unless stated otherwise. In paper III,
Fisher’s exact test (two-tailed) was used to determine sensitivity and specificity.

In paper IV, univariate analysis of quantitative variables was conducted using Student’s t-test, Mann-Whitney U-test or one-way ANOVA, whereas binary categorical variables were analyzed using the chi-square test or Fisher’s exact test according to sample sizes. The Kaplan-Meier method was used to estimate patient and graft survival, and the results are indicated as mean percentages. All statistical tests were two-tailed, and p-values <0.05 were considered significant. Median values with ranges (minimum-maximum) are reported unless stated otherwise. All statistical calculations were made with Statistica software (StatSoft, Inc., Tulsa, USA).
CMV disease in renal transplant patients (I, III, IV)

In Paper I, we retrospectively studied 25 CMV-mismatched (D+/R-) renal transplant patients who received low-dose prophylaxis with VACV (3g/day). The primary endpoint was CMV disease and time to onset of symptoms. CMV disease was considered to be present if a patient had CMV DNAemia, fever ($\geq 38.0^\circ$ C) for >2 consecutive days, and one or more of the following: leukopenia, thrombocytopenia, pneumonitis, gastrointestinal disease, hepatitis, or ophthalmologically detected retinitis. Alternatively, patients were diagnosed with CMV disease if they presented with CMV DNAemia and any kind of illness responsive solely to treatment with ganciclovir or foscarinet. Six of the 25 (24%) patients developed CMV disease within 6 months after transplantation, a number that was about half of that recorded for our historical controls [154]. The time to onset of symptoms was a median 156 days (92-191). None of the patients developed CMV disease during the period of VACV prophylaxis. The symptoms were mild or moderate in five of the six cases, and these patients recovered completely with GCV therapy for 3 weeks. None of the nine patients treated with basiliximab induction and VACV developed CMV disease, whereas one patient outside the protocol, who received basiliximab but not VACV developed a symptomatic CMV-infection.

In paper III, 17 CMV-seropositive renal transplant recipients (D+/R+) were prospectively followed for 1 year post-transplantation. CMV disease in these patients was defined as CMV DNAemia in the plasma, together with fever ($>38.0^\circ$ C) for >2 consecutive days and any clinical symptoms compatible with CMV-infection: leukopenia (below reference values); elevated liver transferases (above reference values); or organ-specific disease, such as colitis or pneumonia, with no other plausible explanation. One patient (6%) developed CMV disease.

In paper IV, we retrospectively studied 102 CMV-mismatched (D+/R-) renal transplant patients who received low-dose prophylaxis with VACV (3g/day). Of these, 26 patients (25%) were diagnosed with CMV disease during the first year post-transplantation (Fig.8). CMV disease was classified into three different categories: CMV syndrome, tissue-invasive CMV disease, and clinical CMV disease. CMV syndrome required a fever ($>38.0^\circ$ C) for >2 consecutive days without other possible cause and detection of CMV.
in blood or serum and any of the following symptoms: fatigue, malaise, arthralgias, decreased renal function, leukopenia, or elevated transaminases. Tissue-invasive disease required any of the above symptoms and detection of CMV in biopsies. Clinical CMV disease was any condition that did not fulfil the criteria listed above but clinical judgment led to treatment with anti-CMV drugs. The time to CMV disease was a median of 124 days (26-191). Seven patients were diagnosed with CMV disease during prophylaxis at a median time of day 60 (26-91), but six of them did not fulfil the criteria for CMV syndrome or tissue-invasive disease.

Figure 8. Incidence of CMV disease. Comparison of the results of paper IV (Present study, VACV 3g) to other comparable studies on D+/R- renal transplant populations with different prophylaxis protocols. The Paya studies are based on a mixed solid organ transplant population, and therefore include more than just renal transplants. The shaded portion of the bars indicates clinical or investigator-treated CMV disease.
CMV resistance (IV)

Resistant CMV was considered to be present if, despite 4 or more weeks of treatment with any anti-CMV drug, the antiviral therapy had to be switched and the CMV load in the blood failed to decline or CMV could be demonstrated in biopsies. One patient (4%) with CMV disease (CMV colitis) fulfilled the criteria and developed a GCV-resistant CMV strain with a mutation in UL97.

Rejection and graft function in renal transplant patients (I, III, IV)

In paper I, biopsy-proven acute rejection (BPAR) was seen in 32% of the patients (8/25), as compared to 38% in our group of historical controls. At 6 months post-transplantation, impaired renal function with creatinine levels >200 umol/l was seen in 20% (5/25), including one patient on hemodialysis.

In paper III, 53% (9/17) of the patients experienced BPAR. Renal function, as measured by serum creatinine levels, was essentially unchanged between 1 year post-transplantation (median level, 138 μmol/l; (range 98-419), and 3 years post-transplantation (median level, 142 μmol/l; (range 101-388).

In paper IV, 17 patients experienced one BPAR, five were diagnosed with two episodes of BPAR, and one was treated for a BPAR on three different occasions during the first year after transplantation. Thus, the total rejection frequency was 22% (Fig.9). The mean time to rejection was 50.2 +/- 58.0 days (median, 28 days; range, 5-236) and the rejection was classified as Banff I (A+B) in 52% and Banff II (A+B) in 48% of the cases. Rejection was generally treated with steroids (n=22), and in six cases (26%) antithymocyte globulin (ATG) was given. The use of basiliximab as induction therapy was correlated with an absence of rejection episodes (p=0.008). One-year graft survival was 95%; the mean serum creatinine level at 1 year after transplantation was 153.8 μmol/l (+/-65.4), and the median was 131 μmol/l (74-485).
Figure 9. Incidence of rejection. Comparison of the results of paper IV (Present study, VACV 3g) to other comparable studies on D+/R- renal transplant populations with different prophylaxis protocols. The Paya studies are based on a mixed solid organ transplant population, and therefore include more than just renal transplants.

CMV DNAemia and graft function in renal transplant patients (III)

In paper III, 16 of the 17 patients (94%) were positive at least once for CMV DNA in the plasma. In these patients, the first positive CMV DNA sample was detected at a median of 40 days (range, 12-227) after transplantation. The maximum level of CMV DNAemia (CMV max) during follow-up in patients without pre-emptive therapy (n=13) was a median of 8,400 (820-2.9 million) copies/ml, detected at a median of 95.5 (52-258) days after transplantation. A correlation was found between the initial viral load and CMV max, but there was no correlation between CMV max and graft function at either 1 or 3 years after transplantation. Two patients with CMV max >1 million copies/ml displayed no symptoms of CMV disease and received no antiviral therapy. Creatinine levels in the three patients with CMV max > 1 million copies/ml were 118, 138, and 180 μmol/l at 1 year and 118, 136, and 151 μmol/l at 3 years after transplantation.
Long-term clinical outcome in D+/R- renal transplant patients (IV)

The 5-year patient survival in the 102 patients (IV) was 92%, comparable to the data from the CTS database (Fig.10, top). Graft survival at 5 years after transplantation was also comparable to the data from the CTS (Fig.10, bottom).

Figure. 10. Patient survival (top) and graft survival (bottom) rates up to 5 years after transplantation. Comparison between all kidney transplants in Uppsala and Europe (EU w/o Uppsala; data from CTS) from September 1998 – June 2007. All patients were CMV D+/R- and received CMV prophylaxis.
Neurotoxic adverse effects of valacyclovir prophylaxis (I, IV)

In paper I, the follow-up period was 6 months and no CNS adverse effects such as hallucinations, confusion, or paranoia were observed.

In paper IV, major neurotoxic adverse effects could be demonstrated in two (2%) of the examined patients (Fig.11). One patient developed confusion on day 31 despite a rather low dose of VACV (500mg thrice daily with a GFR of 30 ml/min). One patient experienced episodes of hallucinations on day 23 when he received GCV iv as a result of ATG-treated rejection, and on day 119 when he received VACV 1g thrice daily despite a diminished GFR (40 ml/min). The neurotoxic effects disappeared in both patients without discontinuation of the VACV prophylaxis. We found no evidence of other major neurotoxic side effects such as paranoia or anxiety. Insomnia, the most common neurotoxic side effect, was observed in 7 patients (7%). The prophylaxis was switched to oral GCV in only one patient, as a result of nephritis with a possible connection to VACV.

![Major neurotoxic adverse effects](image)

Figure 11. Major neurotoxic adverse effects. Comparison of the results of paper IV (Present study, VACV 3g) to other comparable studies on D+/R- renal transplant populations with different prophylaxis protocols.
Frequencies of CMV-specific CD8+ T cells (II, III)

Healthy subjects with latent CMV infection

In paper II, 53 latently infected but otherwise healthy individuals were studied. The tetramers used in the study are displayed in Table 1. The individuals were HLA-matched with at least two of these tetramers. Of the 53 individuals, 51 bound at least one, and 43 bound at least two of the tested tetramers. The frequencies of the different tetramer-binding populations were added, and the average (mean+/−SEM) frequency of CMV-specific CD8+ T cells was 3.5+/−0.8%, or 16+/−3.5 T cells/μl blood. Interestingly, the frequencies of CMV-specific CD8+ cells varied considerably between individuals, but the variation within each individual over time was small. Very high persistent levels of CMV-specific CD8+ T-cell binding to the HLA B*0801ERL tetramer were seen in two subjects: 25.9% (65 cells/μl) and 28.1% (118 cells/μl), respectively (see paper II, Fig.1 H for details). No clinical signs of reactivation or CMV DNAemia could be detected, and the reason for these high numbers remains unknown.

In paper III, 11 healthy subjects were longitudinally sampled during one year. These subjects were positive for HLA A*0201 or B*0701; they also displayed a large variation in CMV-specific CD8+ T cell levels between individuals (0.1-60.2 cells/μl), but the intra-individual variations were low during follow-up.

Healthy subjects with primary CMV infection

In paper II, the frequencies of CMV-specific T cells were investigated in five healthy individuals with primary CMV infection. In these patients, CMV-specific CD8+ T cells were found in high numbers within weeks after the first clinical symptoms. The pattern of T-cell levels during follow-up, expressed as the mean (range), resembled that seen in previous studies of patients with primary Epstein-Barr-virus [155] and immunosuppressed patients with primary CMV [156]. At 1 month after the onset of disease, the frequency HLA A*0101YSE tetramer expression was 35 cells/μl (18.8-50.5), and that for the A*0201NLV tetramer was 83.1 cells/μl (20.5-181.7). At 3 months, the frequencies were 5.1 cells/μl (4.9-5.2) and 18.8 cells/μl (4.3-33.3) for HLA A*0101YSE and A*0201NLV, respectively, and at 6 months, frequencies were down to levels seen in subjects with latent CMV infection.

Immunosuppressed subjects

In paper III, we demonstrated that the levels of CMV-specific CD8+ T cells before transplantation did not differ from those in control subjects. Immediately after transplantation, the T-cell levels decreased rapidly, and at 1 month
after transplantation, the tetraCD8 (both A*0201 and B*0701) counts were lower (median, 6.1 cells/μl; range, 0.3-29.7) than those at baseline. However, at 2 months, the levels were already comparable (median, 11.1 cells/μl; range, 0.6-119.1) to baseline levels, and at 4 months after transplantation, the frequency of CMV-specific T cells was significantly higher (median, 26.1 cells/μl; range, 3.7-145.9) than at baseline. The tetraCD8 levels then remained above baseline throughout the follow-up period, but this difference from baseline values was not statistically significant (Fig.12).

**CD4+ and CD8+ T-cell function (II, III)**

**Healthy subjects with latent CMV infection**

In paper II, functional analyses of the CMV-specific CD8+ T cells from 42 subjects were analyzed by intracellular cytokine staining. The cells were stimulated with a pp65 peptide pool, and the mean number of CMV-specific CD8+ T cells showing IFNγ production was found to be 3.9+/−0.6/μl blood (range, 0-17.8).

**Immunosuppressed subjects**

In Paper III, the levels of IFNγ−producing CD8 (peptide-stimulated) and CD4 (lysate-stimulated) T cells before transplantation did not differ from those in the controls. Immediately after transplantation, the T-cell levels decreased rapidly. At 14 days after transplantation, IFNγCD8 (median, 0.9 cells/μl; range, 0-6.0) and IFNγCD4 (median, 1.8 cells/μl; range, 0-14.4) were already significantly reduced (p<0.05) and continued to decrease thereafter. At 2 months after transplantation, the IFNγCD8 numbers reached a nadir, with a median of <0.03 cells/μl (range, 0-10.4; p<0.05), and the IFNγCD4 reached a plateau, with a median of 1.2 cells/μl (range, 0-10.3; p<0.005). The IFNγCD8 values then rebounded to levels similar to baseline (p=n.s) at 4 months after transplantation, whereas the IFNγCD4 values remained low until 1 year after transplantation (Fig.12).
Figure 12. From the top: The number of tetramer-selected CD8+ T cells, CMV-specific interferon-γ-producing CD8+ T cells and interferon-γ producing CD4+ T cells in renal transplant patients (filled bars, ■) monitored up to 1 year after transplantation, and in controls (unfilled bars, □) monitored for 1 year. The values are expressed as medians and 25%-75% range. The dotted line (---) refers to baseline values obtained immediately before transplantation in patients, and at the start of the study in controls. Significant changes were seen in the transplant group in all T-cell populations, whereas the variation was small in the control group. See text for details.
Frequency and function of CMV-specific CD8+ T cells (II)

When we correlated the frequency of tetra+CD8 T cells with the number of functional (IFNγ-producing CD8+ and CD4+) T cells in paper II, we saw two patterns (Fig.13): A majority of the latently infected healthy individuals displayed low numbers of both tetra+CD8 and functional T cells. One-third of these subjects, however, had high levels of one of these two parameters; none were high in both.

*Figure 13. Relationship between the total number of CMV-specific CD8+ T cells (total tetramer-binding cells) and number of IFNγ-producing CD8+ T cells in subjects eligible for analysis with three or four tetramers. A straight line obtained by least-squares analysis is shown for data in the upper quartile of either of the two parameters, represented by the open symbols (○).*

These findings suggest that CMV remains inactive in most individuals, whereas in a minority of CMV-infected people, an ongoing low-grade replication can occur that may be handled either by producing a small clone of capable CD8+ effector cells or by up-regulating the size of the less effective T-cell clone(s).
CMV-specific CD4+ T cells and CMV DNAemia (III)

In paper III, correlations were sought between the various T-cell populations and CMV$_{\text{max}}$ in patients not receiving pre-emptive therapy (n=13). The relative reduction in IFN$\gamma$CD4 levels at 2 months after transplantation, when compared to baseline values, was found to correlate with the CMV$_{\text{max}}$ (Fig.14). All six patients with IFN$\gamma$CD4 levels <15% of baseline values at 2 months after transplantation developed high-grade DNAemia, with CMV$_{\text{max}}$ >10,000 copies/ml, either at 2 months after transplantation (n=4) or during follow-up (n=2). None of the seven patients with IFN$\gamma$CD4 levels >15% of baseline values at 2 months after transplantation developed high-grade CMV DNAemia. The sensitivity and specificity of this IFN$\gamma$CD4 cut-off level in this group of patients were 100% (p<0.001).

*Figure 14.* Correlation between the relative IFN$\gamma$CD4 reduction from baseline to 2 months after transplantation and CMV$_{\text{max}}$. High-grade CMV DNAemia, >10,000 copies/ml, occurred when the percentage of IFN$\gamma$CD4 cells decreased to below 15% of baseline values.
The absolute number of IFNγCD4 at 2 months after transplantation was directly correlated with the CMV\textsubscript{max}, but no correlation was found at baseline or during the first month after transplantation. However, both patients with undetectable levels of IFNγCD4 at baseline developed high-grade DNAemia. No correlation was found between the CMV\textsubscript{max} and the absolute values for IFNγCD8 or tetraCD8, or the absolute reduction in IFNγCD4, IFNγCD8, or tetraCD8 during the first 2 months after transplantation.

**Immunodominance (II)**

The T-cell response in persons with latent CMV infection is directed against several CMV epitopes. In paper II, we wanted to see whether the frequencies of different CMV epitopes within the same donor were of equal magnitude or whether any of the epitopes displayed immunodominance. In 18 of 37 individuals (49\%) who were able to bind at least two tetramers, the frequency of CD8+ T-cell binding to one of the tetramers was at least 10 times higher than that seen for the tetramer with the second-highest binding frequency. The immunodominant epitope was identified by comparing T-cell frequencies for different tetramers within the same donor. Using these frequencies, we calculated the immunodominance value for each tetramer (Table 2, details in paper II, Fig.2). For example, the A2 tetramer bound with higher frequency in 27 of 57 tetramer pairs examined, and therefore had an immunodominance value of 27/57=0.47. We found that the B*0702\textsubscript{TPR} epitope (0.82) was the most immunodominant of the epitopes available to us.

*Table 2. Immunodominance values based on observations from 53 healthy donors*

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<td>A*0201\textsubscript{SLV}</td>
<td>A*0101\textsubscript{YSE}</td>
<td>B*3501\textsubscript{IPK}</td>
<td>A*0301\textsubscript{TVY}</td>
<td>A*2402\textsubscript{QYD}</td>
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<td>0.82</td>
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DISCUSSION

CMV, termed the “troll of transplantation” by Balfour as long ago as 1979 [157], remains an important pathogen in transplant patients. Both prophylactic and pre-emptive strategies have been developed during the past 20 years to decrease the risk of CMV disease. In 1989, prophylaxis with ACV was demonstrated to be successful in reducing CMV disease [84]. In 1999, VACV 8g/day was shown to yield a significant reduction in CMV disease [86], and in 2004, VGCV was introduced as prophylaxis at many transplant centers after a publication by Paya et al. [92]. On the other hand, pre-emptive strategies involving both GCV and VGCV have also been demonstrated to reduce the incidence of CMV disease [158, 159]. There are pros and cons to both strategies and there is still ongoing debate among different transplant populations and centers about which approach to choose [80, 96, 114, 160]. Recently published evidence-based guidelines [43, 97] recommend universal prophylaxis for patients at the highest risk for CMV disease, whereas preemptive therapy may be most appropriate for those with a moderate or low risk of developing CMV disease. There are, however, still questions to be answered: Issues such as what drug to use, how to respond to adverse reactions and the emergence of resistance, what constitutes an optimal dose, when to start prophylaxis, and how long therapy should be continued still need to be defined.

VACV prophylaxis in the high-risk (D+/R-) population

In the high-risk D+/R- renal transplant population, the risk of developing CMV disease when high-dose VACV prophylaxis is used is comparable to oral GCV [161], whereas the risk with oral GCV is comparable to VGCV [92]. The use of high-dose VACV has been limited because the potential neurotoxic adverse effects associated with this protocol can be profound and have lead to discontinuation of the drug in as much as 6 to 24% of patients in some studies [80, 161], however, in one study high-dose VACV was used without neurotoxic adverse effects [162]. These effects are presumably related to the use of the high VACV dose [163]; we hypothesised that a lower dose of VACV would still be able to prevent CMV disease without causing neurotoxic adverse effects. This prediction was confirmed in paper I and IV, in which we demonstrated that prophylaxis with low-dose VACV, 3g/day,
decreased the incidence of CMV disease without any drug-related neurotoxic adverse effects. These findings have also been supported by other studies [164]. The incidences of CMV disease in paper I and IV was 24% and 25% respectively, and these rates are at the high end of the range reported for similar prophylaxis studies [86, 92]. These high rates may be attributed to our wide-ranging definition of CMV disease; if a stricter definition had been used in paper IV, the incidence would have been 10%.

In general, CMV morbidity was a minor problem, since most infections were easily treated, and in only a few cases was treatment given repeatedly. One observation that deserves comment is the fact that seven patients developed CMV disease during prophylaxis (IV). This frequency is higher than that observed in other prophylaxis studies [86, 165] and could indicate that a low-dose VACV protocol is insufficient in some cases. These patients who developed CMV were, however, easily treated without sequelae, with the exception of the patient with GCV-resistant CMV who developed a severe colitis.

Concerns have been raised about prophylaxis because of the possible increase in late-onset CMV disease and the higher proportion of tissue-invasive disease that has been demonstrated after VGCV prophylaxis [165, 166]. These findings could potentially be explained by the profound viral suppression that is caused by VGCV, which could prevent an effective antigen-induced priming of the host defence. As a result, when anti-viral prophylaxis is withdrawn, the late-onset CMV infection might rapidly develop into CMV disease because of the absence of CMV-specific T cells. If so, VACV, which is not as highly effective as VGCV, could be a more appealing choice because low-level viral replication could more easily occur, priming their CMV-specific immunity.

Two very important endpoints after a renal transplantation, are long-term patient and graft survival rates, and it has been shown that persistant CMV infection can affect long-term graft survival [167, 168]. The impact of CMV disease on long-term graft outcome, however, has been a matter of discussion and studies have produced conflicting results [52, 75, 167]. In paper IV, we demonstrated that low-dose VACV prophylaxis produced results that were equal or better than those from other prophylaxis studies [86, 92, 169] and the CTS database (paper IV, Fig.1 and 2), with 5-year survival rates of >90% (patient) and >80% (graft).

VACV therefore provides an alternative choice for CMV prophylaxis, allowing GCV and VGCV -the current prophylactic drugs- to be reserved for the treatment of CMV disease. This could potentially decrease the likelihood of creating strains that are GCV-resistant.
CMV resistance

The emergence of resistance is a matter of increasing concern; in patients with CMV disease who are infected with drug-resistant virus, the clinical outcome is generally poor, with a mortality rate of up to 20% [108, 114, 115]. It is believed that the higher bioavailability of GCV that follows the administration of VGCV could reduce the risk of GCV resistance [170], but in a recent article by Eid et al. [114], in which VGCV prophylaxis was used in D+/R- solid organ transplant patients, as many as 14% of the patients with CMV disease had drug-resistant virus. The risk of developing drug-resistant CMV in the mismatched renal transplant population has been estimated to be as high as 5% [108], and since the number of renal transplants is high, the need for an optimal prophylactic strategy is clear. One way to reduce GCV-resistant CMV might be to reserve GCV for treatment and switch to other compounds, such as VACV, for prophylaxis. In a study of VACV prophylaxis by Alain et al. [171], a very low incidence of GCV-resistance was demonstrated, and it was postulated that VACV may be less likely to select UL97-mutated GCV-resistant strains than is GCV. This theory is supported by the findings in the present low-dose VACV prophylaxis study (IV), in which we found a very low incidence (1%) of drug-resistant virus.

Strategies in non-high-risk populations

In contrast to CMV mismatched (D+/R-) patients, the risk of developing CMV disease in seropositive patients receiving organs from seropositive donors (D+/R+) is regarded to be low to intermediate [63]. Data from the CTS has also indicated that graft survival in the D+/R+ group is unaffected by the use of prophylaxis (Fig.15). According to current guidelines, patients with low or intermediate risk, based on CMV serostatus, could benefit from pre-emptive therapy [43]. This strategy, however, is logistically demanding, and therapy is often introduced when the patient already has become symptomatic. Also, a large number of patients are monitored and treated “in vain”. It would be better to be able to pinpoint individuals at risk of developing CMV disease, but at present we lack the diagnostic tools to identify these individuals. Therefore, other approaches such as measuring patients’ specific immune status, could be of interest (III).
Figure 15. Graft survival up to 5 years after transplantation in D+/R+ kidney transplant patients with and without CMV prophylaxis.

CMV-specific immunity

In paper II and III, we have used tetramer analysis to enumerate CMV-specific CD8+ T cells. This T-cell population has been demonstrated to be of importance in SCT patients, in whom the number of tetraCD8 T cells has been reported to correlate with the risk of developing CMV disease [172]. In paper II, we found that the inter-individual variation in CMV-specific CD8+ T cells was large, and two healthy latently infected subjects displayed unusually high levels. Several factors could have contributed to these findings: One possibility is that these two patients experienced a local, low-grade reactivation of CMV. Another is the fact that they were slightly older (47 and 70 years of age), since some studies have reported a correlation between age and an increase in CMV-specific T cells [173, 174]. A third, and perhaps the most plausible explanation, is that the functional capacity of this clone was reduced and that an up-regulation of the numbers of T cells is needed to maintain an adequate total immunologic capacity versus CMV. This explanation is supported by the findings in paper II, in which we found a correlation between the frequency and function of CD8+ T cells (paper II, Fig.4); however, this concept requires further study.
Tetramer analysis: pros and cons

Tetramer analysis is rapid and specific and can be performed with small amounts of blood. However, there are limitations to this technique: The patient must have HLA alleles that match the available tetramers, and even though the number of different tetramers has increased recently, there are still a number of alleles lacking. Another disadvantage is that the number of peptides is limited, and even if patients have the right combination of tetramer and HLA, their T-cell clones may be directed against epitopes other than those presented by the tetramer.

These restrictions make it clear that tetramer analysis cannot be used to demonstrate the whole repertoire of CMV-specific T cells in an individual. It can, however, still be of value for monitoring a patient over time, since an increase or decrease in one tetramer-binding population should reflect changes in the total CMV-specific lymphocyte population. One other disadvantage of tetramer analysis is that the number of CMV-specific CD8+ T cells does not necessarily reflect the individual’s level of CMV-specific immunity. This situation was seen in both latently infected subjects (paper II), in whom high numbers of CMV-specific CD8+ T cells were found to correlate with low levels of functional T cells, and in immunosuppressed subjects (paper III), in whom the dynamics of CMV-specific CD8+ T cells differed markedly from those of their IFNγCD8 and IFNγCD4 T cells (paper III, Fig.3). Several studies have tried to use levels of tetramer-binding cells as a marker for clinical outcome in various transplant populations, but the results have proved inconclusive [172, 175-177].

Dynamics of IFNγCMV-specific T cells

When it comes to monitoring CMV-specific immunity, it seems logical to measure the levels of functional T cells, and even though functional assays such as intracellular cytokine staining after pulsing with CMV antigens are time-consuming and laborious, this approach may be more promising. In paper III, we demonstrated that after an initial rapid decrease in all T cells, IFNγCD8 regeneration occurred, but with a delay of approximately one month when compared to the tetraCD8 response. This pattern suggests that the first CD8+ cells may have expanded rapidly but were ineffective and that more effective IFNγCD8 cells were generated over time. Recovery of IFNγCD8 activity has been suggested to be protective against CMV disease in transplant patients [178, 179]. Even though we were unable to demonstrate a direct correlation, the lack of IFNγCD8 response in one of the patients (UA-15, described in paper III, Fig.5), might explain the prolonged DNAemia observed in one patient.
High IFNγCD4 levels have also been shown to correlate with protection from CMV disease [180, 181]. In paper III, the IFNγCD4 response in our transplant patients was severely impaired during the entire first year after transplantation, suggesting that IFNγCD4 restoration in reactivated CMV infection in renal transplant recipients is delayed to a greater extent than had previously been thought [180, 182].

How should CMV-specific T cells be measured?

While previous studies have tried to correlate the percentage of IFNγ-producing CMV-specific cells with clinical CMV complications such as CMV DNAemia and CMV disease [180], we have focused on the absolute number of IFNγ-producing T cells. The reason for taking this approach is that CMV-specific T-cell number vary extensively between individuals with latent CMV infection (paper II, [20]), indicating that there is no general level of T-cell response required to maintain the infection in a latent state. Instead, a balance between the immune system and the virus is apparently established in each individual, and we suggest that the percentage of IFNγCD4 cells, as compared to baseline level, might be a more accurate way of defining the immunological capacity of a specific recipient. Therefore, the relative reduction in T-cell responses rather than the reduction in absolute numbers could be important. This hypothesis is supported by the findings in paper III, in which patients with a rapid and substantial decrease (>85%) in IFNγCD4 during the first 2 months after transplantation had a higher risk of developing high-grade CMV DNAemia.

We suggest that the dynamics of IFNγCD4, and specifically the level of reduction during the first 2 months after transplantation, when compared to baseline, may serve as valuable indicators for predicting the relative risk of CMV DNAemia. These prognostic factors could also be used for identifying subgroups of patients that could benefit from surveillance or prophylactic therapy. Today, prophylactic, pre-emptive, and deferred therapy are all used in various categories of patients with different levels of risk of developing CMV disease [96, 160]. Characterizing the CMV-specific immune responses with baseline samples obtained before the onset of immunosuppression is therefore of highest importance in the development of strategic approaches for pre-emptive or prophylactic therapy, or when monitoring functional T cells in clinical situations involving therapy failure. The dynamic changes in the levels of CMV-specific T-cell responses during the first months after transplantation could possibly even serve as a tool for monitoring the immune status at a more general level, providing the clinician with information for decision-making with regard to immunosuppression levels and/or prophylactic or pre-emptive therapy. These applications will have to be evaluated in larger cohorts of patients, using broadly targeted antigens for T-cell stimulation.
CONCLUSIONS

Paper I

• Low-dose (3g/day) VACV prophylaxis for 90 days post-transplantation reduces the incidence of CMV disease in CMV-seronegative renal transplant patients from 54% to 24%. The graft rejection rate (32%) was similar to those of historical controls (38%).

• Neurotoxic adverse effects like those previously reported with a higher VACV dose (8g/day) were not observed.

• CMV disease was not seen in the subgroup that received basiliximab as induction therapy.

Paper II

• In healthy subjects with a primary CMV infection, high frequencies of CMV-specific CD8+ T cells are seen initially after the infection, but after a few months, the levels are similar to subjects with a latent CMV infection.

• In subjects with latent CMV infection, the frequencies of CMV-specific CD8+ cells vary considerably between individuals, but the intra-individual changes over time are small.

• In subjects with latent CMV infection, the T-cell response is directed against several CMV eiptopes. The B*0702 TPRVTGGGAM epitope was the most immunodominant epitope of those available for testing.
Paper III

- CMV DNAemia is present in >90% of the CMV-seropositive renal transplant patients within the first year after transplantation.

- CMV-specific T-cells decrease rapidly after transplantation. TetraCD8 and IFN$\gamma$CD8 regenerate within 3 months, whereas IFN$\gamma$CD4 recovery is impaired during the entire first year after transplantation.

- The percentage of IFN$\gamma$CD4 T cells at 2 months post-transplantation, as compared to baseline, is strongly correlated with CMV DNAemia.

Paper IV

- Low-dose VACV prophylaxis (3g/day) for 90 days post-transplantation in D+/R- renal transplant patients results in a high 5-year patient and graft survival, comparable to that obtained with other prophylaxis strategies.

- Low-dose VACV prophylaxis reduces the incidence of CMV disease and acute rejection to the same levels as those of other prophylaxis strategies, such as high-dose VACV, oral GCV, or VGCV.

- Major neurotoxic adverse effects are minimal with low-dose VACV prophylaxis.
FUTURE PERSPECTIVES

Despite years of intense research on CMV and its effects, infection with this virus remains a multifaceted problem, and it is evident that much research still needs to be done. The major areas for future research are outlined below.

Among the most important tasks to focus on is the development of a CMV vaccine, with the primary target populations being CMV-seronegative women of childbearing age and high-risk transplantation populations. If a vaccine were successfully developed, a dramatic decrease in congenital CMV disease and transplantation-associated CMV disease would be expected, with a subsequent substantial reduction in morbidity, mortality, and hospital-associated costs. However, recent studies have revealed that vaccine development is very complex, and unfortunately there is no prospect of an effective vaccine becoming available in the immediate future.

In solid organ transplant populations with low or intermediate risk of developing CMV disease, as indicated by their CMV serostatus (e.g., D+/R+), it is of interest to target those patients who are subject to a higher risk for reasons unrelated to CMV serostatus. One potentially useful approach would be to explore CMV-specific immunity further and to establish certain cut-off levels for initiating more active strategies to prevent CMV disease. If prophylactic strategies are used, it is also important to decide whether and how these patients should be monitored after the cessation of prophylaxis in order to reduce late-onset CMV disease.

The development of antiviral drugs is another area in which increased research is needed. During the past 20 years, only a few anti-CMV drugs have been licensed, and the adverse effects of those that are currently available are troublesome. There is an urgent need for more alternatives, both in terms of decreasing adverse effects and as a source of possible combination therapies for minimizing the emergence of resistance. This need is particularly acute because the number of immunosuppressed patients continues to increase, thanks to the availability of more powerful treatment options for patients with both cancer and autoimmune diseases, and to the growth in the SOT and SCT populations.
Finally, more resources need to be channeled into research on the indirect and chronic effects of CMV, such as arteriosclerosis and cancer, and the potential exhaustion of immune defenses in the elderly. The question of whether CMV is a bystander in the chronic inflammatory process or an initiator is a matter for further exploration.

In summary, CMV is a fascinating and multi-talented virus that has evolved for millions of years, collecting a range of powerful tools that enable it to evade a variety of defense mechanisms and infect most of the human population. Another remarkable feature of this virus is its ability to maintain an equilibrium with the host’s immune defense after infection, allowing it to establish life-long persistence in the host. This persistence achieved by co-evolution has been viewed as a disadvantage but may also be of some significance for humans. This alternative remains to be explored, but it is just possible that this little microbial companion may prove vital to our survival for millions of years to come...
ACKNOWLEDGMENTS

This thesis is the sum of many peoples’ hard work, and I would really like to express my sincere gratitude to all those who have contributed and helped me through this process, both directly and indirectly. There are, however, some persons who have contributed a little extra, and I thank you all for that!

Britt-Marie Eriksson, my supervisor. The pace of this project has been close to perfect, with inspiring studies that have had a clinical aspect and relevancy. You have always been confident and believed in me, so thank you for letting me live my kind of life during these years! It has been a long journey but we have learned a lot along the way...

Olle Korsgren, Professor at the Department of Clinical Immunology and my co-supervisor. He always has a smile on his lips, but don’t let that fool you too often... Thank you for our ongoing collaboration and possible future projects.

Jan Sjölin, Professor at the Department of Infectious Diseases. One of the few people that I rank highly in all three areas: research, clinical medicine, and educational skills. A tough negotiator, while still managing to share a smile and a good story. I hope that we can have fun together for many more years to come!

Göran Friman, Professor emeritus at the Department of Infectious Diseases, who has supported me from the start, inspiring me with his vast knowledge of both infectious diseases and wine tasting.

Björn Olsen, our latest Professor at the Department of Infectious Diseases who, despite the little time we have spent together, has already inspired and supported me in scientific matters.

Göran Günther, the head of the Department of Infectious Diseases, who has supported me in both research and management decisions.
Birgitta Sembrant, what can I say... Without you this thesis would never have made it through the formalities! You have a tremendous knowledge of how to get through the red tape, and for you, problems don’t exist!

All co-authors and research facilitators, but especially:

Anna Karin Lidehäll, my predecessor in the CMV thesis work, who, apart from invaluable laboratory work and knowledge in the immunological area, has managed to give birth to two children during the time we worked together, thus giving me some extra time to catch up! You have also been the mastermind behind the immunological figures in the thesis and sometimes more of a co-supervisor (at least in the immunological field!).

Kerstin Claesson, a transplant surgeon who not only read but scrutinized our common manuscript, and then, uncompeled, offered to help me with the chapter on immunosuppression and renal rejection in my thesis.

Gunnar Tufveson, Professor at the Transplant section, with whom I just recently started to collaborate. Another person with a smile below the mustache! I really hope that there will be more mutual research projects in the future.

Ingrid Skarp Örberg, the best transplant coordinator there is, with a fantastic organizational capacity. Without your personal knowledge of all the renal transplant patients and your connections to all the regional hospitals, little of this research would have been done.

Selina Parvin and Tobias Lundberg at the Department of Clinical Immunology, for your hard lab work when all the immunological samples had to be analyzed.

Kåre Bondeson, Jonas Blomberg, and Björn Herrmann at the Department of Clinical Virology, for collaboration and fruitful discussions about the mysteries of CMV and other viruses.

Deborah ”Debbie” McClellan for the fast, humorous and excellent language editing!

I also want to thank all my great colleagues and personnel at the Department of Infectious Diseases who still make my ”working life” interesting and fun, but in particular:
**Elisabeth Löwdin**, my mentor, who has taught me *everything* (almost!) I need to know when it comes to practising infectious disease medicine!

**Mia Furebring**, my partner on the clinical board, with remarkable capabilities. You always prove that it is possible to do more if you just stay focused, not to mention getting things done administratively...

**Ingrid Uhnoo**, my first clinical supervisor, who incessantly demonstrated how thoroughly you could (should?) investigate a patient.

All the amazing secretaries; especially **Marie Sehlbrand**, the ”spider” on the clinical side, who has facilitated clinical work and practical matters since I started at the clinic, **Marianne Söderblom**, for all your help and supportive discussions concerning the schedule and other matters... and finally, **Titti Hamström** for the fantastic and invaluable help with the pictures on the thesis cover!

All the personnel at our regular and emergency wards, especially **Sissi** (our family’s own nurse!), and **Eva Söderberg**, with whom I have worked with since my first scary on-call nights! I have learned a lot from you both!

A great thank you to all my friends in different groups: other colleagues, personnel, relatives, choirmembers, neighbors, soccerteam members, old students, pokerplayers... all you friends who makes life worth living! Among all of these I would especially like to mention:

**Magnus Kaijser** and **Lena Rosenberg** and family, for pleasant, inspiring, and thoughtful conversations and family events, and also for toastmastering the two most important parties of my life, for my wedding and dissertation.

**Katja Fall**, who has demonstrated that even the hardest times can be dealt with without losing a taste for the good life with wine and cheese. I look forward to more of that, maybe in combination with some future CMV studies.

And last, some thanks to the people closest to me:

My second family: **Otto Cars**, also Professor at the Department of Infectious Diseases, who inspired me to start at the clinic, with whom I have had many stimulating discussions about work, research, music, nephews, practical Utö-matters and daughters... I’m also glad that we mutually decided that it would be better for me to start my research in the viral arena, since the fight against bacteria is almost over...
Karin Cars, my mother-in-law, for always stimulating the wine part of my brain, and providing me with clothes refused by the Cars-family members...

Thomas Cars, the nephew with whom you always can have a teasing chat. The amazing combination of a pharmacist, ”practical” wizard, and singer-songwriter whose music have entertained the most memorable moments of my life.

Stefan Cars, my second nephew, who has arranged for me to be able to write much of the thesis in my livingroom.

My dearest brother Krister, with Marie and their two adorable daughters Vera and Vanja. Thank you for being with me almost all my life and for all your skills that have forced me (as a big brother) to develop my own and have kept me on my toes.

My parents Robert and Margareta, who have provided me with the essential genes and environment to manage this kind of life. I especially appreciate the opportunities you have given me in terms of all the musical education, the endless support with childcare, and finally, the fact that you actually moved into our house to take care of my family during the final 2 months while I was finishing the thesis...

And finally: Ninna (or Katarina), for your never-ending energy, all kinds of support, including one figure in the thesis (!), and help with the dissertation party. You are a remarkable companion in life, and I love you!

And my three fantastic children, who with your different personalities always inspire me and remind me what life is really worth living for. You have provided plenty of moments of distraction, reduced my sleeping hours, helped me let go of my frustration, but most of all gived me all the love that I’m really sure I needed!

Till mina tre älsklingar som alla har sett dagens ljus under avhandlingsarbetet: Edla, Dante och Tyra - nu är virusboken klar och vi kan fortsätta upptäcka världen tillsammans!

The studies were conducted with financial support from the Olinder-Nielsen Foundation, Beckman-Coulter Inc. (III) and GlaxoSmithKline (I).
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