CLINICAL MICROBIOLOGY REVIEWS, Jan. 2009, p. 76–98 Vol. 22, No. 1
0893-8512/09/$08.00+0 doi:10.1128/CMR.00034-08
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Immunobiology of Human Cytomegalovirus: from Bench to Bedside

Tania Crough and Rajiv Khanna*

Australian Centre for Vaccine Development and Tumour Immunology Laboratory, Division of Immunology, Queensland Institute of Medical Research, Brisbane, Queensland, Australia

INTRODUCTION

Human cytomegalovirus (HCMV) is a ubiquitous beta human herpesvirus type 5. Compared to other human herpesviruses, HCMV is the largest, with a genome of ~235 kb encoding ~165 genes (56) (Fig. 1 and Table 1). The virion consists of a double-stranded linear DNA core in an icosahedral nucleocapsid, enveloped by a proteinaceous matrix (the tegument) (45). These components are enclosed in a lipid bilayer envelope that contains a number of viral glycoproteins (178). Mature virions range in diameter from 200 to 300 nanometers (178) (Fig. 2).

The tegument compartment contains the majority of the virion proteins, with the most abundant tegument protein being the lower matrix phosphoprotein 65 (pp65), also termed unique long 83 (UL83) (272). Other major tegument proteins include the virion transactivator pp71 (upper matrix protein, UL82 gene product), the herpesvirus core virion maturation protein pp150 (large matrix phosphoprotein, UL32 gene product), the largest tegument protein (UL48 gene product), and the UL99-encoded pp28 (272). In addition, the tegument also contains additional proteins that are present in small amounts and some cellular and viral RNA (178). The function of the tegument proteins can be separated into two classes: (i) proteins that play a structural role and are important for the assembly of virions and the disassembly of the particle during entry and (ii) proteins which modulate the host cell response to infection (178). The host cell endoplasmic reticulum-Golgi intermediate compartment-derived lipid bilayer envelope surrounding the tegument contains at least 20 virus-encoded glycoproteins that are involved in cell attachment and penetration (178) (Fig. 3). These include glycoprotein B (gB), gH, gL, gM, gN, and gO (272). Productive infection leads to the coordinated synthesis of proteins in three overlapping phases based on the time of synthesis after infection, namely, immediate-early (IE) (0 to 2 h), delayed-early (<24 h), and late (>24 h) viral proteins (260) (for more details, see Fig. 3).

TRANSMISSION, LATENCY, AND REACTIVATION

HCMV can be transmitted via saliva, sexual contact, placental transfer, breastfeeding, blood transfusion, solid-organ transplantation (SOT), or hematopoietic stem cell transplantation (SCT) (241). The seroprevalence of HCMV in the human population ranges between 30% and 90% in developed countries, with seroprevalence increasing with age (256). Primary HCMV infection in an immunocompetent host is normally asymptomatic, after which the virus establishes lifelong latency within the host and periodically reactivates. The mech-

* Corresponding author. Mailing address: Tumour Immunology Laboratory, Queensland Institute of Medical Research, 300 Herston Road, Herston (Qld) 4006, Australia. Phone: 61-7-3362 0385. Fax: 61-7-3845 3510. E-mail: rajiv.khanna@qimr.edu.au.

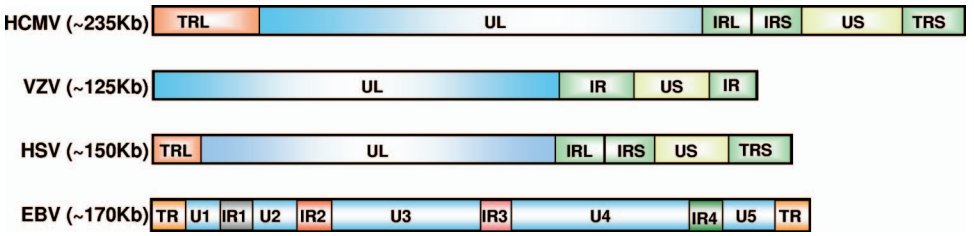

FIG. 1. Comparative schematic genome organizations of human herpesviruses. VZV, varicella zoster virus; HSV, human simplex virus; EBV, Epstein-Barr virus. The lettering within the individual regions of the genome depicts the following features: terminal repeat long (TRL), unique long (UL), unique short (US), internal repeat long (IRL), internal repeat short (IRS), terminal repeat short (TRS), and internal repeat (IR).

anism(s) by which HCMV establishes and maintains latency and reactivates remains poorly understood. During latent infection, the HCMV viral genome is estimated to be carried in between 0.004% and 0.01% of mononuclear cells from granulocyte colony-stimulating factor-mobilized peripheral blood or bone marrow, with approximately 2 to 13 genome copies per infected cell (248). The exact site of latency has not been definitively determined but appears to be in cells of the myeloid lineage (242) (Fig. 4). Genomic viral DNA can be detected in various cell types including monocytes/macrophages (251, 265), lymphocytes (233), $CD34^+$ bone marrow cells (171), immature dendritic cells (DCs) (235), and endothelial cells (95, 244). The presence of genomic DNA in some cell types, however, represents the phagocytosis of intact virions rather than active HCMV replication; the virus can enter the cell, but the transcriptional repression of the major IE (MIE) promoter prevents the production of new virions (245). Rather, the permissiveness of cells for active viral replication is directly related to the state of differentiation, i.e., differentiated cell types permit viral replication, whereas in undifferentiated cells, HCMV can enter, but the cells are nonpermissive for viral replication (245). Nonpermissive cells do nevertheless play an important role in the dissemination of the virus throughout the body.

Elucidating the factors controlling latency is imperative to restricting HCMV disease. Three possible pathways that lead to the establishment of latency have been proposed (46). Firstly, following attachment and entry, HCMV may enter directly into a latent state without de novo viral gene expression. A second possibility is that the virus initiates a productive infection after entry that is prematurely interrupted, subsequently leading to latency. Thirdly, following entry, the virus expresses a subset of viral genes that are not associated with productive infection but that are necessary for the successful establishment of latency. Insights into the mechanisms surrounding latency have been facilitated by studies of both naturally infected cells ex vivo and in vitro experimental model systems, enabling an examination of viral gene expression during latent infection (92, 134, 212). Experimental infection of granulocyte-macrophage progenitor cells have led to the identification of novel HCMV latency-associated transcripts encoded within the MIE promoter region of the HCMV genome (134, 135). These latent transcripts have also been detected in healthy seropositive individuals (136, 144); however, they were dispensable for establishing experimental latent infection in vitro (280), and thus, the functions encoded by MIE latent transcripts remain undefined. A transcript derived from the UL111.5A

TABLE 1. Classification of human herpesviruses

Herpesvirus	Abbreviation		Size (kb)
	Common	Formal	
Alphaherpesvirinae			
Simplexvirus			
Herpes simplex virus type 1	HSV-1	HHV-1	152
Herpes simplex virus type 2	HSV-2	HHV-2	155
Varicellovirus			
Varicella-zoster virus	VZV	HHV-3	125
Betaherpesvirinae			
Cytomegalovirus			
HCMV	HCMV	HHV-5	227–236
Roseolovirus			
Human herpesvirus type 6	HHV-6	HHV-6	159–162
Human herpesvirus type 7	HHV-7	HHV-7	144–153
Gammaherpesvirinae			
Lymphocryptovirus			
EBV	EBV	HHV-4	172–173
Rhadinovirus			
Human herpesvirus type 8	HHV-8	HHV-8	134–138

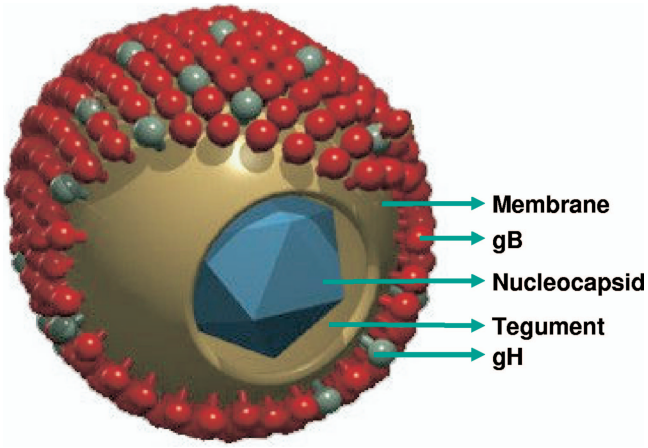

FIG. 2. Virtual three-dimensional model of HCMV showing various components of the virus. (Adapted from http://www.biografix.de/ with permission.)

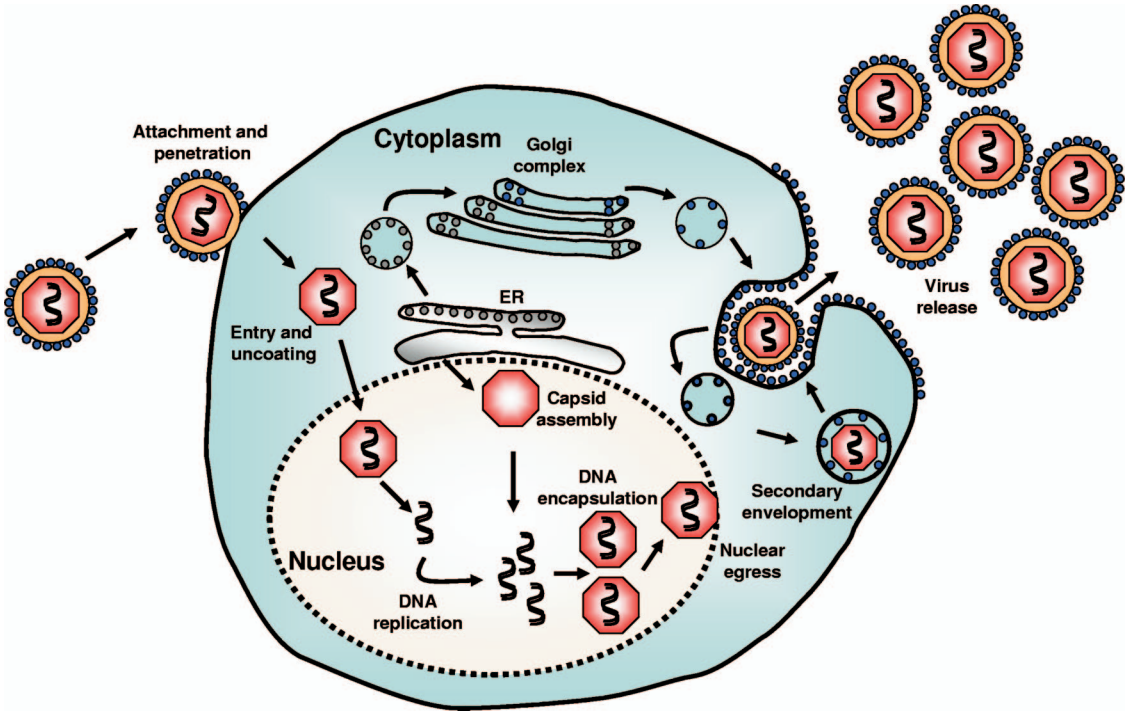

FIG. 3. Life cycle of HCMV in a human cell. HCMV enters human cells either through direct fusion or through the endocytic pathway. The virus attaches to the cell via interactions between viral glycoproteins (e.g., gB and gH) and a specific surface receptor(s) (e.g., platelet-derived growth factor α), followed by the fusion of the envelope with the cellular membrane to release nucleocapsids into the cytoplasm. These nucleocapsids are translocated into the nucleus, where viral DNA is released. This initiates the expression of IE-1/IE-2 genes. Viral replication and maturation follow the stimulation and parallel accumulation of viral synthesis function. This process involves the encapsulation of replicated viral DNA as capsids, which are then transported from the nucleus to the cytoplasm. Secondary envelopment occurs in the cytoplasm at the endoplasmic reticulum (ER)-Golgi intermediate compartment. This is followed by a complex two-stage final envelopment and egress process that leads to virion release by exocytosis at the plasma membrane.

region, encoding a variant of the viral interleukin-10 (IL-10) homologue, has been detected within latently infected granulocyte-macrophage progenitor cells and in naturally infected bone marrow and granulocyte colony-stimulating factor-mobilized blood samples (112). Furthermore, a latency-associated transcript that is antisense to UL81-82 of HCMV has been identified (16), which may inhibit the expression of the UL82 product pp71, which activates viral IE transcription and thus plays a role in initiating lytic infection (38). Studies indeed support the notion that HCMV transiently expresses a unique subset of viral genes in the absence of productive virus replication and that the virus does not initiate a productive infection within cells prior to the establishment of latency (46, 92). Two gene array studies using different models of latent HCMV infection have identified a number of viral genes associated with the establishment of latency, some common to both systems (46, 92). Recently, the UL138 open reading frame (ORF) detected in latently infected $CD14^+$ monocytes and $CD34^+$ progenitor cells from HCMV-seropositive donors was the first viral sequence proven to be functionally required for HCMV latency (91).

The reactivation of HCMV from latency is a key step in the pathogenesis of HCMV infection. HCMV reactivation can be detected in response to immunosuppression, inflammation, infection, or stress (141, 180, 207). The precise mechanism(s) leading to reactivation has not been elucidated; however, tumor necrosis factor alpha (TNF-α) is considered to be a key mediator (72). TNF-α engages the TNF receptor of latently infected cells, resulting in the activation of protein kinase C and NF-κB and, subsequently, the transcription of the HCMV IE genes, which ultimately triggers the onset of virus replication (206, 257). The reactivation of HCMV can also be achieved through stress catecholamines, epinephrine, and norepinephrine, increasing concentrations of cyclic AMP, thus leading to IE enhancer/promoter stimulation (207). Similarly, proinflammatory prostaglandins stimulated in the course of various inflammatory processes also promote viral reactivation through the cyclic AMP pathway (131).

PATHOGENESIS

HCMV rarely causes complications in the healthy individual; however, in the fetus, neonate, and immunocompromised patients, HCMV infection can cause an array of damaging clinical effects. Below is a summary of the incidence, pathogenesis, and clinical features of HCMV infection in various patient populations. More detailed descriptions of the clinical presentation of HCMV infection and pathogenesis are available elsewhere (2, 26, 79, 109, 163, 178, 220, 241, 258).

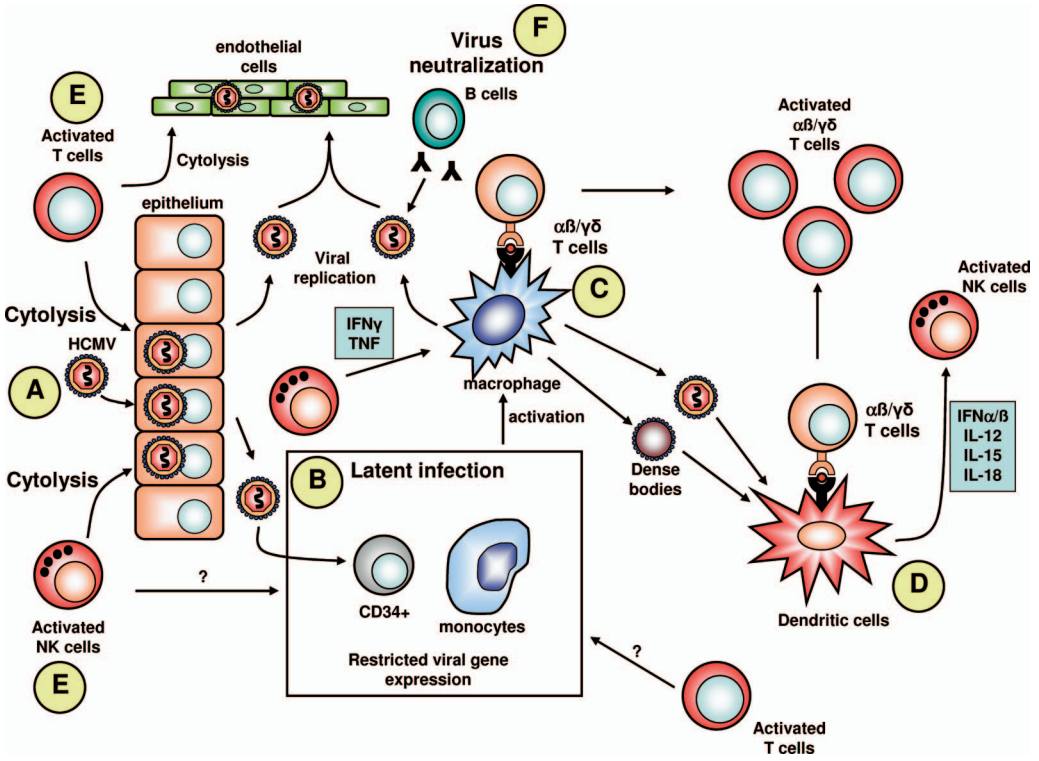

FIG. 4. Immune control of HCMV by innate and adaptive immunity. Primary infection with HCMV in healthy individuals typically initiates with replication in mucosal epithelium (A), after which the virus disseminates to monocytic cells of myeloid lineage including monocytes and $CD34^+$ cells, where it establishes latent infection (B). Restricted viral gene expression is observed in these latently infected cells, thus limiting their immune recognition by effector cells. The differentiation of these virus-infected monocytes into macrophages can initiate productive infection (C). Virus particles or virus-associated dense bodies can be processed by professional antigen-presenting cells (e.g., DCs), which can stimulate antigen-specific T cells (D). In addition, these DCs activated through TLRs can also secrete a range of cytokines/chemokines, which activate the innate arm of the immune system (e.g., NK cells) (D). Virus-infected macrophages can also directly stimulate antigen-specific T cells (C). These activated T cells ($CD8^+$, $CD4^+$, and/or γδ T cells) and NK cells can directly lyse virus-infected cells by cytolysis or block virus replication through the secretion of cytokines such as IFN-γ and/or TNF (E). Another important arm of adaptive immunity involves B cells, which are also activated by the professional antigen-presenting cells and control extracellular virus through antibody-mediated neutralization (F).

Infection of Immunocompetent Adults

Primary HCMV infection in the immunocompetent host is usually asymptomatic and rarely causes illness. In some cases, it can result in a mononucleosis syndrome, which is clinically indistinguishable from primary Epstein-Barr virus (EBV) infection, with fever, myalgia, lymphadenopathy, and hepatomegaly (79, 246) (Table 2). Tonsillopharyngitis, lymphadenopathy, and splenomegaly are less common symptoms of HCMV infection compared to EBV infection (79). Other rare complications of primary HCMV infections include arthralgia and arthritis, ulcerative colitis, pneumonitis, hepatitis, aseptic meningitis, and myocarditis (79).

Congenital and Neonatal Infection

Congenital HCMV infection causes severe morbidity and mortality in newborns and is the leading infectious cause of deafness and a large contributor of neurodevelopmental abnormalities in children (73, 219) (Table 2). The frequency of congenital HCMV infection resulting from primary maternal infection contracted during pregnancy or from the reactivation of HCMV in a seropositive mother during pregnancy is ~0.64% of live births; however, the incidence can vary consid-

TABLE 2. Clinical features of HCMV infection

Patient type	Clinical feature(s)
Healthy individual	Usually asymptomatic; infrequently mononucleosis with fever, myalgia, adenopathy, splenomegaly
Fetus/infant with congenital infection	Jaundice, hepatosplenomegaly, petechiae, microcephaly, hypotonia, seizures, lethargy
SOT recipient	Febrile illness with leukopenia and malaise; pneumonitis; enterocolitis, esophagitis, or gastritis; hepatitis; retinitis; other tissue-invasive disease (nephritis, cystitis, myocarditis, pancreatitis)
Hematopoietic SCT recipient	Pneumonitis; enterocolitis, esophagitis, or gastritis; less commonly retinitis, encephalitis, hepatitis
HIV/AIDS patient	Retinitis; enterocolitis, esophagitis, or gastritis; immune recovery vitritis-posterior segment inflammation; pneumonitis; hepatitis

erably among different study populations (122). The risk of primary infection in a seronegative mother is 1 to 4%, which carries a 30 to 40% risk of congenital infection (122, 254). The risk and severity of HCMV disease are greatest if primary infection in a seronegative mother occurs during the first trimester (192). The reactivation of an HCMV infection during pregnancy can still cause symptomatic congenital infection; however, the risk is lower, as preexisting maternal HCMV antibodies have a protective role against intrauterine transmission (74, 255).

Approximately 10 to 15% of congenitally infected babies are symptomatic at birth, exhibiting intrauterine growth retardation; hepatitis with jaundice and hepatosplenomegaly; thrombocytopenia with petechiae; pneumonitis; and severe central nervous system damage with microcephaly, intracerebral calcifications, chorioretinitis, and sensorineural hearing loss (122, 163) (Table 2). Of those symptomatic infants, a mortality rate of ~30% has been reported, while many others display serious neurological, visual, and hearing impairment (163). The majority of congenitally infected babies are asymptomatic at birth; however, 10 to 17% reportedly subsequently develop hearing defects or neurodevelopmental sequelae (79).

Infection of Immunocompromised Patients

HIV. HCMV is a serious opportunistic infection in immunocompromised individuals such as human immunodeficiency virus (HIV)-infected individuals and transplant patients on immunosuppressive medication due to an impaired adaptive immune system. Prior to the introduction of highly active antiretroviral therapy (HAART) in developed countries, approximately 40% of HIV-infected patients suffered from HCMV disease (258). The incidence of HCMV in patients with AIDS has significantly declined with the availability of HAART, as fewer patients have a $CD4^+$ T-cell count below <100 cells/μl, a threshold below which is a major risk factor for HCMV disease in HCMV-seropositive HIV-infected patients (189, 224). Despite this decline, HCMV infection continues to be problematic for HIV patients, and evidence suggests that it can directly and/or indirectly accelerate the progression to AIDS and death (96, 222, 277).

The most common manifestation of HCMV disease in HIV patients is retinitis, which accounts for ~85% of all cases and is characterized by hemorrhagic retinal necrosis (77, 258, 286) (Table 2). The use of HAART has, however, led to a newly recognized syndrome, "immune recovery vitritis," associated with posterior segment inflammation (119). This occurs almost exclusively in patients with a previous history of HCMV retinitis as the $CD4^+$ T-cell count reconstitutes upon antiretroviral therapy (119). Other manifestations of HCMV-associated disease include enterocolitis, gastritis, esophagitis, hepatitis, and encephalitis, with pneumonitis being a rare cause of lung disease in HIV-infected patients (258).

SOT. Despite improved treatment and surveillance, HCMV is still regarded as being the most significant infectious pathogen in the SOT recipient, and it continues to cause morbidity and mortality after transplantation and be associated with diminished graft survival. More than 50% of SOT recipients show evidence of HCMV infection, with 10 to 50% of patients developing symptomatic disease, depending on the serostatus of the recipient (R) and donor (D) (221). Due to the absence of a host-derived HCMV-specific immune response, the highest risk for infection is the combination of a serologically negative patient receiving an organ from a serologically positive donor (R^-/D^+), with disease being more severe in this group of patients (155). While matching of seronegative recipients to seronegative donors would be ideal, the availability of organs means that this is impractical. Other risk factors for HCMV disease include the type of organ transplantation (241), coinfection with human herpesvirus type 6 (62), and the type and intensity of the immunosuppressive regimen including the use of antibodies to T-cell receptors (TCRs) (20, 201). The administration of antilymphocyte antibodies such as anti-thymocyte globulin or OKT3 results in the release of large amounts of TNF-α and other proinflammatory cytokines, which may be involved in the activation of latent HCMV (72). In addition, the degree of viral load in the transplanted donor organ may also be proportional to the risk of HCMV disease due to the transplanted organ being rich in cells harboring latent or replicating HCMV; however, this notion is yet to be confirmed (12).

Clinically, acute HCMV infection in the immunocompromised SOT patient can manifest as an HCMV syndrome characterized by fever, leukopenia, malaise, arthralgia, and/or mascular rash or as tissue-invasive disease, which presents as hepatitis, pneumonitis, enterocolitis, encephalitis, chorioretinitis, nephritis, cystitis, myocarditis, or pancreatitis (109) (Table 2). Diagnosis of HCMV disease is made according to clinical signs and symptoms in conjunction with the detection of HCMV in the blood and in the involved tissues (109). In addition to directly causing end-organ disease, HCMV has also been associated with a number of damaging indirect effects in SOT patients. HCMV has been implicated in increased graft rejection (70, 94, 223) and is associated with renal artery stenosis in renal transplant recipients (11, 203), accelerated coronary artery stenosis in heart transplant recipients (137, 170), bronchiolitis obliterans in lung transplant recipients (13, 140), and vanishing bile duct syndrome in liver transplant recipients (7, 106, 146, 187); however, a causative role of the virus remains to be established. Furthermore, HCMV infection can also predispose transplant patients to opportunistic superinfection with a range of different microorganisms including *Pneumocystis carinii*, a variety of fungi, and *Listeria monocytogenes* (82, 241).

Hematopoietic stem cell transplantation. Due to a prolonged period of immunodeficiency following allogeneic SCT (allo-SCT), allo-SCT recipients are at significant risk of HCMV infection and disease (Table 2). In contrast to the SOT setting, HCMV infections following SCT are more frequently due to a reactivation of latent virus present in the seropositive recipient than primary infection (33, 40). Primary HCMV infection develops in about 30% of seronegative recipients, whilst reactivation of HCMV occurs in approximately 80% of patients who are seropositive before transplantation (158). The influence of the HCMV serostatus of the donor on the prognosis of an HCMV-seropositive patient remains controversial. Improved survival and reduced transplant-related mortality have been seen in HCMV-seropositive patients receiving grafts from HCMV-seropositive unrelated donors compared to those receiving grafts from HCMV-seronegative donors, potentially

due to the transfer of donor immunity (159). Other studies, however, have not shown any positive effect from using an HCMV-seropositive donor (25, 132). In addition to D/R serostatus, the risk of HCMV infections following allo-SCT is influenced by patient age, source of donor stem cells, degree of human leukocyte antigen (HLA) disparity between the D and R, use of T-cell-depleted grafts or anti-T-cell antibodies, conditioning regimen, posttransplant immunosuppression, time to engraftment, and prophylaxis of acute graft-versus-host disease (26, 102).

During the early (<100 days) SCT period, the most common clinical manifestations of HCMV disease are pneumonitis and enterocolitis (26). The introduction of antiviral therapy specifically targeting HCMV has dramatically reduced the incidence of early HCMV disease after allo-SCT and improved survival in certain high-risk recipients (90, 231). It is, however, associated with significant myelotoxicity and impaired hematological reconstitution and, consequently, higher rates of invasive fungal infections, which are extremely problematic following allo-SCT (33, 151, 184) (Table 2). The onset of late HCMV disease (>100 days post-SCT) has also emerged as a major complication post-allo-SCT and is threatening long-term survival (24, 65). In a study reported previously by Boeckh et al. (26), late HCMV disease developed in 17.8% of patients at a median of 169 days after transplantation, with a mortality rate of 46%. In addition to lung and gastrointestinal tract involvement, HCMV retinitis and encephalitis sometimes manifest in late HCMV disease, yet these are complications rarely observed in early HCMV disease post-SCT (26). Predictors of late HCMV disease include HCMV viral load, lymphopenia, and HCMV-specific T-cell immunodeficiency (26).

IMMUNE RESPONSES TO HUMAN CYTOMEGALOVIRUS

Innate Immunity

The innate immune system plays an important role in the defense against HCMV and also in priming the adaptive immune response. It is becoming increasingly apparent that HCMV is subject to innate sensing by Toll-like receptors (TLRs). The stimulation of TLRs by pathogens such as HCMV activates signal transduction pathways, which induce the secretion of inflammatory cytokines that recruit cells of the innate immune system, and the upregulation of costimulatory molecules such as CD80 and CD86, which are important for the activation of adaptive immunity (27, 48). TLR9 and TLR3 have been proven to be critical components of the innate immune defense against murine cytomegalovirus (MCMV) (60, 107, 264). Upon viral inoculation, their signaling pathways are activated, which leads to the production of alpha/beta interferon (IFN-α/β) by DCs and macrophages and the subsequent activation of natural killer (NK) cells (60, 107, 264) (Fig. 4). HCMV has also been demonstrated to activate and signal through the interaction of gB/gH and TLR2, which triggers inflammatory cytokine production (28, 48, 117).

NK cells are an integral part of innate immunity to cytomegalovirus. They have been shown to be involved in the clearance of experimental MCMV infection (36, 200), and the adoptive transfer of NK cells can provide protection against MCMV (35). Certain strains of mice are resistant to MCMV in vivo but become susceptible upon the depletion of NK cells (225, 226). A single autosomal dominant locus, termed Cmv1, contained in the NK gene complex on mouse chromosome 6 controls both survival and viral titers in the murine spleen (226), and this resistance is mediated by the murine NK cell activation receptor Ly-49H, contained in the NK gene complex (34).

In humans, relatively little is known about the role of NK cells in the immune defense against HCMV. In renal transplant patients, NK activity was shown to increase during both primary and recurrent HCMV infection, indicating that NK cells contribute to recovery from HCMV infection (273). In an isolated case report, NK cell deficiency was associated with severe primary herpesvirus infections, including HCMV disease (22). In a study of 43 patients with HCMV reactivation after allo-SCT in which 12 cases were fatal, the levels of nonspecific NK cell cytotoxicity correlated with the patient's ability to recover from infection (208).

Adaptive Immunity

Humoral responses. The establishment of long-lasting immunity in response to a primary HCMV infection, which serves to control subsequent HCMV reactivation in the host, is vitally important for preventing uncontrolled replication and serious HCMV disease. HCMV is a potent immunogen that triggers strong immune responses from all arms of the immune system. While the contribution of antibodies for protection against and control of HCMV has been debated, evidence does support a role for humoral immunity in the effective immune response against HCMV and MCMV, predominantly in restricting viral dissemination and in limiting the severity of the disease (29, 116) (Fig. 4). The major target for neutralizing antibodies to HCMV is gB, which is involved in cell attachment and penetration (32). It is responsible for at least 50% of the neutralizing antibodies in HCMV-infected individuals (32, 167). gH, which is involved in the fusion of the viral envelope with the host cell membrane, is another target that induces potent virus-neutralizing antibodies (210).

The importance of antibodies is supported by various animal and human studies. In the guinea pig model, passive immunization with guinea pig cytomegalovirus antibodies did not prevent infection, but the antibodies increased the survival of pups (31). Furthermore, the passive immunization of guinea pigs with anti-gB serum (43) and active immunization of pregnant guinea pigs with a recombinant gB vaccine (228) have been shown to decrease guinea pig CMV fetal infection and disease. In humans, the transfer of antibodies from an HCMV-seropositive mother to a newborn infant was shown to be protective against HCMV infection from seropositive blood transfusions (285). It is now well established that women with preconceptual immunity to HCMV transmit infection to the fetus at a lower frequency than women with primary infections (74, 255). In addition, if the antibody response to HCMV is of low avidity and poor neutralizing activity, the probability of the transmission of viral infection from mother to fetus is significantly increased (29).

T-cell-mediated immune responses. The cell-mediated immune response is the predominant mechanism by which HCMV replication is controlled, as with the exception of con-

genital infection, severe HCMV disease occurs almost exclusively in patients with profound cellular immunodeficiency. While the immune response induced by primary infection does not eradicate the virus, it is clear that HCMV-specific $CD8^+$ T cells, $CD4^+$ T cells, and γδ T cells are all important for controlling and restricting viral replication in hosts with persistent infection (Fig. 4).

(i) Role of $CD8^+$ T cells. An essential role for T-cell immunity was first recognized in studies using MCMV models in which the elimination of lymphocytes was coincident with increased levels of reactivation and dissemination of viral infection, and the adoptive transfer of virus-specific $CD8^+$ cytotoxic T lymphocytes (CTL) conferred protection from an otherwise lethal viral challenge (181, 211). The selective depletion of lymphocyte subsets in mice also revealed $CD8^+$ T cells to be the most important component in the immune control of MCMV (200). Furthermore, the depletion of $CD8^+$ T cells with a CD8-specific monoclonal antibody in monkeys infected with simian immunodeficiency virus was coincident with the reactivation of cytomegalovirus (14). In humans, fetal $CD8^+$ T lymphocytes that are mature and functional have been shown to expand in utero in response to a primary HCMV infection (165). In patients with AIDS, IFN-γ-producing HCMV-specific $CD8^+$ T cells appear to be protective against HCMV-associated retinitis (111). Clinical data from bone marrow transplant (BMT) patients also confirm a crucial role for $CD8^+$ T cells in the control of HCMV. The development of HCMV-specific CTL responses following BMT have been shown to correlate with protection (151, 214) and recovery from HCMV disease (208). In a study reported previously by Reusser et al. (214), more than half of patients lacking a detectable anti-HCMV T-cell response developed HCMV disease. Accordingly, pivotal studies by Riddell et al. (216) and Walter and coworkers (276) showed that the infusion of donor-derived HCMV-specific $CD8^+$ T cells effectively restored antigen-specific cellular immunity in allogeneic BMT recipients, with the immune reconstitution coincident with protection from HCMV-associated clinical complications in the recipients (216, 276). A similar importance for $CD8^+$ T-cell immunity has been shown in the SOT setting. Analyses of virus-specific T-cell responses in renal transplant recipients demonstrated the presence of dominant $CD8^+$ T-cell responses that may limit viremia and protect against HCMV disease (209, 213, 238). In lung transplant recipients, the acquisition of HCMV-specific $CD8^+$ T-cell immunity, in addition to $CD4^+$ T-cell immunity, was associated with both freedom from HCMV disease and the preservation of allograft function compared with those who failed to develop HCMV immunity (240). Furthermore, in a study involving heart and lung transplant recipients, high frequencies of IE-1-specific $CD8^+$ T cells were shown to correlate with protection from HCMV disease (37).

The proportion of $CD8^+$ T cells committed to the anti-HCMV response is extraordinarily large. A median of 10% of $CD8^+$ T cells in the peripheral blood of healthy virus carriers and up to 40% of $CD8^+$ T cells in the peripheral blood of elderly individuals can be specific for HCMV antigens (53, 86, 128, 263). It is not understood what makes HCMV so immunogenic so as to provoke such large immune responses and what impact this immune dominance has on the response to other pathogens/antigens. The magnitude of the immune response to HCMV and the presence of large populations of HCMV-specific T cells that can be easily measured ex vivo indeed provide an ideal model for not only studying HCMV but also examining the immune response to persistent viruses. The fine specificity of the $CD8^+$ HCMV-specific T-cell response and the viral proteins to which they are directed has been comprehensively examined in healthy HCMV-seropositive donors. The $CD8^+$ HCMV-specific T-cell response is considerably diverse, with recognition of a variety of structural, early, and late antigens in addition to HCMV-encoded immunomodulators including pp28, pp50, pp150 gH, gB, unique short 2 (US2), US3, US6, US11, UL16, and UL18 (69, 164, 263). Using overlapping 15-mer peptides from all 213 ORFs and ex vivo T-cell assays, other investigators (69, 164, 263) revealed that the $CD8^+$ and/or $CD4^+$ T cells are directed toward more than 70% of the ORFs. These studies in combination with the data reported by other groups revealed that these responses were directed toward HCMV-encoded proteins expressed at different stages of viral replication (IE, early, early-late, and late) and also proteins associated with diverse functions (capsid, matrix/tegument, glycoprotein, DNA/regulatory, and immune evasion) (Fig. 5A). In-depth ex vivo analyses of antigen-specific T-cell responses revealed an interesting hierarchy among virus-encoded proteins. A schematic representation of the hierarchy of the 10 most frequently recognized antigens is shown in Fig. 5B. It can be seen that the most immunodominant antigens to which HCMV-specific $CD8^+$ T cells respond are directed toward include UL123 (IE-1), UL122 (IE-2), and UL83 (pp65). The majority of T-cell studies to date have focused on IE-1 and pp65. It is important to note that the precise antigens critical for controlling HCMV have not been completely determined, and one cannot necessarily assume that the largest detectable response will translate into being the most important for restricting HCMV replication.

A number of studies have examined the impact of chronic HCMV infection on memory T-cell homeostasis and the differentiation phenotype of antigen-experienced $CD8^+$ T cells. Various phenotypic markers, including (but not restricted to) CD45RA, CD45RO, CCR7, CD27, CD28, CD62L, and CD57, in addition to functional markers such as the expression of IFN-γ, granzyme, and perforin have been commonly used to study the differentiation and effector functions of naïve and memory antigen-specific T cells. During acute HCMV infection, the main $CD8^+$ effector T-cell population shows a $CD45RA^-$ $CD45RO^+$ $CD27^+$ $CD28^{+/-}$ $CCR7^-$ phenotype, while in chronic HCMV infection, two types of HCMV-specific T cells appear to exist: $CD45RA^-$ $CD45R0^+$ $CD27^-$ $CD28^-$ $CCR7^-$ effector-memory or $CD45RA^+$ $CD45R0^-$ $CD27^-$ $CD28^-$ $CCR7^-$ terminally differentiated effector T cells re-expressing CD45RA (6, 78). Interestingly, studies of the macaque model have shown that the adoptive transfer of HCMV-specific $CD8^+$ T cells derived from central memory T cells, which express CD62L and CCR7, but not those derived from effector memory T cells persisted long term in the blood and migrated to lymph nodes and bone marrow (19).

Another interesting feature of the $CD8^+$ T-cell response to HCMV is the accumulation of an oligoclonal T-cell repertoire and a reduction in the naïve T-cell pool (57, 205). TCR selection is a highly complex process influenced by various factors. One such factor is the functional avidity of the antigen-specific

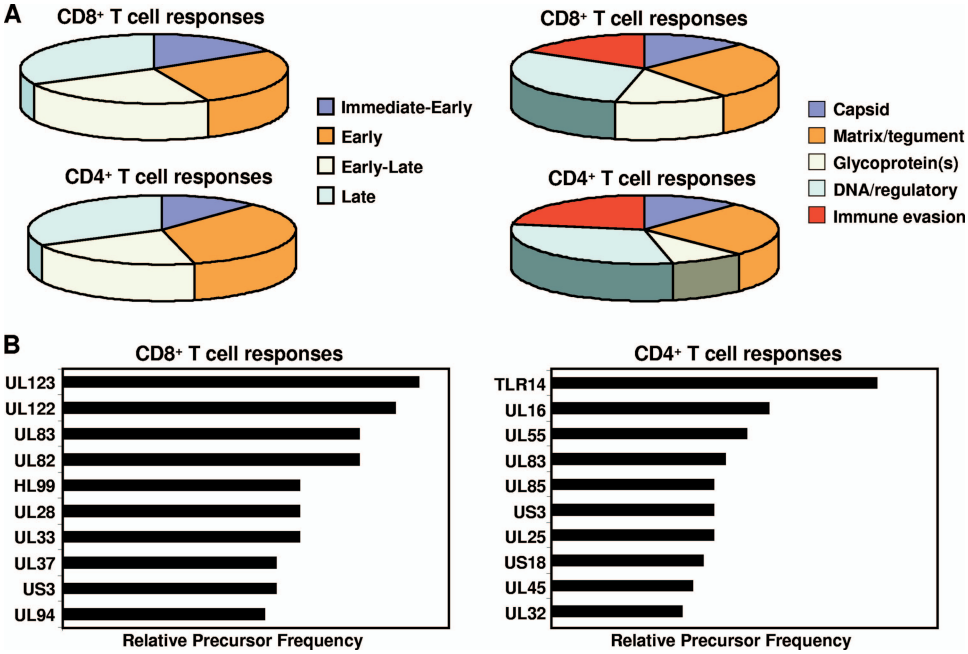

FIG. 5. Distribution of $CD4^+$ and $CD8^+$ T-cell responses within HCMV-encoded proteins. (A) Relative strengths of T-cell responses directed toward HCMV-encoded proteins with respect to expression kinetics (left) or gene function (right). (B) Schematic representation of the magnitude of $CD4^+$ and $CD8^+$ T-cell responses against immunodominant HCMV-encoded ORFs. The data presented in this figure are collated from data reported previously (69, 123–127, 133, 164, 263).

$CD8^+$ T cells. Dominant HCMV-specific clonotypes selected into the long-term memory pool have been shown to display high functional avidity, while subdominant clonotypes which contracted following primary infection were characterized by substantially lower avidity (57, 205). Other factors such as the structural landscape of the HLA-peptide complex and efficiency of endogenous antigen presentation by virus-infected cells have also been shown to influence the selection of the HCMV-specific TCR repertoire (284). Indeed, clonotypes with restricted TCR usage demonstrated more efficient recognition of virus-infected cells and displayed a terminally differentiated phenotype compared to T cells expressing diverse TCR (284). Therefore, high avidity and efficiency of endogenous viral epitope presentation in combination with biophysical characteristics of the HLA-peptide complex are the principal determinants that offer a competitive advantage for selection of the antigen-specific $CD8^+$ T cells into the memory repertoire and possibly for the position in the hierarchy of response. This has implications not only for the control of HCMV but also in the protection against persistent viruses in general.

The total size of the immune response and numbers of T cells in the periphery remain essentially unchanged over time because homeostatic mechanisms are engaged to remove the majority of the expanded population (5, 193). While the quantity of T cells remains relatively stable, the functional capacity and proportionate representation of distinct memory T-cell pools can be dramatically altered (5). The magnitude of the cellular immune response to various pathogens has been studied in donors of different ages, revealing that immunity to viruses such as influenza virus and varicella-zoster virus decreases with progressing age (9, 61). In striking contrast, an accumulation of HCMV-specific $CD8^+$ T cells occurs with age, such that it may represent over 40% of the $CD8^+$ T-cell pool (128, 129, 188). This increase in virus-specific CTL, termed "memory inflation," is a phenomenon also seen with the $CD8^+$ T-cell immune response to MCMV (120) and was very recently shown to also extend to the HCMV-specific $CD4^+$ T-cell response (202). Interestingly, the $CD8^+$ HCMV-specific T-cell expansions are invariably oligoclonal or, in some instances, monoclonal and display a highly differentiated effector memory cell ($CD28^-$ $CD57^+$ $CCR7^-$) phenotype (129). The clonal expansion and differentiated phenotype of the HCMV-specific CTL support the notion that HCMV may significantly contribute to immune senescence, which is characterized by a reduction in levels of naïve cells, the accumulation of clonally expanded $CD28^-$ memory T cells, and a decline in immune responsiveness (129, 193). Indeed, longitudinal-aging studies indicate that HCMV is associated with a cluster of immune parameters termed the "immune risk phenotype," which are predictive of increased mortality in individuals >80 years of age (5, 193). In addition to HCMV seropositivity, the parameters comprising the immune risk phenotype include an inverted $CD4^+/CD8^+$ T-cell ratio due to increased levels of $CD8^+$ T cells, an increased proportion of highly differentiated $CD8^+$ $CD28^-$ T cells, the presence of $CD8^+$ T-cell clonal expansions, and reduced mitogen-stimulated proliferative responses (5, 193). Furthermore, the apparent immunodominance by HCMV may hinder responses to other pathogens, as suggested by findings that HCMV seropositivity is associated with lower success rates for influenza virus vaccination (270) and is a cofactor that enhances progression to AIDS (96, 222, 277).

The general model relating to the establishment of a memory T-cell population in response to a viral infection was

thought to comprise an expansion period during the initial acute phase of infection, followed by a contraction phase once the infection is cleared, after which the surviving virus-specific T cells are maintained as an apparently stable pool of memory T cells (253). In conflict with this model, two studies have revealed that the human T-cell response against latent HCMV infection in fact displays continuous expansions and contractions similar to that seen during the acute phase of infection, albeit at lower levels (53, 64). HCMV-specific memory $CD8^+$ T-cell responses during persistent infection were shown to be highly dynamic, with frequent fluctuations in terms of both function and absolute number despite being part of a stable total T-cell population (53, 64). Furthermore, the fluctuations were synchronous not only between HCMV epitopes but also between HCMV antigens and with responses to the latent and lytic antigens of EBV. An absence of detectable HCMV indicated that the periodic reactivation of these persistent viruses was unlikely to be driving the observed homeostatic T-cell fluctuations. While the phenotype of HCMV-specific T cells in healthy individuals showed little change, a loss of CD62L expression in the total $CD8^+$ T-cell population (not virus-specific T cells) was coincident with the expansion of tetramer-positive virus-specific T cells. The observed fluctuations were therefore attributed to some form of a generalized "bystander" effect of infection, and immune activation was providing heterologous stimulation, which was directly influencing the dynamics of the HCMV- and EBV-specific T-cell population (53, 64).

(ii) $CD4^+$ T cells. While the role of the major histocompatibility complex (MHC) class I-restricted $CD8^+$ T-cell immune response against HCMV is clearly evident, there is increasing evidence that $CD4^+$ T cells are also integral to the control of HCMV infections (66, 239). In mice infected with MCMV, the selective depletion of $CD4^+$ T cells resulted in an increased incidence of recurrent MCMV infection (200). $CD4^+$ T cells have also been shown to contribute to the control of primary MCMV infection in mice that were long-term depleted of $CD8^+$ T cells before infection (115).

In otherwise healthy children who have acquired HCMV, prolonged viral urinary and salivary shedding is linked to a persistent and selective deficiency of HCMV-specific $CD4^+$ T-cell immunity (271). The importance of $CD4^+$ T cells also extends to the transplant setting. Low levels of HCMV-specific $CD4^+$ T cells correlate significantly with susceptibility to infectious complications with HCMV in lung transplant recipients (239). Following renal transplantation, clinical symptoms of HCMV have been shown to be preceded by a decrease in levels of HCMV-specific $CD4^+$ T cells and an increase in viral load, thus suggesting that levels of $CD4^+$ HCMV-specific T cells may be predictive of impending HCMV-associated disease (236). Another longitudinal study involving renal transplant recipients further demonstrated that the HCMV-specific effector memory $CD4^+$ T-cell response was delayed in individuals with symptomatic as opposed to asymptomatic HCMV disease, indicating that effector-memory $CD4^+$ T cells are necessary to control viral replication and for recovery of infection (78). In BMT recipients, the presence of a detectable $CD4^+$ T-helper (Th) response has been associated with protection from HCMV disease (101, 139, 151), and evidence suggests that the recovery of $CD4^+$ HCMV-specific Th cells is required for the endogenous reconstitution of $CD8^+$ CTL (214) and the persistence of adoptively transferred T cells (276). Einsele and colleagues further illustrated the requirement for $CD4^+$ T cells in the control of HCMV (66). In that study, the adoptive transfer of predominantly $CD4^+$ HCMV-specific T-cell lines resulted in a dramatic reduction of viral load in allo-SCT patients (66). A single infusion of T cells resulted in the clearing of HCMV viremia in five out of seven patients, and a second infusion was associated with the resolution of viremia in an additional patient. The transfer of HCMV-specific $CD4^+$ T cells was also coincident with the expansion of $CD8^+$ CTLs, suggesting that without T-cell help, these effectors may not have been activated.

Similar to the $CD8^+$ HCMV-specific T-cell compartment, an extremely high frequency of $CD4^+$ T cells in healthy seropositive individuals is committed to anti-HCMV immunity. HCMV-exposed individuals devote a median of 9.1% of their circulating $CD4^+$ memory T-cell population to this virus (263), with this proportion extending up to 40% in some donors (237). Analysis of the specificity of the HCMV-specific $CD4^+$ T-cell response has revealed broad antigen recognition (Fig. 5). Although gB-specific $CD4^+$ T-cell responses are most frequently detected in healthy individuals (>30%), occasionally, higher number of precursors specific for TRL14 and UL16 can be detected in a small number of individuals (<5%) (263). Interestingly, much of the $CD4^+$ CTL response to gB and gH antigens has been shown to be directed toward highly conserved regions present in both clinical isolates of HCMV and isolates of virus from nonhuman primates (68).

Conventionally, the role of $CD4^+$ T cells in latent infections was considered to be indirect, through the provision of T-cell help in maintaining virus-specific antibody responses (55) and expanding the $CD8^+$ T-cell populations (276). However, studies in fact support a direct role for HCMV-specific $CD4^+$ T cells in controlling infection by killing virus-infected cells. gB-specific $CD4^+$ T cells with cytotoxic activity from healthy seropositive individuals and pregnant women have been successfully expanded in vitro (68, 108). Furthermore, the acquisition of direct cytolytic activity by pp65-specific $CD4^+$ T cells has been shown to occur as a function of the differentiation state (39). Evidence for a direct cytolytic role for gB-specific $CD4^+$ CTL in vivo comes from a study whereby $CD4^+$ T cells directly purified from the blood secreted granzyme B in response to glial cells expressing endogenous gB (103). Characterizations of these gB-specific $CD4^+$ CTL have identified the highly immunodominant peptide epitope DYSNTHSTRYV from gB, which is restricted through HLA DRB*0701 (68).

Recent studies have identified an interesting link between the HCMV-specific $CD4^+$ T-cell response and an unusual presentation of large granular lymphocytosis. Ex vivo analysis of $CD4^+$ T-cell large granular lymphocytosis revealed a highly restricted usage of TCR-Vβ13.1 (81). Garrido and colleagues hypothesized that these monoclonal expansions might be linked to the antigen-driven stimulation of these malignant cells (81). This hypothesis was confirmed by Crompton et al., who showed that TCR-Vβ13.1 $CD4^+$ T cells recognized the HLA DR7-restricted HCMV-specific $CD4^+$ T-cell epitope DY SNTHSTRYV (52). Furthermore, the transient proliferation of an aberrant TCRγ $CD8^+$ clone in a patient with acute HCMV infection was previously reported (168), while

TABLE 3. HCMV-encoded immune evasion proteins and their functions

HCMV protein	Immune evasion function(s)	Reference(s)
gpTRL11	Serves as viral Fc receptor to block ADCC	10, 154, 252
gpUL16	Binds to nonclassical MHC proteins (MICB, ULBP1, and ULBP2) and thus blocks NKG2D-mediated NK cell activation	50
gpUL18	Binds LIR1 and thus interferes with NK cell recognition; also acts as ligand for HLA G (expressed on fetal trophoblast); may have a role in utero	42, 150, 215
gpUL27	Chemokine receptor; postulated to modulate host type I/II interferon effects	148
gpUL33	Orphan chemokine receptor; may be important for viral dissemination	44, 166
gpUL40	Encodes peptide that binds HLA E and thus interferes with NK cell recognition	266
gpUL78	Orphan chemokine receptor; may be important for viral dissemination	44
gpUL83	Inhibits proteasomal processing of IE-1	84, 85
gpUL111a	Homologous to human IL-10; inhibits MHC class I/II expression and lymphocyte proliferation	138
gpUL118	Immunoglobulin G Fc receptor; function not defined	10, 252
gpUL144	TNF receptor homologue; function not defined	17
gpUL146	Potent IL-8-like chemokine (referred to as viral CXC-1); induces chemotaxis of neutrophils	195
gpUS2, gpUS3, gpUS6, gpUS10, and gpUS11	MHC class I/II downregulation through interference in transporters associated with antigen processing and tapasin-dependent peptide loading; ubiquitin-dependent retrograde dislocation of MHC from the endoplasmic reticulum; prevents egress of MHC class I from the endoplasmic reticulum to the Golgi apparatus	4, 76, 113, 114, 281
gpUS28	Chemokine receptor; sequesters CC chemokines and may assist in viral dissemination	80, 130, 183, 261

HCMV has also been linked to the pathogenesis of clonal TCRγ $CD8^+$ T-large granular lymphocyte leukemia (283). The clonotypic composition of the human pp65-specific $CD4^+$ T-cell repertoire has also been shown to be extremely restricted and stable (23). Similarly, clonal restriction has been noted in the rhesus macaque cytomegalovirus (RhCMV) model (204), whereby the presence of a highly polyclonal RhCMV-specific $CD4^+$ T-cell population during primary infection was followed by a narrowing of the antigen-specific clonotypic repertoire in the chronic phase. Interestingly, challenge with RhCMV instigated the reemergence and dominance of RhCMV-specific $CD4^+$ T-cell clonotypes that were detected only during the acute phase of infection. Therefore, the various factors driving the selective pressures on the clonotype of the antigen-specific $CD4^+$ T-cell repertoire and the clinical implications of such clonal restriction certainly warrant further investigation.

(iii) γδ T cells. The γδ T-cell subset comprises <6% of T cells in the blood of healthy humans but represents a more substantial fraction of lymphoid cells in areas of the body exposed to the external milieu, such as the intestinal mucosa (59). Experimental data from mouse models suggest that γδ T cells play an important role in host immunity to viral infections including herpes simplex virus type 1 (234) and also MCMV (41, 186). An accumulation of γδ T cells has been shown to occur in the salivary glands of MCMV-infected mice (41), while the depletion of γδ T cells led to significantly increased MCMV titers (186). In renal transplant patients, a marked increase in levels of circulating γδ T cells from <5 up to 40% of total T cells was coincident with active HCMV infection (58), and delayed γδ T-cell expansion was associated with prolonged and elevated antigenemias and increased severity of HCMV disease (143). Interestingly, γδ T cells specific for HCMV have also been shown to be cross-reactive against intestinal tumor epithelial cells, indicating potential tumor cross-reactivity (98). These studies therefore strongly indicate the involvement of γδ T cells in the anti-HCMV immune response (Fig. 4).

Immune Evasion by HCMV

Analogous to other viruses, including most herpesviruses, HCMV has evolved a multitude of strategies in order to subvert host immune surveillance and defense by both the innate and adaptive arms of the immune system (Table 3). Comprehensive details of these strategies were provided in previous reviews (15, 177, 269, 274), but they are summarized below.

A major evasion mechanism centers on the inhibition of MHC class I-restricted antigen presentation (15). An effective immune response to HCMV is critically dependent upon the generation of antigenic peptides, which can be presented in complex with MHC class I molecules to CTL (51, 97). During the IE phase of an HCMV infection, a CTL response is directed against antigenic peptides derived from a 72-kDa IE-1 transcription factor (84). The matrix protein pp65, which has kinase activity, can, however, phosphorylate the IE-1 protein (84). This in turn selectively blocks the processing and presentation of IE-derived antigenic peptides via the MHC class I pathway and thus prevents an IE-1-specific CTL response (85). In addition, the HCMV genome encodes five proteins, US2, US3, US6, US10, and US11, that block the generation and/or export of MHC class I-peptide complexes and induce a rapid downregulation in MHC class I expression (4, 76, 113, 114, 281). Antigen presentation through the MHC class II pathway is also hindered by HCMV through US2 targeting of the MHC class II DR-α and DM-α molecules for degradation by proteasomes (268) and via the expression of proteins at the IE and delayed-early phases of an HCMV infection (IE/E product), which interfere with the IFN-γ-induced expression of MHC class II molecules (174, 175).

The missing-self hypothesis proposes that NK cells selec-

tively recognize and kill targets that lack cell surface-expressed self-MHC class I products (157). This recognition is mediated by a complex balance of regulatory activating and inhibitory receptors on the surface of NK cells (190). In principle, HCMV-infected cells with downregulated MHC molecules should be vulnerable to NK-mediated lysis. However, HCMV has responded by implementing various tactics to impede NK cell recognition including the expression of virus-encoded MHC class I homologues to act as decoy proteins (177). An example of this is the expression of the nonclassical class I molecule HLA E, which depends on the binding of a signal peptide derived from other host MHC class I molecules and suppresses NK cell recognition by binding the inhibitory CD94/NKG2A receptor (266). The UL40 gene product of HCMV contains a sequence homologous to such signal peptides, which can substitute and upregulate cell surface HLA E expression to protect virus-infected cells (266). A second example is HCMV UL16, which binds a family of human cell proteins called UL16 binding proteins (ULBPs) and also binds MHC class I chain-related gene B (MICB gene) although not MHC class I chain-related gene A (262). These ULBPs are another family of ligands for the human activating NK cell receptor NKG2D, and they have been reported to be upregulated in HCMV-infected cells. Soluble UL16 can block the binding of NKG2D to ULBP1 and ULBP2 and to the MICB gene, consequently preventing the activation of NK cells (50). Other mechanisms designed by HCMV to evade NK cell killing include pp65 inhibition of the NK cell-activating receptor NKp30 (8), HCMV UL122-encoded microRNA that downregulates MICB gene expression and subsequently reduces NK cell killing (259), inhibition of NK cell-mediated lysis by HCMV UL142 (282), and HCMV UL141-mediated blocking of the surface expression of CD155, a ligand for activatory NK receptors (UL141) (267).

HCMV also encodes a variety of other homologues with distinct subversive functions and which mimic the behavior of host proteins to divert the immune response. One such homologue is the human MHC class I homologue UL18, which, like MHC class I, binds β_2-microglobulin and peptide but in contrast shows specific binding only with leukocyte immunoglobulin-like receptor 1, a receptor prominently displayed on monocytes and B cells (49). The binding of leukocyte immunoglobulin-like receptor 1 to UL18 resembles the binding to host MHC class I molecules (42). While the UL18 protein was originally thought to prevent recognition by NK cells, this was later refuted, and the precise biological consequence of UL18 activity during viral infection remains unknown (150, 215). Four genes in HCMV, UL33, UL78, US27, and US28, encode homologues of seven transmembrane G-protein-coupled receptors (44, 166), and of these, US28 encodes a chemokine receptor that binds most human CC chemokines as well as the CX3C chemokine fractalkin (80, 130, 183, 261). HCMV also encodes a homologue (UL111a) of the immunosuppressive cytokine IL-10 (138); a viral TNF receptor (UL144) (17); a potent IL-8-like chemokine, viral CXC-1, which induces the chemotaxis of human peripheral blood neutrophils (UL146) (195); and various antiapoptotic gene products (UL36 and UL37) (87, 247).

VIRUS-SPECIFIC T CELLS FOR MONITORING OF HCMV INFECTION

Predicting clinical HCMV disease in patients is difficult, and rapid and accurate diagnostic tests are critical for the appropriate diagnosis and management of clinical HCMV disease following transplantation. Diagnosis of HCMV disease is made according to clinical signs and symptoms in conjunction with laboratory detection of HCMV in the blood and biopsy isolation of HCMV from the involved tissue(s) (109). There have been significant advances made in the methods for laboratory diagnosis of HCMV, as detailed elsewhere (63, 147, 241). Quantitative PCR methods are now routinely used for determining HCMV loads, and monitoring of viral DNAemia certainly has an important role in the clinical management of transplant patients. However, active HCMV disease does not always correlate with viral load detection, and a proportion of patients exhibit a detectable viral load without developing symptomatic clinical disease and are thus unnecessarily preemptively treated with toxic antiviral mediations (105, 110). Antigen-specific $CD8^+$ T cells are clearly crucial components of the immune response against HCMV. With the recent development of assays that reliably enumerate and assess the phenotype and function of HCMV-specific T cells ex vivo, a major focus has centered on evaluating the diagnostic and clinical utility of measuring the cell-mediated immune (CMI) response, termed "immune monitoring," particularly in the transplant setting, to complement existing quantitative DNA load assays (Fig. 6).

Immune monitoring can employ various ex vivo T-cell assays including peptide-MHC multimers, which facilitate the direct identification and enumeration of HCMV-specific T cells or functional assays such as enzyme-linked immunospot (ELISPOT) and flow cytometric intracellular cytokine staining, which enable the detection of IFN-γ or other cytokine-secreting cells in response to in vitro antigen stimulation (37, 53, 278) (Fig. 6). In addition to these assays, marker analyses to assess the phenotypically defined memory and other functionally distinct populations provide powerful methods for conducting a comprehensive characterization of HCMV-specific T-cell responses (6). Tetramer-based enumeration of HCMV-specific $CD8^+$ T cells has been shown to be a rapid and sensitive tool for identifying SCT recipients at risk of developing HCMV disease, with the reconstitution of HCMV-specific $CD8^+$ T cells to levels of more than 10 cells/μl being protective against HCMV disease (93) (Fig. 6). The appearance of tetramer-positive cells in D^+/R^- lung transplant patients has also been shown to precede detectable HCMV loads (78, 278). Other studies, however, have shown the quantitation of HCMV-specific T cells using MHC-peptide multimers to be of limited value for predicting symptomatic clinical HCMV disease in SOT patients (54). A significant constraint of tetramer-based monitoring is that as it is both HLA and epitope specific, large panels of tetramers are required for this technique to be routinely implemented and applicable to the majority of the population, and in some instances, suitable tetramers may not be available. Furthermore, due to limitations in cell numbers isolated from clinical samples, it is feasible to assess only a limited number of T-cell specificities; therefore,

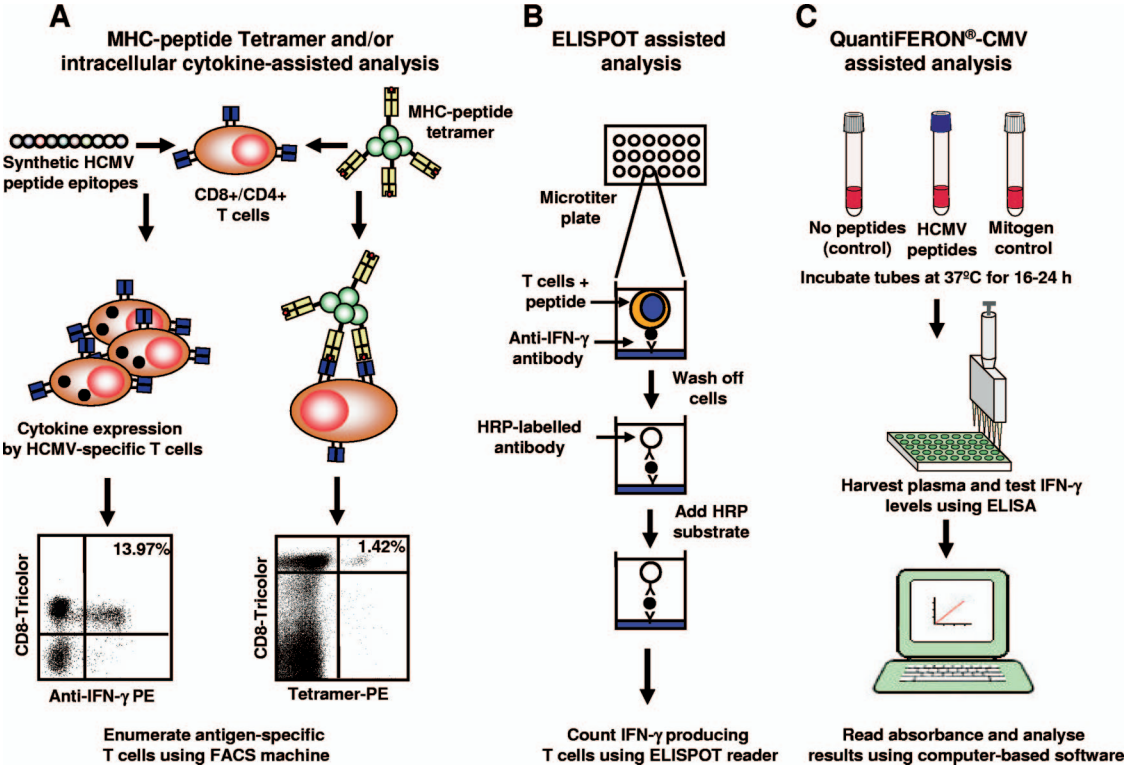

FIG. 6. Ex vivo monitoring of HCMV-specific T-cell responses. (A) Peripheral blood mononuclear cells or whole blood is incubated with either an MHC-peptide tetramer or synthetic peptide epitopes. Following incubation, these cells are processed for flow cytometric analysis for the detection of antigen-specific T cells. For intracellular cytokine staining assays, the T cells were costained with anti-CD3, anti-CD4, anti-CD8, and anti-IFN-γ. For MHC-peptide tetramer analysis, the cells were stained with the MHC-peptide tetramer and anti-CD3, anti-CD4, and anti-CD8. Both these assays can be used to phenotypically characterize antigen-specific T cells using a variety of surface markers (see the text). PE, phycoerythrin; FACS, fluorescence-activated cell sorter. (B) For ELISPOT assays, peripheral blood mononuclear cells were stimulated with synthetic peptide, and IFN-γ was then captured using specific antibodies. This IFN-γ was detected using horseradish peroxidase (HRP)-labeled antibodies, and specific spot-forming cells were analyzed using image analysis software. (C) For the QuantiFERON-CMV assay, whole blood was stimulated with pooled HCMV peptide epitopes or mitogen, and IFN-γ in the plasma was detected and quantitated using standard enzyme-linked immunosorbent assay (ELISA) methodologies. (Panel C courtesy of Cellestis R&D Pty., Ltd.)

global changes to HCMV-specific T-cell responses cannot be monitored.

Various studies have demonstrated that the frequency of IFN-γ-secreting HCMV-specific $CD4^+$ and $CD8^+$ T cells correlates with protection from HCMV infection after transplantation (37, 236) (Fig. 6). Longitudinal analysis of immune responses in SOT patients clearly illustrated that those patients who either showed no evidence of viral recrudescence or showed asymptomatic viral recrudescence maintained stable virus-specific IFN-γ expression by $CD8^+$ T cells (54). In contrast, SOT recipients who were diagnosed with symptomatic viral recrudescence showed significant fluctuations in the levels of IFN-γ-expressing $CD8^+$ T cells. In four of the five symptomatic recipients, the reduction in the level of IFN-γ expression by antigen-specific T cells preceded the clinical diagnosis of active disease. The latter observation was recently supported by the study reported by Mattes and colleagues, whereby impaired frequencies of IFN-γ-secreting pp65-specific $CD8^+$ T cells were predictive of high levels of HCMV replication, and the functional impairment was evident prior to the detection of HCMV DNA (169). Using an IFN-γ intracellular cytokine staining assay, Lozza et al. (160) determined that functional HCMV-specific $CD4^+$ and $CD8^+$ T-cell counts greater than 0.4 cells/μl from patients were considered to be protective, while the absence of T-cell restoration to that level within 30 days following SOT was associated with repeated episodes of HCMV reactivation requiring antiviral therapy (83). Protection from HCMV following transplantation has been shown to correlate with high frequencies of IE-1- but not pp65-specific $CD8^+$ T cells (37); however, this has been disputed by other studies, which indicate that pp65-specific T cells are also important in protecting against clinical HCMV disease (54, 142, 153). Those studies have provided preliminary evidence that quantitative measures of T-cell function may be clinically relevant and could be an effective tool for monitoring disease activity.

The use of phenotypic markers in combination with various functional and other assays has contributed to the identification of functionally distinct T-cell populations at both different stages of T-cell differentiation and different phases of disease. Assessing the diagnostic potential of phenotypic marker analysis has, however, been complicated by the indiscriminate use of cell surface markers, and it is not clear whether quantitative measures of distinct phenotypic populations can function as markers of disease activity that are of adequate sensitivity to detect changes in viral load. A significant increase in the level

of expression of CD38 on HCMV-specific $CD8^+$ T cells in SOT recipients with symptomatic recrudescence has been observed compared to recipients who either showed no viral recrudescence or showed asymptomatic recrudescence (54). This is similar to the HIV setting, whereby the level of CD38 expression on $CD8^+$ T cells has been shown to be a strong predictor of the progression of HIV-associated disease (156, 218). Research indicates that the programmed death 1 (PD-1) marker may also be a useful prognostic indicator of HCMV disease. Levels of PD-1 expression on HCMV-specific tetramer-positive $CD8^+$ T cells have been shown to correlate with levels of plasma HCMV DNAemia, and elevated levels of PD-1 have been reported for liver transplant patients with symptomatic HCMV disease (145). Very recently, the reversion of effector memory T cells to the $CD45RA^+$ phenotype has been demonstrated to correlate with virus clearance from the blood, and a high percentage of HCMV-specific $CD45RA^+$ effector memory T cells was shown to be protective against transmission of HCMV infection to the fetus (152). Therefore, the clinical and diagnostic relevance of these various phenotypic markers in the HCMV setting warrants additional investigation.

While the diagnostic potential in measuring the CMI responses is clear, the diagnostic utility of the various ex vivo T-cell assays such as ELISPOT, MHC-peptide multimers, and flow cytometric-based assays is somewhat restricted by various factors, including the high level of complexity and limited standardization and automation of the techniques together with the significant sample manipulations involved. The application of these techniques in the hospital setting is also constrained by the requirement for specialized equipment or trained personnel. In addition, the viral antigens used for antigenic stimulation directly affect the efficiency and sensitivity of in vitro tests for the detection of HCMV-specific T cells (153). Many studies have used HCMV lysate or overlapping pools of 15-amino-acid peptides spanning the pp65 or IE-1 protein as the antigen source for their ex vivo T-cell assays (37, 209, 239). HCMV lysate has been shown to be less sensitive than overlapping pools of pp65 peptides for the detection of HCMV-specific $CD8^+$ T cells; however, the use of pp65 and IE-1 peptide mixtures also underestimates the actual T-cell response against HCMV (153). Furthermore, as HCMV proteins have different roles in infection and the pathogenesis of disease, a number of HCMV protein antigens may therefore give rise to a protective CTL response. Therefore, analyzing pp65, IE-1, or any one single antigen or epitope in isolation, as has been done in many instances, may be an insufficient predictor of clinical HCMV disease. While it was suggested that the majority of immune responses can be detected by screening with overlapping peptides spanning 19 ORFs in the study reported previously by Sylwester et al., this is still considered to be practically infeasible due the limited number of cells obtained from clinical specimens, particularly following transplantation. Another consideration is that while the most common measure of T-cell function to date has been IFN-γ, other cytokines either in combination with IFN-γ or in isolation may indeed enhance the sensitivity of immune monitoring to predict HCMV disease. These broader issues require consideration before immune monitoring can be effectively translated into the clinical diagnostic laboratory. Recently, some of these limitations have been addressed through the development of the QuantiFERON-CMV assay, a simple and rapid technology which measures HCMV-specific CMI responses by quantitating IFN-γ levels released into the plasma in response to stimulation with defined minimal-length $CD8^+$ HCMV-specific T-cell epitopes from a range of HCMV viral proteins including pp65, pp50, gB, and IE-1 antigens and which is specific for a wide range of HLA class I alleles (275) (Fig. 6). The QuantiFERON-CMV assay has been shown to be of equivalent sensitivity to and, in some cases, more sensitive than the ELISPOT assay and a sensitive and specific test for the detection of virus-specific T-cell responses in both HCMV-seropositive healthy individuals and SOT patients. This assay has also been shown to detect reduced HCMV-specific $CD8^+$ T-cell responses in HIV-infected individuals with a history of HCMV disease (243), and in a proportion of lung transplant patients who developed significant HCMV reactivation in the lung allograft, the QuantiFERON-CMV assay was able to detect a decrease in HCMV immunity in the peripheral blood prior to the episode of HCMV recrudescence in the lung (279). Therefore, this assay may prove to be useful for predicting the likelihood of HCMV disease in immunocompromised HIV-infected and transplant patients and may assist in clinical decisions related to anti-HCMV prophylaxis or therapy.

IMMUNE-BASED STRATEGIES FOR THE PREVENTION AND TREATMENT OF HCMV DISEASE

Exploiting Humoral Immune Responses

Clinically, the passive administration of intravenous immunoglobulins containing high levels of HCMV antibodies (HCMV-specific hyperimmune globulin) can protect against disease in newborns, resulting from the infusion of HCMV-containing blood (250), and can increase virus-specific immunoglobulin G concentrations and avidity and significantly lower the risk of congenital HCMV infection and disease in pregnant women (185). In the transplant setting, the importance of the humoral response is supported by the fact that HCMV infection is more frequent and severe in seronegative SOT recipients of an HCMV-positive organ (155). The administration of HCMV-specific hyperimmune globulin to renal transplant recipients has been shown to reduce the incidence of HCMV-associated syndromes, bacterial or fungal superinfection, and graft loss (249) and also increase the rates of survival of liver transplant recipients (71). In the case of SCT recipients, the relevance of anti-HCMV antibodies remains unclear. The administration of HCMV-specific hyperimmune globulin can be effective in reducing HCMV infection and disease post-allo-SCT, and a correlation between the generation of high titers of glycoprotein-specific neutralizing antibodies in response to viral replication and improved survival has been reported (172, 232). Other studies, however, have failed to show a link between the presence of gB-specific and neutralizing antibodies and either development or recovery from HCMV infection (179) or have shown an association of large amounts of gB antibodies with an unfavorable outcome of manifestation of HCMV disease (162). Immunization with immunoaffinity-purified gB has resulted in neutralizing antibodies and seroconversion in previously seronegative individuals

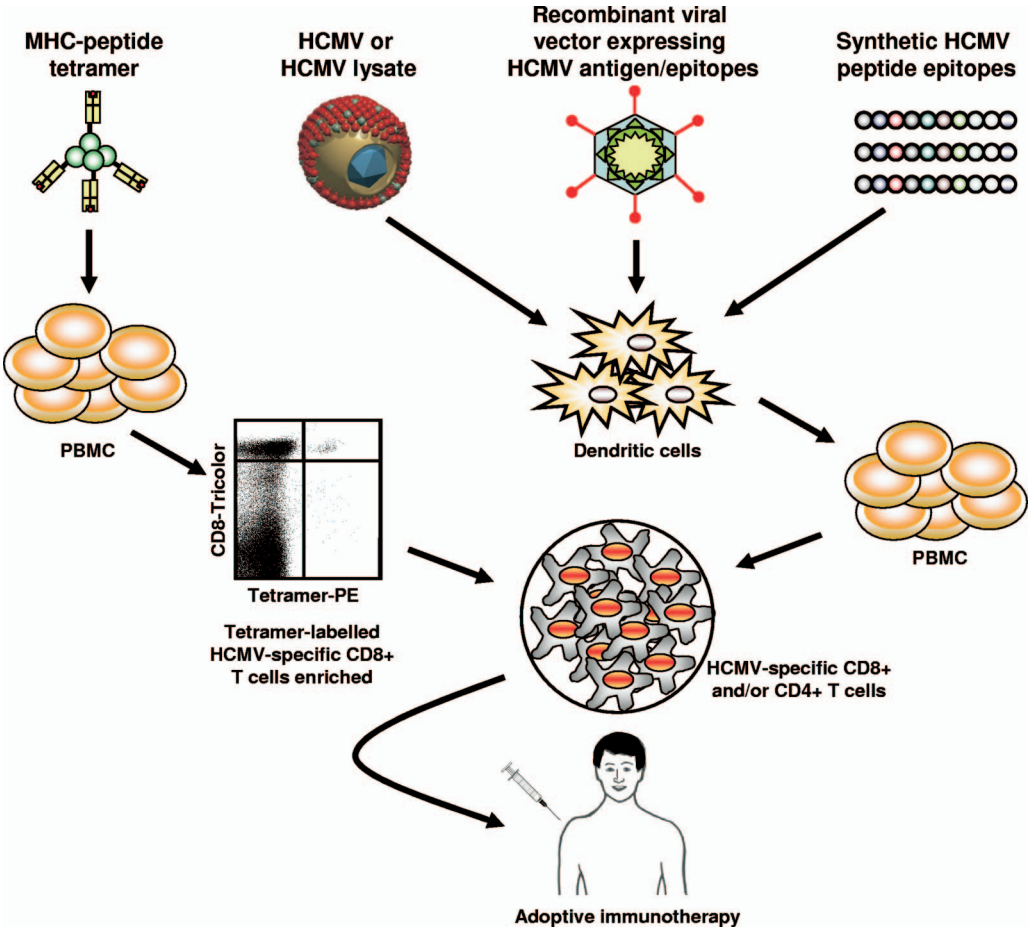

FIG. 7. Strategies for adoptive immunotherapy of HCMV. A number of strategies have been explored for adoptive immunotherapy of HCMV. These include MHC-peptide tetramer enrichment of HCMV-specific T cells or in vitro stimulation of T cells with HCMV viral lysate, recombinant viral vectors, or synthetic peptides. Following enrichment or in vitro expansion, these cells are adoptively transferred into immunocompromised individuals either as a prophylactic or therapeutic treatment. These strategies have been reported previously (47, 66, 173, 194, 216, 217, 276). PE, phycoerythrin; PBMC, peripheral blood mononuclear cells.

(89), while vaccination with recombinant gB induced high levels of neutralizing antibodies; however, the response declined over time (75, 191).

Exploiting Cellular Immune Responses

Attempts to passively restore HCMV cellular immunity in humans were instigated following data from MCMV models in which the adoptive transfer of $CD8^+$ T cells protected against viral challenge (181, 211), together with findings that the recovery of HCMV-specific T-cell immunity was associated with a decreased risk of developing disease after allogeneic BMT (208, 214). A number of strategies have been tested to expand HCMV-specific T cells for immunotherapy (Fig. 7). Riddell and colleagues and Walter et al. pioneered the use of donor-derived HCMV-specific T cells for the prevention of HCMV disease (216, 276). Those studies demonstrated that the adoptive transfer of $CD8^+$ CTL clones specific for structural virion proteins safely and effectively reconstituted HCMV-specific CTL responses in immunodeficient hosts and prevented HCMV viremia and disease. While the transferred T-cell clones were shown to persist for many weeks in vivo, $CD8^+$ CTL activity declined in patients who did not develop a concomitant HCMV-specific $CD4^+$ T-cell response, suggesting that helper T-cell function is required to maintain long-term virus-specific $CD8^+$ T-cell immunity (276). Subsequent studies by Peggs and colleagues (194) showed that the adoptive transfer of virus-specific $CD4^+$ and $CD8^+$ T cells stimulated with autologous HCMV antigen-sensitized DCs readily reconstituted virus-specific immunity with a considerable expansion of HCMV-specific T cells in vivo (Fig. 7). The importance of HCMV-specific $CD4^+$ T-cell immunity was also highlighted by Einsele et al. (66), who achieved significant reductions in HCMV viral loads following the infusion of predominantly $CD4^+$ HCMV-specific T cells in SCT patients with HCMV infection that was unresponsive to antiviral therapy. To overcome major concerns from the regulatory authorities regarding the use of live HCMV virus, Micklethwaite and colleagues used DCs pulsed with HLA class I-restricted epitopes as synthetic peptides to stimulate virus-specific T cells (173) (Fig. 7). This strategy dramatically increased the precursor frequency of antigen-specific T cells; however, the infusion of virus-specific T cells with single-epitope specificity may allow variants to

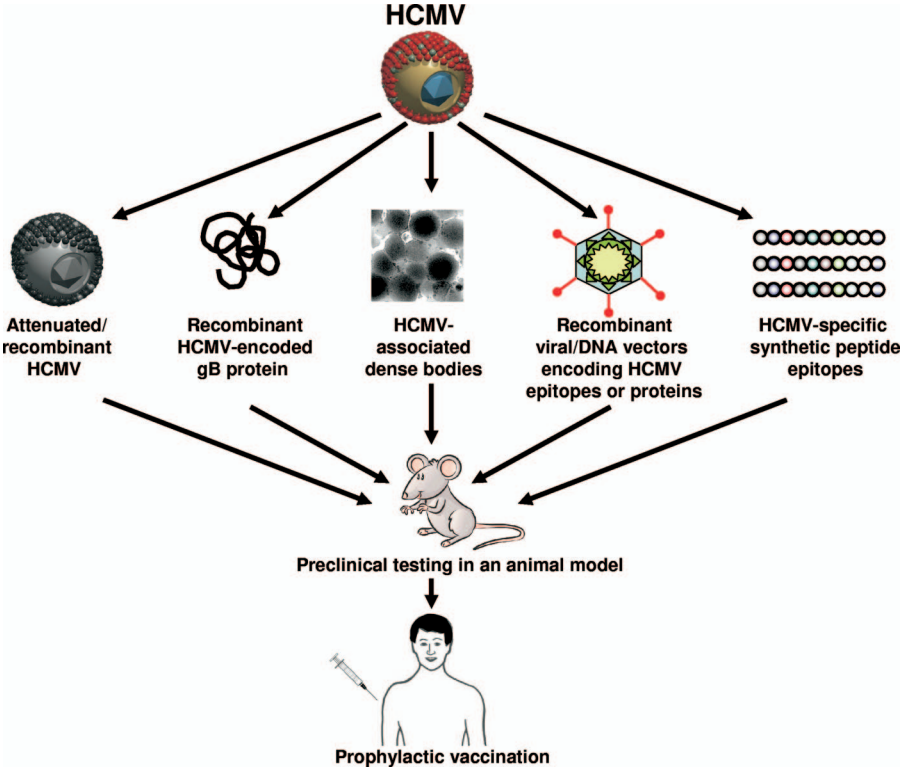

FIG. 8. Human cytomegalovirus vaccine development strategies. Various vaccine strategies have been developed, including live attenuated, recombinant viral proteins, dense bodies, vector vaccine subunit, or synthetic peptide epitopes. These formulations have been extensively tested using different animal models and have shown promising immunogenicity and protective efficacy (21, 30, 100, 228–230). Some of these strategies have already progressed to clinical trials with humans (1, 3, 18, 67, 75, 88, 104, 118, 176, 182, 191, 198, 199).

escape and also limit the use of this technology to patients who carry a single HLA allele. More recently, the use of replication-deficient adenoviral vectors encoding multiple epitopes from HCMV have been used to expand specific T cells that recognize virally encoded antigens expressed at different stages of infection (e.g., early, IE, and late) (217, 288) (Fig. 7).

One of the major limitations of the current strategies for T-cell-based therapies is that most of the methods published to date require prolonged periods (4 to 6 weeks) of stimulation and expansion and also require dedicated good-manufacturing-practice facilities. A number of alternative strategies to overcome this major barrier are currently under investigation. One such strategy was reported by Cobbold et al., who used MHC-peptide tetrameric complexes to select HCMV-specific T cells from the peripheral blood, and the adoptive transfer of these cells (without any in vitro manipulation) resulted in a massive expansion of virus-specific T cells in vivo and induced the clearance of active virus replication in eight of nine stem cell transplant patients (47) (Fig. 7). Although this approach provides exciting prospects for the future application of adoptive immunotherapy, it may be constrained by the availability of clinical-grade MHC-peptide tetramers and also the precursor frequencies of antigen-specific T cells in the peripheral blood. Another alternative strategy to reduce the time scale for the production of T cells is to use HLA-matched allogeneic virus-specific T cells (99, 161). This strategy has been successfully used for the treatment of EBV-associated lymphomas and showed a response rate of >50%, and this therapeutic effect was observed over 6 months after adoptive immunotherapy (99). Our group is currently in the process of establishing an allogeneic T-cell bank specific for HCMV and EBV that will provide "off-the-shelf" therapy for transplant patients. The long-term success of this strategy can be significantly enhanced by generating multispecific T cells that recognize HCMV, EBV, adenovirus, and other infectious agents that cause morbidity and mortality in transplant patients. Indeed, a recent study by Leen et al. at the Baylor College of Medicine has shown that these multiple-virus-specific T cells can be successfully generated and show therapeutic benefit in SCT patients (149).

Prophylactic Vaccination

The development of an effective prophylactic vaccine for HCMV-associated diseases remains a significant challenge. As our knowledge of the immune response to HCMV infection has progressed, various strategies have been explored (Fig. 8), and considerable advances have also been made in the field of HCMV vaccine development. Initial HCMV vaccine development was based on the Jennerian concept of using an attenuated form of the virus as a vaccine (1, 67, 118, 182, 198, 199). A renewed interest in this approach has emerged with the codevelopment of a recombinant technology that has allowed the design of a chimeric virus that may be more immunogenic than the parent viral strain (104, 121). More recently, a number of attempts have been made to design prophylactic HCMV

vaccines that are based predominantly on subunit vaccine technologies. These vaccine formulations have been delivered as recombinant proteins (75, 176, 191) and/or viral vectors (poxvirus/adenovirus) (3, 18, 88, 288). Most of these concepts have been extensively discussed in a number of reviews from our group and others (196, 197, 227, 287). A successful vaccine strategy should ultimately aim to stimulate the innate (TLR, DCs, and NK cells) and adaptive ($CD4^+$/$CD8^+$/γδ T cells) immune responses at the appropriate time. We strongly believe that HCMV vaccine strategies should focus on preventing or controlling HCMV disease rather than preventing infection.

FUTURE DIRECTIONS AND CONCLUDING REMARKS

Significant advances both in the understanding of the immunobiology of HCMV and in the diagnosis and treatment of HCMV disease have been made over the years. There are still many things to learn both about the immune response to HCMV itself and also by using HCMV as a model for how the immune system controls a persistent virus. Some of the areas still requiring further investigation are as follows. What is the mechanism(s) driving the homeostatic fluctuations of $CD8^+$ T cells during latency, and how does this impact the clinical manifestation of HCMV infection in newborn babies, transplant patients, and HIV-infected individuals? The $CD4^+$ T-cell immune response requires additional characterization, including the fine epitope specificity of responses, the phenotype and function of the cells, and more insight into the mechanism of antigen processing and presentation. Further insight into the dynamics and mechanism(s) leading to HCMV $CD4^+$ and $CD8^+$ memory T-cell inflation is needed. What antigen-specific T-cell populations are at the greatest risk for elimination due to HCMV memory T-cell inflation? Does the presence of large numbers of HCMV-specific T cells in healthy individuals or the presence of HCMV-specific T-cell populations with restricted TCR usage increase the susceptibility to other pathogens or malignant conditions? Examination into the factors regulating HCMV immune evasion mechanisms, i.e., what switches the immune evasion mechanisms on or off, particularly those immunomodulators that are also targets of the adaptive immune response, is needed. The control of HCMV relies on the adaptive, humoral, and innate immune response. The interactions that occur between the various arms of the immune system, particularly those between innate and adaptive immunities for the development of the memory HCMV immune response, need to be elucidated. It would also be of significant interest to further elucidate the role of NK cells and γδ T cells in the immune response to both primary and latent HCMV infection. The mechanism by which HCMV may cause or exacerbate graft rejection needs to be examined. Finally, examination of the epitope specificity during the reconstitution of HCMV-specific T cells following allo-SCT needs to be performed. Are the epitope specificities and the TCR diversities of T cells in a seropositive donor the same in the recipient and following transplantation?

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