

## Induction of Immunity against Human Cytomegalovirus

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### Abstract

Most healthy individuals have been exposed to human cytomegalovirus (HCMV) and harbor the virus in a dormant form. However, in situations of immune compromise, HCMV infection is associated with high mortality rates in recipients of bone marrow transplants and with significant morbidity in recipients of solid organ transplants. Conventional vaccination with attenuated HCMV or HCMV proteins fails to prime protective immune responses, presumably because the antigens fail to be presented effectively *in vivo*. Dendritic cells (DC) are professional antigen-presenting cells that can be genetically engineered to direct their ability to induce immune responses toward immunodominant HCMV antigens. DC are a distinct lineage of leukocytes whose function is most notable for their ability to form clusters with T cells and stimulate vigorous cytotoxic T lymphocyte responses. We hypothesize that in this capacity, DC engineered to express HCMV antigens are uniquely positioned to control immunity against HCMV.

**Key Words:** Cytomegalovirus, vaccine, immunity, gene transduction, dendritic cells.

### Background

#### Clinical Human Cytomegalovirus Infection and Immunity

HUMAN CYTOMEGALOVIRUS (HCMV) is the largest member of the human herpesvirus family. By school age, almost all individuals (>90%) have been exposed to HCMV and have developed protective immunity, but a lifelong latent and asymptomatic infection is established with periodic reactivation and viral shedding at mucosal sites. However, symptomatic reactivation of latent HCMV infection is common in

immune-compromised patients and results in severe infections. The incidence of HCMV infection in the first 100 days following allogeneic bone marrow transplantation (BMT) ranges from 30–70% (1–4). In the past, HCMV has been the leading infectious cause of death in BMT recipients, with mortality rates of untreated HCMV pneumonia of 30–60% (1, 5). Significant HCMV morbidity is also frequently associated with solid (kidney, liver, heart) organ transplantation (6–8). In addition, it has been estimated that each year approximately 40,000 newborns in the U.S. are infected prenatally with HCMV and that up to 7,000 of these newborns develop permanent central nervous system damage as a result of the infection (9). Conventional vaccination with attenuated HCMV or HCMV proteins fails to prime protective immune responses, and the standard therapy at present is treatment with ganciclovir, but at a substantial financial cost. Although ganciclovir prophylaxis reduces the incidence of HCMV disease early after organ transplantation, severe neutropenia occurs in 30% of patients and there is an increased incidence of HCMV disease of late onset (>100 days) (10, 11).

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The fundamental role of a competent T lymphocyte response for resolution of acute HCMV infection is supported by the occurrence of progressive infections in individuals with acquired T cell deficiencies. Conversely, after bone marrow transplantation, recovery from HCMV infection correlates with recurrence of antiviral cytotoxic T lymphocyte (CTL) responses. Adoptive transfer of CD8<sup>+</sup> T cells derived from the bone marrow donors reconstitutes cellular immunity to HCMV (12, 13). Studies in humans, using target cells infected with laboratory strains or primary patient isolates of HCMV, have analyzed epitopes recognized by CTLs. Epitopes recognized by the immunodominant CD8<sup>+</sup> CTL clones are conserved in all tested strains, suggesting that genetic heterogeneity of the virus is unlikely to present a significant obstacle to successful cell-mediated immunotherapy (14). CD4<sup>+</sup> T helper cell functions are required for persistence of the CD8<sup>+</sup> CTL response (13, 15). The major role of CD4<sup>+</sup> T cells is production of cytokines which assists in the direction of immune responses toward either predominant-cell-mediated type 1 (Th1) or antibody-mediated type 2 (Th2) responses.

Although cellular immunity in transplant patients correlates with recovery from primary infection (12, 16), humoral immunity probably plays a role in limiting the infection, both by clearing the virus load (17, 18) and by protecting against subsequent viral reactivation (19, 20). However, in several studies with solid organ and BMT recipients, the protective effect of HCMV-specific immunoglobulin has been difficult to demonstrate and is controversial (21).

### Immune Responses Against HCMV Antigens

HCMV contains a 240 kb double-stranded DNA genome with more than 200 open reading frames, the products of only a minority of which have been experimentally characterized (22–25). Although the virion contains more than 30 proteins, with molecular weights ranging from 20–200 kDa, the CTL response against HCMV is limited to a few proteins, with the predominant response directed against the pp65 tegumentum protein. Other components of the virion proteins (pp150, pp28), immediate-early (IE1 and IE2) antigens and the DNA-binding protein (pp52) have been suggested to be targets for CTLs, but at much lower frequencies (26–28).

The pp65 is the major viral tegumentum protein and is produced predominantly during the late phase of gene expression (29). The pp65

protein is a phosphoprotein which constitutes 18% of the virion protein mass, but is dispensable for HCMV replication (30). It is transferred into cells with the HCMV virion at the onset of infection, prior to viral gene expression, suggesting that immunity to viral antigens may be targeted, in part, to structural proteins which do not require *de novo* gene expression (22, 31).

The pp65 antigen is the most extensively studied antigenic target for major histocompatibility complex (MHC) class I restricted CTLs (22, 27, 28, 32–34). Seventy to ninety percent of all CTLs against HCMV have been shown to be specific for pp65 (33). Analysis of the peptide specificity of the pp65-specific CTLs has shown that responses to individual peptide sequences are shared among most human histocompatibility leukocyte antigen (HLA)-A2 seropositive donors, but that for some subjects multiple peptides are recognized throughout the pp65 protein (33). A high level of pp65-specific CTL is maintained over a prolonged period (18–20 months), which may reflect a continued expression of pp65. This supports the idea that periodic reactivation or chronic low level replication of the virus allows for restimulation and maintenance of the CTL memory pool (33). This observation is different from that of other viral infections, such as influenza, in which the CTL response progressively declines over time (35). Therefore, it is conceivable that the demonstrated variations in peptide specificities seen in HCMV-seropositive donors are a result of progressive selection or broadening of the CTL response in favor of particular peptide-HLA class-I associations that control a persistent but latent infection. Thus, a broadened CTL response may be beneficial for obtaining a more sustained effect of vaccination against HCMV, and suggests that designs to stimulate broader CTL responses should be investigated. Furthermore, studies of the fine specificity of the pp65 CTL response from HLA-A2 donors and HLA-A2 transgenic mice have revealed that a common region of the protein (aa 393–507) is recognized in transgenic mice and humans (28, 33). This result suggests that an HLA-A2 transgenic mouse model can be used to study vaccine development, because the obtained CTL responses in transgenic mice, in most cases, are predictive for human CTL responses (28, 36).

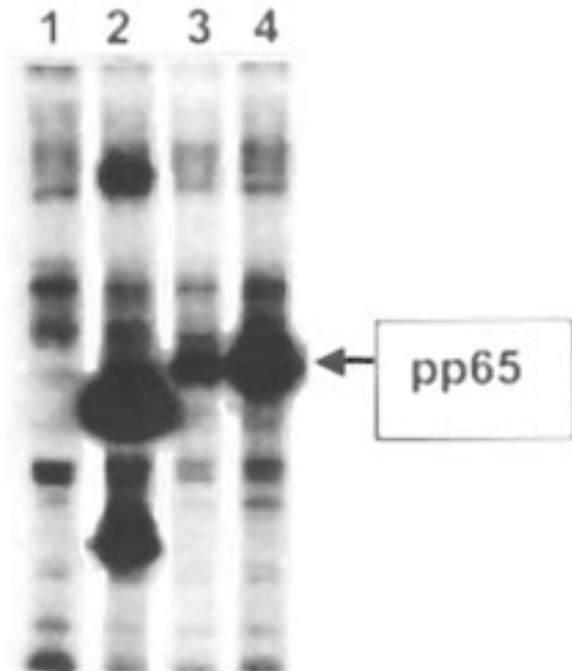
### Immune Evasion by HCMV

HCMV can evade the immune response directed against viral proteins expressed during

the replication cycle. It appears that many genes expressed by HCMV may act in concert to provide an “immune privileged” site for HCMV replication (Table) (37–49). These mechanisms of immune evasion may explain why CTL responses against the pp65 tegument antigen, which does not require *de novo* synthesis, predominate compared to responses to viral antigens produced during the replication cycle when the viral countermeasures are in place.

## Results

To induce the expression of HCMV antigens in primary antigen-presenting dendritic cells (DC), we have developed adenoviral recombinants (Adpp65 and Adpp150) expressing pp65 and pp150 antigens. These recombinants were tested by transient expression in transfected cell lines. Fig. 1 shows HCMV pp65 protein expression after transfection of the Ad-HCMVpp65 plasmid into QT6 cells and immunoprecipitated with pp65-specific antibody. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed abundant expression of pp65 in the two transfected cell cultures (lanes 3 and 4) and compared to un-



**Fig. 1.** Autoradiogram of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showing pp65 expression in QT6 cells after immunoprecipitation with specific antibodies and SDS-PAGE. **Lane 1:** Negative control — untransfected control cells precipitated with anti-pp65 antibody. **Lane 2:** HIV-1 infected cells precipitated with anti-HIV patient serum. **Lanes 3 and 4:** cell lines expressing pp65 precipitated with anti-pp65 antibody. **Methods:** Two adenoviral expression vectors, pACCMVpLpA/pp65 and pACCMVpLpA/pp150, were developed. The genomic HCMV pp65 (1750 bp) and pp150 (3150 bp) sequences were amplified by polymerase chain reaction (PCR) from primers that introduced *Bam*HI and *Xba* I, and *Xba* I and *Hind* III restriction sites, respectively, at the ends of the amplified DNA. Both PCR amplification products were cloned downstream of the CMV major immediate-early promoter (MIEP) in the shuttle plasmid vector pACCMVpLpA. This plasmid contains an adenovirus origin of replication and encapsidation signal upstream of the transgene insertion, and a downstream polyA tail followed by about 3000 bp of adenovirus 5 DNA, to provide a substrate for recombination into the backbone of the adenovirus strain, dl309, in 293 cells. The transgene replaces the adenovirus E1a and E1b gene regions. Recombinant adenovirus was obtained by transfection of linearized plasmid and DNA from adenovirus strain dl309 introduced into 293 cells that constitutively provide the E1a and E1b gene functions for complementation of recombinant virus *in trans*. Recombinant virus plaques were identified by restriction enzyme analysis of candidate strains and designated Adpp65 and Adpp150. After 3 successive plaque purifications and amplification of a representative plaque, high titer ( $10^{10}$  pfu/mL) recombinant virus stocks were purified on CsCl density gradients.

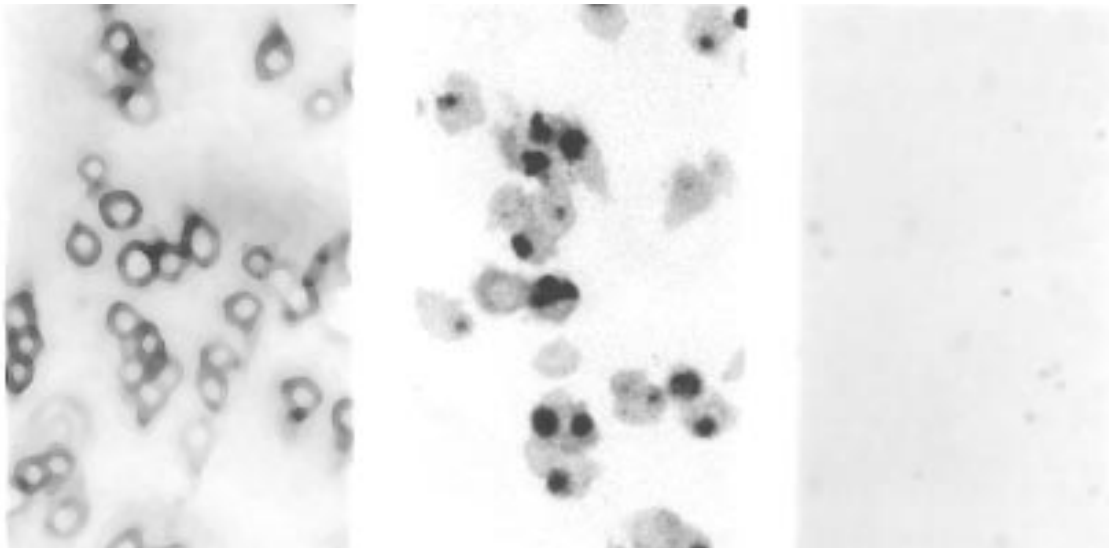
**TABLE**  
*Immune Evasion Induced by HCMV Genes*

Function	HCMV Gene	References
<b>Downregulation of MHC class I</b>	US2, US3, US6 US11	(37–40)
a) Degradation of heavy chains		
b) Retention of heavy chains in ER		
c) Translocation of peptide in ER		
<b>Chemokine trapping</b>		
a) chemokine receptor	US27, US28	(41, 42)
<b>Chemokine homologs</b>	UL146	(43)
<b>TNF receptor</b>	UL144	(44)
<b>IL-10 homolog</b>	UL11a	(45, 46)
<b>IL-10 induction (murine)</b>		
<b>NK cell evasion</b>		
a) MHC homolog	UL36	(47, 48)
<b>Apoptosis inhibitors</b>	UL36	(49)

HCMV = human cytomegalovirus; MHC = major histocompatibility complex; ER = endoplasmic reticulum; TNF = tumor necrosis factor; IL-10 = interleukin-10; NK = natural killer.

transfected control cells (lane 1). Comparable results were obtained with the AdHCMVpp150 plasmid (not shown).

The adenoviral Adpp65 recombinant was next tested in primary cells (Fig. 2) (50). The



**Fig. 2.** Expression of pp65 by human DC transduced with Adpp65 at multiplicity of infection of 100. **(Left)** Late cytoplasmic expression of pp65 by transduced DC. **(Middle)** Early nuclear expression of pp65 by transduced DC. **(Right)** Negative control cells (non-transduced cells). **Methods:** Mature mouse DC were obtained from spleen using techniques established in the laboratory (50).

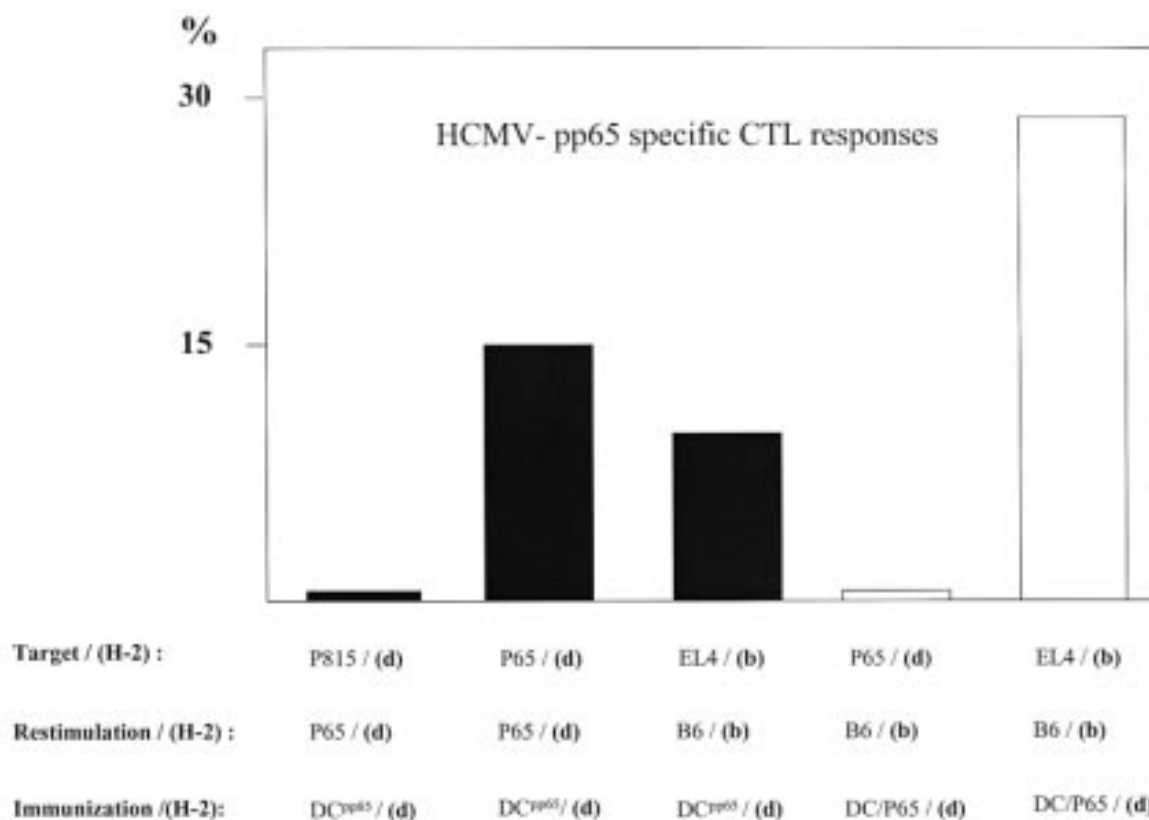
Adpp65 recombinant was used to infect primary dendritic cells *in vitro*, and the results showed that Adpp65 produced efficient (>35%) transduction and high level expression of pp65 in cultures of primary DC. Noteworthy is the fact that the pp65 protein was expressed both in the nucleus and at the cell surface. However, the cell surface expression appeared at a later time point after infection with the recombinant. In several experiments the transduction frequency was comparable (20–35%) to that in previous experiments using adenovirus-mediated expression of lacZ (51).

We next wanted to test the efficacy of pp65-transduced DC in stimulating pp65 CTL responses *in vivo* (Fig. 3). Balb/c (H-2<sup>d</sup>)-derived DC transduced with recombinant adenovirus expressing pp65 were used for *in vivo* immunization of mice to induce anti-pp65 CTL responses. The results showed that strong and specific CTL responses were generated in H-2<sup>d</sup> mice against the CMVpp65 protein after vaccination with  $1 \times 10^6$  live H-2<sup>d</sup> DC expressing pp65 (middle closed column). Furthermore, this antiviral response was higher than that of the response primed against allo-MHC (right closed column). The pp65-specific CTL response is comparable to that of HIV-nef-specific CTL responses reported by using a similar experimental system (52). Conversely, control P815 (H-2<sup>d</sup>) cells which do not express pp65 were not lysed (left closed column). Mice immunized with  $1 \times 10^6$  P65 (H-2<sup>d</sup>) cells which ex-

press pp65, in combination with similar numbers of Balb/c DC (H-2<sup>d</sup>) (not expressing pp65) did not lyse P65 (H-2<sup>d</sup>) target cells when restimulated with H-2 mismatched B6 splenocytes, but an appropriate allo-MLR (mixed leukocyte response) response was generated (open columns).

### Conclusions

One feature of cytomegalovirus (CMV) infection is a transient but severe immunodeficiency that characterizes the initial stages of the infection process (53). Recently, a role of DC in the CMV-associated immune deficiency has become appreciated. The effects of CMV infection with respect to interference with the immune functions of DC is key to the rationale of approaching vaccination against CMV by enhancing DC-mediated immune responses against CMV. CMV has been shown to infect DC (54–58), to impair maturation (57, 58) and antigen presentation by DC (58), and to impair allostimulatory capacity of DC (58). These findings make DC a rational target for gene transfer in the context of CMV immunization. We hypothesize that transplant recipients would obtain improved protection against reactivation of *de novo* CMV infection if effective CTL responses could be generated prior to the scheduled transplant. Likewise, HIV-1 infected individuals might benefit from induction of CMV-directed CLT responses earlier in the disease,



**Fig. 3.** HCMV-pp65-specific CTL responses (%) against target P65 cells (H-2<sup>d</sup>) relative to allo-MHC responses (H-2<sup>b</sup>). **Closed columns:** For induction of pp65-specific CTL, Balb/c DC (H-2<sup>d</sup>) expressing the pp65 recombinant were used for immunization of Balb/c mice (**primary** stimulation) by injection of approximately  $1 \times 10^6$  transduced DC per mouse. After 12 days the splenocytes from the primed mice were restimulated (**secondary** stimulation) *in vitro* with P65 [a P815 (H-2<sup>d</sup>) derivative expressing HCMV pp65], or B6 (H-2<sup>b</sup>) splenocytes: <sup>51</sup>Cr-labeled target cells for CTL assays (% release) were P65 [a P815 (H-2<sup>d</sup>) derivative expressing HCMV pp65], P815 (H-2<sup>d</sup>), or EL4 (H-2<sup>b</sup>). To establish stable cell lines expressing high levels of pp65, a pp65 expression plasmid was transfected into the P815 cell line, along with pSV2neo for neomycin selection. This cell line, P65 (H-2<sup>d</sup>), was the target cell for the CTL assays. **Open columns:** Same experimental setup, except that approximately  $1 \times 10^6$  DC (H-2<sup>d</sup>) with no pp65 expression in combination with a similar number of P65 cells were used as stimulators.

when CD4<sup>+</sup> T cells are not depleted. In fact, studies in Rhesus Macaques infected with pathogenic simian immunodeficiency virus (SIV) have shown CMV-specific CTL activity earlier in the disease (59).

Adenoviral vectors are currently among the most efficient vehicles of gene transfer for both *in vivo* and *in vitro* gene delivery, though their potential use in clinical practice in the future may be limited. One experimental concern with the use of the adenovirus delivery system is the possibility that competing immune responses (e.g., neutralizing antibodies) may be raised against vector-derived antigens rather than the gene of interest (60–62). Our preliminary results using primary DC suggest that competitive anti-adenovirus immune responses are not a limitation for the short-term use of an adenovi-

ral delivery system. This may be because of either the limited number (1–2) of challenges, the limited dose of virus given with washed DC, or a protective effect of DC. In fact, recent evidence suggests that transduced DC can protect against induction of neutralizing antibody responses (Th2 responses) (63, 64). In these reports, administration of adenovirus alone resulted in high titers of antiviral antibodies that inhibited adenoviral infection of 293 cells, whereas the sera of mice injected with infected DC showed only low levels of blocking activity (64). At present there is no explanation for this “protective” effect of DC against antiviral antibodies. It can be speculated that preferential intracellular expression of antigen by DC would favor generation of cellular class I restricted responses. Hamel et al. have recently showed that

adenovirus-transduced DC are able to generate efficient CTL responses against adenovirus and CMV simultaneously (65). The development of adenoviral vectors that are deleted in all viral protein coding sequences (guttled vector) offers the prospect of using less immunogenic vectors.

A different scenario with the use of an adenovirus gene delivery system is the possibility that synthesis of adenovirus gene products will interfere with the expression of pp65 or the subcellular compartmentalization of the pp65 antigen. The gp19 protein encoded within the E3 adenovirus gene region causes intracellular entrapment of MHC class I products, but may also cause nonspecific entrapment of non-HLA-related surface proteins encoded by the vector (66). The strain of adenovirus (dl309) used for recombinant construction in our studies expresses gp19, and entrapment of MHC molecules could render the transduced DC less efficient as antigen/peptide presenting cells. At present, our studies suggest that the pp65 antigen is efficiently expressed in the nucleus, cytoplasm, and cell membrane.

DNA vaccination with plasmids expressing HCMV proteins is a possible future avenue of vaccination. However, recent literature suggests that the mechanisms underlying DNA vaccination, while poorly understood, are in fact dependent on professional antigen-presenting cells such as DC, which are key leukocytes for immune stimulation with DNA (67–72).

Currently several clinical trials of tumor vaccination with *ex vivo*-generated DC are ongoing, but the outcome results are pending (73). Similar studies of HCMV vaccination with *ex vivo*-generated DC may prove to be more efficacious in a system where the antigen is much better defined.

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