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Human Cytomegalovirus pp65- and Immediate Early 1 Antigen-Specific HLA Class I-Restricted Cytotoxic T Cell Responses Induced by Cross-Presentation of Viral Antigens

Zsuzsanna Tabi, Magdalena Moutaftsi, and Leszek K. Borysiewicz

Dendritic cells (DCs) play a pivotal role in the development of anti-viral CD8+ CTL responses. This is straightforward if they are directly infected with virus, but is less clear in response to viruses that cannot productively infect DCs. Human CMV (HCMV) shows strain-specific cell tropism: fibroblast (Fb)-adapted laboratory strains (AD169) and recent clinical isolates do not infect DCs, whereas endothelial cell-adapted strains (TB40/E) result in productive lytic DC infection. However, we show here that uninfected DCs induce CD8+ T cell cytotoxicity and IFN-γ production against HCMV pp65 and immediate early 1 Ags following in vitro coculture with HCMV-AD169-infected Fbs, regardless of the HLA type of these Fbs. CD8+ T cell stimulation was inhibited by pretreatment of DCs with cytochalasin B or brefeldin A, indicating a phagosome/endosome to cytosol pathway. HCMV-infected Fbs were not apoptotic as measured by annexin V binding, and induction of apoptosis of infected Fbs in vitro did not augment CTL induction by DCs, suggesting a mechanism other than apoptosis in the initiation of cross-presentation. Furthermore, HCMV-infected Fbs provided a maturation signal for immature DCs during coculture, as evidenced by increased CD83 and HLA class II expression. Cross-presentation of HCMV Ags by host DCs enables these professional APCs to bypass some of the evasion mechanisms HCMV has developed to avoid T cell recognition. It may also serve to explain the presence of immediate early 1 Ag-specific CTLs in the face of pp65-induced inhibition of Ag presentation at the level of the infected cell.


H

uman CMV (HCMV) is a species-specific β-herpesvirus, a large DNA virus encoding >227 proteins (1). It is a well-adapted virus, establishing lifelong asymptomatic persistence. HCMV has coevolved with the human immune system and developed multiple independent mechanisms to evade recognition by CD8+ CTLs (2–4). One of the main mechanisms of evasion is the direct interference with HLA class I Ag presentation by viral glycoproteins. There are at least four HCMV proteins that interfere with the assembly, maturation, and transport of HLA class I molecules. The immediate early (IE) gene product gpUS3 causes retention of HLA class I molecules in the endoplasmic reticulum (ER), and the late Ag gpUS6 inhibits TAP translocation following peptide binding, whereas gpUS2 and gpUS11 translocate assembled class I molecules back into the cytoplasm where they are rapidly degraded. Due to this blockade, HCMV-infected human fibroblasts (Fbs) have significantly reduced levels of HLA class I expression, impaired Ag presentation, and are resistant to lysis by CD8+ T cells (5). The US2 Ag also down-regulates HLA class II expression, leading to a reduced T cell-stimulatory capacity (6). During HCMV infection, phosphorylation of the abundantly expressed 72-kDa IE 1 Ag has been observed (7). This is mediated by the pp65 tegument protein of HCMV released from virions into the cytosol. This phosphorylation interferes with the presentation of IE1-derived Ags via the HLA class I pathway, and, as a result, pp65- and IE-expressing cells are not recognized and lysed by IE-specific T cells, although they can be killed by T cells directed against other viral Ags (7). Furthermore, HCMV encodes three proteins with homology to CC chemokine receptors: US27, US28, and UL33 (8), which may deplete chemokines from the environment of infected cells. The reduced local availability of chemokines can have a negative effect on the attraction of lymphocytes and activation of CD8+ T cells (9). HCMV also encodes a viral homologue of IL-10 (10), which may have immunosuppressive properties favorable to the virus. However, the most important evasion strategy of HCMV is probably the infection of bone marrow hemopoietic progenitor cells early in their development (11–14), where the virus remains quiescent with limited nonlytic gene expression. At this stage, the virus is invisible to the immune system.

Despite the numerous viral evasion strategies, HCMV-specific effector or memory CD8+ and CD4+ T cells are present in the peripheral blood of healthy seropositive carriers at relatively high frequencies, as demonstrated by limiting dilution analysis (15–18), intracellular cytokine staining (19, 20), and by peptide-HLA tetramer staining (21). To explain how this robust cellular immune response develops in the presence of viral interference with the HLA class I and class II Ag presentation pathways, we examined the possibility that cross-priming via dendritic cells (DCs) enables Ag presentation of epitopes that would otherwise be blocked in the virally infected cell. DCs can acquire Ags from apoptotic tumor cells (22) or influenza virus-infected apoptotic monocytes (23) and present these via the MHC class I pathway. However, DNA viruses, including HCMV, inhibit apoptosis of the infected cells (reviewed in Ref. 24). HCMV IE1 and IE2 proteins inhibit apoptosis...
by modulating the expression of cellular proteins, e.g., NF-κB sub-
units (25, 26), whereas the product of the UL37 gene, vMIA (viral 
mitochondria-localized inhibitor of apoptosis), has broad anti-
apoptotic effects, inhibiting apoptosis at a point downstream of 
caspase 8 activation in a bcl-2-related manner (27).

In this study, we explored whether immature DCs can acquire 
 viral Ags from HCMV-infected Fbs and whether this process is 
 triggered by apoptosis. Our results indicate that cross-presentation 
of HCMV Ags occurs by DCs in the absence of apoptotic markers 
on infected cells. This Ag presentation pathway efficiently 
 bypasses some of the T cell evasion strategies of HCMV and may 
 serve to explain how T cell responses against certain HCMV Ags 
are generated.

Materials and Methods

Donors and cell lines

Four healthy HCMV-seropositive and three -seronegative laboratory vol-
unteers were included in this study. Their HCMV serostatus was deter-
mined by high-sensitivity IgG ELISA (A Department of Medical Microbiol-
ogy, University Hospital of Wales, Cardiff, U.K.). The HLA types of the 
donors and the Fbs used in these experiments were determined by mi-
crolymphocyte cytotoxicity assay (Welsh Blood Transfusion Service, 
Cardiff, U.K.) or by PCR using sequence-specific primers (28) (Table I).

MRC5 cells (human embryonic lung Fbs; European Collection of Cell 
Cultures, Salisbury, U.K.) and human skin Fbs prepared from laboratory 
volunteers (29) were grown in MEM (Life Technologies, Grand Island, 
NY) supplemented with 10% FCS (Life Technologies), 100 IU/ml peni-
cillin, 100 μg/ml streptomycin, 2 mM l-glutamine, 25 mM HEPES buffer, 
and nonessential amino acids. T2 cells (HLA-A2, TAP-negative B cell-
line) were of DC phenotype (CD1a+, CD83+). These cells were of DC pheno-
type (CD1a+, CD83+) and were used for the experiments. Virus titers were 
determined by immunofluorescent staining with a 
supernatant. All cells and virus stocks were negative for contamination 
with mycoplasma as determined by the standard method by infecting PBMC with 
EBV containing B95.8 cell 

Vaccines and viral peptides

HCMV strain AD169 is a laboratory strain adapted to grow in Fbs (30). 
RCMV288 is a mutant strain based on strain AD169 that has a copy of 
a enhanced green fluorescent protein (EGFP) inserted in one copy of the 
HCMV long repeat under the control of the HCMV 
reporter system for monitoring infection (31). HCMV strain TB40/E (kind-
ly provided by Dr. C. Sinzger, University of Tubingen, Tubingen, Ger-
many) is an endothelial cell (EC)-adapted HCMV strain that maintained 
its ability to infect ECs and other cell types including DCs (32). HCMV 
strains were propagated in MRC5 cells. The supernatant of the infected Fbs 
was collected by centrifugation when cells exhibited gross cytopathic ef-
fecnt. The filtered supernatant, stored at −70°C in small aliquots, was used 
for the experiments. The virus titers were determined from the tissue cul-
ture-ID50 values on MRC5 cells. Recombinant adenovirus (rAd)31 is a 
replication-deficient rAd that contains the HCMV major immediate early 
gene (IE1) under the control of its own promoter (33), whereas rAd60 is an 
equivalent (control) adenovirus without a transgene insertion. The adenovo-

In vitro culture of human DCs

PBMC were obtained from peripheral blood by standard Ficoll-Histopaque 
(Life Technologies) separation. The adherent cells were collected in serum-
free RPMI 1640 at 15 × 10^6 cells/well in 6-well trays following a 1-h 
incubation at 37°C and were differentiated into DCs according to the 
standard method (34). Briefly, adherent cells were grown in RPMI 1640 con-
taining 10% FCS and supplements in the presence of 50 ng/ml human 
rGM-CSF (Leucomax; Novartis Diagnostics, East Hanover, NJ) and 
500 U/ml human rIL-4 (BD PharMingen, San Diego, CA). Fresh culture 
medium was added on day 3. After 6 days, nonadherent and loosely ad-
herent cells were collected by vigorous pipetting. More than 90% of the 
cells were of DC phenotype (CD1a+, HLA-DR+, CD14+, CD80+) after 
gating on size and side scatter by FACS to exclude lymphocytes.

Antibodies

The following Abs and fluorescent reagents were used in this study: 
HCMV/IE1-specific mAb ID6-6, HCMV p52 early Ag-specific FITC-conju-
gated mAb (CCh2; Dako, Carpinteria, CA) (35), HCMV pp65-specific 
Ab (BioDesign, Carmel, NY), HCMV glycoprotein B (gB)-specific human 
Fbs (ITC52) (36), mouse IgG1-negative control (Serotec, Oxford, U.K.), 
CD3-PE (BD Biosciences, Mountain View, CA), PKH-26 (Sigma, St.
Louis, MO), CD8-CyChrome, anti IFN-γ-FITC, HLA class II-CyChrome,
annexin V-FITC Apoptosis Detection Kit I (all four from BD PharMingen), 
CD56-PE (Biolclone, Marrickville, Australia), CD83-FITC (Immunotech, 
Luminy, France), and goat anti-mouse IgG (F(ab')2)–FITC (Sigma).

Immunofluorescence, FACS analysis

Detection of HCMV Ags. Fibroblasts grown on 13-mm diameter cover-
slips were fixed with 4% paraformaldehyde, permeabilized with 0.3% 
Tri- 

Fbs were infected with HCMV strain AD169 (both for 20 min at room 
temperature), and labeled with 1 μg/ml goat or mouse IgG for 20 min at 37°C. 

Electron microscopy. HCMV-infected and uninfected MRC5 cells 
were labeled using the annexin V-FITC Apoptosis Detection kit I ac-
cording to the manufacturer’s instructions. Briefly, 5 μl annexin V-FITC and 10 
μl propidium iodide (PI) were added to 10^5 cells in 100 μl labeling buffer.

Intracellular cytokine staining. A total of 1–2 × 10^6 T cells/ml were 
mixed with DCs and/or Fbs, as indicated in the figure legends, at a 10:1 
ratio one hour later, 1 μl/ml GolgiPlug (BD PharMingen) was added for 
5 h. The cells were washed with PBS after the total incubation time of 6 h. 

The samples were first labeled for 5 min at 4°C with CD8-CyChrome and 
in some experiments with CD3-PE followed by fixing with 4% paraform-
aldehyde for 20 min at room temperature. The cells were then perme-

Early apoptosis detection. HCMV-infected and uninfected MRC5 cells 
were labeled using the annexin V-FITC Apoptosis Detection Kit I ac-
cording to the manufacturer’s instructions. Briefly, 5 μl annexin V-FITC and 10 
μl propidium iodide (PI) were added to 10^5 cells in 100 μl labeling buffer.

The cells were incubated for 15 min at 37°C in the dark and analyzed on 

FACSCalibur (BD Biosciences) using CellQuest 3.1 software.

Table I. HLA class I types of donors and Fbs

<table>
<thead>
<tr>
<th>Donors</th>
<th>HLA Class I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seropositive</td>
<td>A*0201, 1; B27, 57</td>
</tr>
<tr>
<td>Seropositive</td>
<td>A*0201, 3; B7, 44</td>
</tr>
<tr>
<td>Seropositive</td>
<td>A*0201, 11; B7, 40</td>
</tr>
<tr>
<td>Seropositive</td>
<td>A*03, 24; B7, 27</td>
</tr>
<tr>
<td>Seronegative</td>
<td>A*0201, 23; B44, B15</td>
</tr>
<tr>
<td>Seronegative</td>
<td>A*0201, 29; B8, w40</td>
</tr>
<tr>
<td>Seronegative</td>
<td>A*0201, 28; B44, 35</td>
</tr>
<tr>
<td>MRC5</td>
<td>A*0201, 29; B7, 44</td>
</tr>
<tr>
<td>Fb1</td>
<td>A1; *0201, B8, 51</td>
</tr>
<tr>
<td>Fb2</td>
<td>A1; *0201, B44, 57</td>
</tr>
<tr>
<td>Fb3</td>
<td>A29, 31; B44, 51</td>
</tr>
</tbody>
</table>
to DCs or to PBMC. Fbs in some of the early experiments were irradiated with 3000 rad; however, a comparative experiment revealed that nonirradiated Fbs were equally efficient in the cross-presentation experiments (data not shown). Thus, for easier interpretation of the results, the irradiation step was omitted. For CTL stimulation in bulk cultures, DCs or Fbs were added at a 1:10 ratio to PBMC at 2 x 10^6 PBMC/ml. For stimulation in 96-well trays, 10^5 DCs and/or HCMV-infected Fbs were added to each well of PBMC as indicated in the figure legends. Cells were collected and counted from DC/Fbs/PBMC bulk cultures 6–7 days later, or cells from 96-well trays were split and added to different targets 9 days later.

T cell cytotoxicity was measured in a standard 51 Cr-release assay using pp65493–507 peptide-pulsed T2 cells or pp65417–426-pulsed autologous BLCL. To determine IE1-specific killing, Fbs were infected with rAd31 or rAd60 (moi = 50 PFU/cell) for 48 h, then labeled with 5 MBq/10^6 cells for 1 h. A total of 1 x 10^5 Fbs or 3 x 10^5 T2 cells or BLCL were added per well into 96-well U-bottom trays. Varying numbers of effector cells in triplicates were incubated with the target cells in 200 µl final volume/well for 4 h (T2 and BLCL) or for 6 h (Fbs), respectively. A total of 25 µl supernatant from each well was harvested onto glass-fiber mats (Wallac, Milton-Keynes, U.K.) and measured on a beta plate counter (Wallac).

Inhibition of Ag presentation

DCs were treated with 5 µg/ml cytochalasin B (Calbiochem, La Jolla, CA) or with 1 µM fura 2 (Molecular Probes) in the form of a fura 2 AM ester (Molecular Probes) at 10^5-10^6 cells/ml for 30 min at 37°C. Following the treatments, DCs were washed three times and incubated overnight with infected Fbs (1:1 ratio) before adding PBMC at a 10:1 PBMC:DC ratio. Intracellular cytokine production by T cells or CTL activity was measured as described above.

Results

Lack of HCMV IE/early gene expression in DCs following infection with HCMV strain AD169

Infection of DCs with the laboratory strain AD169 did not result in the expression of detectable levels of IE Ag, neither at the routinely used 5 moi (Fig. 1A) nor at higher rates of infection (data not shown), as measured 6, 16, and 44 h after infection. However, when DCs were infected under the same conditions with strain TB40/E (moi = 5 PFU/cell), an EC-adapted strain of HCMV, immediate early viral Ag was detected in 3.2% of DCs (Fig. 1B) as early as 6 h after infection, with the rate of Ag-positive cells rising to 53.6% by 44 h after infection. HCMV pp65 was undetectable early (6 h) after infection with both strains. Expression of pp65 was first detectable at 44 h after infection only in DCs infected with strain TB40/E, but not in those infected with strain AD169.

Furthermore, DCs, after infection with strain AD169 (moi = 5 PFU/cell), did not stimulate pp65-specific memory CTLs in vitro from the PBMC of seropositive donors (Fig. 2).

DCs cocultured with HCMV strain AD169 infected Fbs stimulate pp65-specific CTLs

To study whether DCs, while themselves remaining uninfected with HCMV strain AD169 (Fig. 1A), can stimulate HCMV-specific T cell responses in the presence of cells that are productively infected with HCMV strain AD169, DCs from seropositive donors were cocultured with HCMV-infected Fbs and with autologous PBMC. Because the CTL response in healthy seropositive individuals was shown to be dominated by pp65-specific T cells (18), we measured CTL activity against T2 target cells pulsed with the HLA-A*0201-restricted pp65493–507 peptide. DCs cocultured with HCMV-infected Fbs stimulated autologous, HLA-A*0201-restricted, pp65-specific cytotoxic T cells (Fig. 2). DCs preincubated either with HLA-A*0201-matched or with mismatched (allologic) Fbs generated HLA-A*0201-restricted, pp65 peptide-specific CTL responses at similar levels. These CTL responses were higher than that following incubation with HCMV-infected HLA-A*0201 Fbs alone. HCMV-infected allologic Fbs or DCs alone (Fig. 2) did not induce HLA-A*0201-restricted, pp65-specific CTL responses. In identical experiments in which PBMC were obtained from two seronegative HLA-A*0201-positive donors (donors 6 and 7; Table I), HCMV-specific T cell cytotoxicity was not induced (data not shown).

These results indicate that DCs are able to acquire viral Ags from infected Fbs and present them to CD8^+ specific T cells and exclude the possibility that HCMV present in DCs and reactivated by in vitro manipulation could induce these CTL responses.

DCs stimulate CD8^+ CTLs in the presence of low numbers of HCMV strain AD169-infected Fbs

DCs are more powerful than B cells or mononuclear cells in stimulating proliferative or cytotoxic T cell responses both in terms of
maximum response and amount of Ag required (34). To confirm this with respect to HCMV-infected Fbs, they were added in decreasing numbers to a constant number of DCs (2 × 10^5) and PBMC (2 × 10^6). DCs were able to stimulate significant levels of HLA class I-restricted, HCMV pp65 peptide-specific T cells in these cultures in the presence of as few as 2000 Fbs (Fig. 3). HCMV-infected Fbs alone were not capable of stimulating T cells at ratios lower than 200,000 Fbs/culture. (Fig. 3, □). These results indicate that T cell stimulation by DCs is not simply due to high numbers of infected Fbs present in the cultures and support a direct Ag-presenting role for DCs in these experiments.

Stimulation of IE1-specific CTLs by cross-presentation of HCMV Ags

To ascertain that the observed cross-presentation was not unique to the pp65 tegument protein, which can derive from the virion or from dense bodies (viral particles without DNA) without de novo protein synthesis, the CTL response to the 72-kDa IE1 Ag was also studied. IE1 is expressed abundantly in Fbs from 4 h following infection with HCMV, and it is a recognized T cell Ag (15, 20, 37–39). However, the presence of such CTLs in seropositive individuals has not been fully explained, as pp65 inhibits IE1 presentation at the level of the infected cell (7). IE1-specific CTLs were generated in bulk culture (Fig. 4, A and B) or in limiting dilution culture (Fig. 4C) from PBMC of seropositive donors. The results were similar to those obtained with pp65: only a low level of IE1-specific CTLs were induced by HCMV-infected Fbs alone (11% at 50:1 ratio; Fig. 4A), whereas the CTL activity was 42% following coculture of DCs with HCMV strain AD169-infected Fbs (Fig. 4A). When PBMC were cultured in limiting dilution, so that T cell responses at clonal level could be examined (Fig. 4C), fewer wells containing HLA class I-restricted IE1-specific T cells were generated at all cell numbers studied (1–4 × 10^4 PBMC/well) when infected Fbs alone were added, compared with cultures which also contained DCs. The higher levels of specific lysis observed from the individual wells following stimulation of T cells with DCs plus infected Fbs (Fig. 4C, ●) compared with those with infected Fbs alone (Fig. 4C, ▲) may reflect enhanced cell proliferation, resulting in larger clone sizes per well. These results indicate that DCs probably acquire and process several viral Ags from infected cells for Ag presentation.

High frequencies of HCMV-specific CD8+ T cells are stimulated by cross-presentation

The enhanced pp65- and IE1-specific CD8+ CTL induction by DC in the cytotoxicity experiments (Figs. 2–4) was also tested by measuring IFN-γ production by T cells following stimulation of PBMC from seropositive donors with DCs and/or partially infected HLA-matched Fbs (Fig. 5). This approach allowed us to
quantitate the total number of T cells stimulated by DCs and/or infected Fbs. Whereas HCMV AD169-infected Fbs stimulated 0.48% of the CD8+ T cells to produce IFN-γ, DCs plus infected Fbs stimulated 1.38% of the CD8+ T cells. The frequency was only 0.06% when uninfected Fbs were added to DCs (Fig. 5A). Similar results were obtained with three-color staining of lymphocytes with CD3-PE and CD8-CyChrome Abs together before intracellular cytokine staining was conducted as described in Materials and Methods. Stimulation with DCs plus HCMV strain AD169-infected MRC5 (1) induced more IFN-γ-producing cells than that with HCMV strain AD169-infected MRC5 alone (2) or with DCs plus uninfected MRC5 (3) in both (A) CD8+ and (B) CD4+ subsets. A representative of four experiments is shown.

FIGURE 6. FACS analysis following coculture of DCs and Fbs infected with RCMV288, the EGFP-expressing HCMV AD169 strain, to determine cell-to-cell spread of the virus. Immature DCs (donor 7; Table I) were cultured on their own (A) or together (1:1 ratio) with MRC5 cells infected for 48 h with RCMV288 (moi = 5 PFU/cell) (C), or the infected MRC5 cells were cultured on their own (B) for the times indicated on the left. The cells were then collected, stained with CyChrome-conjugated HLA class II Ab, and analyzed by FACS. The cells in the regions marked on the dot plot figures of C are double-positive cells; their frequencies are indicated on the right. The frequency of double-positive cells remained low for the entire period of the experiment (5 days), with the highest level being 0.4% (day 1). A representative of two experiments is shown.

Lack of EGFP expression in DCs following coculture with RCMV288-infected Fbs

Although we have shown that cell-free HCMV strain AD169 does not infect DCs (Fig. 1A), the potential of cell-to-cell spread of the virus during T cell stimulation in the mixed cultures also had to be addressed. For this, we used an HCMV recombinant based on the AD169 strain that encodes EGFP under the control of an early promoter. EGFP provides a strong fluorescent signal that is easy to detect and avoids the potential of nonspecific-Ab binding. Immature DCs were cultured on their own (Fig. 6, column A) or were mixed with equal numbers of MRC5 Fbs infected with RCMV288 (moi = 5 PFU/cell) 48 h earlier (Fig. 6, column C). Infected Fbs were also grown on their own as controls (Fig. 6, column B). The cells from individual wells were harvested at different times after the initiation of cultures (from 1 h to 5 days, as indicated) and labeled with a CyChrome-conjugated anti-HLA class II Ab. FACS analysis was used to detect double-positive cells that express both class II molecules and the GFP at high levels. The small region on the dot plot figures indicates double-positive cells. The results were similar to those we obtained with cell-free AD169 virus (Fig. 1A). Only a small proportion of cells expressed both markers (0.2–0.4%). Because double-positive cells were detected at 0.25% following only 1 h incubation on ice in the presence of the class II Ab (Fig. 6, first row), we suggest that some of the double-positive events observed in the mixed cultures were due to cell adhesion between two cells with different markers and not to infection of DCs with RCMV288. A slight shift of the HLA class II-positive cells on the FL1 axis is also detectable with time in the mixed cultures (Fig. 6, column C; days 4 and 5), and it might be due to phagocytosed material from infected Fbs. Taken together, this coculture experiment of DCs with RCMV288-infected Fbs indicates that if cell-to-cell spread of the virus occurs in the mixed cultures, its level remains below 0.4%.

Low level of infection of DCs with HCMV does not generate significant CTL responses

We used 0.4% (double-positive cells; Fig. 6) as the highest possible rate of infection of DCs with AD169 HCMV in coculture experiments. To determine the functional significance of this low level of DC infection during T cell stimulation, we used the EC-adapted strain of HCMV (TB40/E) to infect DCs. The level of infection with strain TB40/E was measured by FACS following intracellular staining with an Ab to HCMV early Ag as described earlier for strain AD169 (Fig. 1). Strain TB40/E-infected DCs were added at a gradually decreasing proportion (Fig. 7), ranging
additional experiments, similar results were obtained using IFN- 
would enable these DCs to stimulate virus-specific CTLs. In ad-
resulted only in a very low level of CTL activity (Fig. 7; 2.4%). 
DC infection rate in the previous coculture experiment (Fig. 6),
were relatively weak stimulators of CTL even when 20% of the 
DCs were infected. CTL stimulation at 0.4% infection, the possible 
compared with that induced with mixed cultures of DCs and strain AD169-
infected Fbs (□) or with strain AD169-infected Fbs alone (□). Means and SDs 
from triplicate wells at a 20:1 E:T ratio are shown.

from 19.7 (20) to 0.2%, to uninfected DCs, ensuring a constant 
number of DCs in each culture. Strain AD169-infected Fbs alone 
or mixed with DCs were also included in this experiment for com-
parison. Uninfected DCs alone served as negative control. HLA-
B7-restricted HCMV pp65417–426-specific CTL activity was mea-
production by CD8

**Stimulation of class I-restricted CTL responses by cross-
presentation of HCMV Ags is not enhanced by apoptosis of 
HCMV strain AD169-infected Fbs**

DCs have been shown to acquire and cross-present viral Ag to 
CD8
 CTLs from influenza-infected apoptotic cells, but not from 
necrotic cells, or from infected cells treated with an apoptosis inhi-
bitor (23). To determine the role of apoptosis of HCMV-infected 
Fbs in CD8
 T cell stimulation by DCs, when 20% of the 
DCs were infected. CTL stimulation at 0.4% infection, the possible 
DC infection rate in the previous coculture experiment (Fig. 6), 
resulted only in a very low level of CTL activity (Fig. 7; 2.4%). 
This extends our previous observation on the lack of ability of 
HCMV strain AD169 to infect DCs in a way or at a level that 
would enable these DCs to stimulate virus-specific CTLs. In ad-
ditional experiments, similar results were obtained using IFN-γ 
production by CD8
 T cells as a measure of the induced T cell 
response (data not shown).

**Stimulation of CD8
 T cells by cross-presentation is inhibited 
by treatment of DCs with cytochalasin B or brefeldin A**

To gain some insight into the mechanism of the Ag presentation 
described in this paper, DCs were pretreated with cytochalasin B, 
which partially inhibits soluble Ag presentation by disrupting actin 
microfilaments, and with brefeldin A, which blocks secretory pro-
tein traffic and class I Ag presentation (41). Pretreatment of DCs 
with cytochalasin B resulted in a 50% inhibition (Fig. 9A), and that 
with brefeldin A resulted in a 70% inhibition (Fig. 9B) of CD8
 T

da–c) (Fig. 8A, b) and not by Con A (e vs f), a and d show the level of apoptosis in untreated cells. The
numbers represent the proportion of all annexin V
 cells (early and late apoptotic cells). B, PBMC from a seropositive donor (donor 1; Table I) 
were stimulated with autologous DCs and/or HCMV strain AD169-infected 
and apoptotic MRC5 Fbs where apoptosis was induced by Con A treatment for 24 h. The
HLA-A*0201-restricted HCMV pp65-specific 
cytotoxic activity (T2 targets plus pp65 peptide; ■) and the
background lysis (T2, no peptide; □) are shown following stimulation as 
indicated under the columns. DCs with nonapoptotic infected Fbs were the 
most efficient stimulators of CTL. The results are expressed as lytic units (see explanation in Fig. 3 legend). One representative experiment of three is shown.

**Figure 7.** Lack of CTL stimulation with DCs infected with HCMV at a 
low level. PBMC from a seropositive donor (donor 4; Table I) were stimulated 
with autologous DCs (■) that were infected with HCMV strain TB40/E 
(moi = 5 PFU/cell) for 48 h. The level of infection was determined by intra-
cellular staining of DCs with FITC-conjugated HCMV p52-specific Ab. De-
creasing numbers of strain TB40/E-infected DCs were mixed with uninfected 
DCs to maintain a constant DC number in each group. The percentage of 
infectected DCs is indicated on the x-axis. Specific CTL activity was measured 
from the cultures 6 days later against HLA B7-restricted pp65417–426 peptide 
pulsed or unpulsed (data not shown) autologous lymphoblastoid B cells. +, 
CTL activity induced by DCs infected at 0.4% with strain TB40/E, which is 
equivalent to the highest level of double-positive cells described in the legend 
for Fig. 6. HCMV-infected DCs induced only a low level of CTL activity 
compared with that induced with mixed cultures of DCs and strain AD169-
infected Fbs (□) or with strain AD169-infected Fbs alone (□). Means and SDs 
from triplicate wells at a 20:1 E:T ratio are shown.

**Figure 8.** The effect of apoptosis of HCMV strain AD169-infected 
Fbs on CTL stimulation by DCs. A, HCMV-infected (d–f) MRC5 Fbs were 
more resistant than uninfected Fbs (a–c) to apoptosis induced by overnight 
incubation in the absence of serum and glutamine (b vs c) but not by Con 
A (c vs f). a and d show the level of apoptosis in untreated cells. The
numbers represent the proportion of all annexin V
 cells (early and late apoptotic cells). B, PBMC from a seropositive donor (donor 1; Table I) 
were stimulated with autologous DCs and/or HCMV strain AD169-infected 
and apoptotic MRC5 Fbs where apoptosis was induced by Con A treatment for 24 h. The
HLA-A*0201-restricted HCMV pp65-specific 
cytotoxic activity (T2 targets plus pp65 peptide; ■) and the
background lysis (T2, no peptide; □) are shown following stimulation as 
indicated under the columns. DCs with nonapoptotic infected Fbs were the 
most efficient stimulators of CTL. The results are expressed as lytic units (see explanation in Fig. 3 legend). One representative experiment of three is shown.

A (40) (41%); Fig. 8A, c). HCMV-infected Fbs were less sensitive 
to deprivation than uninfected ones (19%; Fig. 8A, e) but were as 
equally sensitive to Con A treatment as uninfected Fbs (Fig. 8A, f; 
53 vs 41%). However, DCs did not stimulate CD8
 T cells more 
efficiently in the presence of HCMV-infected apoptotic Fbs than in 
the presence of nonapoptotic ones (Fig. 8B). This finding suggests 
that DCs are able to acquire viral Ags for induction of specific 
CTLs from HCMV-infected nonapoptotic Fbs.

**Stimulation of CD8
 T cells by cross-presentation is inhibited 
by treatment of DCs with cytochalasin B or brefeldin A**

To gain some insight into the mechanism of the Ag presentation 
described in this paper, DCs were pretreated with cytochalasin B, 
which partially inhibits soluble Ag presentation by disrupting actin 
microfilaments, and with brefeldin A, which blocks secretory pro-
tein traffic and class I Ag presentation (41). Pretreatment of DCs 
with cytochalasin B resulted in a 50% inhibition (Fig. 9A), and that 
with brefeldin A resulted in a 70% inhibition (Fig. 9B) of CD8
 T

![Figure 7](http://www.jimmunol.org/)

![Figure 8](http://www.jimmunol.org/)
the proportion of IFN-\(\gamma\)-producing CD8\(^+\) cells (Fig. 9B). The effect of both inhibitors is reversible; thus, their removal and incubation of DCs in the absence of the inhibitors with HCMV-infected Fbs may be responsible for the partial recovery of DC function. However, the level of inhibition indicates the importance of protein transport from the endoplasmic reticulum in DCs during cross-presentation of CMV Ags. An additional experiment showed that cytochalasin B did not inhibit infection of DC with the EC-adapted HCMV strain TB40/E, either with free virus or in coculture experiments with strain TB40/E-infected Fbs (data not shown), confirming that the cytochalasin B-mediated inhibition was not directed against infectious virus uptake and trafficking to the nucleus within DCs.

**HCMV-infected Fbs provide a maturation signal for DCs**

Optimal cross-presentation of tumor Ags by DCs to CD8\(^+\) T cells requires two steps: 1) uptake of tumor Ag by immature DCs, and 2) a maturation signal for DCs (42). To demonstrate whether HCMV-infected Fbs can provide a maturation signal for DCs, the expression of CD83 on DCs following 2 days coculture with infected Fbs was examined (Fig. 10). DCs were cocultured with uninfected Fbs (Fig. 10a), with HCMV-infected Fbs (Fig. 10b), with autologous PBMC (Fig. 10c), or with infected Fbs and T cells together (Fig. 10d). HCMV-infected Fbs induced maturation of DCs (54.3% expressed CD83 vs 17.2% when uninfected Fbs were added) irrespective of the presence of T cells (45.7%). Up-regulation of HLA class II molecules followed a similar pattern (data not shown). Thus HCMV-infected Fbs provide both the Ag for cross-presentation and the signal for DC maturation, which together result in a successful stimulation of CD8\(^+\) T cells.

**Discussion**

In this study, we have demonstrated that DCs are not permissive for infection with HCMV strain AD169 and do not stimulate CTL responses following “infection” with strain AD169. This is in agreement with findings by others, who also observed lack of infection of DCs with Fb-adapted HCMV strains, and recent clinical isolates in vitro (32, 43). However, we show in this paper that DCs can acquire viral Ags from strain AD169-infected Fbs regardless of the HLA type of the Fbs and present them through their HLA...
Cross-presentation of viral Ags enables the APCs at a population level to bypass evasion mechanisms such as down-regulation of HLA class I molecules (3, 6), inhibition of IE1 Ag presentation by the pp65 tegument protein (7), inhibitory cytokine production (10), and possibly others used by HCMV to avoid recognition by CD8⁺ T cells.

The conventional view of Ag presentation to T cells is that exogenous Ags are presented via MHC class II and endogenous Ags via MHC class I molecules is simplistic and leaves questions unanswered about priming T cell responses to viruses that do not infect professional APCs. The cross-presentation pathway is obligatory in vivo for the initiation of CTL responses to viruses that infect only nonhemopoietic cells (45). Its role should be emphasized and studied more thoroughly also in the stimulation of CTL responses to those viruses that infect bone marrow-derived APCs. Cross-presentation has been described when high concentrations of exogenous soluble or particulate Ags (46), stress protein-associated Ags (47), or Ags derived from apoptotic virus-infected (23, 48) or apoptotic tumor cells (42) gained access to the class I Ag-presenting pathway. We describe here that cross-presentation of HCMV Ags by DCs occurs in the absence of early or late apoptotic markers on infected Fbs, and in vitro induction of apoptosis of HCMV-infected Fbs does not facilitate the process of Ag presentation to CD8⁺ T cells by DCs. The mechanism resulting in CD8⁺ T cell activation following the interaction between DCs and HCMV-infected Fbs is being studied in our laboratory. Because HCMV encodes molecules that block or delay apoptosis to enable virus replication (27), it is likely that cross-priming of specific CTLs by DCs is mediated by nonapoptotic signals expressed by cells infected with HCMV. The possible candidates are 1) cytopathic effects caused by the virus, especially nonapoptotic cell lysis, which would allow release of antigenic material from the infected cells; 2) cell surface molecules that are up-regulated by HCMV on Fbs. CD54 (ICAM-1) and CD58 (LFA-3) (49) may play a role in cell-cell fusion and exchange of intracellular viral particles. This has been shown between polymorphonuclear cells and ECs infected with clinical isolates but not with laboratory strains (50); and 3) decreased level of surface MHC class I molecules and increased production of certain cytokines and chemokines, or a combination of these factors. A recent study on cross-presentation of HCMV pp65 Ag by DC following coculture with apoptotic (TNF-α-treated) strain AD169-infected Fbs concluded that apoptosis was the main factor in cross-presentation of the pp65 Ag (51). However, the possibility of an alternative mechanism cannot be excluded, given the high level (52%) of secondary necrosis of Fbs in the coculture experiments.

Immature DCs are extremely well equipped to capture Ag in tissues. Ag uptake and the inflammatory environment provide signals that mobilize DCs to migrate to secondary lymphoid organs where they stimulate Ag-specific naïve T cells. We observed that coculture of HCMV-infected Fbs with immature DCs causes maturation of DCs as measured by the up-regulation of CD83 and HLA class II molecules. Viral infection of DCs can result in the inhibition of maturation, Ag presentation, or migratory capacity of DCs (52–54). Whether HCMV infection would interfere with the maturation and function of DCs remains to be seen by using virus strains that infect DCs efficiently. Decrease in the viable cell number of DCs 6–8 days following infection with an EC-adapted strain of HCMV has been observed by others (32) and by us, as well as down-regulation of HLA class I molecules on infected DCs (M. Moutaftsi, L. K. Borysiewicz, and Z. Tobi, manuscript in preparation). These preliminary observations also point toward the potential importance of cross-presentation not only in cases in which professional APCs cannot be infected with the virus, but also when the viral infection results in an impairment of the Ag-presenting function. We suggest that infection of DCs with wild-type HCMV in vivo and uptake of viral material in situ from infected tissue can occur simultaneously and may depend on the given environment. Acquisition of noninfected viral material by DCs and cross-presentation of viral Ags to lymph node T cells would increase the host’s chances to develop a strong CTL response 1) without dissemination of infectious virus by DCs, and 2) without being affected by the inhibitory effects of HCMV.

The pp65 protein inhibits the presentation of IE1-derived Ags in target cells (7), but its effect on the stimulation of IE1-specific T cells has not been studied. Indeed, several groups have reported significant levels of T cells specific for IE1 Ags in persistently infected asymptomatic individuals (15, 37) without explaining how it happens in the face of inhibition by pp65. We have shown here that DCs successfully induce IE1-specific CTLs via the cross-presentation pathway, although we did not address the question of whether the weak T cell stimulation by HCMV-infected Fbs alone was due to inhibition by pp65. This still remains an important question.

It is likely that the nonadherent fraction of PBMC used in our experiments contained some DC precursors. These remaining DCs may, in fact, have been inducing the IE1- and pp65-specific CTL responses when HCMV-infected Fbs alone were added to PBMC, given the poor T cell stimulatory capacity of infected Fbs expressing very low levels of HLA class I molecules. The mechanism involved could also have been cross-priming, although at a much reduced level due to the lower number of DCs present. An alternative explanation for the higher stimulatory capacity of DCs could be that they stimulated a different or an additional subpopulation of memory T cells than HCMV-infected Fbs alone. The recently described subpopulations of memory T cells (central and effector memory T cells) (55) with different activation requirements and recirculation pathways would be likely candidates. In conclusion, the data reported here show that DCs present HCMV Ags via HLA class I molecules after acquiring viral Ag from HCMV-infected Fbs. Our results also show that HCMV-infected Fbs provide viral Ags to DCs for cross-presentation to stimulate class I-restricted CTLs in the absence of apoptosis of infected cells. DCs also express maturation markers following the encounter with infected Fbs. These findings have potential implications for generating HCMV-specific T cells for adoptive immunotherapy.

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References


