Macrophage cultures are susceptible to lytic productive infection by endothelial-cell-propagated human cytomegalovirus strains and present viral IE1 protein to CD4⁺ T cells despite late downregulation of MHC class II molecules

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The contribution of CD4⁺ T cells to control of human cytomegalovirus (HCMV) has been shown and infected tissue macrophages might contribute to this response by antigen presentation. As shown previously, CD4⁺ T cells recognize HCMV immediate-early antigen IE1 on glioblastoma cells manipulated to express MHC class II molecules. Here, the possible interference of virus-induced MHC class II downmodulation with the presentation of IE1 by natural target cells was analysed. The capacity of IE1-specific CD4⁺ T-cell clones to recognize HCMV-infected monocyte-derived macrophages was tested. Various HCMV strains were used to achieve efficient infection of macrophages. Activation of CD4⁺ T cells by infected macrophages was evaluated at different time points after infection. Endothelial-cell-adapted HCMV strains efficiently infected cultured human macrophages. However, the immediate-early and early phases of replication were prolonged. Infected cells entered the late replication phase only after 3 days of infection, which was associated with downmodulation of MHC class II molecules at the surface of infected cells. Strong stimulation of IE1-specific CD4⁺ T cells resulted from endogenous de novo antigen production and presentation by infected macrophages during the first 3 days of virus replication, despite MHC class II downmodulation in the late replication phase. Therefore, infected macrophages are assumed to contribute to the antiviral immune response in infected organs.

INTRODUCTION

Human cytomegalovirus (HCMV) establishes lifelong persistence in the human host after primary infection. Viral persistence is due to a balanced state of the host’s antiviral immune reactions and viral mechanisms of immune evasion (Hegde et al., 2003; Johnson & Hill, 1998; Miller et al., 2001), leading to asymptomatic infection in immunocompetent people. Pathology due to HCMV arises mainly when the immune system is deficient, such as in AIDS patients and transplant recipients (Pass, 2001).

Among the various cell types shown to be infected by HCMV in vivo (Sinzger et al., 1993a, b, 1995, 1999), macrophages may play a particular pathogenic role (Sinzger et al., 1996). Macrophages, along with polymorphonuclear cells, have been described as participating as vectors of HCMV dissemination in the blood circulation (Smith et al., 2004), possibly through interaction with endothelial cells (Waldman et al., 1995; Gerna et al., 2000). In vitro, only low-passage isolates and endothelial-cell-propagated ‘endotheliotropic’ strains of HCMV are capable of infecting macrophages efficiently (Jahn et al., 1999; Minton et al., 1994). The UL131–128 region of the HCMV genome has been reported recently as a genetic determinant of endothelial-cell tropism (Hahn et al., 2004) and presumably also governs macrophage tropism. Regarding the underlying mechanisms, endothelial-cell tropism depends on the efficiency of translocation of viral DNA to the nucleus (Sinzger et al., 2000; Slobbe van Drunen et al., 1998) and it is tempting to assume a similar mechanism to explain macrophage tropism.

Little is known about the functional consequences of HCMV infection of macrophages. Surprisingly, although mature macrophages are permissive to the complete replicative cycle in vitro (Ibanez et al., 1991; Lathey & Spector, 1991), significant cytopathology has not been reported (Fish et al., 1996). With regard to the antigen-presenting properties of macrophages, surface expression of MHC class II molecules...
is reduced slightly to about 25 % in macrophage cultures by various HCMV isolates (Fish et al., 1996) and in dendritic-cell cultures by the low-macrophage-tropic strain AD169 (Odeberg & Söderberg-Naucler, 2001). It is not known whether downregulation is more profound after highly efficient infection of macrophage cultures by endothelial-cell-propagated strains and whether such downregulation would affect presentation of viral antigens to CD4$^+$ T lymphocytes.

Strong CD4$^+$ and CD8$^+$ T-lymphocyte immunity against a variety of HCMV antigens has been described (Beninga et al., 1995; Elkington et al., 2004; Kern et al., 1999; Retière et al., 2000). Of these antigens, the immediate-early antigen IE1 has been described in our laboratory as a significant target of the CD4$^+$ T-cell response (Davignon et al., 1995). We have shown that IE1-specific CD4$^+$ T-cell clones can recognize infected U373MG cells induced to express MHC class II by transfection of class II transactivator (CIITA) (Le Roy et al., 2002). Presentation occurs at early time points through endogenous presentation (Le Roy et al., 2002). However, the T-lymphocyte response to infected macrophages has not been studied. Since macrophages are potent antigen-presenting cells (APCs) (Bryant & Ploegh, 2004), we have sought to analyse the interaction of HCMV, macrophages and HCMV-specific CD4$^+$ T cells in a cell-culture model. In particular, this work focused on defining the kinetics of virus replication, cytopathogenicity and MHC class II downregulation in infected macrophages and their capacity to present HCMV IE antigens to CD4$^+$ T cells in vitro.

Here, we have shown that macrophages are highly susceptible to cytopathogenic infection by endothelial-cell-propagated HCMV strains. However, the slow kinetics of virus replication allows a strong CD4$^+$ T-cell response to IE1 resulting from endogenous presentation of this viral antigen.

**METHODS**

**Cells.** Monocytes were enriched by plastic adherence from peripheral blood mononuclear cells (PBMCs) from HCMV-seronegative healthy volunteers, who had given informed consent. PBMCs were isolated by centrifugation of fresh whole blood diluted 1:1 with PBS on Histopaque-1077 (Sigma) for 30 min at 400 $\text{g}$ non-adherent cells were removed by two washes with warm RPMI 1640 and the remaining cells were reincubated with RPMI 1640 plus 10 % FCS until used for infection.

Monocyte-derived macrophages (MDMs) were generated by incubation of monocytes for 7 days at 37 $^\circ\text{C}$ in 5 % CO$_2$ in MDM medium containing 60 % AIM V medium, 30 % Iscove’s modified Dulbecco’s medium, 10 % human serum and 100 µg gentamicin ml$^{-1}$, supplemented with 100 ng granulocyte-macrophage colony stimulating factor (GM-CSF) (Leucomax; Novartis) ml$^{-1}$. For further maturation, MDMs were incubated for 14–21 days in MDM medium without GM-CSF. Cell morphology and cytopathic effects (CPE) were documented by phase-contrast micrographs in a Zeiss Axiosvert microscope.

**Flow cytometry.** The surface phenotype of MDMs was evaluated by flow cytometry. Anti-CD1a, -CD11c, -CD14, -CD80, -CD83, -CD86 and -HLA-DR mAbs coupled to either phycoerythrin or FITC and corresponding isotype controls were purchased from Immunotech Beckman Coulter or Becton Dickinson. For flow cytometry of infected cells, a green fluorescent variant of HCMV TB40/E was used, which allowed direct discrimination of infected cells. MDMs were detached using Accutase (Innovative Cell Technologies), collected and stained with specific mAbs for 20 min at 4 $^\circ\text{C}$. Samples were then analysed using an Epics XL Coulter or FACScalibur (BD Biosciences) apparatus utilizing CellQuest Pro software.

**Viruses strains.** Strains VHL/E and VHL/F, initially propagated from a bone marrow transplant recipient on endothelial cells and fibroblasts, respectively, were kindly provided by J. Waldman (Department of Surgery, University of Chicago, USA). Strains TB40/E and TB40/F were derived in our laboratory from a bone marrow transplant recipient by 22 passages in endothelial cells and fibroblasts, respectively. AD169 is a highly passaged fibroblast-adapted laboratory strain of HCMV. For preparation of virus stocks, human foreskin fibroblasts cells were infected at an m.o.i. of 0-1. Supernatants of infected cultures were harvested 6 days post-infection (p.i.) and stored at −80 $^\circ\text{C}$ after removal of cell debris by centrifugation for 10 min at 2800 $\text{g}$. The infectious titre in HCMV preparations was determined by TCID$_{50}$ assays in fibroblasts grown in 96-well plates.

**Infections.** For cell-free infection of cell cultures, medium was removed and replaced by fresh MEM with 5% FCS 60 min prior to infection. Virus preparations were then added for 90 min at 37 $^\circ\text{C}$. Cells were washed with fresh medium and maintained at 37 $^\circ\text{C}$ in the appropriate medium. For single-step growth curves, MDMs were infected with HCMV preparations as described above at a virus concentration of 10$^5$ TCID$_{50}$ ml$^{-1}$ (m.o.i. = 10). After 90 min of incubation, cultures were washed six times with medium to remove residual virus and cultured for 10 days at 37 $^\circ\text{C}$ in MDM medium containing 60 % AIM V medium, 30 % Iscove’s modified Dulbecco’s medium, 10 % human serum and 100 µg gentamicin ml$^{-1}$. Starting at day 1 p.i., 2 ml supernatant was removed daily from infected-cell cultures, being replaced by 2 ml fresh medium, and stored at −80 $^\circ\text{C}$ for determination of the infectious titre.

**Immunofluorescent detection of HCMV antigens.** For in situ detection of viral antigens in infected cells, indirect immunofluorescent staining was performed. To analyse the kinetics of viral gene expression, mAbs against viral proteins from different phases of the HCMV replicative cycle were used, which displayed nuclear localization. Specifically, mAbs react against the immediate-early proteins IE72 and IE86 (pUL122 and pUL123, mAb E13; Biosoft), the early protein p52 (pUL44, mAb BS510; Biotest) and the late major capsid protein (pUL86, mAb 28-4; kindly provided by W. Brit, University of Alabama at Birmingham, USA) were used. At various time points after infection, MDMs grown in 24-well dishes were fixed with 80 % acetone for 5 min at room temperature. Fixed cells were probed with antibodies against viral antigens for 60 min at 37 $^\circ\text{C}$, followed by incubation with Cy3-conjugated goat anti-mouse Ig Fab$^\gamma$ polyclonal serum (Dianova). Nuclei were counterstained with DAPI. Staining was detected in a Zeiss Axiovert fluorescence microscope with an appropriate filter set (excitation BP 546/12; emission LP 590) and documented by photography. For in situ detection of virus particles, primary antibodies against the late tegument protein pp150 (pUL32, mAb XP1; Dade Behring) were used. This antigen remains associated with the capsid during virus entry. MDMs grown in six-well plates were infected at an m.o.i. of 10 for 24 h. Cells were then trypsinized, scraped from the plate, cytospun and fixed with...
acetone at room temperature. Fixed cells were probed with mAb XP1 for 60 min at 37 °C, followed by incubation with Alexa 488-conjugated goat anti-mouse Ig Fab'2 polyclonal serum (Molecular Probes). Nuclei were counterstained with DAPI. Staining was detected in a Zeiss Axiovert fluorescence microscope with an appropriate filter set (excitation BP 450–490; beam splitter 510; emission LP 515) and documented by photography.

Double immunofluorescence of cell-surface and viral antigens. For detection of surface expression of MHC class II molecules, live-cell cultures were incubated for 1 h on ice with antibodies reactive against HLA-DR (clone CR3/43; Dako) diluted in appropriate medium. Cultures were washed three times with cold medium and incubated with Cy3-conjugated goat anti-mouse Ig Fab'2 polyclonal serum diluted in appropriate medium. Cells were then fixed with 80% acetone for 5 min at room temperature and washed twice with PBS. Viral antigens were detected by incubation with HCMV-specific mAbs followed by Alexa 488-conjugated goat anti-mouse Ig Fab'2 polyclonal serum (Molecular Probes), each at appropriate dilutions, for 1 h at 37 °C. Staining was detected in a Zeiss Axiovert fluorescence microscope with appropriate filter sets for Cy3 staining (excitation BP 546/12; emission LP 590) and Alexa 488 staining (excitation BP 450–490; beam splitter 510; emission LP 515) and documented by photography.

IE1-specific CD4+ T-cell response. IE1-specific, HLA-DR3-restricted, CD4+ T-cell clone FzB1 was obtained by limiting dilution and maintained as described previously (Le Roy et al., 2002). Briefly, FzB1 was restimulated weekly in the presence of phytohaemagglutinin (PHA), IL-2 and allogeneic irradiated PBMCs in culture medium supplemented with 10% AB human serum (Le Roy et al., 2002). MDMs (10^4 per well) were seeded in flat-bottomed Primaria 96-well plates. HCMV (VHL/E) was ultracentrifuged (30 000 g, 30 min) four times to get rid of soluble IE1, as described previously (Le Roy et al., 2002). VHL/E inoculum was then applied to MDM cultures together with FzB1 CD4+ T cells (4 x 10^4 per well). Supernatant was collected 24 h later and/or after 48, 72 and 96 h, aliquotted and frozen at −80 °C until determination of IFN-γ levels.

To test for endogenous production, MDMs were pulsed for 3 h with supernatant from the 48 h infection and fixed with 0.05% glutaraldehyde. This 3 h pulse of MDMs with antigen had been determined previously to be optimal.

RESULTS

The efficiency of infection of MDMs by HCMV is due to strain-dependent efficiency of particle translocation

It is known that HCMV can infect monocyte-derived macrophages productively to some extent. However, to analyse HCMV-induced functional alteration of MDMs, higher infection rates were desirable. As significant inter-strain differences have been described for endothelial and dendritic cells (DCs), various HCMV strains were tested for their capacity to initiate viral gene expression in MDMs. VHL/E and TB40/E represented highly endotheliotropic strains, whereas VHL/F, TB40/F and AD169 represented low-endotheliotropic strains. The virus concentrations of all preparations were normalized on fibroblasts to represent an m.o.i. of 10. The capacity to initiate infection was tested by immunofluorescent detection of viral immediate-early antigens (pUL122 and pUL123) at 24 h after infection of differentiated MDMs with cell-free HCMV preparations. The proportion of MDMs that were infected under these conditions clearly differed in a strain-dependent way. The highly endotheliotropic strains VHL/E and TB40/E infected up to 90% of cells, whereas the efficiency of all low-endotheliotropic strains was <0.1% (Fig. 1a). It is noteworthy that this 1000-fold interstrain difference in MDM tropism was already apparent at the level of immediate-early protein expression.

![Fig. 1. Strain-dependent efficiency of HCMV infection of MDMs. (a) MDMs were infected with HCMV strains VHL/E, VHL/F, TB40/E and TB40/F at an m.o.i. of 10. At 24 h p.i., expression of viral immediate-early antigens (pUL122/123) was detected by indirect immunofluorescence (red nuclear fluorescence). Counterstaining was done with DAPI (blue nuclear fluorescence). (b) Nuclear translocation of incoming virus particles was analysed by indirect immunofluorescence of the viral capsid associated tegument protein pUL32 at 24 h p.i. Green punctate signals represent individual virion particles. Cell nuclei were counterstained with DAPI.](http://vir.sgmjournals.org)
To define further the replication step that determines this phenotype, incoming HCMV particles were visualized by immunofluorescence of the capsid-associated tegument protein pUL32 in MDMs from which adsorbed non-penetrated virus had been removed by trypsin treatment (Fig. 1b). All strains were able to penetrate MDMs, as indicated by the detection of punctate green fluorescent signals. However, only strains that infected MDMs at a high rate were translocated efficiently towards the nucleus after penetration. In contrast, all strains that failed to infect MDMs efficiently remained at the periphery of the cells, despite successful penetration.

In summary, HCMV strains VHL/E and TB40/E proved to be highly MDM tropic, whereas strains VHL/F, TB40/F and AD169 has only a low level of MDM tropism. As a consequence of these results, strains VHL/E and TB40/E were used in subsequent experiments whenever high infection rates were desired.

Endothelial-cell-propagated strains cause CPE in infected MDMs

The course of infection was characterized by analysing the kinetics of viral antigen expression, the release of progeny virus and the induction of morphological CPE by various HCMV strains. Indirect immunofluorescence of viral protein pUL86 from different phases of virus replication revealed that, once initiated, the infection proceeded towards the late stage. However, in comparison with the well-known kinetics in fibroblast cultures (TB40/E and VHL/E behaved identically to AD169 in fibroblasts; data not shown), the early phase appeared remarkably prolonged, as indicated by the fact that the major capsid protein pUL86 was detected only after 4 days of infection (Fig. 2a). Along with the occurrence of this structural viral protein, progeny virus was detected in the supernatant at 4 days p.i. A rise of about 4 log steps in the virus titre from day 3 to day 6 p.i. unequivocally demonstrated productive infection by a highly MDM-tropic strain (TB40/E; Fig. 2b). In contrast, virus production after infection with a low-MDM-tropic strain was approximately 100-fold lower (TB40/F; Fig. 2b). In addition to the occurrence of late viral antigens and progeny virus, CPE became detectable on day 4 p.i., proceeding to almost complete lysis of infected MDM cultures by highly MDM-tropic strains on day 8 of infection (Fig. 2c). In contrast, no CPE was detectable after the low-level infection induced by low-endotheliotropic HCMV strains (Fig. 2c). In summary, the course of infection by highly MDM-tropic HCMV strains was productive and lytic with CPE occurring after 4 days of infection. Whilst it is obvious that virus-induced cell lysis will reduce the capacity of infected MDM cultures to present viral antigens, the kinetics of HCMV-induced CPE raised the question of whether infected MDMs were functional during the non-cytopathic phase of virus replication until day 4 p.i. In particular, we were interested in whether infected MDMs were capable of MHC class II-restricted antigen presentation.

The surface phenotype of HCMV-permissive MDMs

HCMV-permissive MDMs were tested for the expression of markers specific for macrophages and DCs. As shown in Fig. 3, the vast majority of cells expressed both CD14 and CD11c, which are markers of differentiated macrophages (Shortman & Liu, 2002). Surprisingly, a phenotype of mature DCs was suggested by the expression of CD83 (Brigl & Brenner, 2004; Shortman & Liu, 2002). Although expression of markers such as CD80 and CD86 was observed (Fig. 3), it was not as high as is normally observed on mature DCs (data not shown). CD1a was also tested, as it has been shown to show various patterns of expression according to cell differentiation. It is expressed by DCs but not by macrophages, although some DCs are also negative for CD1a (Brigl & Brenner, 2004; Chang et al., 2000). Thus, markers of MDMs suggested that they were able to present antigen to CD4+ T lymphocytes.

Surface expression of MHC class II molecules by infected MDMs

Surface expression of MHC class II molecules is a prerequisite of successful antigen presentation to CD4+ T cells. HCMV infection has been shown to interfere with this process. Therefore, we tested whether MHC class II molecules were downregulated in infected MDMs and when such downregulation would occur. As MDMs adhered strongly to culture dishes and could only be removed after extended treatment with Accutase for 45 min, in situ double immunofluorescent staining was done in addition to FACS analysis to ensure the reliability of the FACS data. For the in situ method, live cells were immunostained for MHC class II molecules in order to stain surface molecules exclusively. Nuclear expression of pUL44, which is specific for the early phase of replication, was then analysed. This technique allowed us to assess individual cells for their MHC class II surface expression in correlation with the stage of infection. For FACS analysis, a green fluorescent variant of HCMV TB40/E was used. This allowed the direct distinction of infected and uninfected cells within the infected culture by analysis of green fluorescence. Thus, the effect of HCMV infection on susceptible cells as well as on non-susceptible bystander cells could be compared with the MHC class II expression in untreated cultures.

There was clear downregulation of MHC class II molecules on infected cells compared with non-infected bystander cells (Fig. 4a, b). However, a subset of infected cells still expressed MHC class II molecules, particularly during the immediate-early and early phases of replication. Only when inclusion bodies appeared within the nucleus of infected cells were MHC class II molecules suppressed below the detection level of the immunofluorescence method (Fig. 4a). Although this finding confirmed that HCMV interferes with MHC class II surface expression, it suggested that infected cells may be functional during the first 3 days p.i. with regard to antigen presentation to CD4+ T cells. Therefore, this aspect was
addressed by functional assays employing HCMV-specific CD4^+ T-cell clones.

**Anti-IE1 CD4^+ T-cell activation by infected MDMs**

We sought to determine whether infected MDMs would be able to present antigen to IE1-specific CD4^+ T cells. We used HLA-DR3-positive MDMs as APCs and HLA-DR3-negative MDMs as controls. As shown in Fig. 5, the HLA-DR3-restricted FzB1 CD4^+ T-cell clone produced IFN-\( \gamma \) in response to infected MDMs. IFN-\( \gamma \) production could be observed as early as day 1 p.i. (Fig. 5a, b). The IFN-\( \gamma \) response increased over time when MDM #1 (Fig. 5a) was used and slightly decreased when MDM #2 (Fig. 5b) was used, probably due to slightly different levels of IE1 expression and kinetics of HLA-DR downregulation from one experiment to another. Thus, it seemed that the CD4^+ T-cell response was still significant, despite the decrease in HLA-DR during the course of infection. To ensure that the CD4^+ T-cell response was against IE1 synthesized by infected MDMs, the virus inoculum was ultracentrifuged to eliminate soluble IE1, as described previously (Le Roy et al., 2002). Control experiments (Fig. 5b) showed that, as expected (Le Roy et al., 2002), the inoculum contained soluble IE1 that could activate FzB1. However, the last wash of ultracentrifuged virus did not contain sufficient amounts of IE1 to be able to activate FzB1. Thus, the secretion of IFN-\( \gamma \) in

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**Fig. 2.** Course of HCMV infection in MDMs. (a) Kinetics of viral antigen expression of HCMV strain TB40/E in endothelial cells after infection at an m.o.i. of 10. Immediate-early antigen (pUL122/123), early antigen p52 (pUL44) and late major capsid protein (pUL86) were detected in MDMs by indirect immunofluorescence (red nuclear staining). The time course of antigen expression in infected endothelial cells is indicated by bars. (b) Single-step growth curve of HCMV strains TB40/E and TB40/F after infection of MDMs at an m.o.i. of 10. Infectivity in supernatants from infected MDMs culture was determined by TCID_{50} assays. (c) CPE induced by HCMV strains TB40/E and TB40/F was documented by phase-contrast microscopy at days 5–8 p.i.
response to infected MDMs was due to activation of FzB1 due to presentation of de novo-synthesized IE1. Production of IFN-γ in the absence of FzB1 was negligible, confirming that, under our conditions, infected MDMs did not produce significant amounts of IFN-γ. As a control, MDMs from an HLA-DR3-negative blood donor (MDM #3) did not present IE1 to FzB1 and no IFN-γ production by FzB1 was observed (Fig. 5b). We then assessed whether endogenous and/or exogenous presentation of IE1 had occurred (Fig. 5c). Supernatant from MDMs infected for 48 h was used as a source of exogenous antigen to pulse uninfected MDMs, which were then fixed. The IFN-γ production by FzB1 was compared with that of infected, fixed MDMs from which the supernatant originated. As seen in Fig. 5(c), IFN-γ secretion under these conditions was approximately 6-fold lower than that observed when FzB1 CD4+ T cells were incubated with infected MDMs, indicating that, although both endogenous and exogenous presentation had occurred, endogenous presentation was predominant at this stage of infection.

Fig. 3. Surface phenotype of HCMV-permissive MDMs. MDMs were cultured for 14 days as described in Methods. Cells were then analysed by flow cytometry using specific phycoerythrin-coupled anti-CD11c, -CD80, -CD83 and -CD86 and FITC-coupled anti-CD1a and -CD14. The results shown are representative of three independent experiments.

Fig. 4. Surface expression of MHC class II molecules on HCMV-infected MDMs. (a) Cultured MDMs were mock infected or infected with HCMV TB40/E at an m.o.i. of 5. After incubation for 2, 3 or 4 days, double immunofluorescent staining was performed for simultaneous detection of MHC molecules and HCMV antigens. Live-cell staining of MHC class II molecules was performed by indirect immunofluorescence with the respective primary antibodies and Cy3-conjugated secondary antibodies (red surface-staining signals). Cells were then fixed and permeabilized with acetone and stained for viral early antigen by indirect immunostaining with mouse mAbs against HCMV pUL44 and Alexa 488-conjugated secondary antibodies (green nuclear-staining signals). (b) Cultured MDMs from two different donors were mock infected (curves with shading beneath) or infected with green fluorescent HCMV TB40/E at an m.o.i. of 10. After incubation for 5 days, cells were harvested by Accutase treatment and surface expression of MHC class II molecules (R-phycoerythrin-labelled anti-MHC class II antibody) on infected (green fluorescent, GFP-positive; dotted lines) and uninfected (GFP-negative; solid lines) cells was analysed by FACS.
Thus, IE1-specific CD4+ T cells are able to recognize and produce IFN-γ in response to IE1 produced endogenously and presented by infected MDMs.

DISCUSSION

Infection of MDMs has been demonstrated to depend on the differentiation state of these cells (Ibanez et al., 1991; Lathey & Spector, 1991) and on the virus strains used for infection (Jahn et al., 1999; Minton et al., 1994). However, little is known about the functional consequences of HCMV infection of macrophages. As professional APCs, macrophages participate in the host’s immune response as targets of the CD4+ response. Therefore, it was of particular interest to determine whether HCMV infection would cause CPE in these cells and whether this would affect the ability of infected macrophages to present viral antigens via the MHC class II pathway.

Here, we have shown that endotheliotropic HCMV strains can also infect MDMs with high efficiency. In contrast, MDMs were almost resistant to infection by low-endotheliotropic strains. Such strains could penetrate MDMs, but were not translocated towards the nucleus and consequently did not initiate viral gene expression. As a result, non-structural viral proteins such as IE1 would not be expected to be presented by MDMs infected with low-endotheliotropic strains. As this analysis focused on the recognition of infected MDMs by IE1-specific CD4+ T cells, only highly MDM-tropic strains were used for the functional analyses. However, even low-MDM-tropic strains might introduce structural proteins into macrophages during penetration and might thus elicit presentation of such input proteins; however, this is outside the scope of the present paper.

HCMV strains TB40/E and VHL/E were translocated to the cell nucleus after penetration and initiated virus replication. Viral immediate-early proteins were detected as early as 2 h p.i. and proteins of the early and late replication phases appeared at 2 and 4 days p.i., respectively, which was suggestive of productive infection. Consistent with the appearance of the viral major capsid protein, progeny virus was released by infected MDMs from day 4 p.i. After 8 days of infection, virus production declined and the infected culture was lysed. Notably, no prominent cytopathogenicity was
detected during the first 4 days of infection. Taken together, the course of infection appeared to be productive, finally leading to cell death. However, the first 4 days of infection open a window for the immune function of infected macrophages. During this time period, many viral proteins of all replication stages are expressed, but there is not yet significant release of infectious progeny virus.

This window might be restricted further by virus interference with the antigen-presentation pathways. In fact, downregulation of MHC class II molecules in infected macrophage cultures or DC cultures has been reported, although the degree of downregulation was moderate (Fish et al., 1996; Odeberg et al., 2003). In particular, when macrophage cultures were infected with non-cytopathic HCMV strains, MHC class II expression was only reduced by 25% at 8 days p.i. and by 80% at 12 days p.i. (Fish et al., 1996). As these studies did not analyse MHC class II expression at the single-cell level, it was unclear whether the observed downregulation occurred on infected cells or on their uninfected bystander cells. We were interested, therefore, in how the cytopathic HCMV strains TB40/E and VHL/E would alter MHC class II expression at the single-cell level. The immunofluorescence data presented here show unequivocally that surface expression of MHC class II molecules was downregulated in infected cells, whereas uninfected bystander cells were unaffected. As these endothelial-cell-propagated strains resemble recent isolates with regard to their cell tropism, productive lytic infection of macrophages with HCMV class II downregulation is assumed to occur in vivo. As a consequence, interaction of infected macrophages with HCMV-specific CD4+ T cells might be impaired within infected organs. On the other hand, MHC class II expression was maintained during the first 2 days of infection. Some degree of reduction occurred only after 3 days of infection and was complete on day 4 p.i. The implication of this result is that infected macrophages might be capable of antigen presentation via the class II pathway, at least during the immediate-early phase of virus replication, and that this might result in stimulation of an antiviral immune response and also in control of infected macrophages by CD4+ T cells.

The question remains as to the cell types represented by the MDM model. Surface markers of permissive MDMs were compatible with those of functional APCs. A mixed macrophage/DC phenotype was suggested by the co-expression of CD83 and CD14, although a more thorough study of specific markers of each population would define more precisely the permissive cell type. In particular, CD1a has been shown to be expressed by immature DCs, diminished on mature DCs and not expressed by macrophages (Brigl & Brenner, 2004). However, CD1a-negative DCs have been described and may display different patterns of cytokine expression (Chang et al., 2000). In conclusion, irrespective of the question of whether in vivo macrophages display exactly the same phenotype, MDMs may serve as a model of professional APCs. In particular, the MDM model will be helpful to reveal the possible interference of MHC class II downregulation with recognition of infected APCs by CD4+ T cells. However, despite some degree of MHC class II downregulation, IE1-specific CD4+ T-cell clones could recognize and respond to infected MDMs. This confirms our previous findings in U373MG–CIITA cell cultures whereby CD4+ produced IFN-γ in response to HCMV infection. In particular, endogenous presentation of IE1 could be established in our previous model at early time points in infection. This led to control of infection by those IE1-specific CD4+ T-cell clones. However, it was unclear whether the finding of target-cell recognition by HCMV-specific CD4+ T cells would also hold true in natural target cells, where MHC class II expression may be altered during virus replication. Thus, the use of CD4+ provided us with a powerful tool in both models of infection (U373MG–CIITA cells or MDMs) to analyse the immediate response at time points where IE1 is produced but mechanisms of MHC class II downregulation are not yet activated.

We have demonstrated that infected MDMs can present IE1 to specific CD4+ T lymphocytes. Downregulation of constitutive MHC class II expression on APCs has been documented widely (Cebulla et al., 2002; Fish et al., 1996; Hegde et al., 2003; Miller et al., 2002; Odeberg & Söderberg-Nauclér, 2001; Odeberg et al., 2003), but no direct consequence has been measured on the anti-HCMV CD4+ T-cell response. In the present study, downregulation of HLA-DR was observed, although a strong CD4+ T-cell response was detectable. Since the kinetics of IE1 expression and MHC class II downregulation are different, presentation of IE1 may precede downregulation of MHC class II molecules, allowing efficient presentation of IE1 by infected MDMs. When downregulation of MHC class II occurs, presentation by infected MDMs is expected to be less efficient. However, at later time points, CPE allows the release of IE1 from infected cells (Le Roy et al., 2002). This may result in the uptake and presentation of IE1 by uninfected APCs.

IE1 is the first protein produced in HCMV-infected cells and was observed as early as 2 h p.i. Therefore, CD4+ T-cell reactivity may target infected cells before CPE and the release of IE1 into the extracellular milieu. This is crucial in view of the early control of infection and reactivation. Although our study was done at a population level and did not analyse cell-to-cell interactions, we can assume that endogenous presentation of IE1 would be effective in controlling infection due to interaction with the infected cell.

Few examples of endogenous presentation of a nuclear protein such as IE1, as shown here, exist and most have resulted from ectopic expression (Oukka et al., 1996; Qi et al., 2000). Endogenous presentation of nuclear protein EBNA1 by MHC class II has been shown recently to depend on autophagy (Paludan et al., 2005). Although the precise mechanism involved in presentation has not been studied in the present paper, our data point to a pathway of presentation used by de novo HCMV-infected APCs.
The present paper extends our previous observations of CD4+ T-cell activation by infected natural APCs. We have demonstrated that, due to the different kinetics of viral antigen expression and virus-induced cytopathogenicity, anti-IE1 CD4+ T cells are capable of recognizing infected APCs, despite MHC class II downregulation.

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