Dendritic cell-induced apoptosis of human cytomegalovirus-infected fibroblasts promotes cross-presentation of pp65 to CD8+ T cells

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An efficient host response to human cytomegalovirus (HCMV) infection may depend on rapid sensing of the infection by the innate immune response prior to deployment of viral immunosubversive functions. Control of HCMV dissemination could be ensured by apoptosis of cells immediately following infection. In the present report, it is demonstrated that changes in the ratio of c-FLIP to FLICE contributed to early sensitivity of HCMV-infected MRC5 fibroblasts to tumour necrosis factor alpha (TNF-α), providing an innate response to infection. Dendritic cells (DCs) co-cultured with HCMV-infected MRC5 cells acquired the ability to secrete TNF-α in an amount sufficient to kill infected fibroblasts. Blockage of TNF-α binding to its receptor on MRC5 cells with soluble TNF-R reduced the number of dead, HCMV-infected fibroblasts ingested by DCs, thus highlighting the impact of the apoptotic state of infected cells for efficient loading of DCs. Those DCs loaded with antigens available early in infection, such as input virion-associated pp65, could then engage antigen processing for cross-presentation to specific CD8+ T cells. Cross-presentation was impaired when MRC5 cells were treated with the pan-caspase inhibitor ZVAD before co-culture with DCs. Altogether, our data suggest that the innate killing capacity of DCs at the early stage of infection plays a role in the activation of anti-HCMV CD8+ T cells.

INTRODUCTION

Co-evolution of viruses with their host cells results in the development of strategies to ensure the survival of both. Apoptosis of infected cells is considered a potential first line of defence to prevent virus spreading. This phase is sustained quickly by initiation of an immune response that is highly dependent on the capacity of dendritic cells (DCs) to prime and activate specific effector T cells. Human cytomegalovirus (HCMV; family Herpesviridae, subfamily Betaherpesvirinae) is associated with lifelong persistent viral infection and has evolved numerous immunoevasion mechanisms (Reddehase, 2002). Accordingly, HCMV subversion could occur at any stage of infection to interfere with both apoptotic pathways and DC functions. On the one hand, HCMV infection is the cause of numerous anti-apoptotic mechanisms, including (i) inhibition of death-receptor signalling by viral proteins such as UL36, a suppressor of caspase-8 activity (Goldmacher et al., 1999), (ii) UL37, an inhibitor of mitochondrial permeability (Skaletskaya et al., 2001), and (iii) blockage of DNA damage-induced apoptosis through disruption of p53- and p73-dependent pathways (Allart et al., 2002). On the other hand, infection of mouse DCs with murine cytomegalovirus in vivo and of human DCs with adapted HCMV strains in vitro impaired their activation and prevented T-cell activation (Andrews et al., 2001; Moutaftsi et al., 2002). We have proposed that early apoptosis of HCMV-infected cells, before expression of viral apoptosis inhibitors, could provide a source of antigens, including virion tegument proteins such as pp65 (UL83), delivered into the cytosol after viral fusion, and immediate-early gene products such as IE1, to uninfected DCs for cross-presentation to CD8+ T cells (Arrode et al., 2000). Moreover, we have shown that cross-presentation of the immunodominant HCMV pp65 antigen was downregulated temporally by soluble factors secreted by cells in the late stage of infection (Arrode et al., 2002), suggesting that the efficiency of cross-presentation could depend on early acquisition of viral antigens by immature DCs (iDCs).

In the present study, we sought to determine whether iDCs in the vicinity of infected fibroblasts could acquire killing activity against infected fibroblasts to ensure their own feeding for early cross-presentation. We show that HCMV infection sensitized fibroblasts to tumour necrosis factor
alpha (TNF-α)-mediated apoptosis in the very early phase after virus inoculation and that, later on, cells recovered their resistance. We demonstrated that expression of c-FLIP, a regulatory protein of the death-initiating signalling complex (DISC) (Krueger et al., 2001), was downmodulated early after infection, whereas procaspase-8 (FLICE) expression was upregulated, suggesting that the c-FLIP : FLICE ratio could be considered a switch that contributes to sensitivity or resistance of infected cells to TNF-α. Further, we demonstrated that internalization of HCMV-infected fibroblasts by iDCs depended on their apoptotic state and was partly due to the pro-apoptotic activity of DC-derived TNF-α. Finally, we provided evidence that cross-presentation was impaired when iDCs were co-cultured with fibroblasts that were treated with the pan-caspase inhibitor ZVAD before infection with HCMV. Altogether, our data suggest that (i) regulation of the expression ratio of c-FLIP : FLICE in infected cells could contribute to the innate immune response against HCMV to reduce virus spreading, (ii) early sensing of infected cells by iDCs could promote their ability to express and secrete death ligands for killing of infected targets, and (iii) the killing activity of iDCs could provide an efficient means to seed uninfected DCs with viral antigens for cross-presentation.

METHODS

Virus and cells. HCMV strain AD169 was propagated in MRC5 human fibroblasts (bioMérieux). Virus titration was determined by p.f.u. titration in human foreskin fibroblasts (ATCC) according to standard procedures. HCMV strain VHLE, which has a tropism for DCs, was kindly provided by C. Sinzger (University of Tübingen, Tübingen, Germany). Virus was collected when cytopathic effects were monitored as described previously (Arrode et al., 1997). MRC5 cells (2 × 10^6 cells ml^-1) from an HCMV-seropositive healthy HLA-A2 donor were cultured in 24-well plates in RPMI medium containing 10% human AB serum, 1% minimal essential medium with non-essential amino acids (Life Technologies) and 10 mM HEPES (Life Technologies). CD8^+ T cells (>90%) directed against a pp65-derived epitope (NLVPMVATV, N9V; Novartis) and interleukin-13 (IL-13, 50 ng ml^-1; IDM). Fresh IL-13 was added again after 4 days culture. Phenotype of iDCs was monitored as described previously (Arrode et al., 2000) and was as follows: CD1a^+, MHC-I^+, MHC-II^+, CD64^+, CD83^+, CD80^low, CD86^low.

Generation of DCs. DCs were obtained from adherent PBMCs in six-well plates (3 × 10^6 cells ml^-1), cultured for 7 days in AIM-V serum-free medium (Life Technologies) supplemented with granulocyte-macrophage colony-stimulating factor (GM-CSF, 100 ng ml^-1; Novartis) and interleukin-13 (IL-13, 50 ng ml^-1; IDM). Fresh IL-13 was added again after 4 days culture. Phenotype of iDCs was monitored as described previously (Arrode et al., 2000) and was as follows: CD1a^+, MHC-I^+, MHC-II^+, CD64^+, CD83^+, CD80^low, CD86^low.

Assay for apoptosis. Cells were cultured in either 24- or six-well culture plates in RPMI 1640 medium (Gibco) containing 10% fetal calf serum and supplemented with glutamax-I, sodium pyruvate, 100 µg penicillin ml^-1 and 100 µg streptomycin ml^-1 (all from Gibco). TNF-α (1000 U ml^-1; R&D Systems) was added to HCMV- or mock-infected cells. Apoptotic cells were detected by labelling with Hoechst 33342 (10 µg ml^-1 in PBS; Sigma). Hoechst-stained, condensed nuclei were counted (100–300 cells for each sample) and results were expressed according to the following formula: percentage of specific apoptosis = (percentage of condensed nuclei from treated cells – percentage of condensed nuclei in untreated cells) × 100. Alternatively, activation of caspase-3 was assessed by using a Caspase-3 apоОer Alert colorimetric assay kit (Dako) or flow cytometry using a specific fluorescein isothiocyanate (FITC)-conjugated anti-active caspase-3 antibody according to the manufacturer’s instructions (R&D Systems). Apoptosis of MRC5 cells in co-culture with DCs was quantified by flow cytometry using APO2.7 reagent (Coulter-Immunotech), known to react with a mitochondrial membrane protein.

Flow cytometry. DCs were incubated for 30 min at 4 °C with the following phycocerythrin (PE)-conjugated mouse antibodies: anti-CD83, anti-CD86, anti-CD80 or their corresponding control isotypes (all from Immunotech) to monitor their activated phenotype. Cell fluorescence was analysed on a Coulter EPICS Elite cell sorter.

Western blotting. Total proteins in the clarified supernatant of cell lysates were quantified by using a MicroBCA assay (BioRad) to allow loading of the gel with identical amounts of proteins, boiled in reducing Laemmli sample buffer and submitted to SDS-PAGE (12.5% acrylamide). A nitrocellulose (Hybond C; Amersham Biosciences) blot of the gel was probed with the following antibodies: mouse anti-FLICE (1:121, 1:500 dilution; Cell Signaling Technology), c-FLIPS rabbit antibodies (H-202, 1:500 dilution; Santa Cruz Biotechnology), c-FLIPL rabbit antibodies (H-150, 1:500 dilution; Santa Cruz Biotechnology) and β-actin mouse mAb (clone AC-15, 1:5000; Sigma). Antibody fixation was revealed by using peroxidase-conjugated appropriate secondary antibodies and a chemiluminescence procedure (ECL; Amersham Biosciences). Western blots were scanned to quantify relative amounts of c-FLIPL, c-FLIPS and FLICE.

Expansion of anti-pp65 CD8^+ T cells from HCMV-seropositive donor PBMCs. PBMCs (2 × 10^6 cells ml^-1) from an HCMV-seropositive healthy HLA-A2 donor were cultured in 24-well plates in RPMI medium containing 10% human AB serum, 1% minimal essential medium with non-essential amino acids (Life Technologies) and 10 mM HEPES (Life Technologies). CD8^+ T cells (>90%) directed against a pp65-derived epitope (NLVPMVATV, N9V; Neosystem) have been obtained as described previously (Vaz-Santiago et al., 2001). Activation of anti-pp65 cytotoxic T lymphocytes (CTLs) was assessed through quantification of secreted gamma interferon (IFN-γ) by ELISA as described below.

Generation of HLA-B7-restricted CD8^+ T-cell lines directed against the T10M peptide (TPRVTGGGAM) has been performed as described previously (Godard et al., 2004).

ELISA for IFN-γ and TNF-α secretion. ELISA for IFN-γ and TNF-α secretion was performed according to the manufacturer’s instructions (Ebioscience). A_{450} was measured on an ELISA apparatus (Dynatech Laboratories).

Assays for cross-presentation. MRC5 cells were infected at the m.o.i. indicated in the figure legends and washed extensively with RPMI medium before addition of DCs. iDCs (2 × 10^5 per well) obtained from HLA-A2 or HLA-B7 donors were added in RPMI medium supplemented with GM-CSF and IL-13 to HCMV-infected or mock-infected MRC5 cells (1:1 ratio) treated or not with ZVAD (10 µM; Calbiochem) and co-cultured for 24 h. Then, DCs were pipetted gently to dissociate them from adherent fibroblasts, washed, plated in duplicate on a 96-well plate at 5000 cells per well and incubated for 24 h in the presence of the anti-pp65 CD8^+ T-cell line at different responder-to-stimulator (R:S) ratios in a final volume of
200 μl. Alternatively, DCs were used either unloaded or after being pulsed overnight with 1 μM N9V peptide in the presence of TNF-α (50 ng ml⁻¹; R&D Systems) for 24 h. To exclude the possibility of direct stimulation of T cells by HLA-A2-positive MRC5 cells that could be recovered during pipetting, MRC5 cells either mock-infected or infected with HCMV or DCs obtained from an HLA-B7 donor and the corresponding HLA-B7 anti-pp65-restricted T cells were used as additional controls.

**RT-PCR analysis.** Total RNA was isolated from cells treated with TRizol reagent (Life Technologies) according to the manufacturer’s instructions. RNA was reverse-transcribed by using SuperScript II reverse transcriptase (Life Technologies), and 3 μg reverse-transcription product was used as a template for PCR amplification (35 cycles at 95°C for 30 s, 62°C for 30 s and 72°C for 1 min) in 50 μl reaction mix containing Tag polymerase (Life Technologies). PCR primers were as follows: actin 5′-CTCTGTGGCAGATCC-ACGAAACT-3′; actin 3′ end, 5′-GAAGCATTGCGGTTGGACAT-3′; UL37 5′ end, 5′-AGGTAGCGTGCCCTGTCGG-3′; UL37 3′ end, 5′-CGACTACACGCCCAGCTGCG; UL36 5′ end, 5′-GAGGAAA-GCCCATGATGCTTC-3′; UL36 3′ end, 5′-CGACTACACGCCCAGCTGCG; IE1 3′ end, 5′-GTGTGATGCTGGCCAAGCGG-3′; IE1 5′ end, 5′-GGGCAGCCTTGCTTCTAGTC-3′.

**Co-cultures of MRC5 cells with DCs.** Adherent MRC5 fibroblasts were harvested from flasks by trypsinization and labelled in tubes with 8 μM PKH26 (Sigma) for 5 min, according to the manufacturer’s protocol. The reaction was stopped by adding fresh serum and washing three times in RPMI medium. Labelled MRC5 cells were counted, centrifuged, and resuspended in RPMI medium containing GM-CSF and IL-13 and supplemented with ZVAD (50 ng ml⁻¹) to decrease apoptosis very early after infection, c-FLIP : FLICE ratios reverted to being lower than those observed in uninfected cells, providing a possible but non-exclusive explanation for resistance of MRC5 cells to TNF-α late after infection. Indeed, the possible involvement of the viral inhibitors UL36 and UL37 must be considered; we addressed expression of these transcripts by RT-PCR analysis, as shown in Fig. 1(c). Significant expression of UL36 observed 48 h after infection and appearance of UL37 transcripts at 96 h p.i. (Fig. 1c) support a possible role for these viral proteins in the late phase of infection under the conditions used in our experiment.

Our data suggest that unlocking of the DISC in HCMV-infected cells, such as MRC5 fibroblasts, could be activated as an immediate line of reaction against infection, provided that pro-apoptotic ligands such as TNF-α are available in their environment. We then asked whether DCs could be a source of TNF-α and thus contribute to killing of infected MRC5.

**RESULTS**

**Modulation of the c-FLIP : FLICE expression ratio sensitizes HCMV-infected fibroblasts to TNF-α-induced apoptosis**

We have shown previously that HCMV infection sensitizes MRC5 fibroblasts to TNF-α-induced apoptosis very early after addition of the virus to the culture medium. To investigate mechanisms involved in this sensitization, MRC5 cells were infected with HCMV strain AD169 and treated with TNF-α at 2 h intervals following addition of the virus. A histogram of Hoechst labelling (Fig. 1a) shows that MRC5 fibroblasts were constitutively resistant, but acquired sensitivity to TNF-α very early after infection [2 h post-infection (p.i.)] that peaked at 8–12 h and then started to decrease (>24 h p.i.). A major regulatory pathway in death-receptor signalling involves c-FLIP, a short-lived, cytoprotective protein acting through blockade of the DISC, an assembling process shared by death receptors of the TNF-R family. Inhibition of procaspase-8 (FLICE) recruitment to the DISC by high levels of c-FLIP is a major blocking mechanism (Kruiger et al., 2001), suggesting that regulation of the c-FLIP : FLICE ratio is essential for the cell balance between resistance or sensitivity to death ligands. Accordingly, we asked whether c-FLIP and FLICE expression was modulated throughout infection of MRC5 cells. Western blotting of c-FLIP shows that both the c-FLIPL and c-FLIPS isoforms were downregulated at time points where MRC5 cells became sensitive to TNF-α (Fig. 1b). At the same time, FLICE expression was upregulated, resulting in a c-FLIP : FLICE ratio known to reverse cells from resistance to sensitivity to TNF-α. These findings suggest that HCMV-induced sensitization of MRC5 cells to TNF-α resulted from unlocking of the DISC. It is noteworthy that, late after infection, c-FLIP : FLICE ratios reverted to being lower than those observed in uninfected cells, providing a possible but non-exclusive explanation for resistance of MRC5 cells to TNF-α late after infection. Indeed, the possible involvement of the viral inhibitors UL36 and UL37 must be considered; we addressed expression of these transcripts by RT-PCR analysis, as shown in Fig. 1(c). Significant expression of UL36 observed 48 h after infection and appearance of UL37 transcripts at 96 h p.i. (Fig. 1c) support a possible role for these viral proteins in the late phase of infection under the conditions used in our experiment.

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**Early secretion of TNF-α by HCMV-conditioned DCs**

iDCs were incubated with HCMV strain AD169, whose replication is abortive in DCs, but is suspected to trigger immediate danger signals. Quantification of TNF-α in DC supernatant by ELISA revealed increased secretion of TNF-α as early as 6 h after infection, reaching a high concentration (>1000 pg ml⁻¹) 24 h after addition of the virus (Fig. 2a). These data confirm the early activation of iDCs that we observed previously by analysing surface expression of CD83, CD80 and CD86 markers (Arrode et al., 2002). Identical results were obtained when cells were infected with HCMV strain VHLE, known to replicate in DCs (Fig. 2a). To determine whether the amounts of TNF-α secreted by HCMV-conditioned DCs were sufficient to kill infected MRC5 cells, a dose–response analysis of active caspase-3-positive cells was performed on cells incubated with recombinant TNF-α. Fig. 2(b) shows that a concentration of TNF-α as low as 1 ng ml⁻¹ could induce apoptosis in about 20% of HCMV-infected cells. We
further assessed TNF-α secretion by DCs in co-culture with strain AD169-infected MRC5 fibroblasts (m.o.i. of 1) that had been treated or not with the pan-caspase inhibitor ZVAD. Fig. 2(c) shows that MRC5 cells secreted low amounts of TNF-α after 6 h infection, as shown in Fig. 2(a), as did iDCs cultured alone. In contrast, when MRC5 cells were co-cultured with DCs, high amounts of TNF-α were secreted, providing evidence that HCMV-conditioned DCs were able to kill infected cells in their vicinity. No secretion of TNF-α by DCs was observed in the presence of non-infected fibroblasts (M-n.i.), in contrast to co-culture with infected cells (M-AD), suggesting that activation of DCs resulted from both sensing of infected neighbouring cells and direct contact of remaining free viruses with the cell membrane. Fig. 2(c) shows that secretion of TNF-α by DCs in co-culture did not depend on the presence of apoptotic infected cells (M-AD versus M-AD-Z), demonstrating that secretion of TNF-α preceded apoptosis.

Efficient internalization of HCMV-infected fibroblasts by iDCs depends on their apoptotic state

To assess whether internalization of infected fibroblasts by iDCs could benefit from their apoptotic state, MRC5 cells were labelled with PKH26, infected with HCMV in the presence or absence of ZVAD and cultured for 6 h before addition of iDCs. Then, cells were co-cultured overnight and recovered for cytofluorimetric analysis. Fig. 3(a) shows that 13 and 11 % of iDCs, as characterized by HLA-DR expression, acquired PKH26 from uninfected MRC5 cells in the absence or presence of ZVAD, respectively. Indeed, treatment of cells with PKH26 induced death of some MRC5 cells (10–20 %), as assessed by using the APO2.7 detection method (data not shown). Accordingly, we can assume that DCs acquired PKH26 from some of these cells that died in a ZVAD-resistant, caspase-independent pathway and were still present in the culture before adding
Fig. 2. Early secretion of TNF-α by HCMV-conditioned DCs. (a) iDCs were incubated with HCMV strain AD169 (●) or VHLE (□) (m.o.i. of 3) for the times indicated and TNF-α was quantified by ELISA on DC supernatants. (b) MRC5 cells were infected with HCMV for 6 h and incubated overnight with increasing amounts of recombinant TNF-α as indicated. Apoptosis of infected MRC5 cells was quantified by using a Caspase-3 apoAlert colorimetric assay kit (Dako). (c) MRC5 cells (M) were infected or not (n.i.) with AD169 (AD) for 6 h in the presence or not of ZVAD (Z). Alternatively, cells were co-cultured with DCs (right panel) at a 1:1 cell ratio and left for 24 h before quantification of TNF-α in the supernatant by ELISA. Error bars indicate SD in (a–c).

Fig. 3. Efficient internalization of HCMV-infected MRC5 cells depends on their apoptotic state. (a) MRC5 cells were labelled with PKH26 and infected (CMV) or not (n.i.) with HCMV for 6 h in the presence or absence of ZVAD. Then, iDCs were added and co-cultured overnight with the MRC5 cells, and capture of PKH26 by HLA-DR-positive iDCs was analysed by flow cytometry. Percentages of PKH26–HLA-DR double-positive cells (upper right) are indicated. (b) The same protocol as described for (a) was used, except that iDCs were incubated with soluble TNFR (TNFR-Fc) before addition to MRC5 cells. Loading of iDCs with PKH26-labelled fibroblasts is represented by percentages as indicated (arrowhead). Data are representative of three independent experiments.
DCs. When iDCs were co-cultured with HCMV-infected MRC5 cells, 38% of iDCs acquired PKH26, in contrast to those that were co-cultured with pan-caspase inhibitor-treated MRC5 cells (15%). These data suggest that internalization of PKH26-labelled MRC5 cells by iDCs was more efficient when cells were infected with HCMV and that it was dependent on their apoptotic state. Accordingly, as HCMV-conditioned DCs were able to secrete TNF-α, we determined how this cytokine could take part in the acquisition process of infected MRC5 cells by iDCs. To this end, MRC5 cells were labelled with PKH26, infected with HCMV for 6 h and co-cultured with iDCs in the presence or absence of soluble TNF-R (TNFR1-Fc). Internalization of PKH26-labelled MRC5 cells by DCs was monitored by cytofluorimetric analysis. Fig. 3(b) shows that the percentage of PKH26-positive DCs was higher from co-cultures with HCMV-infected MRC5 cells (66%) than from co-culture with uninfected cells (37%), as demonstrated in Fig. 3(a). When DCs were pretreated with TNFR1-Fc prior to their addition to HCMV-infected MRC5 cells, the percentage of PKH26-positive DCs decreased (50%), suggesting that TNF-α contributed to killing infected MRC5 cells and that it was dependent on their apoptotic state. Furthermore, we can assert that TNF-α was produced by DCs in the co-culture, as infected MRC5 cells did not produce this cytokine, as assessed by ELISA (Fig. 2c). These data give us arguments favouring a direct role for DCs in killing of infected neighbouring cells for their own feeding. According to our previous experiments demonstrating the ability of DCs co-cultured with fibroblasts in the early stage of infection to induce cross-presentation of anti-pp65 CD8+ T cells (Arrode et al., 2002), we next asked to what extent apoptosis of infected MRC5 cells could take part in this process.

**Apoptosis of fibroblasts in the early stage of infection in co-culture with DCs takes part in cross-presentation of the N9V peptide to T cells**

In accordance with published protocols (Arrode et al., 2002), MRC5 cells were infected with HCMV for various periods of time (6, 24 or 48 h) in the presence or absence of ZVAD; iDCs were added and the co-culture was left for 24 h. DCs were then recovered, added to anti-N9V CD8+ T cells (N9V is an HLA-A2 immunodominant binding peptide from HCMV pp65) at various R:S ratios, and stimulation of T cells was assessed by ELISA quantification of secreted IFN-γ. Fig. 4(a) shows that activation of anti-N9V T cells was stronger in the presence of DCs conditioned with fibroblasts in the early stage of infection (M-6 h p.i.) than with those co-cultured with fibroblasts in the late stage of infection (M-48 h p.i.). These results support our previous demonstration that cross-presentation must take place soon after encounter of DCs with infected cells (Arrode et al., 2002). Moreover, even though incoming pp65 was available very early after addition of the virus (data not shown; Arrode et al., 2000), cross-presentation of the N9V peptide to T cells was impaired when MRC5 cells in the early stage of infection were treated with ZVAD, suggesting that, in DC-MRC5 co-culture, killing of infected fibroblasts is crucial for commitment of DCs to induce T-cell activation.

To address further whether DCs could ensure cross-presentation of pp65 peptide when MRC5 cells were infected with a clinical strain of HCMV, infections were performed with strain VHLE at an m.o.i. of 3 and treated or not with ZVAD, as described above. In this experiment, DCs and CD8+ T cells were generated from an HLA-B7 donor in the presence of the HLA-B7-binding peptide T10M. Fig. 4(b) shows that, as demonstrated previously with the AD169 strain and HLA-A2-restricted T cells, DCs were able to induce activation of these pp65-specific T cells and that pretreatment of MRC5 cells with ZVAD decreased efficiency of cross-presentation. These experiments with HLA-B7-restricted T cells further eliminate the possibility of direct recognition of T cells by infected fibroblasts, which could have been removed during recovery of DCs, and extend our observations to clinical HCMV strains and to an HLA-B7 epitope.

**DISCUSSION**

HCMV infection could potentially be suppressed efficiently if apoptosis could be induced early in the replication cycle, prior to the initiation of viral immunosubversive strategies. In this study, we show that HCMV-infected fibroblasts are sensitized to TNF-α within the first hours following addition of the virus. HCMV infection of MRC5 fibroblasts induces changes in the c-FLIP:FLICE expression ratio that correlate with their sensitivity to apoptosis. We further provided arguments favouring an essential role of this innate response to infection for commitment of DCs to activation of T cells. In this scenario, secretion or surface expression of TNF-α by HCMV-conditioned DCs could contribute to killing infected fibroblasts nearby, allowing the DCs to ingest them. Those DCs loaded with HCMV antigens present in fibroblasts during the early stages of infection, such as pp65, could then engage antigen processing for cross-presentation to specific CD8+ T cells. Unlike some viruses that can commit cells in the early stages of infection to ‘suicide’, HCMV has no overt negative effect on host-cell survival until its late cytopathic effect. Nevertheless, our data show that HCMV could sensitize cells to inflammatory death ligands very early after infection, ensuring an innate control of virus spreading.

It has been demonstrated that small variations in c-FLIP expression levels may decide whether a cell will respond to death ligands (Micheau et al., 2001). The protective activity of c-FLIP is involved in many physiological processes to control sensitivity to death ligands, so modulation of its expression is a main regulatory pathway in immune responses (Kirchhoff et al., 2000; Lens et al., 2002) and tumorigenesis (Igney & Krammer, 2002). This sensor
system could provide the cell with an efficient self-control mechanism to avoid spreading of pathogens. It is therefore not surprising that pathogens usurp this function, as demonstrated by the expression of v-FLIP from human herpesvirus 8 (Thome et al., 1997). Our data suggest that, very early after infection with HCMV, the host cell reacts to virus interference by modulating expression of c-FLIP and FLICE, thus unlocking the DISC and sensitizing cells to death ligands such as TNF-α. Although HCMV-infected cells are protected by UL36 and UL37 anti-apoptotic functions acting on the DISC and mitochondria, respectively, infected cells can be expected to be sensitive to apoptosis before their expression is activated. Our data, demonstrating that significant expression of UL36 and UL37 occurs only at time points later than when infected cells are sensitive to TNF-α, support this hypothesis and are in accordance with a decreased sensitivity of cells to apoptosis in the late stages of infection. Furthermore, the c-FLIP : FLICE expression ratio was reversed in fibroblasts in the late stages of infection, a process that may also contribute to the late resistance to apoptosis. In our scenario, a prerequisite for an early and efficient control of infection is to have pro-apoptotic ligands such as TNF-α available quickly after infection, and infected cells adjacent to stimulated DCs. Interestingly, HCMV is known to be recognized by Toll-like receptor 2 (TLR2) on fibroblasts through interactions with envelope glycoproteins B and H (Boehme et al., 2006), leading to inflammatory-cytokine secretion. Even though this has not been demonstrated for DCs, we can assume that interaction of HCMV with TLR2 could be responsible for TNF-α secretion. This is supported further by the recent demonstration by Weck et al. (2007) that engagement of TLR2 by Pam3Cys on DCs did not impair their capacity to engulf HCMV-infected fibroblasts or to participate in cross-presentation of pp65 to CD8+ T cells in a co-culture model. Even though we have no direct proof that, in our model, other death ligands could be secreted by activated DCs, potentially comparable effects could be induced by TRAIL and/or FasL, and these ligands could also be secreted by other inflammatory cells. This could be of special interest, as MRC5 cells lost their constitutive resistance to TRAIL after infection with HCMV (data not shown). When apoptosis of infected MRC5 cells was monitored in the co-culture by cytometric analysis of PKH26–activated caspase 3 double-labelled cells, it was reduced by 40% in the presence of soluble

Fig. 4. Apoptosis of fibroblasts in the early stage of infection in co-culture with DCs takes part in cross-presentation to anti-pp65 CD8+ T cells. MRC5 cells (M) were infected (p.i.) or not (n.i.) with 1 m.o.i. of (a) HCMV AD169 or (b) HCMV VHLE in the presence or absence of ZVAD for the times indicated. Then, DCs were added and co-cultured for 24 h. DCs were recovered from the mixture and added at R : S ratios as indicated, to (a) anti-N9V HLA-A2-restricted and (b) HLA-B7-restricted anti-pp65 CD8+ T cells. At the end of co-culture (24 h), activation of T cells was monitored through ELISA quantification of IFN-γ. DCs either untreated or treated with TNF-α and pulsed with the N9V peptide (inset) were used as negative and positive controls, respectively, in (a). Data are representative of three independent experiments.
TNF-R (TNFR-Fc; data not shown), suggesting that MRC5 cells died only partly as a result of TNF-α-mediated apoptosis and that other death ligands produced by DCs in the co-culture could take part in the process, e.g. TRAIL. Our data suggest that tissue-resident DCs can sense infected cells, form homotypic clusters, as we observed previously in vitro (Arrode et al., 2002), and become activated, as indicated by TNF-α secretion. Coordination of these early events could provide the host with an efficient method of innate control against infection, but allow DCs to acquire viral antigens contained in dead cells for cross-presentation to CD8$^+$ T cells (Arrode & Davrinche, 2003). Even though cross-presentation by DCs may involve various antigen-uptake mechanisms (Albert et al., 1998; Amigorena, 2002; Binder & Srivastava, 2005; Guermonprez et al., 2003), apoptosis of infected cells and ingestion of dead cells by DCs are thought to provide one of the strongest activation signals for cross-presentation to CD8$^+$ T cells, and even cross-priming of these cells. Our data demonstrating a role for apoptosis of infected fibroblasts in co-culture with DCs in cross-presentation to anti-pp65 CD8$^+$ T cells support this hypothesis. Furthermore, inhibition of apoptosis in fibroblasts in later stages of infection under the effect of UL36 and UL37 proteins could contribute to reducing the efficiency of cross-presentation, thus promoting immuno evasion.

In another field, resistance of retinal pigment epithelial (RPE) cells to apoptosis by death ligands such as FasL and TRAIL has recently been ascribed to enhanced expression of c-FLIP in infected cells, due to the transcriptional activity of IE2 on the c-FLIPL promoter (Chiou et al., 2006). Indeed, following infection of the human RPE cell line ARPE-19 with the AD169 strain of HCMV, the authors demonstrated an increase in c-FLIPL expression from 48 h after infection. Nevertheless, even though we can expect discrepancies between RPE and MRC5 cells due to cell-specific kinetics of IE2 expression, the authors did not assess c-FLIP expression during the first 24 h of infection and an early sensitivity of RPE cells to death ligands could easily have been missed. Anyway, our data confirm the observations made by Chiou et al. (2006) and provide new insights on how HCMV may interfere with apoptosis, leading to survival of infected RPE cells and then to the pathogenesis of retinitis. RPE cells are known to play a role in phagocytosis of dead cells for the maintenance of the retinal environment and we can assume that they could ingest apoptotic bodies from neighbouring HCMV-infected RPE cells. Although the eye is an immune privilege site, under inflammatory conditions RPE cells may acquire the capacity to function as antigen-presenting cells so that recognition by activated CTLs directed against viral antigens could induce their killing. Interestingly, we previously demonstrated an impaired killing of HCMV-infected RPE cells by CTLs directed against incoming as well as newly synthesized pp65, which we ascribed to the sequestration of viral particles into endocytic compartments and the expression of HCMV functions associated with MHC-I downregulation (US2–11) (Allart et al., 2003). Even though cross-presentation of viral antigen contained in dead RPE cells to CTLs may counteract viral immuno evasion, the data of Chiou et al. (2006) suggest that c-FLIP-mediated resistance of infected RPE cells to apoptosis could prevent this rescue mechanism, contributing further to virus spreading and to constitution of HCMV reservoirs within the retina. Finally, our data provide additional clues to explain the prevalence of CD8$^+$ T cells directed against IE1 and pp65 (Bunde et al., 2005; Harari et al., 2004), as both proteins are available in the very early stage after infection, pp65 as incoming and IE1 as an immediate-early gene product, at a time where fibroblasts are sensitive to apoptosis. This hypothesis could be extended to other tegument proteins and IE gene products available at this stage of infection, providing new candidates to be considered in vaccine design and in cellular immunotherapy protocols.

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REFERENCES


