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Processing and MHC class I presentation of human cytomegalovirus pp65-derived peptides persist despite gpUS2–11-mediated immune evasion

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Immune control of human cytomegalovirus (HCMV) infection can be mediated by $CD8^+$ cytolytic T lymphocytes (CTL). Adoptive transfer of antiviral CTL confers protection against HCMV reactivation and disease. The tegument protein pp65 and the immediate-early 1 protein (IE1) are recognized to be major CTL targets, even though during productive infection the viral immunoevasion proteins gpUS2–11 act to suppress major histocompatibility complex (MHC) class I-restricted antigen presentation. Thus it was not clear how infected cells could be labelled with antigenic peptides in the face of immunoevasion. We show here that the immunodominant peptide $pp65_{NLV}$ was presented by MHC class I in cells infected with a gpUS2–11-competent virus. Presentation of $pp65_{NLV}$ was still detectable at 96 h post-infection, although at low levels. Partial suppression of $pp65_{NLV}$ presentation was dependent on the ability of the infecting strain to express gpUS2–11. MHC class I-restricted antigen presentation in HCMV-infected cells (encoding gpUS2–11) exhibited specificity for pp65-derived peptides, as infected fibroblasts did not present the IE1-derived nonapeptide $IE1_{TMY}$. Remarkably, infected cells could restore $pp65_{NLV}$ peptide presentation after acid removal of MHC class I despite gpUS2–11 expression. This recovery was shown to be dependent on proteasome functionality. In contrast to IE1, pp65 peptides are loaded on MHC class I molecules to be transported to the cell surface at early and late times after infection in the face of gpUS2–11-mediated immunoevasion. pp65 is therefore the first example of an HCMV protein only incompletely subjected to gpUS2–11-mediated immunoevasion.

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INTRODUCTION

Infection with human cytomegalovirus (HCMV) affects more than 50 % of the human population. Individuals with immature or compromised immune defence functions are more likely to encounter severe clinical disease conditions. In healthy individuals, however, HCMV is normally efficiently controlled by the immune system (Pass, 2001), and in particular by HMCV-specific $CD8^+$ cytoxic T lymphocytes (CTL) (Reddehase *et al.*, 1985; Reusser *et al.*, 1991). The CTL response is primed against short peptides that are proteolytically processed from intracellular viral proteins and presented on the cell surface by major histocompatibility complex (MHC) class I molecules (Kloetzel, 2004). For this, proteins are labelled by ubiquitination to be targeted to proteasomes for degradation. Peptides of appropriate length are released from proteasomes and processed further at their amino terminus by cytosolic aminopeptidases. Ultimately they are translocated to the endoplasmic reticulum (ER) by the transporter associated with antigen presentation (TAP), where they associate with MHC class I. In some instances, ER-resident proteases contribute to the trimming of antigenic peptides (reviewed by Kloetzel, 2004).

The HCMV genome encodes over 150 proteins, which can potentially serve as target antigens for CTL (Chee *et al.*, 1990). Recent studies showed that memory CTL isolated from HCMV-seropositive individuals display a broad spectrum of target specificity (Elkington *et al.*, 2003; Khan *et al.*, 2005; Sylwester *et al.*, 2005). However, the virus

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also encodes proteins that interfere with the assembly and transport of MHC class I–peptide complexes and reduce class I surface expression (reviewed by Plachter, 1999; Reddehase 2000, 2002; Mocarski, 2004). The glycoprotein US3 (gpUS3) is expressed immediately after infection and prevents peptide loading of MHC class I complexes in the ER (Ahn *et al.*, 1996; Jones *et al.*, 1996; Park *et al.*, 2004, 2006). The viral early glycoproteins US2 (gpUS2) and US11 (gpUS11) mediate retrograde translocation of MHC class I heavy chains from the ER to the cytoplasm, leading to their subsequent proteasomal degradation (Wiertz *et al.*, 1996a, b). Finally, the early-late glycoprotein US6 (gpUS6) leads to a block in peptide transport from the cytosol to the ER through the TAP, thereby preventing the formation of MHC class I–peptide complexes in the ER (Ahn *et al.*, 1997; Hengel *et al.*, 1997; Lehner *et al.*, 1997; Halenius *et al.*, 2006). The question therefore arises as to how HCMV-specific CTL can recognize infected cells expressing the gpUS2–11 immunoevasion proteins.

Adoptive transfer of HCMV-specific CTL has been used successfully to prevent viral reactivation and disease in allogeneic bone marrow and haematopoietic stem-cell transplant recipients (Walter *et al.*, 1995; Rauser *et al.*, 2004; Cobbold *et al.*, 2005). These and additional studies have provided circumstantial evidence that pp65-specific CTL are protective (Riddell *et al.*, 1992; Walter *et al.*, 1995). Therefore, pp65-specific CTL appear to recognize infected cells in the face of HCMV-mediated immunoevasion. In murine cytomegalovirus (MCMV), CTL against the immediate-early protein 1 (IE1) were shown to be protective after adoptive transfer (Reddehase *et al.*, 1987; Pahl-Seibert *et al.*, 2005) and the importance of IE1-specific CTL has recently been confirmed for HCMV (Kern *et al.*, 1999; Bunde *et al.*, 2005).

Considering the medical importance of the immunological mechanisms that lead to protection in HCMV infection, we decided to analyse processing and presentation of pp65 with CTL clones generated in HLA-A2 (human leukocyte antigen A2) transgenic mice. Using these CTL clones, it could be shown that presentation of pp65 by HLA-A2 was only partially impaired by gpUS2–11-mediated immunoevasion. Surprisingly, labelling of infected cells with pp65-derived peptides at later times after infection was only in part caused by stable MHC–peptide complexes on the cell surface. Rather, the presentation of pp65-derived peptides appeared to be the result of continuous translocation of peptide-loaded MHC complexes to the cell surface despite expression of immunoevasion proteins within the cell. pp65 is therefore the first example of a viral protein that is incompletely subjected to HCMV-mediated immunoevasion of MHC class I antigen presentation.

METHODS

Cells. Human MRC-5 cells and primary human foreskin fibroblasts (HFF) were grown in minimal essential medium (MEM; PAA) supplemented with 5–10 % fetal calf serum (FCS; Biochrom AG), 2 mM L-glutamine, 50 mg gentamicin l^{-1} and 0.5 ng basic fibroblast growth factor ml^{-1} (bFGF; Invitrogen). T2 cells were grown in RPMI 1640 medium (PAA) supplemented with 10 % FCS, 2 mM L-glutamine, 50 mg gentamicin l^{-1} and 5 μM β-mercaptoethanol.

CTL lines specific for the HLA-A0201 (A2)-restricted HCMV-derived peptides $pp65_{495–503}$ ($pp65_{NLV}$-CTL) (Wills *et al.*, 1996; Diamond *et al.*, 1997) and $IE1_{297–305}$ ($IE1_{TMY}$-CTL) (Gallez-Hawkins *et al.*, 2003) were generated by immunizing HLA-A2/CD8 double-transgenic (tg) mice (a kind gift of L. Sherman, The Scripps Institute, La Jolla, CA, USA) with 100 μg peptide, suspended in incomplete Freund's adjuvant. Ten days later, spleen cells of primed mice were cultured with irradiated A2-tg lipopolysaccharide-activated spleen cell stimulators that had been pulsed with the respective peptides at 5 μg ml^{-1} in supplement-free RPMI 1640 medium. CTL clones were established by limiting dilution and were cultured as previously described (Theobald *et al.*, 1995).

Viruses and plasmids. Various virus strains were used for infection experiments at an m.o.i. of 5, unless otherwise noted. For infections with viruses expressing both the US2–11 and the UL83 (pp65) gene regions, either the HCMV laboratory strain Ad169 or a bacterial artificial chromosome (BAC) derivative of that strain (AD169-BAC) were used (Hobom *et al.*, 2000). For control, the US2–11 deletion mutant RVΔUS2–11 was employed (Falk *et al.*, 2002). In addition, a pp65 deletion mutant, based on the AD169-BAC, was constructed using Red recombination in *Escherichia coli* strain EL250 as described by Lee *et al.* (2001). A kanamycin resistance gene flanked by FRT (FLP recognition target) sites was amplified from a derivative vector of pCP15 (Cherepanov & Wackernagel, 1995) using primers with 50 bp identity to the nucleotide sequence directly adjacent to the deletion (pp65-frt_fwd: 5′-GCTGCCGCACGAGACGCGACTCCTGCAGAC-GGGTATCCACGTACGCGTGAGGTACCCGGGGATCTTG-3′, pp65-frt_rev: 5′-GGACGTGGGTTTTTATAGAGTCGTCCTAAGCGCGTG-CGGCGGGTGGCTCATCGAGAAACGCAGCTTC-3′). In the resulting BAC clone pHd65, the complete coding sequence of pp65 was deleted except for 150 bp at the 5′ end of UL83 and the stop codon. The reconstitution of BAC-vector-free mutant virus RVHd65 was carried out as described by Hobom *et al.* (2000) for AD169-BAC.

Dense body purification. Dense bodies (DB) were purified from late-stage infected HFF by glycerol–tartrate gradient ultracentrifugation as originally published (Irmiere & Gibson, 1983) and described previously (Pepperl *et al.*, 2000).

Gamma interferon (IFN-γ)-based ELISPOT assays. ELISPOT assays were performed according to published procedures (Miyahira *et al.*, 1995; Frankenberg *et al.*, 2002). The $pp65_{NLV}$-CTL and $IE1_{TMY}$-CTL were used as responder cells. HFF or T2 cells were used as stimulator cells at a number of 10^5 per assay. For inhibition of proteasome function, 10 μM lactacystin or 1 μM epoxomicin (both from Calbiochem) were used. Counting of spots was performed using an SZX-12 microscope (Olympus).

Cytotoxicity assays. Functional consequences on antigen presentation by HLA-A2 were monitored using standard 5 h ^{51}Cr release assays (CRA) with 5×10^3 target cells; $pp65_{NLV}$-CTL were used as effector cells. Cells were employed at different effector to target ratios as indicated. Data represent percentages of specific lysis and are given as the mean value of duplicate cultures.

Peptide stripping by acid wash. To remove MHC class I-bound peptides from the surface of infected HFF, cells were treated with citrate phosphate buffer, pH 3 (0.263 M citric acid and 0.123 M disodium phosphate), modified according to Sugawara *et al.* (1987).

Fibroblasts grown in 10 mm dishes were washed twice with PBS and were then treated for 1 min with 2 ml citrate phosphate buffer, pH 3, at room temperature. Afterwards cells were washed twice with supplement-free MEM medium and once with MEM medium containing 10 % FCS, 2 mM L-glutamine, 50 mg gentamicin l^{-1} and 0.5 ng bFGF ml^{-1}. Subsequently, cells were used for fluorescence-activated cell-sorting (FACS) or IFN-γ-ELISPOT analysis or were further incubated to be analysed at a later time point.

Fixation of stimulator cells. After washing with PBS, stimulator cells were resuspended in 200 µl 0.5 % paraformaldehyde and incubated for 30 min at room temperature. Cells were subsequently washed with PBS and RPMI 1640 medium, supplemented with 10 % FCS, 2 mM L-glutamine, 50 mg gentamicin l^{-1} and 5 µM β-mercaptoethanol, and were then resuspended in an appropriate volume of RPMI 1640 medium, supplemented as mentioned before.

Cell viability assay. To determine the number of viable cells after treatment with proteasome inhibitors, a CellTiter 96 AQueous One Solution cell proliferation assay kit (Promega) was used. The absorbance was measured at 492 nm in a 96-well plate reader.

RESULTS

Generation of pp65- and IE1-specific CTL-clones in HLA-A2 transgenic mice

The aim of this study was to investigate MHC class I presentation of antigenic peptide determinants derived from pp65 during HCMV infection. Presentation of IE1-derived peptides by MHC class I was used as a control. For testing, we chose the HLA-A2-presented nonapeptides $pp65_{495–503}$ ($pp65_{NLV}$) and $IE1_{297–305}$ ($IE1_{TMY}$) (Wills *et al.*, 1996; Diamond *et al.*, 1997; Gillespie *et al.*, 2000; Gallez-Hawkins *et al.*, 2003). To provide a reproducible experimental setting, murine CTL clones rather than human CTL lines were generated in HLA-A2 transgenic mice. The resulting CTL clones $pp65_{NLV}$-CTL and $IE1_{TMY}$-CTL were tested for their specificity in IFN-γ-ELISPOT assays (Fig. 1). Firstly, T2 cells were labelled with both peptides. Both CTL clones were stimulated by their cognate MHC–peptide complex and failed to recognize cells labelled with the mismatched peptide (Fig. 1a). Secondly, $pp65_{NLV}$-CTL were tested for their recognition of HLA-A2-positive HFF that were infected either with the laboratory strain Ad169 (AD169-BAC) or with a pp65-deletion mutant (RVHd65; Fig. 1b). Only those cells infected with the pp65-expressing strain Ad169 stimulated IFN-γ secretion. To test for MHC restriction of the CTL clones, HLA-A2-positive and HLA-A2-negative cells were infected and used as stimulators. Infection was performed with a US2–11-negative HCMV strain (RVΔUS2–11) to avoid suppression of antigen presentation. Only HLA-A2-expressing cells stimulated IFN-γ secretion by both $pp65_{NLV}$-CTL and $IE1_{TMY}$-CTL (Fig. 1c). No detectable stimulation was triggered by infected HLA-A2-negative or by mock-infected cells. This demonstrated that the transgenic CTL-clones were both antigen- and HLA-A2-specific.

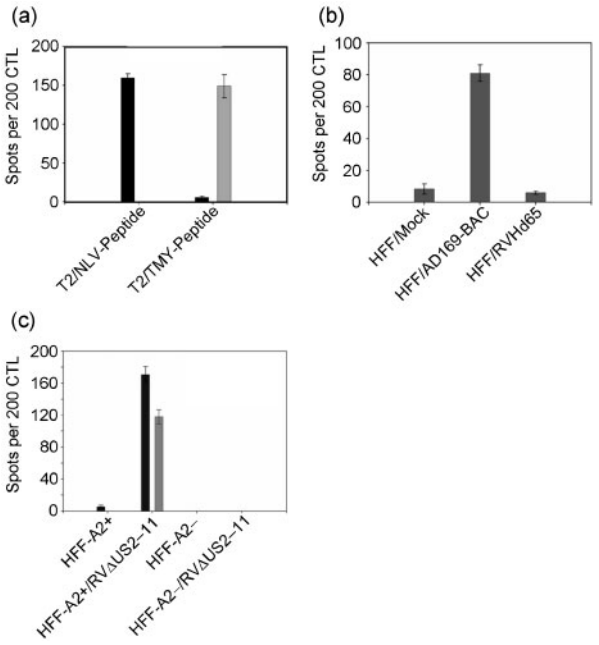

Fig. 1. Analysis of the specificity of $pp65_{NLV}$-CTL and $IE1_{TMY}$-CTL. (a) IFN-γ-ELISPOT analysis of T2 cells, labelled either with $pp65_{NLV}$ or with $IE1_{TMY}$ at a concentration of 10^{-7} M and probed with $pp65_{NLV}$-CTL (black bars) or $IE1_{TMY}$-CTL (grey bars). (b) IFN-γ-ELISPOT analysis of HFF, infected with either a pp65-competent viral strain (AD169-BAC) or with a pp65-negative strain that had been constructed on the genetic background of AD169-BAC (RVHd65), using $pp65_{NLV}$-CTL as responder cells. Cells were infected for 24 h prior to IFN-γ-ELISPOT analysis. (c) IFN-γ-ELISPOT analysis of infected HFF that were either HLA-A2-positive (HFF-A2+) or HLA-A2-negative (HFF-A2–) as stimulator cells. $pp65_{NLV}$-CTL (black bars) and $IE1_{TMY}$-CTL (grey bars) were used as responder cells. Infection was carried out with a viral strain deficient in gpUS2–11 expression (RVΔUS2–11) to ensure recognition by $IE1_{TMY}$-CTL. Results shown are from one representative experiment providing means and SD of triplicate samples.

The immunodominant peptide $pp65_{NLV}$ is presented by MHC class I on infected fibroblasts both at early and late times post-infection (p.i.)

Since we chose to analyse pp65 presentation using the immunodominant, HLA-A2-presented nonapeptide $pp65_{NLV}$, we wanted to test whether recognition of infected cells by $pp65_{NLV}$-CTL followed similar kinetics to those described for polyspecific T-cell lines (McLaughlin-Taylor *et al.*, 1994). MRC-5 human fibroblasts were infected with the HCMV laboratory strain Ad169 and were subjected to CRA (Fig. 2a). MRC-5 cells that had been infected for 16 h proved to be good targets for $pp65_{NLV}$-CTL. Since only low-level *de novo* expression of pp65 can be expected to occur at this time p.i. (Depto & Stenberg, 1989), the $pp65_{NLV}$ peptide must be primarily derived from virion-associated pp65. In contrast, 96 h p.i., cells were no longer lysed by $pp65_{NLV}$-CTL. These results matched the

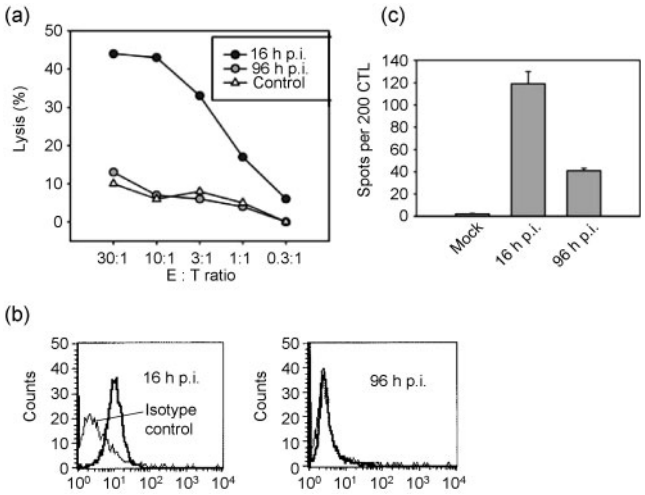

Fig. 2. Kinetics of recognition of HCMV-infected MRC-5 cells by $pp65_{NLV}$-CTL. (a) CRA of MRC-5 cells, infected with an m.o.i. of 10 of the HCMV strain Ad169 for 16 and 96 h, respectively, using $pp65_{NLV}$-CTL as effector cells. Control cells were non-infected. ^{51}Cr release was measured at the indicated effector to target cell ratios (E : T ratio). (b) Cytofluorometric analysis of HLA-A2 mAb BB7-B2–FITC on MRC-5 cells infected with HCMV strain Ad169 for 16 and 96 h, respectively. (c) IFN-γ-ELISPOT using Ad169-infected fibroblasts 16 and 96 h p.i. as stimulator and $pp65_{NLV}$-CTL as responder cells. Results shown represent means and SD of triplicate samples in each case.

downregulation of MHC class I molecules on infected cells at this time p.i. (Fig. 2b). To test whether there might be a stimulation of the CTL by cells 96 h p.i. which did not result in killing, IFN-γ-ELISPOT analyses were carried out. As expected, MRC-5 cells 16 h p.i. stimulated $pp65_{NLV}$-CTL significantly (Fig. 2c). In contrast to CRA, some spots also appeared when 96 h p.i. cells were used for stimulation. This indicates that, in the face of immunoevasion, there are still sufficient HLA-A2-$pp65_{NLV}$ complexes on the surface of fibroblasts 96 h p.i. to stimulate IFN-γ production in CTL.

MHC class I presentation of $pp65_{NLV}$ after exogenous loading of cells by viral particles is sensitive to inhibitors of proteasomal degradation

Introduction of particle-associated pp65 into the MHC class I presentation pathway labels cells for CTL recognition (McLaughlin-Taylor *et al.*, 1994). It remained unclear whether the tegument protein was processed by the proteasome or in a proteasome-independent fashion (Gil-Torregrosa *et al.*, 2000; Del-Val & Lopez, 2002). We found that processing of the $pp65_{NLV}$ after *de novo* synthesis in cells transfected with a pp65 expression plasmid was sensitive to proteasomal inhibition (data not shown). To investigate whether particle-associated pp65 is processed by the same or an alternative processing pathway, HFF were incubated with DB (30 μg per 5×10^5 HFF) from strain RVΔUS2–11 for 12 h while treated with proteasomal inhibitors. Cells were then fixed with paraformaldehyde to prevent further processing and subjected to IFN-γ-ELISPOT analyses using $pp65_{NLV}$-CTL as responder cells (Fig. 3a).

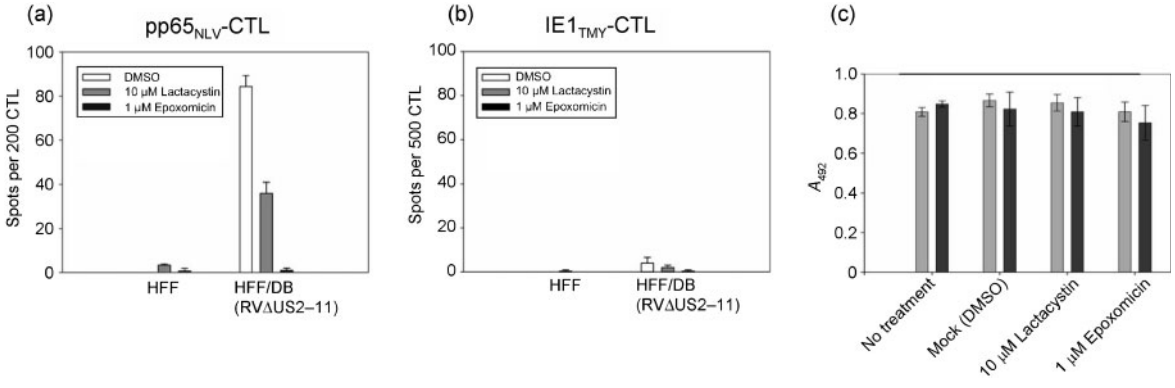

Fig. 3. Impact of proteasome functionality on pp65 processing and MHC class I loading. (a) 5×10^5 HFF were incubated for 12 h with 30 μg DB, prepared from culture supernatants of RVΔUS2–11-infected cells and were treated for 13 h with either DMSO (control) or with the indicated proteasome inhibitors (including a 1 h pretreatment of cells). Subsequently, cells were fixed with paraformaldehyde and were subjected to IFN-γ-ELISPOT analysis, using $pp65_{NLV}$-CTL as responder cells (200 CTL per well). (b) Infection control. HFF were treated as in (a) and were probed with $IE1_{TMY}$-CTL (500 CTL per well) to test for the absence of processing of *de novo*-synthesized antigen under the experimental conditions used. Results in (a) and (b) are shown as means and SD of triplicate samples. (c) Viability control. Cells were treated as in (a) and (b), and were subsequently tested for viability using a cell viability assay. Absorbance at 492 nm represents the content of NADH/NADPH as a measure for the number of viable cells. Results are shown from one representative experiment providing means and SD of five samples. Grey bars, HFF; black bars, DB-treated HFF.

Treatment of DB-loaded cells with lactacystin or epoxomicin resulted in marked reduction of spot numbers, demonstrating that processing of particle-associated pp65 was indeed proteasome dependent. To verify that MHC class I presentation of *de novo*-synthesized pp65 resulting from residual infectious virus in the DB preparation was excluded, parallel cultures were tested with $IE1_{TMY}$-CTL as responders. The expression of IE1 precedes that of pp65 during infection. No significant numbers of spots were observed (Fig. 3b). Cell viability subsequent to proteasome inhibitor treatment and DB exposure was found to be unaffected (Fig. 3c). Furthermore, for control of the impact of fixation on antigen presentation, cells were also labelled with peptide, fixed with paraformaldehyde and used as stimulators in IFN-γ-ELISPOT analysis. Both $pp65_{NLV}$-CTL and $IE1_{TMY}$-CTL were stimulated by peptide-labelled cells that were fixed with paraformaldehyde (data not shown).

Taken together, these results showed that the introduction of particle-associated pp65 into the MHC class I presentation pathway is dependent on the functionality of the proteasome.

The pp65-derived peptide is presented by MHC class I despite gpUS2–11 expression

Recently, HCMV mutants lacking the US2–11 genes have been used to investigate the impact of immunoevasion proteins on MHC class I presentation during infection (Manley *et al.*, 2004; Khan *et al.*, 2005). We used the US2–11 deletion mutant RVΔUS2–11 in comparison with an evasion-competent strain (AD169-BAC) in IFN-γ-ELISPOT analysis to test for the kinetics of MHC class I presentation of $pp65_{NLV}$. Infection of HFF with the US2–11-competent strain resulted in a gradual decrease in spot numbers over time p.i., using $pp65_{NLV}$-CTL as responder cells. In contrast, infection with RVΔUS2–11 resulted in stimulation of IFN-γ secretion by $pp65_{NLV}$-CTL at a comparable level at all time points p.i. tested (Fig. 4a). Thus, the deletion of the US2–11 genes completely restored $pp65_{NLV}$ recognition by CTL, indicating that the impairment of pp65-derived peptide presentation was mediated through immunoevasion proteins. To analyse whether this time frame of MHC class I–peptide presentation was specific to pp65 or was intrinsic to our test system, we repeated the experiment using $IE1_{TMY}$-CTL. In accordance with what had been reported before by others using human IE1-specific CTL lines (Manley *et al.*, 2004; Khan *et al.*, 2005), $IE1_{TMY}$-CTL were not stimulated by Ad169-infected fibroblasts at any time p.i. tested (Fig. 4b). In contrast, RVΔUS2–11-infected fibroblasts were excellent stimulators for IFN-γ production by $IE1_{TMY}$-CTL. Both $pp65_{NLV}$-CTL and $IE1_{TMY}$-CTL induced comparable numbers of spots when incubated with the evasion-negative strain, indicating comparable sensitivity of the CTL clones in this assay. These experiments showed that expression of US2–11 immunoevasion proteins incompletely blocks MHC class I presentation of pp65-derived peptides.

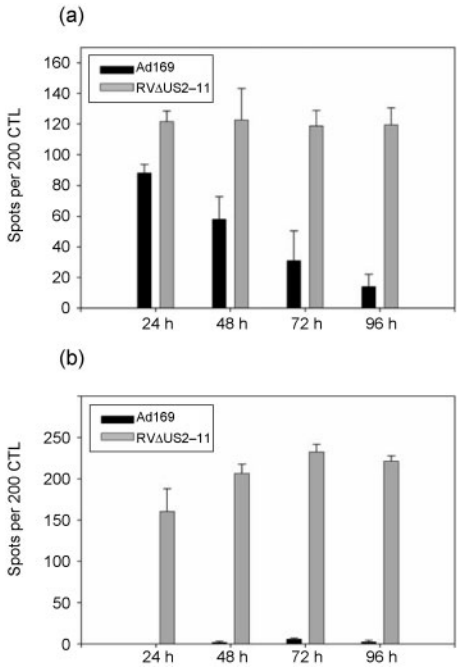

Fig. 4. Time course of pp65- and IE1-specific CTL responses to HFF infected with immunoevasion-competent and immunoevasion-negative HCMV strains. (a) IFN-γ-ELISPOT analysis of HFF infected for various times with the immunoevasion-competent strain Ad169 or the immunoevasion-negative strain RVΔUS2–11. $pp65_{NLV}$-CTL were used as responder cells. (b) Similar experiment as in (a), using the $IE1_{TMY}$-CTL as responder cells. Results are shown from one representative experiment as means and SD of triplicate samples.

Restoration of pp65-derived peptide presentation despite expression of immunoevasion proteins

One possible explanation for continuous pp65 presentation was that US2–11 expression blocked pp65-derived peptide loading and MHC complex translocation to the cell surface efficiently, while continuous $pp65_{NLV}$ presentation was related to metabolically stable complexes at the cell surface, derived from particle-associated antigen. Alternatively, MHC class I peptide loading and surface translocation of pp65-derived peptides could have been subject to an incomplete block by immunoevasion. To test for these alternatives, MHC class I–peptide complexes were removed from the surface of infected cells by acid wash (Sugawara *et al.*, 1987). The capacity of these cells to restore $pp65_{NLV}$ presentation was analysed by IFN-γ-ELISPOT. In a first control experiment, it could be shown that removal of MHC class I was complete immediately after acid treatment, and restoration of MHC class I to the level of untreated cells was entirely accomplished 24 h later, as shown by FACS analysis (Fig. 5a). To ensure the complete removal of functional MHC class I–peptide complexes from the cell surface by acid treatment, HFF were infected for 48 h with the US2–11-negative strain RVΔUS2–11.

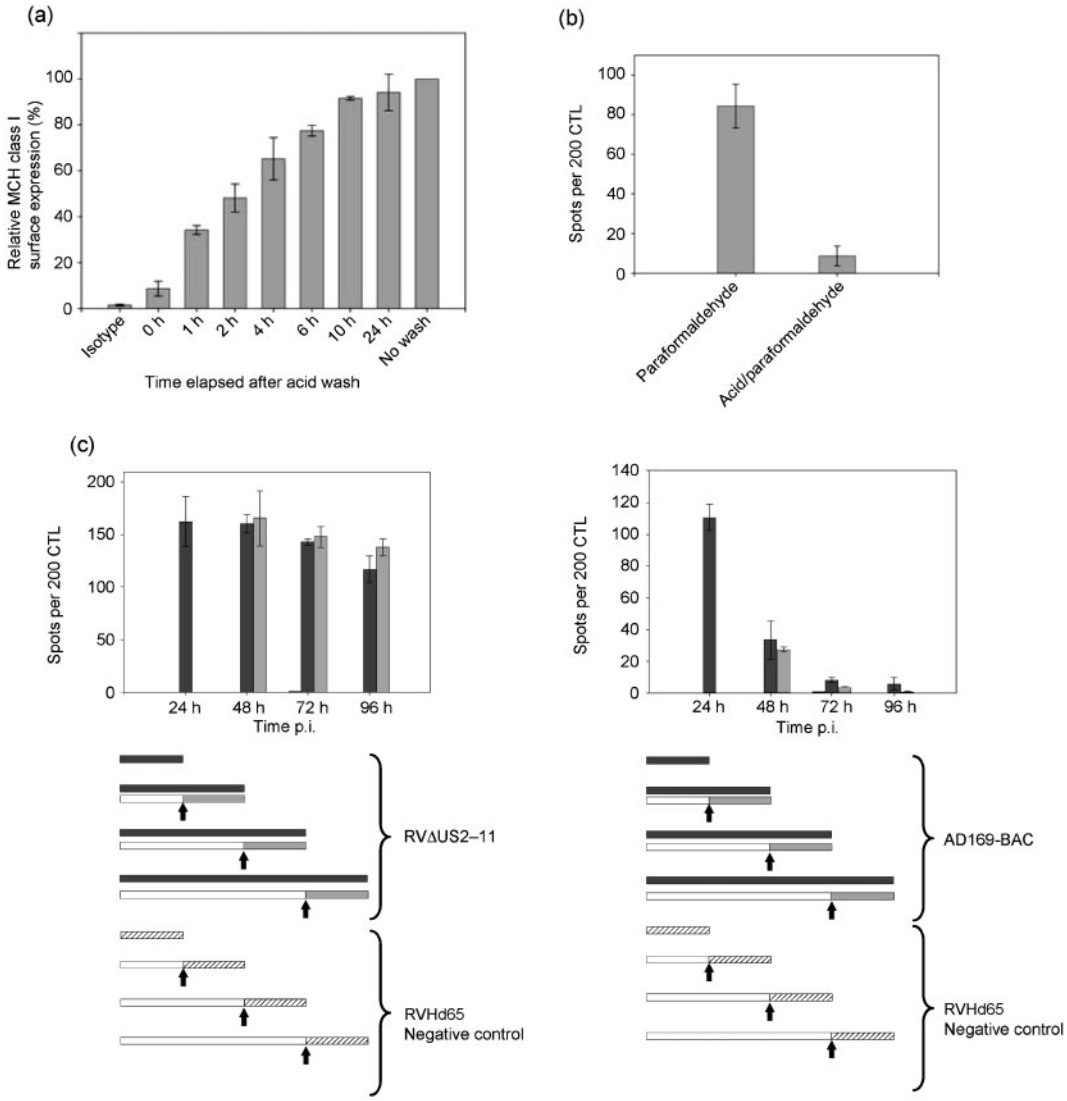

Fig. 5. Analysis of the kinetics of restoration of $pp65_{NLV}$ presentation on infected HFF depending on the expression of immunoevasion proteins gpUS2–11. (a) FACS analysis of MHC class I cell surface expression on infected HFF subsequent to acid wash using mAb W6132. Cells were infected with RVΔUS2–11 for 24 h, acid treated and tested for MHC class I surface expression at the indicated times. MHC class I expression is given as percentage of the expression of untreated control cells ('no wash'). Results shown are means and SD of three independent experiments. (b) IFN-γ-ELISPOT analysis of the removal of MHC class I complexes by acid wash. Cells were infected with RVΔU2–11 for 48 h and were either fixed directly with paraformaldehyde (control) or were acid-stripped and subsequently fixed with paraformaldehyde to prevent further translocation of MHC class I complexes to the cell surface during the following assay period. The cells were used as target cells for IFN-γ-ELISPOT analysis with $pp65_{NLV}$-CTL as responder cells. Results are shown as means and SD of triplicate samples. (c) IFN-γ-ELISPOT of cells infected with the gpUS2–11-negative strain RVΔUS2–11 (left panel) or with the gpUS2–11-competent strain AD169-BAC (right panel). Dark grey bars, HFF infected for the indicated times and subsequently used as stimulator cells for $pp65_{NLV}$-CTL in IFN-γ-ELISPOT analysis. Light grey bars, HFF infected for the indicated times (open bar section below) and then subjected to acid wash to remove MHC class I–peptide complexes from the cell surface; cells were further incubated for another 24 h (light grey section below) and were used as stimulator cells for $pp65_{NLV}$-CTL. For background control, cells were infected with the pp65-negative virus strain RVHd65 and these cells were also subjected to acid wash (open and hatched bars). Results in (c) are representative for several experiments. Means and SD are from triplicate samples.

Cells were fixed using paraformaldehyde, or acid-stripped and subsequently fixed to prevent further processing and MHC translocation. Fixed cells were good CTL targets (Fig. 5b). Acid treatment and paraformaldehyde fixation, however, completely abrogated CTL recognition, demonstrating the efficiency of MHC class I stripping. To test

whether the restoration of MHC class I surface expression correlated with the capacity of the cells to present $pp65_{NLV}$, HFF were infected for 24, 48 and 72 h, respectively, with RVΔUS2–11 (Fig. 5c left panel). Subsequently, cells were either left untreated (control, dark grey bars) or were stripped with acid (light grey bars). In both cases, cells were incubated for another 24 h prior to ELISPOT analysis. The pp65-peptide presentation could be fully restored to the level of untreated cells in each 24 h period after acid wash. This demonstrated that peptide loading and surface translocation of MHC class I–$pp65_{NLV}$ complexes was efficient subsequent to acid wash and was not a limiting step in the chosen experimental setting.

Infection of HFF with the US2–11-competent strain AD169-BAC resulted, as expected, in a gradual decline of recognition by $pp65_{NLV}$-CTL (Fig. 5c, right panel). However, in repeated experiments, acid washes at 24 h resulted in a more than half-maximal restoration of antigen presentation during the following 24 h. Even when acid washes were performed at 48 h p.i., there was restoration of presentation, yet failing to reach the level of untreated cells. $pp65_{NLV}$ presentation was completely abrogated when the acid washes were carried out at 72 h p.i. For negative control, a parallel experiment was conducted with a pp65-negative strain (RVHd65). No recognition of cells infected with this strain was detectable at any of the time points tested (Fig. 5c).

These experiments showed that pp65-derived peptide presentation could be restored in early- and late-infected fibroblasts despite the expression of gpUS2–11 and that this process was, at least in part, due to continuing translocation of MHC class I–$pp65_{NLV}$ complexes to the cell surface. It should be emphasized at this point that $IE1_{TMY}$ presentation was not detectable on HFF infected with an US2–11-competent virus, showing that immunoevasion was fully operative (see Fig. 4). Taken together, these results demonstrated that immunoevasion is only partly effective in preventing $pp65_{NLV}$ presentation.

De novo generation and MHC class I loading of pp65-derived peptides in the face of immunoevasion

One possible explanation for the presentation of $pp65_{NLV}$ in the face of immunoevasion was that pp65 degradation, MHC class I peptide loading of $pp65_{NLV}$ and the translocation of the complex to the cell surface were continuing despite gpUS2–11 expression. Alternatively, recycling of preformed complexes to the cell surface could occur despite immunoevasion. To be able to distinguish between these alternatives, we tested whether restoration of $pp65_{NLV}$ presentation after acid wash was dependent on the functionality of the proteasome. Proteasome functionality would be required for *de novo* processing of $pp65_{NLV}$ from pp65, but not for recycling of preformed peptides. Cells were infected for 24 h at an m.o.i. of 10 with the US2–11-competent strain AD169-BAC. Subsequently, they were acid-washed and analysed in a 20 h IFN-γ-ELISPOT assay (Fig. 6). During the assay period, cells were either mock treated (with DMSO) or treated with the proteasomal inhibitors lactacystin or epoxomicin. Inhibition of proteasomal function almost completely abrogated $pp65_{NLV}$ presentation. To control for the impact of proteasomal inhibitors on CTL function, HFF were labelled with $pp65_{NLV}$, fixed with paraformaldehyde and tested with $pp65_{NLV}$-CTL in IFN-γ-ELISPOT analysis. The CTL were only partially influenced by proteasomal inhibitors. This could not explain the significant reduction of spots seen after treatment of infected cells with these inhibitors. Furthermore, target cell viability was controlled using a commercial proliferation assay (data not shown). Taken together, these experiments showed that $pp65_{NLV}$ can be proteasomally processed, loaded on MHC class I and translocated to the cell surface despite the expression of the US2–11 genes of HCMV.

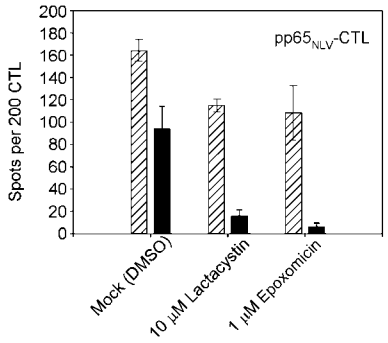

Fig. 6. IFN-γ-ELISPOT analysis of $pp65_{NLV}$ processing by the proteasome during immunoevasion. HFF were infected with the gpUS2–11-competent strain AD169-BAC for 24 h and were then subjected to acid washing. Cells were then analysed by IFN-γ-ELISPOT in the presence of proteasome inhibitors using $pp65_{NLV}$-CTL as responders (black bars). To control for the impact of proteasome inhibitors on CTL function, HFF were labelled with 10^{-5} M $pp65_{NLV}$, fixed with paraformaldehyde and subjected to IFN-γ-ELISPOT analysis in the presence of the diluent (DMSO) or of proteasome inhibitors, as indicated (hatched bars). Means and SD are from triplicate samples.

DISCUSSION

Antiviral CTL have been identified as key players that restrict HCMV replication, terminate infection and control latency (reviewed by Reddehase, 2002). pp65 appears to be exceptional, being an immunodominant antigen that mediates protective immunity against HCMV (reviewed by Reddehase, 2000; Wills *et al.*, 2006). However, this contrasts with *in vitro* findings, demonstrating the efficacy of HCMV immunoevasion proteins (Barnes & Grundy, 1992; Jones *et al.*, 1995; Ahn *et al.*, 1996; Reddehase, 2002). From these investigations, the key question that arose was how infected cells could present pp65-derived peptides

despite immunoevasion, thereby providing the basis for immune recognition and protection mediated by pp65-specific CTL.

Early studies provided evidence that pp65-specific CTL could lyse infected cells from 2 to 66 h p.i. (McLaughlin-Taylor *et al.*, 1994). Here we extended previous findings by showing that (i) pp65-derived peptides are presented by MHC class I in the course of infection, yet to a decreasing extent; (ii) this decrease is completely abrogated by preventing gpUS2–11 expression, indicating that the effect was mediated by these immunoevasion proteins; and (iii) presentation of pp65-derived peptides at early and late times after infection is, at least in part, the result of continuing proteasomal processing and MHC class I loading despite immunoevasion.

MHC class I presentation of pp65-derived peptides can be observed as early as 2 h p.i. (McLaughlin-Taylor *et al.*, 1994) and continues up to 96 h p.i. (this study). However, the capability of cells to present these peptides declined over time. This decrease was shown to be dependent on expression of the US2–11 gene region. It remains to be determined which of the immunoevasion proteins (gpUS2, gpUS3, gpUS6 or gpUS11), or which combination of them, is responsible for the enhanced suppression of pp65-derived peptide presentation by MHC class I over time. In contrast to pp65, we found that presentation of the IE1 peptide was completely suppressed by US2–11 expression, thereby confirming results from previous studies by others (Manley *et al.*, 2004; Khan *et al.*, 2005).

One possible explanation for the presentation of pp65-derived peptide persisting during productive infection could be attributed to the expression of this gene preceding immunoevasion gene expression. However, transcriptional analyses indicate that UL83 mRNA synthesis is restricted to the early and late phases of virus replication (Depto & Stenberg, 1989). Consequently, pp65 is synthesized *de novo* within infected cells only when immunoevasion proteins are present.

As infectious virions contain a significant amount of pp65 (Varnum *et al.*, 2004), particle-associated pp65 may be introduced into the MHC class I presentation pathway before immunoevasion become operative (McLaughlin-Taylor *et al.*, 1994; Pepperl *et al.*, 2000; Pepperl-Klindworth *et al.*, 2003). Virion-associated pp65 could potentially sensitize cells for a prolonged period of time. The half-lives ($t_{1/2}$) of peptide-loaded MHC class I complexes vary considerably, ranging up to over 30 h. Affinity to MHC class I has been found to be one crucial parameter for immunogenicity of a given peptide (Yu *et al.*, 2004; Kambayashi *et al.*, 2004). $pp65_{NLV}$ binds with high affinity to HLA-A2 (Frankenberg *et al.*, 2002). Thus it was theoretically possible that particle-derived $pp65_{NLV}$ was presented throughout infection, while further processing and presentation were inhibited by immunoevasion. However, acid stripping of surface peptide could only transiently prevent $pp65_{NLV}$ presentation, indicating that peptide-loaded MHC class I complexes almost completely replenished the pool that had been removed from the surface. This argues against a significant role for the persistence of stable MHC class I–$pp65_{NLV}$ complexes in the recognition of early and late infected cells by CTL.

However, the results cannot discriminate between whether MHC class I presentation of $pp65_{NLV}$ at later stages of infection was due to prolonged processing and presentation of particle-derived pp65 or *de novo* synthesis of the tegument protein. Particle-associated pp65 appears to be efficiently degraded as evidenced by the rapid loss of staining signal following the exposure of HFF to large amounts of subviral DB (data not shown). It remains questionable, at least, whether particle-associated pp65 could be a relevant source of antigen at early and late times when the UL83 gene is highly active and newly synthesized pp65 is accumulating. Using proteasome inhibition, processing of both particle-derived and *de novo*-synthesised pp65 was observed to be proteasome-dependent. Consequently, following infection, particle-associated pp65 would serve as a source for proteasomal processing and MHC class I loading, whilst, at later stages, the large amounts of *de novo*-synthesized pp65 provide the most abundant potential source of antigenic peptides.

Recycling of MHC class I molecules from the cell surface has been reported (Jondal *et al.*, 1996; Gromme *et al.*, 1999). Reappearance of $pp65_{NLV}$ presentation after acid stripping could have been explained by such a recycling mechanism of peptide-loaded complexes. However, inhibition of proteasomal function abrogated $pp65_{NLV}$ presentation following acid stripping. Therefore, it appears that pp65 degradation by the proteasome and subsequent *de novo* formation of MHC class I–peptide complexes is a prerequisite for $pp65_{NLV}$ presentation in the face of immunoevasion.

Several studies have recently emphasized the diversity of the CTL response against HCMV with respect to target antigens (Elkington *et al.*, 2003; Manley *et al.*, 2004; Khan *et al.*, 2005; Sylwester *et al.*, 2005). Cross-presentation by professional antigen-presenting cells (pAPC) rather than direct presentation of viral antigens has been suggested to explain how CTL could be primed against HCMV proteins which are not presented by infected cells (Sigal *et al.*, 1999; Arrode *et al.*, 2000). This may also explain the high frequencies of IE1-specific CTL in some seropositive donors (Kern *et al.*, 1999; Khan *et al.*, 2002). However, it could not be the reason for the predominance of IE1-specific CTL found in some of the donors as cross-priming of IE1 and pp65, synthesized in permissively infected cells, should be equally effective for both antigens. Recent data from the MCMV model, however, may provide a rationale to understand this phenomenon. Simon *et al.* (2006) showed that $CD8^+$ T cells specific for an antigenic peptide from the MCMV IE1 protein rapidly sense and terminate transcriptional reactivation from viral latency in the lungs. Consequently, downstream early or late gene expression is

prevented and antigenic peptides originating from these genes are not presented. Similar transcriptional reactivation events may occur in humans, allowing for frequent restimulation of pre-existing IE1-specific CTL. Depending on the HLA haplotype of the individual, IE1-derived peptides may be immunodominant and consequently may promote efficient CTL restimulation. Such CTL may then predominate in frequency over pp65-specific CTL, which may require complete reactivation to virus replication for restimulation.

Both pp65 and IE1 have been labelled as immunodominant CTL antigens, based on $CD8^+$ T-cell frequencies in seropositive donors (Wills *et al.*, 2006). However, in the MCMV model, CTL against an immunodominant peptide from the M45 protein failed to protect against infection after adoptive transfer (Holtappels *et al.*, 2004). Consequently, the protective function of CTL against IE1 and pp65 of HCMV cannot be solely deduced from cell frequencies. Yet, both for vaccine development as well as for the design of immunointerventive strategies, it appears important to discriminate between 'protective antigens' and 'non-protective antigens'. Adoptive transfer experiments have provided circumstantial evidence that pp65-specific CTL are protective *in vivo* (Walter *et al.*, 1995; Cobbold *et al.*, 2005). This work shows, for the first time, that loading and cell-surface transport of MHC class I–peptide complexes derived from HCMV antigens can occur in infected cells despite immunoevasion. This was even true in the absence of IFN-γ, known to compensate for the suppression of MHC surface expression by immunoevasion proteins (Benz & Hengel, 2000). Although at this stage it cannot be discriminated whether resistance to immunoevasion was intrinsic to the pp65-derived peptide or whether other structural or non-structural proteins could also evade evasion, the findings may serve as a rationale for future studies on the role of individual HCMV proteins as CTL antigens in the course of immune protection against disease.

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