

Suppression of CD8⁺ T-cell recognition in the immediate-early phase of human cytomegalovirus infection

Julia Hesse,¹ Stefanie Ameres,² Katrin Besold,^{1†} Steffi Krauter,¹ Andreas Moosmann² and Bodo Plachter¹

Correspondence

Bodo Plachter

bodo.plachter@unimedizin-mainz.de

¹Institute for Virology, University Medical Center of the Johannes Gutenberg-University Mainz, Mainz, Germany

²Clinical Cooperation Group Immunooncology, Helmholtz Zentrum München and Ludwig-Maximilians-Universität München, Munich, Germany

Human cytomegalovirus (HCMV) interferes with MHC class I-restricted antigen presentation and thereby reduces recognition by CD8⁺ T-cells. This interference is mediated primarily by endoplasmic reticulum-resident glycoproteins that are encoded in the US2–11 region of the viral genome. Such a suppression of recognition would be of particular importance immediately after infection, because several immunodominant viral antigens are already present in the cell in this phase. However, which of the evasion proteins gpUS2–11 interfere(s) with antigen presentation to CD8⁺ T-cells at this time of infection is not known. Here we address this question, using recombinant viruses (RV) that express only one of the immunoevasins gpUS2, gpUS3 or gpUS11. Infection with RV-US3 had only a limited impact on the presentation of peptides from the CD8⁺ T-cell antigens IE1 and pp65 under immediate-early (IE) conditions imposed by cycloheximide/actinomycin D blocking. Unexpectedly, both RV-US2 and RV-US11 considerably impaired the recognition of IE1 and pp65 by CD8⁺ T-cells, and both US2 and, to a lesser extent, US11 were transcribed under IE conditions. Thus, gpUS2 and gpUS11 are key effectors of MHC class I immunoevasion immediately after HCMV infection.

Received 2 July 2012

Accepted 19 October 2012

INTRODUCTION

Human cytomegalovirus (HCMV) has established a balanced relationship with its host in the course of co-evolution. A key determinant for this is the intricate interplay between immune defence functions on one hand and viral evasion mechanisms on the other (Babić *et al.*, 2011; Hengel *et al.*, 2005; Johnson & Hegde, 2002; Lemmermann *et al.*, 2011; Noriega *et al.*, 2012a; Reddehase, 2002). Of particular interest is how HCMV-infected cells are protected against recognition and killing by cytotoxic CD8 T-lymphocytes (CTLs). A pronounced CTL response is a hallmark of CMV infection, effecting virus control and reactivation from latency (Reddehase *et al.*, 1985; Reusser *et al.*, 1991; Steffens *et al.*, 1998; Walter *et al.*, 1995).

CTLs recognize viral peptides presented at the cell surface in the context of MHC class I molecules. However, early downregulation of MHC I was observed in infected cells, indicating that HCMV has developed means to subvert CTL recognition (Barnes & Grundy, 1992; Yamashita *et al.*, 1993). Endoplasmic reticulum (ER)-resident viral proteins

encoded in the genomic region from US2 to US11 have been found to be instrumental in this downmodulation (Jones *et al.*, 1995; Noriega *et al.*, 2012a). The glycoprotein (gp) US3 binds to MHC I molecules in the ER, leading to their retention (Ahn *et al.*, 1996; Jones *et al.*, 1996; Lee *et al.*, 2000; Zhao & Biegalka, 2003). It also interacts with the ER-resident chaperone tapasin and interferes with peptide loading of MHC I molecules (Park *et al.*, 2004). gpUS2 and gpUS11 mediate retrograde translocation of newly synthesized MHC I heavy chains from the ER to the cytoplasm, leading to their subsequent proteasomal degradation (Wiertz *et al.*, 1996a, b). gpUS6 leads to a block in peptide transport from the cytosol to the ER through the TAP, thereby preventing the formation of MHC I-peptide complexes in the ER (Ahn *et al.*, 1997; Halenius *et al.*, 2006; Hengel *et al.*, 1997; Lehner *et al.*, 1997). Both gpUS8 and gpUS10 interact with MHC I, yet only gpUS10 has been shown to have an effect by targeting the non-classical HLA-G for degradation (Furman *et al.*, 2002; Park *et al.*, 2010; Tirabassi & Ploegh, 2002). In addition to US2–11-encoded proteins, a microRNA, miR-US4-1, has been shown to downregulate the expression of the aminopeptidase ERAP1, which trims peptides within the ER before MHC I loading (Kim *et al.*, 2011). Besides ER-resident viral

[†]Present address: Novartis Pharma AG, Werk Klybeck, Klybeckstrasse 141, CH-4057 Basel, Switzerland.

glycoproteins and miR-US4-1, the tegument proteins pp65 and pp71 have also been suggested to be engaged in MHC I downmodulation (Gilbert *et al.*, 1996; Trgovcich *et al.*, 2006).

Expression of individual members of the US2–11 evasion protein family in HCMV-infected cells is insufficient for complete protection against CTL recognition at early and late times of infection (Besold *et al.*, 2009). Yet, co-expression of these proteins may lead to a pronounced downregulation of MHC I presentation (Besold *et al.*, 2009; Noriega *et al.*, 2012b; Noriega & Tortorella, 2009). This is consistent with the idea that expression kinetics of gpUS2, gpUS3, gpUS6 and gpUS11 overlap considerably at early and late phases of HCMV infection, thereby guaranteeing an optimal protection of infected cells against CTL recognition. In contrast, gpUS3 was reported to be the only evasion protein expressed at immediate-early (IE) times of infection (Ahn *et al.*, 1996; Weston, 1988). This is a critical phase in the lytic cycle of the virus, as both exogenously introduced structural proteins and regulatory IE proteins are present in abundance at this time post-infection (p.i.). Both classes of proteins, in particular pp65 and IE1, serve as dominant sources of antigenic peptides presented by MHC I (Borysiewicz *et al.*, 1988; Jackson *et al.*, 2011; Kern *et al.*, 1999; Manley *et al.*, 2004; Wills *et al.*, 1996). It has been shown that MHC class I molecules are retained in the ER under infection conditions that allow expression of only IE proteins of HCMV (Ahn *et al.*, 1996). It remained, however, unclear whether this was sufficient to protect infected cells from CTL-mediated cytotoxicity immediately after infection and, if so, whether the expression of US3 alone was mediating this effect. Using a set of viral mutants, we investigated this and found that, despite its abundant expression, gpUS3 had limited impact on MHC class I antigen presentation in this IE phase of infection. Unexpectedly, gpUS2 and gpUS11 strongly suppressed MHC I-restricted recognition of HCMV-infected cells at this phase of infection.

RESULTS

Deletion of US2–11 restores presentation of IE1 peptides by MHC I at IE times of HCMV infection

The expression of glycoproteins encoded in the US2–11 gene region of HCMV leads to downregulation of MHC I early in the course of HCMV infection (Jones *et al.*, 1995). To approach the question of whether this downregulation impacts on the recognition of infected cells by CTLs in the initial phases of infection, human foreskin fibroblasts (HFFs) were infected with a US2–11-competent HCMV strain (RV-BADwt) and a US2–11-deficient derivative (RV-JH10) (Fig. 1a). We operationally defined IE conditions in this work by using sequential application of cycloheximide (CX) and actinomycin D (AcD). This exclusively allows expression of viral genes whose transcription is independent of *de novo* synthesis of viral

proteins. Such conditions were established by first treating HFFs with 250 µg CX ml^{−1} for 1 h prior to infection. Cells were then infected in the presence of the drug to allow IE mRNA expression while blocking protein expression. The relatively high concentration of CX was necessary to prevent activation of early or late viral HCMV genes completely at 9 h p.i. (data not shown). After release of the block by washing out CX, cells were treated with AcD for 13.5 h. This prevents *de novo* generation of viral mRNA while allowing expression of IE proteins. Following this, the cells were fixed with paraformaldehyde to prevent any further antigen processing and were subjected to ELISpot analyses, using a CTL clone directed against an HLA-A2-presented peptide from IE1 (IE1_{TMY}-CTLs). Under these conditions, we observed a fourfold higher spot number in response to the US2–11-deficient virus than in response to the US2–11-competent virus (mean of 407 versus 117 spots). Antigen presentation to the CTL clone was abrogated completely in ELISpot analyses in unblocked cells at 1, 3 and 6 h p.i. (data not shown). These results showed that US2–11 genes were indeed interfering with functional antigen presentation under IE conditions.

US3 expression is insufficient to suppress MHC I presentation in the IE phase of infection

gpUS3 is the only member of the US2–11-encoded immunoevasion proteins that had been reported to be expressed at IE times (Greijer *et al.*, 2001; Liu *et al.*, 2002; Weston, 1988). We therefore investigated whether the reduction of IE1 peptide presentation at IE times was mediated by gpUS3. For that purpose, a mutant virus was used that expressed gpUS3, but lacked the US2, US6 and US11 reading frames (Fig. 1a) (Noriega *et al.*, 2012b). HFFs were infected with RV-US3 for 9 h in the presence of CX. Subsequently, cells were kept for 4, 8 or 13.5 h in medium containing AcD. After that, ELISpot analyses with IE1_{TMY}-specific CTLs were performed (Fig. 2a). Surprisingly, US3 expression appeared to have little impact on IE1 presentation under the chosen conditions. Following infection with RV-US3, there was no reduction in presentation, but rather a degree of enhancement compared with the US2–11-deficient control virus RV-JH10. As described in the previous section, infection with the parental virus RV-BADwt strongly suppressed presentation. To confirm that US3 was expressed at the chosen times after infection, total cell RNA was extracted from HFFs that had been infected under CX/AcD-blocking conditions (Fig. 2b). A US3-specific transcript of about 1 kb was detectable in large amounts in both RV-US3- and RV-BADwt-infected cells throughout the observation period. Another band of about 0.7 kb, probably corresponding to the singly spliced mRNA encoded by US3, was also detectable, but was much weaker and appeared to fade over time. Expression levels of the IE1 protein were comparable between the different viruses, excluding the possibility that overexpression of the source of the tested peptide antigen was overriding gpUS3 evasion (Fig. 2c). Taken together, these data suggested that expression

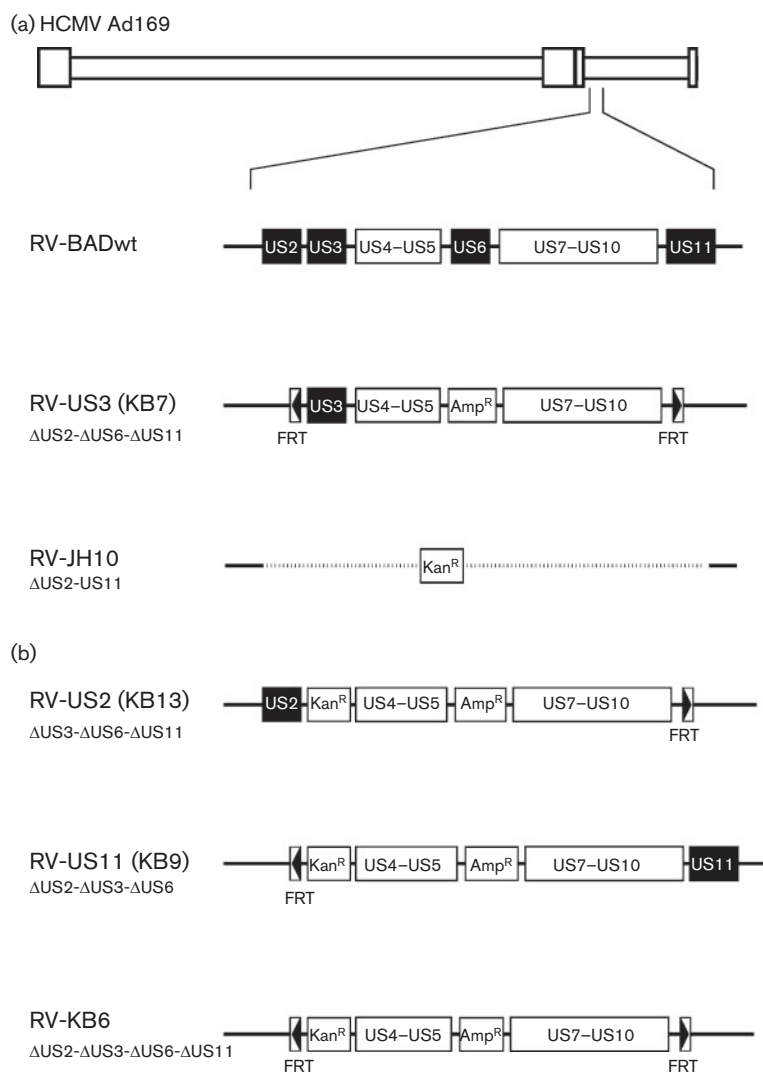


Fig. 1. Schematic representation of HCMV mutants. The HCMV Ad169 genome is shown on top. The region of interest is expanded in the maps below (not drawn to scale). The locations of the ORFs encoding US2, US3, US6 and US11 are shown by black boxes. The kanamycin-resistance (Kan^R) and ampicillin-resistance (Amp^R) genes, used for BAC selection, and the FRT (flippase-recognition target) sites, used in the process of BAC construction, are indicated. (a) Line drawings of the genomic alterations in recombinant viruses RV-US3 (Noriega *et al.*, 2012b) and RV-JH10. (b) Line drawings of the genomic alterations in mutants RV-US2, RV-US11 and RV-KB6, described by Besold *et al.* (2009).

of the US3 gene alone does not diminish MHC I-mediated antigen presentation at IE times of HCMV infection.

US2 and US11 are transcribed at IE times of HCMV infection

A US2–11-competent virus was effective in preventing MHC class I presentation, while RV-US3 was not. It was thus tested whether other members of the US2–11 family of evasion genes were expressed at IE times of infection. For this, HFFs were infected with wild-type (wt) HCMV in the presence of CX to allow transcription exclusively of IE genes. Total cell RNA was isolated and used for Northern blot analyses with US2–11-specific probes (Fig. 3). As expected, US3 was expressed to high levels. Unexpectedly, however, US2 and, to a lesser extent, US11 were also transcribed in the presence of CX. No expression of US6 was seen even after prolonged exposure of the membrane. The blocking conditions used were effective, as no pp65/pp71-specific RNA became detectable. The latter transcript

is known to be expressed at early and late times p.i. (Depto & Stenberg, 1989). These data indicate that both US2 and US11 were transcribed in the absence of *de novo* synthesis of viral proteins, thus fulfilling the definition of IE genes.

US2 and US11 suppress IE1 peptide presentation at IE times of HCMV infection

As US2 and US11 were transcribed under IE conditions, their impact on IE1 peptide presentation was tested. HFFs were infected with mutant viruses that expressed only US2 or US11 (Fig. 1b) (Besold *et al.*, 2009), again applying CX/AcD (Fig. 4). Expression of only US2 led to almost-complete downregulation of IE1 presentation, reaching wt levels. Infection with RV-US11 impaired presentation, yet not as completely as RV-US2. Again, infection with RV-US3 seemed to have little impact on antigen presentation. Co-infection with RV-US2 and RV-US3 or with RV-US11 and RV-US3 did not change the phenotype of the single-mutant viruses expressing US2 or US11, respectively. These

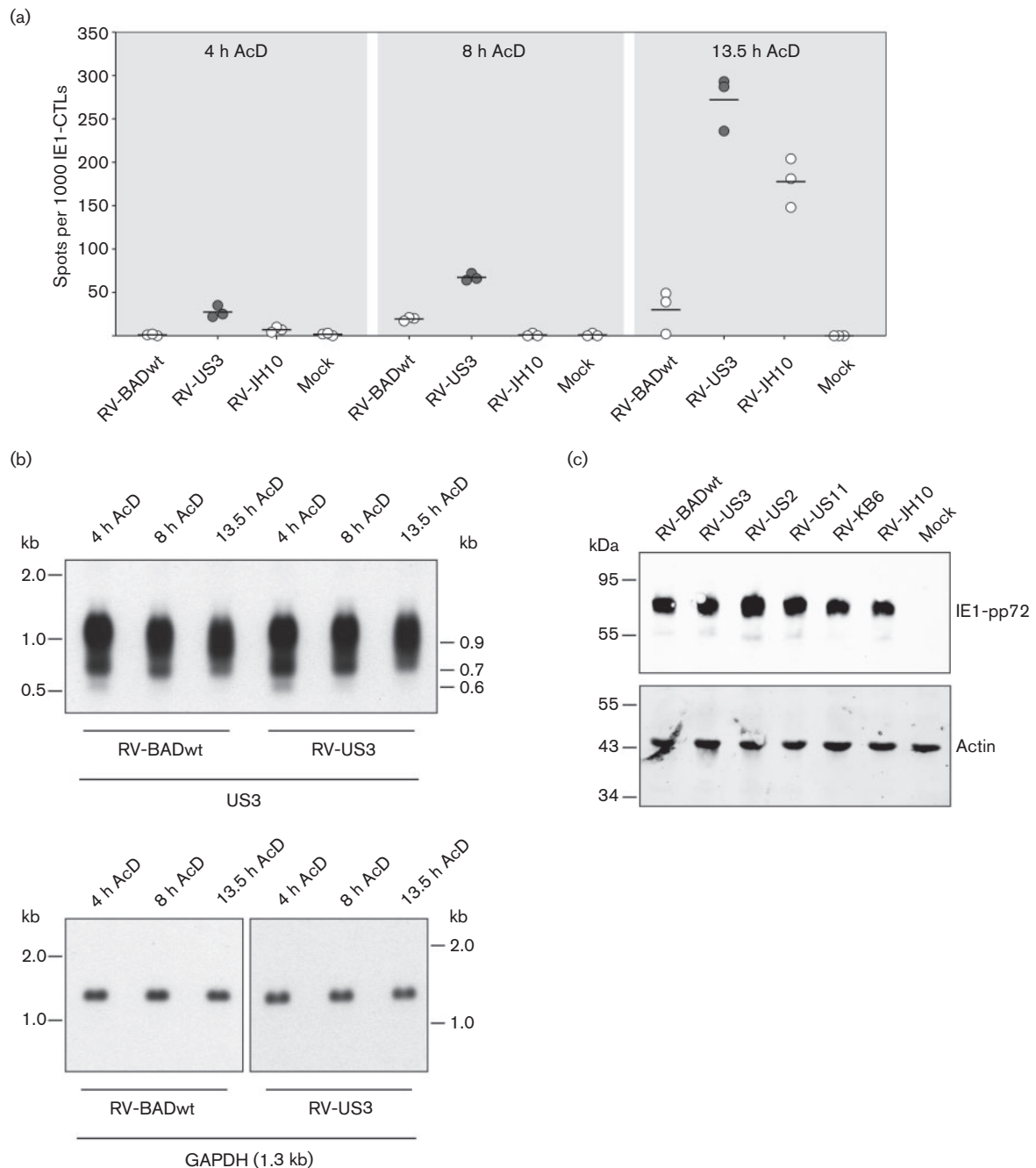


Fig. 2. Analysis of gpUS3-mediated impact on MHC I antigen presentation at IE times of HCMV infection. (a) ELISpot analysis of HFFs infected with the indicated viruses, using IE1_{TMV}-CTL as responder cells. HFFs were infected in the presence of CX at an m.o.i. of 5. Nine hours after infection, CX was removed and AcD was added. The duration of AcD treatment was varied as indicated. Subsequently, cells were fixed with paraformaldehyde and subjected to ELISpot analyses. Shown are the results of triplicate wells (●); mean values are given as horizontal lines. The difference between RV-BADwt and RV-US3 after 13.5 h AcD treatment was reproduced in four independent experiments. (b) Northern blot analysis of US3-RNA expression. Cells were infected with RV-US3 or its parental virus RV-BADwt in the presence of CX at an m.o.i. of 5. Twelve hours after infection, CX was removed and AcD was added. The duration of AcD treatment was varied as in (a). Total cell RNA was then hybridized either to a US3-specific probe or, for control, to a probe specific for cellular GAPDH. For US3 detection, 10 µg total cell RNA was applied on the gel; for GAPDH detection, 5 µg was applied. (c) Immunoblot analysis of IE1-pp72 expression levels following CX/AcD block of infected cells. HFFs were infected with individual mutants in the presence of CX at an m.o.i. of 5. Nine hours after infection, CX was removed and AcD was added for another 13.5 h. After that, cells were lysed and subjected to immunoblot analysis, using the Odyssey system (LI-COR Biosciences).

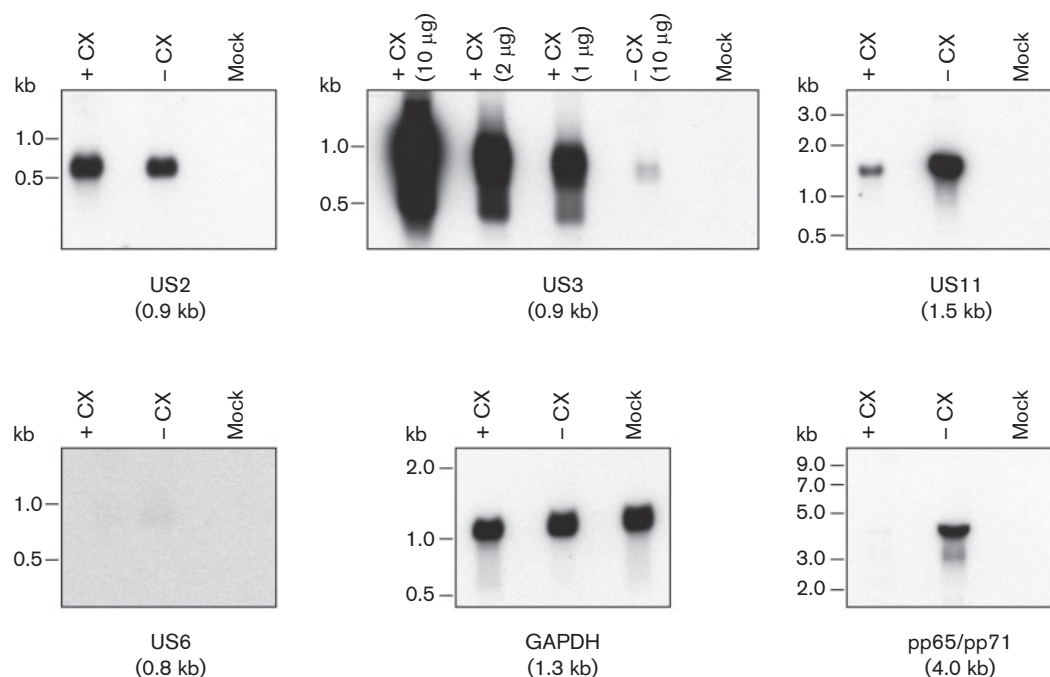


Fig. 3. Northern blot of US2–11 RNA expression. Total cell RNA was isolated from HFFs infected with RV-BADwt in the presence of CX at 125 genomes per cell and incubated for 8 h, except for RNA applied to analysis with the US6 probe, where infection was extended to 14 h. Total cell RNA was hybridized to probes specific for US2, US3, US6 or US11. For control of the block, a probe against the bicistronic early-late RNA encoding pp65 and pp71 was used. Level of loading was controlled by a probe against GAPDH. In all cases, 10 µg total cell RNA was applied to the gel. For US3 detection, 2 µg and 1 µg were also applied in separate lanes.

data indicate that both gpUS2 and gpUS11 have a distinct impact on MHC I antigen presentation immediately after HCMV infection.

Suppression by gpUS2 and gpUS11 in the IE phase is independent of the presented epitope and affects different classes of antigens

The above analyses were conducted using a CTL clone from HLA-A2 transgenic mice, directed against the HLA-A2-restricted IE1_{TMY} peptide. In the next set of experiments, we used human CTL clones obtained from a variety of healthy HCMV carriers. These CTL clones were specific for the HLA-A2-restricted peptides VLEETSVML (VLE) from IE1 or NLVPMVATV (NLV) from the tegument protein pp65. The latter antigen becomes available for MHC I presentation by transfer of protein through virus particles at IE times, without requiring *de novo* synthesis in infected cells (McLaughlin-Taylor *et al.*, 1994). Fibroblasts were infected with the different viruses under CX/AcD inhibition (Fig. 5a, b) and were then incubated with individual T-cell clones. Recognition by each of six IE1_{VLE}-specific T-cell clones from four donors was efficiently suppressed by infecting with RV-US2 or RV-US11, whereas, paradoxically, a certain increase in recognition was seen with RV-US3. This confirmed the data obtained

above with IE1_{TMY}-specific T-cells. Recognition of pp65_{NLV} was also suppressed by gpUS2 and gpUS11, but to a lesser extent than the IE1 peptides, in accordance with an only marginal suppression in wt-infected cells. These results are consistent with and extend our previous findings showing that pp65-derived peptides are presented at early stages of infection, despite the expression of US2, US11 or the whole set of evasion genes (Besold *et al.*, 2009). Expression of US3 had little impact on pp65_{NLV} presentation. These data confirm that both gpUS2 and gpUS11 are highly effective individually in downregulating MHC I antigen presentation immediately after HCMV infection.

Omission of CX/AcD blocking of infected cells renders MHC I antigen presentation susceptible to gpUS3

To investigate whether the CX/AcD block had an impact on the effects imposed by individual evasion proteins, fibroblasts were infected with the mutant viruses without CX/AcD, and then co-incubated with the T-cell clones (Fig. 5c, d). In RV-US2- or RV-US11-infected cells, presentation of the IE1_{VLE} epitope was still fully suppressed under these relaxed conditions, as observed above for stringent IE conditions, and presentation of the pp65_{NLV} peptide was now suppressed more efficiently than under IE conditions.

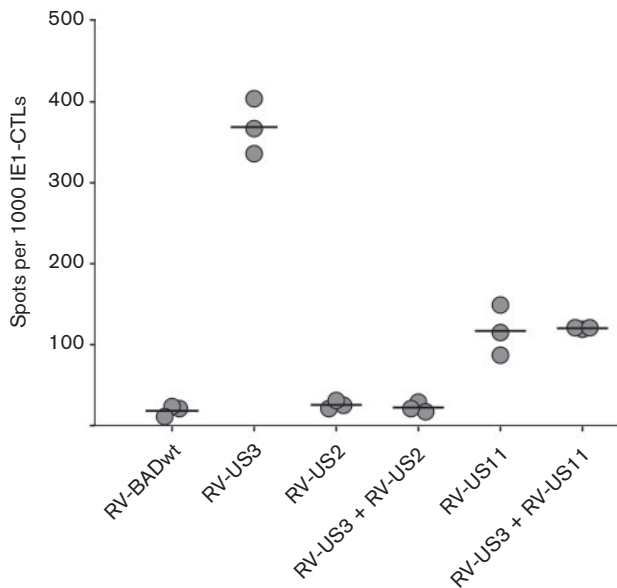


Fig. 4. Analysis of gpUS2- and gpUS11-mediated impact on MHC I antigen presentation at IE times of HCMV infection. ELISpot analysis of HFFs infected with the indicated viruses was performed using IE1_{TMV}-CTLs as responder cells. HFFs were infected and tested as described in the legend to Fig. 2 (a, right panel). Co-infections were carried out using the same infectious dose for each virus as was applied in the single infections. Shown are the results of triplicate wells (●); mean values are given as horizontal lines. The difference between RV-BADwt and RV-US3 was reproduced in four independent experiments. The comparability of RV-BADwt, RV-US2 and RV-US2+RV-US3 was reproduced in two independent experiments.

Surprisingly, gpUS3 expression led to a distinct suppression of the presentation of both peptides in unblocked cells, although the degree of suppression mediated by wt, RV-US2 or RV-US11 was not reached. Taken together, gpUS3 was effective at suppressing HLA-A2-restricted antigen presentation in cells that proceeded into the early phase of HCMV infection, but not in cells that were stalled in the IE phase. In contrast, gpUS2 and gpUS11 appeared to be major suppressors of presentation in the IE phase.

DISCUSSION

The IE phase of HCMV lytic infection is particularly critical with respect to CTL recognition and killing. At this time, structural proteins, such as pp65, delivered with virus particles feed rapidly into the MHC I presentation pathway (Besold & Plachter, 2008; McLaughlin-Taylor *et al.*, 1994). The same may be true for other structural proteins that are prominent targets of CD8⁺ T-cell memory responses (Boppana & Britt, 1996; Elkington *et al.*, 2003; Sylwester *et al.*, 2005). Another Achilles heel of HCMV is the requirement for high-level expression of IE proteins that

are also highly immunogenic (Borysiewicz *et al.*, 1988; Kern *et al.*, 1999; Manley *et al.*, 2004; Sylwester *et al.*, 2005). Peptides from IE1, for example, are already presented to CD8⁺ T-cells at 6 h p.i. (Besold & Plachter, 2008), but may become visible even earlier with IE peptides with higher MHC I affinity. This is a dilemma for the virus, as IE proteins are essential for the initiation of lytic infection, but may mediate killing by CTLs when presented by MHC I. Interestingly, however, infection with a US2–11-competent virus completely abrogated IE1_{TMV} presentation from 1 to 96 h p.i. (Besold & Plachter, 2008). Here we show that immunoevasion is similarly efficient under stringent IE conditions: presentation of different IE1 epitopes is suppressed strongly after infection with US2–11-competent strains, but not after infection with US2–11-deleted virus (Figs 2a, 5a, b). This indicated that one or more functions encoded in the US2–11 region suppressed MHC I presentation efficiently under IE conditions. The most likely candidate to mediate this effect was US3, which had been identified as an IE gene (Greijer *et al.*, 2001; Liu *et al.*, 2002; Weston, 1988). A 22 kDa isoform of gpUS3, encoded by an unspliced transcript, causes MHC I retention in the ER (Ahn *et al.*, 1996; Jones *et al.*, 1996; Lee *et al.*, 2000; Park *et al.*, 2004), whereas a 17 kDa isoform, encoded by a singly spliced transcript, has a dominant-negative effect when expressed together with the full-length protein (Liu *et al.*, 2009; Shin *et al.*, 2006). Unexpectedly, we found that expression of US3 in the absence of US2, US6 and US11 did not diminish the presentation of IE1 epitopes, and interfered only modestly with the presentation of a pp65 epitope. These functional data were concordant with the observation that MHC class I surface expression was not decreased on U373-MG cells stably expressing gpUS3 (Noriega *et al.*, 2012b; Noriega & Tortorella, 2009). Our Northern blot analyses showed abundant expression of the US3 1 kb RNA, probably corresponding to unspliced US3 mRNA (Fig. 2b). A band around 0.7 kb, corresponding to singly spliced US3 RNA, was also present, although with lower intensity. These expression data are in agreement with the results of Liu *et al.* (2002), who detected unspliced and singly spliced US3 RNA after 1 and 2 h of HCMV infection, respectively. Thus, the dominant-negative 17 kDa form of gpUS3 might have competed with the 22 kDa gpUS3 after infection under IE conditions, and prevented interference with CTL recognition.

However, such a dominant-negative effect cannot fully explain our paradoxical observation that the presentation of IE1 epitopes was modestly but consistently increased compared with US2–11-defective virus strains. This effect was specific for IE1 epitopes and was only seen under IE conditions. The underlying mechanism remains unknown at present, but some conjectures can be offered. When other HCMV immunoevasive proteins are absent, degradation of MHC I is not increased in the presence of gpUS3. MHC I molecules bound to gpUS3 in the ER can still acquire peptide (Ahn *et al.*, 1996), and they can still be

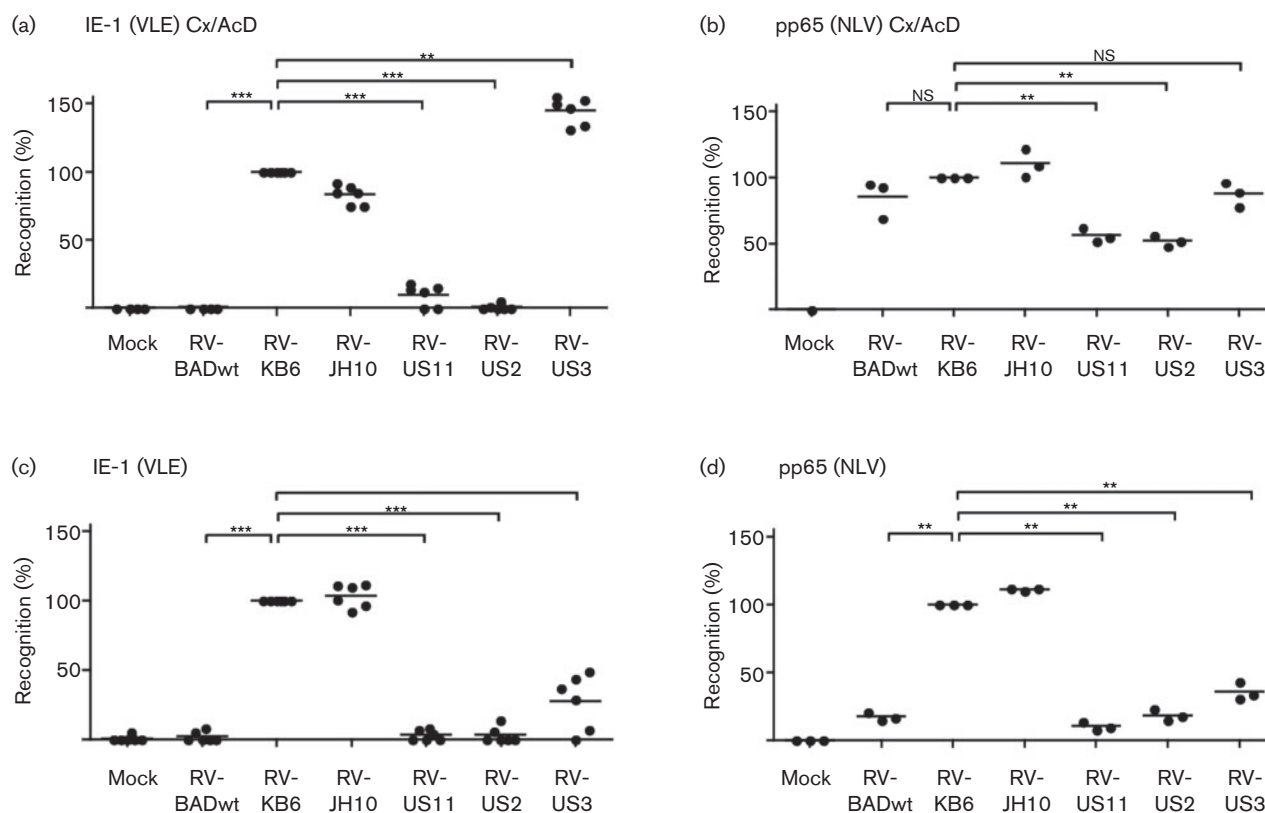


Fig. 5. Analysis of gpUS2-, gpUS3- and gpUS11-mediated impact on MHC I antigen presentation at IE times of HCMV infection, using donor-derived T-cell clones. Six CD8⁺ T-cell clones of four different donors against the HLA-A*0201-restricted peptide VLEETSVML (VLE) from IE1, and three CD8⁺ T-cell clones of two different donors against the HLA-A*0201-restricted peptide NLVPMVATV (NLV) from the tegument protein pp65 were tested by IFN- γ ELISA for their activation by co-cultivation with infected MRC-5 lung fibroblasts. Cells were infected with the indicated viruses under CX/AcD blocking conditions (a, b) or without block (c, d). Cells were then fixed with paraformaldehyde and used as stimulator cells. The IFN- γ release of cells infected with the US2/US3/US6/US11-negative virus RV-KB6 (Fig. 1b) was taken as 100%. One representative experiment of three (CX/AcD blocking) or two (without blocking) independent experiments is shown. Each single data point represents the mean of two or three replicates for a single T-cell clone; different data points for one condition represent different T-cell clones. Horizontal bars indicate the means of these data points. A one-sample *t*-test was used to compare other experimental conditions with the RV-KB6 condition; its results are represented as follows: NS, $P \geq 0.05$; * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0001$.

exported to the cell surface, although more slowly (Jones *et al.*, 1996). Therefore, the massively increased retention times of MHC I in the ER that are likely to result from the high levels of gpUS3 expressed under IE conditions could ultimately favour the loading of empty MHC chains with IE1 peptides, or an exchange of self-peptides by IE1 peptides. The specificity of this effect for IE1-derived but not pp65-derived peptides could indicate that efficient loading of these peptides depends differentially on the peptide-loading complex, whose function is only partially impaired by gpUS3 (Park *et al.*, 2004), or is due to different affinities of the IE1 and pp65 peptides for HLA-A2. Furthermore, HLA-A2 is not among the allotypes that are predicted to interact most strongly with tapasin (Park *et al.*, 2003) and, by implication, with gpUS3 (Park *et al.*, 2004), in contrast to other HLA allotypes expressed in the cells

investigated here (e.g. HLA-B7 and HLA-B44 in MRC-5 fibroblasts). A complete block of export of these other allotypes to the cell surface, occurring under IE conditions with high gpUS3 expression, could therefore release compensatory mechanisms that facilitate, in absolute or relative terms, the export of HLA-A2 peptide complexes to the cell surface and their recognition by T-cells.

Interestingly, the evasive effect of gpUS3 on MHC I presentation was much more pronounced in the absence of stringent IE-restricting conditions (Fig. 5c, d), confirming that HLA-A2 was, in principle, sensitive to gpUS3. This result suggests that a potential competing effect of the 17 kDa gpUS3 is not dominant after the IE phase of infection. Recent evidence shows that gpUS3 may serve as a molecular switch that regulates the impact of gpUS2 and

gpUS11 on MHC class I levels (Noriega *et al.*, 2012b; Noriega & Tortorella, 2009). It remains to be determined how the two isoforms of gpUS3 interact with gpUS2 and gpUS11 in terms of functional immunoevasion.

According to the original report, US2 transcription starts as early as 3 h p.i. (Jones & Sun, 1997). It was not analysed whether expression was independent of *de novo* synthesis of viral proteins in infected cells, thereby meeting the definition of an IE gene. The US11 gene was assigned to the early class of transcripts (Gretch *et al.*, 1988; Jones & Muzithras, 1991). Our results indicate that both US2 and US11 promoters are transcriptionally active without requiring *de novo* synthesis of viral proteins, thus classifying US2 and US11 as IE genes. However, it is known that CX may induce a cellular environment, e.g. by inducing NF- κ B, that may favour or enhance transcription from promoters containing responsive elements. Detailed analyses are required to formally prove that the US2 and US11 promoters are independent of viral *de novo* protein synthesis.

Jochum *et al.* (2012) recently showed that functional mRNA of Epstein–Barr virus (EBV) immunoevasin BNLF2a is contained in EBV virions and mediates protection of infected cells from recognition by EBV-specific CTLs. HCMV virions also include viral mRNAs (Bresnahan & Shenk, 2000; Greijer *et al.*, 2000) and, consequently, a likewise protection of HCMV-infected cells by virion-associated immunoevasin mRNA seems to be conceivable. However, the presence of US2-specific RNAs in HCMV virions has not been reported and conflicting data exist in this respect for US3- and US11-specific RNAs (Bresnahan & Shenk, 2000; Greijer *et al.*, 2000). It remains to be tested whether a part of the functional immunoevasive activity in the IE phase is due to virion-associated mRNAs.

In any case, our functional data show that both gpUS2 and gpUS11 effect their immunoevasive functions very early during the lytic replication cycle of HCMV and thus may be key determinants of the initial MHC class I evasion.

In contrast to IE1, presentation of pp65_{NLV} by cells infected with RV-US2 or RV-US11 was more moderately impaired under IE conditions. The matrix protein pp65 is known to be efficiently introduced into MHC I presentation from incoming particles immediately after infection (Besold *et al.*, 2007; McLaughlin-Taylor *et al.*, 1994). In the first hours of CX-mediated translation block, gpUS2 and gpUS11 are unavailable for interference, while pp65 is processed proteolytically and pp65 peptides are loaded onto MHC I. It was thus expected that presentation of pp65 could only be partially blocked under IE conditions. The nearly complete suppression without a block argues for an early downregulation of pp65-derived peptides mediated by gpUS2 and gpUS11 in infected cells.

The role of gpUS2–11 for the pathogenesis of HCMV is unclear. Because of the species specificity of HCMV, no functional data on the pathophysiological role of HCMV gpUS2–11 are available. In the rhesus monkey CMV, the

US2–11 gene region is conserved and was shown to be important for re-infection, but not for primary infection; nevertheless, this model established that US2–11 interfere with control by CTLs *in vivo* (Hansen *et al.*, 2010). The murine CMV (MCMV) encodes three proteins that impact MHC I surface expression (reviewed by Lemmermann *et al.*, 2011). Infection of BALB/c mice with an MCMV immunoevasion gene deletion mutant resulted in a more rapid control compared with the wt virus (Böhm *et al.*, 2008). This delay in control may result in more efficient horizontal transmission of the virus (Lemmermann *et al.*, 2011). In addition, the MCMV-null mutant established latency less efficiently, leading to a lower load of latent genomes (Böhm *et al.*, 2009). Thus, initial replication of CMVs may be supported by MHC I evasion genes, allowing a higher level of spread to salivary glands for horizontal transmission and to secondary sites where latency is established. Transferring that to the situation in HCMV, such an effect could only be achieved if infected cells are protected early, because of the presence of strongly antigenic targets already at IE times. In this respect, the effective MHC I suppression mediated by gpUS2 and gpUS11 may be pivotal for efficient establishment of latency and for spread of HCMV through salivary glands.

METHODS

Bacterial artificial chromosome (BAC) mutagenesis. The generation of RV-KB6, RV-US2 (RV-KB13), RV-US11 (RV-KB9) and RV-US3 (RV-KB7) has been described elsewhere (Besold *et al.*, 2009; Noriega *et al.*, 2012b). Mutant RV-JH10 was generated by BAC mutagenesis of the HCMV BAC pAD/Cre (Yu *et al.*, 2002), using Red recombination in *Escherichia coli* strain EL250 as described by Lee *et al.* (2001). The entire genomic region encoding the US2–11 genes was deleted by inserting a kanamycin-resistance gene, which was amplified from a derivative of vector pCP15 (Cherepanov & Wackernagel, 1995) using primers with 48–51 bp identity to the nucleotide sequence directly adjacent to the deletion (JH5 fwd: 5'-CTTACAGCTTTTG-AGTCTAGACAGGGTAACAGCCTTCCCTTGTAAGACAGAAGAGC-GCTTTTGAAGCTGGG-3', JH5 rev: 5'-GGGTACTCGTGGCTAGATTATTGAAATAAACCGCGATCCCGGGCGTCGGAATAGGAAC-TCAAGATCCCC-3').

Cells and viruses. Primary human foreskin fibroblasts (HFFs; HLA-A2, A3, B51) and the murine IE1_{TMV}-CTL line were cultured as described previously (Besold *et al.*, 2007). Human CTL clones specific for IE1_{VLE} or pp65_{NLV} were generated by limiting dilution of IE1- or pp65-specific polyclonal T-cell lines (Schub *et al.*, 2009). Polyclonal T-cell lines were prepared from PBMCs by stimulation with mini-LCLs (mini EBV-transformed B-cell lines) expressing pp65 or IE1 (Wiesner *et al.*, 2005). Reconstitution of the wt strain RV-BADwt and the viral mutants, as well as the generation of viral stocks, were performed as described before (Besold *et al.*, 2009). Virus stock titration was performed by quantifying intracellular viral genomes as described previously (Besold *et al.*, 2009) or by counting IE1-positive cells, stained with a mAb against IE1 [p63-27 (Andreoni *et al.*, 1989)] at 48 h p.i. M.o.i. was defined as the number of IE1-positive cells. For gamma interferon (IFN- γ) ELISpot assays or ELISAs, HFFs or MRC-5 lung fibroblasts (HLA-A2, A29, B7, B44) were infected at an m.o.i. of 5. For co-infection experiments, an m.o.i. of 5 was used for each virus. For experiments requiring restriction of gene expression to IE genes in infected cells, CX (250 μ g ml⁻¹) was added 1 h prior to infection

and was maintained until it was replaced 9 h p.i. by AcD ($10 \mu\text{g ml}^{-1}$), which was present for another 13.5 h. After that, cells were fixed using PBS/0.5% (w/v) paraformaldehyde and subjected to ELISpot or ELISA analysis.

IFN- γ ELISpot assay and IFN- γ ELISA. ELISpot assays were performed according to published procedures (Frankenberg *et al.*, 2002; Miyahira *et al.*, 1995). As stimulator cells, either mock- or HCMV-infected HFFs were used, fixed with PBS/0.5% (w/v) paraformaldehyde. Lymphocytes from an IE1_{TMY}-CTL clone, obtained from HLA-A2 transgenic mice, served as responder cells (Besold *et al.*, 2007). For each sample, 5×10^4 antigen-presenting cells were probed with 1000 responder cells. Triplicate samples were analysed. Counting of spots was carried out using an SZX-12 microscope (Olympus). Reactivity of human CTL clones specific for IE1_{VLE} or pp65_{NLV} was tested in IFN- γ ELISA assays (Mabtech). T-cells (4×10^4) and infected MRC-5 fibroblasts (2×10^4) were co-incubated overnight in V-bottom 96-well plates in 200 μl medium. Triplicate (IE1_{VLE}-specific T-cells) or duplicate (pp65_{NLV}-specific T-cells) samples were analysed.

Northern blot analysis. For RNA preparation, cells were infected with an m.o.i. of 5 or with viral culture supernatants, normalized on an equivalent uptake of 125 genomes per cell. The RNA was isolated using an RNeasy mini kit according to the manufacturer's instructions (Qiagen). Northern blot analyses were performed according to the manufacturer's manual (Roche Diagnostics). For electrophoresis, 1–10 μg RNA was loaded on a 1.0–1.3% (w/v) agarose gel, supplemented with 0.6% (w/v) formaldehyde. RNA was transferred to nylon membranes (Roche Diagnostics) and probed with DIG-11-dUTP-labelled DNA probes specific for US2, US3, US6, US11, UL82 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The generation of US2 and US11 probes was described previously (Besold *et al.*, 2009). US3, US6, UL82 and GAPDH probes were generated analogously, using the primers KB30 (5'-TAAATCGCAGACGGGC-GCTCAC-3') and KB31 (5'-GCCGTCTTGTTCCTGAGACTCG-3') for US3 probes, KB32 (5'-CAGGAGCCACAACGTCGAATC-3') and KB33 (5'-CTCTGTCTCCGCGACAACAG-3') for US6 probes, JH7 fwd (5'-GTGAGTCGTGATGCAGAAAC-3') and JH7 rev (5'-CGTAGACCTGACCGACTTAAAC-3') for UL82 probes, and SB46 (5'-TGAGTCTACTGGTGTCTTC-3') and SB47 (5'-GCTTCACCACCT-TCTTGATG-3') for GAPDH probes.

Immunoblot analysis. Immunoblot analyses were performed using the Odyssey system (LI-COR Biosciences) as described previously (Besold *et al.*, 2009), with a few modifications. HFFs were infected at an m.o.i. of 5. For SDS gel electrophoresis, 10% (w/v) polyacrylamide gels, containing 0.1% (w/v) SDS, were used. For blotting, a methanol-reduced transfer buffer [25 mM Tris(hydroxymethyl)aminomethane/192 mM glycine/10% (v/v) methanol] was employed. After blotting, membranes were air-dried for 1 h and reactivated by rinsing in methanol, H₂O and PBS. Membrane blocking was carried out with PBS/5% (w/v) milk powder. Primary antibodies were diluted in PBS/5% (w/v) milk powder/0.1% (v/v) Tween. Rabbit polyclonal antibody directed against human β -actin (Rockland; distributed by Biotrend) was diluted 1:200, and mAb p63-27 against HCMV IE1 (Andreoni *et al.*, 1989) was diluted 1:250. For detection of primary antibody binding, labelled secondary antibodies were used [IRDye 800-conjugated goat anti-mouse IgG (H+L), Rockland; distributed by Biotrend; Alexa Fluor 680-conjugated goat anti-rabbit IgG (H+L), Invitrogen]. Membranes were incubated with secondary antibodies, diluted 1:5000 in PBS/0.1% (v/v) Tween/0.01% (w/v) SDS, in the dark for 2 h.

ACKNOWLEDGEMENTS

We are indebted to Thomas Shenk (Princeton University, Princeton, NJ, USA) for the donation of HCMV BACs, to Neal Copeland and

Chiang Lee (National Cancer Institute, Frederick, MD, USA) for bacterial strains, and to William Britt (Department of Pediatrics, School of Medicine, University of Alabama at Birmingham, Birmingham, AL, USA) for mAbs. This work was supported by grants from the Deutsche Forschungsgemeinschaft: SFB 490, individual project E7 (J.H., K.B. and B.P.), SFB-TR36, individual project A4 (A.M.), and the Clinical Research Group 183, individual project 8 (B.P.). Part of this research was conducted by J.H. in partial fulfilment of the requirements for a doctoral degree from the Johannes Gutenberg University, Mainz, Germany.

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