Exogenous introduction of an immunodominant peptide from the non-structural IE1 protein of human cytomegalovirus into the MHC class I presentation pathway by recombinant dense bodies

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Exogenous introduction of particle-associated proteins of human cytomegalovirus (HCMV) into the major histocompatibility complex (MHC) class I presentation pathway by subviral dense bodies (DB) is an effective way to sensitize cells against CD8 T-cell (CTL) recognition and killing. Consequently, these particles have been proposed as a platform for vaccine development. We have developed a strategy to refine the antigenic composition of DB. For proof of principle, an HCMV recombinant (RV-VM3) was generated that encoded the immunodominant CTL determinant IE1$_{TMY}$ from the IE1 protein in fusion with the major constituent of DB, the tegument protein pp65. To generate RV-VM3, a bacterial artificial chromosome containing the HCMV genome was modified by applying positive/negative selection based on the expression of the bacterial galactokinase in conjunction with λ Red-mediated homologous recombination. This method allowed the efficient and seamless insertion of the DNA sequence encoding IE1$_{TMY}$ in frame into the pp65 open reading frame (UL83) of the viral genome. RV-VM3 expressed its fusion protein to high levels. The fusion protein was packaged into DB and into virions. Its delivery into fibroblasts by these viral particles led to the loading of the MHC class I presentation pathway with IE1$_{TMY}$ and to efficient killing by specific CTLs. This demonstrated that a heterologous peptide, not naturally present in HCMV particles, can be processed from a recombinant, DB-derived protein to be subsequently presented by MHC class I. The results presented here provide a rationale for the optimization of a vaccine based on recombinant DB.

INTRODUCTION

Infection with human cytomegalovirus (HCMV; family *Herpesviridae*, subfamily *Betaherpesvirinae*) may cause significant morbidity and mortality in individuals with immature or compromised immune-defence functions, but proceeds asymptomatically in most healthy adults (Pass, 2001). Prevention of HCMV infection or disease by a vaccine has been ranked as a high-priority goal (Stratton *et al.*, 2001). Although several vaccine approaches have been developed, none have yet entered routine clinical practice (Pepperl-Klindworth & Plachter, 2006; Plotkin, 2004; Schleiss & Heineman, 2005; Zhong & Khanna, 2007). We have demonstrated that HCMV dense bodies (DB)

provide a promising basis for vaccine development (Pepperl *et al.*, 2000; Pepperl-Klindworth *et al.*, 2002).

DB are enveloped, subviral particles devoid of capsids and viral DNA. They are released from infected fibroblast cultures and enter cells presumably via the normal HCMV entry processes. The major constituent of DB is pp65 (ppUL83) (Varnum *et al.*, 2004), which is a prominent target of the CD4 and CD8 T-cell (CTL) responses following natural infection (Beninga *et al.*, 1995; McLaughlin-Taylor *et al.*, 1994; Wills *et al.*, 1996). Although pp65 by itself appears to be an attractive antigen for the design of a vaccine, other HCMV proteins may also be important and should thus be considered (Reddehase, 2002). One of these, as far as the CTL response is concerned, is the regulatory immediate-early 1 protein (IE1) (Borysiewicz *et al.*, 1988; Bunde *et al.*, 2005; Kern *et al.*, 1999; Lacey *et al.*, 2006; Pahl-Seibert *et al.*, 2005; Simon *et al.*, 2006). However, IE1 is not contained in viral

Supplementary methods describing HCMV BAC recombineering using *galK* positive/negative selection are available with the online version of this paper.

particles. Previously, we showed that a fusion protein consisting of pp65, green fluorescent protein and neomycin phosphotransferase was packaged into virions and DB during infection with an HCMV recombinant (Pepperl-Klindworth *et al.*, 2003). However, the efficacy of this approach was limited. More importantly, it remained unclear whether peptides from the recombinant protein were presented in an immunologically relevant way by DB-treated cells.

Here, we provide proof of principle that recombinant DB (recDB) can be designed to mediate the loading of heterologous antigenic peptides into the major histocompatibility complex (MHC) class I presentation pathway. As proof of principle, a DNA sequence encoding an immunodominant determinant of the IE1 protein (Gallez-Hawkins *et al.*, 2003) was inserted into the UL83 open reading frame (ORF) by using an HCMV bacterial artifical chromosome (BAC) clone. This was achieved by using λ Red-mediated recombination in conjunction with positive/negative selection based on the bacterial galacto-kinase (*galK*) gene (Warming *et al.*, 2005), a method adopted here for the first time to modify a herpesviral genome. The resulting recombinant virus, RV-VM3, encoded a pp65 fusion protein that was expressed with similar kinetics to wild-type (wt) pp65. The fusion protein was packaged into recombinant virions and DB, and these particles were shown to be immunologically active in sensitizing target cells for CTL recognition.

METHODS

Cells and viruses. Human foreskin fibroblasts (HFF) and CTL lines were grown as described previously (Besold *et al.*, 2007). For mutagenesis, the HCMV strain RV-HB5 was used as parental strain (Borst *et al.*, 1999). RV-HB5 carries the BAC vector inserted into the US2–US6 region of HCMV strain Ad169, thereby deleting these genes. For control purposes, the viruses RV-ΔUS2–11 (Falk *et al.*, 2002) and RVHd65 (Besold *et al.*, 2007) were employed. RV-ΔUS2–11 is deleted in the US2–US11 gene region and is based on RV-HB5. RVHd65 is deleted in the UL83 (pp65) gene region of Ad169-BAC, which is a US2–US6 positive revertant of RV-HB5 (Hobom *et al.*, 2000). Reconstitution of viruses from BAC clones was performed as described by Hobom *et al.* (2000). Viral supernatants were titrated by counting IE1-positive cells stained with a monoclonal antibody (mAb) against IE1 (p63-27; Andreoni *et al.*, 1989).

Plasmids. For generation of the recombinant BAC construct, plasmids p*galK* (Warming *et al.*, 2005) and pK65-1 were used. pK65-1 is a cloning vector based on pUC19 that contains nucleic acid sequences encoding the C-terminal part of pp65, the HLA-A2-presented peptide IE1$_{TMY}$ (IE1$_{297-305}$) (Gallez-Hawkins *et al.*, 2003) and a c-*myc* epitope tag. To ensure efficient proteasomal processing, the minimal nonapeptide sequence was supplemented with additional amino acids flanking IE1$_{TMY}$ (TSDACMM**TMYGGISLL**SEFC). The IE1-derived peptide sequence corresponds to the respective sequence of the HCMV Ad169 genome described by Chee *et al.* (1990). The DNA sequence from pK65-1, inserted into UL83 of pHB5 by recombineering (recombination-mediated genetic engineering), was the following: 5′-ACTAGTGACGCTTGTATGATGACCATGTACG-GGGGCATCTCTCTCTTAAGTGAGTTCTGTGAACAAAAACTCA-TCTCAGAAGAGGATCTGTG-3′.

BAC recombineering using *galK* positive/negative selection. Modification of HCMV BACs was performed according to the procedure of Warming *et al.* (2005). The procedure used is detailed in the Supplementary Methods, available in JGV Online.

DB purification. DB were purified from the supernatant of late stage-infected HFF by glycerol tartrate gradient ultracentrifugation as described by Irmiere & Gibson (1983).

Immunoblotting, immunofluorescence and antibodies. SDS-PAGE, immunoblotting and immunofluorescence analyses were carried out as described previously (Schmolke *et al.*, 1995). mAbs directed against pp65 (65-33), against glycoprotein B (gB) (27-287; Utz *et al.*, 1989) and against IE1 (p63-27; Plachter *et al.*, 1993) (all kindly provided by W. Britt, University of Alabama, Birmingham, AL, USA), and against the c-*myc* epitope (9E10; Covance) were used for detection. For determination of viral infectivity, the number of IE1-positive cells (IE1p72 fluorescence) after infection was quantified as described by Andreoni *et al.* (1989).

Electron microscopy. Six days after infection of 3×10^6 HFF with an m.o.i. of 2–3, cells were prefixed in a 2.5 % glutaraldehyde (Sigma-Aldrich), 0.1 M sucrose, 0.1 M cacodylate buffer (pH 7.3) (Roth) for 1 h at room temperature. After three washes for 10 min in 0.1 M cacodylate buffer, cells were post-fixed for 1 h in 1 ml 2 % OsO$_4$, 0.1 M sucrose and 0.1 M cacodylate buffer, dehydrated in a graded series of increasing concentrations of ethanol, incubated twice for 10 min each in propylene oxide (Serva) and embedded in 1 : 1 propylene oxide : araldite resin (Plano) solution. Ultrathin sections were cut with a Leica Ultracut S microtome and were counterstained with 2 % aqueous uranyl acetate (Sigma-Aldrich). Sections were analysed in a FEI Tecnai 12 BioTwin transmission electron microscope (Philips) and imaged with an SCCD SIS MegaView III camera (Soft Imaging System).

Gamma interferon (IFN-γ)-based ELISpot assay and cytotoxicity assay. ELISpot assays and cytotoxicity assays were performed as described previously (Besold *et al.*, 2007; Frankenberg *et al.*, 2002). CTL lines specific for the HLA-A0201 (A2)-restricted HCMV-derived peptides pp65$_{495-503}$ (pp65$_{NLV}$-CTL) (Diamond *et al.*, 1997; Wills *et al.*, 1996) and IE1$_{297-305}$ (IE1$_{TMY}$-CTL) (Gallez-Hawkins *et al.*, 2003) were used in these analyses. The CTL lines had been generated by immunizing HLA-A2/huCD8 double-transgenic (tg) mice (Besold *et al.*, 2007).

RESULTS

Modification of the HCMV genome by *galK*-mediated recombineering

galK-mediated recombineering was introduced as a versatile method to generate seamless mutations or insertions in mouse BACs (Warming *et al.*, 2005). We investigated whether this method was applicable to the precise modification of the large HCMV genome needed to generate recDB. For this, the BAC pHB5, containing the HCMV genome (Borst *et al.*, 1999), was transformed into the *galK*-negative *Escherichia coli* strain SW102. This strain allows temperature-induced λ Red-mediated recombination and *galK*-mediated positive/negative BAC selection (Warming *et al.*, 2005).

For insertion of the IE1$_{TMY}$–*myc* fusion peptide, a position close to the C terminus of pp65, adjacent to proline 548, was

chosen. In a first step, the *galK* gene was amplified from p*galK* and inserted into pHB5 by λ Red-mediated recombination (Fig. 1a). Correct insertion of *galK* in the resulting BAC-VM3-galK was verified by PCR amplification (data not shown), restriction-enzyme analysis and Southern blot hybridization (Fig. 1b, c). In a second step, a PCR fragment was generated that encoded the $IE1_{TMY}$–*myc* fusion peptide, flanked by 50 nt homologous to the UL83 ORF on each side of the insertion site of the *galK* gene. The fragment was recombined into BAC-VM3-galK (Fig. 1a) to obtain BAC-VM3. Supplementation of minimal medium plates, containing glycerol as carbon source, with 2-deoxygalactose (DOG) enabled the selection of BAC clones defective for *galK* expression. Ten DOG-resistant colonies were picked and tested by colony PCR. PCR fragments of a length corresponding to a correct insertion of the $IE1_{TMY}$–*myc* coding DNA were found in six of 10 clones tested. The correct insertion of the desired fragment was verified by restriction analysis, Southern blot hybridization (Fig. 1b, c) and DNA sequencing (data not shown). Subsequently, DNA of BAC-VM3 was transfected into HFF and recombinant virus RV-VM3 was reconstituted for further analysis. This showed that the positive/negative selection based on a single selectable marker gene, namely *galK*, in combination with λ Red-mediated recombination is an efficient method for rapid generation of seamless HCMV mutants.

Growth kinetics of RV-VM3

Previous work had shown that pp65 is dispensable for growth in cell culture (Schmolke *et al.*, 1995). Consequently, growth kinetics similar to those of the parental strain were expected for RV-VM3. To test this, multiple-cycle growth curves of RV-HB5 and RV-VM3 were determined by a quantitative real-time PCR approach, using TaqMan technology (TaqMan ABI Prism 7700 Sequence Detector; Applied Biosystems). The amount of viral DNA in cell-culture supernatants was taken as an equivalent for viral particles released from infected cells. HFF were infected at an m.o.i. of 0.1, as determined by IE1p72 fluorescence. DNA was extracted from culture supernatants at different times post-infection (p.i.) and TaqMan analysis was carried out in triplicate. The growth kinetics of RV-VM3 resembled those of the parental strain RV-HB5 (Fig. 2), although HFF cultures infected with RV-VM3 appeared to contain slightly increased levels of viral DNA compared with RV-HB5-infected cultures.

Expression level and packaging of the pp65–IE1$_{TMY}$ fusion protein

To test the level of expression of the pp65–IE1$_{TMY}$ fusion protein in HCMV RV-VM3-infected cells, immunoblotting analyses were performed. Cell lysates from HFF infected with either RV-VM3 or the parental strain RV-HB5 were obtained at 4 days p.i. To be able to compare the levels of fusion protein expression from the viruses, the amount of
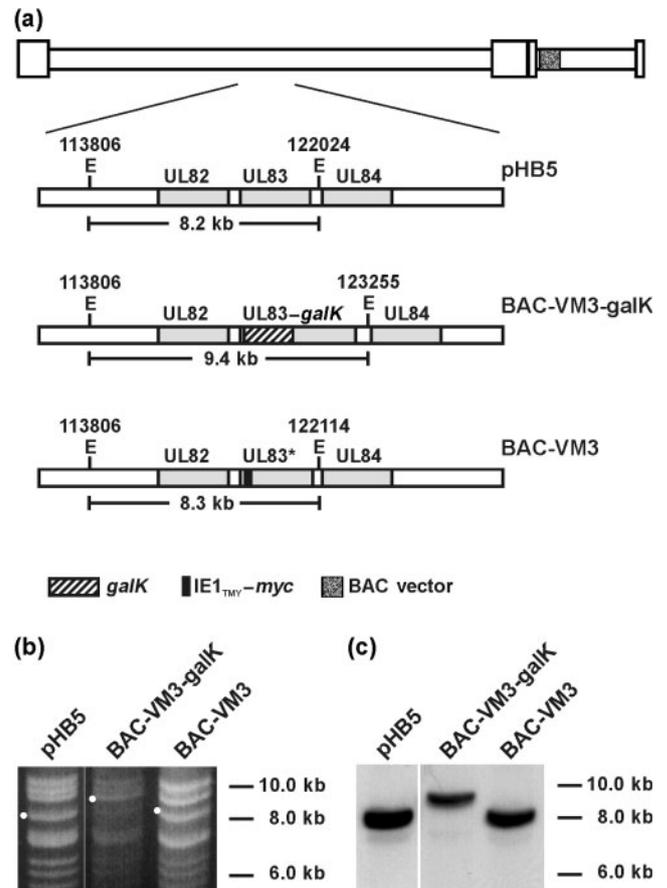
Fig. 1. Genetic modification of HCMV BACs by using *galK* selection. (a) Structure of the parental HCMV BAC pHB5, the intermediate construct BAC-VM3-galK and the final construct BAC-VM3. The HCMV genome is depicted above, with the location of the BAC vector indicated by a shaded box. Sites for cleavage with *Eco*NI (E) are indicated, together with their relative position on the DNA sequence of the HCMV Ad169 genome (Chee *et al.*, 1990). The bars between the respective *Eco*NI sites, as shown below each BAC clone, represent the sizes of the fragments expected in restriction analysis and Southern blotting. UL83*, ORF UL83 containing the IE1–*myc* sequence. (b) Agarose gel analysis of the different BAC clones. The DNA was cleaved with *Eco*NI and submitted to 0.6 % agarose gel electrophoresis. The restriction fragments containing the coding sequences for pp65, pp65–GalK or pp65–IE1$_{TMY}$ fusion proteins are indicated by white dots. (c) Southern blotting analysis of the DNA from the different BAC clones, cleaved with *Eco*NI and transferred to nylon membranes. The DNA was probed with a 500 bp, digoxigenin-labelled PCR fragment from UL83.

gB (mAb 27-287) was taken as an internal standard for viral protein expression. The fusion protein was labelled with the pp65-specific antibody 65-33 or an antibody directed against the *myc* tag (9E10). Both antibodies showed comparable binding patterns (Fig. 3a). The amount of pp65–IE1$_{TMY}$ detectable in RV-VM3-infected
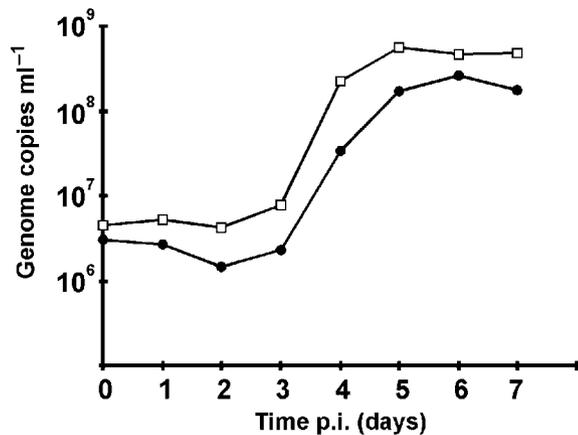
Fig. 2. Quantitative PCR analysis of DNA from virions released into the supernatant of infected cells. HFF were infected at an m.o.i. of 0.1 with RV-HB5 (●) or RV-VM3 (□). Aliquots of cell-culture supernatants were removed at the indicated time points p.i. Viral DNA of each aliquot was extracted and subjected to a TaqMan quantitative PCR analysis. PCR was performed on triplicate samples.

cells was similar to the amount of pp65 detectable in cells infected with RV-HB5, based on normalization with gB.

To analyse DB formation in RV-VM3-infected cells, ultrastructural analysis was performed. HFF were infected at an m.o.i. of approximately 2–3 with RV-HB5 or RV-VM3 and were prepared for electron microscopy at 6 days p.i. Cells infected with RV-HB5 showed the typical formation of cytoplasmic DB, as reported previously for HCMV (Craighead et al., 1972; Severi et al., 1988; Topilko & Michelson, 1994). These electron-dense, enveloped particles varied in size, measuring two to three times the diameter of enveloped virions (Fig. 3b, upper panel). Fibroblasts infected with RV-VM3 showed the formation of DB in the cytoplasm, similar to wt DB (Fig. 3b, lower panel). As seen in RV-HB5-infected cells, multivesicular structures delineated by membranes, containing virions and DB components, were found occasionally. However, about half of the cells infected with RV-VM3 displayed the formation of large, meshwork-like structures in the cytoplasm. These structures were apparently not associated with larger numbers of virus particles and were not seen after infection with RV-HB5 (data not shown). Their nature is unclear at this point and their characterization warrants further investigation. Nevertheless, these analyses showed that formation of cytoplasmic DB can proceed despite the insertion of the IE1–myc sequence in RV-VM3.

To test whether recDB were released from RV-VM3-infected fibroblasts, cell-culture supernatants were collected at 7 days p.i. and subjected to glycerol tartrate
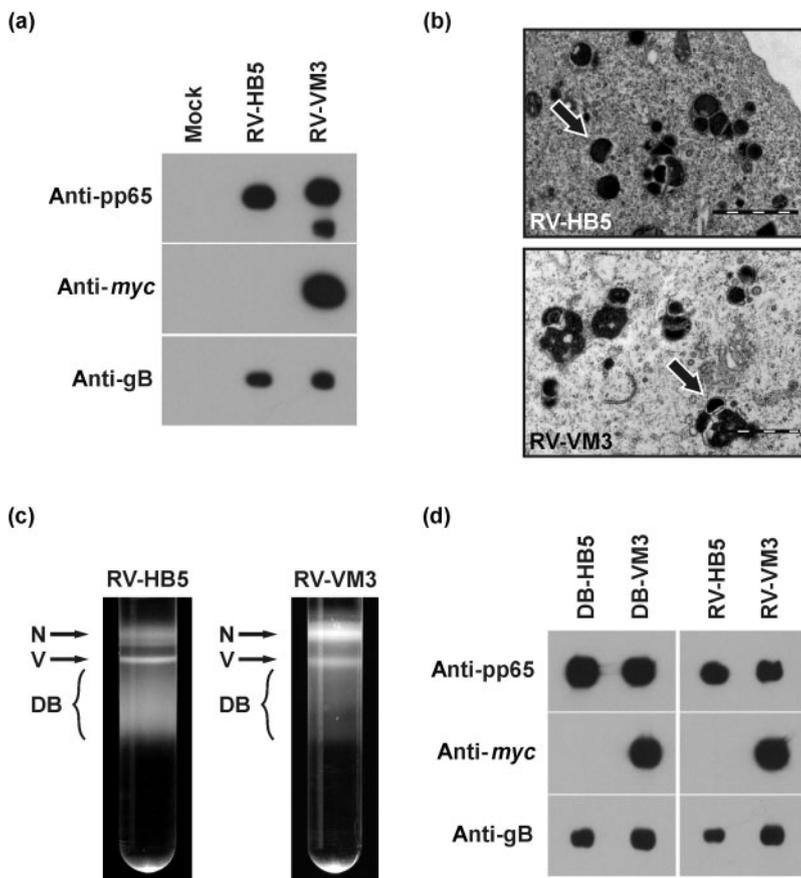
Fig. 3. Analysis of fusion-protein synthesis and packaging into recDB. (a) Immunoblotting analysis of pp65 or the pp65–IE1$_{TMY}$ fusion protein in infected HFF. Cell lysates from RV-HB5- and RV-VM3-infected cells were collected at 4 days p.i. and analysed by immunoblotting. The amount of protein loaded on each lane was normalized against HCMV gB. (b) Electron microscopic analysis of HFF infected with RV-HB5 or RV-VM3. Cells were infected for 6 days with RV-HB5 or RV-VM3, fixed and processed further for transmission electron microscopy. Arrows indicate cytoplasmic DB. Bars, 1 μm. (c) Separation of viral particle fractions released from infected HFF after 7 days by gradient centrifugation. Positions of the bands containing non-infectious enveloped particles (N), virions (V) and DB are indicated next to the gradients. (d) Immunoblotting analysis of the DB and virion fractions using antibodies against pp65 (65-33) and against the myc tag (9E10). The amount of protein loaded on each lane was normalized against gB (mAb 27-287).

gradient ultracentrifugation. Gradients obtained from culture supernatants of RV-HB5-infected cells, used as a control, revealed the typical banding pattern of non-infectious enveloped particles, infectious virions and DB (Irmiere & Gibson, 1983; Pepperl et al., 2000) (Fig. 3c). A similar pattern was observed with culture supernatant from RV-VM3-infected cells; however, lower numbers of DB were detected compared with RV-HB5. To analyse the packaging of the fusion protein, the virion and DB fractions were collected and subjected to immunoblotting analysis. The amount of protein loaded onto the poly-acrylamide gel was determined with antibodies against pp65 (65-33) and against the myc tag (9E10) and was normalized with gB (mAb 27-287), which is contained in all three types of particle. Cells infected with RV-VM3 packaged the pp65–IE1$_{TMY}$ fusion protein into both virions and DB, with no significant difference from cells infected with the parental strain RV-HB5 (Fig. 3d). Taken together, these results indicated that recDB were formed in and released by RV-VM3-infected cells and that the content of pp65–IE1$_{TMY}$ and pp65 in DB from RV-VM3 and RV-HB5, respectively, was comparable, relative to the amount of gB taken as standard.

Analysis of the subcellular localization of the pp65–IE1$_{TMY}$ fusion protein

To analyse whether the pp65–IE1$_{TMY}$ fusion protein expressed from RV-VM3 followed the same pattern of subcellular localization as wt pp65 at various stages of infection, immunofluorescence staining and subsequent laser-scanning microscopy were performed. After infection with the parental strain RV-HB5, pp65 was located mainly in the cell nucleus at 1 and 2 days p.i. Cytoplasmic staining became apparent at 3 days p.i. and increased at 4 and 5 days p.i. However, some pp65 still appeared to be retained in the nucleus at these late stages of infection (Fig. 4). In RV-VM3-infected cells, the pp65–IE1$_{TMY}$ fusion protein was largely nuclear at 1 and 2 days p.i. The shift to the cytoplasm became visible at 3 and 4 days p.i. Surprisingly, in contrast to the wt situation, pp65 staining appeared to be completely cytoplasmic at 5 days p.i. Signal intensity was comparable for the two viruses at each time point tested. Cells infected with the pp65 deletion mutant RVHd65 (Besold et al., 2007), used as a negative control, showed no specific staining. These experiments demonstrated that the kinetics of intracellular transport of the pp65–IE1$_{TMY}$ fusion protein in RV-VM3-infected cells resembled those of wt pp65. However, differences became evident in the extent to which pp65 or the pp65–IE1$_{TMY}$ fusion protein was exported from the nucleus at late stages of viral infection.

MHC class I presentation of the IE1$_{TMY}$ peptide derived from viral particles

Previous studies provided evidence that peptides derived from exogenously delivered, particle-associated HCMV proteins are presented by MHC class I molecules without
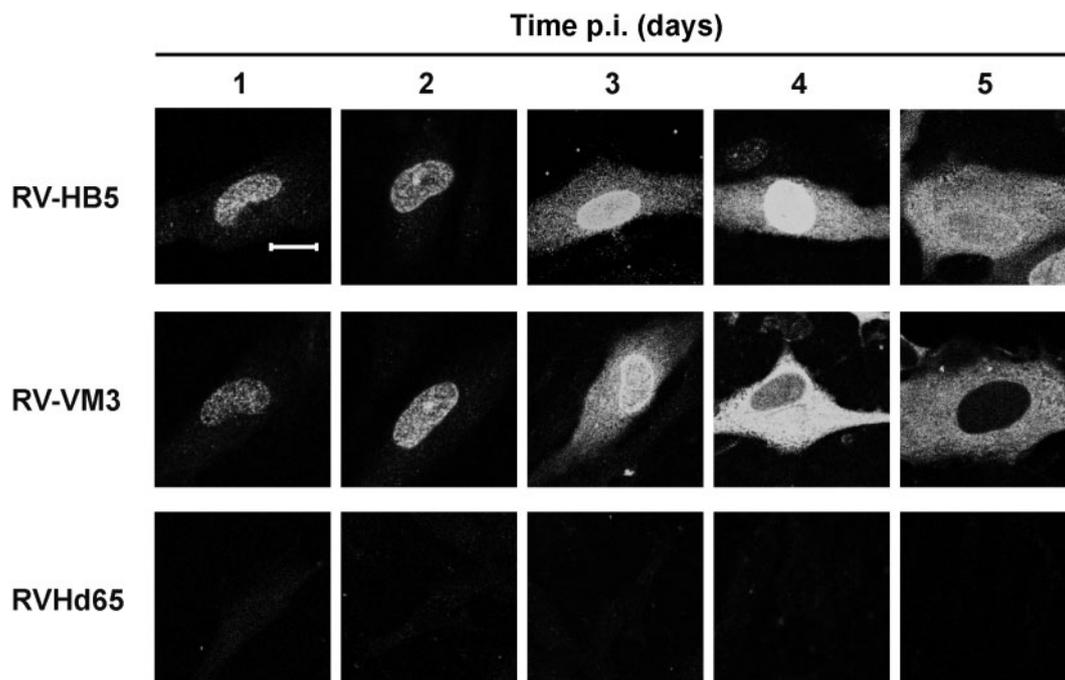
Fig. 4. Laser-scanning immunofluorescence analysis of the subcellular distribution of the pp65–IE1$_{TMY}$ fusion protein in infected HFF. Cells were infected at an m.o.i. of 2 with RV-HB5 or RV-VM3 and at an m.o.i. of 3 with RVHd65. Cells were fixed at the indicated time points and stained with mAb 65-33, directed against pp65. Bar, 10 μm.

requiring *de novo* protein synthesis (Besold *et al.*, 2007; Manley *et al.*, 2004; McLaughlin-Taylor *et al.*, 1994; Pepperl *et al.*, 2000; Reddehase *et al.*, 1984; Riddell *et al.*, 1991). It was previously unknown whether peptides not usually included in viral particles would also be introduced into MHC class I presentation.

To test this, cells were infected for 24 h with the HCMV strains RV-HB5 and RV-VM3 (Fig. 5a). Cells were then subjected to IFN-γ ELISpot analysis using pp65$_{495–503}$-specific CTL (pp65$_{NLV}$-CTL) and IE1$_{297–305}$-specific CTL (IE1$_{TMY}$-CTL) as responder cells (Besold *et al.*, 2007). As a control for the ability of the two CTL clones to respond to antigen presentation, cells were also infected with RV-ΔUS2–11, a variant that lacks the genomic region encoding the HCMV immune-evasion proteins (Falk *et al.*, 2002).

Cells infected with RV-HB5 stimulated pp65$_{NLV}$-CTL to secrete IFN-γ, but failed to stimulate IE1$_{TMY}$-CTL. As immune-evasion proteins US2–US6 are deleted in

RV-HB5, these data indicated that gpUS11, the only remaining immunoevasin, was sufficient to suppress IE1$_{TMY}$ presentation, but failed to suppress pp65$_{NLV}$ presentation. In contrast, infection of HFF with RV-VM3 resulted in the stimulation of IFN-γ secretion from both IE1$_{TMY}$-CTL and pp65$_{NLV}$-CTL. Infection with the control virus RV-ΔUS2–11 stimulated IFN-γ secretion from both pp65$_{NLV}$-CTL and IE1$_{TMY}$-CTL.

As RV-VM3 differed from RV-HB5 only in the expression of the pp65–IE1$_{TMY}$ fusion protein, a likely interpretation of these results is that the peptide presented on RV-VM3-infected cells was derived from the pp65–IE1$_{TMY}$ fusion protein and hence was delivered through the particle-entry process rather than from *de novo*-synthesized wt IE1 protein.

To prove that particle-derived proteins can serve as a source for IE1$_{TMY}$ peptide processing and presentation, DB were prepared from culture supernatants of RV-ΔUS2–11-,
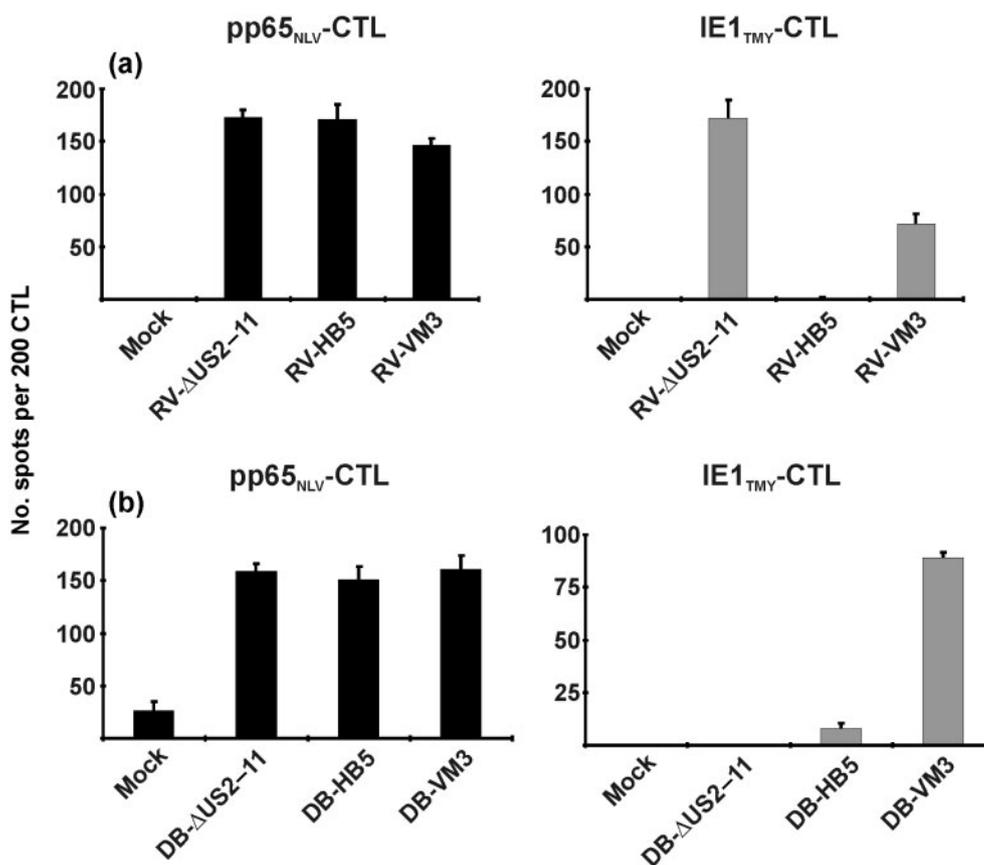
Fig. 5. IFN-γ ELISpot analysis of MHC class I presentation by HLA-A2[+] HFF infected with different viruses or treated with DB from different viral strains. (a) Cells were infected with the indicated viruses with an m.o.i. of 10. After 24 h incubation, they were used as stimulator cells in the ELISpot assay, using either pp65$_{NLV}$-CTL or IE1$_{TMY}$-CTL as responder cells. (b) Cells were incubated for 24 h with 10 μg UV-irradiated DB from RV-ΔUS2–11 (DB-ΔUS2-11), RV-HB5 (DB-HB5) or RV-VM3 (DB-VM3), and were subsequently used as stimulator cells (see above). Mean+SD values of triplicate measurements are represented.

RV-HB5- and RV-VM3-infected cells (DB-ΔUS2–11, DB-HB5 and DB-VM3). The purified DB, although non-infectious by virtue of their morphogenesis, were UV-irradiated to reduce residual infectivity from contaminating infectious virions. They were then incubated with HFF for the stimulation of CTL in IFN-γ ELISpot analyses. Using pp65$_{NLV}$-CTL as responders, comparable spot numbers became apparent for DB-HB5, DB-ΔUS2–11 and DB-VM3 (Fig. 5b). This demonstrated that the pp65$_{NLV}$ peptide was processed from particle-associated fusion protein and presented by HLA-A2, comparable to its processing from wt pp65. Consequently, the introduction of IE1$_{TMY}$ as a second HLA-A2-presented, immunodominant peptide did not influence processing and presentation of pp65$_{NLV}$. As expected from the absence of the IE1 protein in wt HCMV DB (Varnum *et al.*, 2004), HFF incubated with either DB-ΔUS2–11 or DB-HB5 failed to stimulate IE1$_{TMY}$-CTL in the ELISpot assay (Fig. 5b). Furthermore, the complete failure of DB-ΔUS2–11 to activate IE1$_{TMY}$-CTL, in contrast to infection with the respective virus, demonstrated the efficiency of the removal of residual infectivity from the DB preparations by UV irradiation [compare Fig. 5(a) and (b)]. Cells treated with DB-VM3 induced activation of IE1$_{TMY}$-CTL significantly, indicating efficient presentation of the cognate peptide present in DB-VM3.

To corroborate these findings further with an independent type of assay, target cells prepared with DB-VM3 were tested for presentation of IE1$_{TMY}$ in a cytolysis assay, using IE1$_{TMY}$-CTL as effector cells (Fig. 6). Whilst pp65$_{NLV}$-CTL, employed as a positive control, lysed DB-HB5 and DB-VM3 target cells with indistinguishable dose–response curves, IE1$_{TMY}$-CTL only lysed target cells treated with DB-VM3. These results confirmed that processing of the pp65$_{NLV}$ peptide from the fusion protein was comparable with its processing from the authentic pp65. Importantly, the IE1$_{TMY}$ peptide was also processed successfully from the fusion protein to be presented by MHC class I.

Taken together, both assays for antigen presentation showed unequivocally that a heterologous peptide that is not usually present in virion proteins can be processed proteolytically from a recombinant, particle-derived protein of HCMV and can be introduced into the MHC class I presentation pathway.

DISCUSSION

In previous studies, we showed that DB are highly immunogenic and thus provide a promising basis for the development of an HCMV vaccine (Pepperl *et al.*, 2000). One major attribute of DB, i.e. the abundance of the major T-cell antigen pp65 (Varnum *et al.*, 2004), renders these particles attractive for the development of a vaccine. However, recent studies showed that non-structural antigens, such as IE proteins, may also contribute significantly to the priming of a natural T-cell response
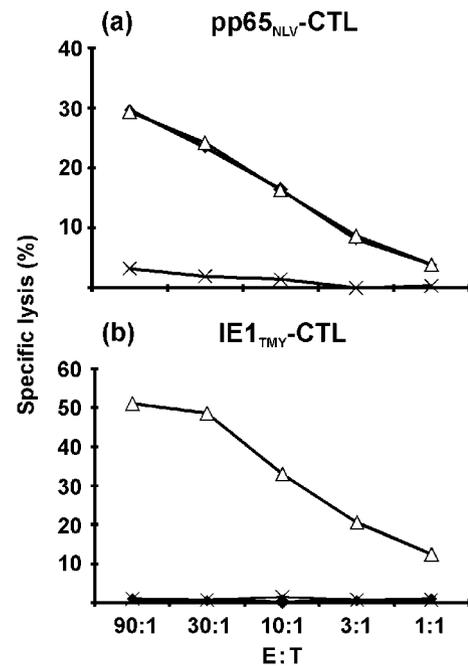
Fig. 6. Chromium-release assay of CTL-mediated cytolysis of DB-treated cells. HLA-A2$^+$ HFF were left untreated ($\times$) or were incubated for 24 h with 20 μg UV-irradiated DB from either RV-HB5 (DB-HB5, $\blacklozenge$) or RV-VM3 (DB-VM3, $\triangle$). Cells were used as targets in a standard 5 h chromium-release assay at the indicated effector : target cell (E : T) ratios. pp65$_{NLV}$-CTL and IE1$_{TMY}$-CTL were used as effector cells. Reported cytolytic activities represent mean values of specific lysis from triplicate cultures.

against HCMV in humans (Elkington *et al.*, 2003; Khan *et al.*, 2005; Manley *et al.*, 2004; Sylwester *et al.*, 2005). It was thus mandatory to show that DB can be modified to include additional antigens not normally present in these particles and that these antigens can be introduced successfully into the MHC class I presentation pathway.

DB can accommodate a heterologous polypeptide of 55 kDa, if this protein is expressed in fusion with pp65 (Pepperl-Klindworth *et al.*, 2003). However, packaging efficiency of the fusion protein was limited in this case. Here, we have extended these investigations to show (i) that a pp65 fusion protein containing an immunodominant peptide from IE1 is packaged into recDB in amounts comparable to wt pp65, (ii) that the IE1$_{TMY}$ peptide is presented efficiently on recDB-treated cells, implying its processing from the particle-associated fusion protein, and (iii) that this IE1 presentation does not interfere with the presentation of an immunodominant peptide from pp65, also presented by HLA-A2.

RV-VM3 was comparable to the parental strain, RV-HB5, with regard to replication kinetics. Levels of the fusion protein within cells and within purified viral particles also appeared to be unaltered compared with wt pp65, when normalized to gB. Consequently, the fusion protein was

expressed with kinetics comparable to wt pp65 and was packaged efficiently into particles. Furthermore, like wt pp65 from RV-HB5, the fusion protein from RV-VM3 accumulated predominantly in the cytoplasm of late-stage RV-VM3-infected cells. However, the yield of DB that could be recovered by gradient centrifugation was reduced compared with the parental strain RV-HB5. By using indirect immunofluorescence analysis in combination with conventional microscopy, pp65 appeared to be concentrated in dot-like structures in the cytoplasm of RV-HB5-infected, but not RV-VM3-infected, cells (data not shown). It is unclear at this stage how these structures relate to the formation and release of DB. However, these results suggest that the efficiency of release of DB may be linked to the subcellular localization of pp65 and this needs to be investigated further.

Infection of HFF with RV-VM3, but not with the parental strain RV-HB5, led to the presentation of $IE1_{TMY}$ by MHC class I. One explanation for this difference could be that RV-VM3 and RV-HB5 are distinct in their capacity to express the IE1 gene. The viruses, however, differ only in the insertion made in pp65. As there is no evidence that pp65 interferes with IE1 gene expression (B. Plachter, unpublished), it is unlikely that the modification within the tegument protein results in alteration of immediate-early gene expression. Furthermore, RV-VM3 showed replication kinetics similar to those of its parental strain, also arguing against an impairment of IE gene expression, which would affect viral replication. A more reasonable explanation is that, after infection with RV-VM3, $IE1_{TMY}$ was processed from the particle-associated pp65–$IE1_{TMY}$ fusion protein, rather than from de novo-synthesized IE1. Presentation of $IE1_{TMY}$ was suppressed by immune evasion – presumably by gpUS11, the only remaining recognized function that downregulates MHC class I antigen presentation to be expressed – in pHB5-infected cells. It is very likely that gpUS11 also suppressed presentation of de novo-synthesized IE1 in RV-VM3-infected cells. The finding that incubation of HFF with non-infectious DB-VM3 also resulted in stimulation of $IE1_{TMY}$-CTL confirmed this concept of exogenous introduction of $IE1_{TMY}$ by the fusion protein. Consequently, $IE1_{TMY}$ presentation appears to be restored in the face of immune evasion by introducing the peptide into cells as part of a structural component of viral particles.

The ELISpot reading after infection with the HCMV RV-VM3-infected sample was lower than that detected with the virus RV-ΔUS2–11, used to control CTL reactivity. Direct comparison of the two viruses, and hence comparison of the efficiency of presentation, is complicated by the fact that RV-VM3 differs from RV-ΔUS2–11 in both expression of gpUS11 and formation of the fusion protein. As emphasized above, presentation of peptides derived from the IE1 protein seems to be repressed by gpUS11 in RV-VM3-infected cells. Consequently, presentation of $IE1_{TMY}$ on these cells is the result of processing of particle-associated pp65–$IE1_{TMY}$ fusion protein. As there

is no virus available that expresses the fusion protein, but lacks US2–US11, one can only speculate on the effect of immune evasion on processing and presentation of $IE1_{TMY}$ from the exogenous fusion protein and whether presentation would be enhanced if all evasion proteins were lacking. However, ELISpot numbers were also limited when non-infectious DB-VM3 were used for loading of the cells. No HCMV-induced suppression of MHC class I presentation is expected to occur in these cells. Quantitative comparison of $IE1_{TMY}$ presentation on infected and recDB-loaded cells is impossible in this experimental setup. However, there appears to be a factor limiting processing and/or presentation efficiency of $IE1_{TMY}$ from the exogenous fusion protein compared with the presentation of the peptide from de novo-synthesized IE1 in RV-ΔUS2–11-infected cells.

A likely explanation could be that efficient de novo IE1 protein synthesis, driven by the HCMV major immediate-early promoter/enhancer, led to the formation of a large protein pool in infected cells. This may well have exceeded the amount of fusion protein that was introduced by viral particles and thus may have resulted in higher rates of processing and presentation of $IE1_{TMY}$ in RV-ΔUS2–11-infected cells. Alternatively, differences in the proteasomal processing efficiency of the IE1-derived and fusion protein-derived $IE1_{TMY}$ peptide could have accounted for the differences in presentation. Although care was taken in the cloning strategy to provide amino acids flanking $IE1_{TMY}$ in IE1 to ensure proper proteasomal processing, differences in proteolysis of IE1 versus the fusion protein cannot currently be formally excluded.

Interestingly, cells infected with HCMV RV-VM3 or incubated with DB-VM3 presented both immunodominant CTL determinants, namely the $pp65_{NLV}$ peptide and the $IE1_{TMY}$ peptide, concurrently in the context of HLA-A2. This showed that peptides that are not usually present in virus particles could be included in recDB, to be presented efficiently by the same MHC class I molecule, and that introduction of $IE1_{TMY}$ did not influence processing or presentation of $pp65_{NLV}$. Consequently, fusion of immunodominant peptides to pp65 for the generation of recDB may be a reasonable approach to vaccine development.

This study provides evidence that a heterologous peptide can be introduced into recDB by fusion to pp65. The $IE1_{TMY}$ peptide is presented by MHC class I after exposure of cells to these particles. However, for the outbred human population, more than one MHC class I-restricted peptide and, in addition, also MHC class II-restricted peptides from non-structural HCMV proteins may have to be included. IE1 appears to be a preferential target for both CD8 and CD4 T-cell responses in humans (Davignon et al., 1996; Elkington et al., 2003; Kern et al., 1999; Khan et al., 2005; Sylwester et al., 2005). Consequently, fusion of IE1 to pp65 would potentially generate an excellent recDB-based vaccine candidate. Such a fusion protein, expressed by

insect cells, was effective at stimulating the expansion of anti-HCMV CD4 and CD8 T-cell clones from HCMV-seropositive donors *in vitro* (Vaz-Santiago *et al.*, 2001). However, including the complete IE1 polypeptide for the design of a vaccine may be subject to safety concerns, as IE1 is known to activate both cellular and viral genes (Mocarski *et al.*, 2007). Consequently, it may be more reasonable to include only portions of the protein that are known to be essential targets of the CD8 and CD4 T-cell response.

Careful design of the fusion protein to be included in a recDB vaccine is mandatory. We have shown previously that sequences encoding up to 55 kDa can be added to UL83, maintaining assembly and release of DB (Pepperl-Klindworth *et al.*, 2003). As also seen in both this work and the present study, however, the size of the fusion partner and the location chosen for insertion in the primary structure of pp65 may be critical and limiting for the capacity to package the fusion protein. Current work is focusing on identifying an insertion site within pp65 that will allow packaging of larger fusion proteins with a higher efficiency in formation of recDB. One prerequisite for this is the ability to modify the HCMV genome in *E. coli* by using *galK*-mediated selection.

The development of BAC technology was a major breakthrough in the genetic analysis of cytomegaloviruses (Borst *et al.*, 1999; Messerle *et al.*, 1997). We have adopted *galK*-mediated positive/negative selection in combination with λ Red-mediated recombination (Warming *et al.*, 2005) for seamless insertion of the TMY peptide into an HCMV BAC clone. *galK* can be used for both positive and negative selection. Any functionally critical mutation in *galK* will be suppressed during positive selection, thus reducing the level of background for the negative selection significantly. Furthermore, the use of *galK* enables seamless insertion of the heterologous sequence into pp65 without leaving operational traces. Thus, the combined features of *galK*-based recombineering facilitate systematic mutagenesis of the HCMV genome.

In conclusion, the *galK*-based selection procedure proved to be a versatile method to mutate the large HCMV genome. It may also be useful for the modification of other herpesviral genomes. The experiments presented here provide proof of principle that a heterologous MHC class I peptide antigen can be packaged into recDB and that this peptide can be introduced efficiently into the MHC class I presentation pathway. On the basis of this knowledge, recDB can be refined further in their antigen composition to establish a novel vaccine candidate.

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