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ORIGINAL INVESTIGATION

Refinement of strategies for the development of a human cytomegalovirus dense body vaccine

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Abstract Development of a vaccine against human cytomegalovirus (HCMV) infection has been identified as a high priority goal in biomedical research, yet no vaccine has been licensed until now. Recombinant subviral dense bodies (recDB) are a promising basis for the establishment of such a vaccine. In this article, strategies for the generation of recDB, based on recombination-mediated genetic engineering of the 230 kb HCMV DNA genome in *E. coli* are outlined. Analysis of viral mutants that were constructed in this process provided the proof-of-principle that heterologous antigens can be packaged into recDB and that these particles prime CD8 T cell responses against the recombinant antigen upon their application to HLA-A2 transgenic mice.

Keywords Cytomegalovirus · Vaccine · Dense bodies · pp65 · IE1

Introduction

Infections with human cytomegalovirus (HCMV) continue to be important complications of allogeneic hematopoietic stem cell transplantation (HSCT) [1]. Prenatal HCMV infection, in addition, is the leading cause of mental retardation and sensorineural hearing loss in infants in the Western world [2, 3], and it may also contribute to morbidity and mortality in newborns in developing countries [4]. Numerous studies have found a strong correlation of immature or impaired immune defence functions with morbidity and mortality second to HCMV infection or reactivation [3]. Cytolytic CD8 T lymphocytes (CTL) have been denominated as key effectors that control viral infection and limit viral reactivation, both in animal models and in clinical studies [5–7]. Consequently, work performed to develop immunotherapeutic or prophylactic intervention strategies against HCMV disease has focused on the enhancement of CD8 and CD4 T cell responses [8–10]. Although the role of T lymphocytes in the prevention of prenatal HCMV infection and disease remains controversial, there is no doubt that improved reconstitution of CD8 and CD4 T cells is a primary goal of HCMV specific immunotherapy in allogeneic HSCT [1, 11].

Success of therapeutic tumor intervention applying allogeneic HSCT is frequently compromised by HCMV reactivation [1]. Depletion of donor-derived lymphocytes to reduce graft-versus-host disease is one risk factor in this context, as antiviral T cells may be concomitantly depleted [12]. Selective removal of only the alloreactive T cells may be a way to retain antiviral CD8 responses [13, 14]. Transfer of antiviral T cell lines, generated by in vitro stimulation prior to transfusion, has been shown to be another option to reconstitute HCMV immune responses [7–9]. However, logistic limitations and the possible risk of transfer of alloreactive cells together with HCMV specific cells have prevented introduction of such strategies as a clinical routine. T cell receptor (TCR-) based ex vivo sorting of antiviral CD8 T cells and subsequent adoptive transfer of such cells has been shown to be effective to

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reduce the incidence of cytomegalovirus induced disease in a mouse model [15, 16], and it has also been applied in first clinical studies [10]. However, a number of questions regarding the adoptive transfer of HCMV-specific T lymphocytes remain unanswered. Most importantly, the effectiveness of such a procedure to confer lasting protection against HCMV disease after HSCT remains undetermined. Furthermore, it may not be possible to generate HCMV specific T-lymphocytes from HCMV-seronegative donors. One way to improve the potency of the HCMV specific adoptive T cell transfer for immunoprophylaxis is the use of a vaccine for in vivo stimulation of transferred T cells.

Several vaccine candidates have been introduced, none of which has entered clinical routine, yet [17–20]. We have focused on the development of a vaccine based on subviral dense bodies (DB) [21, 22]. DB enter cells in a way comparable to virions and thereby deliver their proteinaceous content into the cytoplasm. As exemplified for the major component of DB, the pp65 (ppUL83), peptides derived from this tegument protein are efficiently introduced into the MHC class I presentation pathway without requiring de novo protein synthesis [21]. Although pp65 by itself is a prominent target of the CD4 and CD8 T cell responses following natural infection [23–25], other proteins like the non-structural, regulatory IE1-protein (ppUL123) are also targeted by the immune system [26, 27]. Consequently, inclusion of such antigens in a vaccine formulation may be important to induce a broad immune response [15, 26–30]. We have started a project to broaden the antigenic content of DB. Viral mutants that expressed pp65 in fusion with a heterologous polypeptide were generated. Cells infected with these viruses released recombinant DB (recDB) containing the fusion protein, thus proving the feasibility of the approach [31]. This strategy, however, had to be refined with respect to the numbers of recDB released from infected cells. In addition, the methodology of conventional recombination in eukaryotic cells, used for these experiments, may be critical in the development of a vaccine for human application.

The aim of the work described in this article was to define a location within pp65 that allowed fusion with a heterologous polypeptide while retaining efficient release of recDB from HCMV infected cells. For this, we used two different variations of recombination-mediated genetic engineering (recombineering) of bacterial artificial chromosome (BAC) clones of the virus to modify the 230 kb HCMV genome in *E. coli* [32–35]. For the sake of this experimental approach, we decided to fuse fragments of the IE1 protein of HCMV, containing HLA-A2 presented peptide determinants, to pp65. For analysis, an antibody tag for an epitope from the c-myc gene was included in some of the recombinants. The respective viral mutants were reconstituted in human foreskin fibroblasts (HFF) and were subsequently analysed for synthesis and release of recombinant particles by infected HFF.

Materials and methods

Generation of BAC clones by positive selection using kanamycin resistance (Kan^R)

The generation of HCMV BACs, using positive selection, was performed according to Lee et al. [34].

Cloning of BAC-SR1

Primers SR1 and SR2 (Table 1) were used to amplify the $IE1_{(288–402)}$ fragment and to introduce a Kozak-ATG as well as the restriction sites for *Xho*I and *Bam*HI. The PCR fragment was cloned into the *Xho*I and *Bam*HI digested vector pCP-o-15-link2 [36], resulting in pCP-IE1 with the FRT-Kan^R-FRT cassette upstream of the IE1 fragment. For homologous recombination, the FRT-kanamycin-IE1 region was amplified using primers SR4 and SR5 to insert the IE1 fragment in frame at the N-terminus of pp65 of BAC pHB5 [33].

Cloning of BAC-VR1

For the construction of the C-terminal pp65 fusion protein, the primer pair SR9/SR10 (Table 1) was used to amplify the FRT-Kan^R-FRT cassette from the plasmid template pCP-IE1. The *Nsi*I cleaved PCR-fragment was inserted into *Nsi*I cleaved pBamHIR, a pBluescript based plasmid containing the *Bam*HI R fragment [37] of HCMV with the complete pp65 gene. The resulting plasmid pBamHIR-Kan, with the FRT-Kan^R-FRT cassette downstream of pp65 was digested with *Nhe*I and ligated with the *Nhe*I digested PCR-fragment of the template plasmid pCP-IE1 and primers SR11/SR12. This step adds the $IE1_{(288–402)}$-fragment to the C-terminus of pp65. The construct was termed pBamHIR-IE1-Kan. This plasmid was used as template with primers SR13/SR14 to amplify the PCR-fragment for the homologous recombination into pHB5.

Cloning of BAC-EP0, BAC-EP1, BAC-EP2

To generate pp65-fusion proteins labelled with a myc tag, a PCR product was generated using pBamHIR-Kan as template and the SR16/SR17 primer pair (Table 1) for amplification. The resulting fragment was inserted into a TA cloning vector and the plasmid was termed pK65. Plasmid pK65 contained the 3′ end of UL83 with an

Table 1 Sequences of the oligonucleotides used for PCR-amplification

Name	Used to construct	Sequence
SR1	pCP-IE1	GAGACTCGAGCCACCATGAAGGTCACTAGTGACGCTTGTATG
SR2	pCP-IE1	GAGA GGA TCC TGA CAC CAG AGA ATC AGA GGA GC
SR4	RV-SR1	CCCAGTACGGATATCATTTCGGGACAACGGCGCCAGCGCG ACTCCATTGACACCAGAGAATCAGAGGAG
SR5	RV-SR1	CAGAGGGCGCGCCGCTCAGTCGCCTACACCCGTACGCGCAGGCA GCTAATTAAGGCGCGCCGGTACC
SR9	pBamHIR-Kan	GAGAATGCATCGCCTCGACGCCCAAAAAGCACCGAGGTGC TAGCTAATTAAGGCGCGCCGGTAC
SR10	pBamHIR-Kan	GAGAATGCATCATGGTGGCTCGAGAAACG
SR11	pBamHIR-IE1-Kan	GAGAGCTAGCAAGGTCACTAGTGACGCTTGTATG
SR12	pBamHIR-IE1-Kan	GAGAGCTAGCTCATGACACCAGAGAATCAGAGGAG
SR13	RV-VR1	CCGAATTGGAAGGCGTATGG
SR14	RV-VR1	TGTTCCGCTTCCTTTAGCAG
SR16	pK65	GCCTTGCCCGGGCCATGCATCGCCTCGACGCCCAAAAAGC ACCGAG
SR17	pK65	GGACGTGGGTTTTTATAGAGTCGTCCTAAGCGCGTGCGGCGGGTGGC CATGGTGGCTCGAGAAACGCAGCT
SR22	pK65-1 for pBAC-EP1 cloning	GAGAGCTAGCACTAGTGACGCTTGTATGATG
SR23	pK65-1 for pBAC-EP1 cloning	GAGAGCTAGCTCACAGATCCTCTTCTGAGATGAGTTTTTGT TCAGGCCGCTTGGCCAGCATC
SR24	pK65-2 for pBAC-EP2 cloning	GAGAGCTAGCACTAGTGACGCTTGTATGATG
SR25	pK65-2 for pBAC-EP2 cloning	GAGAGCTAGCTCACAGATCCTCTTCTGAGATGAGT TTTTGTTCACAGAACTCACTTAAGAGAGAG
SR26	pK65-0 for pBAC-EP0 cloning	CTAGCGAACAAAAACTCATCTCAGAAGAGGATCTGTGAG
SR27	pK65-0 for pBAC-EP0 cloning	CTAGCTCACAGATCCTCTTCTGAGATGAGTTTTTGTTCG

adjacent FRT-Kan^R-FRT cassette. In order to construct the pK65-0 plasmid, the complementary oligonucleotides SR26 and SR27, which carried the nucleic acid sequence of a c-myc tag and a stop codon flanked by *Nhe*I restriction sites, were annealed. This annealing product was then digested with *Nhe*I and sub-cloned into a *Nhe*I digested pK65 plasmid.

The sequences coding for the IE1 peptides $IE1_{(288–309)}$ and $IE1_{(309–328)}$ were amplified by PCR using the pCP-IE1 plasmid with the respective forward primers (SR24, SR22) containing a *Nhe*I restriction site, and with the respective reverse primers (SR25, SR23), containing sequences of a c-myc tag, a stop codon and a *Nhe*I restriction site. The pK65-1 plasmid, carrying the coding sequence for the $IE1_{(288–309)}$ peptide, and the pK65-2 plasmid, containing the coding sequence for the $IE1_{(309–328)}$ peptide, were obtained by sub-cloning the different *Nhe*I digested PCR products into the *Nhe*I digested pK65 plasmid. To fuse the different IE1 fragments at the C-terminus of pp65 into the HCMV BAC, PCR fragments were generated using primers SR16/SR17 and plasmid pK65-1 resulting in BAC-EP1, or plasmid pK65-2 for BAC-EP2, or plasmid pK65-0 for BAC-EP0. The correct nucleotide sequences of all plasmids and BACs were verified by sequencing the modified regions. All used primers are listed in Table 1.

Results

Construction of recombinant viruses expressing N- and C-terminal fusion proteins of pp65

In a first attempt to optimise the generation of recDB, different portions of the IE1 protein carrying one or more immunodominant HLA-A2 presented determinants were fused to the N- and C- terminus of pp65 (Fig. 1a–c). Using this technology, several recombinant viruses were established (Fig. 1d). The C-terminal part of pp65 was chosen to fuse either the $IE1_{TMY}$ determinant ($IE1_{(297–304)}$, TMYGGISLL) [38] or the $IE1_{VLE}$ determinant ($IE1_{(316–324)}$, VLEETSVML) [39, 40]. The single nonamer peptides were extended by flanking amino acids ($IE1_{(288–309)}$ and $IE1_{(309–328)}$) to ensure proper proteolytic processing. Furthermore, the IE1 peptides were labelled with a c-myc epitope tag to facilitate analysis (recombinants RV-EP1 and RV-EP2). A pp65 fusion protein, containing the minimal myc-tag was also generated for control (RV-EP0). Lastly, a

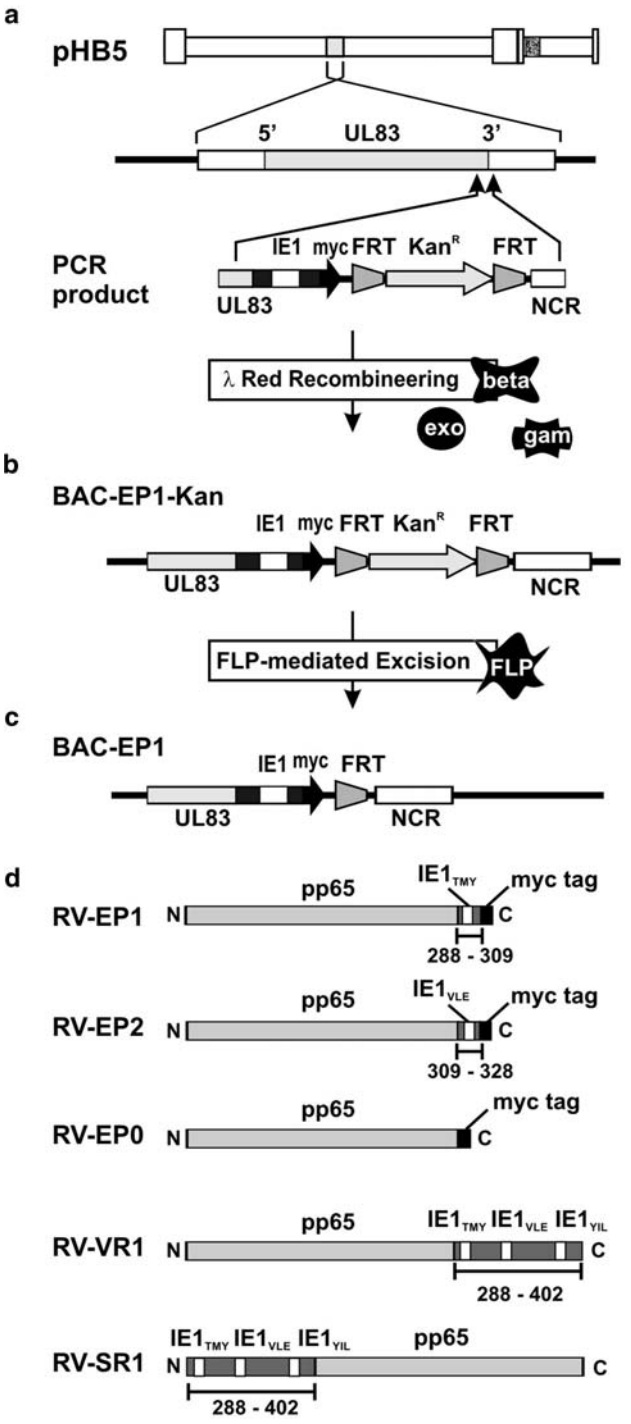


Fig. 1 Genetic modification of HCMV BACs using positive selection by Kan^R. As an example, the cloning procedure for BAC-EP1 is shown. For λ-Red-recombineering, *E. coli* strain EL250 was used. These bacteria carry the λ-Red genes exo, beta and gam under heat-inducible promoter control and an arabinose inducible FLP-recombinase gene, both as chromosomal integrates [34]. **a** Structure of the PCR-product used for homologous recombination into the C-terminus of the pp65 gene of the HCMV BAC pHB5 by recombineering. **b** Structure of the intermediate BAC clone BAC-EP1-Kan. Bacteria harbouring this BAC were induced for FLP expression by the addition of arabinose. **c** Structure of the final construct BAC-EP1 after excision of FRT-Kan^R. This BAC served as the basis for the reconstitution of RV-EP1. **d** structure of the pp65 fusion proteins expressed from the different recombinant viruses. The portions of IE1 are indicated by *dark grey boxes* and are represented with respect to the IE1 protein sequence below each bar. The different HLA-A2 presented peptides from IE1 are shown as *white boxes*. The pp65 sequence is represented by *light grey bars*, the myc tag sequence by *black boxes*. *NCR* non-coding region of the UL83 gene; *FLP* flip recombinase

longer IE1 polypeptide ($IE1_{(288–402)}$) was selected, containing both $IE1_{TMY}$ and $IE1_{VLE}$ as well as the sub-dominant determinant $IE1_{YIL}$ ($IE1_{(354–363)}$, YILGADPLRV) [41], all presented by HLA-A2 (RV-VR1). The same fragment was also fused to the very N-terminus of pp65 [42] (Fig. 1d; RV-SR1). After FLP-mediated excision of Kan^R from each BAC, the correct insertion was verified by nucleotide sequencing and the recombinant viruses were reconstituted in HFF.

Expression and packaging of the N- and C-terminal pp65-IE1 fusion proteins in infected cells

The expression of the fusion proteins in infected HFF was verified by immunoblot analysis and indirect immunofluorescence. No expression of the fusion protein from RV-SR1 could be detected in crude lysates from infected cells (data not shown). This virus was thus excluded from further analysis. In contrast, all C-terminal fusion proteins were expressed in HFF infected with the respective mutant (Fig. 2a). No significant differences in the steady-state levels of these proteins became apparent.

To analyse the subcellular localisation of the fusion proteins, indirect immunofluorescence analyses were performed (Fig. 2b). Cells infected with RV-HB5 showed the well-established nuclear localisation of pp65 at 2 days p.i. and the cytoplasmic shift at 4 d.p.i.. In cells infected with RV-VR1, pp65 was only vaguely detectable at 2 days p.i., and was localized in the cytoplasm at 4 days p.i.. In cells infected with the RV-EP0 and RV-EP1, the fusion protein was translocated from the nucleus to the cytoplasm during later stages of infection. This was comparable to the parental strain. In contrast, in cells infected with RV-EP2, the fusion protein remained mainly in the nucleus. To test whether the fusion proteins of the different viral mutants were incorporated into extracellular progeny viral particles, the different particle fractions were separated by glycerol–tartrate gradient centrifugation of culture supernatants from infected cells [43] (data not shown). The gradient obtained from RV-HB5 infected cells showed the typical banding pattern of HCMV viral particles, consisting of NIEPs, virions and DB. In the gradients from RV-VR1, RV-EP1 and RV-EP2 infected cells, the NIEPs and virion bands, but no

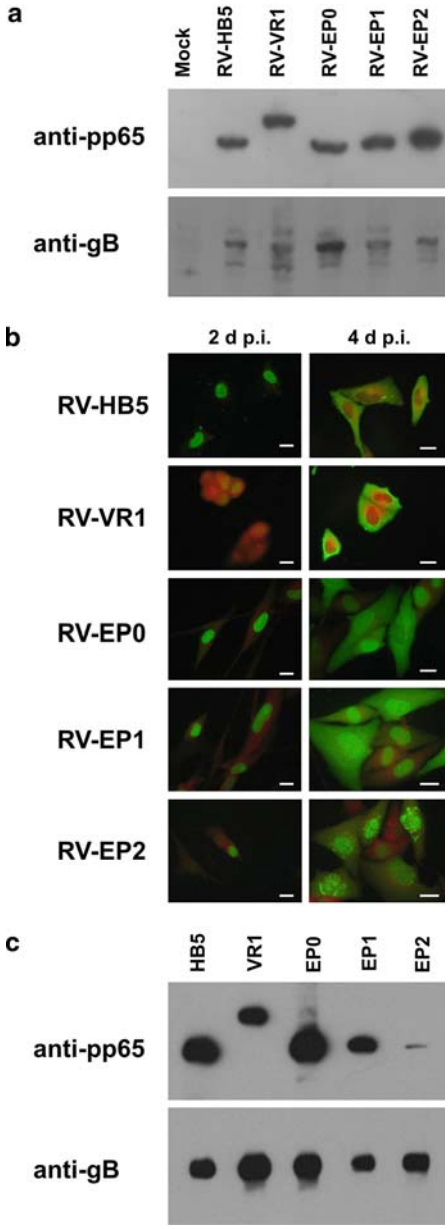


Fig. 2 Analysis of pp65 fusion protein expression, subcellular distribution in infected HFF, and content in the extracellular NIEPs fractions. **a** Immunoblot analysis of pp65 and pp65 fusion protein expression in infected HFF. Cell lysates from infected cells were collected at 4 d.p.i. and were analysed by immunoblotting. The amount of protein loaded on each lane was normalized against HCMV gB (mAb 27-287). The fusion proteins were detected with an antibody against pp65 (mAb 65-33). **b** Immunofluorescence analysis of the subcellular distribution of the pp65 fusion proteins in infected HFF. Cells were infected at an m.o.i. of 2–3 with the different viruses. Cells were fixed at 2 and 4 d.p.i. and stained with mAb 65-33. Immunofluorescence analyses were carried out as described in the accompanying article [51]. *White bars* represent 10 µm. **c** Immunoblot analysis of the amount of fusion protein packaged in the extracellular NIEPs fractions, collected by glycerol–tartrate gradient centrifugation [43]. For staining, mAbs 65-33 and 27-287 were used

DB were detectable. Only in the RV-EP0 gradient, a faint smear of DB became apparent. Because bands corresponding to DB were absent in the gradients from RV-VR1, RV-EP1 and RV-EP2 and because the material from the virion fractions was not sufficient for further analysis, only bands from the NIEPs fractions were collected and subjected to immunoblot analyses (Fig. 2c). The pp65-c-myc fusion protein from RV-EP0 appeared to be packaged with similar abundance compared to the wild type pp65 tegument protein. In contrast to that, lower amounts of the fusion proteins from RV-VR1, RV-EP1 and RV-EP2 were detected in the respective NIEPs fractions. These results showed that infection with these three recombinant viruses leads to decreased packaging of the respective pp65-IE1 fusion proteins into viral particles compared to infection with wild type RV-HB5. Taken together, fusion of heterologous partners to the very C-terminus may allow packaging of the fusion protein into NIEPs and virions to various extents, but it may be inappropriate for the generation of recDB.

Ultrastructural analysis of infected cells

Results obtained by gradient centrifugation and immunoblot analyses suggested that the formation of DB containing the pp65-IE1 fusion proteins was impaired in cells infected with RV-VR1, RV-EP1 or RV-EP2. To corroborate this on the ultrastructural level, electron microscopy was performed as described before [44] on 6-day infected HFF (Fig. 3). Analysis of the nuclei of RV-HB5 infected cells showed electron-dense structures which likely corresponded to the nuclear capsid assembly sites and, in close proximity, the three types of capsids (Fig. 3). In the cytoplasm of RV-HB5 infected HFF, DB and multivesicular bodies (MVB) [45] were present in large numbers. The wild type DB, which were enclosed by a membrane, appeared as round, uniform and electron-dense particles with a size varying between 300 and 500 nm (white arrows in Fig. 3b). Similar electron-dense structures were found in RV-EP0 infected cells. However they were larger (between 300 nm and 1 µm) and appeared to be less frequent compared to RV-HB5. Some of the different electron-dense structures were composed of a mixture of capsids, vacuoles and electron-dense masses, and apparently were losing their homogeneity and their shape (grey arrows in Fig. 3c). These cytoplasmic accumulations reached sizes up to 5 µm. It remains unclear, whether they are related to DB. In contrast to cells infected with RV-HB5 and RV-EP0, cells infected with the other recombinant viruses did not display any formation of DB, although some large electron-dense structures without any specific organisation were observed in the cytoplasm of RV-EP1 and RV-VR1 infected cells

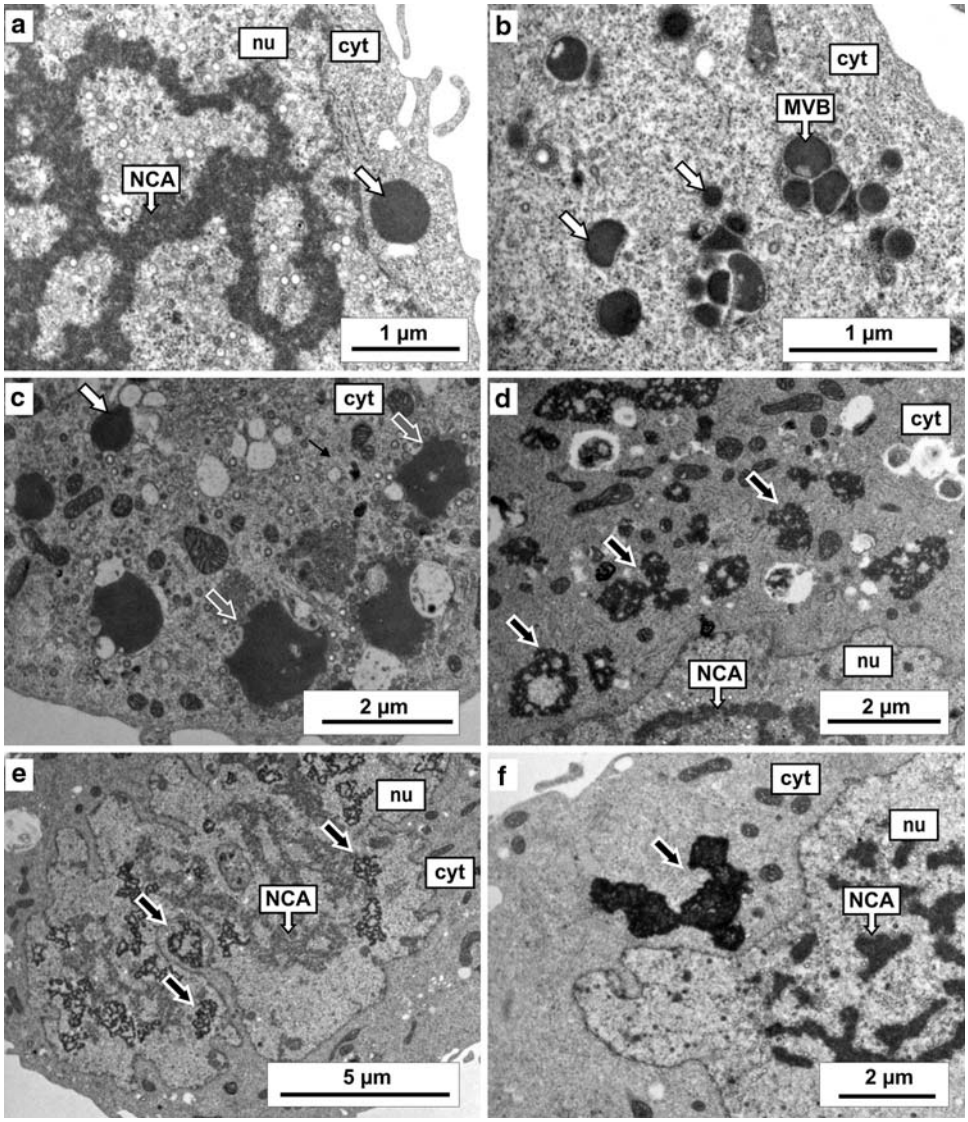


Fig. 3 Electron micrographs of HFF infected with recombinant viruses. HFF were infected with RV-HB5 (**a**, **b**), RV-EP0 (**c**), RV-EP1 (**d**), RV-EP2 (**e**) and RV-VR1 (**f**) at an m.o.i. of 2–3. Six days after infection, cells were processed for ultrastructural examination as published [44]. Cytoplasmic DB (*white arrows*); electron dense round aggregates (*grey arrows*); electron dense aggregates (*black arrows*); *NCA* nuclear capsid assembly site; *cyt* cytoplasm; *nu* nucleus; *MVB* multivesicular bodies (composed of virions and DB, and surrounded by a membrane)

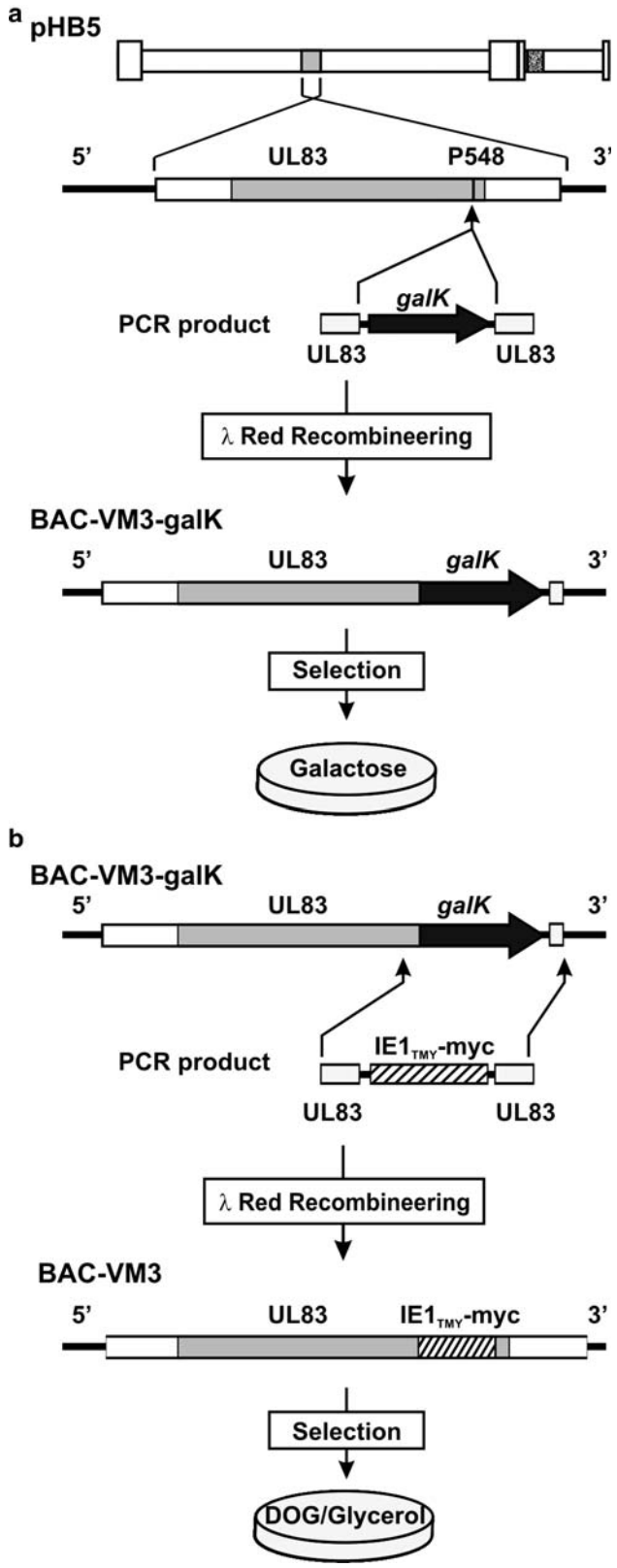
(black arrows in Fig. 3d, f). These aggregates were spread out all over the cytoplasm and reached sizes of up to 7 µm, whereas the electron-dense accumulations in RV-EP2 infected cells were mainly located in the nuclei (black arrows in Fig. 3e). These analyses confirmed that the minor alteration of adding small peptides to the very C-terminus of pp65 already prevented the formation of DB in the cytoplasm of HFF infected with RV-VR1, RV-EP1 and RV-EP2, but not the production of infectious virions.

Internal seamless fusion of heterologous peptide sequences to the viral pp65 by *galK* mediated recombineering

The experiments described above provided evidence that fusion of IE1-derived polypeptides to both C- and N-terminus of pp65 disturbed synthesis of recDB by infected cells. To test, if internal insertion of the heterologous peptide was appropriate to retain DB formation, an alternative technique, employing the bacterial galactokinase (*galK*) for both positive and negative selection, was used for HCMV BAC recombineering [35]. This method allows precise and seamless modification of the target BAC (Fig. 4). By this, HCMV mutant RV-VM3 was established expressing the $IE1_{TMY}$-myc fusion peptide (as in RV-EP1) at position P548 of pp65 [44].

Immunologic potential of RV-VM3-dense bodies

In a recent paper, we could demonstrate by immunofluorescence analysis that the fusion protein of RV-VM3 behaves like wt pp65 with respect to its subcellular localization in infected HFF. Furthermore, RV-VM3-infected cells release both virions and DB that contain the fusion protein. The $IE1_{TMY}$ peptide is efficiently presented on the surface of



◀ **Fig. 4** Genetic modification of HCMV BACs by using *galK*-mediated positive/negative selection. **a** Structure of the PCR-product used for insertion of the *galK*-gene into position P548 of pp65 of BAC pHB5, using λ-Red-recombineering in *E. coli* strain SW102, and structure of the intermediate BAC clone BAC-VM3-galK. Proline 548 (P548) was chosen for targeting, as this location had been successfully used before to generate recombinant DB [31]. Bacteria harbouring this BAC-VM3-galK were selected by supplying galactose as carbon source. **b** structure of the PCR fragment containing $IE1_{TMY}$-myc (as in RV-EP1), used to replace the *galK* gene, and structure of the final construct BAC-VM3. Primers for amplification have been published elsewhere [44]. BAC-VM3 was isolated by adding 2-deoxygalactose (DOG) for negative selection of *galK* expression and glycerol. Recombinant virus RV-VM3 was reconstituted by transfecting BAC-VM3 into HFF [44]

cells exposed to the recombinant particles [44]. However, one critical question remained open, namely whether an immune response against the recombinant antigen could be mounted after immunization with DB from RV-VM3 (DB-VM3).

To test this, 10–12-week-old HLA-A2 transgenic mice (HHD mice) [46] were immunized once intraperitoneally with 6 µg DB from RV-HB5 (wt-DB) or DB-VM3, or with PBS as negative control. Lymphocytes were prepared from the spleens at day 7 after immunization and were cultured for two successive rounds of weekly restimulation in medium containing IL-2 and the specific peptide. The cells were subjected to an IFN-γ ELISpot analysis after the first and second restimulation, using peptide loaded RMA-S HHD stimulator cells [46, 47]. CD3ε-redirected ELISpot-analysis [48] was used to determine the total number of cells in the effector cell population that were able to respond with IFN-γ secretion (data not shown). The percentage of peptide specific CD8 T cells was calculated in the overall responding T cell population. Spleen cells from animals that were exposed to wt-DB or DB-VM3 were significantly stimulated with $pp65_{NLV}$ (Fig. 5a). Stimulation of cells from DB immunized mice with $IE1_{TMY}$ did not result in significant expansion of IE1-specific T cells. This was an expected result, as DB do not contain IE1. In contrast, splenocytes from animals injected with DB-VM3 were stimulated by the $IE1_{TMY}$ to about 60% of the total inducible population after 1 week and reached 100% after 2 weeks (Fig. 5b). These results provided the first proof that a heterologous antigenic determinant, not naturally present in DB, can prime a CD8 T cell response if expressed in fusion with pp65 and packaged into recDB.

Discussion

In this communication, we have described experiments aiming at the modification of the protein composition of HCMV DB by fusing portions of a heterologous protein to pp65. Whilst the expression of N- and C-terminal pp65 fusion proteins in cells infected with the respective HCMV mutant viruses did not result in the release of significant numbers of recDB, the results obtained may well extend

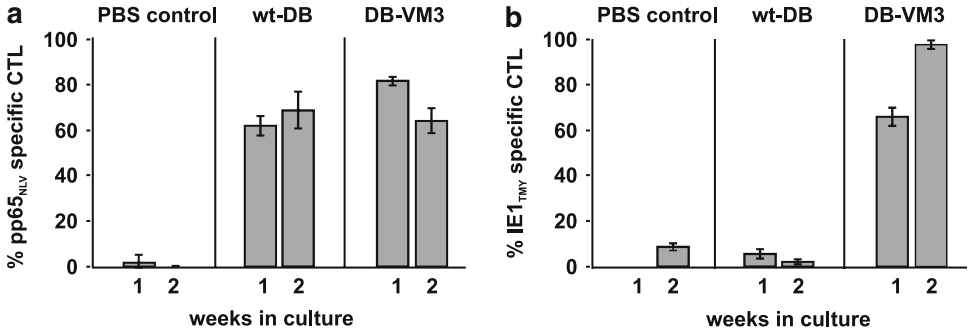


Fig. 5 ELISpot-analysis of CD8 T cells from DB-immunized HLA-A2 transgenic HHD mice. Ten to twelve-week-old HLA-A2 transgenic HHD mice [46] were immunized intraperitoneally (i.p.) with 6 µg of purified DB of RV-HB5 (wt-DB) or of RV-VM3 (DB-VM3). Five animals were included in each group. PBS was applied to a control group. One week after immunization, spleen cells were prepared and restimulated with 10^{-9} M of either $pp65_{NLV}$ or $IE1_{TMY}$ peptide in the presence of 0.2 ng/ml IL2. Cells were cultured for 1 week. A fraction of the cells was then subjected to an IFN-γ ELISpot analysis [44] using 10^5 RMA-S HHD cells as stimulators loaded with 10^{-9} M $pp65_{NLV}$ or $IE1_{TMY}$, respectively. The remaining cells were restimulated once again, cultured for another week and then subjected to another IFN-γ ELISpot analysis. **a** ELISpot-analysis of $pp65_{NLV}$-specific CTLs. **b** ELISpot-analysis of $IE1_{TMY}$-specific CTLs. The percentage of peptide specific CD8 T cells was calculated in the overall reactive effector cell population determined by polyclonal stimulation via CD3ε [48]

our understanding of the biology of pp65 and of HCMV particle morphogenesis.

Fusion of a heterologous polypeptide following the first ATG codon downstream of the transcription initiation site of the pp65 gene, as defined by Rüger et al. [42], led to a complete abrogation of pp65 protein expression in RV-SR1 infected cells. The reason for the lack of expression is unclear at the current stage. Depto and Stenberg [49] reported that, in transient transfection analyses, the octamer sequence ATTTCGGG found at position −51 with respect to the proposed transcription start site of the pp65 gene was essential for promoter activation. They identified the same sequence at position +93 and an inverted version at +67. There was no analysis performed on the relevance of these downstream sequences with respect to activation of the pp65 promoter. Both sequences were translocated, however, to a far downstream position by constructing the fusion gene in RV-SR1 (Fig. 1). Consequently, disrupting the downstream promoter structure could interfere with the expression of the N-terminal fusion protein. Alternatively, there may be additional transcription start sites downstream of position +93 that could be more important than the one described before [42].

The fusion proteins, with the exception of that of RV-EP2, were packaged into NIEPs. However, extracellular DB were, if at all, detectable only after infection with RV-EP0. From these analyses, it can be concluded that the formation of at least NIEPs containing pp65 fusion proteins was unaffected in RV-VR1-, RV-EP0-, and RV-EP1-infected cells. It may be assumed that also virion formation was unimpaired, although formal analyses still have to be performed to prove this. No DB formation, as compared with wt-infected cells, became apparent on the ultrastructural level. In accordance, cells infected with a pp65 negative virus released virions and NIEPs, but no DB [50]. These results point towards a dichotomy in the ability of infected cells to package the pp65 fusion protein into DB and NIEPs. In contrast to the current understanding, this may indicate that formation and maybe also release of DB and NIEPs (or virions) follow separate pathways.

The fusion protein expressed by RV-EP2 was found in low amounts in extracellular NIEPs, although its expression level was indistinguishable from that of the fusion proteins of the other viruses. As a striking difference, the RV-EP2 fusion protein remained mainly in the nucleus at late times p.i.. Consequently, the defect of packaging of this protein in progeny particles seems to be related to an impaired nuclear export of this polypeptide. This cannot be explained by differences in size or position of the IE1-peptide fused to pp65, as both were identical for RV-EP1 and RV-EP2. Alternatively, the primary structure of the protein fused to the very C-terminus of pp65 may influence nuclear export and, as a consequence, packaging of the recombinant protein into viral particles. Further work has to be conducted to investigate this issue.

The work described in this article was experimental in nature to identify insertion sites within pp65, optimal for formation and release of recDB. However, it was not designed to procure a final vaccine candidate to be used in humans. For a final formulation, inclusion of undesired sequences like myc-tags or FRT-sites should be avoided. Using the positive selection procedure of BAC modification in combination with FLP-mediated excision will always leave behind operational traces, such as FRT-sites close to the insertion site. In contrast, employing *galK* for both positive and negative selection allows precise and seamless modification of the HCMV strain used for vaccine production and thus may be preferentially used to generate recDB.

The antigens, however, that should be included in such a recDB vaccine to provide a sufficient breadth of the immune response still need to be defined. For this, the diversity of human MHC class I haplotypes and of the antiviral CD8 T cell responses have to be considered. We could show here for the first time, yet, that recDB can prime a CD8 T cell response against an HLA-A2 presented model peptide, not normally contained in DB. Significant numbers of IE1-specific CD8 T cells could be detected after one or two rounds of in vitro restimulation, whereas the ex vivo frequency of these cells was very low (data not shown). Nevertheless, even very low numbers of ex vivo isolated IE1-specific CD8 T cells—namely 400 cells—are sufficient to effectively control CMV infection, as has been shown in the murine CMV infection model [15]. In conclusion, the results of these initial immunization experiments are encouraging. However, further studies with the aim to refine the strategy of stimulating an HCMV specific T lymphocyte response, including serial immunizations and the use of adjuvant, need to be conducted.

Outlook

Development of vaccines against human herpesviruses has proven to be a formidable endeavour. A wisdom that was accomplished in this process is that complex problems may not be successfully met by simple solutions. Translating this into biology means that complex infections, like that with herpesviruses, may not be successfully addressed with vaccines relying on single antigens. More elaborate formulations are needed to confer reliable protection. In the case of HCMV, DB may be a basis to achieve this noble goal of preventing disease by a vaccine. Recent implementation of recombineering methods to genetically engineer the HCMV genome in bacteria provides the opportunity to optimise the antigenic content of these particles. Different recDB can then be tested in vitro, e.g. for their capacity to stimulate human HCMV specific T cells. These approaches will help to finally establish a candidate vaccine. Such a vaccine may then be tested for its immunoprophylactic potential in combination with the adoptive transfer of TCR-sorted antiviral T lymphocytes. This strategy will preferentially be explored in murine challenge models and subsequently be investigated for its benefit on the level of clinical studies in HSCT patients.

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