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Optimized recombinant dense bodies of human cytomegalovirus efficiently prime virus specific lymphocytes and neutralizing antibodies without the addition of adjuvant

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ABSTRACT

Control of human cytomegalovirus (HCMV) infection correlates with the reconstitution of antiviral T lymphocytes in haematopoietic stem cell transplant recipients. A vaccine to foster this reconstitution and to ameliorate the severe consequences of HCMV reactivation is yet unavailable. This work focused on providing a rationale for the amendment of the yields and the antigenic composition of a vaccine, based on subviral dense bodies (DB) of HCMV. Modified DB were generated that contained the HLA-A2 presented IE1 model peptide TMYGGISLL, integrated at different positions in the major DB protein pp65. Insertion at position W175 of pp65 allowed efficient formation of recDB in the cytoplasm of infected cells and resulted in considerable yields of these particles. Even in the absence of adjuvant, these particles proved to be highly immunogenic with respect to CD8 and CD4 T cell and neutralizing antibody responses.

1. Introduction

Infection with the human cytomegalovirus (HCMV) is a major cause of disease in patients following solid organ or haematopoietic stem cell transplantation (HSCT) [1–9]. Transmission of the virus during pregnancy, in addition, is one of the most frequent causes of lasting sequelae in the newborns in industrialized countries [10,11]. The development of an HCMV vaccine has thus been identified as a high priority goal [12]. One aim of such a vaccine would be to prevent infection in the mother or, at least, to block transplacental transmission. Induction of neutralizing antibodies is considered essential to achieve this [10]. Protection against viral reactivation and control of infection in transplant recipients is, in contrast, considered to be afforded by cellular responses, particularly mediated through CD8 T cells [13–15]. Addressing lymphocyte responses by vaccination is thus an option to ameliorate the consequences of viral reactivation of HCMV in the post-transplant period [16]. Despite promising approaches to develop an HCMV vaccine, there is, however, still no licensed formulation available [10,17–19].

Infection of culture fibroblasts with HCMV results in the formation of subviral, non-infectious particles, termed dense bodies (DB). These enveloped particles are released in large amounts from HCMV infected culture fibroblasts. They contain dominant antigens of both the T lymphocyte as well as of the neutralizing antibody response against the virus [20,21]. We could show in previous studies in mice that native DB are suitable to induce considerable levels of neutralizing antibodies as well as CD4 and CD8 lymphocyte responses in mice without addition of adjuvant [22]. Consequently, efforts were made to further develop DB as an HCMV vaccine. In this study, particular focus was laid on the development of a vaccine strategy to interfere with viral infection and disease in HSCT recipients. Since such a vaccine should primarily address the CD8 T cell response, we attempted to broaden the spectrum of MHC-class I presented peptides contained in DB to be able to address multiple class I alleles. Recent experiments from our laboratory provided the proof-of-concept that DB can be modified in their antigenic content [23,24]. This was achieved by inserting a heterologous MHC-class I presented peptide from the viral IE1 protein into the major DB component pp65. Human foreskin fibroblasts (HFF), infected with HCMV recombinants that expressed the hybrid protein showed the formation of recombinant DB (recDB). The recDB, containing the fusion protein were released from infected HFF [23]. Application of these particles to HLA-A2 transgenic HHD mice induced a $CD8^+$ T lymphocyte response, which became apparent after *in vitro* peptide stimulation of isolated T cells [24]. However, HCMV specific T cells could not be detected when $CD8^+$ T cell fractions were tested directly *ex vivo*. This indicated that priming was ineffi-

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cient with these particles. Further to that, the yield of these recDB from infected HFF cultures was low. Consequently, this particular formulation appeared inadequate for further development.

Previous investigations revealed that the insertion site for heterologous peptides sequences into pp65 was critical for the efficiency of subsequent DB formation. To further refine the DB based strategy for HCMV vaccine development, a study was initiated to identify insertion sites within pp65, which would be more appropriate for the insertion of heterologous peptides into this tegument protein. One insertion site within pp65 could be identified that allowed efficient formation and release of recDB. These recDB primed CD8 T cell responses, which became readily detectable by *ex vivo* Elispot analyses of CD8 T cell fractions. They also induced CD4 T lymphocytes as well as neutralizing antibodies.

2. Materials and methods

2.1. Cells

Primary HFF, CTL lines and T2 cells were cultured as described before [25]. RMA-S cells [26] were grown in RPMI 1640 medium (PAA Laboratories, Cölbe, Germany) supplemented with 10% FCS, 2 mM L-glutamine, 50 mg/l gentamicin and 5 μM β-mercaptoethanol.

2.2. Plasmids and viruses

For mutagenesis of the viral DNA, the HCMV bacterial artificial chromosome (BAC) pHB5 [27] was used. Mutagenesis of pHB5 was performed according to the galK positive/negative selection procedure [28] as described elsewhere [23]. The DNA sequence inserted into the UL83 open reading frame encoded the HLA-A2 presented peptide $IE1_{TMY}$ [$IE1_{297-305}$], flanked by additional amino acids to enable accurate proteasomal processing. The additional polypeptide fused to pp65 reads TSDACMMTMYGGISLLSEFC, with the HLA-A2 presented nonapeptide underlined. Viral reconstitution from BAC clones was performed according to Hobom et al. [29].

Virus stocks were generated, titrated and quantified either by counting IE1-positive cells at 48 h p.i. or by TaqMan DNA PCR analysis of extracellular viral genomes as described in Ref. [30]. HFF were infected with 10 genomes/cell for 7 days.

2.3. Dense body purification, indirect immunofluorescence analysis, electron microscopy and immunoblotting

DB were purified from late-stage infected HFF by glycerol–tartrate gradient ultracentrifugation as originally published by Irmiere and Gibson [31] and described previously in Ref. [22]. Indirect immunofluorescence analysis was carried out as described in Ref. [32]. The pp65 was labelled by using the monoclonal antibody (mAb) 65-33 (kindly provided by W. Britt, University of Alabama, Birmingham, AL, USA) and FITC-conjugated secondary antibodies (DAKO, Hamburg, Germany). The nucleus was counterstained with DAPI. Data from immunofluorescence analyses were collected at a magnification of 1000-fold. Electron microscopy was performed using a Leo 906E microscope (Zeiss) as previously described in Ref. [23]. For immunoblotting, protein samples were denatured under reducing conditions, separated by SDS-PAGE and transferred onto nitrocellulose membranes (Millipore, Schwalbach, Germany) by electroblotting at 400 mA for 1 h 45 min. The membranes were incubated with antibodies against pp65, β-actin (Rockland, Gilbertsville, PA, USA) and glycoprotein B (gB) [33]. Western blots were probed with anti-mouse or anti-rabbit secondary antbodies, conjugated to ALEXA Fluor 680 (Invitrogen, Karlsruhe, Germany) or IRdye 800 (Rockland). Blotted proteins were deteced and quantified using the Odyssey infrared imaging system (LI-COR, Lincoln, NE, USA).

2.4. Interferon-γ Elispot assay of HFF incubated with recDB

Interferon-γ (IFN-γ) enzyme linked immunospot (Elispot) assays were performed as described before in Refs. [25,34]. CTL lines specific for the HLA-A0201 (A2) restricted HCMV-derived peptides $pp65_{495-503}$ ($pp65_{NLV}$-CTL) [35,36] and $IE1_{297-305}$ ($IE1_{TMY}$-CTL) [37] were used in these analyses. The CTL lines had been generated by immunizing HLA-A2/huCD8 double-transgenic (tg) mice [25].

2.5. HLA-A2 transgenic mouse model

8–12 week-old HLA-A2 transgenic mice (HHD mice [38]) were immunized intraperitoneally with 6 μg of DB of RV-HB5 (DB-HB5), RV-SB3 (DB-SB3) or RV-SB6 (DB-SB6), respectively, or with PBS. Lymphocytes were prepared from the spleens at day 7 after immunization. CD8 T cells were enriched by MACS sorting and the frequency of IFN-γ secreting cells was analysed by Elispot directly *ex vivo*, using peptide loaded RMA-S HHD or T2 stimulator cells [24]. For this, Elispot plates from Millipore (Schwalbach/Ts., Germany) were used. The Elispot assay was performed according to the manufacturer's recommendations. Frequencies of responding cells were determined by linear regression analysis as described by Böhm et al. [39].

2.6. CD4 T cell analysis

For determining the activation of CD4 T cells, BALB/cJ mice were immunized intraperitoneally with 20 μg of the respective DB. Seven days after immunization, the spleens of each group were pooled (DB-HB5: 2 mice; DB-SB3: 3 mice) and the lymphocytes were isolated. Cells were seeded in 24-well plates in RPMI 1640, supplemented with 10% FCS, L-glutamine (100 mg/l), gentamicin (50 mg/l) and 5 μM β-mercaptoethanol (99%) at a density of 10^6 cells/ml. Cell suspensions were directly restimulated by adding 20 μg of the respective DB. After stimulation for 24 h, splenocytes were washed with ice cold PBS and stained with an FITC-coupled anti-CD4 mAb (GK1.5; BD Biosciences) and a phycoerythrin-coupled CD62L specific mAB (MEL-14; Immunotools) or the respective isotype control antibody (Ab) for 20 min at 4 °C. After additional washing with ice cold PBS, the expression of CD62L on $CD4^+$ T cells [40] was analysed on a FACS Canto (BD Biosciences) as described before in Ref. [41].

2.7. Analysis of neutralizing antibody responses

BALB/cJ mice were immunized twice intraperitoneally within a 2 week interval with 20 μg of the respective DB or, for control, with PBS. Blood from immunized animals was collected 2 weeks after the second application by puncture of the retrobulbar vessels of the eyes. Samples were allowed to clot at 4 °C overnight. For the clarification of the sera, samples were centrifuged at 4 °C for 10 min at 8000 × *g*. Sera were transferred to a sterile tube. HFF were seeded on 96 well plates, which were precoated with 01% gelatine o./n. at 37 °C [42]. Neutralization assays were performed according to Andreoni et al. [43], as described before in Ref. [22].

3. Results

3.1. Insertion site selection is critical for formation of cytoplasmic DB

The potential of a vaccine candidate to be further developed critically depends on the yield that can be achieved for further up-

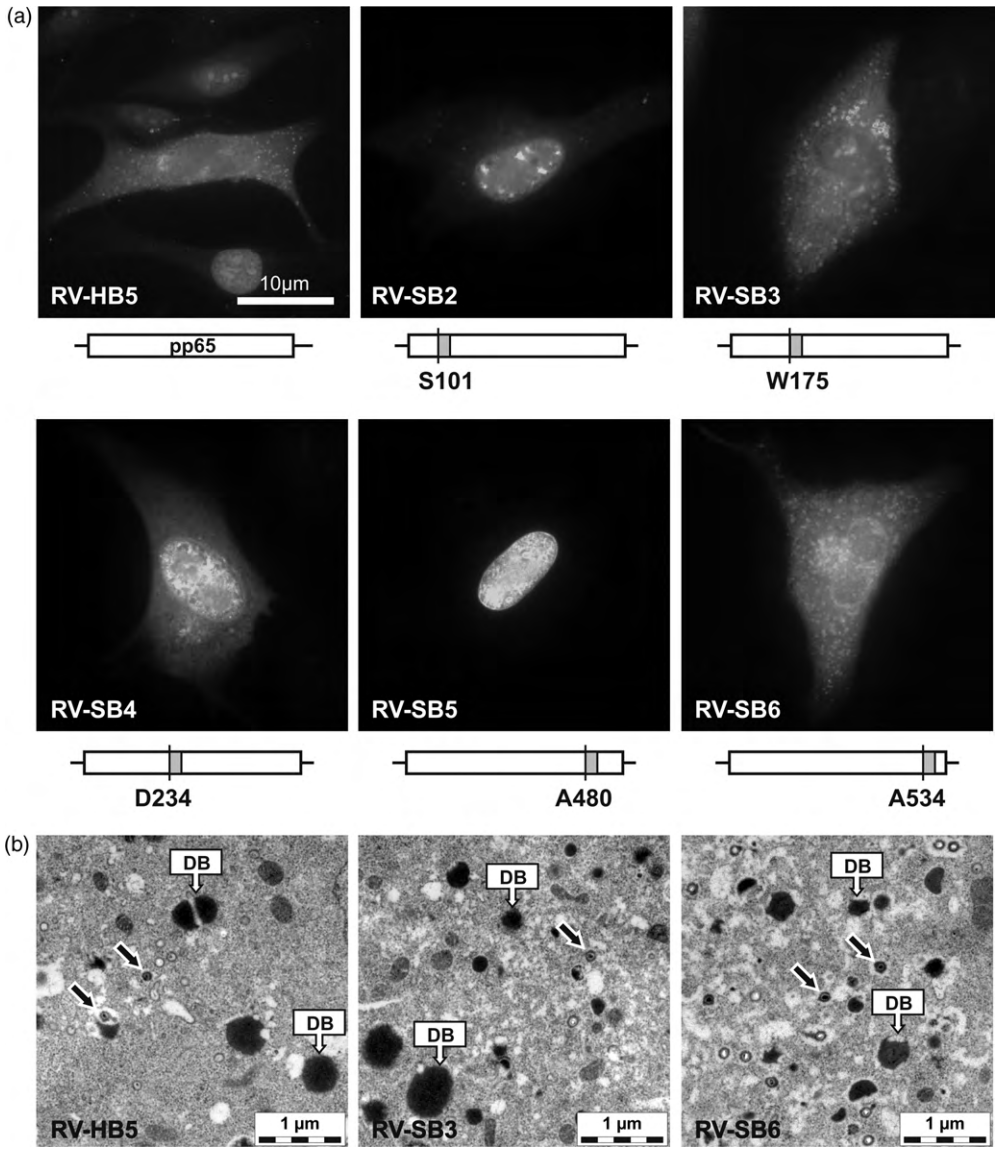

Fig. 1. Indirect immunofluorescence analysis and electron microscopy of HFF, infected with HCMV recombinants. (a) Cells were infected with the indicated viruses for 4 days and were subsequently processed for immunofluorescence staining with pp65-specific mAb 65-33. The configuration of the recombinant pp65, expressed by each virus is indicated below the micrographs. The N-terminal amino acid of pp65, flanking the insertion site is denoted. (b) Transmission electron microscopy analysis of RV-HB5-, RV-SB3- and RV-SB6-infected HFF. Cells were infected for 6 d.p.i. before being processed for conventional electron microscopy. In cells infected with all three viruses, DB appeared in the cytoplasm as electron dense spheres. C-Capsids in the cytoplasm are marked by black arrows.

scaling. We could recently show that DB can be modified in their antigenic content by inserting a heterologous peptide sequence to the tegument protein pp65 [23]. However, cells infected with the respective recombinants of HCMV released only limited amounts of recDB. We thus tested the hypothesis that insertion at other sites within pp65 would enhance the yield of recDB. Five sites in different regions of the molecule were selected for insertion (Fig. 1a). The inserted peptide consisted of 20 amino acids encompassing the HLA-A2 presented nonapeptide TMYGGISLL ($IE1_{TMY}$) from the HCMV IE1 protein [37]. Recombinant viruses were generated by modifying the HCMV BAC pHB5 [27], using the galK-based selection procedure [23,24]. Recombinant BACs were analysed for accuracy by restriction endonuclease digestion and nucleotide sequencing of the insertion site (data not shown). Reconstitution of recombinant viruses was subsequently performed by transfection of the BACs into HFF. The resulting viruses were tested for DB formation. One hallmark of HCMV infection of HFF is the cytoplasmic accumulation of DB, which can be visualized by indirect immunofluorescence analysis (IFA). Consequently, HFF were infected with the newly generated mutants and IFA was performed at 4 days post-infection (d.p.i.), using a pp65-specific antibody for detection (Fig. 1a). Virions were seen as small fluorescent dots within the cytoplasm whereas DB were visible as larger fluorescent dots. Only cells infected with the mutants RV-SB3 and RV-SB6 showed the formation of cytoplasmic DB in a way comparable to the formation of DB in parental-virus infected cells. In contrast, no or only little DB formation was seen in RV-SB2, RV-SB4 and RV-SB5 infected cells. Note that in these cells, the nucleo-cytoplasmic translocation of pp65, which is typically seen in late-stage infected HFF was impaired. These results indicated that site selection for insertion of heterologous peptide sequences within pp65 was critical for recDB formation. Based on the results the viruses RV-SB3 and RV-SB6 were chosen for further analysis.

To verify the synthesis of DB after infection of HFF with RV-SB3 and RV-SB6, transmission electron microscopy was performed. Cells were infected for 6 days and subsequently processed for

analysis. Cytoplasmic DB became apparent in both RV-SB3- and RV-SB6-infected cells (Fig. 1b). The formation of these particles was comparable to the formation of wild-type DB (wt-DB) in cells, infected with parental RV-HB5. These data showed that insertion of the heterologous sequence in at position 175 of pp65 in RV-SB3 and at position 534 of pp65 in RV-SB6 did not disturb the potential for DB formation.

3.2. Recombinant viruses RV-SB3 and RV-SB6 are comparable to the parental strain with respect to pp65-expression and genome release

Expression levels of pp65 in RV-SB3- and RV-SB6-infected HFF were tested by immunoblot analysis. Cells were infected with either virus or with parental RV-HB5 for 2 or 4 days, respectively. Cells lysates were subjected to quantitative immunoblot analysis, using the Odyssey infrared imaging system. The amount of cellular actin was taken as an internal standard (Fig. 2a). Expression levels of modified pp65 in RV-SB3- and RV-SB6-infected cells were slightly reduced at 2 days p.i., compared to levels of wt-pp65 in cells infected with parental RV-HB5. However, at 4 days p.i., the levels of the two fusion proteins appeared to be even higher compared to wt-pp65 in RV-HB5 infected cells. This indicated that sufficient protein was synthesized in RV-SB3- and RV-SB6-infected cells to direct the synthesis of recDB. To analyse progeny release from infected HFF, viral DNA was quantified in the culture medium as a surrogate marker [30]. Cells were infected with 10 genome copies/cell. Culture supernatant was collected in daily intervals until 7 days p.i.. Viral DNA released into the supernatant was quantified using quantitative PCR analysis (Fig. 2b). In these analyses, both RV-SB3 and RV-SB6 proved generate progeny to similar levels as the parental RV-HB5 strain.

3.3. RecDB are released from infected HFF and contain the modified versions of pp65

To analyse whether RV-SB3- and RV-SB6-infected cells released recDB, culture supernatant was collected at 6 days p.i. and subjected to glycerol-gradient ultracentrifugation. A banding pattern, comparable to that of wt-HCMV (RV-HB5) was detectable in the gradients from RV-SB3- and RV-SB6-infected cells (Fig. 2c). Bands represented non-infectious enveloped particles (NIEPs), virions and DB [44]. In several independent experiments, the amount of DB-SB6 appeared to be lower, compared to the amount seen for DB-SB3. Measuring protein concentrations of recDB, the average yield of DB-SB3 of three independent preparations was 1.35 μg/ml supernatant versus 0.77 μg/ml supernatant of DB-SB6 (data not shown).

Finally, packaging of the fusion proteins was analysed by immunoblot of gradient purified virions and DB. Normalization was performed using the viral envelope glycoprotein B (gB) as internal standard. No differences in the packaging of pp65 into virions or DBs were found when the recombinant strains were compared to their parental strain (Fig. 2d). Taken together these results indicated that DB-SB3 and DB-SB6 were released from infected cells and that both particle types contained the modified pp65.

3.4. Both $pp65_{NLV}$ and $IE1_{TMY}$ are presented by DB-SB3- or DB-SB6-treated HFF

One goal of using recDB for vaccine development would be to support cytotoxic T cell reconstitution in patients following transplantation. These cells are of critical importance for the prevention of viral reactivation and disease ([13,15] reviewed in Ref. [16]). We thus tested, whether the recDB were capable to introduce the IE1-derived peptide into the MHC-class I presentation pathway of HFF. Cells were treated with recDB and were subsequently sub-

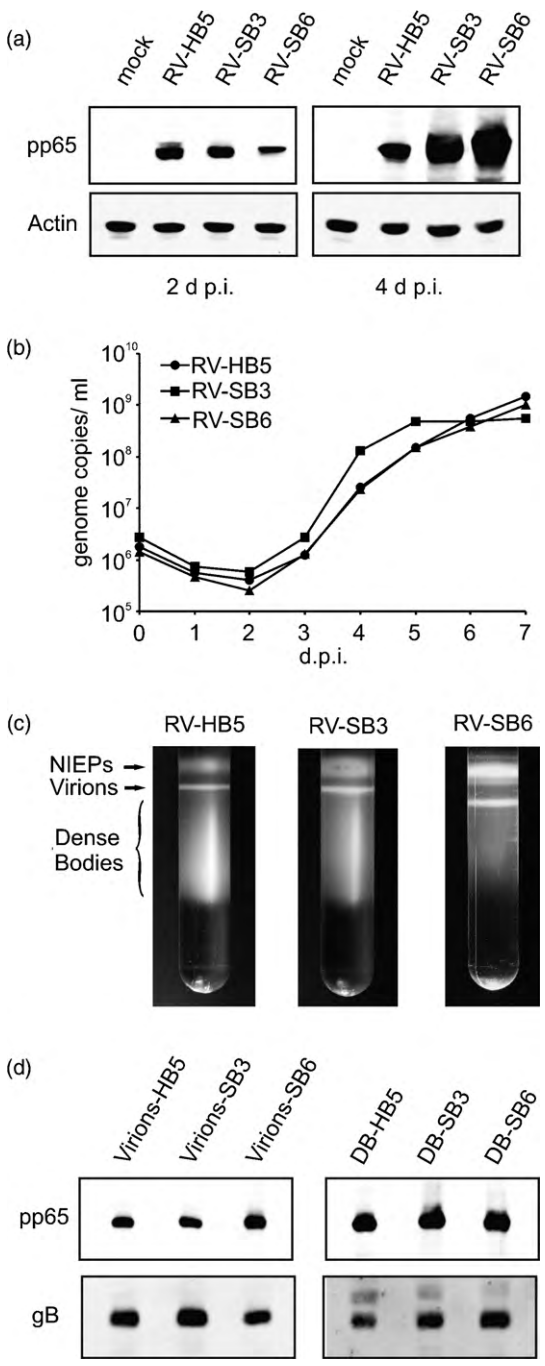

Fig. 2. Expression of pp65 proteins, release of particles from infected cells and efficiency of packaging of pp65 variants into virions and DB. (a) Immunoblot analysis of 2 and 4 days infected HFF. Cells were infected at an m.o.i. of 2 and were collected at the indicated times for analysis. Filters were probed with pp65-specific mAb 65-33. Amounts of protein in each lane were normalized against β-actin. (b) Quantitative TaqMan DNA PCR analysis of viral genomes in the cell culture supernatant of infected HFF. Cells were infected at an m.o.i. of 10 genomes/cell. Culture supernatants were collected at the indicated time points and frozen. PCR analysis was performed in parallel in the same assay. (c) Glycerol tartrate gradients of particles, released in the culture supernatant. Bands representing NIEPs, virions and DB are depicted. (d) Immunoblot analysis of pp65, packaged in recDB or virions. Fractions obtained by collecting the virions- and DB-bands from the gradients were analysed by immunoblot, using mAb 65-33 or, as internal standard, using a mAb against the viral gB.

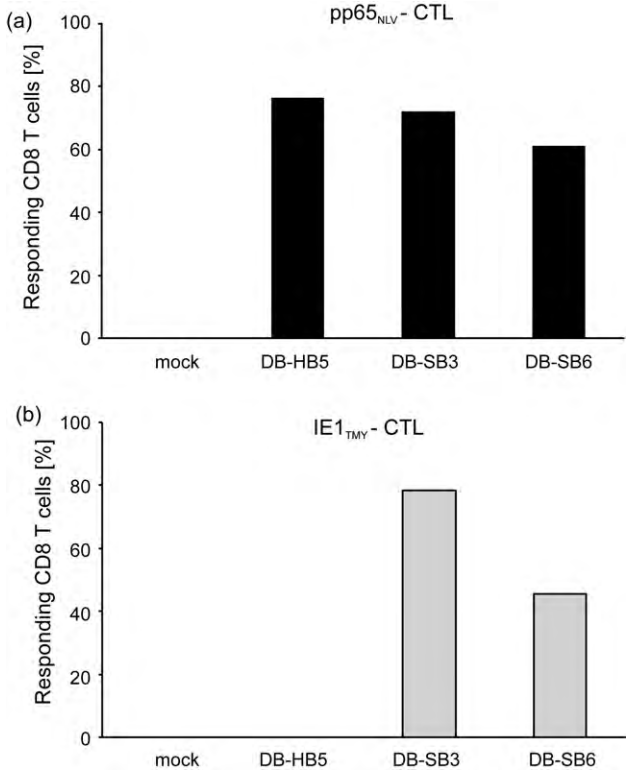

Fig. 3. IFN-γ Elispot analysis of MHC-class I presentation by HLA-A2 positive HFF, treated with recDB. Cells were treated with the indicated DB and were subsequently used as stimulator cells in IFN-γ Elispot analysis, using either $pp65_{NLV}$-CTL (a) or $IE1_{TMY}$-CTL (b) as responder cells. Columns represent means of spots from three independent wells. Results shown are representative of two independent experiments.

jected to IFN-γ Elispot analysis (Fig. 3). As responder cells, a CTL clone against $IE1_{TMY}$ was used [25]. For control, a CTL clone specific for the $pp65_{NLV}$-peptide ($pp65_{NLV}$-CTL) was employed. Both DB-SB3- and DB-SB6-treated cells presented $IE1_{TMY}$ and $pp65_{NLV}$. The $pp65_{NLV}$-presentation was comparable between recDB and wt-DB, indicating that the insertion of the IE1-derived sequence did not impair pp65-presentation in HFF. Treatment of cells with DB-SB3 led to numbers of responding $IE1_{TMY}$-CTL to levels that were comparable to the pp65-specific response. However, treatment of cells with DB-SB6 led to a markedly reduced number of positive spots in the IE1-specific assay. This indicated that the ability of HFF to present the IE1-peptide after DB-incubation was sensitive to the site, where the peptide was inserted into pp65.

3.5. *Immunization with recDB primes considerable frequencies of $IE1_{TMY}$-specific and $pp65_{NLV}$-specific CD8 T cells*

Previous experiments had shown that recDB are suitable to induce $IE1_{TMY}$-specific CD8 T cells in mice. These cells were, however, only detectable after *in vitro* stimulation of CD8 T cell fractions with the cognate peptide. No responding CD8 T cells were detected directly *ex vivo*, indicating that the total number of specific cells, and thus the overall response to those recDB was low [23]. To evaluate the immunological potential of the newly established recDB, HLA-A2 transgenic HHD mice were immunized with the different DB in the absence of adjuvant. Cells from immunized mice were also tested directly *ex vivo*. For this, the $CD8^+$ fractions of spleen cells were separated by MACS sorting and tested in Elispot analysis, using peptide loaded antigen presenting cells. CD8 T cells, specific for $IE1_{TMY}$ could be detected after immunization with both DB-SB3

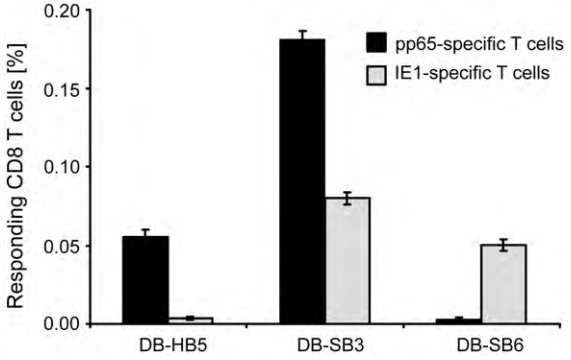

Fig. 4. *Ex vivo* IFN-γ Elispot analysis of CD8-enriched spleen cells from HHD mice, immunized with recDB without adjuvant. RMA-S cells, loaded with the cognate peptides were taken as stimulator cells. Bar sizes represent most probable frequencies of IFN-γ secreting CD8 T cells, as determined by linear regression analysis, described by Böhm et al. [39]. Error bars indicate 95% confidence intervals. Results shown are representative of three independent experiments.

and DB-SB6 (Fig. 4). The IE1-specific response primed by DB-SB3 appeared to be stronger than the response induced by DB-SB6, but both reached clearly detectable levels. After immunization with DB-SB3, CD8 T cells reactive against the $pp65_{NLV}$ could be detected to a roughly threefold level, compared to wt-DB. Surprisingly, however, $pp65_{NLV}$-specific CD8 T cells were almost undetectable after immunization with DB-SB6. This result was confirmed upon repeating the experiment and indicated that the CD8 T cell response, primed against the immunodominant $pp65_{NLV}$-peptide by immunizing with DB-SB6 was inefficient (Fig. 4). Comparable results were obtained when T2 cells were chosen for antigen presentation (data not shown). Taken together these experiments proved the potential of DB-SB3 to prime a CD8 T cell response against a heterologous antigenic peptide. Consequently, the position 175 of pp65 was identified as a suitable insertion site for the further development of the vaccine.

3.6. *Immunization with DB-SB3 stimulates CD4 T cell and neutralizing antibody responses*

It could previously be shown that wt-DB stimulated CD4 T cell as well as neutralizing antibody responses [22,45]. Consequently, DB-SB3 were tested for the conservation of these immunogenic properties. In a first approach, BALB/cJ mice were immunized with DB-SB3 or wt-DB. Splenocytes were isolated from these animals at day 7 after application. Cells were plated and wt-DB or DB-SB3 were added to the cultures. After 24 h of antigenic stimulation, reduced expression of CD62L on CD4 T cell fractions as an indicator for activation was measured by FACS [40] (Fig. 5) A portion of the CD4 T cells from both wt-DB and DB-SB3-immunized animals displayed a $CD62L_{low}$ phenotype. This showed that both wt-DB and DB-SB3 stimulated CD4 T cells.

Next, the induction of virus specific neutralizing antibodies by DB-SB3 was tested. For this, DB were applied twice intraperitoneally in a 2 weeks interval to BALB/cJ mice. Blood was collected 2 weeks after the second injection and the respective sera were tested in a microneutralization assay [22,43]. Sera obtained from wt-DB- and DB-SB3-immunized animals showed a completely identical capacity of virus neutralization, as evidenced by the reduction of cells expressing the IE1-antigen (Fig. 6). This confirmed the potential of DB to induce an antiviral neutralizing antibody response and proved that this property had been retained despite the genetic modification that was used to generate DB-SB3.

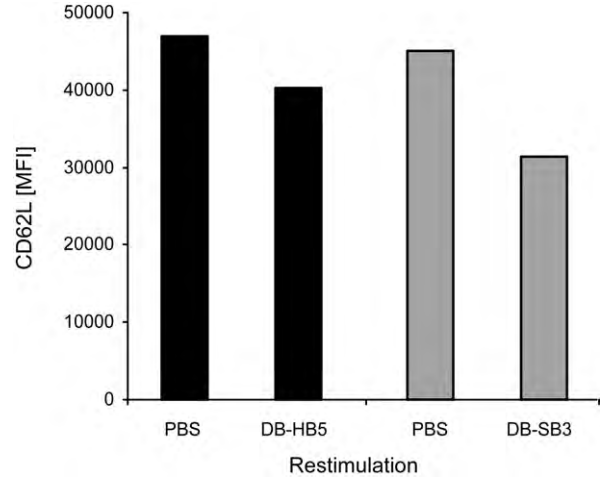

Fig. 5. Stimulation of CD4 T cells by DB-SB3. BALB/cJ mice were immunized intraperitoneally with DB-SB3 (3 mice) or wt-DB (2 mice) without adjuvant. Splenocytes were collected, pooled and plated for 24 h in the presence of DB or PBS. Reduced expression of CD62L was measured on CD4 T cell fractions by FACS as an indicator for T cell activation. Black bars, immunization with wt-DB. Grey bars, immunization with DB-SB3. MFI, mean fluorescence intensity.

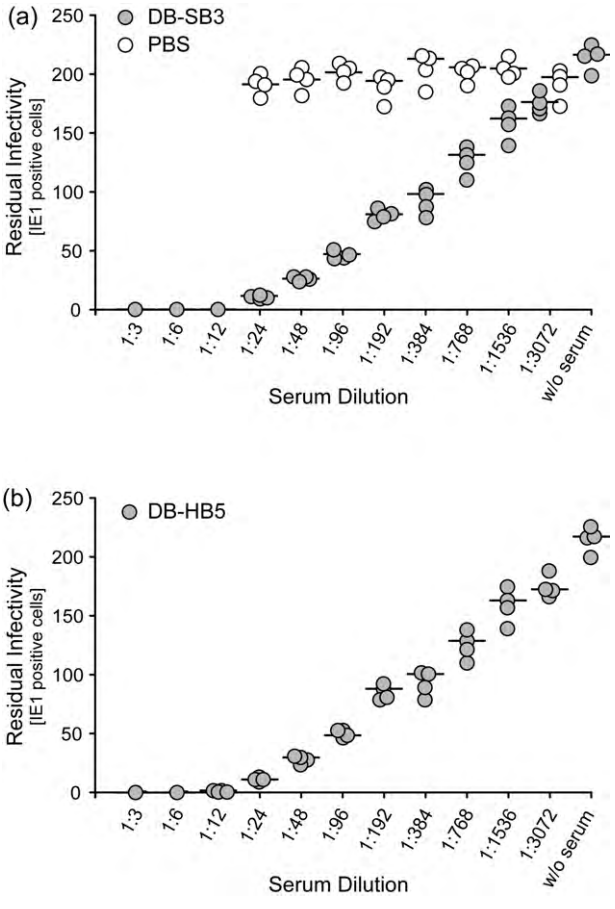

Fig. 6. Induction of virus-neutralizing antibodies by immunization with DB-SB3. BALB/cJ mice were immunized twice intraperitoneally in a 2 weeks interval with DB-SB3 or wt-DB. The sera of these animals were collected 2 weeks after the second injection and analysed by a microneutralization assay. Residual infectivity was determined by counting IE1-positive cells per well and was plotted against serum dilutions. Symbols represent data from independent samples. Median values are indicated by horizontal bars. Grey symbols, immunization with DB, open symbols, immunization with PBS.

4. Discussion

HCMV is a pathogen for all seasons [10], affecting different groups of patients. From numerous studies addressing the immune effectors that afford protection, it appears evident that an ideal vaccine against HCMV should induce both antiviral neutralizing antibodies and T lymphocytes [10,17,18,46]. There is, however, concern if such an universal HCMV vaccine may ever be established. A recent clinical study showed that alphavirus replicons expressing HCMV proteins were well tolerated and induced sustained cellular and humoral responses [19]. Another study employed purified gB as vaccine and provided encouraging results, as significant levels of HCMV neutralizing antibodies could be induced [18]. These vaccines hold promise to be used for the prevention of congenital HCMV infection. In this study, we focused on the development of a DB based vaccine to preferentially ameliorate the consequences of viral reactivation following transplantation. Cytotoxic T lymphocytes are known to be essential for the control of viral reactivation and the limitation of viral infection in this particular setting [13,15]. This study consequently focused on the identification of a strategy to optimize a DB based vaccine with respect to the induction of CD8 T cell responses by generating recombinant HCMV, expressing a chimeric pp65.

Previous studies had shown that the selection of the site in pp65 for insertion of heterologous sequences is critical with respect to the efficiency of DB formation and release by infected cells [23,24,47]. Little is known about the function of pp65 and its domains, important for function, proper folding of the protein and for its packaging into particles. Consequently, there is no rationale for insertion site selection that would definitely spare relevant regions within pp65. Insertion site selection was thus performed in a way to avoid both regions conserved in beta-herpesviruses and regions with predicted secondary structures (α-helices and β-sheets). This was done assuming that conserved regions, α-helices and β-sheets could be structurally and functionally important and should not be destroyed. However, most of these mutants failed to efficiently form recDB, indicating that this strategy could not identify proper insertion sites. Opposed to that, insertion sites in RV-SB3 (pp65-175) and RV-SB6 (pp65-534) were randomly chosen and proved to be more appropriate for recDB formation. The suitability of the position 175 was later verified by the insertion of the model peptide SIINFEKL from ovalbumin, thereby generating recombinant DB containing a pp65-SIINFEKL fusion protein (unpublished data). However, additional experiments are warranted to investigate, if larger heterologous peptides may also be inserted at this site without impairing the yield of recDB formation and release.

Protein quantification of purified DB revealed that synthesis and/or release of DB-SB6 was less efficient compared to DB-SB3. In contrast, DB-SB3 preparations reached about 40–50% of the yield obtained with wt-DB. However, the yields obtained with a given virus varied considerably between experiments. No attempts were made to optimize the DB preparation procedures for individual recDBs. It is thus reasonable to assume that the yields of recDB can be significantly increased by the development of a standardized procedure which is optimized for a specific type or recDB.

Exogenous introduction of chimeric pp65 by DB-SB3 and DB-SB6 into HFF led to efficient presentation of both the $pp65_{NLV}$- and $IE1_{TMY}$-peptides. Application of these particles to HLA-A2 transgenic HHD mice primed a CD8 T cell response against both peptides that could be detected after *in vitro* expansion of these T cells with the cognate peptide. Similar results have also been obtained in previous experiments, using recDB with insertion of the IE1-peptide at position 548 of pp65 [23,24]. However, using the latter recDB, CD8 T cells against either peptide were undetectable directly *ex vivo*, suggesting that priming was inefficient. Application of DB-SB3, in

contrast, led to readily detectable frequencies of $pp65_{NLV}$-specific and $IE1_{TMY}$-specific CD8 T cells without restimulation. This indicates that these recDB were highly immunogenic and induced T cells against both the endogenous and the heterologous peptide.

Immunization of HHD mice with DB-SB6 also primed a detectable CD8 T cell response against the heterologous $IE1_{TMY}$-determinant, although this response was reduced compared to DB-SB3. Surprisingly, however, in repeated experiments, a $pp65_{NLV}$-specific response could not be detected *ex vivo* for this mutant. This indicates that CD8 T cell responses against the $pp65_{NLV}$ were induced in only low frequencies, which were below the detection limits of the assay. In support of this, restimulation of $pp65_{NLV}$-specific T cells *in vitro* was delayed compared to $IE1_{TMY}$-specific cells (data not shown). The reason for this poor priming of a response against the immunodominant $pp65_{NLV}$-peptide in DB-SB6 is unclear at the current stage. Mutation of the peptide sequence imposed by the genetic manipulation of the virus could be excluded by nucleotide sequencing of the coding region (data not shown). Competition of the two HLA-A2 presented peptides in priming are unlikely, since both $pp65_{NLV}$-CTL and $IE1_{TMY}$-CTL could be detected after immunization with DB-SB3. Interference of the newly inserted peptide with proteasomal processing of $pp65_{NLV}$ also appears unlikely. Although flanking sequences have been shown to be critical for proteasomal processing [48–50], this has only been reported for regions within close proximity of a given antigenic peptide. However, $pp65_{NLV}$ and $IE1_{TMY}$ are separated by roughly 30 amino acids in the primary structure of pp65-SB6. To our knowledge, direct interference of proteasomal processing of antigenic peptides over a spacer region of 30 amino acids has never been demonstrated.

Another explanation for the failure of DB-SB6 to induce $pp65_{NLV}$-CTL could be that the insertion of the IE1-peptide at position 534 of pp65 altered the structure of the tegument protein in a way to impede its access to the proteasome. Since $IE1_{TMY}$ is closer to the carboxyterminus of the protein than $pp65_{NLV}$, the former peptide could be more readily accessible to proteolytic degradation compared to the latter. However, again, this is merely speculative at this point and further analyses would be warranted to elucidate the poor priming by DB-SB6.

A successful HCMV vaccine candidate should address not only CD8 T cell responses, but should also induce CD4 T lymphocytes. The latter cells were shown to be essential for a sustained CD8 T cell response in transplant recipients [15]. The DB-SB3 were stimulated CD4 T cell responses. Consequently, these particles appear attractive for the development of a therapeutic vaccine for HSCT recipients. For the prevention of congenital HCMV infection, however, induction of neutralizing antibodies is critical. The DB-SB3 induced virus-neutralizing antibodies to levels that were indistinguishable from the levels induced by wt-DB. Thus DB-SB3 may also be attractive for further development for a more widely applicable vaccine.

In summary, we have defined a site at position 175 of pp65, which is suitable to accommodate heterologous peptide antigens without impairing recDB formation and release by infected cells. We further showed that injection of one single dose of these recDB without adjuvant induced significant CD8 T cell responses, which were readily detectable without *in vitro* restimulation. This study thus provides a rationale to further develop DB-SB3 as a first candidate to be tested in clinical trials.

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