Evaluation of recombinant modified vaccinia Ankara virus-based rhesus cytomegalovirus vaccines in rhesus macaques

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Abstract A vaccine consisting of rhesus cytomegalovirus (RhCMV) pp65-2, gB and IE1 expressed via modified vaccinia Ankara (MVA) was evaluated in rhesus macaques with or without prior priming with expression plasmids for the same antigens. Following two MVA treatments, comparable levels of anti-gB, pp65-2 and neutralizing antibody responses, and pp65-2- and IE1-specific cellular immune responses were detected in both vaccinated groups. Similar reductions in plasma peak viral loads were observed in these groups compared to untreated controls. This study demonstrates the immunogenicity and protective efficacy of rMVA-based RhCMV subunit vaccines in a primate host

and warrants further investigation to improve the efficacy of subunit vaccines against CMV.

Keywords Cytomegalovirus vaccine · Rhesus macaques · Modified vaccinia Ankara virus

Introduction

Development of an effective vaccine for human cytomegalovirus (HCMV) has been designated a “level 1” priority by the Institute of Medicine because of the devastating impact of congenital infection by HCMV which can have lifelong consequences [19]. The strict species-specificity of the virus requires assessment of vaccine strategies in a cognate animal model. Rhesus CMV (RhCMV) infection of rhesus macaques (*Macaca mulatta*—Mmu) strongly recapitulates HCMV infection in humans in terms of the similarity of viral genome, persistence, pathogenesis and host immune responses [2–4, 26, 27], therefore, it represents a clinically relevant model to evaluate HCMV vaccine strategies.

Measurement of natural immunity to HCMV supports targeting the predominant neutralizing antibody response antigen gB and the strongly recognized cellular immunogens pp65 and IE1 as the optimal antigens in a vaccine formulated to prevent infection and control disease [28]. Previously, a study in Mmu demonstrated that immunization with DNA vaccines expressing RhCMV pp65-2 and truncated gB with deletion of transmembrane and intracellular domains (gBΔ) stimulated antigen-specific CD8+ T cell and antibody responses and provided limited protection against challenge [25]. A notable observation of this study was that a combination of DNA priming administered five times in 50 weeks followed by a heterologous boost (in this case, by the virus challenge) stimulated a rapid and profound

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increase in neutralizing antibody titers. In this report, we compared an approach in which recombinant modified vaccinia Ankara (rMVA) expressing RhCMV pp65-2, gB and IE1 antigens was administered twice to Mmu versus an identical schedule of rMVA administration preceded by a single DNA prime. Our primary goal was to establish whether these sub-unit vaccines are effective for controlling an experimentally applied CMV infection and to test whether DNA priming enhances the effectiveness of rMVA to stimulate protective immunity in this model.

Results

RhCMV-rMVA construction and expression

As a first step, the genes encoding RhCMV₆₈₋₁ pp65-2 and gB were amplified from previously described plasmid expression vectors [25, 27] and engineered into the pZWIIA MVA transfer vector (Rh-gB-pp65-pZWIIA, Fig. 1a), using established protocols [21]. Similarly, the RhCMV IE1 gene was amplified from a plasmid (pND/IE1) containing the cDNA (exons 1–4) for the RhCMV₆₈₋₁ IE1 [11] and engineered into the pLW51 MVA transfer vector (Rh IE1-pLW51, [22]).

rMVA expressing either both RhCMV gB and pp65-2 (Rh gB-pp65-MVA) or IE1 alone (Rh IE1-MVA) was generated on BHK-21 cells via homologous recombination.

The protein expression levels for RhCMV gB, pp65-2 and IE1 in infected BHK-21 cells were confirmed by Western blot using polyclonal antibodies to RhCMV gB, pp65-2, and IE1 by chemiluminescence detection (ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK) (Fig. 1b).

Although the genetic stability of the rMVA constructs was not directly evaluated, previous work using the same recombination plasmid pZWIIA to express HCMV soluble gB and/or pp65 and IE1 exon-4 demonstrated that the resultant rMVA constructs were stable through four passages [20, 21]. The genetic instability of rMVA that has been observed usually happens when attempting to express certain integral membrane proteins at high levels, which may be due to the toxicity of over-expressed proteins [23]. The genetic stability of rMVA expressing membrane proteins can be enhanced after truncating the cytoplasmic domain [22, 23]. In this report, we used the same strategies and methods to construct the rMVAs expressing RhCMV truncated gB, pp65-2 and IE1. RhCMV gB, pp65-2 and IE1 are homologues of HCMV gB, pp65 and IE1 and the results in Fig. 1b confirm expression of the foreign inserts. Aliquots of the same rMVA stocks used to demonstrate expression in infected tissue culture cells (Fig. 1) were used to infect rhesus macaques.

Vaccination-induced immune responses

Six age-matched RhCMV-seronegative Mmu (2 years of age) were divided into three groups—DNA prime/rMVA/

Fig. 1 rMVA construction and expression in vitro. **a** Construction of two rMVA constructs containing either truncated RhCMV gB and pp65-2 genes (Rh gB-pp65-MVA) or IE1 gene alone (Rh IE1-MVA). FL1 and FL2 represent distinct flanking MVA DNA sequences abutting either deletions II (Del II) or III (Del III). DRs are identical direct repeat sequences used for intragenomic homologous recombination to remove the marker genes, *gus* and *EGFP*. The expression of RhCMV gB, pp65-2 and IE are under the control of synthetic vaccinia promoters, Psyn I and II, respectively. Rh gB-pp65-pZWIIA and Rh IE1-pLW51 are transfer plasmids. **b** Western blot detection of Rh gB, pp65-2 and IE1 protein from Rh gB-pp65-MVA and Rh IE1-MVA infected BHK-21 cells. Lanes 1 and 5 cell lysate of Rh gB-pp65-MVA #1 infected BHK-21 cells; lanes 2 and 6 cell lysate of Rh gB-pp65-MVA #2 infected BHK-21 cells; lanes 3, 7 and 10 cell lysate of wild type MVA infected BHK-21 cells as (–) negative control; lanes 4 and 8 cell lysate from uninfected BHK-21 cells; lane 9 cell lysate of Rh IE1-MVA infected BHK-21 cells

rMVA boost (DNA/rMVA), rMVA prime/rMVA boost (rMVA) and untreated control. Initially, DNA/rMVA animals were immunized intramuscularly and intradermally with a mixture of three plasmids (in pND vector [13]) expressing RhCMV pp65-2, gB Δ , and IE1 at week 0, following a previously published protocol [25]. Subsequently, all the DNA/rMVA and rMVA animals were injected intramuscularly with a mixture of Rh gB-pp65-MVA and Rh IE1-MVA (5×10^8 pfu of each) at weeks 6 and 12 (Fig. 2). The elicited immune responses were measured by antigen-specific ELISA, an EGFP-fluorescence intensity reduction-neutralizing assay using an EGFP-RhCMV [6], and intracellular cytokine staining (ICS). The ICS was performed as previously described protocols [5] to detect the frequency of CD4+ and CD8+ T cells that express IFN- γ or TNF- α upon re-stimulation with a pool of overlapping peptides (15-mers overlapping by 11 amino acids) comprising full-length IE1 or pp65-2 antigen [26]. Briefly, freshly isolated 1.0×10^6 peripheral blood monocytes (PBMCs) were stimulated for 6 h with the peptide pool or medium in the presence of monoclonal antibodies to CD28 and CD49d. Brefeldin A was added to the culture for the final 5 h of stimulation. SEB stimulated PBMCs were used as positive control. The stimulated cells were first surface stained with conjugated antibodies to CD3 and CD8, or CD3 and CD4 for 30 min at 4°C. After fixation and permeabilization, the cells were incubated with conjugated anti-cytokine monoclonal antibodies for 30 min at 4°C. The stained cells were then washed, and resuspended in 1% paraformaldehyde. Three hundred thousand lymphocyte events were collected on a FACS ARIA instrument (Becton–Dickinson). Data were analyzed using Flowjo software (version 8.1, Treestar, Ashland, OR).

DNA/rMVA and rMVA animals exhibited different kinetics of antigen-specific and neutralizing antibody responses (Fig. 3). As expected, single DNA immunization was not effective by itself, but effectively served to prime the animals for rMVA boosting (0–6 weeks, Fig. 3). Following the first rMVA vaccination (6–12 weeks, Fig. 3), both DNA/

rMVA animals generated higher levels of anti-gB, pp65-2 and IE1 antibodies and 50% neutralizing titers (NT₅₀, 527 and 108, respectively) than Mmu receiving rMVA without DNA priming (Fig. 3). The two Mmu, only receiving rMVA, had low but detectable anti-gB responses and only one exhibited demonstrable NT₅₀ (Animal #4: 55) (Fig. 3). Anti-gB and pp65-2 antibody levels and NT₅₀ rose sharply in both groups after the second rMVA immunization such that there were no differences between the two vaccine groups (12–18 weeks, Fig. 3). For the DNA/rMVA animals, the second rMVA boost stimulated peak anti-gB and anti-pp65-2 antibody responses within 1 week to levels higher than those observed after the first boost at weeks 7–8, but not peak IE1 antibody and NT₅₀ (185 and 77, respectively). In contrast, the NT₅₀ in the rMVA group were appreciably higher than those observed after the first rMVA immunization (294 and 122, respectively). rMVA immunization alone did not induce detectable anti-IE1 antibodies (Fig. 3).

IE1- and pp65-2-specific CD4+ and CD8+ T cells were also detected in DNA/rMVA and rMVA animals with different kinetics and magnitudes (Fig. 4). DNA priming followed by rMVA boosting generated both pp65-2- and IE1-specific CD4+ and CD8+ T cells mostly at 1–2 weeks after the first MVA booster, when a positive response was defined as a 0.01% increase of each stained cell population compared to the pre-vaccination baseline. The second rMVA immunization only stimulated transient increases prior to challenge. For the rMVA group, IE1- and pp65-2-specific CD4+ and CD8+ T cell responses in both animals were frequently detectable after the second rMVA immunization with the peak responses at week 14 or 18.

Plasma viral loads post-challenge

To evaluate the protective efficacy of the two vaccine strategies, all the animals were challenged intravenously (IV, 2.0×10^5 pfu) and subcutaneously (SC, 6×10^5 pfu) with RhCMV₆₈₋₁ 6 weeks after the second rMVA vaccination.

Fig. 2 DNA and rMVA vaccination regimen and RhCMV challenge. Six age-matched macaques were divided into three groups—DNA/rMVA, rMVA, and untreated control group. The DNA/rMVA animals were first primed with three DNA plasmids by intradermal (ID, 50 μ g of each plasmid) and intramuscular (IM, 150 μ g of each plasmid) needle injection at week 0. At weeks 6 and 12, all of the DNA/rMVA and rMVA animals were immunized with rMVA vaccines (5×10^8 pfu of

each rMVA vaccine). At week 18, all three groups of animals were intravenously (IV, 2.0×10^5 pfu) and subcutaneously (SC, 6.0×10^5 pfu) challenged with RhCMV strain 68-1. A quantitative real-time PCR (qPCR) was performed on the plasma samples collected prospectively from the time of challenge to measure RhCMV viral loads (RhCMV VL)

Fig. 3 Antigen-specific antibody and neutralizing antibody responses in DNA/rMVA-, rMVA-vaccinated and control monkeys. Antibody responses to RhCMV gB, pp65-2 and IE1 were analyzed by antigen-specific ELISA. Absorbance units at 450 nm (A_{450}) were obtained at a dilution of 1:100 of plasma samples. 50% neutralizing titers (NT_{50}) were titrated by an EGFP fluorescence reduction-neutralizing assay and were reported as the logarithmic values of the reciprocal endpoint dilution. The arrows indicate the time of DNA, rMVA and RhCMV administration and the horizontal dashed lines represent the threshold for a positive antibody response

The plasma viral DNA loads were evaluated by real-time PCR [12] (Fig. 5a). The reduction levels of peak viral loads in vaccines were calculated as $(1 - \text{peak viral loads of vaccinee} / \text{peak viral loads of control animal}) \times 100\%$. All four vaccinees exhibited different levels of reduction in peak plasma RhCMV genome copy numbers compared to the controls (Fig. 5b). One animal in each vaccine group (animals #2 and #3) had modest reductions (25.5–77.6%), while the other animal in each group (animals #1 and #4) had profound reductions (90.8–98.9%). Both of these latter animals were the same animals that had the higher NT_{50} titers at 1 week after the second rMVA immunization and the time of challenge. Although statistical tests did not show a significant correlation between the reduction levels of peak plasma viral loads and NT_{50} titers at 1 week after the second rMVA immunization (Spearman $r = 0.80$, $P = 0.33$) and at the time of challenge (Spearman $r = 0.80$, $P = 0.33$) in these four vaccinees, this may be due to the small numbers of Mmu. All four vaccinated animals initially cleared virus from plasma by day 10, compared to days 13 and 17 for the two control monkeys (Fig. 5a). Transient detection of RhCMV in plasma (less than 3.5×10^3 copy number/ml) was noted for two of the vaccinees and one of the control animals (data not shown).

In addition to plasma viral loads, skin biopsies of two sites of RhCMV inoculation (SC) were obtained on days 7 and 10 post-challenge to evaluate RhCMV replication at

local inoculation sites. IE1-expressing cells in skin tissue slides were analyzed by immunohistochemistry. No significant differences were observed between either the vaccinated and control groups (data not shown).

Immune responses post-challenge

All the vaccinees exhibited vigorous memory antibody responses post-challenge. These included anti-IE1 antibodies in the rMVA animals, which had no detectable antibodies prior to challenge (Fig. 3). The relative increases in NT_{50} of DNA/rMVA and rMVA animals at 1 and 2 weeks post challenge, compared to the time of challenge, were 24 to 44-fold and 101 to 453-fold, respectively (Fig. 3). It was also noted that the NT_{50} of DNA/rMVA animals at 2 weeks post-challenge were approximately two- to fivefold above those of the rMVA animals. The normative range of NT_{50} in long-term RhCMV seropositive macaques is 231–3,348 ($N = 24$, mean 973, median 833) (unpublished data). DNA/rMVA and rMVA/rMVA animals produced comparable NT_{50} at 1-week post-challenge and higher NT_{50} at 2-week post-challenge as compared to long-term naturally infected Mmu, whereas the control animals had comparable NT_{50} after 4 weeks post-challenge. RhCMV challenge stimulated a rapid increase in the frequencies of IE1- and pp65-2-specific CD8+ T cells in one of the DNA/rMVA treated Mmu (animal #2) and the frequency of pp65-2-specific CD8+ T

Fig. 4 RhCMV pp65-2- and IE1-specific T cell responses in DNA/rMVA-, rMVA vaccinated and control monkeys. The intracellular cytokine staining was used to detect the frequency of CD4+ and CD8+ T cells that express IFN- γ or TNF- α upon re-stimulation with a pool of overlapping peptides comprising full-length pp65-2 or IE1. The frequencies of IFN- γ - and TNF- α -positive CD4+ and CD8+ T cells were shown after deducting the baseline frequencies at the pre-vaccination or challenge. The *arrows* indicate the time of DNA, rMVA and RhCMV administration

cells in one of the rMVA treated animals (animal #4) at 1-week post-challenge (Fig. 4). The frequencies were comparable to or higher than those observed in two unvaccinated naturally RhCMV-seropositive animals, suggesting a memory T cell response in these animals. One unvaccinated control produced a very high frequency of IE1-specific CD8⁺ T cells at 7 weeks of challenge.

Discussion

In this report, we demonstrate that rMVA, either given as the sole vaccine or part of a heterologous prime/boost, can result in a dramatic decrease in viral load after RhCMV challenge in the Mmu model. Noteworthy was the need for only a single application of DNA to effectively prime the

Fig. 5 Plasma viral loads post-challenge. **a** The kinetics of plasma viral loads in DNA/rMVA-, rMVA-vaccinated and control animals. RhCMV genomic DNA in plasma was measured by a real-time PCR and was shown as RhCMV copy numbers per milliliter. **b** The reduction of peak viral loads in vaccinees (percentage) compared to the peak viral loads of control animals #5 and #6, respectively. The NT₅₀ titers of each vaccinee at 1 week post-second rMVA and at the day of RhCMV challenge was shown at the bottom

immune system for a heterologous boosting to produce higher levels of immunogenicity than observed with three DNA applications without viral boosting [20].

Modified vaccinia Ankara has been established as a safe and potent antigen delivery system. Promising data obtained from both nonhuman primates and clinical trials have shown that a combination of DNA prime and rMVA boost is effective in stimulating effective cellular immune responses and providing high protective efficacy against HIV/SIV and malaria infection [7]. In this study, following the first rMVA boosting, the monkeys primed with only a single application of DNA plasmids elicited earlier and stronger antibody and cellular immune responses than those without priming. However, similar magnitudes of anti-gB, pp65-2 and neutralizing antibodies and IE1- and pp65-2-specific CD4+ and CD8+ T cells were detected in both vaccinated groups after the second rMVA boost, with the exception of anti-IE1 antibodies, which were not detectable in rMVA animals.

Based on the level of IE1 expression in infected tissue culture cells (Fig. 1b), it was somewhat surprising that rMVA immunization in the absence of DNA priming did not stimulate detectable antibody responses. However, the results were not unprecedented. Other studies have shown that a recombinant canarypox expressing HCMV gB did not stimulate immune responses in vaccinated humans, despite expression both in vitro and in small animals (mice and guinea pigs) [1, 10]. Comparable to our study, the canarypox construct did prime the immune system as demonstrated by the memory response generated after infection with the live attenuated Towne vaccine. In our study, prior immunization with DNA or RhCMV infection post rMVA vaccination similarly demonstrated that RhCMV IE1 was recognized in vivo in the context of rMVA.

Both approaches provided comparable protection against challenge. It was also noted that the frequencies of pp65-2- and IE1-specific CD4+ and CD8+ T cells in both vaccine groups generally were low, and strong memory T cell responses were only found in one animal in each vaccination group post-challenge. The reasons for the relatively low level of IE1- and pp65-2-specific T cell responses in this study are unknown. Other studies have demonstrated readily detectable levels of T cell responses to these RhCMV proteins in naturally infected or vaccinated Mmu [25, 26]. Naturally infected Mmu with IE1 and pp65-2 responses were included as positive controls for all of the cellular immune assays. Future studies will address whether augmentation of vaccine-induced responses to IE1 and pp65-2 correlate with increased protection against RhCMV challenge.

An observation of interest in this study was the apparent influence of neutralizing antibody on the containment of viral challenge in which the levels of peak plasma viral load reduction were related to the levels of neutralizing antibodies prior to challenge. Although a larger numbers of Mmu are needed to confirm this observation, it further indicates the protective role of neutralizing antibodies against CMV [14, 17, 18, 24] and a gB-based vaccine strategy [15, 17, 25]. Natural immunity studies have suggested that a vaccine that stimulates immunity equivalent to levels associated with natural infection could reduce the morbidity due to congenital CMV infection by ~80% [8, 9]. While the employment of either DNA/rMVA or rMVA to deliver RhCMV gB did not induce a relevant natural level, long-lived neutralizing antibody response, other approaches including co-administration of GM-CSF, an adjuvant for the induction of NT [16], either expressed via DNA or MVA are being explored.

In summary, this study for the first time demonstrates the capacity of rMVA-based CMV vaccines to induce CMV-specific antibody and cellular immune responses in Mmu, and the protective efficacy in reducing plasma viral loads

following challenge. The data highlight the potential of rMVA-based CMV vaccines for humans and warrant continuing investigation of the DNA/rMVA approach in a larger number of Mmu to further test the possibility that subunit vaccines can interrupt horizontal transmission.

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