A cytomegalovirus-based vaccine provides long-lasting protection against lethal ebolavirus challenge after a single dose

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Running title: Single dose CMV vaccine gives durable Ebola protection

Abstract

Ebolavirus is a highly lethal hemorrhagic disease virus that most recently was responsible for two independent 2014 outbreaks in multiple countries in Western Africa, and the Democratic Republic of the Congo. Herein, we show that a cytomegalovirus (CMV)-based vaccine provides durable protective immunity from ebolavirus following a single vaccine dose. This study has implications for human ebolavirus vaccination, as well as for development of a 'disseminating' vaccine to target ebolavirus in wild African great apes.

The original zoonotic source of the 2014 *Zaire ebolavirus* (ZEBOV) outbreak in Western Africa is currently unclear (1, 2). Following transmission into the human population, the chain of ebolavirus infection is maintained by human-to-human transmission. Contact with wild animals serves as a main conduit for the initial zoonotic transmission of ebolavirus into the

human population (2-7). Fruit bats are believed to be a main source of human infection, and direct contact or exposure to environments inhabited and frequented by bats has been associated with human outbreaks (2, 4, 7). Great apes (western lowland gorillas and chimpanzees) are a second significant source of transmission due, in large part, to the bushmeat trade which drives humans and wild animals together within an environment conducive to zoonotic transmission (i.e., hunting and butchering) (3-5). Consistent with the importance of this route for zoonotic transmission of ebolavirus, a 2014 ZEBOV outbreak in the Boende Health Zone in the Equateur Province in the Democratic Republic of Congo, independent from the West Africa epidemic, was a result of handling and preparation of bushmeat (8). Ebolavirus is highly lethal in African great apes, and is regarded as a major threat to the survival of chimpanzees and gorillas in the wild (3, 5, 9-12). Vaccination of great apes has been proposed as one strategy to decrease the transmission of ebolavirus to humans, whilst at the same time also protecting these wild animal populations from the devastating effects of ebolavirus (4, 13, 14). We recently proposed the use of a CMV-based 'disseminating' vaccine as one approach to achieve vaccine coverage in the inaccessible and hostile environment of African tropical forest regions, where application of conventional vaccines using baiting/individual darting strategies may prove more difficult, if not impossible (14). CMV is a species-specific β-herpesvirus that is benign except in the immunocompromised host, such as individuals undergoing iatrogenic immunosuppression, AIDS patients (prior to HAART) and the neonate (15). CMV is also highly immunogenic, and has shown promise for development as a vaccine vector platform (16-20). We hypothesize that amongst other ebolavirus vaccine platforms, the established ability of CMV to spread easily through its host population regardless of CMV immune status (14, 21-24) makes this vector platform suited for development as a 'disseminating' ebolavirus vaccine that could spread ebolavirus-specific immunity from animal-to-animal without the need for direct vaccination of every individual. CMVs are extremely host specific (25, 26). In a previous

study we showed the ability of a single dose of a murine CMV (MCMV) expressing a CD8 T cell epitope from nucleoprotein (NP) of ZEBOV (MCMV/ZEBOV-NP_{CTL}) to induce durable ZEBOV-specific $CD8^+$ T cell immunity for at least 33 weeks (> 8 months) post-vaccination (14). In this earlier study, mice vaccinated with MCMV/ZEBOV-NP_{CTL} were protected against disease when challenged with a lethal ZEBOV dose of mouse-adapted ZEBOV (ma-ZEBOV) at 6 weeks post-boost. Previous studies using MCMV recombinants expressing pathogen target epitopes (influenza A and lymphocytic choriomeningitis virus) have shown long-lasting protective immunity (27). In the current study, we wanted to assess whether MCMV/ZEBOV-NP_{CTL} was able to afford durable protective immunity against a lethal ZEBOV challenge after only a single vaccine dose. We reasoned that the capacity to provide such long-lasting protective immunity would be an attractive if not essential quality for development of CMV as either a 'disseminating' vaccine for use in wild African great ape populations, or as a human CMV-based vaccine for conventional use. Animal use complied with the Guide for the Use and Care of Laboratory Animals, USDA Animal Welfare Regulations, PHS Policy on Humane Care and Use of Laboratory Animals and other relevant regulations. All procedures received prior approval by IACUC committees at RML, DIR, NIAID, NIH and OHSU. Figure 1 shows a schematic of the mouse-adapted (ma)-ZEBOV challenge study using MCMV/ZEBOV-NP_{CTL} vaccinated mice. To assess whether vaccine-induced immunity provided durable protection, we challenged mice at 119 days (17 weeks) post-vaccination. This time of challenge was based on the observation that most previous mouse studies (ours included (14)) have only looked at short-term protection, within 6 weeks following the last vaccine dose (28-30). Briefly, female C57BL/6 mice were vaccinated intra-peritoneally (IP) with either MCMV/ZEBOV-NP_{CTL} (Clone 5A1) ($5x10^5$ plaque-forming units, pfu), parental MCMV wild-type (MCMV WT), or vaccine diluent (2% FBS in DPBS) (Mock). Excepting a mouse receiving MCMV WT (which died during the vaccine phase) $CD8^+$ T cell responses were assessed in mice (n = 4-5) 8/9 and 14 weeks after vaccination

(Figure 2B & C). The gating strategy is shown for a representative MCMV/ZEBOV-NP_{CTL} vaccinated mouse in Figure 2A. Consistent with our earlier study, MCMV/ZEBOV-NP_{CTL} induced ZEBOV NP-specific $CD8^+$ T cells, which were not observed in either MCMV WT or Mock controls. All MCMV WT and MCMV/ZEBOV-NP_{CTL}, but not Mock groups also had responses against MCMV endogenous proteins M38 and M45 as expected. At week 17 (approx. 4 months) post-vaccination, age-matched mice (n=14) were challenged with $1x10^3$ LD_{50} ma-ZEBOV (IP). An additional control group of mice (n=14) received the 'benchmark' VSVΔG/ZEBOVGP vaccine (31) to serve as a vaccine efficacy control. Vaccine efficacy was assessed on the basis of morbidity (clinical symptoms and weight loss) and survival (Figure 3). Weight was monitored in mice until day 17 post-challenge, or until all animals had succumbed to ZEBOV disease. Surviving mice were then followed until days 28 or 29 post-challenge, at which time they were humanely euthanized. All MCMV WT and Mock control mice showed signs of severe ma-ZEBOV disease with clinical symptomology (ruffled hair, reduced mobility and weight loss). 100% of Mock and 90% of MCMV WT mouse groups perished as a result of ZEBOV-associated disease by day 7 post-challenge (Figure 3A). In contrast, no ZEBOV disease was observed in MCMV/ZEBOV-NP_{CTL} vaccinated mice. Although not statistically significant, MCMV/ZEBOV-NP_{CTL} vaccinated mice did show a slight loss in weight suggesting that immunity was not sterilizing in all mice (Figure 3B), which is consistent with results from the earlier study (14). Together, these results indicate that a CMV-based ZEBOV vaccine can provide long-term protection from ZEBOV-associated disease and mortality following only a single inoculation at least 119 days (approx. 4 months) post-vaccination. Although a role for antibodies cannot be formally discounted in this protection, the expression of only a single CD8 T cell ZEBOV epitope by MCMV/ZEBOV-NP_{CTL}, the absence of detectable ZEBOV antibodies in vaccinated mice prior to challenge (Table 1) and the presence of ZEBOV NP-specific $CD8^+$ T cell responses (Figure 2) are consistent with the mode of protection induced by the CMV vector as being

primarily T cell mediated. CMV has been shown to induce T cell responses shifted towards 'effector' memory (T_{EM}) that are primed for immediate 'effector' function at mucosal/epithelial tissue sites (32-34). We previously showed that ZEBOV NP $CD8^+$ T cell responses had T_{EM} characteristics based on similarity in kinetics of expansion as a MCMV 'inflationary' endogenous protein (M38) (14). Using the same study group from this earlier published study (Figure 2 in (14)), splenocytes were harvested at days 442 and 444 (> 14 months) following a single MCMV/ZEBOV-NP_{CTL} IP vaccination ($1x10^5$ pfu). Antigen-specific $CD8^+$ T cell responses were then phenotyped into T_{EM} and T_{CM} on the basis of CD44, a marker of antigen-experience (35), and KLRG-1, a marker of CMV- as well as other herpesvirus-specific $CD8^+$ T_{EM} found consistently upregulated to high levels on these cells (36, 37). As shown in Figure 4, ZEBOV NP-specific $CD8^+$ T cell responses were comparable to the T_{EM}-biased responses directed against M38 rather than to the central memory (T_{CM}) responses against M45. In summary, we show that a CMV-based ebolavirus vaccine can provide durable immunity for at least 119 days following only a single vaccine dose. These findings have important implications for development of CMV as a disseminating vaccine to prevent ebolavirus in great apes, and possibly a human CMV (HCMV)-based ebolavirus vaccine for humans. Studies ongoing will determine whether these results translate to protection in the macaque ebolavirus challenge model, regarded as the 'gold standard' for vaccine efficacy assessment in a model representative of ebolavirus infection in great apes, including humans.

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Figure 1. Schematic showing mouse groups and sampling regimen in ma-ZEBOV challenge study of MCMV/ZEBOV-NP_{CTL}. C57BL/6 ($H2^b$-restricted) mice were immunized using a single IP dose of $5x10^5$ pfu of MCMV/ZEBOV-NP_{CTL}. Control groups received MCMV WT or diluent (Mock). Splenocytes were harvested for analysis of T cell responses in groups of mice at times indicated (week 8/9: days 56, 58, 65 post-vaccination, and prior to challenge: days 96 and 100 post-vaccination). Antigen specific T cells were assayed by using ICS with a 6 hour incubation in the presence of BFA with peptide. After 119 days (> 4 months) post-vaccination, mice were challenged with $1x10^3$ LD_{50} ma-ZEBOV IP and disease course was followed for 28 days. VZVΔG/ZEBOVGP vaccinated mice served as a vaccine efficacy control group, and received a single IP dose of VZVΔG/ZEBOVGP ($5x10^5$ pfu) prior to the ma-ZEBOV challenge (47 days later).

Figure 2. $CD8^+$ T cell responses following immunization with MCMV/ZEBOV-NP_{CTL}. Female C57BL/6 $H2^b$-restricted mice were immunized IP using a single inoculation of $5x10^5$ pfu of MCMV/ZEBOV-NP_{CTL}. Control groups received MCMV WT ($5x10^5$ pfu) or diluent (Mock). Splenocytes were harvested for analysis of T cell responses. (A) Schematic showing gating strategy for ICS. NP-specific T cells for a representative MCMV/ZEBOV-NP_{CTL} vaccinated mouse is shown. (B) 8/9 weeks (days 56, 58 and 65 post-vaccination), and (C) week 14 (days 98 and 100 post-vaccination). T cells were analyzed by using ICS with a 6 hour incubation in the presence of BFA with indicated peptide as previously described (14). Human prostate-specific antigen (PSA) is an irrelevant control peptide (20), and NP (peptide pool) is an overlapping peptide pool (15-mer, 5 amino acid overlap) representing the full length ZEBOV NP protein. All mice receiving MCMV had $CD8^+$ T cell responses against MCMV M38 and M45, MCMV endogenous 'inflationary' and 'non-inflationary' antigens, respectively. Mock-infected mice showed no MCMV-specific T cell responses as expected. All MCMV/ZEBOV-NP_{CTL} immunized mice showed significant CD8-restricted T cell

responses against the NP target antigen (2-tailed t-test, $p<0.05$) consistent with previous results (14). All mice were 29 weeks old at time of vaccination other than the Mock group assessed at Week 14, which were 21 weeks old. • = not tested.

Figure 3. Efficacy of MCMV/ZEBOV-NP$_{CTL}$ vector against ma-ZEBOV challenge following a single inoculation at day -119. Age matched groups of C57BL/6 mice (n=10) were vaccinated with a single IP administration of $5x10^5$ pfu of MCMV/ZEBOV-NP$_{CTL}$. Additional groups received either diluent (Mock), or VSVΔG/ZEBOVGP (positive control for vaccine efficacy, given 47 days prior to challenge). After 119 days, mice were challenged with 10^3 LD_{50} ma-ZEBOV (IP). Data represent (A) Percent survival. (B) Body weight change over time post-challenge. For body weight, groups were weighed daily until 17 days post-EBOV challenge, or until all animals in a group had succumb to ZEBOV disease. Vaccination with MCMV/ZEBOV-NP$_{CTL}$ had a significant impact on survival from ma-ZEBOV challenge compared to MCMV WT control ($p <0.0001$) using a Log-rank (Mantel-Cox) Test. MCMV WT and Mock groups showed a significant decrease in bodyweight compared to MCMV/ZEBOV-NP$_{CTL}$ (p-value at least <.05) from day 3 onwards using a one-tailed t-test. No significant differences were seen in body weight between MCMV/ZEBOV-NP$_{CTL}$ and VSVΔG/ZEBOVGP groups at any time post-challenge. All mice were 21 weeks old at time of vaccination.

Figure 4. MCMV/ZEBOV-NP$_{CTL}$ induces T_{EM}-biased responses against ZEBOV NP. 129S1/SvlmJ/Cr $H2^b$-restricted mice were immunized (IP) with a single dose ($1x10^5$ pfu) of MCMV/ZEBOV-NP$_{CTL}$ (clone 5D1). These mice are the same groups that were serially followed for T cell responses through week 33 post-vaccination in reference (14). (A) At days 442 and 444 (> 14 months) post-vaccination, splenocytes were harvested and $CD8^+$ T cell responses were determined by ICS using a 6 hour incubation in the presence of BFA with

peptides (NP, M38 or M45). (B) ZEBOV NP-specific $CD8^+$ T cell (IFN^+/TNF^+) responses were characterized into T_{EM} and T_{CM} on the basis of CD44 and KLRG-1 expression. M38 and M45 responses served as controls for T_{EM} and T_{CM}-biased responses, respectively. All responses were normalized against cells incubated in the absence of peptide. Typical response (B and C) and (D) average responses in total mice tested (n=6) with SD shown. Populations were compared using 1-way ANOVA with Bonferroni's Post Test.

Table 1. Total anti-ZEBOV VLP IgG antibody titre in mouse blood samples pre- and post-challenge. VLPs (GP/NP/VP40) were used as the source of antigen. Pre-challenge Mock samples were used to establish background values. Samples were deemed positive if the signal was greater than the mean of pre-challenge Mock values plus four standard deviations. An 'in house' anti-VP40 antibody was used as the positive control. NT = not tested. Samples from 4 mice of each experimental group were analyzed.