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A live-attenuated RhCMV/SIV vaccine shows long-term efficacy against heterologous SIV challenge

Scott G. Hansen[1], **Emily E. Marshall**[1,†], **Daniel Malouli**[1], **Abigail B. Ventura**[1], **Colette M. Hughes**[1], **Emily Ainslie**[1], **Julia C. Ford**[1], **David Morrow**[1], **Roxanne M. Gilbride**[1], **Jin Y. Bae**[1], **Alfred W. Legasse**[1], **Kelli Oswald**[2], **Rebecca Shoemaker**[2], **Brian Berkemeier**[2], **William J. Bosche**[2], **Michael Hull**[2], **Jennie Womack**[1], **Jason Shao**[3], **Paul T. Edlefsen**[3], **Jason S. Reed**[1], **Ben J. Burwitz**[1], **Jonah B. Sacha**[1], **Michael K. Axthelm**[1], **Klaus Früh**[1], **Jeffrey D. Lifson**[2], **Louis J. Picker**[1,*]

[1]Vaccine and Gene Therapy Institute and Oregon National Primate Research Center, Oregon Health & Science University, Beaverton, OR 97006;

[2]AIDS and Cancer Virus Program, Frederick National Laboratory for Cancer Research, Frederick, MD 21702;

[3]Statistical Center for HIV/AIDS Research and Prevention, Vaccine and Infectious Disease Division, Fred Hutchinson Cancer Research Center, Seattle, WA 98109.

Abstract

Previous studies have established that strain 68–1-derived Rhesus cytomegalovirus (RhCMV) vectors expressing simian immune deficiency virus (SIV) proteins (RhCMV/SIV) are able to elicit and maintain cellular immune responses that provide protection against mucosal challenge with highly pathogenic SIV in rhesus monkeys (RM). However, these efficacious RhCMV/SIV vectors were replication- and spread-competent, and therefore have the potential to cause disease in immune-compromised subjects. To develop a safer CMV-based vaccine for clinical use, we attenuated 68–1 RhCMV/SIV vectors by deletion of the Rh110 gene encoding the pp71 tegument protein (ΔRh110), allowing for suppression of lytic gene expression. ΔRh110 RhCMV/SIV vectors are highly spread-deficient *in vivo* (~1000-fold compared to the parent vector) yet are still able to superinfect RhCMV$^+$ RM and generate high frequency effector-memory-biased T cell

*To whom correspondence should be addressed: pickerl@ohsu.edu.

†Current address: Vir Biotechnology, 4640 SW Macadam Avenue, Portland, OR 97239

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responses. Here, we demonstrate that ΔRh110 68–1 RhCMV/SIV expressing homologous or heterologous SIV antigens are highly efficacious against intravaginal (IVag) SIV_{mac239} challenge, providing control and progressive clearance of SIV infection in 59% of vaccinated RM. Moreover, among 12 ΔRh110 RhCMV/SIV-vaccinated RM that controlled and progressively cleared an initial SIV challenge, 9 were able to stringently control a second SIV challenge ~3 years after last vaccination, demonstrating the durability of this vaccine. Thus, ΔRh110 RhCMV/SIV vectors have a safety and efficacy profile that warrants adaptation and clinical evaluation of corresponding HCMV vectors as a prophylactic HIV/AIDS vaccine.

Summary:

Highly attenuated pp71-deleted RhCMV/SIV vectors elicit immune responses that stringently protect 59% of vaccinated monkeys from SIV challenge.

INTRODUCTION

Although the advent of antiretroviral therapy and other preventative interventions have greatly reduced the number of new infections and AIDS-related deaths from their peak incidence, epidemiologic modelling suggests that an efficacious HIV vaccine will still be necessary to reduce the annual incidence of new HIV infections to a degree commensurate with ending the epidemic (1,2). However, despite many decades of concerted effort, vaccine platforms capable of eliciting protective immune responses against HIV or its nonhuman primate counterpart SIV are few, as these viruses are highly immune-evasive and either lack susceptibility to natural immunity, or rapidly escape immune responses that are initially effective (3). We hypothesized a number of years ago that immune control of these viruses might be possible if infection could be immediately intercepted at portals of viral entry and sites of early spread by pre-established, effector-differentiated $CD8^+$ T cell responses, either in place in these sites (resident memory cells) or rapidly recruited from the blood (circulating effector-memory cells), without the need for the “too little, too late” process of anamnestic memory T cell expansion, effector differentiation and trafficking to sites of infection (4). Since human CMV (HCMV) and RhCMV naturally elicit and maintain effector-memory T cell responses having these properties, we investigated the possibility of exploiting CMV as a vaccine vector, using RhCMV/SIV vectors based on the 68–1 RhCMV strain in the RM-SIV model as proof-of-principle (5).

In a series of reports, we demonstrated that a 68–1 RhCMV/SIV vaccine expressing SIV Gag, Rev/Nef/Tat, Pol and Env was able to super-infect naturally RhCMV-infected RM and elicit and indefinitely maintain SIV-specific $CD4^+$ and $CD8^+$ T cell responses that closely mimicked the characteristics of responses to RhCMV itself: high frequency, widely distributed in lymphoid and non-lymphoid sites and effector-differentiated (highly effector-memory-biased) (6–8). Insert-specific antibody (Ab) responses were absent in most vaccinated RM, and unexpectedly, the $CD8^+$ T cell responses elicited by these vectors manifested unconventional epitope targeting characterized by extraordinary breadth and response restriction by either major histocompatibility complex II (MHC-II) or MHC-E, but not MHC-Ia, an immunologic feature that was found to be related to genetic changes in the 68–1 RhCMV strain associated with adaptation to *in vitro* fibroblast culture (9,10). Although

the significance of this unusual $CD8^+$ T cell antigen (Ag) recognition remains an area of active investigation, the hypothesis that pre-established circulating and tissue-based, effector-differentiated cellular immune responses might be more efficacious than conventional memory responses is supported by multiple SIV challenge studies showing that over half of 68–1 RhCMV/SIV-vaccinated RM manifested an early and complete control of SIV_{mac239} infection after mucosal challenge. Protected RM were found to be definitively infected after challenge, but viral spread appeared to be completely arrested prior to establishment of a permanent viral reservoir, and the infection was progressively cleared over the ensuing months, until protected RM became indistinguishable by both virologic and immunologic criteria from animals that were never challenged (8,11).

This “control and clear” vaccine effect against highly pathogenic SIV has not been reported for any other vaccine modality, and if translatable to humans, has the potential to contribute to control of the HIV epidemic, either as a stand-alone vaccine or in combination with Ab-targeted vaccines (12). However, translating CMV-based vectors as a prophylactic vaccine in humans requires careful consideration of safety. Although the vast majority of HCMV infections in people and RhCMV infections in monkeys are clinically inapparent, these viruses have the capacity to cause serious disease in settings of immune deficiency, with maternal to fetal transmission being of particular concern (13). 68–1 RhCMV, the parent strain of vectors used in the above-described efficacy studies, lacks subunits of a pentameric glycoprotein complex which facilitate viral entry into most non-fibroblast cells (14,15) and, presumably as a result of this restricted tropism, demonstrates reduced viremia, shedding and horizontal transmission compared to wildtype (WT) RhCMV (16,17). Nevertheless, 68–1 RhCMV retains the ability to disseminate in infected RM, transmit from one monkey to another, and has the potential to cause disease (18–20). “68–1-like” HCMV vectors thus still carry some potential risk for vaccine-mediated disease in otherwise healthy populations. The challenge then becomes whether CMV can be further genetically attenuated such that it retains the ability to super-infect, elicit and maintain effector-differentiated T cell responses (including the unconventionally targeted $CD8^+$ T cell responses) and “control and clear” protective efficacy, while losing the ability to widely disseminate in the body, spread from individual to individual, and to cause disease in settings of immunodeficiency. Moreover, any genetic attenuation must be stable, minimize the likelihood for reversion by mutation or recombination, allow vector manufacture at scale, and involve a virologic mechanism that is conserved between RhCMV, where the concepts will be tested, and HCMV, which will serve as the basis of any clinical vector.

In a companion report (20), we describe a CMV attenuation strategy based on deletion of the *Rh110* gene (RhCMV ortholog to HCMV UL82), which encodes pp71, a tegument phospho-protein which functions to disperse and/or degrade the host intrinsic immunity protein death-domain associated protein (DAXX). DAXX functions in nuclear ND10 bodies to repress transcription of viral immediate early (IE) genes, which are critical for early and late CMV gene expression, and thus, viral genome replication, assembly and egress (21–27). In the absence of viral pp71, DAXX represses lytic CMV replication, and the infection becomes and remains latent. Although DAXX can be overcome at high multiplicities of infection *in vitro*, ΔRh110 (Δpp71) RhCMV is highly spread-deficient *in vivo*, with infection largely restricted to the inoculating dose at the site of inoculation and draining

lymph nodes (20). In contrast to parental 68–1 RhCMV, ΔRh110 68–1 RhCMV was not shed in urine, nor transferred to new hosts by close contact or adoptive cell transfer, and this attenuation was stable over time with no signs of reversion *in vivo*. Despite this attenuation, the SIV insert-specific T cell immunogenicity of ΔRh110 68–1 RhCMV/SIV vectors was similar to its *Rh110*-intact counterpart in terms of magnitude, durability, effector-memory phenotype and function, and for the $CD8^+$ T cell responses, both in breadth and unconventional epitope targeting (20). Here, we investigate whether spread-deficient ΔRh110 68–1 RhCMV/SIV vectors can provide the same "control and clear" protection against homologous SIV challenge as their spread-competent *Rh110*-intact counterparts, and additionally assess whether these attenuated 68–1 RhCMV/SIV vectors can protect against challenge with a heterologous SIV strain.

RESULTS

Experimental Design and ΔRh110 RhCMV/SIV Vector Immunogenicity.

We previously reported vaccination of cycling female RM with spread-competent (*Rh110*-intact), strain 68–1 RhCMV/SIV vectors expressing SIV Gag, Rev/Tat/Nef (RTN), 5'-Pol, 3'-Pol, and Env (with inserts primarily based on SIV_{mac239} gene sequences). This vaccination provided stringent, aviremic (except for transient plasma viremia in early infection), long-term (>52 weeks) control of intravaginal (IVag)-introduced SIV_{mac239} infection in 8 of 16 vaccinees vs. 0 of 18 controls (8). Here, we sought to determine 1) whether this homologous efficacy would extend to vaccination with an attenuated (spread-deficient) ΔRh110 68–1 RhCMV/SIV vector set expressing the same predominantly SIV_{mac239} sequence inserts (ΔRh110/SIV_{mac239}), and 2) the extent to which a ΔRh110 68–1 RhCMV/SIV-vectored vaccine with heterologous SIV_{smE660}-sequence inserts (ΔRh110/SIV_{smE660}) would provide protection against the same SIV_{mac239} challenge regimen. We elected a heterologous vaccine rather than heterologous challenge approach because available heterologous challenge strains – the SIV_{smE660} swarm or SIV_{smE543} clone – are sufficiently different in key immunobiologic characteristics from SIV_{mac239} to make direct efficacy comparisons difficult (28, 29). To this end, two ΔRh110 RhCMV/SIV vector sets were constructed from the parental 68–1 RhCMV bacterial artificial chromosome (BAC), each with the coding region of the *Rh110* (pp71) gene replaced with the SIV insert (Gag, 5'-Pol, 3'-Pol, RTN, and Env), derived from either the SIV_{mac239} sequence or an SIV_{smE660} consensus sequence (fig. S1A). Divergence between the SIV_{mac239} and SIV_{smE660} amino acid sequences averages 15% across all inserts (Fig. S2), differences that approximate the variation between single clade–based HIV vaccines and circulating HIV isolates within that clade (30). Two groups of 14 female RM each were vaccinated twice (week 0, 14) with the set of 5 ΔRh110/SIV_{mac239} (Group 1) or ΔRh110/SIV_{smE660} vectors (Group 2) expressing Gag, RTN, 5'-Pol, 3'-Pol, or Env inserts by subcutaneous administration of 5×10^6 plaque-forming units (pfu) per vector (Fig. 1A). Immunogenicity was followed for 60 weeks post-initial vaccination, at which time repeated, limiting dose, IVag SIV_{mac239} challenge was initiated for both vaccine groups and a cohort of unvaccinated controls (Group 3; n = 20). Immunogenicity and outcome of Groups 1 and 2 were also compared to our previously reported cohort of female RM (Group 4) vaccinated with a set of WT (*Rh110*-intact) 68–1

RhCMV/SIV vectors (WT 68–1/SIV_{mac239}) expressing the same SIV_{mac239} sequence inserts that were IVag SIV_{mac239} challenged by a similar limiting dose protocol (8).

RhCMV vectors are T cell-targeted vaccines, with little to no ability to elicit insert-specific Ab responses (7, 8, 31), and in keeping with this, only 3 of 28 RM in Groups 1 and 2 (all in Group 2) showed detectable SIV Env-specific Abs after vaccination, and these 3 responses were very low titer (Fig. S3A). In contrast, using flow cytometric intracellular detection of CD69 and either or both of TNF and IFN-γ as the indicator of Ag-triggered T cells responding to pools of consecutive, overlapping, SIV_{mac239} sequence 15mer peptides, all RM in both ΔRh110/SIV vector-vaccinated groups developed $CD4^+$ and $CD8^+$ T cell responses in blood to all SIV inserts (Fig. 1B; **top panel**). In both Group 1 and Group 2, the overall response peaked 2–4 weeks following initial or boost vaccinations, prior to establishing a stable steady-state within ~12 weeks of the second vaccination that was maintained for the duration of the vaccine phase. During the plateau-phase of the vaccine response (defined here as weeks 30–58 post-initial vaccination), total SIV-specific, $CD4^+$ and $CD8^+$ T cell response frequencies in peripheral blood, as measured by the response to SIV_{mac239} sequence peptides, were significantly higher overall in Group 1 RM, given ΔRh110/SIV_{mac239} vectors, than in Group 2 RM, given ΔRh110/SIV_{smE660} vectors ($P < 0.001$; Fig. 1B; **bottom panel**). This difference in overall response magnitude in blood was primarily driven by differences in the responses to Env and RTN (Fig. 1B; **bottom panel**), the SIV inserts with the most divergence between the SIV_{mac239} and SIV_{smE660} sequences (fig. S2).

We also determined the memory differentiation phenotype of the SIV Gag-specific $CD4^+$ and $CD8^+$ T cells at plateau phase in Group 1 and Group 2 RM by intracellular cytokine staining (ICS), delineating central memory T cells (T_{CM}), transitional effector-memory T cells (T_{TrEM}), and effector-memory T cells (T_{EM}) by their expression of CCR7 vs. CD28 (Fig. 1C). This analysis showed a predominance of effector-differentiated cells (T_{TrEM} + T_{EM}) that was similar in both vaccine groups. In keeping with this, SIV-specific $CD4^+$ and $CD8^+$ T cells were enriched in bronchoalveolar lavage fluid samples (BAL, used as an accessible effector site), and despite using SIV_{mac239} sequence peptides for this analysis, the magnitude of the overall and individual SIV insert-specific, $CD4^+$ and $CD8^+$ T cell responses in BAL were not different for Group 1 and Group 2 RM (Fig. 1D).

Both *Rh110*-intact and *Rh110*-deleted 68–1 RhCMV vectors elicit $CD8^+$ T cell responses that are entirely unconventional in their MHC restriction (with epitopes presented by MHC-E or MHC-II, not MHC-Ia). Moreover, RM vaccinated with SIV_{mac239} Gag-expressing 68–1 RhCMV vectors invariably respond to a set of universal MHC-E- and MHC-II-restricted $CD8^+$ T cell epitopes [so-called “supertopes” (9, 10, 20)]. All RM in Group 1 and Group 2 manifested $CD8^+$ T cell responses to all 4 of the previously characterized SIVgag supertopes tested (2 MHC-E-restricted; 2 MHC-II-restricted), and the magnitudes of all these universal responses (3 of which were sequence identical in both the SIV_{mac239} and SIV_{smE660} inserts and one of which was different by 3 amino acid substitutions; fig. S1B) were not different between Group 1 and Group 2 RM (Fig. 1E).

We next compared the magnitude and phenotype of responses elicited by the ΔRh110/SIV_{mac239} vectors (Group 1) with results from our previously reported cohort of female RM vaccinated with WT 68–1/SIV_{mac239} vectors (Group 4) (8). As shown in Figs. 1B–E, neither the magnitude (blood or BAL), nor the T_{EM} + T_{TrEM} skewing (blood) of the various SIV-specific $CD8^+$ T cell responses, including supertope-specific responses, were different between the 2 groups. However, the magnitude of plateau-phase SIV-specific $CD4^+$ T cell responses in blood and BAL were significantly higher ($P < 0.001$ for both), and in blood, the SIVgag-specific $CD4^+$ T cell responses were significantly more T_{EM} + T_{TrEM}-biased (i.e., lower $\%T_{CM}$; $P = 0.013$), in the Group 4 RM compared to the Group 1 RM. These observations suggest that the restricted spread of the ΔRh110 vectors (20), and likely, diminished Ag availability, modestly reduced $CD4^+$ T cell immunogenicity and effector differentiation, while having little to no effect on $CD8^+$ T cell responses.

To explore heterologous T cell immunity with RhCMV vectors, we directly compared the ability of vaccine-elicited T cell responses of both Group 1 and Group 2 RM to recognize and respond to SIV_{mac239} vs. SIV_{smE543} sequence peptides. The SIV_{smE660} swarm-derived SIVsmE543 clone (29) is 96% identical to the SIV_{smE660} consensus amino acid sequence and has a similar 15% overall amino acid sequence divergence from the SIV_{mac239}, see fig. S2. For $CD8^+$ T cells, we also examined responses to autologous $CD4^+$ T cells infected with the cloned SIV_{mac239} or SIV_{smE543} viruses (Fig. 2). $CD4^+$ T cells from ΔRh110/SIV_{mac239}-vaccinated Group 1 RM showed no difference in their plateau phase responses to matched (SIV_{mac239}) vs. mismatched (SIV_{smE543}) peptide mixes, whereas $CD8^+$ T cells from the same RM showed a significant reduction (average = 31%; $P = 0.017$) in the overall frequency of cells able to respond to the mismatched peptides (Fig. 2A). For ΔRh110/SIV_{smE660}-vaccinated Group 2 RM, both $CD4^+$ and $CD8^+$ T cells recognized mismatched peptides significantly less well than matched peptides, with the reduction in the magnitude of the $CD4^+$ and $CD8^+$ T cell response to mismatched peptides being ~21% ($P < 0.001$) and ~44% ($P = 0.002$) less, respectively, than for matched peptides (Fig. 2B). Thus, mismatch between the insert sequence and stimulating peptide sequence modestly reduced the magnitude of ΔRh110/$SIV_{mac239/smE660}$ vector-elicited $CD8^+$ T cell responses. Importantly, however, $CD8^+$ T cells from both Group 1 and Group 2 RM showed equivalent ability to recognize autologous $CD4^+$ T cells infected with SIV_{mac239} or SIV_{smE543} virus clones (Fig. 2C), suggesting that at the level of SIV-infected cell recognition, the breadth of the $CD8^+$ T cell responses generated by both ΔRh110/SIV_{mac239} and ΔRh110/SIV_{smE660} vector sets was able to overcome sequence mismatch in individual epitopes.

Efficacy of ΔRh110 RhCMV/SIV Vectors.

To determine if spread-deficient ΔRh110/$SIV_{mac239/smE660}$ vectors retain the ability to mediate the characteristic “control and clear” protection demonstrated by spread-competent WT 68–1/SIV_{mac239} vectors in previous reports (7, 8, 12), we subjected the vaccinated Group 1 and Group 2 RM, and the unvaccinated Group 3 RM, to repeated (up to 12 challenges at 2–4 week intervals), limiting dose (100 focus-forming units for first 6 exposures; 300 for last 6 exposures) IVag SIV_{mac239} challenge. The goal was to establish infection “take” in each RM (at which time challenges were stopped), and then determine non-protection vs. protection by the presence or absence of progressive SIV infection after

infection establishment (7, 8, 12). Since protected RM may or may not manifest detectable viremia after challenge, SIV infection "take" is confirmed by the onset of *de novo* T cell responses to SIV Vif, an SIV Ag not included in any of the RhCMV/SIV vectors (7, 8). In our challenge system, SIV Vif-specific T cells ($CD4^+$ and $CD8^+$) appear in blood 2–3 weeks post-productive SIV challenge, allowing attribution of successful (infection "take"-positive) challenges when successive challenges are 2 or more weeks apart. With this approach we were able to establish productive SIV infection in 13/14, 14/14, and 17/20 RM after up to 12 challenges in Groups 1–3, respectively, with no statistically significant difference in the rate of infection acquisition in the 3 challenge groups or in the overall ΔRh110 68–1 RhCMV/SIV vector-vaccinated cohort vs. unvaccinated controls (fig. S4).

In keeping with previous observations on WT 68–1/SIV_{mac239} vector efficacy (7,8), the outcome of productive SIV challenge was strikingly different in the vaccinated Groups 1 and 2 vs. the unvaccinated Group 3. Whereas all 17 SIV-infected unvaccinated control RM manifested typical systemic SIV infection, 7 of 13 Group 1 RM (54%; $P = 0.0004$) and 9 of 14 Group 2 RM (64%; $P < 0.0001$) showed the onset of *de novo* SIV Vif-specific T cell responses in the absence of SIV viremia ($n = 3$ and $n = 5$ for Groups 1 and 2, respectively) or with plasma viremia positive at only a single time point ($n = 4$ for both Groups 1 and 2; Fig. 3A,B). To confirm the take of SIV infection in the presumptively protected (SIV Vif response-positive) RM without detectable plasma viremia, we performed adoptive transfer of bone marrow (BM) cells alone or BM cells plus PBMC obtained after the onset of SIV Vif-specific T cell responses from 6 of these RM (3 each from Group 1 and Group 2) into SIV-naïve recipients (Fig. 3C). As shown in the figure, adoptive transfer of cells from all 6 donor RM resulted in the onset of typical SIV_{mac239} infection in recipient RM, demonstrating the presence of fully replication-competent SIV_{mac239} in the donor RM and confirming stringent SIV control in these animals. Also, in keeping with previous results (7,8), there was no reduction in chronic phase plasma viremia in unprotected, vaccinated RM relative to unvaccinated controls, consistent with the "all or none" nature of RhCMV/SIV vaccine efficacy. The degree (% protected) and pattern of efficacy observed in Group 1 and Group 2 were not significantly different from the previously reported efficacy of WT 68–1/SIV_{mac239} vector-vaccinated RM (Group 4) subjected to a similar challenge protocol [56% with initial stringent control; (8)]. Of note, across all protected vs. unprotected Group 1 plus Group 2 RM, efficacy was not predicted by the magnitude of overall or individual insert, SIV_{mac239} peptide-specific $CD4^+$ or $CD8^+$ T cell responses, or supertope-specific $CD8^+$ T cell responses in blood at peak post-prime, peak post-boost or at vaccine response plateau phase, or by the magnitude of $CD8^+$ T cell recognition of SIV_{mac239}-infected $CD4^+$ T cells at vaccine response plateau phase (fig. S5).

As previously shown for protection against SIV_{mac239} challenge mediated by spread-competent WT 68–1/SIV_{mac239} vectors, the stringent control of SIV_{mac239} infection mediated by the ΔRh110/$SIV_{mac239/smE660}$ vectors occurred in the absence of an increased (boosted) SIV Gag- or Pol-specific T cell response in blood post-infection (fig. S6), and without development or boosting of an SIV Env-specific Ab response (fig. S3B). The lack of T cell response boosting was also observed post-infection in unprotected (viremic) vaccinated RM, indicating that the lack of increased T cell responses in protected RM was not due to limitation in SIV Ag availability. However, vaccinated, unprotected RM

developed high titer SIV Env-specific Ab responses after challenge (similar to unvaccinated controls) indicating that the lack of such Ab responses in protected vaccinated RM is almost certainly a function of SIV Ag limitation due to early arrest of infection (keeping Ag levels below the threshold needed for Ab response generation). The conclusion that vaccinated, protected RM have early arrest of viral spread after initial take of infection, sharply limiting the extent of SIV infection, is also supported by the lack of the activation of circulating monocytes [as measured by increased interferon-induced expression of CD169; (11,32,33)] specifically in protected RM (fig. S7). Taken together, these results demonstrate that spread-deficient ΔRh110/$SIV_{mac239/smE660}$ vaccines manifest efficacy equivalent to their spread-competent counterparts, which is not affected by a sequence mismatch between vector insert and challenge strain.

SIV Dynamics in ΔRh110 RhCMV/SIV Vector-Vaccinated, Protected RM.

We have previously demonstrated that in RM protected by WT 68–1/SIV_{mac239} vector vaccination, the arrest of SIV infection occurs after initial dissemination via both lymphatic and hematogenous routes, the latter including seeding of liver, spleen and BM. Over extended follow up, cells harboring SIV slowly disappear from all tissue sites until both virologic and immunologic evidence of SIV infection is lost (8). To determine if RM protected by spread-deficient ΔRh110/SIV_{mac239} or ΔRh110/SIV_{smE660} vector vaccination have similar post-infection SIV dynamics, we quantitated cell-associated SIV DNA and RNA in blood and BM of all protected RM in Groups 1 and 2 for up to 60 weeks following SIV infection. As shown in Fig. 4A,B, as expected, overtly infected control RM showed abundant cell-associated SIV RNA and DNA in both blood and BM at all tested time points. In contrast, vaccine-protected RM in both Groups 1 and 2 manifested only sporadic detection of cell-associated virus in blood over 60 weeks of observation (Fig. 4C), consistent with the arrest of progressive SIV infection in these monkeys. Most striking, however, were the SIV dynamics in BM, previously shown to be a common site of early SIV spread in 68–1 RhCMV/SIV vector-protected RM (8). As shown in Fig. 4D, all but 1 of the 16 protected Group 1 and Group 2 RM manifested cell-associated SIV RNA in BM 4 weeks after infection, comparable to unvaccinated controls, and cell-associated SIV DNA was also detected in the majority of these BM samples. Similar quantities of cell-associated SIV RNA and DNA were detected in most of the BM samples from these RM at week 8 as well, but starting at week 12, there was a clear decline in cell-associated SIV in BM, and by week 20, SIV RNA and DNA were below the limit of detection in all BM samples from all RM. The difference in the number of SIV RNA- and DNA-positive samples from <20 weeks and ≥20 weeks post-infection was significant ($P < 0.0001$; Barnard's exact test of binomial proportions).

To more globally assess the "total body" SIV infection burden in vaccine-protected Group 1 and Group 2 RM, we longitudinally followed SIV Vif-specific T cell responses as an *in vivo* circulating immunologic "biosensor" to detect residual SIV infection-related Ag production in these animals, all of which were aviremic except for rare low-level viral blips prior to week 34 post-infection (Fig. 5A). As noted above, SIV Vif-specific T cell responses are generated and maintained by SIV infection-derived Ag; in WT 68–1/SIV_{mac239} vector-vaccinated RM, we have previously associated decline in these responses with progressive

clearance of SIV reservoirs (8). All Group 1 and 2 protected RM showed a similar overall pattern of SIV Vif-specific response dynamics characterized by increasing or stable, high frequencies of SIV Vif-specific $CD4^+$ and $CD8^+$ T cells over the first 6–12 weeks post-infection. Thereafter, there is a slow but unequivocal decline in these frequencies that starts no later than week 20 and continues to extinction (e.g., response below detection limit in blood) over the subsequent 1–2 years (Fig. 5B), a pattern that is strikingly similar to data with the WT 68–1/SIV_{mac239} vaccine (8). Indeed, the slope of decline of SIV Vif-specific $CD4^+$ and $CD8^+$ T cell responses in ΔRh110/SIV vaccine-protected Group 1 and Group 2 RM was not significantly different from that of RM protected by spread-competent WT 68–1/SIV_{mac239} vectors (Fig. 5C; Wald test: $F_{2,582} = 0.097$ and 2.10 for $CD4^+$ and $CD8^+$, respectively).

To confirm that the observed loss of SIV Vif-specific T cell responses reflected “total body” SIV clearance, we selected 4 of the ΔRh110/$SIV_{mac239/smE660}$-vaccinated, long-term protected RM (>100 weeks post-infection; 2 RM each from Group 1 and Group 2) for detailed virologic and immunologic analysis at necropsy. Three of these 4 RM (RM #7, #9, #10) had previously manifested a single plasma viral blip early after infection and subsequently remained aviremic, whereas the 4th RM (RM #8) was aviremic throughout its course. All 4 of these RM had developed and then lost robust SIV Vif-specific T cell responses, while maintaining stable (vaccine maintained) SIV Gag- and Pol-specific T cell responses (fig. S8). At necropsy, all animals had SIV Gag- and Pol-responsive T cells in all tissues examined (Fig. 6A). In contrast, SIV Vif-specific T cell responses were predominantly negative in 3 of 4 RM (RM #7, #8, #9), with above-threshold responses in only a few tissues, and in the other RM (RM #10), were present as low frequency responses (predominantly $CD8^+$) in multiple sites (Fig. 6B). Cell-associated SIV RNA and DNA were quantified by nested quantitative RT-PCR/PCR (8) in extensively sampled tissues from the 4 ΔRh110/$SIV_{mac239/smE660}$ vaccine-protected RM (Fig. 6C) and for comparison, tissues from 2 ΔRh110/SIVgag vector-vaccinated RM never exposed to SIV (Fig. 6D) and 1 unvaccinated RM with progressive SIV infection (Fig. 6E). Both of the ΔRh110/SIVgag-vaccinated, unchallenged control RM were negative for SIV DNA and RNA in all tissues, whereas, as expected, the RM with progressive SIV infection manifested high amounts of both, with SIV RNA ~2 logs higher than DNA. All 4 ΔRh110/$SIV_{mac239/smE660}$ vector-vaccinated RM manifested detectable, albeit low-level, cell-associated SIV DNA in 5 or more tissues with 28% (98/235) of samples positive overall (vs. 0 of 114 samples in vaccinated, unchallenged controls; $P < 0.0001$ using Barnard’s exact test of binomial proportions). In contrast, cell-associated SIV RNA was detectable in only 1 RM, 2.6% of overall samples (9 of 345 vs. 0 of 114 samples in controls, P = NS). To determine if this detection of SIV DNA/RNA reflected replication-competent virus, we performed co-culture analysis on a total of 1120 tissue specimens sampled from the 4 protected RM (Fig. 6F). Only 6 of these specimens (0.5%), from 2 of the 4 RM, were SIV+ upon co-culture (5 in RM #2; 1 in RM #4 vs. 270/274 SIV+ co-cultures in the unvaccinated control RM). We next combined 56–100 million cells from the necropsy tissues of each of these 4 protected RM and then adoptively transferred these cells into SIV-naïve recipient RM and found no transfer of SIV infection in any of the 4 recipient RM (Fig. 6G), observations consistent with the majority of SIV DNA signals detected in tissues at necropsy representing replication-incompetent proviruses (34).

Finally, we repeated the adoptive transfer experiment using cells collected at late time points from 4 different ΔRh110/$SIV_{mac239/smE660}$ vaccine-protected, always aviremic RM (RM #1 and #2 from Group 1; RM #4 and #5 from Group 2) that were previously shown (early after the onset of protection) to harbor replication-competent SIV by adoptive transfer. A total of 10^8 pooled cells from BM, lymph node, or blood, collected at 60–102 weeks post-infection from these RM were administered to 4 SIV-naïve recipients, with no take of SIV infection detected in the recipient RM (Fig. 7). Taken together, these results provide compelling evidence that replication-competent SIV declines over time in ΔRh110 68–1 RhCMV/SIV-vaccinated, long-term protected RM such that after ~2 years, lymphoid cells infected with replication-competent SIV are very rare or undetectable.

Re-challenge of RhCMV/SIV Vector-Protected RM.

We next addressed the question of whether 68–1 RhCMV/SIV vector-vaccinated RM retain the capacity to clear a second SIV challenge after control and progressive clearance of an initial challenge, and if so, whether RM vaccinated with spread-competent vs. spread-deficient vectors differ in this regard. To this end, we followed 8 protected RM from our previously reported cohort of WT 68–1/SIV_{mac239} vector-vaccinated animals (Group 4)(8) and 12 ΔRh110/$SIV_{mac239/smE660}$ vaccine-protected RM from this study (5 from Group 1; 7 from Group 2) for at least 2 years after initial SIV infection. All RM developed and then lost SIV Vif-specific $CD4^+$ and $CD8^+$ T cell responses in blood during this follow-up, while retaining stable frequencies of (vaccine-elicited) SIV Gag- and Pol-specific $CD4^+$ and $CD8^+$ T cell responses (fig. S9). We then initiated the same repeated limiting dose, IVag SIV_{mac239} challenge protocol used in the first challenge. All RM were infected by this challenge protocol, as indicated by the redevelopment of SIV Vif-specific $CD4^+$ and $CD8^+$ T cell responses (Fig. 8A). Strikingly, 4 of 5 Group 1 RM, 5 of 7 Group 2 RM, and 7 of 8 WT/ 68–1 SIV_{mac239} vector-vaccinated RM were protected after this second SIV challenge, again showing either no viremia or only transient viremia (Fig. 8B). The twice-protected Group 1 and Group 2 RM included RM #1, #2, #4, and #5, which were previously shown to lack transferable SIV prior to second challenge. BM and/or PBMC samples from these 4 RM were collected after the (second) onset of SIV Vif-specific T cell responses and were inoculated into 4 additional SIV-naïve RM. All of the 4 recipient RM became SIV-infected (Fig. 8C), indicating the presence of replication-competent SIV in these aviremic animals, and thereby confirming a second, stringently controlled SIV infection. Overall, 16 of the 20 re-challenged RhCMV/SIV vector-vaccinated RM were protected a second time. Although this degree of efficacy (80%) is higher than the overall efficacy of initial challenge (58%), this difference did not quite achieve statistical significance ($P = 0.06$). These data confirm that both spread-competent and spread-deficient (ΔRh110) RhCMV/SIV vectors are able to maintain efficacy for ~3 years after last vaccination and can provide protection against more than one SIV challenge.

DISCUSSION

HCMV infection is ubiquitous, especially in resource poor settings, and although HCMV persists for life in infected individuals, the vast majority of these individuals will never develop CMV disease due to immune control of viral spread after primary infection and

upon reactivation from latency (35–37). The vast majority of such $HCMV^+$ individuals would not be expected to develop symptomatic infection upon administration of an HCMV-based vaccine, even one with WT replication and spread capacity. However, in the setting of prophylactic vaccination of large populations, non-HCMV-infected individuals, potentially including immunocompromised subjects, would possibly be exposed to such a WT HCMV-based vaccine, either through direct administration or potentially spread from a vaccinated subject, and a subset of such individuals would be at risk of developing overt HCMV disease (38,39). To mitigate this risk, we have sought to make a CMV-based vaccine safer by identifying an attenuation strategy that would substantially limit vector spread within and between hosts and thereby preclude disease in vaccinated individuals, spread to (and within) the fetus of pregnant subjects, and shedding in secretions (to prevent person-to-person spread). The strategy should preserve the ability of the vector to super-infect CMV^+ individuals, productively infect sufficient numbers of cells to prime robust T cell responses and persist long-term to provide the antigenic stimulation needed for maintaining effector-memory differentiation. In a companion paper (20), we provide evidence that ΔRh110 RhCMV may strike such a balance in RM, showing an ~1000-fold reduction in vector spread *in vivo*, no vector shedding in secretions, and no animal-to-animal spread with close contact or leukocyte transfusion. This vector still retains the ability to elicit insert-specific T cell responses that are comparable in magnitude, phenotype, function, epitope-targeting, and durability as *Rh110*-intact RhCMV vectors. Furthermore, by insertion of the SIV antigens into the *Rh110* locus we also eliminate the possibility of reversion to WT by homologous recombination with the endogenous virus present in CMV-infected hosts.

Here, we performed a large vaccination and challenge trial of ΔRh110 RhCMV/SIV vectors to extend the immunogenicity analysis to a larger cohort of RM vaccinated with these attenuated vectors, and to determine protection from highly pathogenic SIV_{mac239} challenge. We also expanded our analysis of ΔRh110 RhCMV/SIV vectors to include determination of the extent to which mismatch between the vector insert sequence and SIV challenge strain would affect SIV-infected cell recognition by vector-elicited T cells and vaccine efficacy, as such mismatch will be invariably present in any clinical application of this vaccine. These results confirm that ΔRh110/SIV_{mac239} vectors elicit insert-specific $CD8^+$ T cell responses that are essentially indistinguishable in magnitude, phenotype, and durability from that of WT 68–1/SIV_{mac239} vectors. $CD8^+$ T cell responses elicited by ΔRh110 RhCMV vectors expressing SIV_{mac239} vs. SIV_{smE660} sequence inserts in blood were reduced in magnitude by 30–40% when tested on mismatched sequence peptides, but these responses were equivalent in their ability to recognize SIV_{mac239}-infected and SIV_{smE543}-infected autologous $CD4^+$ T cells. Thus, while epitope recognition by the unconventionally (MHC-E- and MHC-II-) restricted (9,10)$CD8^+$ T cells elicited by ΔRh110 RhCMV/SIV vectors can be modestly compromised by sequence divergence, the breadth of these responses is sufficiently great to ensure equivalent recognition of cells infected by divergent SIV strains. Interestingly, ΔRh110 RhCMV/SIV vector-elicited $CD4^+$ T cells were largely unaffected by insert-target sequence mismatch (0–20% reduction), but were significantly reduced, albeit modestly, in both magnitude and effector-memory bias after the boost vaccination relative to *Rh110*-intact RhCMV/SIV vector-elicited responses. This modest reduction is consistent with the interpretation that RhCMV vector-elicited $CD4^+$ T cell responses may be more

sensitive to reduction in overall Ag availability than the corresponding $CD8^+$ T cell responses.

Of primary importance, we found that the extent and pattern of protection afforded by ΔRh110/SIV vector vaccination, irrespective of sequence match vs. mismatch between vector insert and challenge virus, was essentially identical to that of *Rh110*-intact vectors. In our previous analysis of WT 68–1/SIV_{mac239} vector vaccination, 56% of RM were protected after initial challenge and 50% after 1 year (8). This degree of efficacy was not significantly different from 59% overall efficacy of the ΔRh110/SIV vectors observed in the present study, with all these protected RM showing both initial and long-term protection. Interestingly, the percentage of protected RM was actually higher for monkeys given the challenge-mismatched ΔRh110/SIV_{smE660} vectors (64%) compared to RM given the challenge-matched ΔRh110/SIV_{mac239} vectors (54%). Although this difference was not statistically significant, the finding that heterologous efficacy is as good as or better than homologous efficacy is an encouraging sign for clinical translation. Moreover, the characteristics of protection after ΔRh110/$SIV_{mac239/smE660}$ vaccination were very similar to that of the WT 68–1/SIV_{mac239} vaccine. Animals acquired SIV, but except for rare viral blips, there was complete elimination of viremia, which is consistent with replication arrest. SIV was stringently controlled prior to systemic immune activation, as demonstrated by a lack of monocyte activation, anti-Env Ab production, and in the absence of boosting of the vaccine-stimulated T cells. Taken together, these data indicate that vaccine-elicited immune protection can be achieved with substantially reduced levels of RhCMV vector spread.

We have previously demonstrated that WT 68–1/SIV_{mac239} vector-protected RM show progressive loss of SIV infection over time, and this viral clearance process is particularly well-documented in the current analysis of ΔRh110/$SIV_{mac239/smE660}$ vector-vaccinated RM. We demonstrate loss of detectable cell-associated SIV RNA/DNA detection in BM over the first 20 weeks post-infection and decline in SIV Vif-specific $CD4^+$ and $CD8^+$ T cells in blood to below the threshold of detection over 1–2 years. Most strikingly, RM that were able to transmit infection to naïve recipients by transfer of cells obtained early after the onset of protection no longer transmitted infection 1–2 years later. Four ΔRh110/SIV vector-protected RM (2 each given vectors with matched – Group 1 – vs. mismatched – Group 2 – SIV inserts) were taken to necropsy ~2 years after infection for extensive tissue analysis. Although PCR analysis demonstrated these 4 RM had more SIV DNA than 2 ΔRh110/SIVgag-vaccinated, but never SIV-challenged controls, SIV RNA and co-culturable virus was largely undetectable, and adoptive transfer of cells from tissues from these RM did not transfer SIV infection. Taken together, these results suggest a vanishingly small amount of residual infectious SIV in these ΔRh110/$SIV_{mac239/smE660}$ vector-protected RM. The residual SIV DNA in these 4 necropsied RM did, however, appear to be somewhat more than in our previous analysis of WT/SIV_{mac239} vector-protected RM. This finding and the more frequent detection of low frequency SIV Vif-specific T cells in tissues of the current RM relative to the previously studied RM (8) is consistent with the conclusion that viral clearance was not quite complete in these animals. This does not necessarily indicate a difference in the extent or kinetics of viral clearance between WT 68–1/SIV_{mac239} and ΔRh110/$SIV_{mac239/smE660}$ vector-protected RM, as the WT 68–1/SIV_{mac239} vector-vaccinated RM studied previously at necropsy were males infected by intrarectal challenge

(as opposed to females being infected via IVag challenge), and 4 of these 6 previously studied animals were taken to necropsy after >1000 days post-infection, compared to ~700 days in the current study. Indeed, given the apparent dependence of SIV Vif-specific T cell responses on SIV Vif Ag production by SIV-infected cells, the observation that the slope of decline of SIV Vif-specific T cell responses in WT 68–1/SIV_{mac239} and ΔRh110/$SIV_{mac239/smE660}$ vector-protected RM were not significantly different (both $CD4^+$ and $CD8^+$) suggests that rate of SIV infection clearance was broadly similar with both vaccines. This is in keeping with our previous hypothesis that SIV clearance in RhCMV/SIV vector-protected RM predominantly results from arrest of infection prior to seeding a long-lived SIV reservoir and the subsequent decline of the less durable reservoir that is initially seeded (11). Although this implies that vaccine-elicited T cell responses are not actively clearing the viral reservoir, these responses very likely contribute to maintaining stringent replication control while the residual viral reservoir spontaneously declines, and if this is the case, WT 68–1/SIV_{mac239} and ΔRh110/$SIV_{mac239/smE660}$ vector-elicited responses appear to be equivalent in this activity.

Finally, we directly compared the outcome of a second round of SIV challenge in RM that were previously protected by WT 68–1/SIV_{mac239} or ΔRh110/$SIV_{mac239/smE660}$ vector vaccination and subsequently cleared the initial infection, as assessed by extinction of their SIV Vif-specific T cell responses over ~2 years. Remarkably, 80% of these re-challenged RM, across all vaccine groups, were able to control this second challenge, with re-infection and aviremic control demonstrated in 4 protected RM by conversion of the adoptive transfer assay of SIV infection from negative before the second challenge to positive after, in the absence of viremia. These data indicate that WT/SIV_{mac239} and ΔRh110/$SIV_{mac239/smE660}$ vectors can maintain efficacy for up to ~3 years after last vaccination, with the striking stability of the SIV-specific T cell responses elicited by these vectors suggesting that the potential for efficacy might extend for considerably longer periods, perhaps lifelong. However, it should also be noted that SIV-specific T cell response magnitude in blood did not correlate with outcome in the first challenge for the ΔRh110/$SIV_{mac239/smE660}$ vector-vaccinated RM, and that 20% of previously RhCMV/SIV vector-protected RM were not protected after the 2^{nd} challenge, despite maintaining stable SIV-specific T cell responses. Thus, there is either an element of stochasticity to protection, or some unmeasured aspect of the innate or adaptive immune response to vaccination that is required for efficacy, and this parameter or parameters can vary over time.

The “control and clear” protection against highly pathogenic SIV_{mac239} challenge afforded by RhCMV/SIV vectors is unique and offers an alternative mechanism for a clinically useful prophylactic HIV/AIDS vaccine, either alone or in combination with an Ab-targeted vaccine designed to reduce HIV acquisition (12). The ability to substantially limit vector spread while preserving both the extent (%protected) and durability of efficacy is a critically important step in clinical translation of the CMV vector platform, as is the demonstration that RhCMV/SIV vector efficacy can tolerate the equivalent of an intra-clade sequence mismatch between vaccine insert and challenge virus strain without loss of efficacy. However, a major limitation of this study is that CMVs are species-specific viruses and a clinical vector for vaccination of humans against HIV will be based on HCMV, not the orthologous, but distinct, RhCMV. The pp71 protein is encoded by *UL82* in HCMV, and

although the RhCMV and HCMV pp71 proteins have similar function, *UL82* deletion in HCMV results in a more pronounced growth defect *in vitro* than *Rh110* deletion in RhCMV, suggesting a ΔUL82 HCMV might be more attenuated *in vivo* in people than ΔRh110 RhCMV in monkeys (20). While this additional attenuation increases the margin of safety for clinical testing, it might also reduce immunogenicity, or more likely, increase the dose required to achieve full immunogenicity – issues that can only be resolved through human testing. Despite this potential concern, the results presented in this study strongly support the further development of pp71-deleted, 68–1-like HCMV/HIV vectors as prophylactic vaccines for HIV/AIDS.

MATERIALS AND METHODS

Study Design

The primary objective of this study was to determine whether attenuated (spread-deficient) ΔRh110 68–1 RhCMV/SIV vectors expressing homologous or heterologous SIV Ag inserts would, relative to unvaccinated controls, provide cycling female RM stringent post-acquisition control of SIVmac239 infection, administered by repeated, limiting dose IVag challenge. Based on previous experience with WT 68–1 RhCMV/SIV vectors (6–8), we randomly assigned n = 48 cycling female RM assigned to one of three vaccine groups as follows: n = 14 ΔRh110 /SIVmac239 (Group 1), n = 14 ΔRh110 /smE660 (Group 2), and n = 20 unvaccinated (Group 3). This group size was anticipated to allow us to resolve 20% protection at 90% power, pooling the vaccine groups. Although only the RM with take of infection (animals with SIVvif T cell response induction and either cell-associated SIV in tissue or plasma viremia post-challenge) were considered for evaluation of protection (n = 13 Group 1, n = 14 Group 2, n = 17 Group 3), our criteria for stringent SIV control (aviremic infection) was met in 16/27 vaccinated RM (59%), allowing us to proceed to our secondary objectives of determining the extent of viral clearance over time in these protected RM, and the ability of previously protected RM to control a second infection. At the end of an ~2 year observation period, during which time SIVvif responses in blood in all protected RM decayed to below the level of detection and all virologic assays reverted to (or remained) negative, the 16 protected RM were arbitrarily assigned to either necropsy for comprehensive tissue analysis of residual SIV (n = 4; 2 each from Group 1 and Group 2) or to repeat SIV challenge (n = 12; 5 from Group 1, 7 from Group 2). The latter analysis was also performed on long-term protected RM vaccinated with WT 68–1 RhCMV/SIV vaccine from our previous report (8). All the described RM experiments were performed once, and all results from these experiments are included in the presented data (no data were excluded as outliers). All plasma and cell-associated viral load assays were assayed by blinded analysts; however, due to logistical constraints, other staff were not blinded to treatment assignments. Primary data are reported in data file S1.

Statistical Analysis

We compared the fraction of protected RMs between treatment groups and challenges using Barnard's exact test of binomial proportions. To compare time-to-event data, we used the Mantel-Haenszel log-rank test. To examine SIV dynamics in vaccinated and protected RMs, we fit linear models of T cell responses with time and treatment group as independent

variables. Slope analyses of SIV Vif-specific T cell responses are described below. For all other comparisons, we used non-parametric Wilcoxon rank-sum tests for both paired and unpaired comparisons, and Kruskal-Wallis for comparisons of more than two groups. Neutralizing Ab titers were $\log_{10}$-transformed and normalized to baseline prior to computation of the area under curve (AUC) and compared using Wilcoxon rank sum tests. For log transformations when zeros were present, a small positive constant smaller than any nonzero value was added to all values prior to log transformation. For comparisons of AUC for percent responses, the data were also baseline-subtracted prior to AUC calculation. For all analyses of SIV dynamics including those described below for SIV Vif-, Gag-, and Pol-specific T cell responses, we also fit confirmatory models to account for variation among individual RMs using linear mixed, which confirmed our analysis in each case. All statistical analyses were conducted in R version 3.2.2 using the following R package versions: lmtest 0.9.34, zoo 1.8.1, survival 2.42.3, Exact 1.7, and lme 1.1.17. All P-values are based on two-sided tests and unadjusted except where noted. Adjusted P-values were computed using the Holm procedure for FWER control. Boxplots in Fig. 1 and Fig. S5 show jittered points and a box from 1st to 3rd quartiles (IQR) and a line at the median, with whiskers extending to the farthest data point within 1.5*IQR above and below the box, respectively.

For analyses of SIV-specific T cell responses, we log-transformed the responses prior to fitting to account for variance over time. For Vif-specific responses, we used Wald tests to compare models with and without specific time/group interaction terms to determine whether SIV clearance rate differed by vaccine. Models were fit using all data points in range after defining start and stop time points for each analysis according to the following predetermined procedure: we determined the start point as the time when the relevant mean response over all RMs reached its first peak before declining. The end point was the first time point after the start point at which the mean response was below the threshold for “return to baseline,” which we determined by taking the mean plus three standard deviations of all response values in the plateau phase (beyond 96 days after infection). For SIV Gag- and Pol-specific $CD4^+$ and $CD8^+$ T cell responses, we used Wald tests to compare individual slopes to 0.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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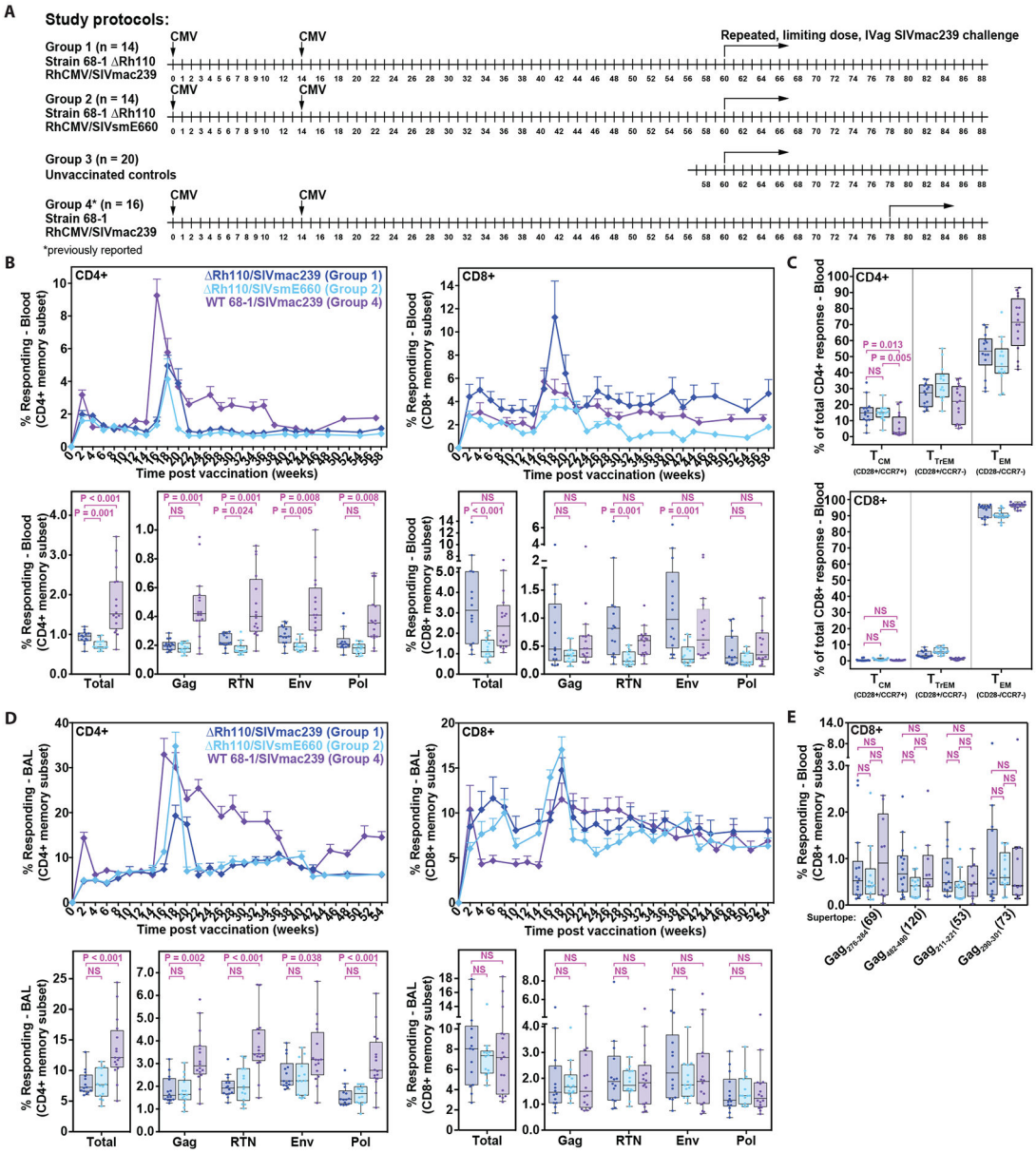


Figure 1: Immunogenicity of ΔRh110 RhCMV/SIV vectors.

(**A**) Schematic of the RM groups analyzed in this study. (**B**) Longitudinal and plateau-phase analysis of the vaccine-elicited, SIV Gag, Rev/Tat/Nef (RTN), Pol, and Env insert-specific $CD4^+$ and $CD8^+$ T cell responses in peripheral blood. In the top panel, the background-subtracted frequencies of cells producing TNF and/or IFN-γ by flow cytometric ICS assay to peptide mixes comprising each of the SIV inserts (SIV_{mac239} sequence) within the memory $CD4^+$ or $CD8^+$ T cell subsets were summed for overall responses with the figure showing the mean (+ SEM) of these overall responses at each time point. In the bottom panel, boxplots compare the overall and individual SIV insert-specific $CD4^+$ and $CD8^+$ T cell response frequencies between the vaccine groups at the end of the vaccine phase (each data point is the mean of response frequencies in all samples from weeks 30–58 post-first vaccination). Two-sided Wilcoxon rank-sum tests were used to compare the significance of

differences in plateau-phase response frequencies between Group 1 and Group 2 (SIV_{mac239} vs. SIV_{smE660} inserts in ΔRh110 68–1 vectors), and between Group 1 and Group 4 (SIV_{mac239} inserts in WT 68–1 vs. ΔRh110 68–1 vectors). (**C**) Boxplots compare the memory differentiation of the vaccine-elicited $CD4^+$ and $CD8^+$ memory T cells in peripheral blood responding to SIV Gag peptide mix (SIV_{mac239} sequence) with TNF and/or IFN-γ production at the end of vaccine phase (week 54 for Groups 1 and 2; week 60 for Group 4). Memory differentiation state was based on CD28 and CCR7 expression, delineating central memory (T_{CM}), transitional effector-memory (T_{TrEM}), and effector-memory (T_{EM}), as designated. Two-sided Wilcoxon rank-sum tests were used to compare the significance of differences in the fraction of responding cells with a T_{CM} phenotype (reciprocal of fraction with effector differentiation - T_{TrEM} + T_{EM}). (**D**) Same analysis as in B, but for responses in lung airspace (BAL). Each data point for the boxplots is the mean of response frequencies in all samples from weeks 30–54 post-first vaccination (**E**) Boxplots show plateau-phase analysis (each point is the average of all samples between weeks 24–30 post-first vaccination) of the vaccine-elicited $CD8^+$ T cell responses to SIV Gag supertopes (SIV_{mac239} sequence; Fig. S1B) in peripheral blood of Group 1, Group 2, and Group 4 RM by the same ICS assay described above. $Gag_{276–284}$ (69) and $Gag_{482–490}$ (120) are MHC-E-restricted supertopes; $Gag_{211–222}$ (53) and $Gag_{290–301}$ (73) are MHC-II-restricted supertopes (9,10). Statistical testing performed as described in **B**. In all panels, n = 14, 14, and 16 respectively for Groups 1, 2 and 4, except Group 4 in panel **E** where n = 10. Analyses were adjusted for multiple comparisons across inserts (**B, D**), epitopes (**C**), and supertopes (**E**) using the Holm method, and P-values ≤0.05 were considered significant. Analyses of total responses (**B, D**) were not adjusted.

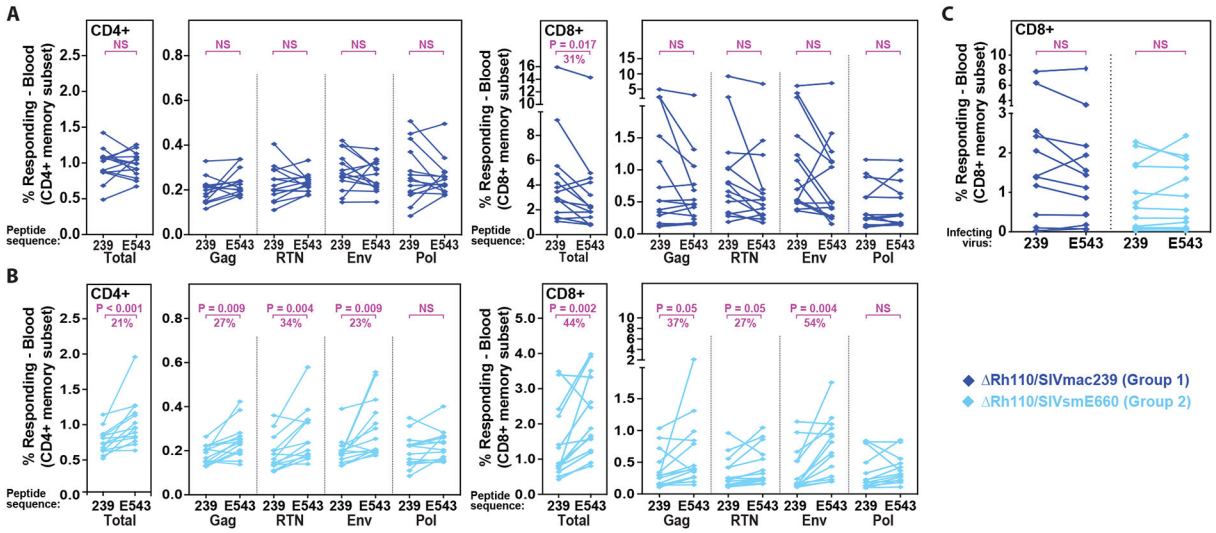


Figure 2: Cross-recognition by ΔRh110 RhCMV/SIV$_{mac239}$ and RhCMV/SIV$_{smE660}$ vector-elicited T cells.

(**A,B**) Flow cytometric ICS analysis of SIV-specific CD4$^+$ and CD8$^+$ T cell response frequencies (using TNF and/or IFN-γ readout in memory subset) in the blood of Group 1 (n = 14; SIV$_{mac239}$ inserts) and Group 2 (n= 14; SIV$_{smE660}$ inserts) RM in plateau phase (week 44 after first vaccination) comparing recognition of matched vs. mis-matched peptide mixes (SIV$_{mac239}$ vs. SIV$_{smE543}$; see Fig. S2), including overall (summed) responses and responses to each SIV insert. Two-sided paired Wilcoxon rank-sum tests were used to compare the significance of differences in matched vs. mismatched peptide mix recognition. Unadjusted (total responses) or Holm-adjusted (each insert-specific response) P-values ≤0.05 were considered significant. When significant differences were observed (reduction in response frequencies with mismatched peptide mixes), the median effect size (% reduction with mismatch) is shown. (**C**) ICS analysis of CD8$^+$ T cell recognition of autologous CD4$^+$ T cells infected with the SIV$_{mac239}$ vs. SIV$_{smE543}$ viruses (after background subtraction of the response to mock-infected autologous CD4$^+$ T cells) in plateau phase (between weeks 49–57 post-first vaccination). Statistical analysis performed as described above, with n = 12 and 13, for Groups 1 and 2, respectively.

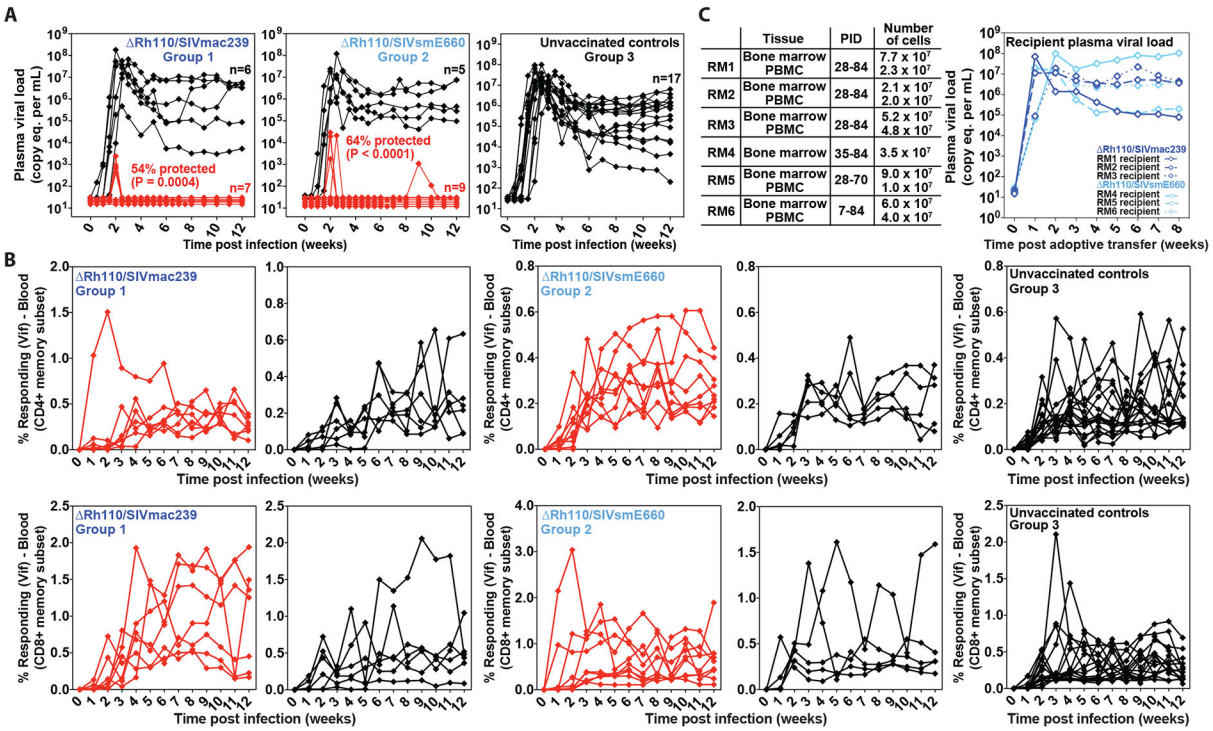


Figure 3: Efficacy of ΔRh110 RhCMV/SIV vectors.

(**A,B)** Assessment of the outcome of effective challenge by longitudinal analysis of plasma viral load (**A**) and *de novo* development of SIV Vif-specific $CD4^+$ (**B**, top panel) and $CD8^+$ (**B**, bottom panel) T cell responses. RM were challenged until the onset of any above-threshold SIV Vif-specific T cell response, with the SIV dose administered 2 or 3 weeks prior to this response detection considered the infecting challenge (week 0). RM with sustained viremia were considered not protected (black); RM with no or transient viremia were considered protected (red) (8). The fraction of protected RM in the vaccinated groups (Groups 1 and 2, n = 13 and 14, respectively) were compared to that of the unvaccinated group (Group 3, n = 17) by Barnard's exact test of binomial proportions, with the P-values shown in (A). (**C**) BM cells and PBMC were collected and cryopreserved from ΔRh110/$SIV_{mac239/smE660}$ vaccine-protected RM without any detectable viremia (RM #1, RM #2, RM #3 from Group 1; RM #4, RM #5, RM #6 from Group 2) at the indicated time points post-effective challenge (left panel; PID – post-infection day). Cells were thawed and administered intravenously (left panel) to 6 SIV-naïve RM to assess the presence of replication-competent SIV with the plasma viral dynamics in recipient RM shown (right panel).

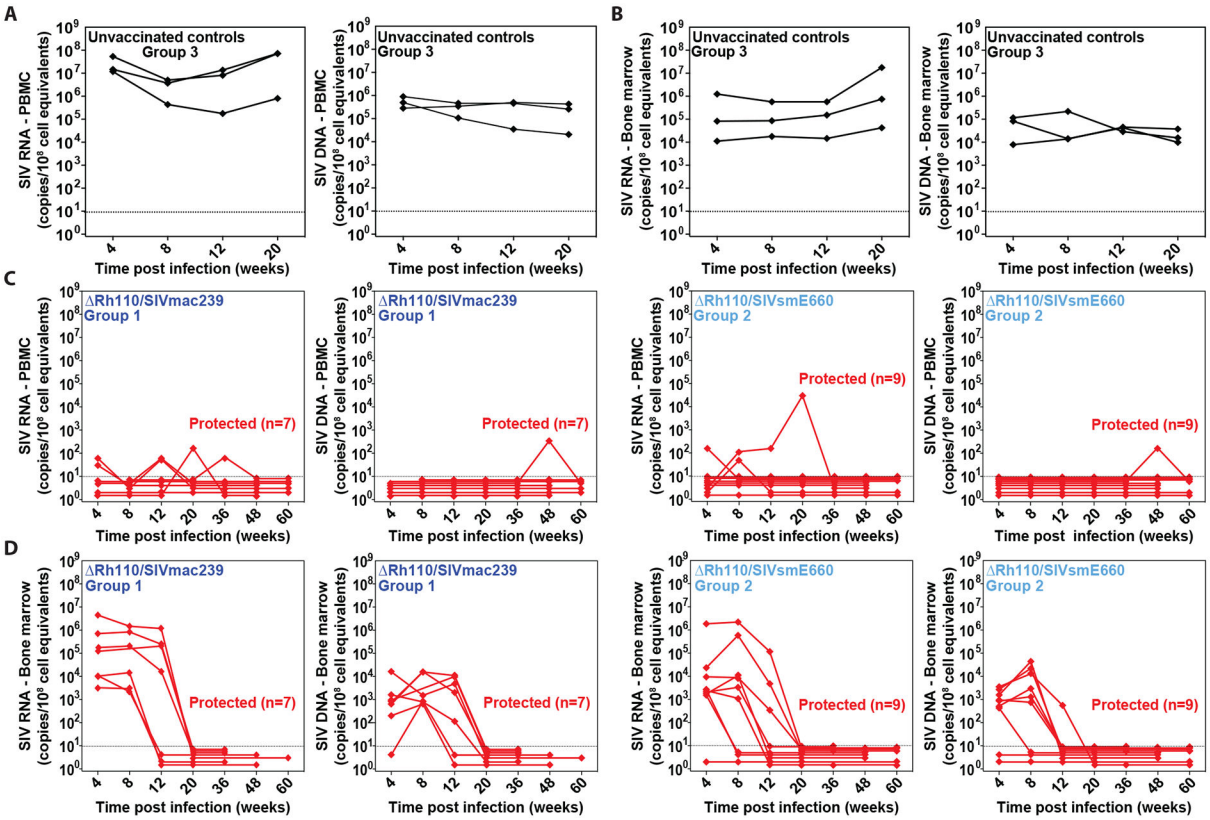


Figure 4: Clearance of cell-associated SIV in the BM of ΔRh110 68–1 RhCMV/SIV vector-protected RM.

(**A**–**D**) Longitudinal analysis of PBMC-associated (**A,C**) and BM cell-associated (**B,D**) SIV RNA (left panels) and DNA (right panels) from 3 randomly selected unvaccinated RM with progressive infection (**A,B**), and all 16 ΔRh110/SIV$_{mac239/smE660}$ vector-protected RM in Groups 1 and 2 (**C,D**).

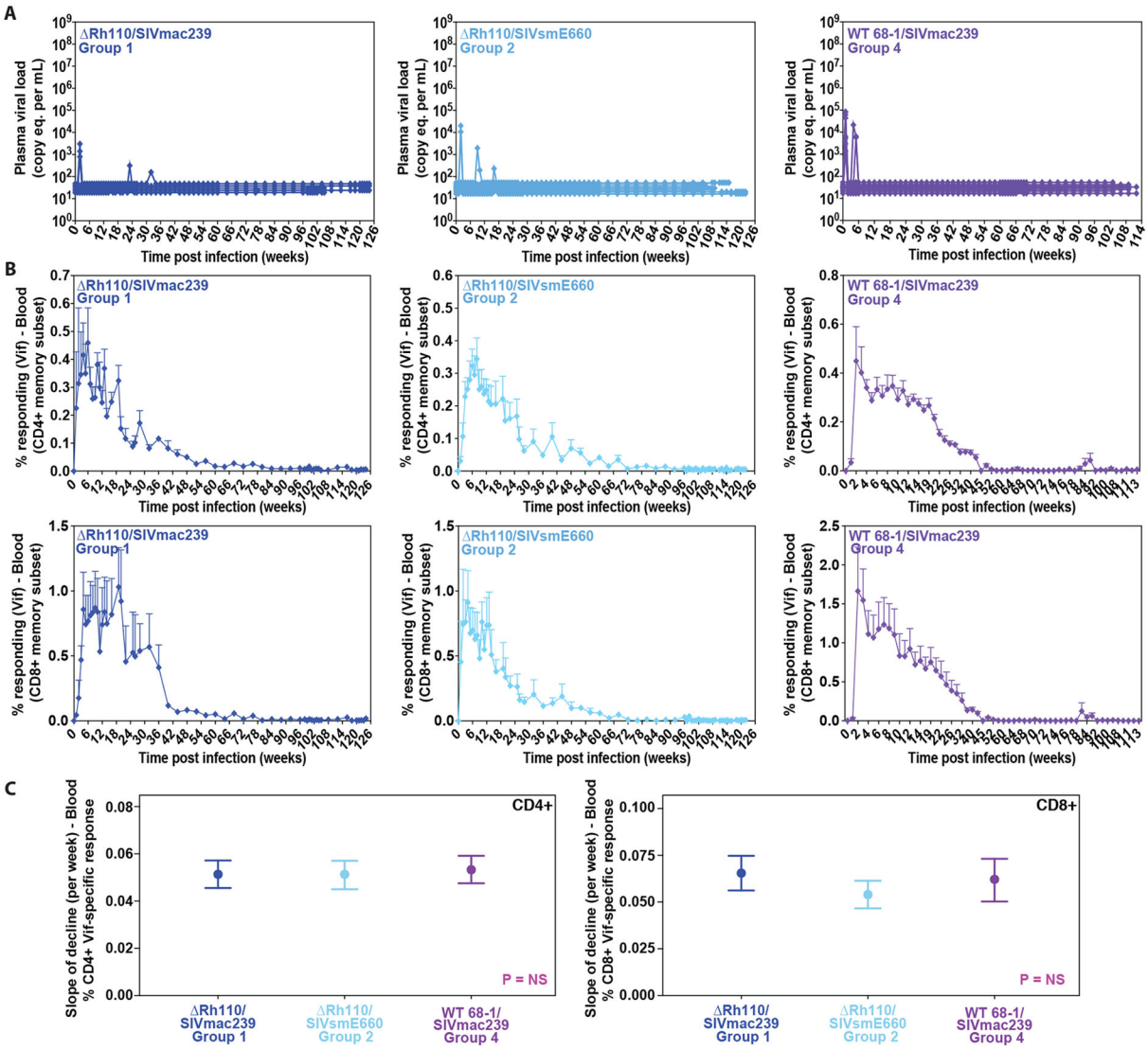
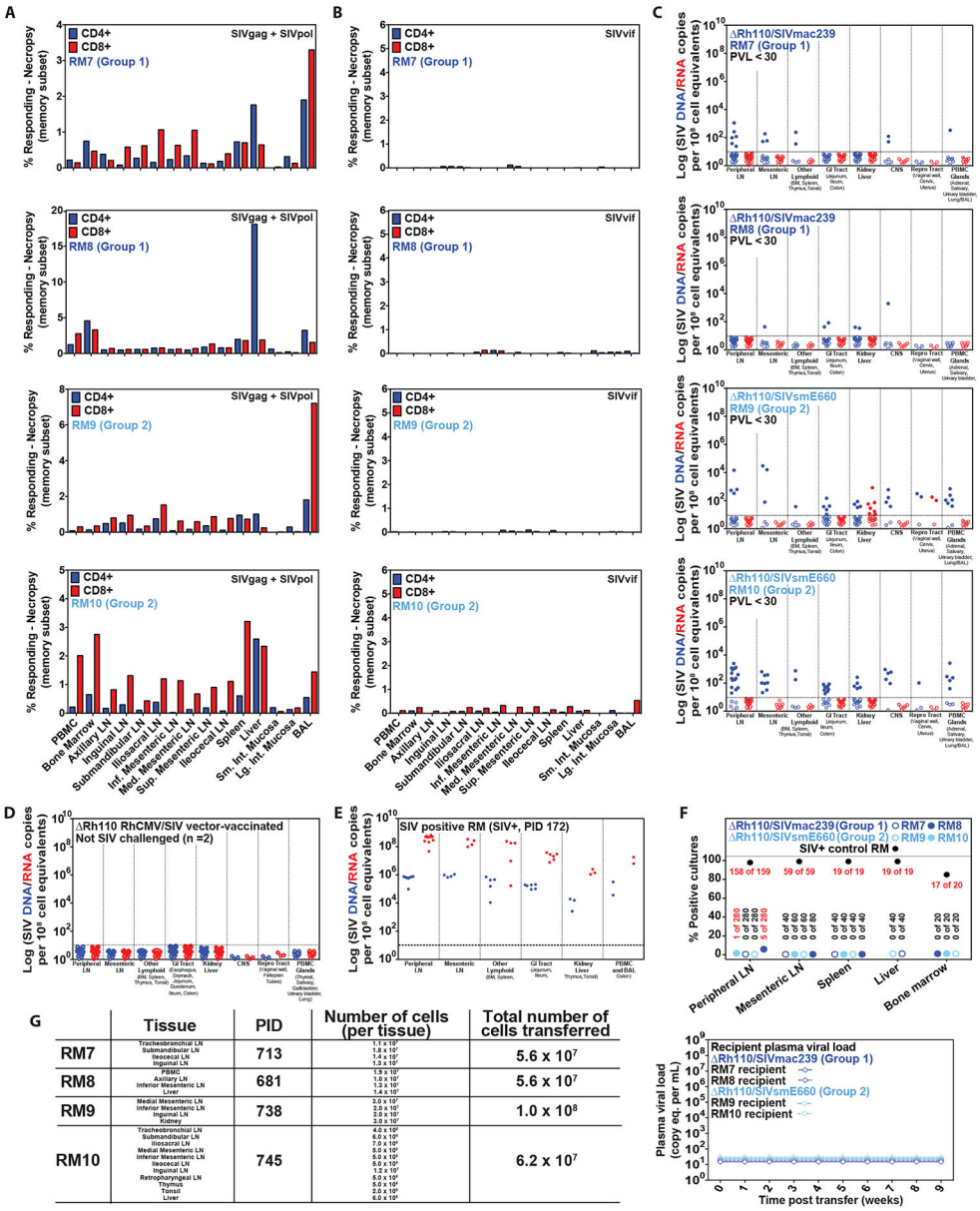


Figure 5: Loss of circulating SIV infection-induced, SIV Vif-specific T cells in ΔRh110 68–1 RhCMV/SIV vector-protected RM

(**A**) Long-term longitudinal analysis of plasma viral load in ΔRh110/$SIV_{mac239/smE660}$ vector-protected (left and middle panels for Groups 1 and 2, respectively) and WT 68–1/ SIV_{mac239} vector-protected RM (Group 4, right panel, (8)). (**B**) Long-term longitudinal analysis of SIV Vif-specific $CD4^+$ (top panels) and $CD8^+$ (bottom panels) among the same groups of ΔRh110 and WT 68–1 RhCMV/SIV vector-protected RM with the figure showing the mean (+ SEM) of these SIV Vif-specific T cell response frequencies in the memory subset at each time point. (**C**) Wald tests comparing the slope (± 95% confidence intervals) of decline of log-transformed SIV Vif-specific $CD4^+$ (left panel) and $CD8^+$ (right panel) T cell response frequencies. Calculation of slopes is described in Materials and Methods. In all analyses, n = 7, 9, and 8 for Groups 1, 2 and 4, respectively.



G

	Tissue	PID	Number of cells (per tissue)	Total number of cells transferred
RM7	Tracheobronchial LN Submandibular LN Ileocecal LN Inguinal LN	713	1.1 x 10⁷ 1.6 x 10⁷ 1.4 x 10⁷ 1.3 x 10⁷	5.6 x 10⁷
RM8	PBMC Axillary LN Inferior Mesenteric LN Liver	681	1.9 x 10⁷ 1.9 x 10⁷ 1.3 x 10⁷ 1.4 x 10⁷	5.6 x 10⁷
RM9	Medial Mesenteric LN Inferior Mesenteric LN Inguinal LN Kidney	738	3.0 x 10⁷ 2.0 x 10⁷ 2.0 x 10⁷ 3.0 x 10⁷	1.0 x 10⁸
RM10	Tracheobronchial LN Submandibular LN Iliosacral LN Medial Mesenteric LN Inferior Mesenteric LN Ileocecal LN Inguinal LN Retropharyngeal LN Thymus Tonsil Liver	745	4.0 x 10⁶ 6.0 x 10⁶ 7.0 x 10⁶ 5.0 x 10⁶ 5.0 x 10⁶ 5.0 x 10⁶ 1.3 x 10⁷ 5.0 x 10⁶ 5.0 x 10⁶ 2.0 x 10⁷ 6.0 x 10⁶	6.2 x 10⁷

Figure 6: Necropsy analysis of ΔRh110 68–1 RhCMV/SIV vector-protected RM.

(**A**–**C**) Analysis of SIV Gag+Pol-specific (**A**) and SIV Vif-specific (**B**) $CD4^+$ and $CD8^+$ T cell response frequencies by flow cytometric ICS (using SIV_{mac239} peptides mixes; see Fig. 1), and tissue-associated SIV DNA and RNA by nested qPCR/RT-PCR (**C**) in tissues of 4 $\Delta Rh110/SIV_{mac239/smE660}$ vector-protected RM (RM #7 and RM #8 from Group 1; RM #9 and RM #10 from Group 2) taken to necropsy at 713 days (RM #7), 681 days (RM #8), 738 days (RM #9) and 745 days (RM #10) post-infection. (**D,E**) Analysis of tissue-associated SIV DNA and RNA in tissues of 2 ΔRh110 68–1 RhCMV/SIVgag (SIV_{mac239} sequence insert) vector-vaccinated RM that were taken to necropsy 531 and 763 days post-vaccination without SIV challenge (negative controls; **D**), and one SIV_{mac239}-infected RM with progressive infection taken to necropsy 172 days post-infection (positive control; **E**). In **C**–**E**, each data point indicates an independent tissue sample of the indicated tissue type and the

dotted lines indicate the detection threshold. (**F,G**) Assessment of residual replication-competent SIV in cell suspensions obtained from the indicated tissue samples by *in vitro* co-culture analysis (**F**) and by adoptive transfer of cells into 4 SIV-naïve RM (**G**).

	Tissue	PID*	Number of cells (per tissue)	Total number of cells transferred
RM1	LN PBMC	673-728	3.6×10^7 6.4×10^7	1.0×10^8
RM2	Bone marrow LN PBMC	420-714	1.1×10^7 1.5×10^7 7.4×10^7	1.0×10^8
RM5	Bone marrow LN PBMC	420-673	1.0×10^7 4.1×10^7 4.9×10^7	1.0×10^8
RM6	LN PBMC	588-714	4.0×10^7 6.0×10^7	1.0×10^8

*First challenge

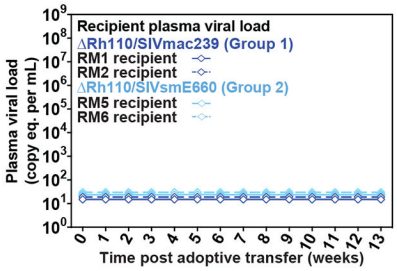
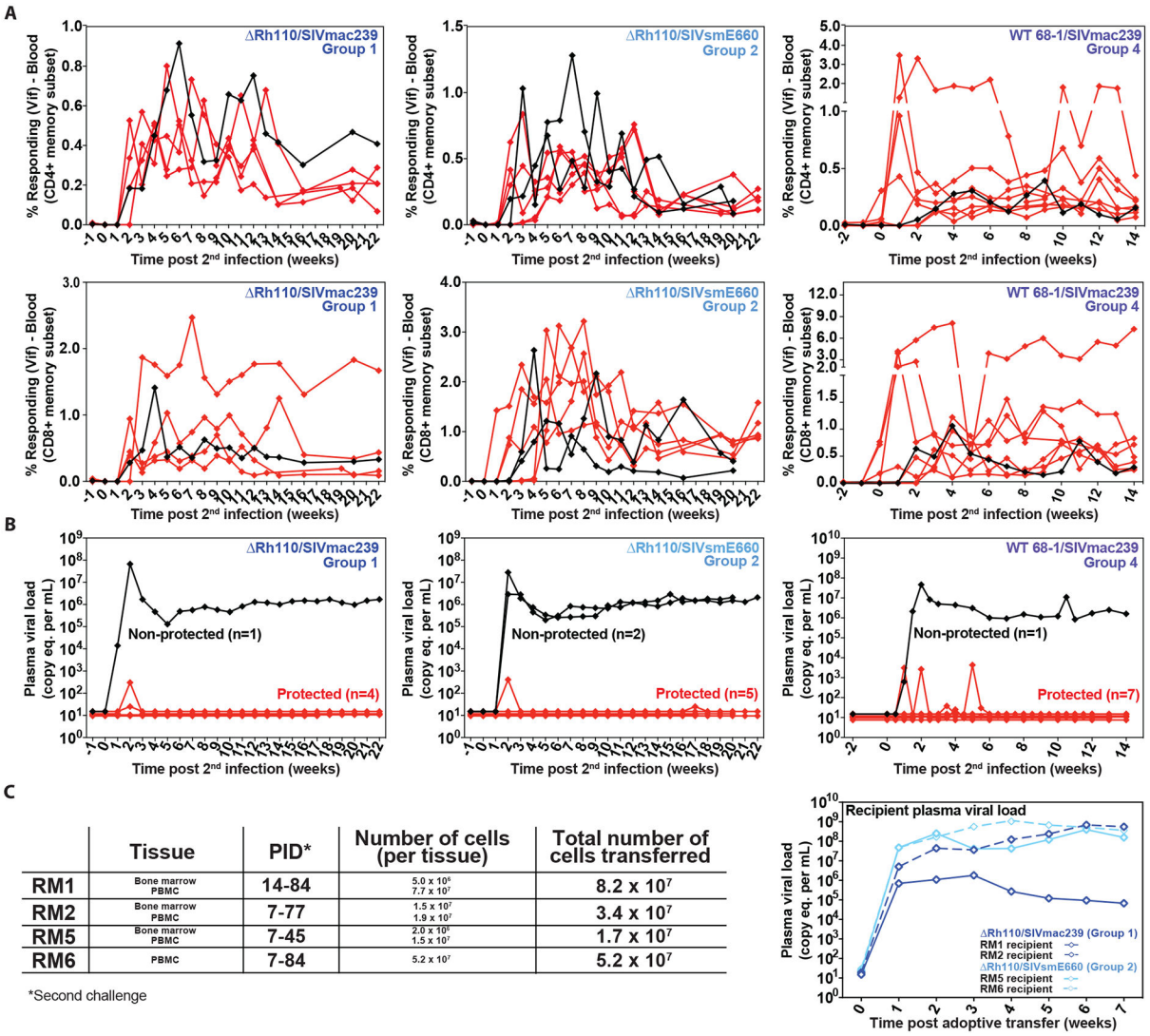


Figure 7: Loss of transferable SIV in long-term ΔRh110 68–1 RhCMV/SIV vector-protected RM.

Second assessment of replication-competent SIV by adoptive transfer of cells from 4 long-term ΔRh110/$SIV_{mac239/smE660}$ vector-protected RM (RM #1 and RM #2 from Group 1; RM #5 and RM #6 from Group 2) that were previously shown to harbor replication SIV by the same assay.



	Tissue	PID*	Number of cells (per tissue)	Total number of cells transferred
RM1	Bone marrow PBMC	14-84	5.0×10^8 7.7×10^7	8.2×10^7
RM2	Bone marrow PBMC	7-77	1.5×10^7 1.9×10^7	3.4×10^7
RM5	Bone marrow PBMC	7-45	2.0×10^8 1.5×10^7	1.7×10^7
RM6	PBMC	7-84	5.2×10^7	5.2×10^7

*Second challenge

Figure 8: Resistance of ΔRh110 68–1 RhCMV/SIV vector-protected RM to repeat SIV challenge.

(**A, B**) Outcome of repeat SIV_{mac239} challenge of long-term 68–1 RhCMV/SIV vector-protected RM (n = 5, 7 and 8 for Groups 1, 2 and 4, respectively) by longitudinal analysis of *de novo* SIV Vif-specific $CD4^+$ and $CD8^+$ T cell responses (**A**) and plasma viral load (**B**) with protected and non-protected RM defined as described in Fig. 3. (**C**) Third assessment of replication-competent SIV by adoptive transfer of cells from RM #1, RM #2, RM #5 and RM #6 after effective re-challenge (re-induction of SIV Vif-specific T cell responses) with repeated aviremic protection.