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# Enhancing safety of cytomegalovirus-based vaccine vectors by engaging host intrinsic immunity

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## Abstract

Rhesus cytomegalovirus (RhCMV)-based vaccines maintain effector-memory T cell responses ($T_{EM}$) that protect ~50% of rhesus monkeys (RMs) challenged with simian immunodeficiency virus (SIV). Because human CMV (HCMV) causes disease in immune-deficient subjects, clinical translation will depend upon attenuation strategies that reduce pathogenic potential without sacrificing CMV's unique immunological properties. We demonstrate that "intrinsic" immunity can be used to attenuate strain 68–1 RhCMV vectors without impairment of immunogenicity. The tegument proteins pp71 and UL35 encoded by *UL82* and *UL35* of HCMV counteract cell-intrinsic restriction via degradation of host transcriptional repressors. When the corresponding RhCMV genes, *Rh110* and *Rh59*, were deleted from 68–1 RhCMV (ΔRh110 and ΔRh59), we observed only a modest growth defect *in vitro*, but *in vivo*, these modified vectors manifested little to no amplification at the injection site and dissemination to distant sites, in contrast to parental 68–1

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**AUTHOR CONTRIBUTIONS**: KF and LJP conceived the attenuated RhCMV vector strategy, supervised experiments, analyzed and interpreted data, and wrote the paper assisted by SGH, EEM and DM. SGH planned and performed animal experiments and immunologic assays, assisted by RMG, CMH, ABV, EA, ANS, JCF and DB. EEM and DM constructed and quality-tested recombinant RhCMV constructs, assisted by JW. DS and CNK planned and supervised RhCMV quantification by PCR/RT-PCR assisted by CK. AWL and MKA managed the animal care and procedures. PTE performed all statistical analyses.

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**DATA AND MATERIALS AVAILABILITY**: All data associated with this study are present in the paper or Supplementary Materials. Vector primer sequences have been deposited in Genbank (see Supplementary Materials for accession numbers). The computer code used to perform statistical analysis is available at https://doi.org/10.5281/zenodo.3242804. RhCMV/SIV vectors can be obtained through a materials transfer agreement.

RhCMV. ΔRh110 was not shed at any time post-infection and was not transmitted to naïve hosts by either close contact (mother to infant) or by leukocyte transfusion. In contrast, ΔRh59 was both shed and transmitted by leukocyte transfusion, indicating less effective attenuation than pp71 deletion. Importantly, the T cell immunogenicity of ΔRh110 was essentially identical to 68–1 RhCMV with respect to magnitude, $T_{EM}$ phenotype, epitope targeting, and durability. Thus, pp71 deletion preserves CMV vector immunogenicity while stringently limiting vector spread, making pp71 deletion an attractive attenuation strategy for HCMV vectors.

## One sentence summary:

Live-attenuated, spread-deficient rhesus CMV-vectors retain T cell immunogenicity.

## INTRODUCTION

The beta-herpesviruses HCMV and RhCMV are ubiquitous in human and RM populations, respectively (1,2). Although these viruses are specifically adapted to their respective host species, they share orthologous genomes and a remarkably similar biology, reflecting millions of years of host-viral co-evolution (3,4). Infection usually occurs in the first year of life and is asymptomatic in the vast majority of subjects, with viral replication and spread controlled by host immunity, particularly virus-specific T cell responses (5,6). At the same time, CMV uses complex immune evasion strategies to prevent host immunity from clearing the infection or inhibiting its ability to spread to new hosts, including super-infection of $CMV^+$ hosts, resulting in permanent infections with one or more viral strains (7–9). CMV and their primate hosts thus establish a stable equilibrium characterized by 1) a low viral burden in infected individuals with mostly latent infection (albeit with occasional reactivation and sufficient shedding to maintain efficient host-to-host transmission) resulting in persistent infection, and 2) an extra-ordinarily high frequency of $CD4^+$ and $CD8^+$ CMV-specific T cells with a unique $T_{EM}$ -biased phenotype and function that contains the infection and thus tissue damage and disease (6,10,11).

Although other viruses can establish persistent infection, the lifelong, high frequency, tissue-based, $T_{EM}$-biased T cell responses established by CMV are unique, which raised the possibility of exploiting CMV as a vaccine vector for other infectious diseases (12–14). In particular, we hypothesized that recombinant CMV expressing heterologous inserts would pre-position potent, effector-differentiated responses to a different pathogen in the portal of entry and sites of early spread of that pathogen. This early interception (prior to implementation of its immune evasive programs) would potentially have superior efficacy for immune evasive pathogens than typical vaccine-elicited memory T cell responses that require anamnestic expansion over many days, if not weeks, to mount peak effector responses in tissues (14). Indeed, we demonstrated that the cellular immune responses elicited by strain 68–1 RhCMV vectors expressing SIV inserts intercept and completely control SIV replication in the first week post-challenge in ~50% of vaccinated RMs, without anamnestic expansion of SIV-specific immune responses (15–17). Most remarkably, the residual infection in protected RMs was progressively cleared over weeks to months, ultimately leaving these RMs indistinguishable by virologic and immunologic criteria from vaccinated RMs that were never challenged (17). RhCMV vectors with *Mycobacterium*

*tuberculosis* (*Mtb*) inserts elicit analogous $CD4^+$ and $CD8^+$ $T_{EM}$ responses to *Mtb*, and RMs vaccinated with these vectors show a nearly 70% reduction in *Mtb* disease after Erdman strain challenge, with 40% of vaccinated animals showing no detectable granulomatous disease at all (18).

These preclinical data support consideration of the possible development of CMV-vectored HIV and *Mtb* vaccines for use in people, creating an imperative to develop and test HCMV-based vectors in the clinic (19,20). However, clinical development of HCMV-based vectors for prophylactic use in healthy individuals will require a high standard of safety. Although HCMV does not cause overt disease in the vast majority of infections, the potential for symptomatic primary infection in HCMV-naïve individuals, and for serious disease in immunocompromised subjects or in mother-to-fetus transmission (21), is sufficiently high to contraindicate the use of unmodified HCMV vectors in a prophylactic vaccine setting. We therefore sought to develop an attenuation strategy that would reduce or, if possible, eliminate the pathogenic potential of HCMV, yet retain the unique, persistent immunogenicity, as well as the ability to super-infect $CMV^+$ individuals. Furthermore, it is preferable for clinical vectors to not be transmissible by close contact or even transfusion, so as to prevent unintentional infection of individuals for which vaccination might pose a safety risk.

Encouragingly, mouse CMV (MCMV) rendered unable to spread from initially infected cells nonetheless maintained strong cell-mediated immunity, suggesting that viral dissemination may not be required for immunogenicity (22–24). However, it remains unclear whether highly debilitated RhCMV or HCMV vectors can establish and maintain persistent secondary infections in monkeys or humans. Indeed, the HCMV strain Towne, attenuated by serial *in vitro* passaging, seems to have lost its ability for persistent immune induction as indicated by steadily declining T cell responses (25). In addition, chimeras of Towne and a fibroblast-adapted derivative of the primary isolate Toledo were unable to establish secondary infections in $HCMV^+$ individuals (26), and the T cell responses elicited by these chimeras in $HCMV^-$ individuals did not display the CMV-typical $T_{EM}$ phenotype and declined over time (27,28). These data suggest that traditional attenuation strategies may yield suboptimal HCMV vectors that lose the ability to maintain persistent immune stimulation.

Here, we examine a new strategy for CMV attenuation based on exploiting host proteins that provide “intrinsic” immunity. This term describes cellular intrinsic defense mechanisms involving host proteins such as death domain-associated protein (DAXX), alpha-thalassemia X-linked mental retardation protein (ATRX), BCL-associated factor 1 (BclAF1) and promyelocytic leukemia protein (PML) that combine to form nuclear ND10 bodies (29). ND10 proteins repress transcription of viral immediate early (IE) genes, which are critical for early (E) and late (L) gene expression and genome replication (30, 31). To counteract intrinsic immunity, herpesviruses encode proteins that eliminate or disperse ND10 components (32). HCMV evades intrinsic defenses through viral tegument proteins released during viral entry, thus facilitating IE expression (33). The tegument protein pp71, encoded by *UL82*, mediates the degradation of DAXX (34–36) and, together with UL35, BclAF1 (37) in the infected cell nucleus. Intrinsic immune repression of IE expression might enable

the establishment and maintenance of latency by silencing viral genes needed for lytic replication (38–40). Thus, by eliminating viral counter-mechanisms targeting intrinsic immunity, it might be possible for CMV vectors to maintain persistent infection and long-term immune stimulation, while severely hampering lytic replication, cell-to-cell spread, and distant dissemination. To examine this possibility, we characterized the *in vivo* replication, spread, dissemination, and immunogenicity of RhCMV vectors lacking *Rh110* and *Rh59*, the RhCMV orthologs of HCMV *UL82* and *UL35*.

## RESULTS

### RhCMV pp71 mediates DAXX degradation and supports RhCMV growth *in vitro.*

HCMV pp71 has two DAXX interaction domains that are required for localization of pp71 to ND10 bodies (41) where pp71 dislocates ATRX and mediates the degradation of DAXX by the proteasome (38). Although Rh110, the predicted RhCMV pp71 ortholog, shares 41% identity with HCMV pp71 (42), these DAXX interaction domains are not conserved. Nevertheless, when RhCMV pp71 was expressed in telomerized rhesus fibroblasts (TRFs) under doxycycline (DOX) control, steady-state DAXX was reduced upon removal of DOX (Fig. 1A). When we examined the localization of rhesus DAXX, ATRX, and PML by immunofluorescence, we observed punctate nuclear co-staining of these proteins in the absence of pp71. Upon pp71 expression, both ATRX and DAXX no longer localized in punctate dots, whereas pp71 partially co-localized to PML bodies (Fig. S1). Thus, despite lacking canonical DAXX-interaction domains, RhCMV pp71 degrades and displaces DAXX from PML bodies similar to HCMV pp71 (32).

As reported for HCMV (31,35,36,43,44), DAXX and BclAF1 were reduced during early times of infection with parental strain 68–1 RhCMV (68–1) compared to uninfected cells (Fig. 1B) [unless otherwise noted, all RhCMV constructs are based on bacterial artificial chromosome (BAC)-derived strain 68–1 (45)]. This degradation was mediated by pp71, since DAXX abundance, and to some extent, BclAF1 abundance were restored in fibroblasts infected with 68–1 RhCMV lacking pp71 (ΔRh110) (Fig. 1B, Fig. S2A,B). At the high multiplicity of infection (MOI) used in this experiment, deletion of pp71 did not affect the kinetics or expression of IE, E, or L proteins, similar to previous reports showing that DAXX repression of HCMV gene expression is overcome at high MOI (36,43). This growth defect could be reversed by knockdown of DAXX expression (Fig. S2C), suggesting increased anti-viral activity by DAXX being the main reason for the observed growth defect and consistent with increased IE expression reported for HCMV upon DAXX siRNA treatment (36).

Interestingly, pp71-deleted RhCMV could be recovered upon BAC transfection of fibroblasts without the need for complementation, and high titer stocks could be generated in the absence of pp71 complementation. In contrast, UL82-deleted HCMV could only be grown in pp71-complementing cells (46) and guinea pig CMV lacking the UL82 homolog could not be recovered unless complemented (47). To determine the impact of pp71 deletion and complementation on viral growth *in vitro*, we compared 68–1 virus production upon infection of fibroblasts with RhCMV in which Rh110 had been replaced with SIVgag (ΔRh110/SIVgag) so that transcription was regulated by the native Rh110 promoter.

ΔRh110/SIVgag was generated on complementing cells, which results in pp71 incorporation into the virus particle (Fig. S2D), or without complementation (−/−). There was little or no growth impairment of complemented or uncomplemented ΔRh110/SIVgag at high MOI (1 and 0.1), whereas a 10- to 100-fold reduction of viral yield was observed at low MOI (0.01 and 0.001) (Fig. 1C). In contrast, a 10- to 100-fold reduction in viral growth was reported for complemented or uncomplemented HCMV at high MOI and ~5-log reduction in viral growth at low MOI (46) suggesting that abrogation of pp71 expression impacts growth of RhCMV less severely than that of HCMV. Nevertheless, pp71 deletion clearly reduced spreading of virus from infected cells to neighboring cells since the average plaque sizes of complemented and uncomplemented ΔRh110/SIVgag at day 7 post-infection at low MOI (0.001) were strongly reduced compared to 68–1 in TRF, but not in TRF-pp71 (Fig. 1D). Thus, although pp71 is not absolutely required for RhCMV growth *in vitro,* it substantially enhances the efficiency of viral spreading in fibroblast cultures.

### ΔRh110 RhCMV is spread-deficient *in vivo* but remains immunogenic for T cell responses.

To examine whether deletion of pp71 would affect viral dissemination *in vivo,* we subcutaneously inoculated RhCMV-naïve RMs with $10^7$ plaque-forming units (PFU) of 68–1 expressing SIVgag in the left arm and complemented ΔRh110-expressing SIVrev/tat/nef/int (rtni) in the right arm (Fig. S2A). The viral constructs used in each of the *in vivo* experiments in this report are schematically depicted in Fig. S3. RMs were necropsied at 14, 21, or 28 days post-infection (dpi) and viral copy numbers were determined by ultra-sensitive, nested qPCR specific for SIVgag or SIVrtni sequences in multiple tissue samples (17, 49). SIVgag-containing genomes were detectable in most tissues in the RMs necropsied at 14 dpi, with particularly high genome copy numbers observed at the site of inoculation and associated draining lymph nodes, the contralateral injection site, and salivary glands (Table 1A), whereas genome copy numbers were progressively lower in RMs necropsied at 21 and 28 dpi, in keeping with progressive immune control of infection, but were still detectable in most tissues. In notable contrast, genomes for ΔRh110/SIVrtni were barely detectable in the same tissues at 14 dpi and below detection limits in almost all tissues at 21 and 28 dpi (Table 1A). Of note, similar frequencies of SIVgag- and SIVrtn-specific $CD4^+$ and $CD8^+$ T cell responses were measured in lymphoid and non-lymphoid tissues of all 3 RMs, with the possible exception of some tissues at 14 dpi where SIVgag-specific T cell responses were modestly higher (Fig. S4A). Thus, deletion of Rh110 markedly limited the genome replication and dissemination of RhCMV during primary infection, but had little to no effect on the initial development of vector-elicited, SIV-specific T cells.

The inability to detect ΔRh110/SIVrtni by ultra-sensitive PCR at four weeks post-primary infection could indicate that RhCMV lacking pp71 was unable to persist, and thus might be unable to maintain immune stimulation. However, in two RhCMV-naïve RMs given $10^7$ PFU of ΔRh110, the magnitude and kinetics of T cell responses to RhCMV IE1 and pp65a in peripheral blood mononuclear cells (PBMCs) and bronchoalveolar lavage (BAL) cell preparations were comparable to that of 4 RhCMV-naïve RMs inoculated with $10^7$ PFU of 68–1 through 231 dpi (Fig. 2A,B). In contrast to 68–1, ΔRh110 was not shed in the urine of these monkeys at any time point (Fig. 2C), confirming the *in vivo* attenuation of ΔRh110.

Thus, despite marked inhibition of viral spread *in vivo*, lack of pp71 did not affect viral immunogenicity in the first 7 months after primary infection.

A key feature of CMV vectors is their ability to overcome pre-existing anti-CMV immunity, permitting the use of CMV vectors regardless of prior CMV infection (7), which must be preserved in any attenuation strategy. To determine whether ΔRh110 retained this capability, we administered RhCMV-seropositive ($RhCMV^+$) RMs with $10^7$ PFU of 68–1/SIVrtni (subcutaneously, right arm) and the same dose of complemented ΔRh110/SIVgag (subcutaneously, left arm), and monitored their dissemination by qPCR in tissues of RMs necropsied at 14, 21 or 28 dpi. As shown in Table 1B, pre-existing immunity substantially reduced genome copy numbers of 68–1/SIVrtni in all tissues when compared to nonimmune RMs (Table 1A), consistent with anti-CMV immunity profoundly limiting replication and dissemination of Rh110-intact 68–1 RhCMV. However, similar to seronegative RMs, ΔRh110 was not detectable or barely detectable in any tissues at 14 dpi, except for the inoculation site, and in the RMs necropsied at 21 and 28 dpi, this vector was below the limit of detection in all tissues. Again, SIVgag- and SIVrtn-specific $CD4^+$ and $CD8^+$ T cell responses were detectable in most tissues of all 3 RMs and were of comparable magnitude (Fig. S4B), indicating that even in the setting of pre-existing anti-CMV immunity, the development of RhCMV vector-elicited, insert-specific T cell responses was not compromised by the Rh110 deletion. We also compared the spread of uncomplemented ΔRh110 with pp71-complementation, which would provide the incoming vector with pp71 in its tegument and increase the efficiency of the first round of vector replication. At 14 dpi, both complemented ΔRh110/SIVgag and uncomplemented ΔRh110/SIVrtni were largely undetectable in tissues in two $RhCMV^+$ RMs (Table 1C), confirming the profound spread deficiency of pp71-deleted vectors.

Persistent CMV infections, including infections with 68–1 RhCMV vectors, elicit and indefinitely maintain high frequency $T_{EM}$, likely reflecting continuous or frequently recurring antigen exposure (13,15,18,48,50,51). To determine whether, despite limited spreading, pp71-deficient RhCMV maintained $T_{EM}$ with the same phenotype and function as 68–1 in CMV-immune RMs, we co-inoculated four $RhCMV^+$ RMs with complemented ΔRh110/SIVrtni or 68–1/SIVpol-5' so as to directly compare their immunogenicity in the same RM. 68–1/SIVpol-5' was shed into urine at 28 dpi in all 4 RMs, and found in all urine samples thereafter, as expected (7), whereas ΔRh110/SIVrtni was not detected in urine at any time point through 490 dpi (Fig. S5). Despite this notable difference, the average peak and plateau phase frequencies of SIVrtn-specific $CD4^+$ and $CD8^+$ T cells elicited by ΔRh110/SIVrtni in blood and BAL were similar, if not higher, than the corresponding SIVpol-specific T cell responses elicited by 68–1/SIVpol-5' (Fig. 3A; Fig. S6). Moreover, the circulating SIVrtn-specific $CD4^+$ and $CD8^+$ T cell responses elicited by ΔRh110 displayed the same highly $T_{EM}$-biased phenotype (CCR7-, CD28-; Fig. 3B) as 68–1 vector-elicited responses and overlapping patterns of cytokine production that were commensurate with the $T_{EM}$ phenotype (high TNF-α, IFN-β, MIP-1β; low IL-2; Fig. 3C). We also previously reported that 68–1 RhCMV vectors elicit $CD8^+$ T cells that very broadly target highly unconventional epitopes restricted by either MHC-II or MHC-E (52, 53). This broad, unconventional epitope targeting is not affected by pp71 deletion (Fig. 3D). Thus, the magnitude, durability, phenotype, cytokine synthesis function, and $CD8^+$ T cell epitope

targeting of ΔRh110-elicited SIV-specific T cell responses were essentially indistinguishable from that elicited by parental 68–1 RhCMV. We therefore conclude that, despite our inability to detect ΔRh110 in tissues 4 weeks following inoculation, pp71-deficient RhCMV persists in tissues at levels that support the maintenance of abundant highly $T_{EM}$-biased T cells.

To confirm that ΔRh110 RhCMV vectors could be used repeatedly in the same RM, either to boost pre-existing T cell responses or elicit new T cell responses to different inserts, we inoculated 2 RMs that had previously been vaccinated with ΔRh110/SIVrtni with both a second dose of ΔRh110/SIVrtni and with a different pp71-deleted vector expressing an SIVenv insert. As shown in Fig. 4A, this repeat, dual vaccination resulted in both a transient boosting of the SIVrtn-specific $CD4^+$ and $CD8^+$ T cell responses, and the *de novo* induction of an SIVenv-specific $CD4^+$ and $CD8^+$ T cell response of similar magnitude. This outcome is essentially identical to the previously reported boosting and repeated *de novo* infection behavior of pp71-intact 68–1 RhCMV (15). Moreover, the magnitude of both the SIVrtn- and SIVenv-specific $CD4^+$ and $CD8^+$ T cell responses remained stable for at least 4 years (Fig. 4A), at which point immune monitoring was discontinued. Thus, pp71-deleted RhCMV vectors retain the unique ability of this vaccine platform to sequentially induce and maintain T cell responses to different antigens in the same host.

CMV vectors are T cell-targeted vaccines, and RhCMV vectors elicit few, if any, insert-specific antibodies (Abs) in RMs (15–18). However, these vectors have the potential to elicit/boost Ab responses to RhCMV itself, raising the question of whether ΔRh110 vectors differ from parental 68–1 vectors in this activity. To address this, we compared RhCMV-specific Ab responses in cohorts of $RhCMV^+$ RMs that were vaccinated with either a 68–1 (n=16) or ΔRh110 (n=14) RhCMV/SIV vector set composed of 5 vectors, each expressing one SIVmac239 insert (gag, env, rtn, pol-5' and pol-3') and subcutaneously administered at a dose $5\times10^6$ PFU per vector. Abs to whole RhCMV viral lysates were measured by ELISA prior to, and 4, 6, and 12 weeks post-vaccination. As shown in Fig. S7, both cohorts showed a significant ($p<0.001$), but transient, boost in antibody titers, the magnitude of which was not different between the monkeys vaccinated with ΔRh110 vs. 68–1 vectors. Thus, when administered in a high-dose vaccination regimen, pp71-deleted RhCMV vectors were able to boost antibody responses to a similar degree as the parental 68–1 vectors.

To determine the extent to which pp71 deletion affects the minimal dose required for T cell immunogenicity, we first inoculated 3 cohorts of seropositive RMs (n=3 each) with $10^2$, $10^4$, or $10^6$ PFU of pp71-complemented ΔRh110/SIVgag and monitored the development of $CD4^+$ and $CD8^+$ T cell responses to SIVgag. As shown in Fig. 4B, ΔRh110/SIVgag induced *de novo* SIVgag-specific T cell responses at $10^4$ and $10^6$ PFU, but not at $10^2$ PFU. To further refine the dose required for immunogenicity, we re-inoculated the 3 RMs that had failed to respond to $10^2$ PFU with $10^3$ PFU ΔRh110/SIVgag, and for comparison, we inoculated all 9 RMs with $10^1$ PFU of 68–1/SIVpol-5' (Fig. 4B). Remarkably, despite the extremely low dose of 68–1 administered, all 9 RMs manifested SIVpol-specific $CD4^+$ and $CD8^+$ T cell responses, and at $10^3$ PFU, ΔRh110/SIVgag also induced both $CD4^+$ and $CD8^+$ SIVgag-specific T cells. Since this experiment was conducted with ΔRh110 vectors grown on complementing cells, we also determined the minimal dose required for uncomplemented

ΔRh110 while, at the same time, confirming the minimal effective dose for complemented ΔRh110. We directly compared the immunogenicity of three different ΔRh110 constructs in three RhCMV$^+$ RMs: 1) pp71-complemented ΔRh110/SIVenv at $10^3$ PFU, 2) uncomplemented ΔRh110/SIVgag at $10^3$ PFU, and 3) uncomplemented ΔRh110/SIVrtni at $10^4$ PFU. As shown in Fig. 4C, the $10^3$ PFU dose was immunogenic (elicited SIVenv-specific T cells) for the pp71-complemented ΔRh110/SIVenv vector, but not for the uncomplemented ΔRh110/SIVgag vector. However, at the $10^4$ PFU dose, the uncomplemented ΔRh110/SIVrtni vector was immunogenic, indicating that pp71 complementation provides a 1-log dose sparing.

### No evidence for recombination of ΔRh110 RhCMV/SIV vectors with endogenous RhCMV.

One concern with using CMV vectors in CMV$^+$ hosts is the possibility of recombination between the vector and the endogenous virus that would revert the attenuation. Such *in vivo* recombination has been reported for alpha-herpesvirus vaccines and naturally circulating varicella zoster strains (54,55), and deep sequence analysis of primary HCMV isolates shows signs of past recombination events (56). To investigate propensity for recombination with endogenous virus, we designed three vectors each carrying two SIV antigens as immunogenic markers in different parts of the RhCMV genome. In all three vectors, SIVenv replaced Rh110, whereas SIVpol-5' replaced the coding sequence of three different RhCMV genes – *Rh19*, *Rh107,* and *Rh192* (corresponding, respectively, to the HCMV RL11 gene family, *UL78* and *US12*) – in different parts of the genome. Each gene locus was shown to support insert expression and to be dispensable for vector replication *in vitro* (Fig. S8). Any repair of ΔRh110 by homologous recombination would result in a virus that lacks SIVenv, but retains SIVpol, and repair of Rh110 by other types of recombination would yield a pp71-intact vector with one or both of the SIV inserts included, any of which would likely be dissemination-competent and therefore expected to appear in the urine over time. Upon inoculation of two RhCMV$^+$ RMs with each of the three recombinants, we observed *de novo* induction and long-term maintenance of CD8$^+$ T cell (Fig. 5A–C) and CD4$^+$ T cell (Fig. S9A–C) responses to both SIVenv and SIVpol indicating "take" and persistence of all three vectors, and functional expression of both inserts in each vector. In general, the magnitude of the SIVpol- and SIVenv-specific CD4$^+$ and CD8$^+$ T cell responses over >800 days of follow-up was similar in each RMs (with the possible exception of modest reduction in the CD8$^+$ T cell response to Rh107-regulated pol), suggesting that T cell immunogenicity is not highly dependent on promoter type or location in the RhCMV genome. Most importantly, immunoblots of co-cultures of viruses isolated from urine of all 6 RMs were consistently negative for SIV antigens over the >800 days of observation (Fig. 5D), suggesting that the Rh110 deletion was not repaired by recombination with endogenous virus in any of these RMs over a >2-year period.

### Lack of ΔRh110 RhCMV/SIV vector transmission with close contact.

Transmission of RhCMV is highly efficient with essentially 100% of conventionally raised RMs becoming RhCMV$^+$ in the first year of life (2). In keeping with this, we observed serial transmission of SIV-specific cellular immunity (using *de novo* SIV-specific T cell responses as a surrogate for vector transmission) from a 68–1/SIV vector-vaccinated dam to her newborn infant, and then, after weaning, from the infant to a cohoused cage mate (Fig. 6A,

Fig. S10A). In contrast, the nursing infants of 5 naturally RhCMV-infected dams inoculated with ΔRh110 vectors ($10^6$ PFU each of 5 different vectors expressing SIVenv, pol-5', pol-3', gag and rtn), did not acquire SIV-specific $CD4^+$ or $CD8^+$ T cell responses to any insert, although in all 5 instances, *de novo* responses to RhCMV were observed indicating that endogenous RhCMV was transmitted from mother to infant (Fig. 6B–F, Fig. S10B–F, G). Thus, pp71-deleted RhCMV vectors are either not shed at all in urine, saliva, or breast milk or, if shedding occurs, the amount is insufficient to mediate transmission to even a highly susceptible naïve host over prolonged periods of close contact.

## Attenuation analysis of Rh59 (UL35)-deleted RhCMV/SIV vectors.

To determine whether the lack of viral dissemination, viral shedding and host-to-host transmission of pp71-deleted vectors was unique to this deletion or extended to other viral proteins that counteract intrinsic immunity, we evaluated the attenuation resulting from deletion of Rh59, the homolog of HCMV UL35, which cooperates with pp71 to counteract the cellular restriction factor BclAF1 (37). Similar to deletion of *UL82*, deletion of *UL35* results in a modest growth defect at low MOI of HCMV (57). *Rh59* was either deleted from 68–1/SIVrtni or the *Rh59* open reading fame (ORF) was replaced with SIVgag (Fig. S11). Also similar to ΔRh110, we were able to recover both ΔRh59/SIVrtni and ΔRh59/SIVgag without the need for complementation. Interestingly, however, RhCMV lacking *Rh59* showed a 10- to 100-fold reduction of viral yield at all MOIs tested (Fig. 7A) suggesting that deletion of *Rh59* affects viral replication even at high MOI. This growth defect was also reflected by reduced plaque size measured at 7 dpi (Fig. 7B).

To evaluate ΔRh59 vector spread *in vivo*, we performed necropsies on 3 $RhCMV^+$ RMs at 14, 21, and 28 days after subcutaneous inoculation with $10^7$ PFU of ΔRh59/SIVgag (right arm) and the same amount of 68–1/SIVrtni in the left arm. As shown in Table 2, the genome copy numbers of ΔRh59/SIVgag were at or below detection limits in all tissues tested at all 3 time points, whereas 68–1/SIVrtni was detected at all time points in multiple tissues. As demonstrated for ΔRh110, SIVgag-specific $CD4^+$ and $CD8^+$ T cell responses in tissues at necropsy elicited by ΔRh59 were similar to the SIVrtn-specific responses elicited by 68–1 in the same RM (Fig. S12A). Longitudinal analysis of ΔRh59 vs. 68–1-elicited SIV-specific $CD4^+$ and $CD8^+$ T cells in blood (Fig. 7C), and BAL (Fig. S12B) further showed similar response magnitude and durability in two RMs over 250 days, suggesting that deletion of *Rh59* did not affect vector immunogenicity despite substantially decreased dissemination *in vivo*.

## ΔRh59, but not ΔRh110, RhCMV/SIV vectors are shed and transmitted upon leukocyte transfer.

To further compare the relative attenuation of ΔRh110 and ΔRh59 *in vivo*, we first inoculated $RhCMV^+$ RMs with $5\times10^6$ PFU of ΔRh59/SIVrtni and ΔRh110/SIVenv, and then 175 days later, inoculated these RMs with 68–1/SIVpol-5' and ΔRh110 68–1.2/SIVgag. Compared to 68–1 RhCMV, 68–1.2 RhCMV displays broader cell tropism due to repair of Rh157.5 and Rh157.4 (homologous to the pentameric receptor complex subunits UL128 and UL130 in HCMV), as well as the anti-apoptotic gene *Rh61/60* (*UL36* in HCMV) (58). ΔRh110 68–1.2/SIVgag was thus generated to determine whether increased anti-apoptosis

activity and the ability to more efficiently infect non-fibroblast cells due to an intact pentamer would counter the attenuation resulting from pp71 deletion. All 4 vectors induced *de novo*, persistent $CD8^+$ (Fig. 8A) and $CD4^+$ (Fig. S13A) T cell responses to their respective SIV inserts in all 4 RMs that were similar in magnitude in all tissues (Fig. S13B). In keeping with previous results, 68–1/SIVpol-5' appeared in urine by 56 dpi (day 231 of the experiment) and at every subsequent time point, whereas ΔRh110/SIVenv was not detected in the urine at any time point through 742 dpi (Fig. 8B). Similarly, ΔRh110 68–1.2 /SIVgag was not found in urine through 567 dpi (day 742 of the experiment), indicating that repair of the pentameric complex and anti-apoptotic genes did not reverse the attenuation of pp71 deletion. However, in sharp contrast, ΔRh59/SIVrtni appeared in urine in one RM at 91 dpi, and in all RMs by 112 dpi, and was found in all subsequent urine samples (Fig. 8B). Thus, despite the *in vitro* growth deficiency and strongly reduced *in vivo* dissemination, Rh59-deficient RhCMV was shed from infected RMs, albeit with a modest delay compared to 68–1 RhCMV.

CMV can readily spread from infected individuals to naïve recipients by blood transfusion or bone marrow transplantation, likely from latent viral reactivation in myeloid lineage cells, particularly monocytes (59–61). To more stringently compare the *in vivo* spread deficiency of ΔRh59 and ΔRh110, we adoptively transferred by intravenous infusion 8 or $19 \times 10^6$ bone marrow cells plus $30 \times 10^6$ peripheral blood leukocytes from 2 RMs inoculated with ΔRh59/SIVrtni, ΔRh110/SIVenv, 68–1/SIVpol-5', and ΔRh110 68–1.2/SIVgag to two naturally RhCMV-infected, but vector-naïve, RMs, and then monitored these recipient RMs for induction of SIV-specific T cell responses indicating vector transmission (Fig. 8C). Strikingly, SIVpol- and SIVrtn-specific T cell responses rapidly appeared in both RMs. The SIVpol-specific responses arising from transmission of the 68–1 vector peaked a week earlier than the SIVrtn-specific responses arising from transmission of the ΔRh59 vector, whereas SIVenv and SIVgag responses were never detected, consistent with a lack of transmission of both 68–1- and 68–1.2-derived ΔRh110 vectors. These data indicate that ΔRh110 vectors were either not present in the transferred cells or that these attenuated viruses were unable to reactivate from latency and disseminate despite being transferred in myeloid cells. These data indicate that the long-term *in vivo* attenuation afforded by deletion of *Rh110* (pp71) is greater than that afforded by deletion of *Rh59* (UL35).

## DISCUSSION

The goal of this study was to identify a strategy that would enhance the safety of CMV-based vectors while maintaining the unique immunologic features that characterize the CMV vector platform, in particular the establishment and maintenance of high frequency $T_{EM}$ in both CMV-naïve and naturally CMV-infected individuals. Since it is very likely that the indefinite maintenance of $T_{EM}$ requires long-term Ag exposure provided by persistent viral infection (62), attenuation strategies that eliminate viral persistence would not be suitable for this purpose. In this regard, the replication-deficient HCMV with *in vivo*-abrogated expression of IE1/2 and UL51 currently being developed as an HCMV vaccine (63) would not be expected to maintain high frequency $T_{EM}$ with appropriate long-term functionality. Moreover, since it is very likely that the unique phenotype and function of CMV vector-elicited responses reflect the composite interaction of the virus' many immunomodulatory

programs with the host immune system (64,65), attenuation strategies that interfere with these programs also run the risk of adversely affecting the quality of the CMV vector-elicited immune responses. For instance, we previously demonstrated that deletion of the gene region encoding RhCMV homologs of the MHC-I evasins US2–11 resulted in vectors that lost the ability to super-infect (7), whereas retention of natural killer cell-activating ligands by RhCMV ORF Rh159 (UL148) was required for primary infection (66). Therefore, in planning our CMV vector attenuation strategy, we sought an attenuation approach that would down-modulate, but not eliminate, CMV vector infectivity by markedly reducing its capacity to spread from cell-to-cell upon lytic replication or reactivation, but that would otherwise preserve its immunomodulatory capabilities and its ability to persist. Our goal was a stably attenuated vector that would be able to exceed the threshold required for persistent immunogenicity, but would be sufficiently spread restricted such that the infection would be kept well below the level required to cause disease, and even more stringently, below the level necessary to transmit vector from one individual to another. We also sought to accomplish this goal with discrete, difficult-to-reverse genetic modifications based on well-understood mechanisms of action at the molecular and cellular levels so as to avoid, as far as possible, unexpected off-target impacts on vector biology and function.

In view of these immunobiological and virological considerations, genetic deletion of viral tegument proteins such as pp71 that counter host intrinsic immunity was an attractive candidate attenuation strategy. First, attenuation based on deletion is much more difficult to reverse *in vivo* than attenuation based on point mutations or on adding attenuating genetic elements, especially with our strategy of replacing the pp71 coding sequence with the heterologous Ag. This strategy not only results in a more naturally regulated expression of the transgene than use of heterologous promoters, but also eliminates the possibility of reversing vector attenuation by homologous recombination with endogenous virus.

Second, the host intrinsic immune mechanisms under consideration are highly conserved in mammals and the relevant components, such as DAXX, are essential genes that are expressed in every cell type (29,67). These features increase the likelihood of successful translation from RMs to humans, and reduce concerns that genetic heterogeneity in the human population would reduce the effectiveness of this strategy in subpopulations, potentially placing subgroups of individuals at higher risk for adverse events after vaccination. Indeed, the universality of these mechanisms had led to them being proposed as a general approach to attenuate herpes viral vaccines and vectors (68).

Third, the pattern of *in vitro* attenuation reported for Δpp71 HCMV – reduction in viral growth at low MOI, but not high MOI (46) – also appeared to be advantageous for a $T_{EM}$-targeted vaccine, as it suggested a relatively specific and early defect in viral infectivity that greatly reduces, but does not eliminate, the capacity for lytic, productive infection. Pp71 deletion does not appear to irrevocably inactivate CMV or change its fundamental immunobiology, but rather, appears to place a barrier to the triggering of its lytic genetic program that limits viral production and subsequent cell-to-cell spread during initial infection or upon subsequent reactivation from latency. Some Δpp71 CMV-infected cells (perhaps those exposed to high MOI) might bypass this restriction and progress to lytic infection, but pp71-deleted progeny of those few cells that produce virus would be severely

handicapped in their ability to productively infect new cells, potentially resulting in a smoldering, low-level infection that persists, but cannot efficiently expand or disseminate. Moreover, Δpp71 CMV may go directly into latency (38,39), potentially maintaining the capacity for subsequent insert expression either during latency or in response to reactivation stimuli, and therefore providing for persistent or periodic Ag production and presentation from all infected cells, whether or not they are able to engage in productive infection (64).

These promising *in vitro* characteristics do not, however, indicate whether the degree to which a Δpp71 CMV vector would manifest the necessary balance between the infectivity and persistence (needed for immunogenicity) and the spread-restriction (needed for safety) would be appropriate for optimal vector function. The level of spread of Δpp71 *in vivo* might be too high or too low, resulting in insufficient attenuation or insufficient immunogenicity, respectively. In this regard, the relatively modest *in vitro* attenuation of ΔRh110 vectors relative to their HCMV counterparts (46), even at very low MOI, raised concern that the Δpp71 RhCMV vector design might be insufficiently attenuated in the RM model. However, this turned out not to be the case, as pp71-deleted 68–1 vectors showed a remarkable degree of *in vivo* attenuation relative to pp71-intact RhCMV based on strain 68–1. Whereas 68–1 RhCMV showed high level replication at the site of inoculation ($>10^9$ genome copies/$10^7$ cell equivalents at day 14) and robust dissemination in primary infection, co-injected ΔRh110 was barely detectable with a high-sensitivity assay at 14 dpi, and was essentially undetectable in tissues taken later. Indeed, the numbers of ΔRh110 genome copies were so low in both primary and super-infection (with no difference between primary and super-infection, in contrast to 68–1), that it remains unclear whether there was any spread of ΔRh110 *in vivo*; the genome copies detected at day 14 might simply reflect the ΔRh110 genomes from the injected (high dose) inoculum and/or the virus produced within the cells infected by this inoculum. The robust attenuation of ΔRh110 was also supported by our findings that whereas 68–1 is readily shed in urine and efficiently transferred from RM to RM with close (mother-to-infant) contact or blood cell transfusion, ΔRh110 was never found in urine and was not transferred from RM to RM with either close contact or cell transfer. These data may indicate that pp71 is essential for *in vivo* RhCMV infectivity, such that ΔRh110 would be too attenuated for use as an effective vector. However, this concern is allayed by comparable 68–1 and ΔRh110 $CD4^+$ and $CD8^+$ T cell immunogenicity, even in the setting of super-infection. Moreover, as shown in the companion article, we demonstrate that ΔRh110 vectors show efficacy against viral challenge that is as good or better than 68–1 vectors (69).

The only clear immunologic difference between ΔRh110 and parental RhCMV vectors was the dose required to establish immunogenicity. Whereas 68–1 can establish fully immunogenic super-infection with 10 injected PFU, the (uncomplemented) ΔRh110 vector required $10^4$ PFU to establish immunogenicity in $RhCMV^+$ RMs. This observation is consistent with the hypothesis that the primary defect of ΔRh110 is a reduction in its ability to spread from cell-to-cell. The lack of any shedding or transmission of ΔRh110 suggests that this vector never effectively seeds shedding sites such as the salivary glands and kidneys, or long-term latency sites such as hematopoietic stem cells and their myeloid lineage progeny in the bone marrow and in the circulation (60, 61). This, in turn, implies that robust CMV vector immunogenicity can be achieved and maintained with a local infection

(sites of inoculation and draining lymph nodes) initiated by $10^4$ PFU, and therefore possibly involving only thousands of infected cells or fewer.

The magnitude of the SIV-specific T cell responses elicited by the different effective doses of ΔRh110, with or without complementation, and by the pp71-intact 68–1 RhCMV were similar, suggesting that a threshold level of vector infection and associated insert expression is required to trigger immunogenicity, but once this threshold is achieved, the magnitude of resultant immune response is dose independent. Since complemented ΔRh110 has pp71 in its tegument to facilitate the first round of replication, the comparison between 68–1 and uncomplemented ΔRh110 better reflects the effect of pp71 deletion on *in vivo* spread, indicating that pp71 deletion compromises vector spread by at least 1000-fold *in vivo* (10,000 PFU vs. 10 PFU), a 10- to 100-fold higher deficit than the reduction of viral growth observed with low MOI *in vitro*.

It is also noteworthy that the Rh59 (UL35)-deleted RhCMV vector, which showed an MOI-independent 1–2-log growth defect *in vitro* and a similar spread deficiency after *in vivo* inoculation as ΔRh110, did not show the same overall attenuation as ΔRh110, as ΔRh59 was both shed in urine and readily transferred to naïve RMs by leukocyte transfer. Thus, even though UL35 is involved in countering the same intrinsic immune mechanism as pp71, the contribution of this protein to inactivating the host PML repressor complex would therefore appear to be less than pp71, at least for RhCMV. Alternatively, pp71 could support *in vivo* spread by countering additional host defense mechanisms. For instance, it was recently reported that pp71 of HCMV counters the innate immune signaling adaptor STING (70). From a vaccine development standpoint, the biologic phenotype of ΔRh59 (ΔUL35) RhCMV, though adequately immunogenic, is not satisfactorily attenuated for a primary CMV attenuation strategy.

Most of the ΔRh110 constructs analyzed in this study were based on BAC-cloned strain 68–1 (45) which shows CMV-typical signs of fibroblast adaptation including deletion of UL128 and UL130 homologous subunits of the tropism-determining pentameric receptor complex (Rh157.5 and Rh157.4), and a defect in the anti-apoptotic UL36 homolog Rh60/61 (4,71,72). These mutations are associated with reduced viremia, shedding and transmission of 68–1 compared to low passage RhCMV isolates (73), and thus potentially contribute to the attenuation of ΔRh110. However, repair of the homologs of UL128, UL130 and UL36 in strain 68–1.2 (58), did not enable Rh110-deleted RhCMV to be shed or transmitted by transfusion. This observation suggests that increased surveillance by intrinsic immunity resulting from Rh110 deletion substantially increases the attenuation afforded by the other deletions (particularly pentameric complex ablation) that spontaneously occurred in 68–1 during tissue culture adaptation.

The low level of *in vivo* infection manifested by ΔRh110 reduces the likelihood of co-infecting the same cell as endogenous RhCMV and potential repair of the Rh110 deletion by recombination. Homologous recombination of a ΔRh110 vector in which the Ag insert replaces the Rh110 coding sequence with an endogenous RhCMV would be expected to yield a pp71-intact, Ag-less vector and a pp71-deleted, Ag-containing version of the endogenous RhCMV strain, not an insert-expressing wildtype vector with the potential to

spread. Thus, such a recombination event would have no consequence for either the vaccine recipient or unvaccinated close contacts. It is, however, theoretically possible that pp71 expression might be restored in Rh110-deleted RhCMV by non-homologous recombination, leaving a spread-competent CMV that also expresses the vaccine insert. While this would not subject the vaccine recipient to additional risk, since this individual already harbors wildtype CMV, it could lead to shedding and consequent transmission to unvaccinated close contacts of an insert-containing CMV vector. However, in experiments designed to detect Rh110 repair by either homologous or non-homologous gene exchange, we saw no evidence of such attenuation-reversal in 6 superinfected RMs followed over 800 days. These data suggest that if such Rh110-repairing recombination occurs at all, it is likely to be a very infrequent event. We would also note that the most likely outcome of Δpp71 CMV vector co-infection with a wildtype CMV is complementation of the Δpp71 CMV virions, as reported for MCMV lacking essential genes (74), which would increase their infectivity. However, since the vast majority of available target cells would not be infected, this enhancement, like complementation during *in vitro* production, would be lost in subsequent rounds of infection and therefore would not be expected to meaningfully reduce Δpp71 CMV vector attenuation.

Taken together, our data support the general conclusion that the indefinite maintenance of T cell immunity by CMV vectors can be uncoupled from viral spread within individual hosts and viral dissemination among hosts. A limitation of our study is that we only evaluated attenuated vectors in immunocompetent animals where RhCMV is non-pathogenic. Nevertheless, the results strongly suggest that the markedly reduced capacity to spread will also limit the ability of attenuated vectors to cause disease while preserving full CMV vector immunogenicity. Our findings in RhCMV are reminiscent of the finding in the mouse model where it was demonstrated that single-cycle MCMV viruses (i.e., viruses that replicate their genomes but are unable to generate infectious progeny) generated the MCMV-typical $T_{EM}$ inflation over time and protected against MCMV challenge (23,24), and are in contrast with findings in most other replication-defective viral vector systems in development (75). Although, so far, this finding is limited to animal models, the fact that similar observations were made in both murine and rhesus models, bodes well for the development of safe, HCMV-based vaccines for the human population. Moreover, the “pp71 deletion by insert replacement” design is a viable vector design strategy for initial assessment of attenuated HCMV vectors in humans. Given that *UL82* (pp71)-deleted HCMV is more growth restricted *in vitro* than RhCMVΔRh110 (5-log vs. 2-log reduction relative to 68–1 at low MOI) (46), it is likely that a HCMVΔUL82 vector will be, if anything, more attenuated in humans than ΔRh110 in RMs, offering an extra margin of safety at the possible expense of requiring a higher dose for immunogenicity.

## MATERIALS AND METHODS

### Study Design.

The objective of this study was to evaluate the impact of viral attenuation by deletion of RhCMV genes that, when present, counteract host immune responses. *In vitro* studies were performed with life-extended primary rhesus fibroblasts to determine the impact of viral

gene deletions on host cell protein expression and viral growth. The number of independent experiments and the number of replicates per experiment are indicated in the figure legends. Animal studies were approved by the Institutional Animal Care and Use Committee. To minimize the number of animals used in these experiments, most were designed with the goal to generate qualitative rather than quantitative comparisons with non-attenuated vectors. Because we observed highly consistent results among the 2–4 animals per group, these low numbers were sufficient to determine whether attenuated viral vectors lack viral shedding, viral transmission, and recombination with endogenous virus, while maintaining the ability to re-infect and elicit as well as maintain unconventionally restricted T cell responses. Furthermore, most key observations, e.g., lack of shedding, and all immunological parameters, were independently observed in multiple experiments whereas others, such as lack of spontaneous transmission, were independently confirmed by distinct experimental designs. To obtain quantitative comparisons with non-attenuated vectors, such as measurements of genome copy numbers or the determination of the minimal immunogenic dose, animal numbers were minimized by including internal controls, i.e., animals were co-inoculated with non-attenuated vectors. Primary data are reported in data file S1.

### Statistical Analysis.

We used non-parametric testing to determine differences between groups. In comparisons of two groups only, we used two-sided Wilcoxon rank-sum tests with $\alpha=0.05$ to detect differences between the groups. In comparisons of more than two groups, we used the Kruskal-Wallis rank-sum test to detect any differences between groups with $\alpha=0.05$. If any differences were detected, we performed post-hoc two-sided Wilcoxon rank-sum tests with $\alpha=0.05$ on the pairwise differences between groups. No multiplicity adjustments were performed for post-hoc analyses. All statistical analyses were performed using R (v. 3.2.2).

## Supplementary Materials

Refer to Web version on PubMed Central for supplementary material.

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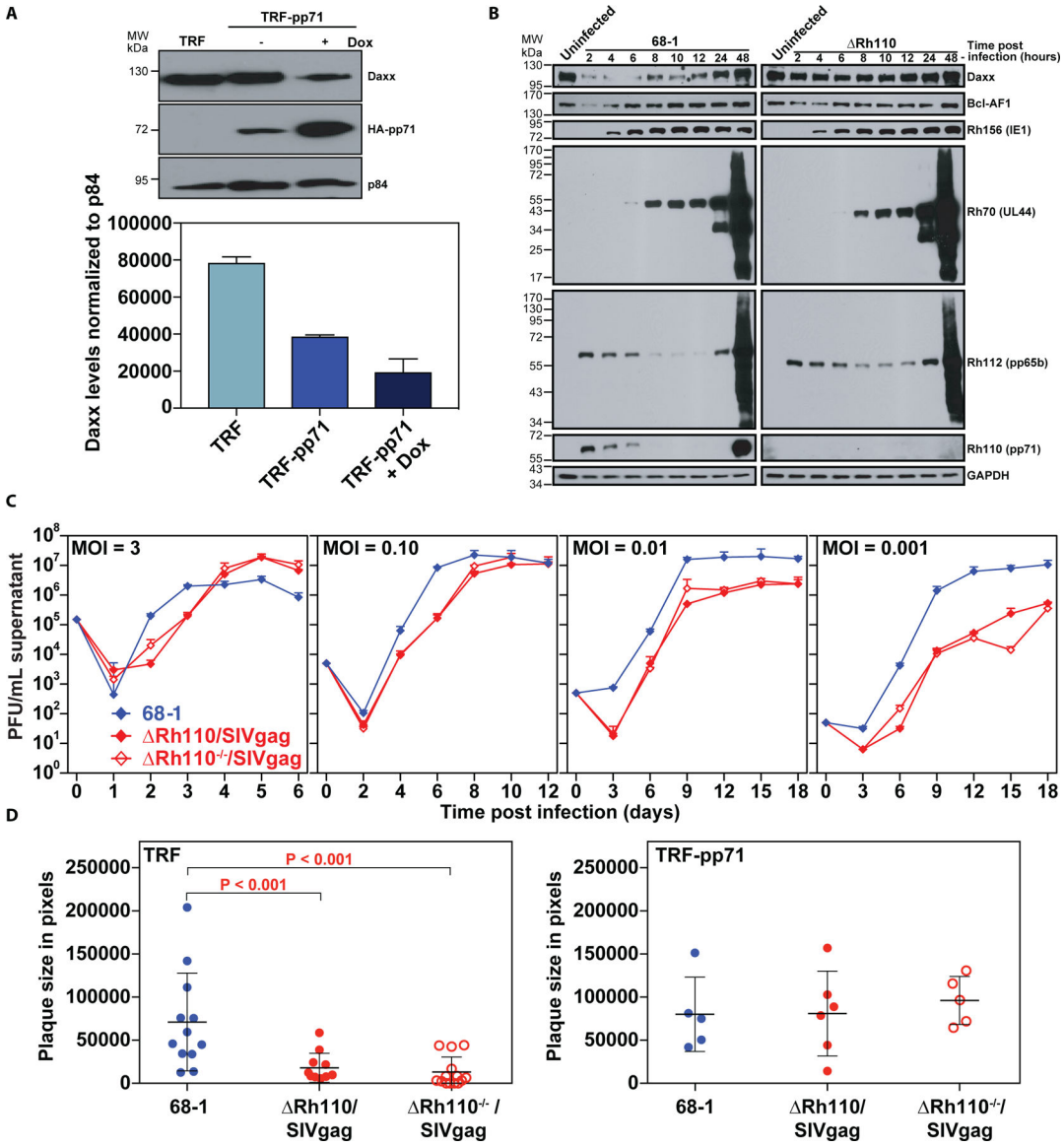


**Figure 1: RhCMV pp71 degrades DAXX and absence of pp71 results in *in vitro* growth deficiency**

(**A**) TRFs or TRF-pp71 were untreated (−) or treated with 10 μg/ml doxycycline (+) to induce RhCMV pp71 expression for 24 hrs. Nuclear lysates were harvested and analyzed by immunoblot using anti-hemagglutinin (HA) antibodies to detect epitope-tagged pp71. The cellular nuclear matrix protein p84 was analyzed as a loading control. The graph shows mean (+ SD) with n = 2. (**B**) TRFs were infected with 68–1 or ΔRh110 (MOI=3) or left uninfected and cell lysates were harvested at the indicated time points, electrophoretically separated, and subjected to immunoblots with antibodies to the indicated host and viral proteins. Each time point represents an independent infection. (**C**) TRFs were infected with 68–1 or either complemented or uncomplemented ($^{-/-}$) ΔRh110/SIVgag at the indicated MOI. Supernatants were harvested at the indicated times and titered by $TCID_{50}$ (median tissue culture infectious dose) on TRF-pp71. Average titers from two experimental and two technical replicates (+ SD) are shown. (**D**) TRFs or TRF-pp71 were infected with 68–1,

complemented ΔRh110/SIVgag, or uncomplemented ΔRh110 ($\Delta Rh110^{-/-}$) at MOI = 0.001. Plaques were analyzed at 7 dpi using phase microscopy and plaque size was measured using Adobe Photoshop. Individual plaque sizes, as well as average (+/− SD) from one of two experiments are shown. The Kruskal-Wallis (KW) test was used to determine the significance of differences between the 3 groups [P = 0.0002, left; P = not significant (NS), right] with the Wilcoxon rank sum test used to perform pair-wise analysis if KW P-values were ≤0.05; brackets indicate pair-wise comparisons with two-sided Wilcoxon P-values ≤0.05. P-values ≤0.05 are considered statistically significant.

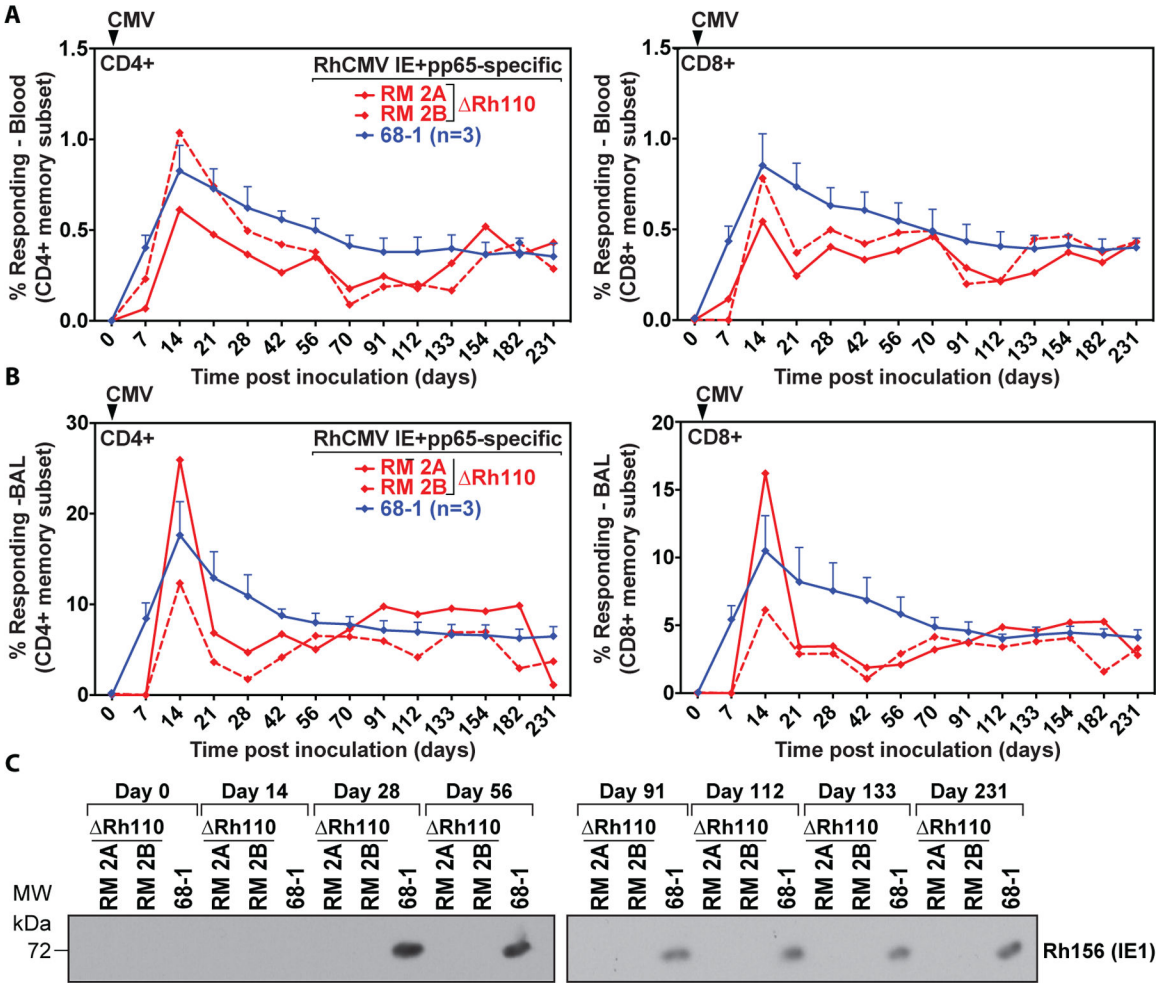


**Figure 2: Rh110 (pp71)-deleted RhCMV retains T cell immunogenicity but is no longer shed in urine.**

(**A**) Frequencies of RhCMV-specific $CD4^+$ and $CD8^+$ T cell responses in PBMCs were determined at the indicated time points in two RM inoculated with $10^7$ PFU of ΔRh110 and three RMs given the same dose of 68–1 RhCMV. RhCMV IE1- and pp65a-specific T cells were determined by flow cytometric ICS after stimulation with mixes of consecutive, overlapping peptides comprising the RhCMV IE1 and pp65a proteins using intracellular expression of CD69 and either or both of TNF-α and IFN-γ to define Ag-specific T cells. Shown are response frequencies to IE1 and pp65a within the memory subset after background subtraction for each of the two ΔRh110-inoculated RMs (RM 2A and RM 2B) and the mean (+ SEM) of these response frequencies for the three RMs given 68–1 RhCMV. (**B**) Frequencies of RhCMV-specific T cell responses in bronchoalveolar lavages (BAL) were determined as in panel A in the same animals at the indicated time points. (**C**) Urine was isolated at the indicated days post-infection from RM 2A and RM 2B or one RM inoculated with 68–1. The presence of virus in co-cultures was determined by immunoblot for RhCMV IE1 (see Materials and Methods).

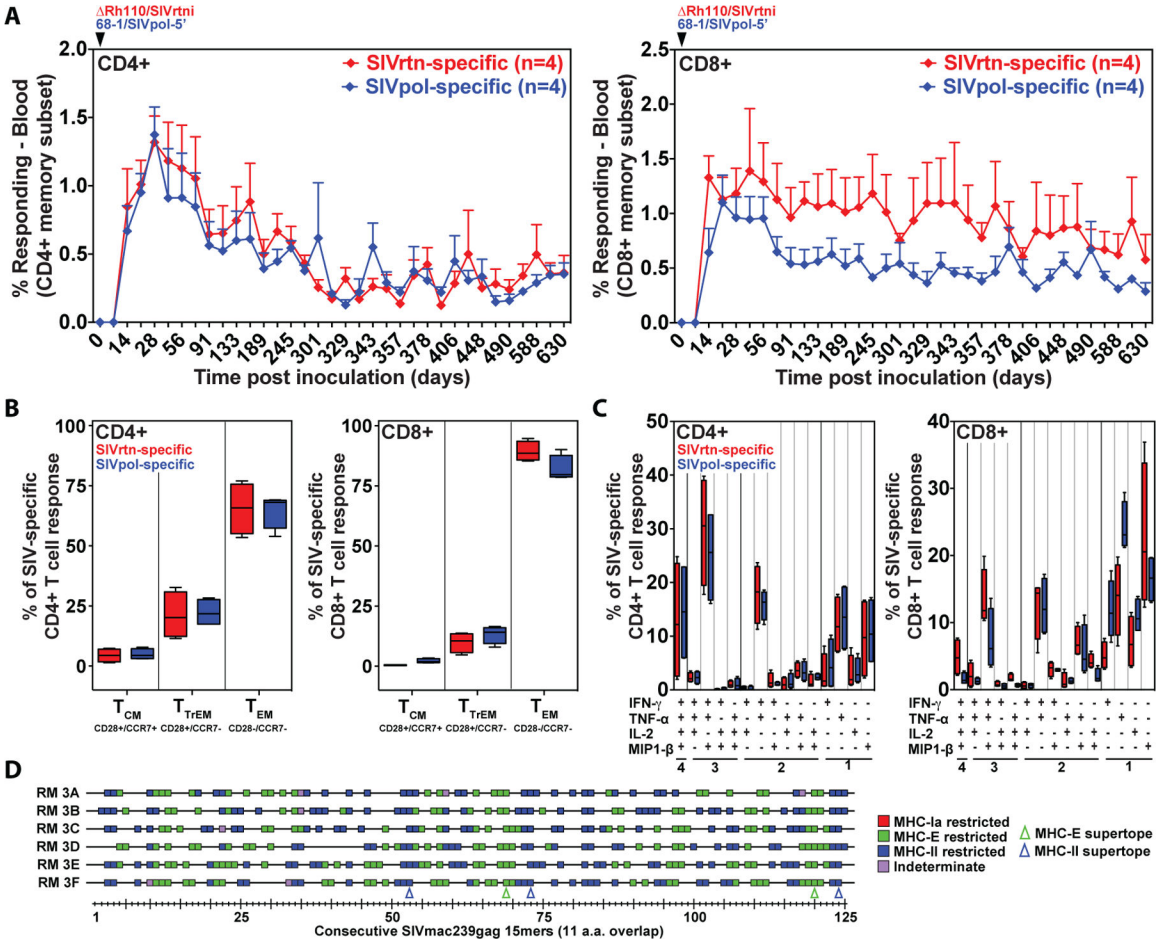


**Figure 3: Durability, functional phenotype, and epitope targeting of SIV insert-specific T cell responses elicited by Rh110 (pp71)-deleted RhCMV vectors in naturally RhCMV-infected RMs.**

(**A**) Four naturally RhCMV-infected RMs were co-inoculated with $10^7$ PFU of 68–1/SIVpol-5' and the same dose of ΔRh110/SIVrtni and flow cytometric ICS was used to follow the magnitude of the $CD4^+$ and $CD8^+$ T cell responses in peripheral blood to SIVpol and SIVrtn peptide mixes as described in Fig. 2. The mean + SEM of SIVpol- and SIVrtn-specific response frequencies within the memory $CD4^+$ (left panel) and $CD8^+$ (right panel) T cell populations are shown. (**B**) Boxplots compare the memory differentiation of the RhCMV-elicited $CD4^+$ and $CD8^+$ memory T cells in PBMCs (of the same RM shown in panel A) responding to SIVpol or SIVrtn with TNF-α and/or IFN-γ production at 630 dpi. 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. The Wilcoxon rank sum test was used to pairwise compare differences between the fraction of SIVpol- and SIVrtn-specific $CD4^+$ and $CD8^+$ T cells within each memory subset, with P = NS for all comparisons. (**C**) Boxplots compare the frequency of RhCMV-elicited $CD4^+$ and $CD8^+$ memory T cells in PBMCs of the same RM shown in panel A responding to SIVpol or SIVrtn peptides with TNF-α, IFN-γ, IL-2, and MIP1-β production, alone and in all combinations at 630 dpi. The Wilcoxon rank sum test was used to pairwise compare differences between the fraction of SIVpol- and SIVrtn-specific $CD4^+$ and $CD8^+$ T

cells expressing 1, 2, 3 or 4 cytokines, with P = NS for all comparisons. (**D**) SIVgag-specific $CD8^+$ T cells in the peripheral blood of six ΔRh110/SIVgag-inoculated RMs were epitope-mapped using a flow cytometric ICS assay (CD69, TNF-α, IFN-γ readout, as described above) to detect recognition of each consecutive, overlapping 15-mer peptide comprising the SIVgag protein. Peptides resulting in specific $CD8^+$ T cell responses are indicated by a box, with the color of the box designating MHC restriction as determined by blocking with the anti-pan-MHC-I mAb W6/32, the MHC-E blocking peptide VL9 and the MHC-II blocking peptide CLIP, as previously described (52,53). The blue and green arrowheads indicate the positions of previously identified MHC-II- and MHC-E-restricted SIVgag supertopes, respectively.

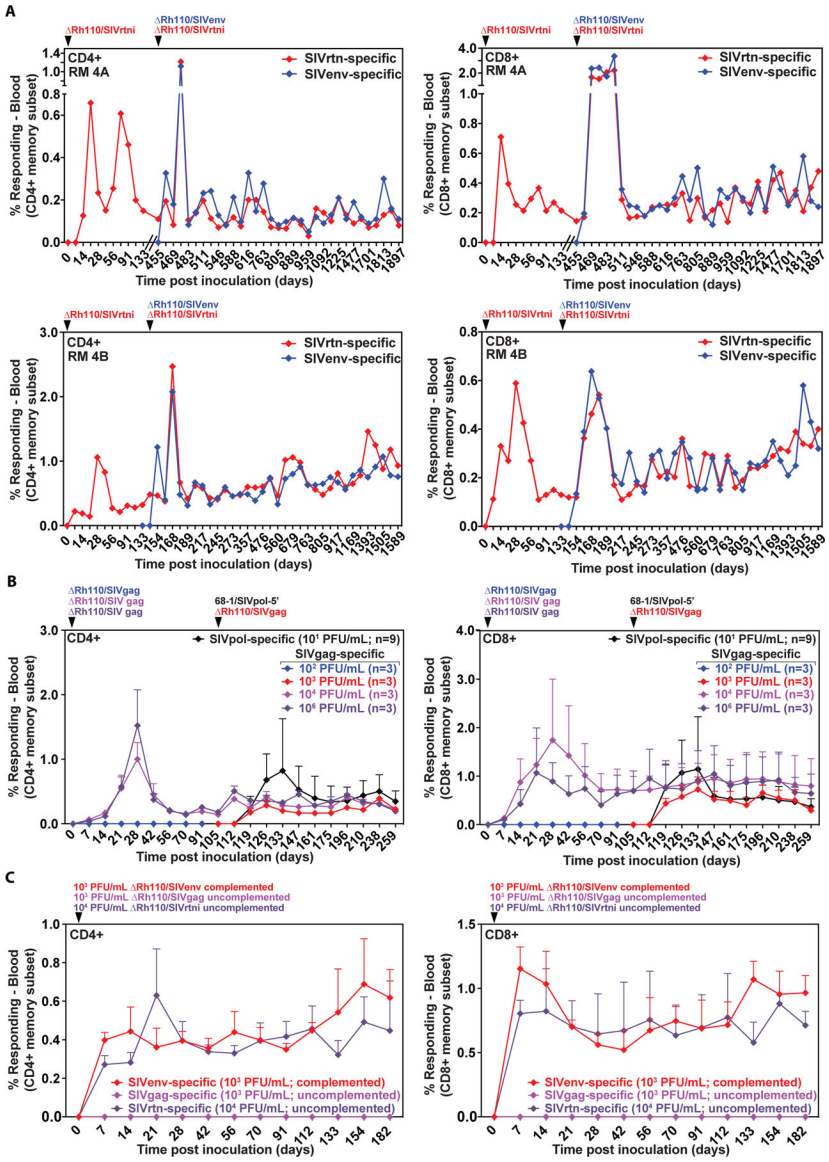


**Figure 4: Potency of Rh110 (pp71)-deleted RhCMV vectors in superinfection.**

(**A**) Two naturally RhCMV-infected RMs were inoculated with $10^7$ PFU of ΔRh110/SIVrtni on day 0 and again on day 455 (for RM 4A) or day 133 (for RM 4B), the latter inoculation in combination with $10^7$ PFU of ΔRh110/SIVenv. The panels show longitudinal analysis of the SIVrtn- and SIVenv-specific $CD4^+$ and $CD8^+$ T cell response frequencies among PBMCs determined by flow cytometric ICS (CD69, TNF-α, IFN-γ readout) for each animal. (**B**) At time point 0, three groups (n=3 per group) of naturally RhCMV-infected RMs were subcutaneously inoculated with the indicated dose ($10^2$, $10^4$, and $10^6$ PFU) of ΔRh110/SIVgag, complemented for pp71 by growing in TRFs-pp71. The three RMs given $10^2$ PFU did not manifest a detectable SIVgag-specific T cell response through 112 days of observation and were re-inoculated with $10^3$ PFU dose of the same vector. At the same time, all 9 RMs were inoculated with $10^1$ PFU of 68–1/SIVpol-5'. The figures show longitudinal analysis of the mean + SEM of SIVgag- and SIVpol-specific $CD4^+$ and $CD8^+$ T cell

response frequencies among PBMCs, determined as described in Panel A. (**C**) Three naturally RhCMV-infected RMs were subcutaneously inoculated with $10^3$ PFU of pp71-complemented ΔRh110/SIVenv, $10^3$ PFU of ΔRh110/SIVgag, and $10^4$ PFU ΔRh110/SIVrtni, with the latter two vectors grown in TRFs and thus not complemented for pp71. SIVenv-, SIVgag-, and SIVrtn-specific $CD4^+$ and $CD8^+$ T cell responses within the peripheral blood memory compartment were followed by flow cytometric ICS as described above (mean + SEM shown at each time point).

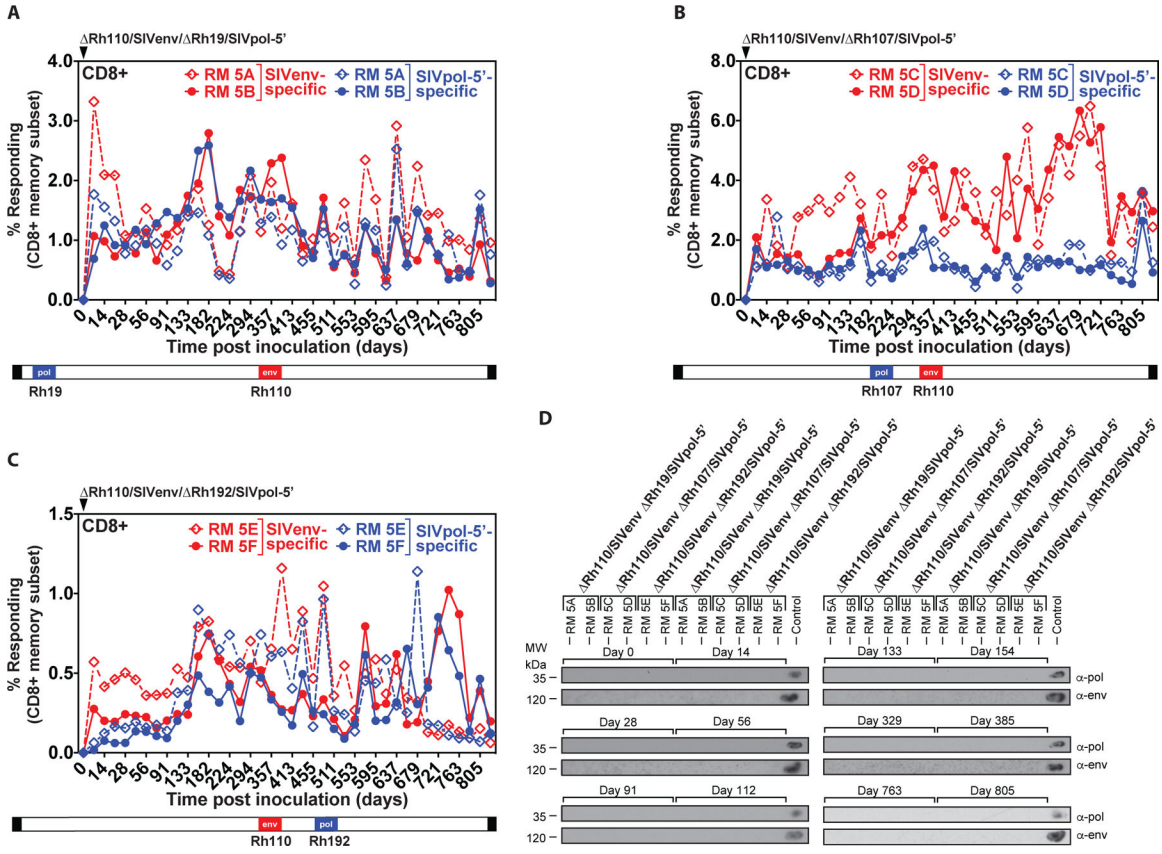


**Figure 5: Genetic stability of Rh110 (pp71)-deleted RhCMV vectors in the setting of superinfection.**

Two naturally RhCMV-infected RMs were each inoculated with $10^7$ PFU of each dual insert-expressing ΔRh110/SIVenv/ΔRh19/SIVpol-5' (**A**), ΔRh110/SIVenv/ΔRh107/SIVpol-5' (**B**), or ΔRh110/SIVenv ΔRh192/SIVpol-5' (**C**) and flow cytometric ICS was used to follow the SIVenv- and SIVpol-specific $CD8^+$ T cell responses in peripheral blood, as described in Fig. 2. Dashed and solid lines each delineate the individual RM among the RM pairs given each vector. The relative position of the SIV antigens replacing endogenous genes in the viral genome is shown schematically below the graphs. $CD4^+$ T cell responses from the same RM are shown in fig. S9. (**D**) Urine samples from the indicated time points post-vector inoculation were analyzed for vector shedding by viral co-culture followed by western blot analysis of SIVpol-5' (top panels) or SIVenv (bottom panels) expression. Urine from RMs that previously received 68–1/SIVpol-5' and 68–1/SIVenv vectors was included as a positive control.

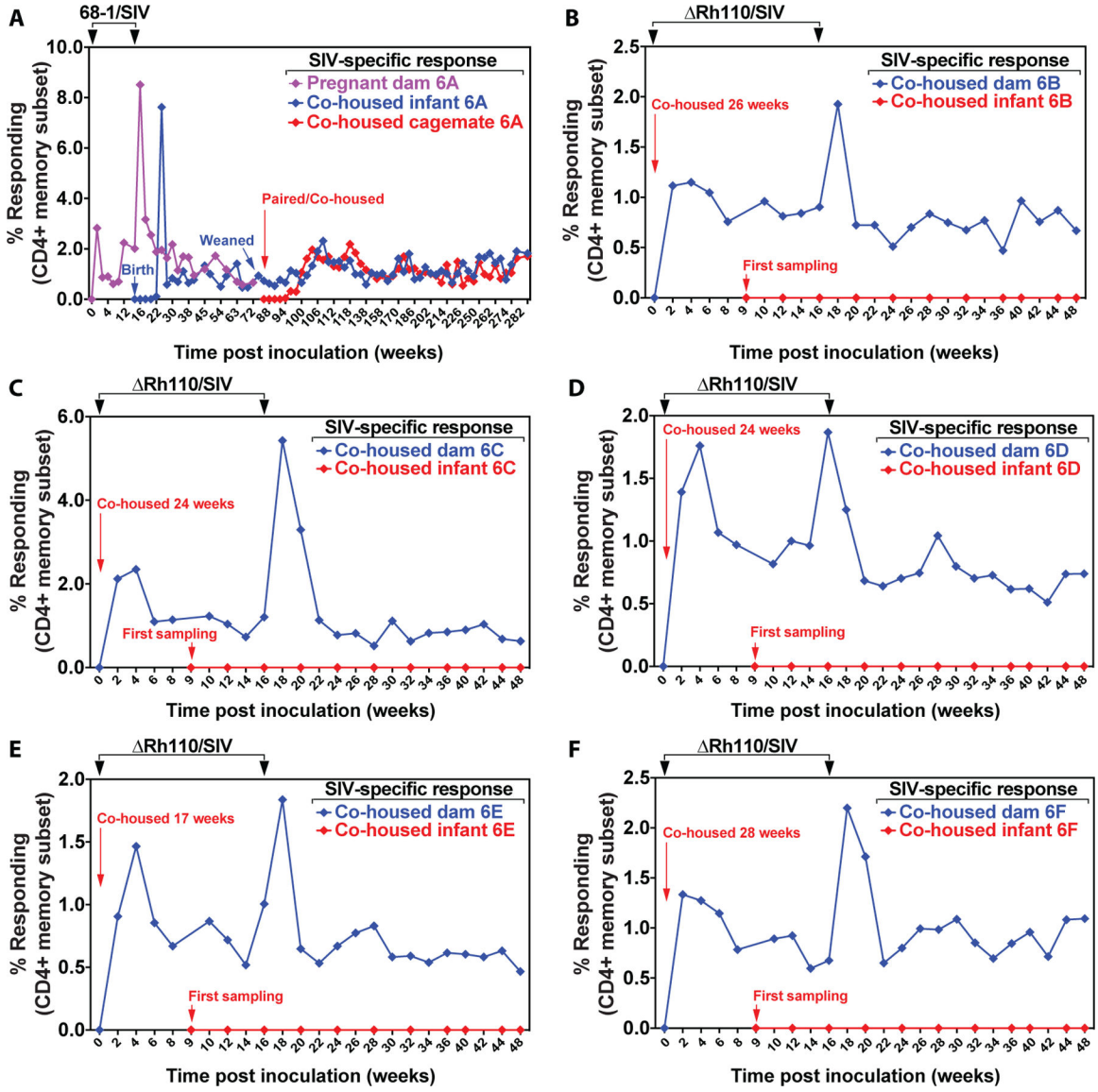


**Figure 6: Lack of maternal-infant transmission of Rh110 (pp71)-deleted RhCMV vectors.**

**(A)** A naturally RhCMV$^+$ pregnant dam was inoculated twice, as shown, with a panel of five 68–1 vectors ($5\times10^6$ PFU each) expressing SIVenv, SIVgag, SIVrtni, SIVpol-3', or SIVpol-5′. The dam gave birth to a healthy infant at 16 weeks post first inoculation. The infant was co-housed with, and nursed from, the inoculated dam for 88 weeks, at which time the infant was weaned and co-housed with another, already naturally RhCMV$^+$ juvenile RM. The mother, infant, and co-housed cagemate were followed for total SIV-specific CD4$^+$ T cell responses (SIVgag + pol + rtn + env) in peripheral blood by flow cytometric ICS with the response frequencies in the memory compartment shown. (**B to F**) Five naturally RhCMV-infected female RMs were inoculated with a panel of five ΔRh110 vectors ($5\times10^6$ PFU each) expressing SIVenv, SIVgag, SIVrtni, SIVpol-3', or SIVpol-5' while nursing 17–28-week-old infants and were re-inoculated 16 weeks with later with the same vectors at the same dose. Vaccinated mothers and nursing infants were co-housed for a total of 48 weeks.

Both mothers and infants were longitudinally followed for total SIV-specific $CD4^+$ T cell responses by flow cytometric ICS. For A-F, $CD8^+$ T cell responses are shown in fig. S10.

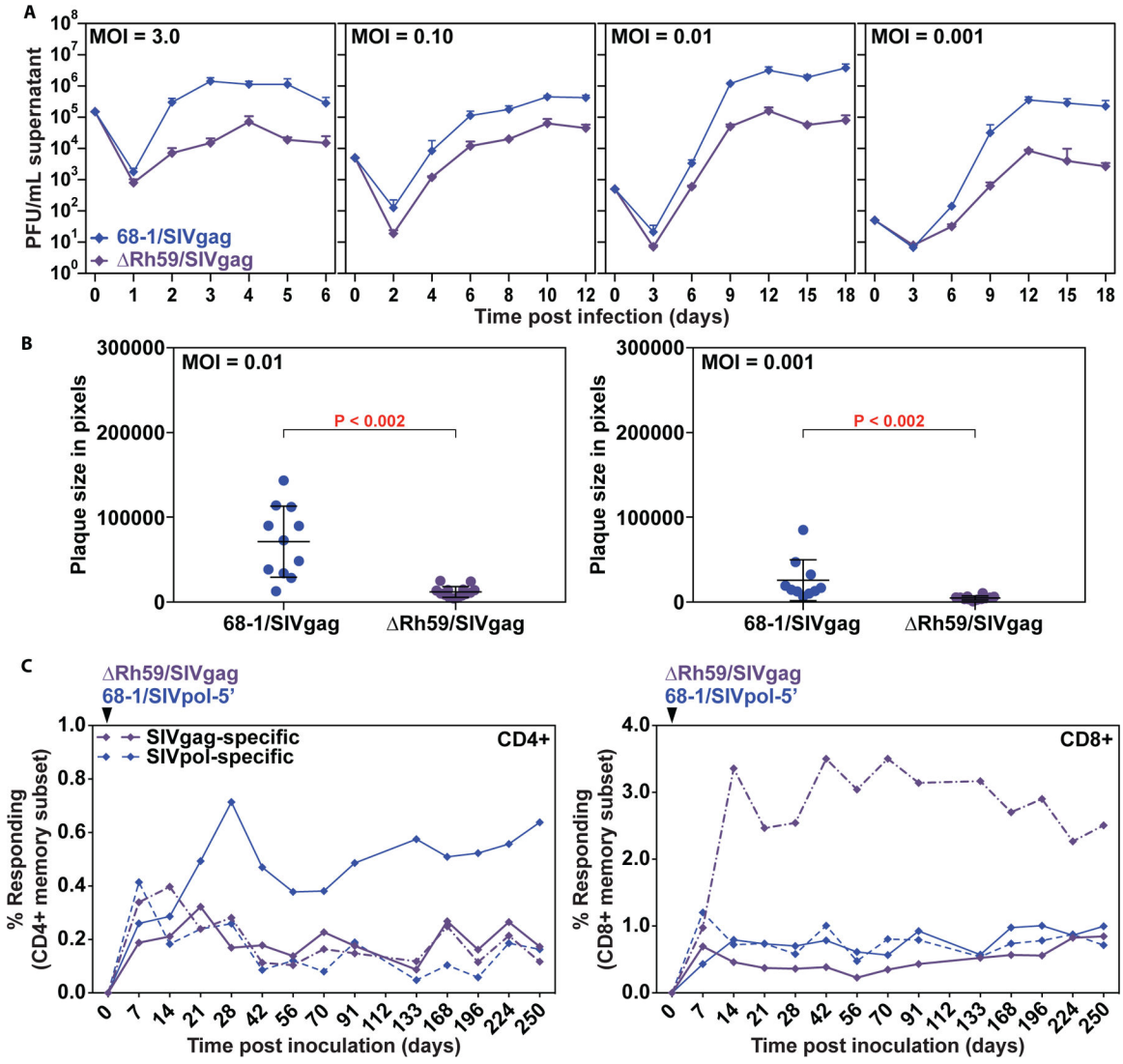


**Figure 7: Deletion of the UL35 ortholog Rh59 from RhCMV results in growth deficiency *in vitro* while maintaining immunogenicity *in vivo*.**

(**A**) TRFs were infected with 68–1/SIVgag or ΔRh59/SIVgag at the indicated MOI. The supernatant was harvested at the indicated dpi and titered by $TCID_{50}$ on TRFs. Average titers from two experimental and two technical replicates (+ SD) are shown. (**B**) TRFs were infected with 68–1/SIVgag or ΔRh59/SIVgag at MOI = 0.01 or 0.001. Plaques were analyzed at 7 dpi using phase microscopy and plaque size was measured using Adobe Photoshop. Individual plaque sizes, as well as average (+/− SD) from one of three experiments are shown. Statistical significance was determined by the Wilcoxon rank-sum test with $P \leq 0.05$ considered significant. (**C**) Two RMs were co-inoculated with $10^7$ PFU of 68–1/SIVpol-5' and ΔRh59/SIVgag, and the T cell responses to peptide mixes comprising SIVpol and SIVgag were longitudinally monitored in peripheral blood by flow cytometric ICS (CD69, TNF-α, IFN-γ readout) with the response frequencies in the memory compartment shown for each RM (one designated by solid lines, the other by dashed lines).

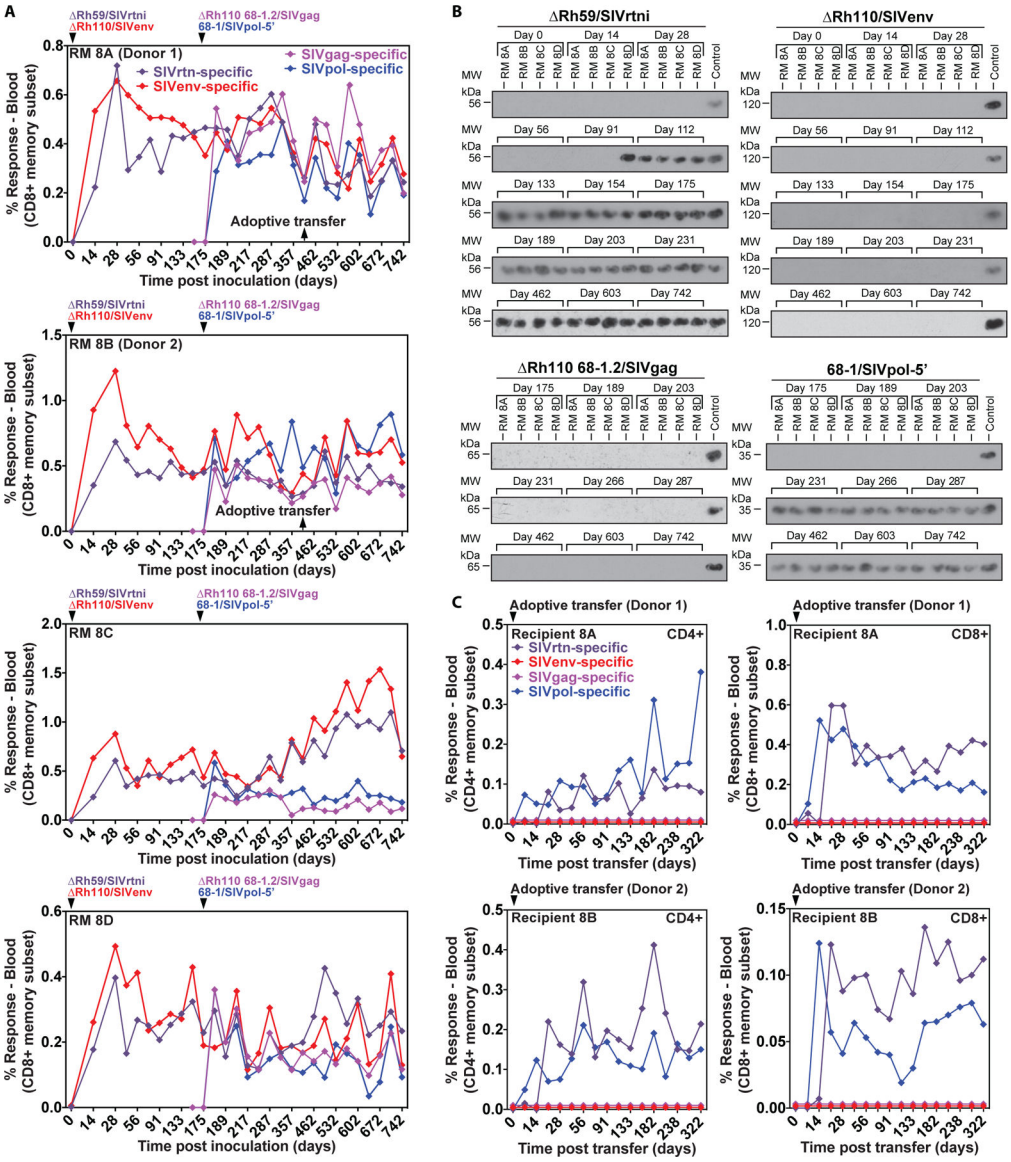


**Figure 8: Comparison of the shedding and transmission upon leukocyte transfer of Rh110 (pp71)-deleted, Rh59 (UL35)-deleted and 68–1 RhCMV vectors.**

(**A**) Four RMs were co-inoculated with $10^6$ to $10^7$ PFU of ΔRh59/SIVrtni, pentameric complex-repaired ΔRh110 68–1.2/SIVgag, ΔRh110/SIVenv, and 68–1/SIVpol-5' at the designated time points and the $CD8^+$ T cell responses to peptide mixes comprising each of the SIV antigens were longitudinally monitored in peripheral blood by flow cytometric ICS (CD69, TNFα, IFN-γ readout), with the response frequencies in the memory compartment shown (see also fig. S13B). (**B**) Immunoblots of viral co-cultures from urine samples obtained at the indicated days. Each of the SIV inserts carries a different epitope tag, allowing specific identification of each vector using tag-specific mAbs (see Materials and Methods). (**C**) Bone marrow and blood leukocytes from two of the RMs shown in **A** ($1.9 \times 10^7$ bone marrow and $3.0 \times 10^7$ blood cells from RM 8A (donor 1); $0.8 \times 10^7$ bone marrow and $3.0 \times 10^7$ blood cells from RM 8B (donor 2); obtained at the indicated time point) were transferred to two naturally $RhCMV^+$ (but vector-naïve) RMs to test the ability of leukocyte

transfer to transmit each vector to a new host. Vector infection of the new host was determined by longitudinal assessment of $CD4^+$ and $CD8^+$ T cell responses to each of the four different SIV inserts, as described in (A).

## Table 1:
## Rh110 (pp71) deletion reduces dissemination of RhCMV vectors.

**(A)** Three RhCMV-naïve RMs (T1A-1, T1A-2, T1A-3) were co-inoculated with $10^7$ PFU each of 68–1/SIVgag (left arm) and ΔRh110/SIVrtni (right arm). One RM each was necropsied at 14, 21, or 28 dpi and viral genome copy numbers per $10^7$ cell equivalents were determined in the indicated tissues using ultra-sensitive nested qPCR specific for SIVgag (68–1) or SIVrtni (ΔRh110). (**B**) Three naturally RhCMV-infected RMs (T1B-1, T1B-2, T1B-3) were co-inoculated with $10^7$ PFU each of 68–1/SIVrtni (left arm) and ΔRh110/SIVgag (right arm). One RM each was necropsied at 14, 21, or 28 dpi and viral genome copy numbers per $10^7$ cell equivalents in the indicated tissues were determined using ultra-sensitive nested qPCR specific for SIVgag (ΔRh110) or SIVrtni (68–1). (**C**) Two naturally RhCMV-infected RMs (T1C-1 and T1C-2) were co-inoculated with $10^7$ PFU each of complemented ΔRh110 and uncomplemented ΔRh110 in different arms, with the SIVgag and SIVrtni inserts used to mark the complemented and uncomplemented vectors, respectively, in RM1 and the reverse in RM2. Both RMs were necropsied at 14 dpi and viral genome copy numbers were determined using ultra-sensitive nested qPCR specific for SIVgag vs. SIVrtni. Normalized to $1\times10^7$ cell equivalents. WT, wild type; GI, gastrointestinal; LN, lymph node; PLN, parietal lymph node.

| **Table 1A** | **Primary infection: 68–1 vs. ΔRh110** | | | | | |
|---|---|---|---|---|---|---|
| | **RM T1A-1** | | **RM T1A-2** | | **RM T1A-3** | |
| **Tissue type** | **14 dpi** | | **21 dpi** | | **28 dpi** | |
| | **68–1/SIVgag** | **ΔRh110/ SIVrtni** | **681/SIVgag** | **ΔRh110/ SIVrtni** | **68–1/SIVgag** | **ΔRh110/ SIVrtni** |
| Skin Inj site-right (ΔRh110) | **19,975,462** | **<1** | **13,227** | **2** | **<1** | **<1** |
| Skin Inj site-left (WT) | **1,013,192,441** | **3** | **14,453** | **<1** | **<1** | **<1** |
| Axillary LN-right (ΔRh110 draining) | **4,547** | **<1** | **<1** | **<1** | **7** | **<1** |
| Axillary LN-left (WT draining) | **17,687,789** | **<1** | **29,484** | **<1** | **5,771** | **<1** |
| PLN (except draining LN) | **265,298** | **49** | **3** | **<1** | **40** | **<1** |
| Mesenteric LN | **<1** | **1** | **5** | **<1** | **5** | **<1** |
| GI Tract | **34** | **5** | **8** | **<1** | **64** | **<1** |
| Liver/Gallbladder | **11** | **<1** | **<1** | **<1** | **41** | **<1** |
| Heart/Lung/Kidney/BAL | **66** | **4** | **77** | **<1** | **<1** | **<1** |
| BM/Spleen/Tonsil | **4** | **3** | **9,736** | **<1** | **<1** | **<1** |
| Neuro/Endocrine | **14** | **<1** | **8** | **<1** | **<1** | **<1** |
| Genitourinary tract | **125** | **14** | **14,075** | **<1** | **17** | **<1** |
| Salivary Glands | **13,262** | **<1** | **1,242** | **<1** | **11** | **<1** |
| PBMCs | **<1** | **3** | **<1** | **<1** | **<1** | **<1** |

| Table 1B | Superinfection: 68–1 vs. ΔRh110 | | | | | |
|---|---|---|---|---|---|---|
| | RM T1B-1 | | RM T1B-2 | | RM T1B-3 | |
| Tissue type | 14 dpi | | 21 dpi | | 28 dpi | |
| | 68–1/SIVrtni | ΔRh110/ SIVgag | 68–1/SIVrtni | ΔRh110/ SIVgag | 68–1/SIVrtni | ΔRh110/ SIVgag |
| Skin Inj site-right (WT) | **47** | **5** | **16,502** | **<1** | **376** | **<1** |
| Skin Inj site-left (ΔRh110) | **11** | **354** | **7** | **<1** | **355** | **<1** |
| Axillary LN-right (WT draining) | **117** | **<1** | **7** | **<1** | **509** | **<1** |
| Axillary LN-left (ΔRh110 draining) | **<1** | **<1** | **<1** | **<1** | **<1** | **<1** |
| Peripheral LN | **1,434** | **<1** | **41** | **<1** | **2** | **<1** |
| Mesenteric LN | **<1** | **1** | **1** | **<1** | **<1** | **<1** |
| GI tract | **3** | **<1** | **<1** | **<1** | **12** | **<1** |
| Liver/Gallbladder | **1** | **<1** | **9** | **<1** | **9** | **<1** |
| Heart/Lung/Kidney/BAL | **5** | **<1** | **<1** | **<1** | **8** | **<1** |
| BM/Spleen/Tonsil | **15** | **5** | **<1** | **<1** | **<1** | **<1** |
| Neuro/Endocrine | **<1** | **<1** | **3** | **<1** | **3** | **<1** |
| Genitourinary tract | **3** | **<1** | **4** | **<1** | **1,047** | **<1** |
| Salivary gland | **23** | **1** | **<1** | **<1** | **<1** | **<1** |

| Table 1C | ΔRh110: Complemented vs. Uncomplemented | | | | |
|---|---|---|---|---|---|
| | RM T1C-1 | ← 14 dpi → | RM T1C-2 | | |
| Tissue type | Uncomplemented ΔRh110/SIVrtni | Complemented ΔRh110/SIVgag | Tissue type | Uncomplemented ΔRh110/SIVgag | Complemented ΔRh110/SIVrtni |
| Skin Inj site-right (compl.) | **<1** | **<1** | Skin Inj site-right (uncompl.) | **<1** | **<1** |
| Skin Inj site-left (uncompl.) | **13** | **<1** | Skin Inj site-left (compl.) | **<1** | **<1** |
| Axillary LN-right (compl. draining) | **<1** | **<1** | Axillary LN-right (uncompl. draining) | **<1** | **<1** |
| Axillary LN-left (uncompl. draining) | **17** | **<1** | Axillary LN-left (compl. draining) | **<1** | **<1** |
| PLN (except draining LN) | **<1** | **<1** | PLN (except draining LN) | **<1** | **<1** |
| Mesenteric LN | **<1** | **<1** | Mesenteric LN | **<1** | **<1** |
| GI Tract | **<1** | **<1** | GI Tract | **<1** | **<1** |
| Liver/Gallbladder | **<1** | **<1** | Liver/Gallbladder | **<1** | **<1** |
| Heart/Lung/ Kidney/BAL | **<1** | **<1** | Heart/Lung/ Kidney/BAL | **<1** | **<1** |
| BM/Spleen/Tonsil | **<1** | **<1** | BM/Spleen/Tonsil | **<1** | **<1** |
| Neuro/Endocrine | **<1** | **<1** | Neuro/Endocrine | **<1** | **<1** |
| Genitourinary tract | **5** | **<1** | Genitourinary tract | **<1** | **2** |
| Salivary Glands | **<1** | **<1** | Salivary Glands | **<1** | **<1** |
| PBMCs | **<1** | **<1** | PBMCs | **<1** | **<1** |

## Table 2:
## Rh59 (UL35) deletion reduces dissemination of 68–1 RhCMV vectors.

Three naturally RhCMV-infected RMs (T2–1, T2–2, T2–3) were co-inoculated with $10^7$ PFU each of 68–1/SIVrtni (left arm) and ΔRh59/SIVgag (right arm). One RM was necropsied at 14, 21, or 28 dpi and viral genome copy numbers per $10^7$ cell equivalents in the indicated tissues were determined using ultra-sensitive nested qPCR specific for SIVrtni (68–1) or SIVgag (ΔRh59).

| Table 2 | Super infection: 68–1 vs. ΔRh59 | | | | | |
|---|---|---|---|---|---|---|
| | RM T2–1 | | RM T2–2 | | RM T2–3 | |
| Tissue type | 14 dpi | | 21 dpi | | 28 dpi | |
| | 68–1/SIVrtni | ΔRh59/SIVgag | 68–1/SIVrtni | ΔRh59/SIVgag | 68–1/SIVrtni | ΔRh59/SIVgag |
| Skin Inj site-right (ΔRh59) | **12** | **<1** | **193** | **<1** | **36,110** | **7** |
| Skin Inj site-left (68–1) | **541** | **<1** | **23,793** | **<1** | **4,480,601** | **<1** |
| Axillary LN | **3** | **<1** | **13** | **<1** | **2,515** | **7** |
| Peripheral LN | **<1** | **<1** | **<1** | **<1** | **18** | **<1** |
| Liver/Gallbladder | **<1** | **<1** | **<1** | **<1** | **2** | **<1** |
| Heart/Lung/Kidney/BAL | **2,933** | **<1** | **3** | **<1** | **6** | **<1** |
| Bone marrow/Spleen/Tonsil | **6** | **<1** | **<1** | **<1** | **8** | **<1** |
| Neuro/Endocrine | **3** | **<1** | **<1** | **<1** | **4** | **1** |
| Genitourinary tract | **<1** | **<1** | **3** | **<1** | **9** | **<1** |
| Salivary gland | **2** | **<1** | **4** | **<1** | **3** | **<1** |

Normalized to $1\times10^7$ cell equivalents