

VACCINES

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

Emily E. Marshall^{1*†}, Daniel Malouli^{1*}, Scott G. Hansen^{1*}, Roxanne M. Gilbride¹, Colette M. Hughes¹, Abigail B. Ventura¹, Emily Ainslie¹, Andrea N. Selseth¹, Julia C. Ford¹, David Burke¹, Craig N. Kreklywich¹, Jennie Womack¹, Alfred W. Legasse¹, Michael K. Axthelm¹, Christoph Kahl^{1‡}, Daniel Streblow¹, Paul T. Edlefsen², Louis J. Picker^{1§}, Klaus Früh^{1§}

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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 immunodeficient 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 (Δ Rh110 and Δ Rh59), 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 RhCMV. Δ Rh110 was not shed at any time after infection and was not transmitted to naïve hosts either by close contact (mother to infant) or by leukocyte transfusion. In contrast, Δ Rh59 was both shed and transmitted by leukocyte transfusion, indicating less effective attenuation than pp71 deletion. The T cell immunogenicity of Δ Rh110 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.

INTRODUCTION

The betaherpesviruses human cytomegalovirus (HCMV) and rhesus cytomegalovirus (RhCMV) are ubiquitous in human and rhesus monkey (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 (i) 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 (ii) an extraordinarily high frequency of CD4⁺ and CD8⁺ CMV-specific T cells with a unique effector memory (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 preposition potent, effector-differentiated responses to a different pathogen in the portal of entry and sites of early spread of that pathogen. This early interception (before implementation of 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). We demonstrated that the cellular immune responses elicited by strain 68-1 RhCMV vectors expressing simian immunodeficiency virus (SIV) inserts intercept and completely control SIV replication in the first week after challenge in ~50% of vaccinated RMs, without anamnestic expansion of SIV-specific immune responses (15–17). 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-vectorized 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

¹Vaccine and Gene Therapy Institute and Oregon National Primate Research Center, Oregon Health & Science University, Beaverton, OR 97006, USA. ²Statistical Center for HIV/AIDS Research and Prevention, Vaccine and Infectious Disease Division, Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA.

*These authors contributed equally to this work.

†Present address: Vir Biotechnology, 4640 SW Macadam Avenue, Portland, OR 97239, USA.

‡Present address: Atara Biotherapeutics, 611 Gateway Boulevard Suite 900, South San Francisco, CA 94080, USA.

§Corresponding author. Email: pickerl@ohsu.edu (L.J.P.); fruehk@ohsu.edu (K.F.)

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. 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), α -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 countermechanisms 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 costaining of these proteins in the absence of pp71. Upon pp71 expression, both ATRX and DAXX no longer localized in punctate dots, whereas pp71 partially colocalized 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 because DAXX abundance and, to some extent, BclAF1 abundance were restored in fibroblasts infected with 68-1 RhCMV lacking pp71 (Δ Rh110) (Fig. 1B and fig. S2, A and 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 antiviral activity by DAXX being the main reason for the observed growth defect and consistent with increased IE expression reported for HCMV upon DAXX small interfering RNA (siRNA) treatment (36).

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 (Δ Rh110/SIVgag) so that transcription was regulated by the native Rh110 promoter. Δ Rh110/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 Δ Rh110/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 affects growth of RhCMV less severely than that of HCMV. Nevertheless, pp71 deletion reduced spreading of virus from infected cells to neighboring cells because the average plaque sizes of complemented and uncomplemented Δ Rh110/SIVgag at day 7 post-infection at low MOI (0.001) were strongly reduced compared to 68-1 in TRFs 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.

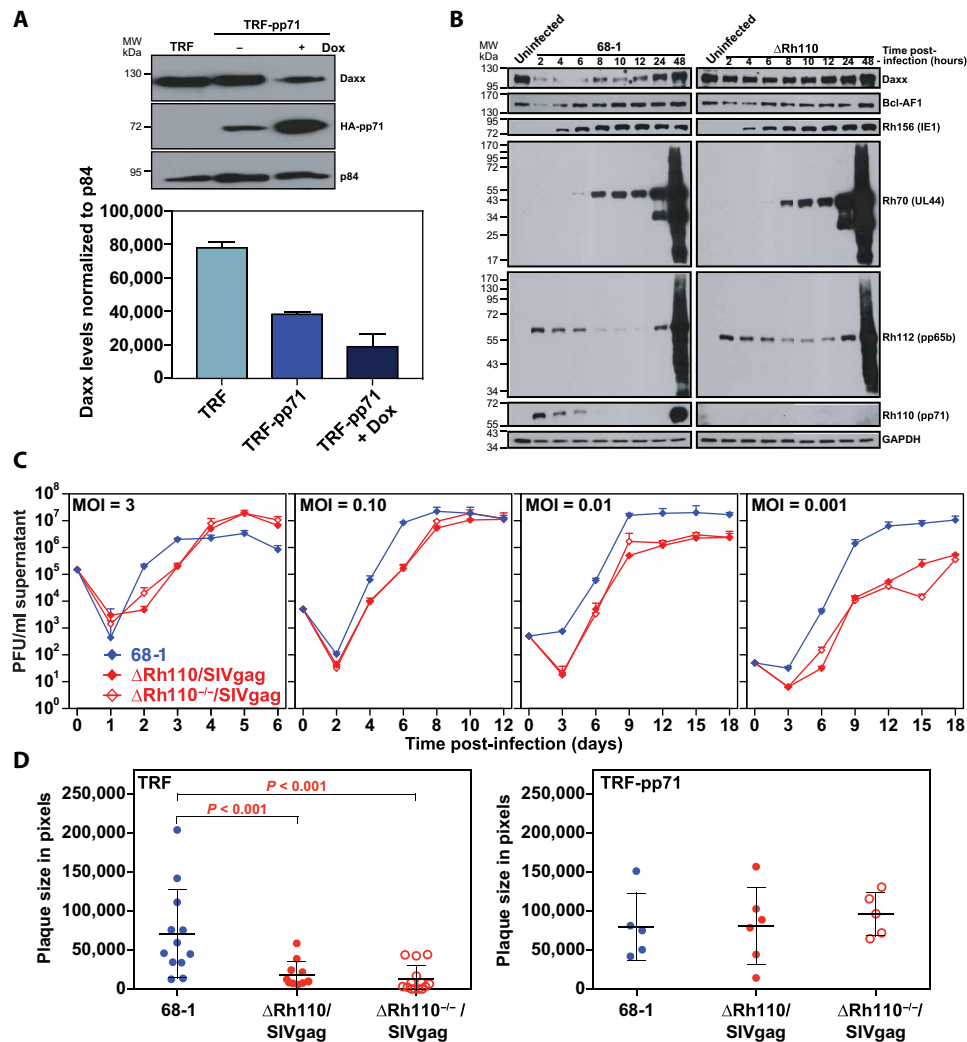


Fig. 1. RhCMV pp71 degrades DAXX and absence of pp71 results in in vitro growth deficiency. (A) TRFs or TRF-pp71 were untreated (–) or treated with DOX (+) (10 µg/ml) to induce RhCMV pp71 expression for 24 hours. Nuclear lysates were harvested and analyzed by immunoblot using anti-hemagglutinin (HA) Abs 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 Δ Rh110 (MOI = 3) or left uninfected, and cell lysates were harvested at the indicated time points, electrophoretically separated, and subjected to immunoblots with Abs 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 ($-/-$) Δ Rh110/SIVgag at the indicated MOI. Supernatants were harvested at the indicated times and titered by TCID₅₀ (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 Δ Rh110/SIVgag, or uncomplemented Δ Rh110 (Δ 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 (\pm SD) from one of two experiments, are shown. The Kruskal-Wallis (KW) test was used to determine the significance of differences between the three groups [$P=0.0002$, left; P = not significant (NS), right], with the Wilcoxon rank sum test used to perform pairwise analysis if KW P values were ≤ 0.05 ; brackets indicate pairwise comparisons with two-sided Wilcoxon $P \leq 0.05$. $P \leq 0.05$ is considered statistically significant.

Δ Rh110 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 10⁷ plaque-forming units (PFU) of 68-1 expressing SIVgag in the left arm and complemented Δ Rh110-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 ultrasensitive, nested quantitative polymerase chain reaction (qPCR) specific for SIVgag or SIVrtni sequences in multiple tissue samples (17, 48). 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 Δ Rh110/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 nonlymphoid tissues of all three 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 Δ Rh110/SIVrtni by ultrasensitive PCR at 4 weeks after 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 10⁷ PFU of Δ Rh110, 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 four RhCMV-naïve RMs inoculated with 10⁷ PFU of 68-1 through

231 dpi (Fig. 2, A and B). In contrast to 68-1, Δ Rh110 was not shed in the urine of these monkeys at any time point (Fig. 2C), confirming the in vivo attenuation of Δ Rh110. Thus, despite marked inhibition of viral spread in vivo, the 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 preexisting anti-CMV immunity, permitting the use of CMV vectors

Table 1. Rh110 (pp71) deletion reduces dissemination of RhCMV vectors. (A) Three RhCMV-naïve RMs (T1A-1, T1A-2, and T1A-3) were co-inoculated with 10⁷ 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 10⁷ cell equivalents were determined in the indicated tissues using ultrasensitive nested qPCR specific for SIVgag (68-1) or SIVrtni (ΔRh110). (B) Three naturally RhCMV-infected RMs (T1B-1, T1B-2, and T1B-3) were co-inoculated with 10⁷ 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 10⁷ cell equivalents in the indicated tissues were determined using ultrasensitive 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 10⁷ 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 ultrasensitive nested qPCR specific for SIVgag versus SIVrtni. Normalized to 1 × 10⁷ cell equivalents. WT, wild type; GI, gastrointestinal; LN, lymph node; PLN, parietal lymph node.

A. Primary infection: 68-1 versus ΔRh110						
Tissue type	RM T1A-1		RM T1A-2		RM T1A-3	
	14 dpi		21 dpi		28 dpi	
	68-1/SIVgag	ΔRh110/SIVrtni	68-1/SIVgag	ΔRh110/SIVrtni	68-1/SIVgag	ΔRh110/SIVrtni
Skin injection site—right (ΔRh110)	19,975,462	<1	13,227	2	<1	<1
Skin injection 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

B. Superinfection: 68-1 versus ΔRh110						
Tissue type	RM T1B-1		RM T1B-2		RM T1B-3	
	14 dpi		21 dpi		28 dpi	
	68-1/SIVrtni	ΔRh110/SIVgag	68-1/SIVrtni	ΔRh110/SIVgag	68-1/SIVrtni	ΔRh110/SIVgag
Skin injection site—right (WT)	47	5	16,502	<1	376	<1
Skin injection 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

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C. Δ Rh110: Complemented versus uncomplemented					
Tissue type	RM T1C-1		← 14 dpi →	RM T1C-2	
	Uncomplemented Δ Rh110/SIVrtni	Complemented Δ Rh110/SIVgag		Tissue type	Uncomplemented Δ Rh110/SIVgag
Skin injection site—right (complemented)	<1	<1	Skin injection site—right (uncomplemented)	<1	<1
Skin injection site—left (uncomplemented)	13	<1	Skin injection site—left (complemented)	<1	<1
Axillary LN—right (complemented draining)	<1	<1	Axillary LN—right (uncomplemented draining)	<1	<1
Axillary LN—left (uncomplemented draining)	17	<1	Axillary LN—left (complemented 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

regardless of previous CMV infection (7), which must be preserved in any attenuation strategy. To determine whether Δ Rh110 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 Δ Rh110/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, preexisting 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, Δ Rh110 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 SIVrtni-specific CD4⁺ and CD8⁺ T cell responses were detectable in most tissues of all three RMs and were of comparable magnitude (fig. S4B), indicating that even in the setting of preexisting 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 Δ Rh110 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 Δ Rh110/SIVgag and uncomplemented Δ Rh110/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, 49–51). 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 Δ Rh110/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 four RMs and found in all urine samples thereafter, as expected (7), whereas Δ Rh110/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 SIVrtni-specific CD4⁺ and CD8⁺ T cells elicited by Δ Rh110/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 and fig. S6). Moreover, the circulating SIVrtni-specific CD4⁺ and CD8⁺ T cell responses elicited by Δ Rh110 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 tumor necrosis factor- α (TNF- α), interferon- γ (IFN- γ), and macrophage inflammatory protein 1 (MIP-1 β), and low interleukin-2 (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 major histocompatibility complex class II (MHC-II) or MHC-E (52, 53). This broad, unconventional epitope targeting is not affected by pp71 deletion (Fig. 3D).

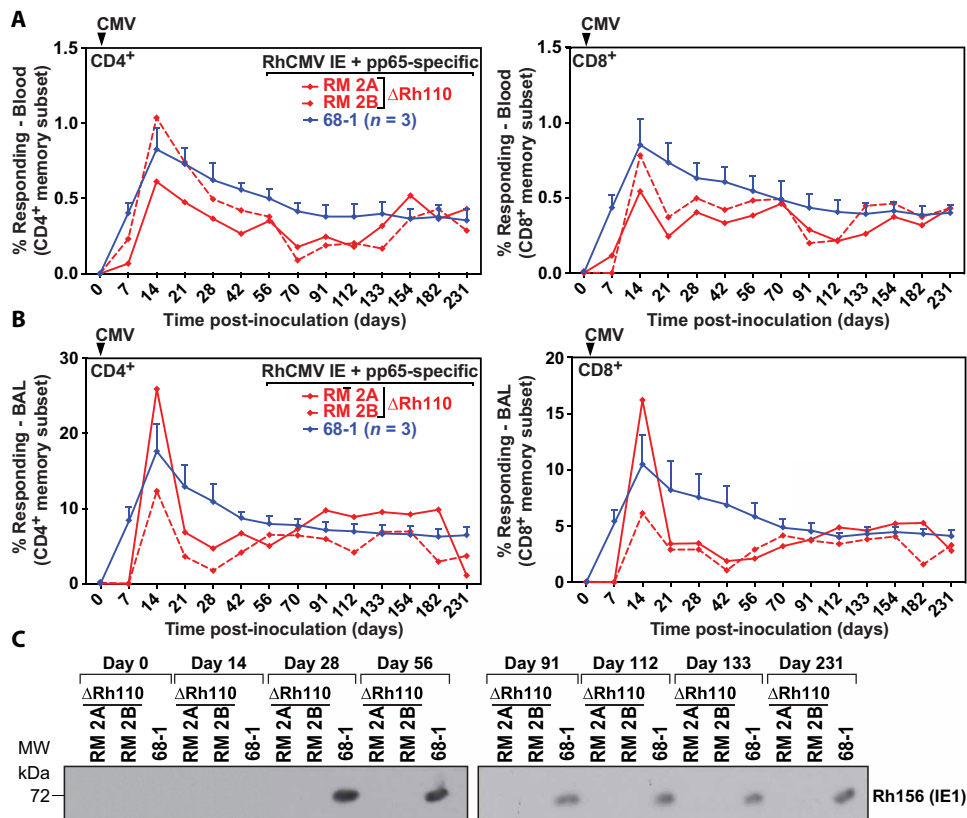


Fig. 2. Rh110 (pp71)-deleted RhCMV retains T cell immunogenicity but is no longer shed in urine. (A) Frequencies of RhCMV-specific CD4⁺ and CD8⁺ T cell responses in PBMCs were determined at the indicated time points in two RMs inoculated with 10⁷ PFU of ΔRh110 and three RMs given the same dose of 68-1 RhCMV. RhCMV IE1- and pp65a-specific T cells were determined by flow cytometric intracellular cytokine staining (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 TNF-α and IFN-γ to define Ag-specific T cells. 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 are shown. (B) Frequencies of RhCMV-specific T cell responses in bronchoalveolar lavages (BAL) were determined as in (A) in the same animals at the indicated time points. (C) Urine was isolated at the indicated dpi from RM 2A and RM 2B or one RM inoculated with 68-1. The presence of virus in cocultures was determined by immunoblot for RhCMV IE1 (see Materials and Methods).

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 after inoculation, pp71-deficient RhCMV persists in tissues at levels that support the maintenance of abundant, highly T_{EM}-biased, RhCMV- and insert-specific T cells.

To confirm that ΔRh110 RhCMV vectors could be used repeatedly in the same RM, either to boost preexisting T cell responses or elicit new T cell responses to different inserts, we inoculated two RMs that had previously been vaccinated with ΔRh110/SIVrtm both with a second dose of ΔRh110/SIVrtm 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 SIVrtm-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 SIVrtm- 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 five vectors, each expressing one SIVmac239 insert (gag, env, rtm, pol-5', and pol-3') and subcutaneously administered at a dose of 5 × 10⁶ PFU per vector. Abs to whole RhCMV viral lysates were measured by enzyme-linked immunosorbent assay (ELISA) before and 4, 6, and 12 weeks after vaccination. As shown in fig. S7, both cohorts showed a significant (P < 0.001), but transient, boost in Ab titers, the magnitude of which was not different between the monkeys vaccinated with ΔRh110 versus 68-1 vectors. Thus, when administered in a high-dose vaccination regimen, pp71-deleted RhCMV vectors were able to boost Ab 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 three cohorts of seropositive RMs (n = 3 each) with 10², 10⁴, or 10⁶ 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 10⁴ and 10⁶ PFU but not at 10² PFU. To further refine the dose required for immunogenicity, we reinoculated the three RMs that had failed to respond to 10² PFU with 10³ PFU ΔRh110/SIVgag, and for comparison, we inoculated all nine RMs with 10¹ PFU of 68-1/SIVpol-5' (Fig. 4B). Despite the extremely low dose of 68-1 administered, all nine RMs manifested SIVpol-specific CD4⁺ and CD8⁺ T cell responses, and at 10³ PFU, ΔRh110/SIVgag also induced both CD4⁺ and CD8⁺ SIVgag-specific T cells. Because 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: (i) pp71-complemented ΔRh110/SIVenv

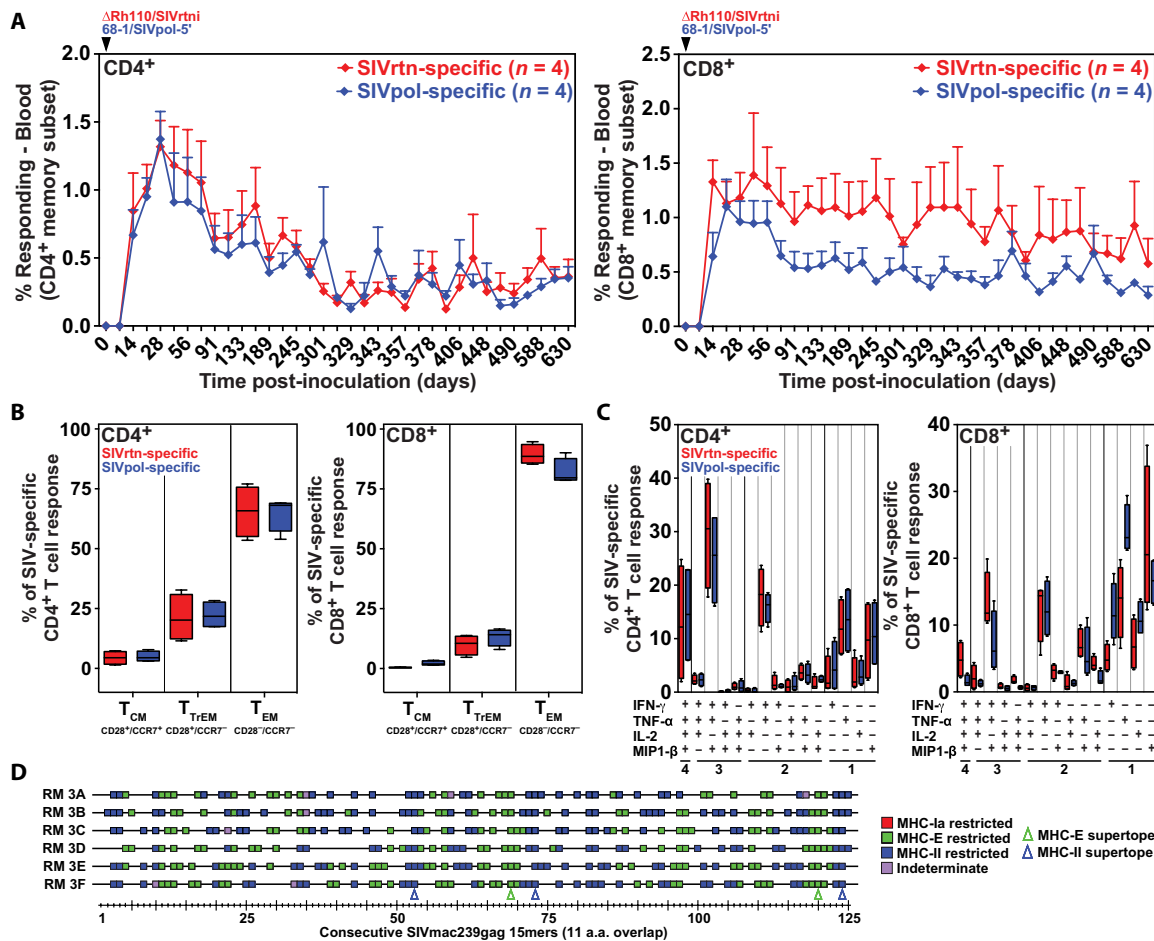


Fig. 3. Durability, functional phenotype, and epitope targeting of SIV insert-specific T cell responses elicited by Rh110 (pp71)-deleted RhCMV vectors in naturally RhCMV-infected RMs. (A) Four naturally RhCMV-infected RMs were co-inoculated with 10^7 PFU of 68-1/SIVpol-5' and the same dose of Δ Rh110/SIVrtm, and flow cytometric ICS was used to follow the magnitude of the CD4⁺ and CD8⁺ T cell responses in peripheral blood to SIVpol and SIVrtm peptide mixes as described in Fig. 2. The mean + SEM of SIVpol- and SIVrtm-specific response frequencies within the memory CD4⁺ (left) and CD8⁺ (right) 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 (A)] responding to SIVpol or SIVrtm 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 SIVrtm-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 (A) responding to SIVpol or SIVrtm 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 SIVrtm-specific CD4⁺ and CD8⁺ T cells expressing one, two, three, or four cytokines, with $P = NS$ for all comparisons. (D) SIVgag-specific CD8⁺ T cells in the peripheral blood of six Δ Rh110/SIVgag-inoculated RMs were epitope-mapped using a flow cytometric ICS assay (CD69, TNF- α , and 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 monoclonal Ab (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.

at 10^3 PFU, (ii) uncomplemented Δ Rh110/SIVgag at 10^3 PFU, and (iii) uncomplemented Δ Rh110/SIVrtm 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 Δ Rh110/SIVenv vector but not for the uncomplemented Δ Rh110/SIVgag vector. However, at the 10^4 PFU dose, the uncomplemented Δ Rh110/SIVrtm vector was immunogenic, indicating that pp71 complementation provides a 1-log dose sparing.

No evidence for recombination of Δ Rh110 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 alphaherpesvirus 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

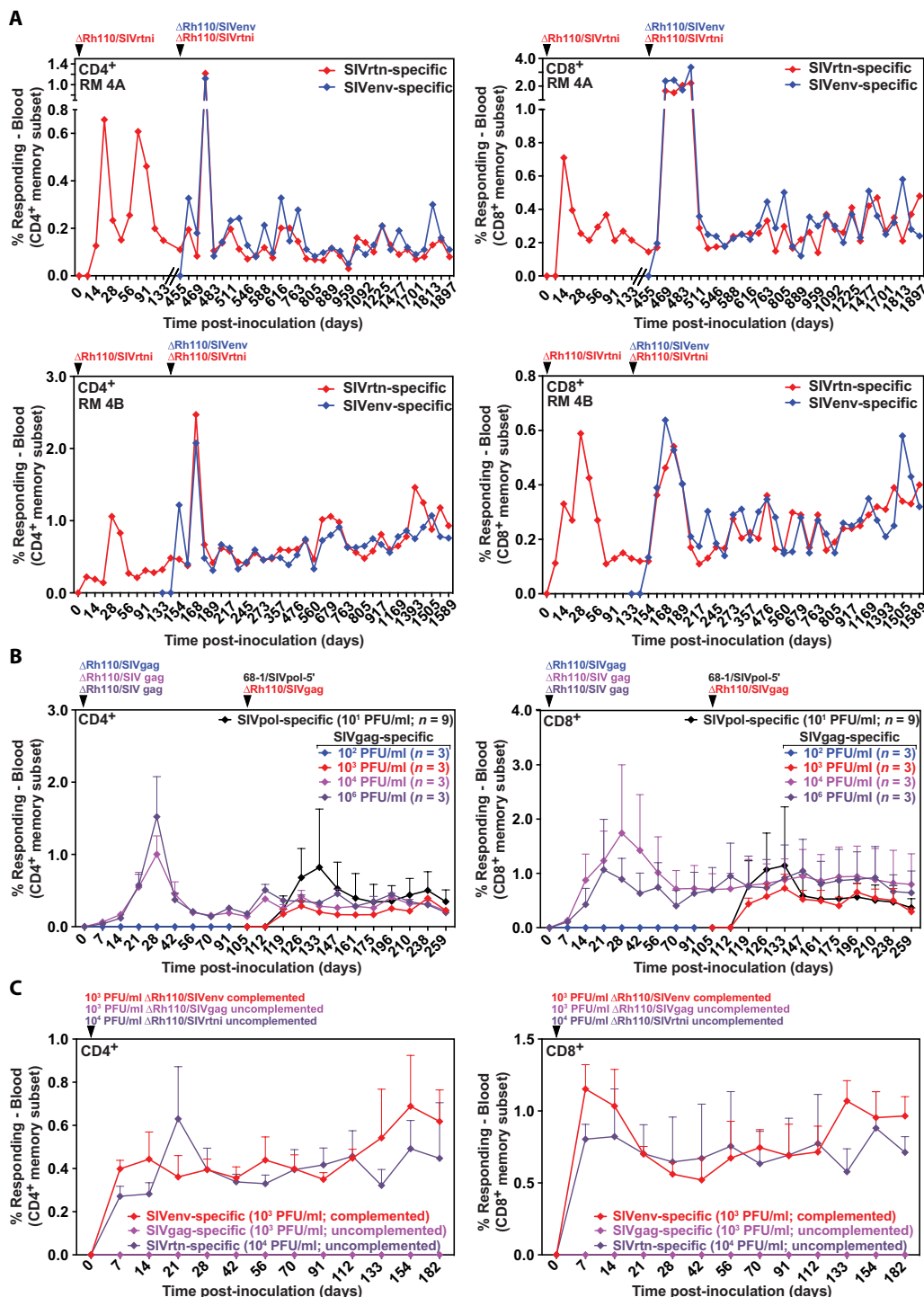


Fig. 4. Potency of Rh110 (pp71)-deleted RhCMV vectors in superinfection. (A) Two naturally RhCMV-infected RMs were inoculated with 10^7 PFU of Δ Rh110/SIVrtni on day 0 and again on day 455 (for RM 4A) and day 133 (for RM 4B), the latter inoculation in combination with 10^7 PFU of Δ Rh110/SIVenv. The panels show longitudinal analysis of the SIVrt- and SIVenv-specific CD4⁺ and CD8⁺ T cell response frequencies among PBMCs determined by flow cytometric ICS (CD69, TNF- α , and 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 Δ Rh110/SIVgag, complemented for pp71 by growing in TRF-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 reinoculated with 10^3 PFU dose of the same vector. At the same time, all nine RMs were inoculated with 10^1 PFU of 68-1/SIVpol-5'. The figures show longitudinal analysis of the mean \pm SEM of SIVgag- and SIVpol-specific CD4⁺ and CD8⁺ T cell response frequencies among PBMCs, determined as described in (A). **(C)** Three naturally RhCMV-infected RMs were subcutaneously inoculated with 10^3 PFU of pp71-complemented Δ Rh110/SIVenv, 10^3 PFU of Δ Rh110/SIVgag, and 10^4 PFU Δ Rh110/SIVrtni, with the latter two vectors grown in TRFs and thus not complemented for pp71. SIVenv-, SIVgag-, and SIVrt-specific CD4⁺ and CD8⁺ T cell responses within the peripheral blood memory compartment were followed by flow cytometric ICS as described above (mean \pm SEM shown at each time point).

dispensable for vector replication *in vitro* (fig. S8). Any repair of Δ Rh110 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. 5, A to C) and CD4⁺ T cell (fig. S9, A to 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 RM (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. Immunoblots of cocultures of viruses isolated from urine of all six RMs were consistently negative for SIV antigens over >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 Δ Rh110 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 cagemate (Fig. 6A and fig. S10A). In contrast, the nursing infants of five naturally RhCMV-infected dams inoculated with Δ Rh110 vectors (10^6 PFU each of five 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 five instances, *de novo* responses to RhCMV were observed, indicating that endogenous RhCMV was transmitted from mother to infant (Fig. 6, B to F, and fig. S10, B to F and G). Thus, pp71-deleted RhCMV vectors are not shed at all in urine, saliva, or breast milk or, if shedding

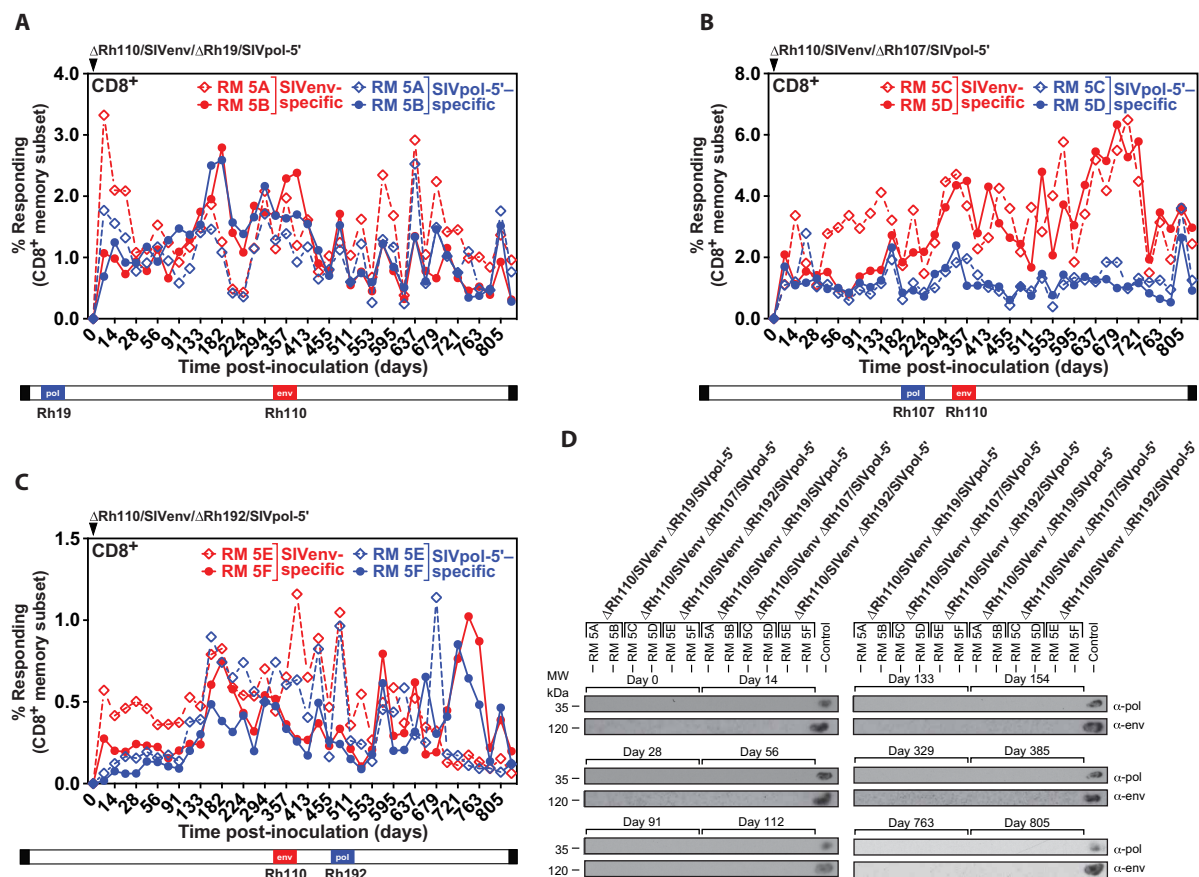


Fig. 5. Genetic stability of Rh110 (pp71)-deleted RhCMV vectors in the setting of superinfection. Two naturally RhCMV-infected RMs were each inoculated with 10^7 PFU of each dual insert-expressing Δ Rh110/SIVenv/ Δ Rh19/SIVpol-5' (A), Δ Rh110/SIVenv/ Δ Rh107/SIVpol-5' (B), or Δ Rh110/SIVenv/ Δ Rh192/SIVpol-5' (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 after vector inoculation were analyzed for vector shedding by viral coculture, followed by Western blot (WB) analysis of SIVpol-5' (top) or SIVenv (bottom) expression. Urine from RMs that previously received 68-1/SIVpol-5' and 68-1/SIVenv vectors was included as a positive control.

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 deleted from 68-1/SIVrtni, or the *Rh59* open reading frame (ORF) was replaced with SIVgag (fig. S11). Also similar to Δ Rh110, we were able to recover both Δ Rh59/SIVrtni and Δ Rh59/SIVgag without the need for complementation. 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 Δ Rh59 vector spread in vivo, we performed necropsies on three RhCMV⁺ RMs at 14, 21, and 28 days after subcutaneous inoculation with 10^7 PFU of Δ Rh59/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 Δ Rh59/SIVgag were at or below detection limits in all tissues tested at all three time points, whereas 68-1/SIVrtni was detected at all time points in multiple tissues. As demonstrated for Δ Rh110, SIVgag-specific CD4⁺ and CD8⁺ T cell responses in tissues at necropsy elicited by Δ Rh59 were similar to the SIVrtn-specific responses elicited by 68-1 in the same RM (fig. S12A). Longitudinal analysis of Δ Rh59 versus 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.

Δ Rh59, but not Δ Rh110, RhCMV/SIV vectors are shed and transmitted upon leukocyte transfer

To further compare the relative attenuation of Δ Rh110 and Δ Rh59 in vivo, we first inoculated RhCMV⁺ RMs with Δ Rh59/SIVrtni

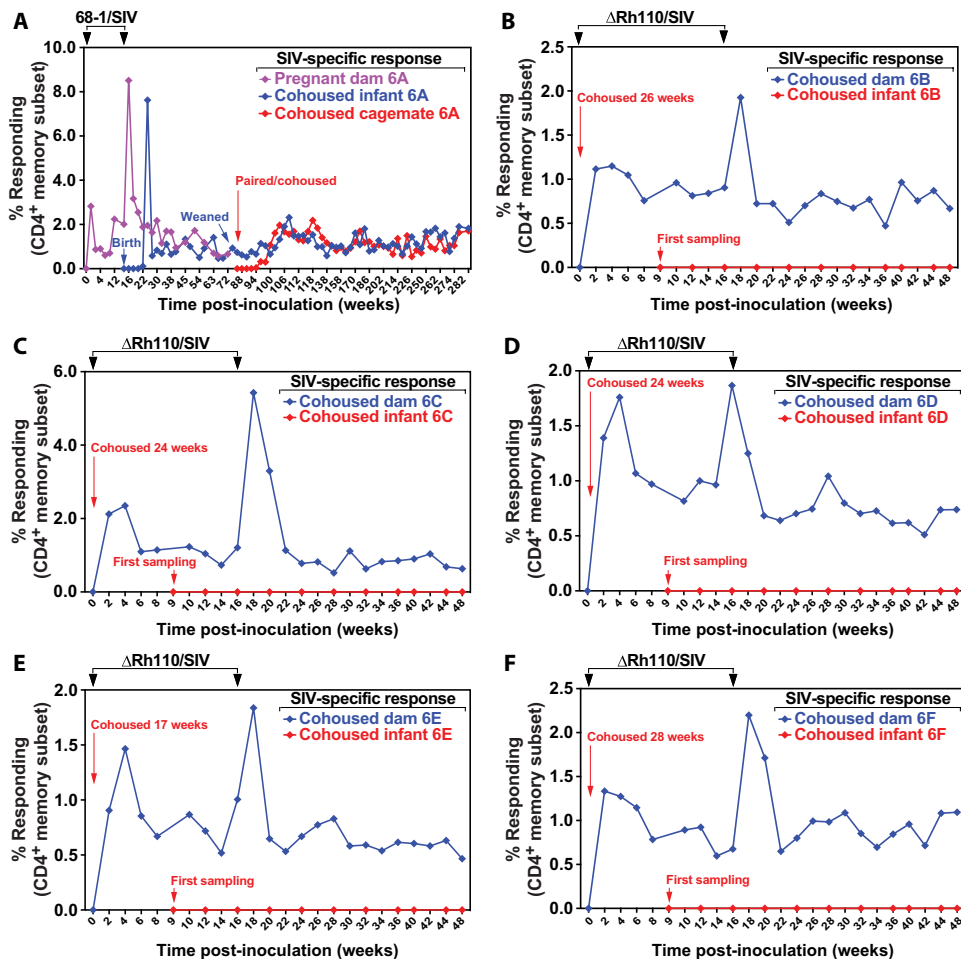
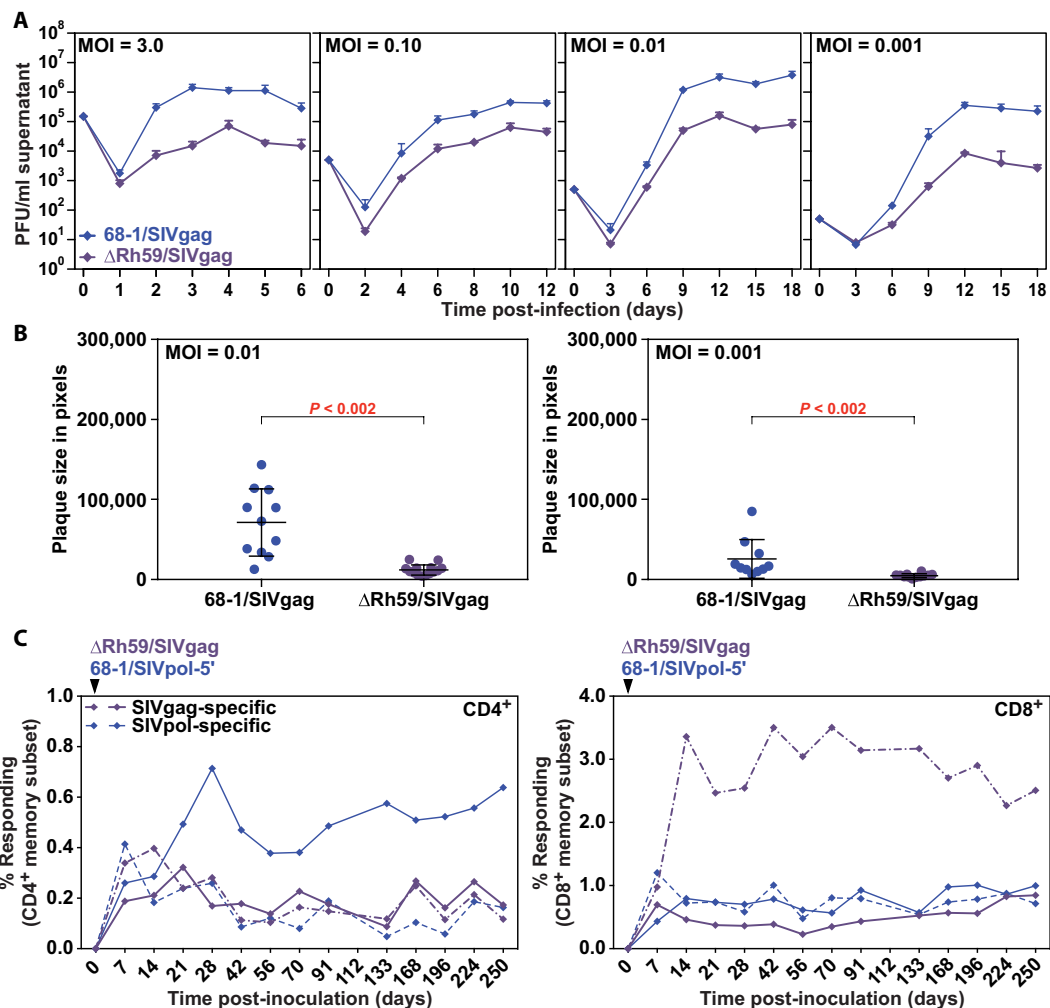


Fig. 6. Lack of maternal-infant transmission of Rh110 (pp71)-deleted RhCMV vectors. (A) A naturally RhCMV⁺ pregnant dam was inoculated twice, as shown, with a panel of five 68-1 vectors (5×10^6 PFU each) expressing SIVenv, SIVgag, SIVrtni, SIVpol-3' or SIVpol-5'. The dam gave birth to a healthy infant at 16 weeks after the first inoculation. The infant was cohoused with, and nursed from, the inoculated dam for 88 weeks, at which time the infant was weaned and cohoused with another, already naturally RhCMV⁺ juvenile RM. The mother, infant, and cohoused 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 Δ Rh110 vectors (5×10^6 PFU each) expressing SIVenv, SIVgag, SIVrtn, SIVpol-3', or SIVpol-5' while nursing 17- to 28-week-old infants and were reinoculated 16 weeks later with the same vectors at the same dose. Vaccinated mothers and nursing infants were cohoused 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) to (F), CD8⁺ T cell responses are shown in fig. S10.

and Δ Rh110/SIVenv and then, 175 days later, inoculated these RMs with 68-1/SIVpol-5' and Δ Rh110 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 antiapoptotic gene *Rh61/60* (*UL36* in HCMV) (58). Δ Rh110 68-1.2/SIVgag was thus generated to determine whether increased antiapoptosis activity and the ability to more efficiently infect nonfibroblast cells due to an intact pentamer would counter the attenuation resulting from pp71 deletion. All four vectors induced de novo, persistent CD8⁺ (Fig. 8A) and CD4⁺ (fig. S13A) T cell responses to their respective SIV inserts in all four RMs that were similar in magnitude in all tissues (fig. S13B). In keeping with

Fig. 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 Δ Rh59/SIVgag at the indicated MOI. The supernatant was harvested at the indicated dpi and titered by TCID₅₀ on TRFs. Average titers from two experimental and two technical replicates (\pm SD) are shown.

(B) TRFs were infected with 68-1/SIVgag or Δ Rh59/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 and average (\pm 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 Δ Rh59/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 , and 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).



previous results, 68-1/SIVpol-5' appeared in urine by 56 dpi (day 231 of the experiment) and at every subsequent time point, whereas Δ Rh110/SIVenv was not detected in the urine at any time point through 742 dpi (Fig. 8B). Similarly, Δ Rh110 68-1.2/SIVgag was not found in urine though 567 dpi (day 742 of the experiment), indicating that repair of the pentameric complex and antiapoptotic genes did not reverse the attenuation of pp71 deletion. However, in sharp contrast, Δ Rh59/SIVrtnei 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 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 Δ Rh59 and Δ Rh110, we adoptively transferred by intravenous infusion 8×10^6 or 19×10^6 bone marrow cells plus 30×10^6 peripheral blood leukocytes from two RMs inoculated with Δ Rh59/SIVrtnei, Δ Rh110/SIVenv, 68-1/SIVpol-5', and Δ Rh110 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). SIVpol- and SIVrtnei-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 SIVrtnei-specific responses arising from transmission of the Δ Rh59 vector, whereas SIVenv and SIVgag responses were never detected, consistent with a lack of transmission of both 68-1- and 68-1.2-derived Δ Rh110 vectors. These data indicate either that Δ Rh110 vectors were 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. Because it is very likely that the indefinite maintenance of T_{EM} requires long-term Ag exposure provided by persistent viral infection

Table 2. Rh59 (UL35) deletion reduces dissemination of 68-1 RhCMV vectors. Three naturally RhCMV-infected RMs (T2-1, T2-2, and T2-3) were co-inoculated with 10^7 PFU each of 68-1/SIVrtni (left arm) and Δ Rh59/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 ultrasensitive nested qPCR specific for SIVrtni (68-1) or SIVgag (Δ Rh59). Normalized to 1×10^7 cell equivalents.

Tissue type	Super infection: 68-1 versus Δ Rh59					
	RM T2-1		RM T2-2		RM T2-3	
	14 dpi		21 dpi		28 dpi	
	68-1/SIVrtni	Δ Rh59/SIVgag	68-1/SIVrtni	Δ Rh59/SIVgag	68-1/SIVrtni	Δ Rh59/SIVgag
Skin injection site—right (Δ Rh59)	12	<1	193	<1	36,110	7
Skin injection 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

(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, because 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. 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 Δ pp71 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 Δ pp71 CMV-infected cells (perhaps those exposed to

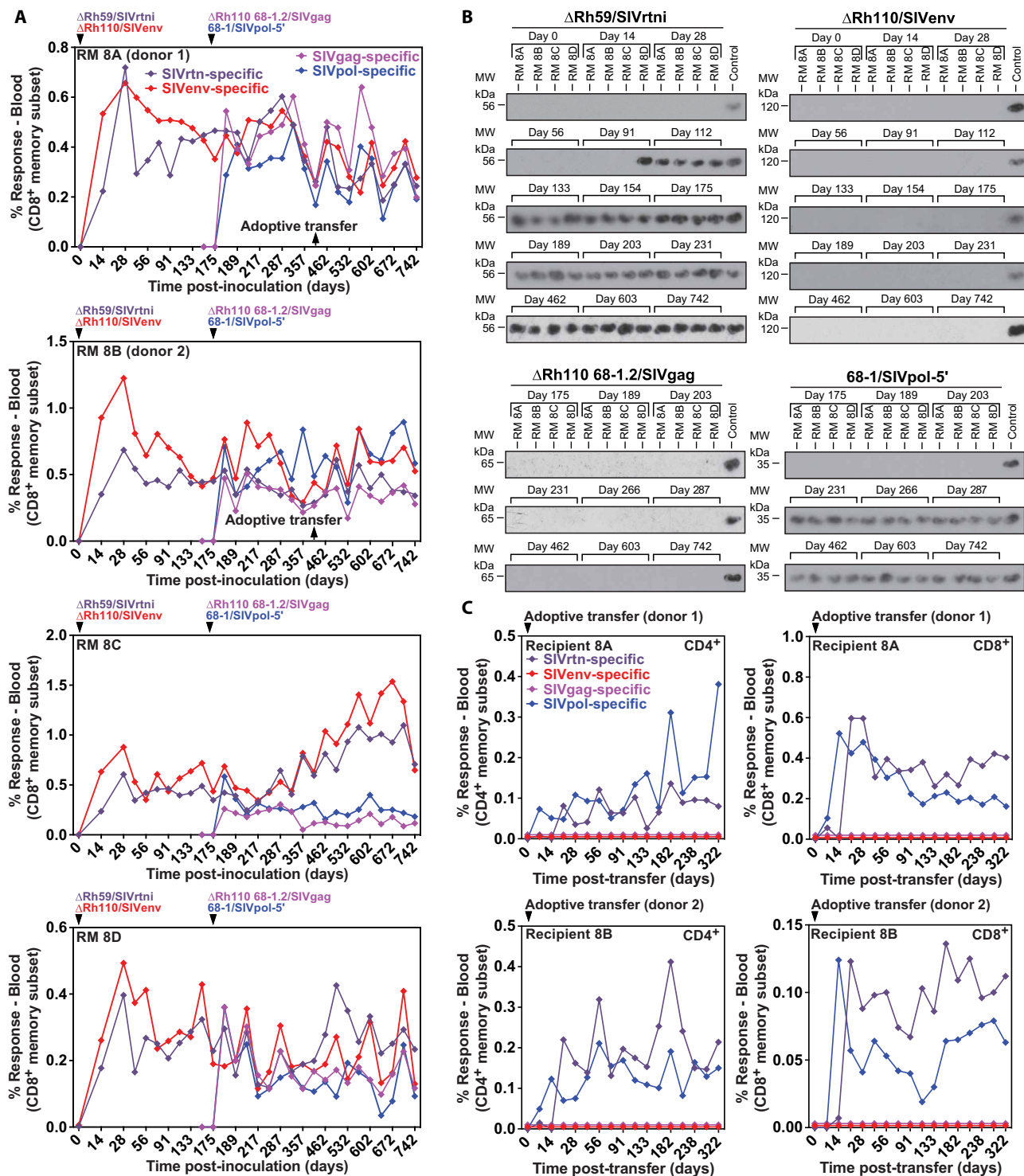


Fig. 8. Comparison of the shedding and transmission upon leukocyte transfer of Rh110 (pp71)-deleted, Rh59 (UL35)-deleted, and 68-1 RhCMV vectors. (A) Four RMs were co-inoculated with 10^6 to 10^7 PFU of Δ Rh59/SIVrtni, pentamer complex-repaired Δ Rh110 68-1.2/SIVgag, Δ Rh110/SIVenv, and 68-1/SIVpol-5' 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, TNF- α , and IFN- γ readout), with the response frequencies in the memory compartment shown (see also fig. S13B). (B) Immunoblots of viral cocultures 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) [1.9×10^7 bone marrow and 3.0×10^7 blood cells from RM 8A (donor 1); 0.8×10^7 bone marrow and 3.0×10^7 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).

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, Δ pp71 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 Δ pp71 CMV vector would manifest the necessary balance between infectivity and persistence (needed for immunogenicity) and spread restriction (needed for safety) would be appropriate for optimal vector function. The level of spread of Δ pp71 *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 Δ Rh110 vectors relative to their HCMV counterparts (46), even at very low MOI, raised concern that the Δ pp71 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, coinjected Δ Rh110 was barely detectable with a high-sensitivity assay at 14 dpi and was essentially undetectable in tissues taken later. The numbers of Δ Rh110 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 Δ Rh110 *in vivo*; the genome copies detected at day 14 might simply reflect the Δ Rh110 genomes from the injected (high dose) inoculum and/or the virus produced within the cells infected by this inoculum. The robust attenuation of Δ Rh110 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, Δ Rh110 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 Δ Rh110 would be too attenuated for use as an effective vector. However, this concern is allayed by comparable 68-1 and Δ Rh110 CD4⁺ and CD8⁺ T cell immunogenicity, even in the setting of super-infection. Moreover, as shown in the companion article, we demonstrate that Δ Rh110 vectors show efficacy against viral challenge that is as good or better than 68-1 vectors (69).

The only clear immunologic difference between Δ Rh110 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) Δ Rh110 vector required 10^4 PFU to establish immunogenicity in RhCMV⁺ RMs. This observation is consistent with the hypothesis that the primary defect of Δ Rh110 is a reduction in its ability to spread from cell to cell. The lack of any shedding or transmission of Δ Rh110 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 Δ Rh110, with or without complementation, and by the pp71-intact 68-1 RhCMV was 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. Because complemented Δ Rh110 has pp71 in its tegument to facilitate the first round of replication, the comparison between 68-1 and uncomplemented Δ Rh110 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 versus 10 PFU), a 10- to 100-fold higher deficit than the reduction of viral growth observed with low MOI *in vitro*.

The Rh59 (UL35)-deleted RhCMV vector, which showed an MOI-independent 1- to 2-log growth defect *in vitro* and a similar spread deficiency after *in vivo* inoculation as Δ Rh110, did not show the same overall attenuation as Δ Rh110, as Δ Rh59 was both shed in urine and readily transferred to naïve RMs by leukocyte transfer. Thus, although 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 Δ Rh59 (Δ UL35) RhCMV, though adequately immunogenic, is not satisfactorily attenuated for a primary CMV attenuation strategy.

Most of the Δ Rh110 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-passaged RhCMV isolates (73), and thus potentially contribute to the attenuation of Δ Rh110. 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 Δ Rh110 reduces the likelihood of coinfecting the same cell as endogenous RhCMV and potential repair of the Rh110 deletion by recombination. Homologous recombination of a Δ Rh110 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 wild-type 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 nonhomologous recombination, leaving a spread-competent CMV that also expresses the vaccine insert. Although this would not subject the vaccine recipient to additional risk, because this individual already harbors wild-type 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 nonhomologous gene exchange, we saw no evidence of such attenuation reversal in six superinfected RMs followed over 800 days. These data suggest that if such Rh110-repairing recombination occurs at all, then it is likely to be a very infrequent event. We would also note that the most likely outcome of Δ pp71 CMV vector coinfection with a wild-type CMV is complementation of the Δ pp71 CMV virions, as reported for MCMV lacking essential genes (74), which would increase their infectivity. However, because 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 Δ pp71 CMV vector attenuation.

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 nonpathogenic. 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 to 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 RhCMV Δ Rh110 (5-log versus 2-log reduction relative to 68-1 at low MOI) (46), it is likely that a HCMV Δ UL82 vector will be, if anything, more attenuated in humans than Δ Rh110 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 nonattenuated vectors. Because we observed highly consistent results among the two to four 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 reinfect 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 nonattenuated 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 nonattenuated vectors. Primary data are reported in data file S1.

Statistical analysis

We used nonparametric 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

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Materials and Methods

Fig. S1. Redistribution of corepressors by RhCMV pp71.

Fig. S2. Description and characterization of pp71-deleted RhCMV vectors.

Fig. S3. Summary of recombinant RhCMV/SIV vectors used *in vivo*.

Fig. S4. Tissue distribution of SIV insert-specific CD4⁺ and CD8⁺ T cell responses elicited by Δ Rh110 versus 68-1 vectors.

Fig. S5. Urine shedding of Δ Rh110 versus 68-1 vectors.

Fig. S6. T cell responses in lung airspace (BAL cells) upon super-infection with pp71-deleted RhCMV vectors.

Fig. S7. Boosting of RhCMV Ab responses by Δ Rh110 (Δ pp71) RhCMV vectors.

Fig. S8. Description and characterization of dual insert-expressing, pp71-deleted RhCMV vectors.

Fig. S9. CD4⁺ T cell responses to dual antigen insert-expressing, pp71-deleted RhCMV vectors.

Fig. S10. Lack of maternal-infant transmission of pp71-deleted vectors.

Fig. S11. Description and characterization of Rh59 (UL35)-deleted RhCMV used in this study.

Fig. S12. SIV antigen-specific CD4⁺ and CD8⁺ T cell responses elicited by Δ Rh59 versus 68-1 RhCMV vectors in tissue sites.

Fig. S13. T cell responses to SIV antigens expressed by Δ Rh110 and Δ Rh59 vectors.

Data file S1. Primary data.

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Acknowledgments: We thank T. Shenk (Princeton University) for reagents and J. Shao (Fred Hutchinson Cancer Research Center) for help with statistical analysis. We also thank the OHSU molecular virology support core and monoclonal Ab core for technical service, P. Smith for help with the ELISA for RhCMV Abs, A. Townsend for figure preparation, and A. Sylwester, S. Hagen, N. Whizin, K. Randall, A. Selseth, and L. Boshears for other technical or administrative assistance. **Funding:** This work was supported by the Bill & Melinda Gates Foundation–supported Collaboration for AIDS Vaccine Discovery (OPP1033121 to L.J.P.), the National Institute of Allergy and Infectious Diseases (NIAID) (R01 AI095113, P01 AI094417, and R37 AI054292 to L.J.P.; R01 AI059457 to K.F.; and U42 OD023038 to M.K.A.), and the NIH Office of the Director (U42OD010426 and P51OD011092). **Author contributions:** K.F. and L.J.P. conceived the attenuated RhCMV vector strategy, supervised experiments, analyzed and interpreted data, and wrote the paper, assisted by S.G.H., E.E.M., and D.M. S.G.H. planned and performed animal experiments and immunologic assays, assisted by R.M.G., C.M.H., A.B.V., E.A., A.N.S., J.C.F., and D.B. E.E.M. and D.M. constructed and quality-tested recombinant RhCMV constructs, assisted by J.W. D.S. and C.N.K. planned and supervised RhCMV quantification by PCR/reverse transcription PCR, assisted by C.K. A.W.L. and M.K.A. managed the animal care and procedures. P.T.E. performed all statistical analyses. **Competing interests:** OHSU, L.J.P., E.E.M., S.G.H., and K.F. have a substantial financial interest in Vir Biotechnology Inc., a company that may have a commercial interest in the results of this research and technology. L.J.P., S.G.H., and K.F. are co-inventors of patent PCT/US2011/036657 “Recombinant RhCMV and HCMV vectors and uses thereof” licensed to Vir. L.J.P., S.G.H., and K.F. have received compensation for consulting for Vir. The potential individual and institutional conflicts of interest have been reviewed and managed by OHSU. **Data and materials availability:** All data associated with this study are present in the paper or the Supplementary Materials. Vector primer sequences have been deposited in GenBank (see the 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.

Submitted 3 December 2018
Resubmitted 14 March 2019
Accepted 13 June 2019
Published 17 July 2019
10.1126/scitranslmed.aaw2603

Citation: E. E. Marshall, D. Malouli, S. G. Hansen, R. M. Gilbride, C. M. Hughes, A. B. Ventura, E. Ainslie, A. N. Selseth, J. C. Ford, D. Burke, C. N. Kreklywich, J. Womack, A. W. Legasse, M. K. Axthelm, C. Kahl, D. Strelow, P. T. Edlefsen, L. J. Picker, K. Früh, Enhancing safety of cytomegalovirus-based vaccine vectors by engaging host intrinsic immunity. *Sci. Transl. Med.* **11**, eaaw2603 (2019).

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

Emily E. Marshall, Daniel Malouli, Scott G. Hansen, Roxanne M. Gilbride, Colette M. Hughes, Abigail B. Ventura, Emily Ainslie, Andrea N. Selseth, Julia C. Ford, David Burke, Craig N. Kreklywich, Jennie Womack, Alfred W. Legasse, Michael K. Axthelm, Christoph Kahl, Daniel Streblow, Paul T. Edlefsen, Louis J. Picker and Klaus Früh

Sci Transl Med 11, eaaw2603.
DOI: 10.1126/scitranslmed.aaw2603

Building a safer CMV vector

Vaccine vectors based on cytomegalovirus (CMV) show strong T cell induction and protection against a multitude of pathogens. However, CMV can be harmful to people who are immunodeficient or immunosuppressed. Marshall *et al.* genetically modified rhesus CMV to allow engagement of host intrinsic immunity. The modified Δ Rh110 vector did not spread once administered to nonhuman primates but still induced robust T cell immunity. Hansen *et al.* showed in a simian immunodeficiency virus (SIV) challenge model that the Δ Rh110 vector provided equivalent protection to the parental vector, enabling control and progressive clearance of virus from more than half of the vaccinated primates. Most protected animals that were rechallenged 3 years later were able to control the second challenge, demonstrating the durability of this vaccine. Mutations in the human CMV vector should lead to a potent but restrained CMV that could be used widely in people.

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