Supplementary Information

Effector-memory T cell responses are associated with protection of rhesus monkeys from mucosal SIV challenge

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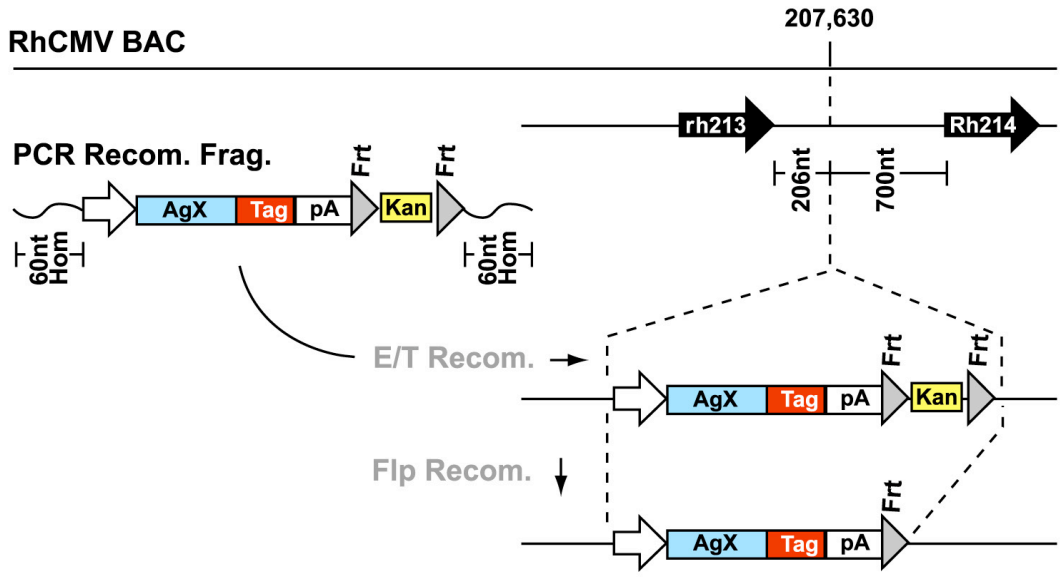
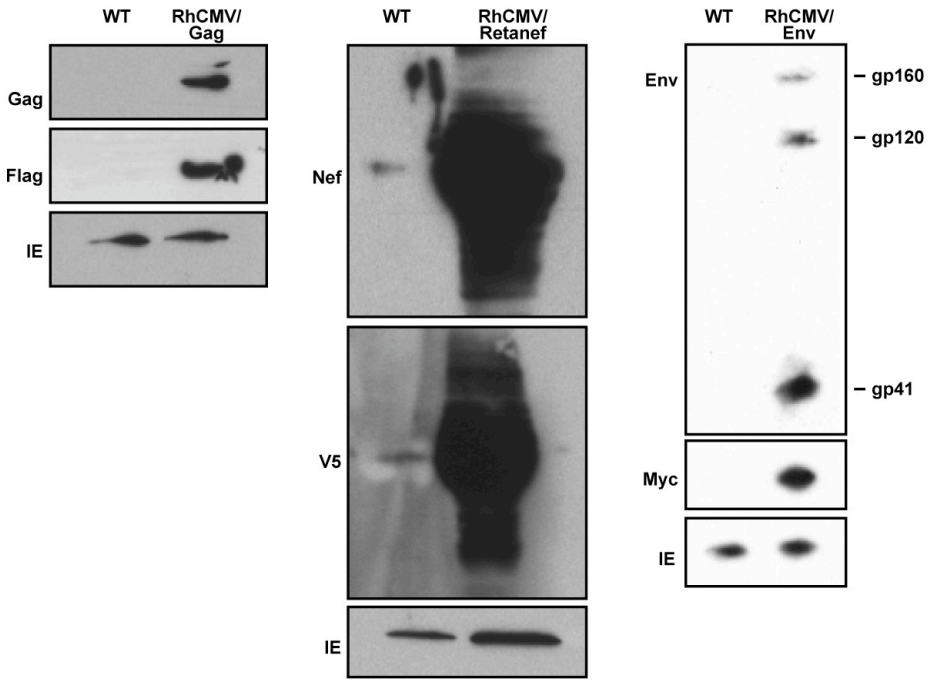
SUPPLEMENTAL FIGURES

Figure S1



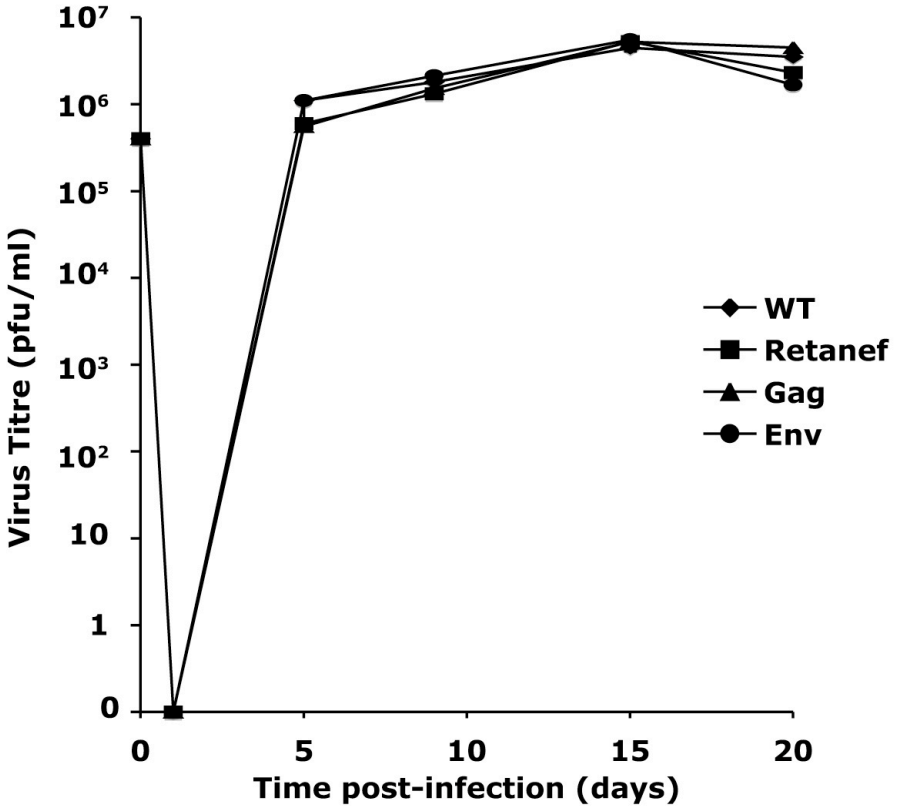
Supplementary Figure S1: Schematic illustrating the construction and characterization of RhCMV/SIV vectors. SIV antigen-expressing cassettes (codon-optimized for expression in mammalian cells) were inserted into RhCMV BAC (pRhCMV/BAC-Cre) at nucleotide 207,630 within the non-coding region between rh213 and Rh214 by E/T recombination. The pRhCMV/BAC-Cre BAC contains the entire RhCMV strain 68-1[1] and utilizes a Cre recombinase system to remove the BAC cassette from the viral genome during virus reconstitution in mammalian cells[2,3]. Consequently, RhCMV/SIV vectors contain the entire RhCMV genome, and are wt, other than for presence of the SIV expression cassette. The SIV(env) and SIV(gag) (p57) inserts are based on SIVmac239 sequences with the SIV(env) construct containing additional mutations within the 3' untranslated region to eliminate the rev-response element, and within the signal peptide to target the protein to the cytoplasm[4]. Retanef is a fusion construct comprised of rev, tat and nef mutagenized to decrease toxicity[5]. The nef sequence in this construct is SIVmac239-derived, whereas the rev and tat are based on SIVmac251 sequences. To achieve maximal expression, SIV(gag) and SIV(retanef) expression were placed under the control of the human EF1α promoter[6]. The EF1α promoter has been shown to result in high levels of expression in a cell-type independent manner. To avoid toxicity resulting from over-expression of a hydrophobic protein, SIV(env) was placed under control of the human CMV gH promoter[7], which is expressed only at late times of CMV infection. Following selection of recombinant BACs on the basis of kanamycin resistance, the Kan^R marker was removed by flp-mediated recombination. Recombinant RhCMV/SIV BACs were characterized by digestion with EcoR1 followed by electrophoresis and Southern blot analysis using P^{32}-labeled DNA probes against the SIV expression cassette (not shown). [open arrow, promoter (either EF1α or human CMV gH); AgX, SIV antigen; Tag, unique epitope tag; pA, polyadenylation site; Kan, kanamycin resistance gene for selection in bacteria; grey triangle, Frt flp-recombinase recognition site)].

Figure S2



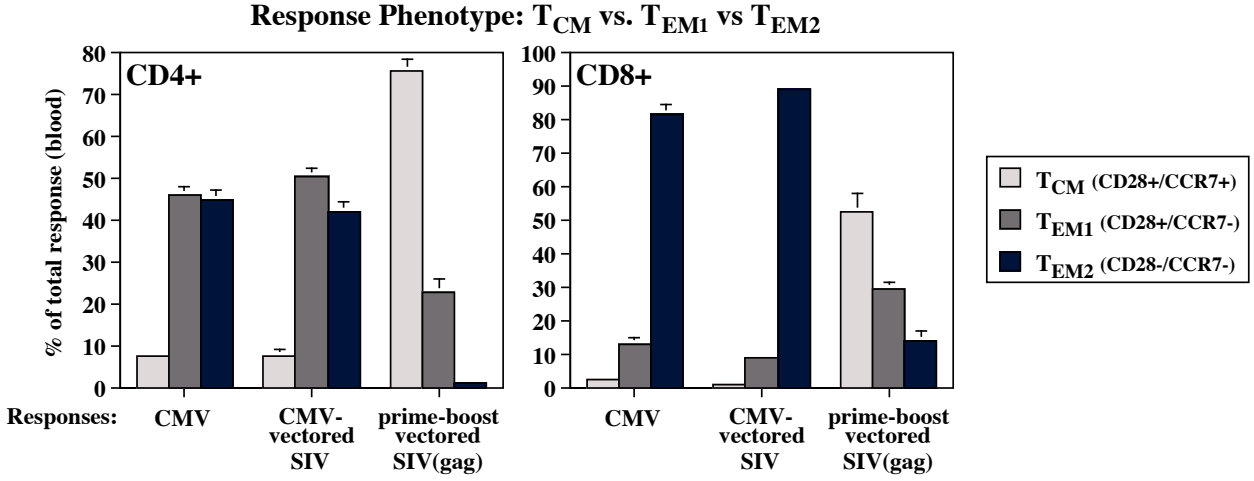
Supplementary Figure S2: RhCMV vectors highly express SIV proteins. Rhesus fibroblasts were infected with either wildtype (WT) RhCMV or each RhCMV/SIV vector, and lysates were harvested after visualization of extensive cytopathic effect. Proteins were separated by SDS-PAGE electrophoresis, transferred and assayed by western immunoblot for the presence of SIV antigens using epitope and SIV antigen-specific antibodies. Western analysis was performed using the following SIV antigen and epitope tag-specific antibodies: anti-V5 (1:2,000) (Invitrogen), M2 anti-FLAG (Sigma-Aldrich), anti-Myc (Clontech), anti-Nef (NIH AIDS Repository), anti-Gag (NIH AIDS Repository), and anti-gp160 (1:200) (NIH AIDS Repository). Expression levels of RhCMV immediate-early IE antigen using a RhCMV IE-specific antibody (1:1,000) was used as a measurement of RhCMV infection.

Figure S3



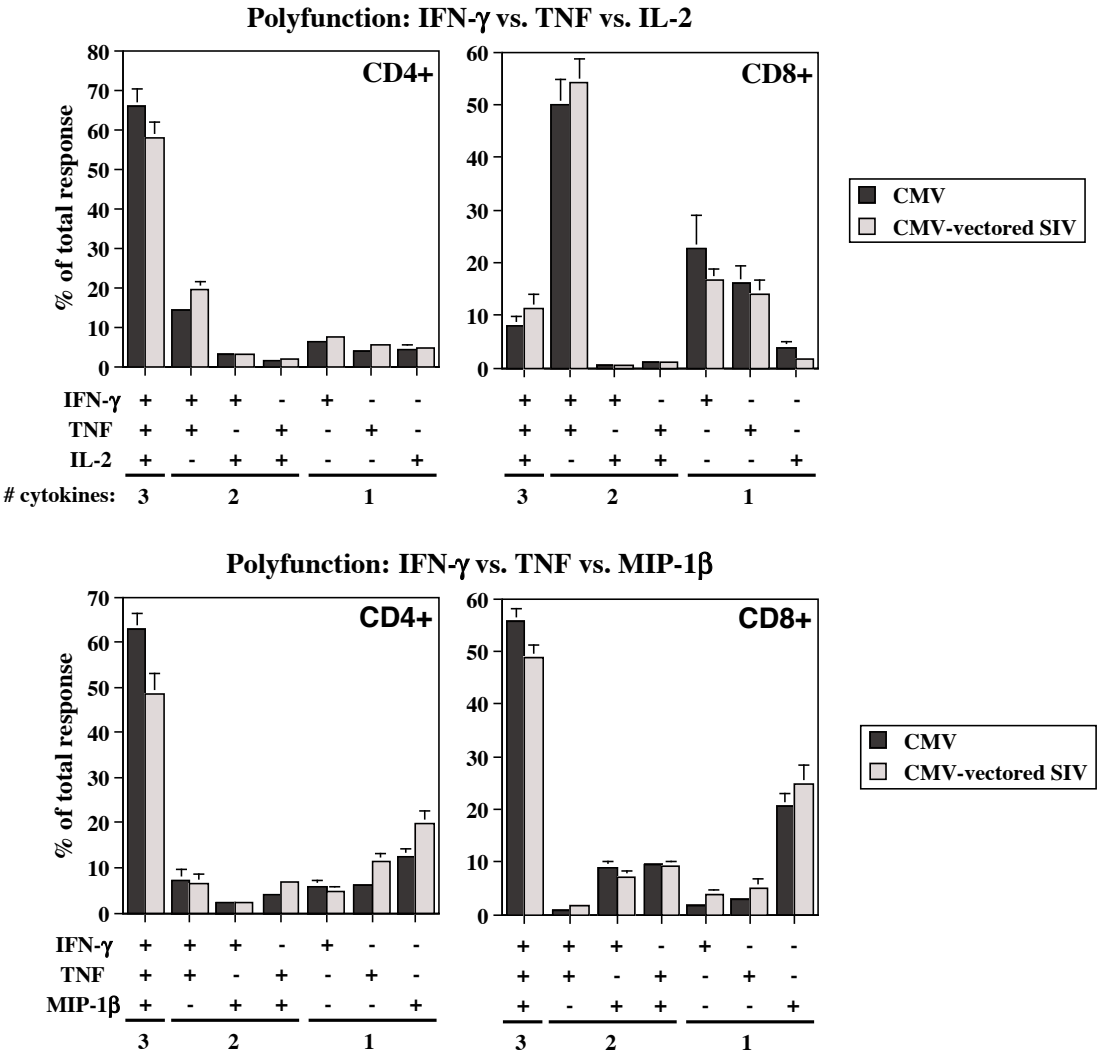
Supplementary Figure S3: RhCMV vectors replicate with wildtype (WT) kinetics. Multi-step growth analysis of each RhCMV/SIV vector vs. WT RhCMV on rhesus fibroblasts infected at a multiplicity of infection of 0.1. Samples were collected at the days indicated post-infection and titred by plaque assay.

Figure S4



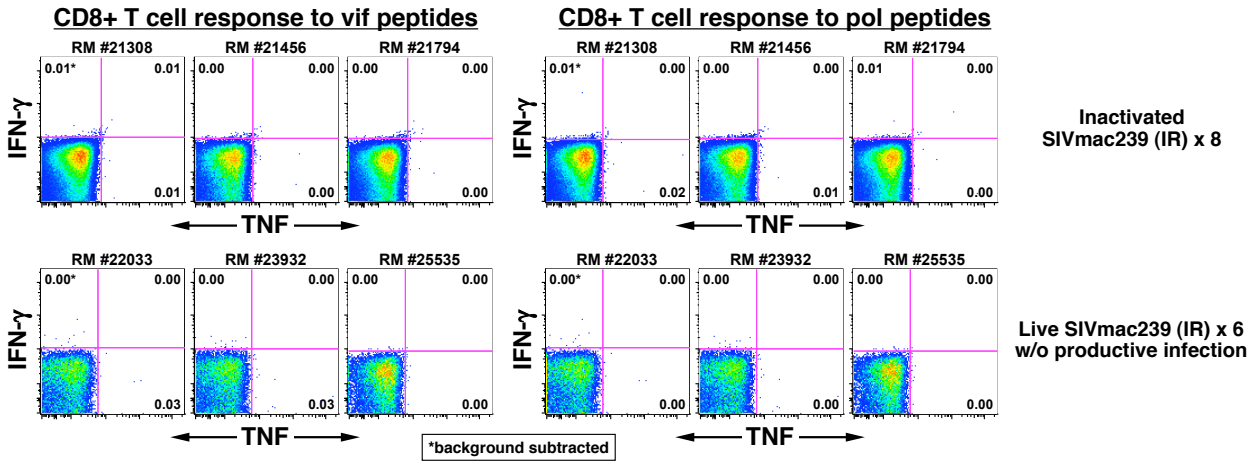
Supplementary Figure S4: CMV-vectored, SIV-specific T cell responses maintain a T_{EM}-bias similar to that of native CMV-specific T cells and distinct from the T_{CM} phenotype developed by SIV-specific T cells elicited by a protein prime-Ad5 boost vaccine regimen. Long-term, chronic-phase (>300 days), RhCMV-vectored, SIV-specific $CD4^+$ and $CD8^+$ T cell responses (SIV Gag and Rev/Nef/Tat) and the $CD4^+$ and $CD8^+$ T cell responses to wildtype RhCMV lysate in the same RM were phenotyped for CD28 and CCR7 expression as shown in **Fig. 2a**. Analyzed responses were at least 0.25% of the overall $CD4^+$ and $CD8^+$ population, and included 7 $CD4^+$ SIV-specific responses, 7 $CD4^+$ RhCMV-specific responses, 18 $CD8^+$ SIV-specific responses, and 9 $CD8^+$ CMV-specific responses. Also studied were 9 $CD4^+$ and $CD8^+$ SIV Gag-specific responses that were generated by a Gag protein prime followed by Ad5(gag) boost regimen (with responses analyzed 105-128 days after the Ad5(gag) boost). Mean $\pm$ SEM of the proportions of these responses with the designated T_{CM}, T_{EM1} and T_{EM2} phenotypes are shown. Mixed effects statistical analysis revealed that the subset distribution of RhCMV-vectored, SIV-specific responses was not significantly different from the RhCMV-specific responses (both $CD4^+$ and $CD8^+$), but that the subset distribution of both of these response types was distinct from that of SIV Gag-specific responses generated by the prime-boost regimen ($p < .0001$).

Figure S5



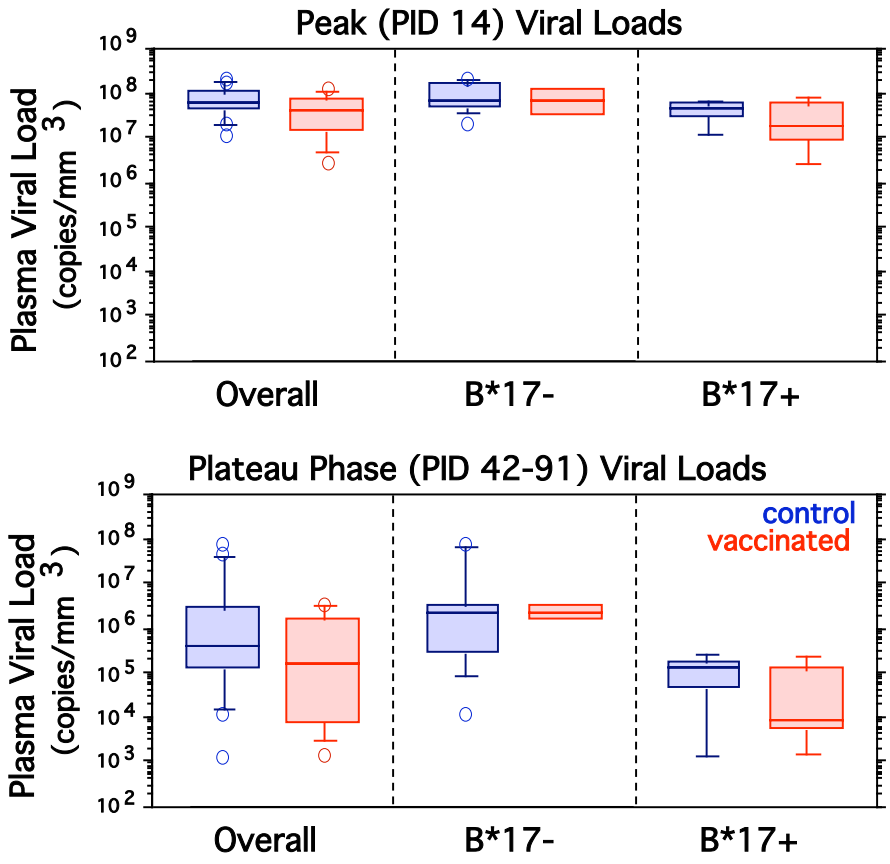
Supplementary Figure S5: CMV-vectored, SIV-specific T cell responses have a similar effector response program to that of native CMV-specific T cells. Long-term, chronic-phase (>500 days) RhCMV-vectored, SIV-specific $CD4^+$ and $CD8^+$ T cell responses (SIV Gag and Rev/Nef/Tat), and RhCMV-specific $CD4^+$ and $CD8^+$ T cell responses in the same RM were analyzed for their coordinate expression of TNF, IFN-γ, and IL-2, and separately, TNF, IFN-γ, and MIP-1β as shown in **Fig. 3a/b**, and the proportions of total responding cells producing these cytokines alone or in combination were determined. For this experiment, we evaluated RhCMV-specific $CD4^+$ T cell responses using the response to whole RhCMV lysate, but for $CD8^+$ T cell responses, we measured the response overlapping peptides comprising the RhCMV immediate early-1 protein as a representative $CD8^+$ T cell-targeted RhCMV antigen. Analyzed responses were at least 0.25% of the overall $CD4^+$ or $CD8^+$ T cell population, and the overall analysis included 6-10 different responses for each group. The figure shows the mean ± SEM of the proportions of these responses with the designated functional responses. Mixed effects statistical analysis revealed that the subset distribution of RhCMV-vectored, SIV-specific responses was not significantly different from the RhCMV-specific responses for both $CD4^+$ and $CD8^+$ subsets.

Figure S6



Supplementary Figure S6: Unvaccinated control RM do not develop measurable SIV-specific CD8+ T cell responses with repeated low dose exposure to inactivated SIV virions or to live virions in the absence of overt infection. Four of 12 RhCMV vector-vaccinated RM resisted progressive infection during repeated low dose, mucosal SIVmac239 challenge, and during the challenge period, all 4 of these RM developed relatively robust $CD8^+$ T cell responses to SIV proteins (Pol and Vif) that were not in the vaccine. These findings imply an at least transient infection with the challenge virus in these protected RM, but it remains theoretically possible that uptake of viral antigens from the challenge preparation could, via cross-presentation, initiate such an immune response. To assess this possibility, we administered an AT2-inactivated SIVmac239[8] preparation to 3 additional naïve RM, using an equivalent dose to that used in the live virus challenge of the vaccinated and control RM (dose normalized by genome copy number), and the same intra-rectal challenge protocol. The top panel shows FCICA of SIV Pol- and Vif-specific responses among peripheral blood $CD8^+$ T cells from these 3 RM one week after the 8^{th} weekly challenge with inactivated SIV (at which time, all 4 of the vaccinated, protected RM had clear SIV Pol- and Vif-specific responses with the same assay). $CD4^+$ T cells from these RM were similarly unreactive with these peptide mixes, and both $CD4^+$ and $CD8^+$ T cells from these 3 RM were also found to be non-reactive to SIV Gag, Rev/Nef/Tat and Env peptides (but were robustly reactive to both SEB and RCMV lysate, validating the assay). We also asked whether such SIV-specific T cell responses could develop in unvaccinated RM that failed to become infected during the initial intra-rectal exposures to live, low dose SIVmac239 (which would imply that aborted infection is common, and not necessarily related to vaccine generated immune responses). The bottom panel of the figure shows FCICA of SIV Pol and Vif $CD8^+$ T cell responses in 3 RM intra-rectally exposed to live SIVmac239 for 6 exposures without progressive infection, but that subsequently became infected upon additional exposures. As with the RM given AT2-inactivated SIV, both $CD4^+$ and $CD8^+$ T cells from these RM were non-reactive to all SIV peptides at this time point (Pol, Vif, Rev/Nef/Tat, Gag and Env), but responsive to positive controls.

Figure S7



<u>Supplementary Figure S7</u>: CMV-vectored, SIV-specific T_{EM} responses do not significantly alter acute and early plateau phase viral dynamics in RM that manifest progressive infection. The plasma viral load data presented in Fig. 4c is here reconfigured as box plots showing the distribution of peak and plateau phase viral loads (the former occurring at day 14 following the presumed infecting dose; the latter representing the geometric mean of plateau phase viral load values from day 42 to 91 after the presumed infecting dose) among control RM (n= 16; blue) vs. vaccinated RM <u>with</u> progressive infection (n = 8; red; note that these data <u>exclude</u> the 4 RM that resisted progressive infection). Within both of these groups, we identified 5 RM positive for the *Mamu* B*17 MHC allele that has been previously associated with superior SIV control[9], and thus have further subdivided these data by the presence or absence of this allele. Statistical analysis using general linear models confirm a positive effect of the B*17 allele on plateau phase viral loads (p = .0008), but did not show significant differences between the control and vaccinated groups overall or subdivided by the presence or absence of B*17. These data suggest that once progressive systemic infection is initiated, RhCMV-vectored, SIV-specific T_{EM} responses are unable to manifest significant virologic control. Significantly, however, the presence of these responses (which include SIV-specific $CCR5^+$, $CD4^+$ T cells) does not appear to facilitate or accelerate viral dynamics.

SUPPLEMENTAL METHODS

FCICA of PBMC and BAL. For T cell stimulation, the antigens studied included titred whole RhCMV lysates (68-1 strain) or overlapping 15mer peptides representing the SIVmac239 Gag, Env, Rev/Nef/Tat, Pol and Vif proteins or the RhCMV Immediate Early-1 protein, all used in the presence of co-stimulatory monoclonal antibodies (mAbs) CD28 and CD49d (BD Biosciences). Co-stimulation in the absence of antigen served as background control. Cells were incubated with antigen and co-stimulation alone for 1 hr, and then in the presence of the secretion inhibitor brefeldin A (10μg/ml; Sigma-Aldrich) for an additional 8 to 11 hrs. For determination of CD107 externalization, the stimulation procedure was modified by inclusion of the conjugated mAb to CD107 in the stimulatory culture and by using brefeldin A (5μg/ml) in combination with monensin (0.7μg/ml; BD Biosciences) for secretion inhibition. After surface and intracellular staining with conjugated mAbs, polychromatic (6 to 10 parameter) flow cytometric analysis was performed on an LSR II Becton Dickinson instrument. List mode multiparameter data files were analyzed using the FlowJo software program (version 6.3.1; Tree Star, Inc.). The following mAbs were used in designated combinations: a) from BD Biosciences, L200 (CD4; AmCyan), SP34-2 (CD3; Alexa700, PacBlu), SK1 (CD8α; TruRed, APC-Cy7), DX2 (CD95; PE), D21-1351 (MIP-1β; PE), M-A251 (IL-2; PE), H4A3 (CD107a; FITC), H4B4 (CD107b; FITC), 25723.11 (IFN-γ; APC, FITC), 6.7 (TNF; APC, FITC), b) from Beckman Coulter, mAbs CD28.2 (CD28; PE-Texas Red) and L78 (CD69; PE), and c) from R&D Systems, MAb 150503 (anti-CCR7; which was conjugated to biotin using a Pierce Chemical Co. biotinylation kit, and visualized with streptavidin-Pacific Blue from Invitrogen).

SUPPLEMENTAL REFERENCES

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