Established infections with the human and simian immunodeficiency viruses (HIV and SIV, respectively) are thought to be permanent with even the most effective immune responses and antiretroviral therapies only able to control, but not clear, these infections. Whether the residual virus that maintains these infections is vulnerable to clearance is a question of central importance to the future management of millions of HIV-infected individuals. We recently reported that approximately 50% of rhesus macaques (RM; Macaca mulatta) vaccinated with an SIV protein-expressing recombinant alphaherpesvirus (SIV-αHV) vector manifest durable, aviraemic control of infection with the highly pathogenic strain SIVmac239 (ref. 5). Here we show that regardless of the route of challenge, SIV vector-elicted immune responses control SIVmac239 after demonstrable lymphatic and haematogenous viral dissemination, and that replication-competent SIV persists in several sites for weeks to months. Over time, however, protected RM lost signs of SIV infection, showing a consistent lack of measurable plasma- or tissue-associated virus using ultrasensitive assays, and a loss of T-cell reactivity to SIV determinants not in the vaccine. Extensive ultrasensitive quantitative PCR and quantitative PCR with reverse transcription analyses of tissues from SIV-αHV vector-protected RM necropsied 69–172 weeks after challenge did not detect SIV RNA or DNA sequences above background levels, and replication-competent SIV was not detected in these RM by extensive co-culture analysis of tissues or by adoptive transfer of 60 million haematolymphoid cells to naive RM. These data provide compelling evidence for progressive clearance of a pathogenic lentiviral infection, and suggest that some lentiviral reservoirs may be susceptible to the continuous effector memory T-cell-mediated immune surveillance elicited and maintained by alphaherpesvirus vectors.

Clinical and experimental observations have suggested that HIV and SIV infections might be vulnerable to immune control or pharmacological clearance in the first few hours to days of infection, before both the viral amplification needed for efficient mutational escape and the establishment of the highly resilient viral reservoir that sustains the infection. Cytomegalovirus (CMV) vectors were designed to exploit the viral amplification needed for efficient mutational escape and the logical clearance in the first few hours to days of infection, before both SIV infections might be vulnerable to immune control or pharmacological clearance. We sought to define more precisely the spread and dynamics of SIV infection in RM that controlled the infection as a consequence of RhCMV/SIV vector vaccination, and in particular, the extent to which residual SIV was eventually cleared from these animals.

To establish the extent of SIV spread early after the onset of RhCMV/SIV vector-mediated control, we studied a group of five RM vaccinated with RhCMV vectors containing SIV gag, Rev/Tat/Nef, Env and Pol (but not Vif) inserts that were taken to necropsy within 24 days of controlling plasma viraemia after intrarectal inoculation with SIVmac239. All of these RM had measurable SIV RNA in plasma for one or two weekly time points after challenge, followed by at least three consecutive weekly samples with plasma SIV RNA below 30 copy equivalents (equiv.) per ml, and at the time of necropsy, below 5 copy equiv. ml⁻¹, as measured by an ultrasensitive assay (Fig. 1a). Infection was confirmed by the de novo development of cell-mediated responses against SIV Vif (not included in the vaccine) in all RM (Fig. 1b and Supplementary Fig. 1a). As previously described, protection occurred without anamnestic boosting of vaccine-elicted SIV-specific CD8⁺ T-cell responses in blood (Fig. 1b), and at necropsy, robust CD4⁺ and CD8⁺ T-cell responses to SIV proteins included in the RhCMV/SIV vaccine vectors were identified (Supplementary Fig. 1b). We then used ultrasensitive, nested quantitative PCR (qPCR) and quantitative PCR with reverse transcription (qRT–PCR) assays to quantify SIV DNA and RNA, respectively, in the tissues of these protected RM, in comparison with tissues from three unchallenged, RhCMV/SIV vector-vaccinated RM (SIV controls), two unvaccinated RM with productive SIV infection (one progressor and one elite controller) and three RM with SIV infection suppressed with antiretroviral drug treatment (ART) (Fig. 1c, Supplementary Figs 2–4 and Supplementary Table 1). Two of the five RhCMV/SIV vector-protected RM showed levels of SIV DNA and RNA approaching the very low level background signal observed for SIV control RM. However, the other three showed readily measurable SIV RNA, not only in rectal/colonic mucosa (portal of entry), but also in lymph nodes draining the portal of entry (iliac and mesenteric lymph node groups), as well as in sites of presumed haematogenous spread: bone marrow, spleen and liver. The level of SIV RNA in the tissues of these three RM was less than that seen in progressive infection, but comparable to that in the elite SIV controller and in ART-suppressed SIV infection. Notably, however, levels of tissue-associated SIV DNA in the RhCMV/SIV vector-protected RM were all substantially lower than in the RM with elite control and ART suppression, probably reflecting virological control before, rather than after, peak viral replication.
Duration of infection after intravaginal inoculation has been reported to require the infection within the mucosa. By contrast, the development of SIV as well as bone marrow, spleen and liver, before stringent control. portal of entry and establish infection in draining lymph nodes, as strate that in RhCMV/SIV vector-protected RM, SIV can escape the significantly less than found in either progressive or ART-suppressed SIV festing only minimal interferon-stimulated gene expression, signifi-

cantly less than found in either progressive or ART-suppressed SIV infection, we were able to recover replication-competent SIV from iliosacral lymph nodes and spleen in all five of the RhCMV/SIV vector-protected RM at necropsy using ultrasensitive quantitative PCR and RT–PCR. BM, bone marrow; LN, lymph nodes; PID, post-infection day; SIM, small intestine mucosa.

in the RhCMV/SIV vector-protected RM, and the limited time for SIV DNA+ cells to accumulate in these RM before necropsy. Although these data suggest a much smaller SIV reservoir in the RhCMV/SIV vector-protected RM than in the SIV+ controls, including the RM with ART-suppressed SIV infection, we were able to recover replication-competent SIV from iliosacral lymph nodes and spleen in all five of the RhCMV/SIV-protected RM taken to early necropsy (and from bone marrow and mesenteric lymph nodes in three of these five RM), including the two RM with near background levels of SIV RNA by nested qRT–PCR (Table 1). This replication-competent SIV was found in tissues manifesting only minimal interferon-stimulated gene expression, significantly less than found in either progressive or ART-suppressed SIV infection (Supplementary Fig. 5). Taken together, these data demonstrate that in RhCMV/SIV vector-protected RM, SIV can escape the portal of entry and establish infection in draining lymph nodes, as well as bone marrow, spleen and liver, before stringent control.

After intrarectal inoculation, SIV infection has been reported to spread to draining lymph nodes within 4 h (ref. 11), a rate of dissemination that may preclude SIV-specific effector memory T cells from containing the infection within the mucosa. By contrast, the development of SIV infection after intravaginal inoculation has been reported to require proteins that were (Gag plus Pol) or were not (Vif) included in the RhCMV/SIV vectors, shown before and after the onset of the controlled SIV infection. The response frequencies (mean ± s.e.m.) were normalized to the response frequencies immediately before SIV infection for the vaccine-elicited SIV Gag- and Pol-specific responses, and to the peak frequencies after SIV infection for the de novo SIV Vif-specific responses. c, Analysis of tissue-associated SIV DNA and RNA (copy equiv. per 10^8 cell equiv.) in the five RhCMV/SIV vector-protected RM at necropsy using ultrasensitive quantitative PCR and RT–PCR. BM, bone marrow; LN, lymph nodes; PID, post-infection day; SIM, small intestine mucosa.

*24 weeks after ART initiation.

Table 1 | Replication-competent SIV by inductive co-culture at necropsy

| Animal Virus | Plasma SIV (copy equiv. mL^-1) | Duration of infection at necropsy (weeks) |
|--------------|-------------------------------|------------------------------------------|
| Rh23657      | SIVmac239 SIVmac239 SIVmac251 | 74                                       |
| Rh21582      | SIVmac239 SIVmac239 SIVmac251 | 28                                       |
| Rh25708      | 110,000 510 11                | 63*                                      |

| Early term | Medium and long term |
|------------|---------------------|
| SIV+ cultures/total cultures |
| Distal LN | 80/80 32/80 4/49 |
| Iliosacral LN | 40/40 9/40 0/0 |
| Mesenteric LN | 80/80 2/80 3/80 |
| Spleen | 40/40 1/40 5/40 |
| Liver | ND 0/20 2/19 |
| Bone marrow | ND 0/20 0/20 |
| Total positive | 240/240 44/280 14/248 |

30 copy equiv. ml

| RhCMV/SIV vector-protected |
|---------------------------|
| Rh22613 Rh24506 Rh24557 Rh22230 Rh22930 |
| SIVmac239 SIVmac239 SIVmac251 SIVmac239 SIVmac239 |
| <2 <3 <3 <2 <5 |
| Rh24514 Rh26467 Rh24552 Rh24272 Rh24399 Rh24250 |
| SIVmac239 SIVmac251 SIVmac239 SIVmac239 SIVmac239 SIVmac239 |
| <1 <1 <1 <1 <1 <1 |

Table 1 | Replication-competent SIV by inductive co-culture at necropsy

| Progressor Elite controller ART-suppressed |
|----------|---------------------|
| Rh23657 | SIVmac239 SIVmac239 SIVmac251 |
| Rh21582 | SIVmac239 SIVmac239 SIVmac251 |
| Rh25708 | 110,000 510 11 | 63* |

| Early term | Medium and long term |
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| SIV+ cultures/total cultures |
| Distal LN | 80/80 32/80 4/49 |
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| Mesenteric LN | 80/80 2/80 3/80 |
| Spleen | 40/40 1/40 5/40 |
| Liver | ND 0/20 2/19 |
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| SIVmac239 SIVmac239 SIVmac251 SIVmac239 SIVmac239 |
| <2 <3 <3 <2 <5 |
| Rh24514 Rh26467 Rh24552 Rh24272 Rh24399 Rh24250 |
| SIVmac239 SIVmac251 SIVmac239 SIVmac239 SIVmac239 SIVmac239 |
| <1 <1 <1 <1 <1 <1 |
Five of these nine protected female RM manifested a second episode of transient plasma viremia within the first 12 weeks after initial control, but overall, the fraction of protected female RM (followed for at least 30 weeks) with such plasma viral blips (56% versus 100%; \( P = 0.02 \) by Fisher’s exact test) and the number of blips per RM (0.7 versus 6.0; \( P < 0.0001 \)) by two-sided Wilcoxon rank sum test were less than that observed in RhCMV/SIV vector-vaccinated male RM protected against intrarectal challenge \(^5\). Other characteristics of protection in these intravaginally challenged, RhCMV/SIV vector-vaccinated female RM were identical to those previously reported for RhCMV/SIV vector-mediated protection of male RM against intrarectal challenge \(^5\), including development of de novo SIV Vif-specific CD4\(^{+}\) and CD8\(^{+}\) T-cell responses, lack of an anamnestic boost of the vaccine-elicited SIV-specific CD4\(^{+}\) or CD8\(^{+}\) T cells, lack of SIV Env seroconversion, and lack of CD4\(^{+}\) T-cell depletion at mucosal effector sites (Fig. 2b and Supplementary Figs 9, 11 and 12). To determine whether SIV infection spread from the cervical/vaginal mucosa in the nine RhCMV/SIV vector-protected female RM, we biopsied bone marrow, peripheral lymph nodes (axillary/inguinal) and small intestinal mucosa for nested qRT–PCR and qPCR analysis of SIV RNA and DNA, respectively, at 5, 9, 17 and \( > 30 \) weeks after infection. Notably, in the first 9 weeks of infection, five of these nine RM manifested levels of SIV RNA in bone marrow comparable to levels seen in uncontrolled SIV infection, but, whereas in uncontrolled infection SIV RNA levels were similarly high in peripheral blood mononuclear cells (PBMCs), lymph nodes and intestinal mucosa, SIV RNA was either not detected or detected only at very low levels in these sites in the RhCMV/SIV vector-protected RM (Fig. 2c).

Moreover, in contrast to uncontrolled infection, SIV DNA was inconsistently detected in the samples from the RhCMV/SIV vector-protected RM, and by \( 40 \) weeks after infection, all nine of the RhCMV/SIV vector-protected RM had at least one sample set in which both SIV RNA and DNA were below the level of detection in all sites. In eight of these RM (excluding Rh20363, see below), all samples obtained subsequent to \( 30 \) weeks after infection showed SIV RNA and DNA levels below the level of detection, with the exception of one PBMC sample with low-level SIV RNA (454 copy equiv. per \( 10^8 \) cells). The differences in the frequency of SIV detection in samples obtained at 5, 7 and 17 weeks versus \( > 30 \) weeks after infection from these eight RM were highly significant (\( P = 0.002 \) for all samples, \( P = 0.0006 \) for bone marrow by two-sided Wilcoxon rank sum tests).

The ability to detect tissue-associated SIV early, but not later, after infection in these eight stably protected female RM, particularly in bone marrow, is consistent with initial spread and subsequent control and progressive clearance of SIV. In accordance with this, the frequencies of circulating SIV Vif-specific T cells, which are elicited and maintained by antigen derived from SIV infection (rather than the vaccine), progressively declined in these RM until these responses were no longer detectable (Fig. 2b and Supplementary Fig. 11). However, despite having no detectable SIV RNA or DNA in PBMCs and tissue samples at week 17, and declining SIV Vif-specific T-cell responses, one animal (Rh20363) showed the emergence of low-level productive SIV infection at week 31 after infection (Fig. 2a). The boosting of SIV-specific CD4\(^{+}\) and CD8\(^{+}\) T-cell responses (Fig. 2b and Supplementary Fig. 11), including de novo CD8\(^{+}\) T-cell responses to canonical Mamm-a-A*01-restricted SIV epitopes (Supplementary Fig. 13), the appearance of cell-associated RNA and
DNA in subsequent PBMCs, lymph node and intestinal samples (Fig. 2c), and the induction of increased plasma and PBMC-associated SIV loads with experimental in vivo CD8⁺ lymphocyte depletion (Supplementary Fig. 14) indicates that this RM spontaneously converted from a unique state of stringent viral containment with little or no continuing viral replication to a different state characterized by continuing, but low-level SIV replication (consistent with conventional ‘elite’ immunological control). In keeping with this, sequence analysis of the breakthrough virus 3 weeks after initial viral rebound showed little evolution from the initial SIVmac239 sequence except, notably, a putative escape mutation in the Tat-SL8 epitope sequence, consistent with early escape from the Tat-SL8-specific T-cell responses that developed after viral rebound at week 31 (Supplementary Figs 13 and 15). Given the enormous breadth of RhCMV/SIV vector-elicited CD8⁺ T-cell responses, this limited sequence evolution suggests that the loss of aviraemic control in Rh20363 was more likely due to inadequate immune surveillance of residual infection than mutational escape. Experimental CD8⁺ lymphocyte depletion was also performed on three RhCMV/SIV vector-protected female RM that retained aviraemic control, and in keeping with previous analysis of CD8⁺ lymphocyte depletion of RhCMV/SIV vector-vaccinated male RM protected after intrarectal challenge⁵,⁹, this treatment did not induce detectable plasma viraemia (Supplementary Fig. 14). However, one of these RM (Rh21176) transiently manifested unequivocal detection of SIV RNA (10 out of 10 replicates positive) and replication-competent SIV (7 out of 20 co-cultures positive) in lymph nodes at day 10 after CD8⁺ lymphocyte depletion, demonstrating the presence of at least local, very low level residual SIV infection in this RM after 52 weeks of stringent control. In contrast to Rh20363, Rh21176 maintained aviraemic control, indicating that this RM’s immune system either controlled or eliminated residual foci of SIV replication.

The finding that RhCMV/SIV vector-protected RM are able to control haematogenous SIV dissemination after both intrarectal and intravaginal challenge suggested that the immune responses elicited by these vectors might provide protection even when mucosal surfaces are bypassed. To assess this possibility, we challenged six RhCMV/SIV-vaccinated RM with low dose, intravenous SIVmac239, and found that two of these six RM manifested the same pattern of control observed after mucosal challenge—a transient, low-level viraemia associated with the development of an SIV Vif-specific T-cell response, and detection of SIV RNA in bone marrow (high level) and/or PBMCs (low level) early, but not late, after infection (Supplementary Fig. 16). Taken together, these data indicate that (1) RhCMV/SIV vector-elicited immune responses can mediate protection regardless of the route of SIV challenge, (2) viral control is both local and systemic, and (3) replication-competent SIV can persist in several sites for weeks to months in protected RM (even when aviraemic), but seems to decline over time.

To determine the ultimate fate of residual SIV in RhCMV/SIV vector-protected RM, we followed a total of ten protected RM for 69–180 weeks after infection (Fig. 3a, b). In all of these RM, plasma viral blips became increasingly infrequent over time, with no blips observed after 70 weeks. The frequency of the SIV infection-dependent, SIV Vif-specific CD8⁺ T cells in blood also progressively declined in all RM until these responses were no longer detectable (Supplementary Fig. 17). In contrast to the SIV Vif-specific CD8⁺ T-cell responses, the SIV-specific CD8⁺ T-cell responses elicited by the RhCMV/SIV vectors remained stable, including high frequencies of CD8⁺ T cells capable of recognizing autologous SIV-infected CD4⁺ T cells (Supplementary Fig. 18). Analysis of six of these medium- to long-term protected RM at necropsy, including one RM that was CD8⁺ lymphocyte-depleted 10 days before necropsy (Supplementary Fig. 19), confirmed the systemic loss of SIV Vif-specific T cells, and the maintenance of RhCMV vector-elicited, SIV-specific T cells (Supplementary Fig. 20). Most importantly, ultrasensitive, nested qRT–PCR and qPCR analysis of ≥54 tissues per animal (ten replicates per tissue, including extensive sampling of all tissues shown to contain SIV in the short-term RhCMV/SIV vector-protected RM) revealed extremely low to absent levels of SIV RNA and DNA that were indistinguishable from measurements in unchallenged RhCMV/SIV-vaccinated (SIV⁻) controls (Fig. 3c, d, Supplementary Figs 2 and 21 and Supplementary Tables 1 and 3). Moreover, despite extensive sampling (>240 cultures per animal), no replication-competent SIV was isolated by co-culture analysis from the lymphoid tissues of these RM (Table 1). Finally, we asked whether the adoptive transfer of a total of 6 × 10⁷

Figure 3 | Virological analysis of medium- to long-term RhCMV/SIV vector-mediated protection. a, b, Plasma viral load (measured as log(copy equiv. per ml)) profiles of ten RhCMV/SIV vector-vaccinated RM that controlled SIV infection after intrarectal challenge (eight long term (a) and two medium term (b)). The limit of detection for all pre-terminal plasma viral load assays is 30 copy equiv. ml⁻¹, the limit of detection for the ultrasensitive assay used on the terminal sample of the study was ≤1 copy equiv. ml⁻¹. Note that one of the RM with medium-term protection (Rh26467) was CD8⁺ lymphocyte-depleted 10 days before the terminal sample. c, d, Quantification of tissue-associated SIV DNA and RNA in four long-term and two medium-term protected RhCMV/SIV-vaccinated RM studied at necropsy, including the CD8⁺ cell-depleted RM (Rh26467). e, Assessment of residual replication-competent, cell-associated SIV in medium- and long-term protected RM by adoptive transfer of 6 × 10⁷ haematolymphoid cells (3 × 10⁷ blood leukocytes and 3 × 10⁷ lymph node cells or, in one transfer from Rh26467, represented by the open symbol, 3 × 10⁷ bone marrow leukocytes and 3 × 10⁷ splenic cells) to SIV-naive RM with SIV infection in the recipient RM delineated by plasma viral load. Cell transfers from RM with conventional elite SIV control and ART-suppressed SIV infection resulted in rapid onset of SIV infection in the recipient RM, but no SIV infection was observed in RM receiving cells from medium- to long-term RhCMV/SIV vector-protected RM (including Rh26467, analysed both before and after CD8⁺ cell depletion).
haematolymphoid cells (3 × 10⁷ each of peripheral blood leukocytes and lymph node cells, or 3 × 10⁵ each of bone marrow leukocytes and spleen cells) from three SIV⁺ control RM (two with ART-suppressed infection and one elite controller), and five medium- or long-term RhCMV/SIV vector-protected RM (including one RM tested before and after CD8⁺ cell depletion) would initiate infection in SIV-naïve RM. Remarkably, although cells from the SIV⁺ controls, including ART-suppressed RM, rapidly initiated SIV infection in the SIV-naïve recipients (manifested by the onset of SIV replication and induction of SIV Vif-specific T-cell responses), no evidence of SIV infection was observed in the SIV-naïve recipients receiving cells from the medium- and long-term RhCMV/SIV vector-protected RM (Fig. 3e and Supplementary Fig. 22). Taken together, these data provide strong evidence that after being unequivocally infected with SIV, these RhCMV/SIV vector-vaccinated RM cleared detectable infection, such that by all measured criteria (lack of plasma viral blips, absence of Vif-specific T-cell responses, extensive ultrasensitive qRT-PCR and qPCR and coculture analysis, and adoptive transfer) these RM were indistinguishable from RhCMV/SIV vector-vaccinated controls that had never been exposed to SIV. Although we cannot rule out residual virus below our level of detectability, or in tissues not examined, these data strongly support progressive immune-mediated clearance of an established lentivirus infection, leading to a situation meeting criteria for a functional cure12 and consistent with possible viral eradication.

In the past 5 years, the HIV/AIDS vaccine field has concluded that a prophylactic HIV/AIDS vaccine must prevent or eliminate HIV infection, as it is thought that any residual infection runs a high risk of eventual progression13. Our demonstration here that the virus-specific, effector memory T cells maintained by a persistent vector can shut down productive SIV infection, and by maintaining immune surveillance over time, functionally cure and possibly eradicate this infection, indicates that an effector memory T-cell-targeted HIV/AIDS vaccine could (by itself, or combined with antibody-targeted approaches) provide meaningful long-term efficacy. Our results also suggest that an effector memory T-cell-targeted vaccine might contribute to HIV cure strategies. Although the SIV reservoirs that initially develop in RhCMV/SIV vector-vaccinated controllers are smaller in size, and possibly different in character from HIV/SIV reservoirs in the setting of ART administration initiated in chronic infection, it is conceivable that the indefinitely persistent, unconventionally targeted viral-specific T cells elicited and maintained by CMV vectors—alone or in combination with agents designed to activate HIV gene expression12–14—might exert potent immune pressure on cells with any HIV protein expression (including expression of viral antigen by stochastically activated, latently infected cells) and thereby facilitate depletion of residual HIV reservoirs in patients on suppressive ART. It is also possible that these responses might stringently control recrudescence 'rebound' infection after ART withdrawal in a manner analogous to their control of primary SIV infection in this study. In summary, the ability of CMV vectors to implement continuous, long-term, and potent antipathogen immune surveillance makes them promising candidates for vaccine strategies intended to prevent and cure HIV/AIDS, as well as other chronic infections.

METHODS SUMMARY

RhCMV/SIV vectors expressing SIV Gag, Rev/Tat/Nef, Pol and Env or irrelevant control inserts were used as described13. RM were challenged with SIVmac239 by intrarectal, intravaginal or intravenous inoculation using a repeated (weekly), limiting dose protocol13. RM were considered infected after detection of plasma SIV RNA ≥ 30 copy equiv. ml⁻¹ and the de novo development of T-cell responses to SIV Vif, an SIV antigen not included in the RhCMV/SIV vectors. Selected RM were depleted of CD8⁺ lymphocytes, as described14. SIV-specific T-cell responses were measured in mononuclear cells from blood and tissues by flow cytometric intracellular cytokine analysis16. Levels of SIV RNA and DNA in tissue/cell preparations were quantified, respectively, by an ultrasensitive, nested qRT–PCR and qPCR approach1. Replication-competent SIV was detected in mononuclear cells by inductive co-cultivation assays, as described15. To ascertain the presence of occult replication-competent SIV below the level of detection of the co-cultivation assay, 60 million cells from blood, lymph nodes, bone marrow or spleen from RhCMV vector-protected RM or RM with spontaneously controlled or ART-suppressed SIV infection were adoptively transferred by intravenous infusion to SIV-naïve RM, with infection in the recipient RM determined as described above.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions S.G.H. planned and performed animal experiments, and analysed immunological and virological data, assisted by A.B.V., C.M.H., R.M.G., J.C.F., M.S.L., A.N.G. and N.W. and J.D.L. planned and performed SIV quantification, assisted by K.O., R.S. and Y.L. B.J.B. and J.B.S. performed infected cell recognition assays. B.F.K. performed sequencing analysis. J.D.E. performed immunohistological studies. S.L.F., J.M.T., A.W.L. and M.K.A. managed the animal protocols. J.A.N. and K.F. supervised CMV vector design and development. J.D.L. planned and supervised SIV quantification and any associated references are available in the online version of the paper.

New SIVmac239 sequences reported in this manuscript are accessible in GenBank under accessions KF430957, KF430958 and KF430959. Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details accompany the full-text HTML version of the paper at www.nature.com/nature. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to J.D.L. (lifsonj@mail.nih.gov) or L.J.P. (pickerl@ohsu.edu).
METHODS
Rhesus macaques. Ninety-nine purpose-bred male and female RM (M. mulatta) of Indian genetic background were used with the approval of the Oregon National Primate Research Center Animal Care and Use Committee, under the standards of the US National Institutes of Health Guide for the Care and Use of Laboratory Animals. These animals were specific pathogen-free as defined by being free of coccopithecine herpesvirus 1, D-type simian retrovirus, simian T-lymphotrophic virus type 1, rhesus rhadinovirus, and Mycobacterium tuberculosis. MHC-1 genotyping for common Mmm alleles such as Mmm-A’01, Mmm-A’02 and Mmm-B’08-B’17 was performed by sequence-specific priming PCR, as described29. The 99 RM included 15 RhCMV/SIV vector-vaccinated RM (7–10 years of age) with aviremic control of SIV infection (14 with SIVmac239, 1 with SIVmac251) after intrarectal challenge (5 with short-term follow up; 10 with medium- to long-term follow up); 52 RM (4–19 years of age) vaccinated with RhCMV/SIV or control RhCMV vectors or left unvaccinated before SIVmac239 challenge (42 intravaginal, 10 intravenous); 12 SIV+ RM (5–12 years of age; 8 with progressive SIVmac239 infection, 1 with spontaneously controlled SIVmac239 infection, 3 with ART-suppressed SIVmac251 infection); and 20 SIV-naive RM (4–14 years of age) used as negative controls (these include 4 RM vaccinated with RhCMV/SIV vectors) or as SIV- and RhCMV-vector-naive recipients in the adoptive transfer experiments. Early (<1 year) follow-up of eight of the RhCMV/SIV vector-vaccinated RM with long-term, aviremic control of SIV infection was previously reported25, with this study extending that follow-up from 1 to >3 years. RhCMV/Gag, Rev/Tat/Nef, Env and Pol-1 and Pol-2 vectors were administered subcutaneously at a dose of $5 \times 10^7$ plaque forming units per vector. The control antigen-expressing RhCMV vector was used at a total dose of $2.5 \times 10^7$ plaque forming units to match the total dose of the RhCMV/SIV vectors. RM were vaccinated twice with RhCMV vectors, 14 weeks apart. All SIV challenges (intrarectal, intravaginal and intravenous) used a repeated limiting dose protocol with dosing designed to require >1 challenge for infection of $>60\%$ of challenged RM, and to infect all or nearly all challenged RM with $>10$ (weekly) challenges for intrarectal or intravaginal inoculation (300 focus-forming units) and $>3$ (every third week) challenges for intravenous inoculation (0.2 focus-forming units). RM were considered SIV-infected (and challenge discontinued) with the onset of plasma viral load $>30$ copy equiv. ml$^{-1}$ and the de novo development of CD$^+$ and CD$^-$ T-cell responses to SIV Vif, an SIV antigen not included in the RhCMV/SIV vectors. RM were considered controllers if plasma viral load became undetectable ($<30$ copy equiv. ml$^{-1}$) within 2 weeks of the initial positive plasma viral load and was then maintained below threshold for three consecutive determinations. RM with progressive SIV infection were followed for 20 weeks after infection, or if progression was rapid, until the onset of AIDS. ART’s consisted of two reverse transcriptase inhibitors (20 mg day$^{-1}$ tenofovir, 50 mg day$^{-1}$ emtricitabine), an integrase inhibitor (240 mg day$^{-1}$ raltegravir) and a protease inhibitor (600 mg twice daily darunavir boosted with 100 mg twice daily ritonavir). Selected RM were depleted of CD$^+$ lymphocytes by administration of 10, 5, 5 and 5 mg per kg body weight of the CD8a monoclonal antibody M-T807R1, a modified version of the cM-T807 humanized monoclonal antibody M-T807 (Life Technologies) and 2.5 ml of this ‘nested’ reaction was transferred to 20 ml of real-time PCR reaction mix with 1 U Platinum Taq polymerase (Life Technologies) and 2.5 ml of this reaction was transfected to 20 ml of real-time PCR reaction mix with 1 U Platinum Taq polymerase. For DNA determinations, the preamplification reactions were 20 ml in volume, comprising 10 ml sample and 10 ml reaction cocktail; 2 ml of this ‘nested’ reaction was transfected to 20 ml of real-time PCR reaction mix. As previously described, for both RNA and DNA determinations, 12 replicate reactions were tested per sample including a spike of RNA or DNA internal control sequence standard in two of the twelve reactions to assess overall amplification efficiency and assess potential inhibition of the PCR or RT-PCR reactions. The amount of DNA or RNA standard added to replicate reactions to monitor inhibition and PCR performance was typically 10–100 copies, depending on the anticipated level of SIV sequences present. Samples showing greater than a five cycle shift in amplification of the spiked standard, compared to amplification in the absence of specimen nucleic acid, corresponding to less than 74% overall amplification efficiency, were diluted and re-assyayed. Quantitative determinations for samples showing amplification in all replicates were derived directly with reference to a standard curve. Quantitative determinations for samples showing fewer than 10 positive amplifications in replicates were derived from the frequency of positive amplifications, corresponding to the presence of at least one target copy in a reaction, according to a Poisson distribution of a given median copy number per reaction. To avoid false positives in biopsy material, in which the specimen size and total number of specimens is limited, we required a minimum of two positive reactions out of ten for a sample to be considered positive. The presence of inducible, replication-competent SIV in mononuclear cell preparations derived from different tissue sites at necropsy was detected by co-cultivation of $2 \times 10^3$ unfractonated cells from each tissue with $2 \times 10^5$ CEMx174 cells (20 replicates per tissue, cell numbers permitting). CEMx174 cells obtained from NIH AIDS Repository were used as target cells. After 18 days of culture, cells were stained for CD3, CD4 and intracellular SIV gag-p27 (monoclonal antibody 55-2F12), with positive cultures based on $\geq 0.5\%$ CEMx174 cells with intracellular SIV Gag expression over background by flow cytometry.

Immunological assays. SIV-specific CD$^+$ and CD$^-$ T-cell responses were measured in blood and tissues by flow cytometric intracellular cytokine analysis, as previously described in detail37. To determine T-cell responses to SIV peptide mixtures or individual peptides, mononuclear cells were incubated with mixes of overlapping 15-amino-acid peptides comprising SIV proteins or individual epitopic 8- to 10-amino-acid peptides (with every individual peptide always at 2 $\mu$g ml$^{-1}$) and stained with antibodies specific for fluorochrome-conjugated monoclonal antibodies including: SP34-2 (CD3; Pacific Blue, PerCP-Cy5.5), L200 (CD4; AmCyte), SK-1 (CD8a; APC, PerCP-Cy5.5), CD28.2 (CD28; PE; PE-TexasRed), DX2 (CD95; APC; PE), 15053 (CCR7;
Antigen-presenting cells in both CD4+ and TNF-α cytokines. After subtracting background, the raw response frequencies were determined by their intracellular expression of CD69 and either or both of IFN-γ and TNF were directly phenotyped with respect to the memory markers CD28 and CCR7 (refs 5, 9).

Tissues of SIV Env-specific antibodies were determined by neutralization of tissue culture-adapted SIVmac251 using a luciferase reporter gene assay23.

Immunohistochemistry. Immunohistochemistry was performed using a biotin-free polymer approach (Golden Bridge International) on 5-μm tissue sections mounted on glass slides, which were dewaxed and rehydrated with double-distilled H2O. Heat-induced epitope retrieval was performed by heating sections in 0.01% citraconic anhydride containing 0.05% Tween-20 in a pressure cooker set at 122–125°C for 30 s. Slides were incubated with blocking buffer (TBS with 0.05% Tween-20 and 0.5% casein) for 10 min. For APOBEC3G, slides were incubated with rabbit anti-APOBEC3G (1:100; Sigma HPA004627) diluted in blocking buffer overnight at 4°C. Slides were washed in 1× TBS with 0.05% Tween-20, endogenous peroxidases blocked using 1.5% (v/v) H2O2 in TBS, pH 7.4, for 10 min, incubated with rabbit polyclin-2 horseradish peroxidase (HRP) and developed with impact DAB (3,3′-diaminobenzidine; Vector Laboratories). For ISG15 staining, after the heat-induced epitope retrieval step, the slides were loaded on an Intell iPATH autostainer (Biocare Medical) and stained with optimal conditions determined empirically that consisted of a blocking step using blocking buffer (TBS with 0.05% Tween-20 and 0.5% casein) for 10 min and an endogenous peroxidase block using 1.5% (v/v) H2O2 in TBS, pH 7.4, for 10 min. Rabbit anti-ISG (1:250; Sigma HPA004627) was diluted in blocking buffer and incubated for 1 h at room temperature. Tissue sections were washed and developed using the Rabbit Polink-1 HRP staining system (Golden Bridge International) according to manufacturer’s recommendations. Sections were developed with impact DAB (Vector Laboratories). All slides were washed in H2O, counterstained with haematoxylin, mounted in Permount (Fisher Scientific), and scanned at high magnification (×200) using the ScanScope CS System (Aperio Technologies), yielding high-resolution data from the entire tissue section. Representative regions of interest (500 mm2) were identified and high-resolution images extracted from these whole-tissue scans. The percentage area of the T-cell zone that stained for APOBEC3G and ISG15 was quantified using Photoshop CS5 and Fovea tools.

Statistical analysis. The RM used in the vaccine efficacy analysis were randomly assigned to vaccine groups with randomization stratified to balance groups for expression of protective MHC alleles. All reported experiments were conducted once and are reported fully. The criteria for categorizing post-challenge RM into ‘protected’ and ‘unprotected’ groups was established previously22. Experimenters were not explicitly blinded to the treatment assignments of the RM, nor were the analyses conducted by blinded investigators. All statistical analyses were conducted using non-parametric and model-independent analysis procedures either for the main analysis or as a sensitivity analysis, and in every sensitivity analysis the result was qualitatively consistent with the main analysis. No tests depended on an assumption of equal variance across compared groups. The only exceptions were the time series analyses, which were conducted with two model-based (regression) approaches; the residuals of these analyses were evaluated and found to be consistent with homoscedasticity and normality requirements, and the results were consistent across approaches. For comparisons of continuous–valued data from independent samples, we applied bivariate Mann–Whitney U tests24, also known as Wilcoxon rank sum tests. For comparisons of dichotomous values across groups, we applied Fisher’s exact tests25. We estimated confidence bounds for binomial proportions using the Wilson score method, as described in Agresti and Coull26. We compared group means of positivity frequencies for which we have repeated binary measures on individual RM using mixed effects logistic regression (with individual RM mean deviations from group means modelled as a normally-distributed random effect). We compared confidence intervals for RM groups to confidence intervals for individual RM (from other groups) by directly determining overlap of the intervals (and we used the estimated random effect variance and estimated group means to conduct z-tests in sensitivity analyses, which yielded consistent results). We compared Kaplan–Meier curves using the log-rank test27. We conducted time series analyses using standard linear regression with time as the primary predictor, and we used Gaussian first-order autoregressive process models in sensitivity analyses, which yielded consistent results. All tests were conducted as two-tailed tests with a type-I error rate of 5%. We used the R statistical computing language28 for all statistical analyses.

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CORRIGENDUM

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Corrigendum: Immune clearance of highly pathogenic SIV infection

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The Acknowledgements section of this Letter should have included the following sentence: “We acknowledge the contribution of M. A. Jarvis to the design, construction and initial in vitro characterization of all the strain 68.1-derived RhCMV vectors used in this study, including both previously published RhCMV/SIV vectors and a previously unpublished vector expressing an Mycobacterium tuberculosis Ag85B–ESAT6 fusion protein, used as a negative control in this study.” S.L.P. and J.M.T. did not reply when asked to approve the wording of this Corrigendum. The HTML version of the paper has been corrected.
Addendum: Immune clearance of highly pathogenic SIV infection

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Here we provide additional information about the specificity and validation of the simian immunodeficiency virus (SIV) DNA and RNA assays reported in this Letter. As described, we used quantitative PCR and quantitative PCR with reverse transcription (qPCR and qRT–PCR, respectively) assays targeting sequences in the SIV gag gene for quantification of SIV RNA and DNA. For measurements of plasma viremia (SIV RNA), we used a standard qRT–PCR assay that has been extensively used in the field. For measurements of cell- and tissue-associated SIV DNA and RNA, we used nested qPCR and qRT–PCR assays. In our Letter, we report the evaluation of rhesus cytomegalovirus (RhCMV)-based vaccine vectors that include one vector with an SIV gag sequence insert. As shown in Fig. 1, sequence identity between the SIV gag insert in the RhCMV/Gag vector and the SIV gag-targeted primer and probe sequences for the standard qRT–PCR assay used for plasma SIV RNA measurements (sGAG21, sGAG22 and sGAG23 probes) raises the possibility that transcripts from the vaccine insert could have been detected by the assay we used.

For the nested assay, designed mismatched of the outer reverse primer (SIVnestR01) and the codon-optimized portion of the vaccine gag insert sequence and specific priming of the reverse transcription step of the qRT–PCR assay is intended to prevent potential cross reactivity. Biological features of the RhCMV vectors used are expected to minimize the prospects of detection of vaccine insert-derived SIV gag RNA in plasma or insert-derived SIV gag DNA or RNA sequences in cell or tissue samples.

To test empirically for possible detection of vaccine insert-derived SIV gag RNA sequences in plasma, we evaluated 394 plasma samples from RhCMV/Gag-vaccinated, SIV-unexposed rhesus macaques using our standard (non-nested) SIV RNA qRT–PCR assay. All 394 samples were negative. These results support the interpretation that under the experimental conditions reported, the standard assay does not detect vaccine insert-derived sequences in the plasma of vaccinated animals, and that SIV RNA detected after SIV challenge in such animals is derived from SIV infection.

To evaluate empirically the potential detection of vaccine insert sequences in tissues by the nested qPCR and qRT–PCR assays that we used, we surveyed a total of 291 tissue samples obtained at necropsy from five rhesus macaques that were vaccinated with a regimen that included RhCMV/Gag vectors, but were not challenged with SIV, including three animals described in our Letter, and two additional animals not previously reported. Out of 2,550 total nested PCR or RT–PCR reactions, we detected 4 (0.16%; 95% confidence interval (CI) 0.06–0.40%) and 42 (1.65%; 95% CI 1.22–2.22%) reactions positive for SIV DNA and RNA, respectively. We compared these frequencies to results obtained from the five rhesus macaques described in our Letter that were vaccinated with RhCMV/Gag vectors and challenged with SIV, that then showed evidence of SIV infection, and were necropsied shortly after demonstrating control of plasma viremia (<5 SIV gag RNA copies per millilitre at necropsy). The frequencies of

Figure 1 | Sequence of SIV gag PCR/RT–PCR assay primers/probe and vaccine insert. The nucleic acid sequence for the first 640 base pairs of the native SIVmac239 gag gene (‘SMI239’) is shown aligned with the sequence of the SIV gag insert used in the RhCMV/Gag vector (‘GAG.FLAG’). The gag-targeted primers and probe are indicated in colour on the strands that were used in qPCR- and qRT–PCR-based viral nucleic acid quantification assays. Dashes indicate sequence identity and only base differences from the SIVmac239 gag gene are shown in the sense strand of the RhCMV/Gag vector. The primers and probe are listed as follows. The ‘dP’ and ‘dK’ bases were incorporated to minimize non-biased coverage of non-SIVmac239 isolates with divergent sequences at the relevant positions in other studies using different viruses. Forward primer, sGAG21: 5′-GTCTCGGCTCAT(dP)TGTTTATTG-3′; reverse primer, sGAG22: 5′-GAGATCAG(dK)TGTTCTGCGAT(dP)TGTTTATTG-3′; probe, psGAG23: 5′-FAM-CTTC(dP)TACGAT(dK)TGTTTATTG-3′. Probe, psGAG23: 5′-FAM-CTTC(dP)TACGAT(dK)TGTTTATTG-3′. Probe, psGAG23: 5′-FAM-CTTC(dP)TACGAT(dK)TGTTTATTG-3′. Probe, psGAG23: 5′-FAM-CTTC(dP)TACGAT(dK)TGTTTATTG-3′. Probe, psGAG23: 5′-FAM-CTTC(dP)TACGAT(dK)TGTTTATTG-3′. Probe, psGAG23: 5′-FAM-CTTC(dP)TACGAT(dK)TGTTTATTG-3′. Probe, psGAG23: 5′-FAM-CTTC(dP)TACGAT(dK)TGTTTATTG-3′. Probe, psGAG23: 5′-FAM-CTTC(dP)TACGAT(dK)TGTTTATTG-3′. Probe, psGAG23: 5′-FAM-CTTC(dP)TACGAT(dK)TGTTTATTG-3′. Probe, psGAG23: 5′-FAM-CTTC(dP)TACGAT(dK)TGTTTATTG-3′. Probe, psGAG23: 5′-FAM-CTTC(dP)TACGAT(dK)TGTTTATTG-3′. Probe, psGAG23: 5′-FAM-CTTC(dP)TACGAT(dK)TGTTTATTG-3′. Probe, psGAG23: 5′-FAM-CTTC(dP)TACGAT(dK)TGTTTATTG-3′. Probe, psGAG23: 5′-FAM-CTTC(dP)TACGAT(dK)TGTTTATTG-3′. Probe, psGAG23: 5′-FAM-CTTC(dP)TACGAT(dK)TGTTTATTG-3′. Probe, psGAG23: 5′-FAM-CTTC(dP)TACGAT(dK)TGTTTATTG-3′. Probe, psGAG23: 5′-FAM-CTTC(dP)TACGAT(dK)TGTTTATTG-3′. Probe, psGAG23: 5′-FAM-CTTC(dP)TACGAT(dK)TGTTTATTG-3′. Probe, psGAG23: 5′-FAM-CTTC(dP)TACGAT(dK)TGTTTATTG-3′. Probe, psGAG23: 5′-FAM-CTTC(dP)TACGAT(dK)TGTTTATTG-3′. Probe, psGAG23: 5′-FAM-CTTC(dP)TACGAT(dK)TGTTTATTG-3′. Probe, psGAG23: 5′-FAM-CTTC(dP)TACGAT(dK)TGTTTATTG-3′. Probe, psGAG23: 5′-FAM-CTTC(dP)TACGAT(dK)TGTTTATTG-3′. Probe, psGAG23: 5′-FAM-CTTC(dP)TACGAT(dK)TGTTTATTG-3′. Probe, psGAG23: 5′-FAM-CTTC(dP)TACGAT(dK)TGTTTATTG-3′. Probe, psGAG23: 5′-FAM-CTTC(dP)TACGAT(dK)TGTTTATTG-3′. Probe, psGAG23: 5′-FAM-CTTC(dP)TACGAT(dK)TGTTTATTG-3′. Probe, psGAG23: 5′-FAM-CTTC(dP)TACGAT(dK)TGTTTATTG-3′. Probe, psGAG23: 5′-FAM-CTTC(dP)TACGAT(dK)TGTTTATTG-3′. Probe, psGAG23: 5′-FAM-CTTC(dP)TACGAT(dK)TGTTTATTG-3′.
positive reactions for the vaccinated but non-SIV challenged animals were significantly lower than the results observed for tissue specimens from the vaccinated and SIV-challenged animals necropsied shortly after demonstrating control of SIV infection (early protected monkeys), which showed 59 (2.65%; 95% CI 2.06–3.40%) and 531 (23.81%; 95% CI 22.09–25.62%) positive DNA and RNA reactions, respectively, out of 2,230 total nested PCR and RT–PCR reactions ($P < 2.2 \times 10^{-16}$ for DNA, $P = 3.7 \times 10^{-16}$ for RNA, by Fisher’s exact test). These results support the interpretation that under the experimental conditions reported, the exquisitely sensitive nested qPCR and qRT–PCR assays we used detect only very low frequencies of reactions positive for SIV DNA and RNA in tissues from animals vaccinated with SIV gag insert-containing vaccines but not challenged with SIV, probably reflecting background false positive reactions, and that the significantly higher levels of SIV DNA and RNA detected after SIV challenge of vaccinated animals are derived from SIV infection. This interpretation is corroborated by other evidence of an initial SIV infection in the challenged, vaccine-protected monkeys including T cell responses to SIV antigens not included in the vaccine (for example, SIV Vif), and rescue of replication-competent virus by culture.

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