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Induction of CD8$^+$ Regulatory T Cells Protects Macaques against SIV Challenge

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SUMMARY

Efforts to develop a vaccine against HIV have so far met with limited success. Given that CD4$^+$ T cell activation drives the initial burst of viral replication, we explored in macaques whether an oral vaccine comprised of *Lactobacillus plantarum*, a commensal bacterium that favors immune tolerance, and inactivated simian immunodeficiency virus mac239 (SIVmac239) would induce CD4$^+$ T cell unresponsiveness/tolerance toward SIV antigens and thereby prevent the establishment of SIV infection. The tolerogenic vaccine induced MHC-Ib/E-restricted CD8$^+$ regulatory T cells (Tregs) that suppressed SIV-harboring CD4$^+$ T cell activation and ex vivo SIV replication in 15 of 16 animals without inducing SIV-specific antibodies or cytotoxic T lymphocytes. Of 16 macaques that were intrarectally challenged with SIVmac239 or heterologous strain SIVB670, 15 were sterilely protected. In four macaques that were re-challenged intravenously, plasma SIV levels peaked slightly and then dropped to undetectable levels, although the animals subsequently harbored intracellular SIV DNA. Infusion of CD8 antibodies confirmed the role of CD8$^+$ Tregs in preventing/suppressing SIV in vivo. These findings suggest a new avenue of research toward developing an HIV-1 vaccine.

INTRODUCTION

Efficient vaccines against viral infections generally elicit virus-specific neutralizing antibodies and sometimes also cytotoxic T lymphocytes (CTLs) that prevent virus infection and/or eradicate the virus rapidly after it enters the body (Plotkin, 2010). So far, however, this process does not seem to work for HIV type 1 (HIV-1) infection, as shown by the failure to generate efficient long-lasting HIV-specific immunity via either neutralizing antibodies or CTLs induced by the vaccine prototypes that have been tested over the last 20 years (Fauci et al., 2008). Recently, the ALVAC-AIDSVAX vaccine trial showed that vaccinated individuals had a modest and short-term decreased incidence of HIV-1 acquisition (Rerks-Ngarm et al., 2009). However, more disturbing are the results of the STEP study, which showed an initial increase in virus acquisition in the group of vaccinated individuals compared with the placebo group (Buchbinder et al., 2008; Duerr et al., 2012). It is known that CD4$^+$ T cell activation drives the initial burst of viral replication that leads to virus dissemination in vivo (Andrieu and Lu, 1995; Fauci, 1996). Therefore, the enhanced risk of infection observed in the STEP study could potentially be explained by the fact that the vaccine generated HIV-1-specific CD4$^+$ T cells that on one hand may help CTLs to eradicate the virus, and on the other hand provide a substrate for efficient HIV-1 replication (Douek et al., 2002). Indeed, it has previously been shown that mucosal SIV transmission could be prevented in macaques by the local administration of an anti-inflammatory agent that prevented immune activation (Li et al., 2009). Against this background, we propose here that induction of HIV-1-specific tolerance might prevent the establishment of HIV-1 infection through suppression of CD4$^+$ T cell activation.

To test this hypothesis, we used the model of the simian immunodeficiency virus (SIV) in macaques of Chinese origin, which best mimics HIV-1 infection in humans (Chen et al., 2011; Ling et al., 2002; Marcondes et al., 2006; Stahl-Hennig et al., 2007). We assessed whether an oral vaccine that favors immune tolerance and consists of *Lactobacillus plantarum* (LP) (Kleerebezem et al., 2003), a digestive tract commensal bacterium (Grangette et al., 2005; van Baarlen et al., 2009), and inactivated SIV would induce CD4$^+$ T cell unresponsiveness/tolerance toward SIV antigens, which would in turn inhibit viral replication in vivo.

RESULTS

Induction of SIV-Specific Tolerance by an Oral Vaccine

In the first set of experiments, eight Chinese macaques were immunized via the intragastric route with aldrithiol-2 (AT-2)/heat-inactivated SIVmac239 (iSIV) (Rossio et al., 1998) formulated with LP, whereas control animals received LP only (four macaques) or iSIV only (four macaques). No difference was observed in the distribution of the classical major histocompatibility complex 1 (MHC-1) genotypes of the 16 animals (Table S1). No side effect was observed after the vaccine was administered.

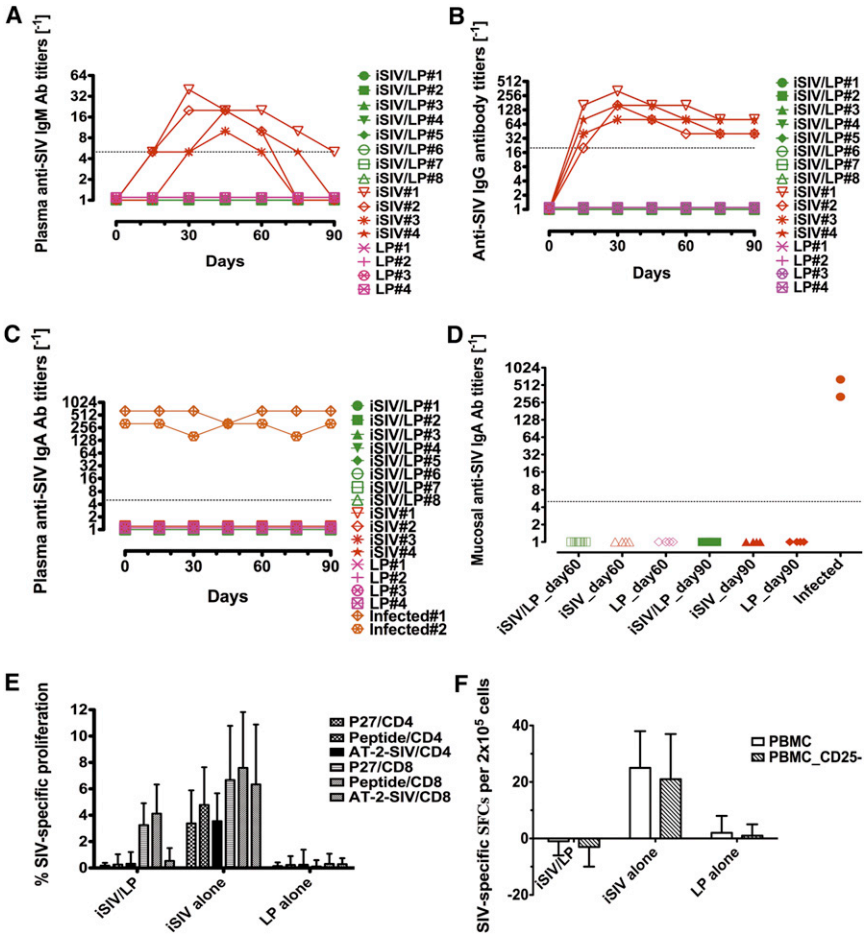

Figure 1. Humoral and Cellular Immune Responses following Intragastric Immunization with AT-2/iSIV and LP

(A) Plasma SIV IgM antibody titers.

(B) Plasma SIV IgG antibody titers.

(C) Plasma SIV IgA antibody titers.

(D) Anti-SIV IgA in the rectum washing at 60 days postimmunization.

(E) SIV (Gag p27, Gag peptides or AT-2 SIV)-specific proliferation (mean ± SD) of $CD4^+$ and $CD8^+$ T cells at 60 days postimmunization. See also Figure S1.

(F) SIV Gag-specific IFN-γ-releasing T cells (mean ± SD) at 60 days postimmunization. See also Table S1.

Because tolerance generally includes antigen-specific unresponsiveness of both the humoral and cellular arms of immunity, we looked first at SIV antibodies. We were unable to detect SIV-specific antibodies in the LP/iSIV-immunized monkeys, whereas we detected low titers of SIV immunoglobulin M (IgM) and IgG antibodies in the plasma of iSIV-treated controls (Figures 1A and 1B). Similarly, SIV IgA antibodies were not detected in the plasma and rectum washings of LP/iSIV-immunized monkeys or iSIV-treated controls, whereas they were detected in SIV-infected monkeys (Figures 1C and 1D). Due to the absence of reagents, SIV secretory-IgA antibodies were not checked. We then tested for SIV-specific cellular responses. Two months after immunization, SIV Gag-p27- and Gag-peptide-specific $CD4^+$ T cell proliferation was not detected in any of the eight iSIV/LP-immunized animals, whereas it was present in the four iSIV-treated controls ($p < 0.01$). In contrast, SIV Gag-specific $CD8^+$T cell proliferation was observed in both the eight iSIV/LP-immunized monkeys and the four iSIV-treated controls ($p > 0.05$; Figure 1E and Figure S1). Interferon γ (IFN-γ)-secreting T cells (CTLs) were also not detected upon SIV Gag p27 stimulation in the eight iSIV/LP-immunized animals, but were found (although at a low frequency) in the four iSIV-treated controls ($p < 0.01$; Figure 1F). We also depleted $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) from the peripheral blood mononuclear cells (PBMCs) of immunized macaques using an CD25 monoclonal antibody. This had no effect on p27-specific T cell unresponsiveness in vitro (Figure 1F). Furthermore, we showed that there was no specific interleukin 10 (IL-10) or transforming growth factor β (TGF-β) release upon stimulation with SIV Gag p27 or Gag peptides in either the iSIV/LP-immunized or control animals (data not shown).

Suppression of SIV-Specific $CD4^+$T Cell Activation by Nonclassical MHC-Restricted $CD8^+$T Cells

We next investigated whether this form of immune tolerance involves a suppression of activation of SIV-positive $CD4^+$ T cells. Because infected $CD4^+$ T cells present Gag antigen derived from entering virions, i.e., before viral integration and viral protein expression (Sacha et al., 2007a), we pulsed fresh PBMCs from iSIV/LP-immunized macaques and control animals with nonreplicative (AT-2-treated) SIVmac239. As expected (Vatakis et al., 2009), ~5% of the $CD4^+$ T cells of all of the monkeys were "infected" by AT-2-treated (killed) SIV, as evidenced by intracellular p27 staining (Figures 2A and 2B). We then stimulated PBMCs overnight with staphylococcal enterotoxin B (SEB) and CD3/CD28 antibodies in the presence or absence (after depletion) of autologous $CD8^+$ T cells and assayed for $p27^+CD4^+$ T cells bearing the early activation/proliferation marker Ki-67. We found that the percentage of activated ($Ki\text{-}67^+$) $p27^+CD4^+$ T cells was much lower in PBMCs from iSIV/LP-immunized macaques than in PBMCs from LP- or iSIV-treated animals (mean ± SD, 8.9% ± 2.7% versus 49.1% ± 9.1% or 53.4% ± 7.0%, $p < 0.01$). Moreover, in PBMCs from iSIV/LP-immunized macaques, the percentage of $Ki\text{-}67^+p27^+CD4^+$ T cells was also much lower in the presence than in the absence of autologous $CD8^+$ T cells (mean ± SD, 8.9% ± 2.7% versus 54.5% ± 6.3%, $p < 0.01$). By contrast, in PBMCs from LP- or iSIV-treated animals, the percentages of $Ki\text{-}67^+p27^+CD4^+$ T cells remained high in either the presence or absence of autologous $CD8^+$ T cells (49.1% ± 9.1% and 53.4% ± 7.0%, respectively, $p > 0.05$; or 52.9% ± 7.4% and 57.5% ± 8.4%,

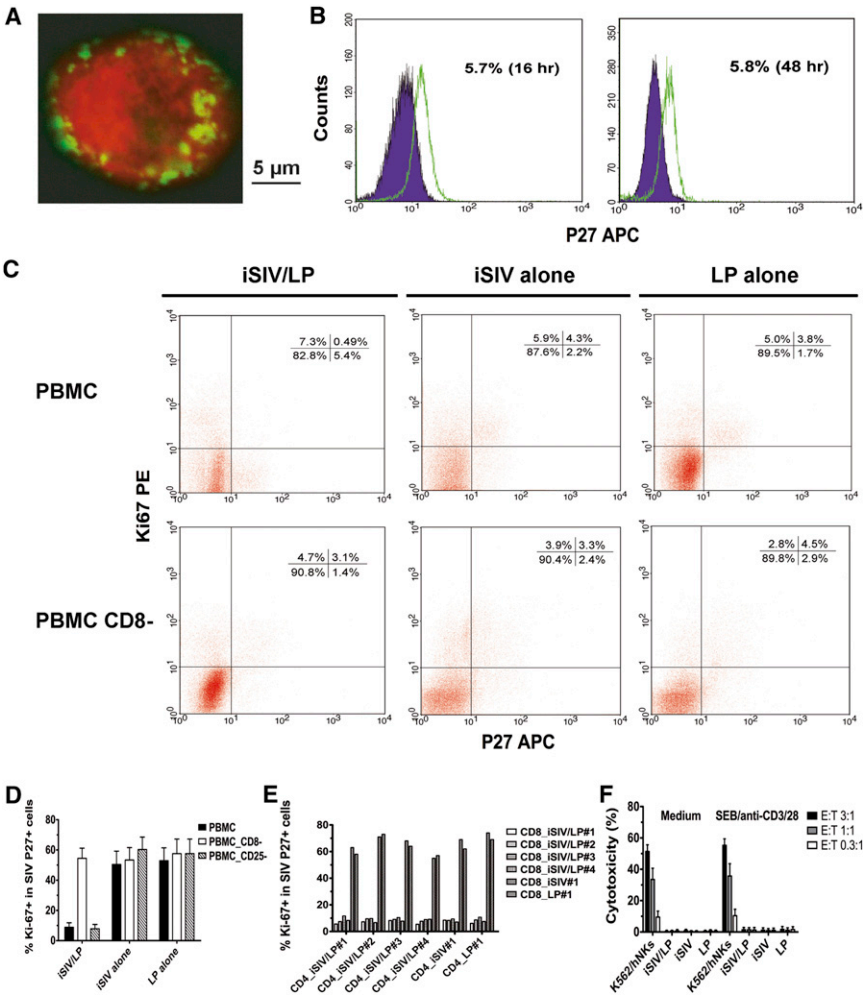

Figure 2. Suppression of SIV-Specific CD4$^+$ T Cell Activation by Nonclassical MHC-Restricted CD8$^+$ T Cells at 60 Days following Intragastric Immunization with iSIV and LP

(A) SIV Gag p27 (green) in the cytoplasm (red) of nonreplicative (AT-2-treated) SIV-pulsed quiescent CD4$^+$T cells visualized by fluorescent confocal microscopy.

(B) SIV Gag p27$^+$ cells in nonreplicative SIV-pulsed quiescent CD4$^+$ T cells following 16 and 48 hr poststimulation analyzed by flow cytometry (SIV-pulsed cells in green and nonpulsed cells in violet).

(C) Representative data for the activation marker Ki67 and SIV Gag p27$^+$ cells within gated CD4$^+$ T cells in nonreplicative SIV-pulsed PBMCs (depleted or not of CD8$^+$T cells) at 48 hr poststimulation by flow cytometry.

(D) Expression (mean ± SD) of Ki-67 within SIV Gag p27$^+$ cells on the gated CD4$^+$ T cells in AT-2 SIV-pulsed PBMCs (depleted or not of CD8$^+$ or CD25$^+$T cells) taken from iSIV/LP-immunized animals and iSIV or LP-treated animals.

(E) Expression of Ki-67 within SIV Gag p27$^+$ (AT-2 SIV-pulsed) CD4$^+$ T cells in the presence of autologous or allogenic CD8$^+$T cells.

(F) Cytotoxicity (mean ± SD) of AT-2 SIV-pulsed CD4$^+$ T cells in the presence of CD8$^+$ T cells or of K562 in the presence of human natural killer cells (controls) with or without SEB and anti-CD3/CD28 stimulation.

respectively, $p > 0.05$; Figures 2C and 2D). We also showed that iSIV/LP-induced CD8$^+$ T cells operated through a nonclassical MHC-restricted mechanism, because they suppressed the activation of nonreplicative SIV-pulsed CD4$^+$ T cells from other iSIV/LP-immunized monkeys as well as those from control animals ($p > 0.05$; Figure 2E). Moreover, because the depletion of CD25$^+$ cells did not modify the suppressive activity of PBMCs on p27$^+$CD4$^+$T cell activation (Figure 2D), we concluded that Tregs had no role in this suppression process. Finally, we controlled for the absence of CD4$^+$ T cell lysis ($p > 0.05$) using a highly sensitive cytotoxicity assay (Marchetti et al., 1996; Figure 2F). Together, these results support the conclusion that a noncytotoxic mechanism underlies the CD8$^+$ T cell-mediated suppression.

Suppression of Virus Replication by MHC-Ib/E Restricted CD8$^+$ T Cells

Having demonstrated that adding LP to iSIV turned a classical SIV-specific immune response into an SIV-specific, CD8$^+$ T-dependent tolerance, including the suppression of SIV-positive CD4$^+$ T cell activation, we further studied whether the SIV-specific CD8$^+$ T cells would suppress the replication of SIV in freshly infected CD4$^+$ T cells ex vivo. We purified CD8$^+$ T cells from PBMCs from the iSIV/LP-immunized macaques and added them to autologous CD4$^+$ T cells acutely infected with SIVmac239. They were then stimulated overnight with SEB and CD3/CD28 antibodies. Over a period of 5 days, the CD8$^+$ T cells conferred a strong antiviral activity in the coculture ($p < 0.001$) (Figure 3A). If the CD8$^+$T cells were added more than 48 hr poststimulation (i.e., at a time when SIV-specific CD4$^+$ T cell activation was well established), they could no longer inhibit viral replication ($p < 0.001$) (Figure 3B). This strongly suggests that the antiviral effect of the CD8$^+$ T cells was generated by suppression of SIV-specific CD4$^+$ T cell activation. We also demonstrated that the CD8$^+$ T cell-mediated antiviral activity required cell-to-cell contact ($p < 0.001$) (Figure 3C) and was a classical MHC-unrestricted activity (as was the case for suppression of CD4$^+$ T cell activation), as shown by the strong inhibition of viral replication by CD8$^+$ T cells on acutely infected CD4$^+$ T cells from other immunized animals in the group or from control animals ($p < 0.001$) (Figure 3D).

Finally, we showed that the CD8$^+$ T cell-mediated antiviral activity was blocked by an MHC-Ib/E antibody but not by an ia/ABC antibody, indicating a nonclassical MHC-Ib/E-restricted CD8$^+$T cell activity ($p < 0.001$) (Figure 3E). Clearly, T cell receptor (TCR) expression by CD8$^+$ T cells is essential for recognition of MHC-Ib/E-peptide complexes carried by target CD4$^+$ T cells (Sarantopoulos et al., 2004; Van Kaer, 2010). By depleting cell subsets in vitro with antibody-conjugated magnetic microbeads,

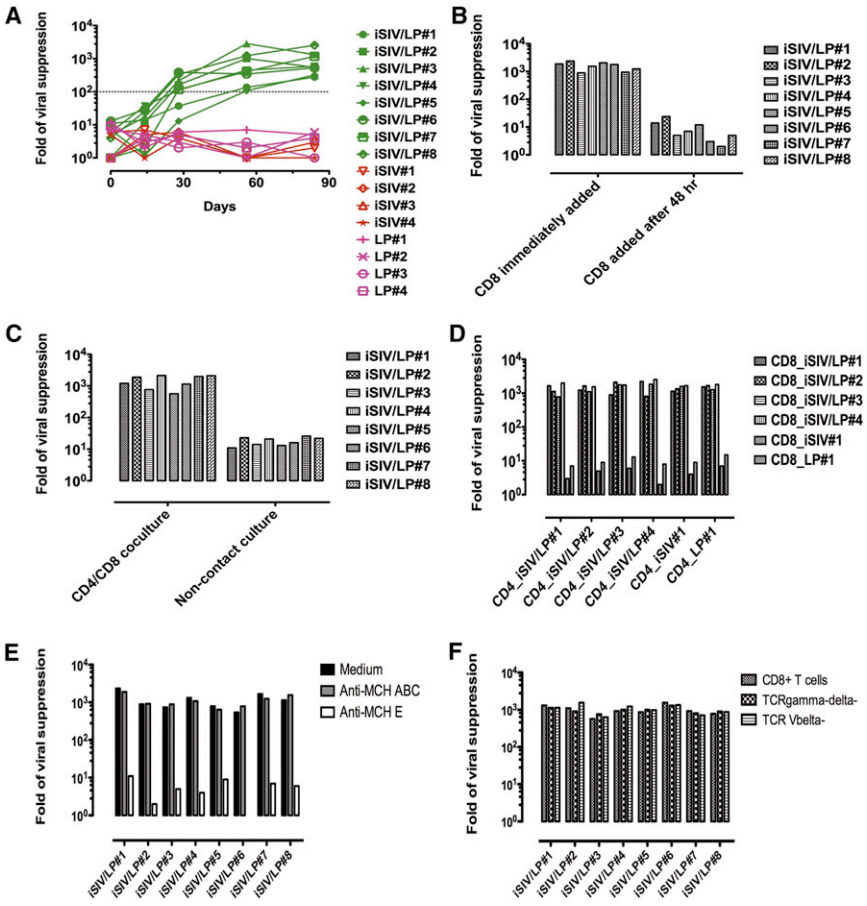

Figure 3. In Vitro Antiviral Activity of CD8$^+$T Cells following Intragastric Immunization with iSIV and LP

(A) Anti-SIV activity (fold of viral suppression) of CD8$^+$ T cells.

(B–F) Anti-SIV activity of CD8$^+$ T cells after 60 days following oral immunization in a delayed (B), insert (C), or allogenic (D) culture system, in the presence of MHC-Ia/ABC or MHC-Ib/E antibodies (E), and in CD8$^+$ T cells depleted of TCR$\gamma\delta^+$ or Vβ8$^+$ subset (F).

See also Figure S2 and Table S2.

we showed that TCR$\gamma\delta$ and Vβ8 were not involved in suppression of viral replication ($p > 0.05$) (Figure 3F), suggesting that TCR$\alpha\beta$ plays a central role in the recognition of MHC-Ib/E-peptide on infected CD4$^+$T cells. However, even though we tested for a large number of CD8$^+$T-cell-associated CD antigens (by depleting CD8$^+$ T cells with human antibodies cross-reacting with membrane CD antigens of nonhuman primates), we were not able to identify any CD antigen associated with MHC-Ib/E-restricted CD8$^+$ T cell activity ($p > 0.05$) (Table S2). Finally, in an additional experiment, we found lower peripheral blood myeloid dendritic cell (mDC) and plasmacytoid DC (pDC) counts in protected (iSIV/LP-immunized) animals as compared with infected control animals ($p < 0.01$ and < 0.05 for mDC and pDC, respectively) or naive animals ($p < 0.001$ for both mDC and pDC) (Figure S2).

Protection of Macaques from Homologous and Heterologous SIV Challenges

Having established that oral immunization with iSIV/LP induced an SIV-specific tolerance that suppressed viral replication ex vivo, and that both suppression of CD4$^+$ T cell activation and inhibition of viral replication resulted from the activity of non-IFN-γ-secreting TCR$\alpha\beta$ MHC-Ib/E-restricted CD8$^+$ T cells, we sought to determine whether such a tolerogenic vaccine would protect macaques from mucosal SIV infection. To that end, we challenged the eight iSIV/LP-immunized and eight control animals intrarectally with a high dose (100,000 $TCID_{50}$) of SIVmac239 3 months after immunization (Chen et al., 2011). Surprisingly, the eight immunized animals were fully protected without any detectable level of plasma SIV RNA or PBMC proviral DNA. By contrast, the eight control animals were all infected ($p < 0.001$) (Figure 4A).

We further wanted to observe whether the animals that were protected against intrarectal SIV challenge could also be protected against an intravenous challenge. Two months after the intrarectal challenge, four out of the eight monkeys were challenged via the intravenous route with 200 $TCID_{50}$ of virus. All showed a slight peak of SIV replication ($\leq$200 DNA copies/million PBMCs and 200 SIV copies/ml of plasma) at day 10 postchallenge. However, by day 30, plasma SIV RNA had become undetectable ($\leq$10 copies/ml) and PBMC SIV DNA had decreased to $\leq$10 copies/million cells, showing the absence of virus replication in vivo. In contrast, two naive animals that received the same intravenous SIVmac239 challenge were fully infected ($p < 0.001$) (Figure 4B). The four remaining monkeys were intrarectally rechallenged (100,000 $TCID_{50}$) and, as expected, remained fully protected ($p < 0.001$) (Figure 4C).

To test whether vaccinated monkeys would also be protected from a heterologous challenge, 8 months later we further challenged the four intrarectally rechallenged monkeys and the four intravenously rechallenged monkeys with 100,000 $TCID_{50}$ of the antigenically distinct SIVB670 via the intrarectal route. The eight animals remained fully protected, as shown by their undetectable SIV B670 DNA and RNA levels, whereas two control naive animals were infected by the same SIV B670 challenge ($p < 0.001$). This demonstrated that the SIV-specific tolerogenic vaccine was cross-protective, presumably through preventing the activation of CD4$^+$ T cells that were infected by another SIV strain (Figure 4D).

Longevity of Vaccine-Induced Protection

We further immunized a new group of 16 macaques (eight with iSIV/LP, four with iSIV, and four with LP) (Table S3) to study the

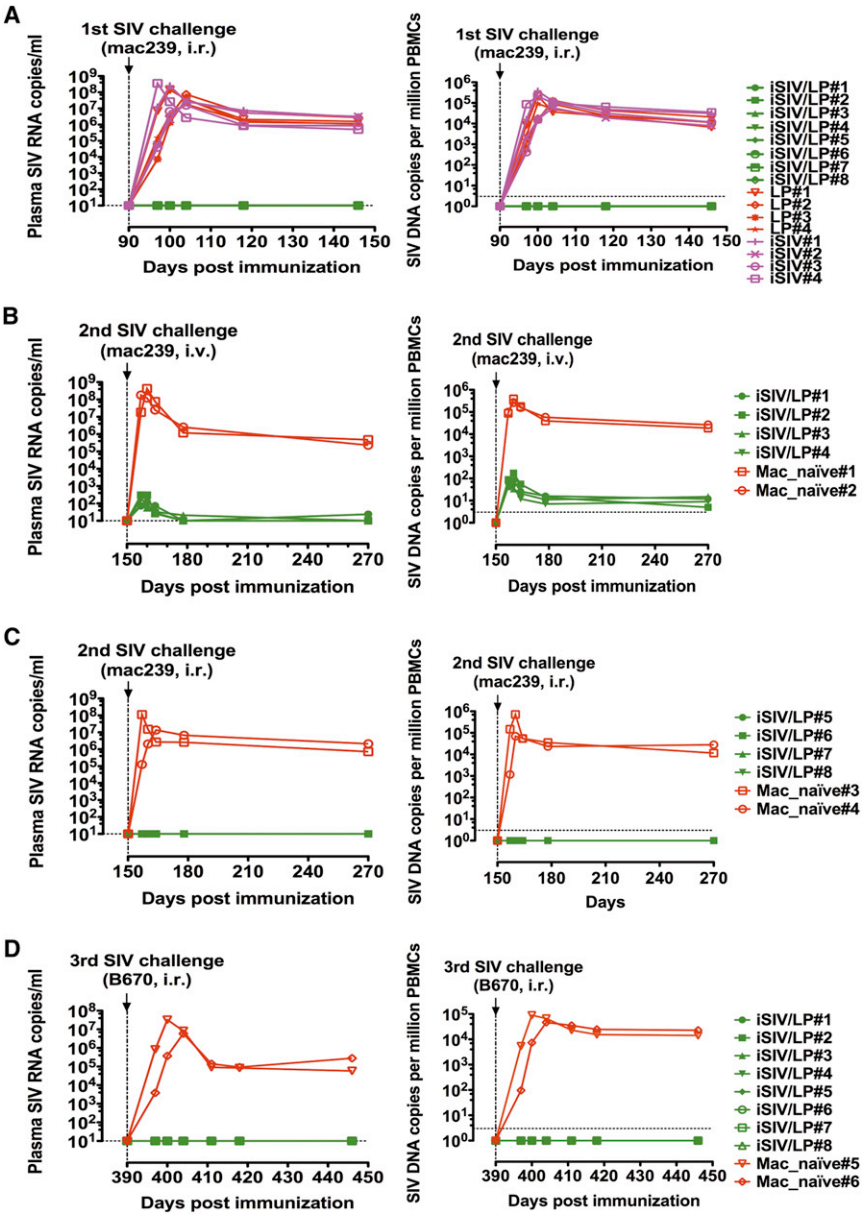

Figure 4. Prevention of SIV Infection in iSIV/LP-Immunized Macaques Challenged with Different Pathogenic SIV Strains

(A–D) Plasma and PBMC viral loads of rhesus macaques intragastrically immunized with iSIV and LP after (A) a first challenge performed intrarectally with SIVmac239 (100,000 $TCID_{50}$) in eight macaques and in eight control monkeys treated with LP alone or iSIV alone, (B) a second SIVmac239 challenge performed intravenously (200 $TCID_{50}$) in four immunized monkeys and two naive monkeys, (C) a second SIVmac239 challenge performed by intrarectal route in the four remaining immunized monkeys and two naive monkeys, and (D) a third intrarectal challenge performed intrarectally with SIVB670 in the eight immunized macaques and two additional naive monkeys.

longevity of the protection. The ex vivo antiviral activity of cells from this group was followed regularly. We observed that seven of the eight animals that received iSIV/LP maintained a high ex vivo antiviral activity of $\geq$100-fold compared with controls for up to 420 days ($p < 0.001$) (Figure 5A). As expected, no antiviral activity was detected in cells from animals treated with iSIV or LP alone.

On day 420 postimmunization, all 16 animals were intrarectally challenged with 100,000 $TCID_{50}$ of SIVmac239. Seven out of the eight iSIV/LP-immunized animals had a sterile immunity as indicated by the absence of any SIV RNA and DNA emergence in plasma, PBMCs (Figures 5B and 5C), rectal mucosa lymphocytes (measured from day 1 postchallenge), or pelvic lymph nodes (Figure S3). Interestingly, one iSIV/LP-immunized macaque as well as all four of the iSIV-treated and all four of the LP-treated monkeys became fully infected ($p < 0.001$; Figures 5B and 5C; Figure S3). Moreover, the evolution of the ex vivo antiviral activity of the eight vaccinated monkeys allowed us to predict, from day 360 postimmunization (i.e., 60 days before their challenge), which one of the eight immunized monkeys would not be protected (Figures 5A and 5B).

Role of $CD8^+$ T Cells in Suppressing SIV Replication In Vivo

In order to confirm the role of $CD8^+$ T cells in vivo, we temporarily depleted $CD8^+$ T cells from peripheral blood and lymphoid organs in the eight already challenged and protected monkeys in the first group of animals. Three intravenous injections of a mouse-human chimeric monoclonal CD8 antibody, cMT-807 (Centocor), were administered over a period of 1 week at days 300, 304, and 307 postimmunization (Figure 6A), and viral RNA and DNA were monitored. In the four macaques that had been rechallenged by the intrarectal route, no viral RNA or DNA was detected, demonstrating again their sterile protection. Interestingly and by contrast, a strong viral replication was observed in the four animals that had previously been challenged intravenously and harbored proviral DNA. In these animals, at the nadir of $CD8^+$ T cell depletion (day 15), virus replication peaked at 10^6 RNA copies/ml of plasma, and lymph node proviral loads reached 10^4 DNA copies/10^6 cells ($p < 0.001$). However, by days 60–90, as the four animals recovered baseline $CD8^+$T cell concentrations, virus replication was again brought under control, as indicated by plasma SIV RNA and PBMCs and lymph node SIV DNA returning to baseline levels ($p > 0.05$) (Figures 6B and 6C). This confirmed the unique role of the vaccine-induced $CD8^+$ T cells

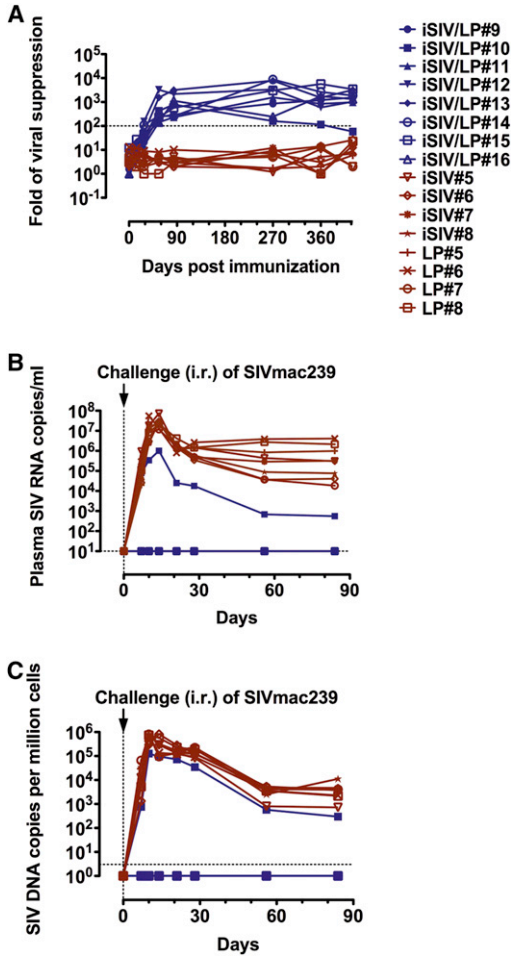

Figure 5. In Vitro and In Vivo CD8⁺Treg-Mediated Antiviral Activity after Intragastric Immunization with iSIV and LP in a Second Set of 16 Macaques

(A) Anti-SIV activity (fold of viral suppression) of CD8⁺ T cells during 60–420 days postimmunization.

(B) Plasma viral loads >90 days after intrarectal challenge.

(C) Cellular SIV DNA loads >90 days after intrarectal challenge.

See also Figure S3 and Table S3.

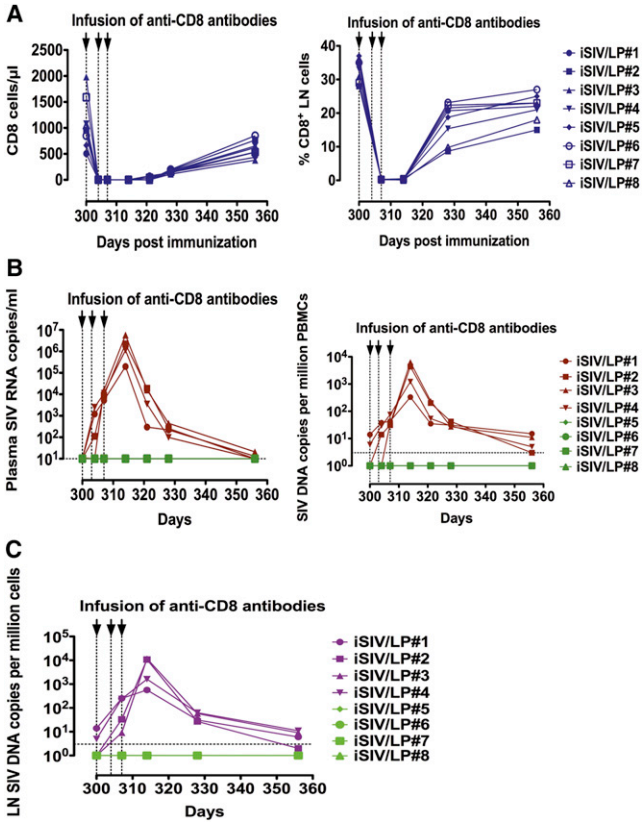

Figure 6. Depletion of Peripheral Blood and Lymph Node CD8⁺T Cells in Eight iSIV/LP-Treated Macaques by Infusion of the CD8 Antibody cMT807

(A) Peripheral blood CD8⁺ T cell counts and lymph node CD8⁺T cell percentage (%) before and after three injections of cMT807.

(B) Plasma and cellular viral loads before and after three injections of cMT807 in the eight iSIV/LP-immunized monkeys. Four macaques (iSIV/LP #1–4, in red) were previously intravenously challenged and four (iSIV/LP #5–8, in green) were previously intrarectally challenged.

(C) Lymph node SIV DNA loads before and after three injections of cMT807. Four macaques (iSIV/LP #1–4, in violet) were previously intravenously challenged and four (iSIV/LP #5–8, in green) were previously intrarectally challenged.

in controlling viral replication in intravenously SIV-challenged animals in which replication-competent virus remained latent, presumably in quiescent memory CD4⁺ T cells (Willerford et al., 1990). A similar state of latency has been observed in HIV-1 patients treated with highly active antiviral therapy (Finzi et al., 1997).

Role of CD8⁺ T Cells in Protecting Macaques against Intrarectal SIV Challenge

To further define the role of CD8⁺ T cells in protecting against mucosal (rectal) challenge, we redepleted CD8⁺ T cells in the four macaques (#5–8) that previously had been sterilely protected through three intrarectal challenges (performed 3, 5 and 13 months postimmunization). The CD8 antibody (cMT-807) was administered 36 months postimmunization over a period of 1 week (days 0, 4, and 7) to temporarily deplete the CD8⁺ T cells in the same manner as described above. However, in this set of experiments, 2 hr after the first administration of CD8 antibody, the four monkeys were challenged intrarectally with 100,000 $TCID_{50}$ of SIV239. As before, the cMT-807 treatment dramatically reduced the percentage of CD8⁺ T cells in PBMCs (Figure 7A). Consistent with our previous observations regarding the role of CD8⁺ T cells, all of the animals became infected. Indeed, at the nadir of the CD8⁺T cell count, the plasma viral loads of the four animals peaked at $\sim 10^4 - 10^6$ RNA copies/ml (Figure 7B) and $\sim 10^3 - 10^4$ copies of SIV DNA per million PBMCs (Figure 7C). By weeks 4–7, when the CD8⁺ T cell concentrations in the four monkeys had recovered to almost normal levels, plasma SIV RNA dropped to almost undetectable levels (10–50 copies/ml). This

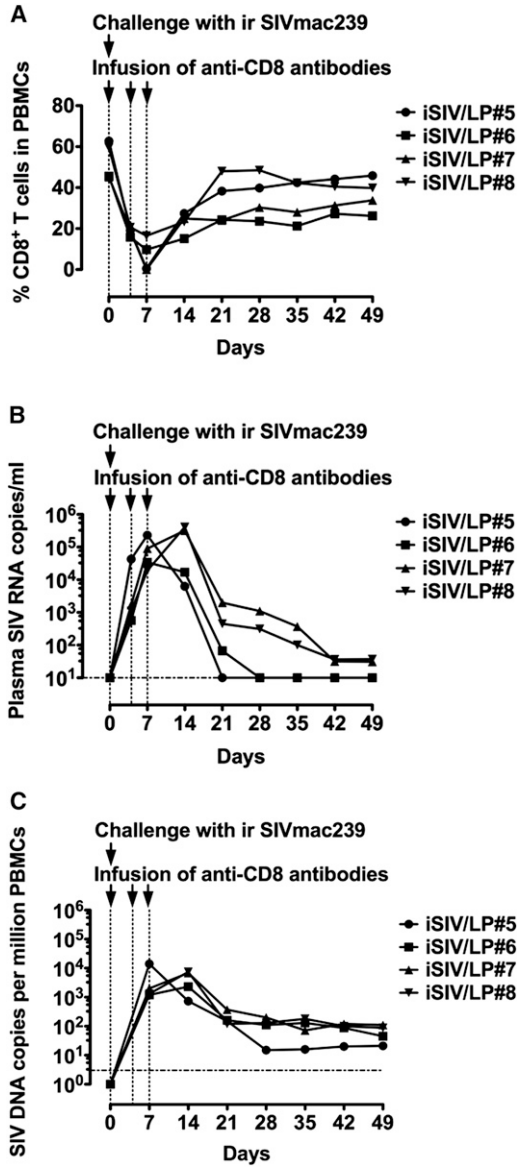

Figure 7. Intrarectal Challenge of SIVmac239 in Four iSIV/LP-Immunized and Protected Macaques after Infusion with the CD8 Antibody cMT807

(A) Peripheral blood $CD8^+$T cell counts (%) before and after three injections of cMT807.

(B) Plasma SIV RNA loads before and after three injections of cMT807.

(C) SIV DNA loads in PBMCs before and after three injections of cMT807.

experiment further demonstrated the central role of $CD8^+$ T cells in preventing initial infection at the mucosal barrier in immunized animals. When the $CD8^+$ T cells were depleted, the virus replicated freely in lymphoid organs, but as soon as the vaccine-induced $CD8^+$ T cells recovered, they again strongly controlled viral replication. Consistent with the above observations regarding intravenous challenge, the recovered animals now contained SIV DNA in target cells (Figure 7C), but without obvious viral replication (Figure 7B).

DISCUSSION

In the SIV-macaque model, we have demonstrated that infection can be prevented by inducing immunological tolerance against the infecting agent. The administration of iSIVmac239 and LP, a nonpathogenic commensal bacterium of the digestive tract that is known to induce immunologic tolerance, stimulated the macaques to develop a thus far unrecognized type of SIV-specific tolerance. This tolerance was characterized by the suppression of SIV-specific antibody and CTL responses, and activation of a subset of $CD8^+$ T cells that are SIV-specific, non-cytolytic, and MHC-Ib/E restricted. These cells apparently have the ability to suppress $CD4^+$ T cells activated by SIV and thereby prevent the establishment of productive SIV infection both in vivo and in vitro.

These suppressive noncytolytic MHC-Ib/E-restricted $CD8^+$ T cells represent a class of $CD8^+$ Tregs that has not been described previously in the context of any vaccination or in SIV or HIV infection. Thus far, the only Tregs that have been proposed to be associated with HIV infection are $CD4^+$ Tregs ($CD25^+FoxP3^+CD4^+$ T cells) that are known to be involved in some models of immune tolerance (Faria and Weiner, 2005); however, their role in HIV infection is not clearly understood. On the one hand, the expansion of $CD4^+$ Tregs has been shown to be associated with the suppression of HIV-specific $CD4^+$ T cell responses and disease progression (Aandahl et al., 2004; Nilsson et al., 2006; Weiss et al., 2004), whereas on the other hand, $CD4^+$ Tregs have been associated with protection from productive infection, $CD4^+$T cell activation, and disease progression in both humans (Card et al., 2009; Chase et al., 2008; Legrand et al., 2006) and nonhuman primates (Jacquelin et al., 2009; Kornfeld et al., 2005). In the study presented here, the ex vivo removal of $CD25^+FoxP3^+CD4^+$ T cells by a CD25 antibody did not modify either the suppression of $CD4^+$T cell activation or viral replication.

Except for the fact that they are noncytolytic, the $CD8^+$ Tregs observed here resemble those that target and eliminate abnormally activated antigen-specific $CD4^+$ T helper cells in the mouse model (Kim et al., 2010; Sarantopoulos et al., 2004), where the inhibitory interaction depends on recognition of surface Qa-1 (corresponding to MHC-Ib/E in macaques and to HLA-E in humans) expressed by aberrantly activated target cells (Kim et al., 2011; Nagarajan et al., 2012). Similar $CD8^+$ Tregs have also been implicated in the control of autoimmune type 1 diabetes in humans (Jiang et al., 2010).

Because monkeys challenged via the intrarectal route were negative for both SIV RNA in plasma and SIV DNA in PBMCs, our results suggest that virus infection was arrested before nuclear integration occurred. In quiescent $CD4^+$ T cells, virus penetration is followed within 2 hr by the presentation at the plasma membrane of Gag/Pol and other protein epitopes derived from incoming virions (Sacha et al., 2007a), whereas Env and Nef proteins need de novo synthesis (Sacha et al., 2007b). The subsequent phases of the infectious process, including reverse transcription and proviral DNA integration, develop inefficiently in quiescent $CD4^+$ T cells but very efficiently in activated $CD4^+$ T cells (Korin and Zack, 1999; Zack et al., 1990). This is in keeping with the notion that the early

activation of a small founder population of infected $CD4^+$ T cells at the portal of entry is required for the local expansion and establishment of systemic infection (Haase, 2011; Zhang et al., 2004). This early event potentially provides vaccine-induced $CD8^+$ T cells the opportunity to arrest the infectious process before proviral DNA integration occurs. We observed that the withdrawal of vaccine-induced $CD8^+$ T cells from PBMC cultures before $CD4^+$ T cell activation allowed the $CD4^+$ T cells to become activated and viral replication to proceed. Similarly, in vivo depletion of $CD8^+$ T cells performed at the time of intrarectal challenge allowed the $CD4^+$ T cells to be activated and the virus to replicate. Such replication, however, was brought under control as the $CD8^+$ T cells recovered in lymphoid organs and the blood stream. We showed in cultures ex vivo that the inhibition of viral replication was directly attributable to this class of $CD8^+$ Tregs. This is highly likely to be the case in vivo as well; however, direct evidence is lacking because we were restricted to depletion of the whole $CD8^+$ T cell population rather than the Treg subset specifically.

So far, we have not identified any CD antigen specifically associated with the vaccine-induced $CD8^+$ Tregs observed in this study. The phenotypic and molecular characteristics of these $CD8^+$ Tregs therefore require further study. Further studies are also necessary to elucidate how peripheral DCs are downregulated in vaccinated monkeys, because previous publications have reported that DC expansion is downregulated in vivo by classical Tregs (Kim et al., 2007; Liu et al., 2009).

In intravenously challenged monkeys, the transient peak of viral replication followed by the residual presence of cellular SIV DNA at a low level suggested that infectious SIV particles that entered the body by this route encountered activated $CD4^+$ T cells, probably in secondary lymphoid organs, that allowed the virus to complete the first cycles of replication. We assume, however, that any newly released virions then entered nonactivated (quiescent) $CD4^+$ T cells that were prevented from becoming activated by the LP/iSIV-generated $CD8^+$ Tregs. Given that in vitro, SIV antigen-specific $CD8^+$ Tregs of immunized macaques were effective enough to inhibit the superantigen SEB and CD3/CD28 antibody activation of infected $CD4^+$ T cells (Figures 2C–2E), it is likely that in vivo, the activation of SIV-infected $CD4^+$ T cells by SIV-unrelated stimuli known to upregulate viral replication (Andrieu and Lu, 1995; Fauci, 1996) will also be inhibited.

The protective effect of this innovative immunization regimen against SIV challenge, together with the identification of a correlate of protection in vitro, is quite striking. Given that SIV and HIV require activated immune cells in which to replicate, this tolerogenic vaccine approach may offer an exciting new avenue in HIV vaccine research. Moreover, the de novo induction of this class of $CD8^+$ Tregs could potentially be used therapeutically to suppress HIV replication and prevent disease progression in place of antiviral therapy. Furthermore, the tolerogenic vaccine approach described here could potentially be exploited in the management of a wide range of immune disorders and is likely to have profound implications across immunology generally.

EXPERIMENTAL PROCEDURES

Animals

Colony-bred rhesus macaques (*Macaca mulatta*) of Chinese origin were housed at the Nonhuman Primate Laboratory of the Gaoyao Experimental Animal Center (Guangdong, PRC) in accordance with the National Institutes of Health's "Guide for the Care and Use of Laboratory Animals." All animals were in good health, 2–4 years old, and weighed 4–6 kg, and were seronegative for SIV, simian retrovirus, simian T cell lymphotropic virus 1, hepatitis B virus, and herpesvirus simiae (B virus).

Viral Preparation

SIV was produced in CEM174 cells inoculated with SIVmac239 (gift of P.A. Marx; Chen et al., 2011). The culture supernatants were collected at peak virus production and inactivated first with 250 μM AT-2 (Sigma-Aldrich, St. Louis) (Rossio et al., 1998) and then by heating at 56°C for 30 min. The inactivated virus was inoculated to CEM174 cells to verify the 100% inhibition of viral infectivity.

Bacterial Preparation

LP (ATCC8014) was cultured at 37°C in MRS medium with a rotation rate of 200 rpm. To obtain LP at the logarithmic (midlog) phase of bacterial culture, bacteria were cultured until they reached an optical density at 600 nm of 1.0 with a final LP concentration of $\sim 10^{10}$ cfu/ml (~3.5 hr) (van Baarlen et al., 2009).

Oral Delivery of the Vaccine

In the first study, 16 monkeys that had fasted overnight were anesthetized with intramuscular tiletamine hydrochloride and zolazepam (0.7 mg/kg). Eight animals (iSIV/LP #1–8) were then intragastrically administered 30 ml of a preparation containing 4×10^7 copies/ml of iSIV and 3×10^9 cfu/ml of living LP in maltodextrin (20%) solution. Monkeys received 25 ml of the same preparation intragastrically every 30 min for 3 hr. The same protocol was performed over five consecutive days. Four control animals (LP #1–4) received living LP alone, and the remaining four (iSIV #1–4) received only iSIV according to the same protocol.

In the second study, 16 new animals were recruited on the same screening basis: eight animals (iSIV/LP #9–16) received the same dose of the same vaccine, four (LP #5–8) were administered living LP alone, and four (iSIV #5–8) received only iSIV.

Assay for Antibody Responses to SIV

Plasma SIV IgG, IgM, and IgA antibodies were titrated by an immunofluorescence antibody assay (IFA) (Tsai et al., 1993) using fluorescein isothiocyanate (FITC)-conjugated goal anti-macaque IgG (Sigma), IgM (ADI, San Antonio, TX, USA), or IgA (ADI) antibodies. The sensitivity of the IFA assay was a titer of 20 for IgG and a titer of 5 for IgM and IgA. When a plasma sample was below the assay sensitivity, a value of one was assigned. Mucosal secretions were collected by washing the rectum with PBS using a catheter for gastric instillation as described previously (Méderlé et al., 2003). Anti-SIV IgA titers in the rectum were measured by the IFA assay.

Flow Cytometry

Flow-cytometry analysis was carried out with FACScalibur (BD Biosciences, San Jose, CA, USA) using the following fluorescence-labeled monoclonal antibodies: CD3-FITC (clone SP34-2), CD3-PE-Cy7, CD4-PE or CD4-PerCP-Cy5 (clone MT477), CD8-PerCP (clone RPA-T8), and Ki67-PE (BD Biosciences). FITC-conjugated P27 monoclonal antibody (Fitzgerald, Concord, MA, USA) or biotin-conjugated P27 monoclonal antibody (Fitzgerald) coupled with APC-SAv (BD Biosciences) was used for the intracellular staining of SIV-infected cells. Phycoerythrin (PE)-conjugated monoclonal antibodies against TCRγδ (clone B1), Vβ8, and CD antigens CD7, CD16, CD28, CD62L, CD95, CD122, CD137, CD150, CD183, CD184, CD195, CD196, CD197, CD226, CD272, and CD305 were purchased from BD Biosciences; PE-conjugated monoclonal antibodies against CD antigens CD11a, CD25, CD27, CD39, CD101, CD129, CD215, CD277, and CD357 were purchased from BioLegend (San Diego, CA, USA); and PE-conjugated monoclonal antibodies against CD

antigens CD127, CD247, and CD279 were purchased from eBioscience (San Diego, CA, USA).

SIV-Specific Cell Proliferation Assay

PBMCs were obtained from fresh blood by centrifugation through Ficoll-Hypaque (Sigma). The proliferation of SIV-specific $CD4^+$ or $CD8^+$ T cells was evaluated by flow cytometry using the carboxy-fluorescein diacetate, succinimidyl ester (CFSE) labeling assay (Molecular Probes, Eugene, OR, USA). CFSE-labeled cells were stimulated for 5 days with 10 μg/ml recombinant SIV core protein P27 (ImmunoDiagnostics, Woburn, MA, USA), 2 μg/ml SIV Gag 15-mer peptides (GLS, Shanghai, PRC), 10^9/ml AT-2-inactivated SIV, or medium alone.

SIV-Specific ELISpot Assay

IFN-γ and IL-10 ELISpot assays were carried out in uncultured PBMCs in the presence or absence of SIV P27 using a commercial kit (Cell Sciences, Canton, MA, USA). A TGF-β1 ELISpot kit was purchased from R&D Systems (Minneapolis). The data were read with an automated ELISpot reader (AID, GmbH, Straβberg, Germany). The number of SIV P27-specific spot-forming cells (SFCs) was calculated by subtracting the nonspecific SPCs in the presence of medium alone.

T Cell Suppression Assay

Fresh PBMCs, depleted (or not) with either CD8 or CD25 by magnetic-bead-conjugated CD8 or CD25 antibodies (Miltenyi Biotec, Auburn, CA, USA), were pulsed with nonreplicative (AT-2-treated) SIVmac239 (at a viral concentration of 10^{10}/ml) for 2 hr at 37°C in a 5% CO_2 incubator. Pulsed cells were treated overnight with SEB (2.5 μg/ml; Sigma) and CD3 (2.5 μg/ml)/CD28 (2.5 μg/ml) antibodies (BD Biosciences) in RPMI 1640 culture medium (Sigma) supplemented with 1% fetal calf serum (Sigma) and 2% BSA (Sigma). Simultaneous intracellular stainings of SIV P27 and Ki-67 were performed 48 hr after the nonreplicative SIV pulse in order to determine the percentage of T cell activation ($Ki\text{-}67^+$) within infected ($P27^+$) cell populations. The $P27^+$ cells were visualized with a fluorescent confocal microscope (Leica TCS SP2; Leica Microsystems CMS GmbH, Mannheim, Germany) using FITC-conjugated P27 antibody (Fitzgerald) with cytoplasmic staining by Evans Blue (Sigma).

T Cell Cytotoxicity Assay

Both purified $CD8^+$ T cells (effector cells) and purified $CD4^+$T cells pulsed with 10^{10} AT-2-treated SIVmac239 (target cells) were labeled with 40 nM 3,3′dihexyloxacarbocyanine ($DiOC_6$) (Marchetti et al., 1996) (Molecular Probes) for 10 min at 37°C. Target cells were labeled with PerCP-Cy5-conjugated anti-CD4 (BD Bioscience) for 20 min on ice. Effector cells were washed three times and then mixed with target cells in a U-bottomed, 96-well plate at different effector/target ratios (3:1, 1:1, and 0.3:1) in triplicate. K562 cells (target) with APC-conjugated anti-CD32 (BD Bioscience) and purified $CD56^+$ (NK) cells (effector) from four healthy donors were included as an assay control. After 4 hr of incubation at 37°C in the presence of SEB and anti-CD3/anti-CD28, cells were harvested and analyzed by flow cytometry. Percent cytotoxicity was calculated as follows: 100 × (% of total apoptotic target cells − % of spontaneous apoptotic target cells)/(100 − % of spontaneous apoptotic target cells).

SIV Suppression Assay

Autologous $CD4^+$ T cells from each animal were purified by magnetic positive labeling (MicroBeads; Miltenyi Biotec) and then acutely infected with SIVmac239 (10^{-3} multiplicity of infection) in the presence or absence of magnetically purified $CD8^+$ T cells at a CD4/CD8 ratio of 1:3, and then stimulated with SEB and CD3/CD28 antibodies for 16 hr. After washing, the cells were cultured in quadruplicates in 96-well plates. Cultures were maintained in a final volume of 200 μl per well of RPMI 1640 medium containing 100 IU of human rIL2 (Roche Diagnostics GmbH, Mannheim, Germany) for 5 days. Viral loads were measured by a real-time RT-PCR in culture supernatants collected at day 5 (see below). Fold suppression was determined as the geometric means of viral concentration in the culture supernatants from the infected $CD4^+$ target cells only divided by the geometric means of viral concentration in the supernatants from the mixed $CD8^+$ and $CD4^+$ T cells.

In some experiments, $CD8^+$ and $CD4^+$ T cells were cultured without cell-to-cell contact by means of a Multiwell Insert System (BD Biosciences; CD8 in the insert well and CD4 in the bottom well), $CD4^+$ T cells were cocultured with allogenic $CD8^+$ T cells in order to determine the correlation between viral suppression and MHC restriction, and $CD8^+$ and $CD4^+$ T cells were also cocultured in the presence of MHC-ABC (BioLegend) or MHC-E (Cell Science) antibodies to define the modes of MHC restriction. To define the subsets of $CD8^+$ T cells associated with antiviral activity, $CD8^+$ T cells were purified from PBMCs immediately after their depletion with PE-conjugate TCRγβ, Vβ8, or other CD antigen antibodies using anti-PE microbeads through an LD column (Miltenyi Biotec).

Viral Challenges

Three months after oral administration of the vaccine or the controls, the 16 animals of the first study were inoculated intrarectally with 2,500 MID_{100} (100,000 $TCID_{50}$) of pathogenic SIVmac239. Two months later, four vaccinated monkeys were rechallenged via the intrarectal route (100,000 $TCID_{50}$) and four others were intravenously rechallenged with 5 MID_{100} (200 $TCID_{50}$) of SIVmac239. As controls, two monkeys received an intrarectal challenge and other two received an intravenous challenge. These infectious doses generally result in a systemic infection in 100% of Chinese rhesus macaques with a peak plasma viral load (10^7–10^9 vp/ml) by days 10–14.

Thirteen months after the intragastric immunization (i.e., 8 months after the second challenge), the eight animals received a third intrarectal challenge with 100,000 $TCID_{50}$ of pathogenic SIVB670 (a distinct strain of SIV provided by F. Villinger, Emory University School of Medicine, Atlanta, GA, USA). SIVB670 was propagated in vitro using primary macaque PBMCs, and the first passages of the original viral stocks were used for challenge. Again, two additional untreated macaques (Mac-naive #5–6) received in parallel an intrarectal challenge of SIVB670 as control.

In the second study, the 16 animals were intrarectally challenged on day 420 postimmunization with 100,000 $TCID_{50}$ of SIVmac239.

Viral Loads

Cell-associated SIV DNA or plasma/supernatant SIV RNA was quantified by a quantitative PCR or RT-PCR with primers (sense, 5′-GAGGAAAAGAA ATTTGGAGCAGAA-3′; antisense, 5′-GCTTGATGGTCTCCCACACAA-3′) and probe (5′-FAM-AAAGTTGCACCCCCTATGACA TTAATC AGATGTTA-TAM RA-3′) for SIVmac239 and primers (sense, 5′-TTGAGCCCTGGG AGGTTCT-3′; antisense, 5′-GCCAAGTGCTGGTGAGAGTCT-3′) and probe (5′-FAM-AACA CCCAGGCTCTACCTGCTAGTGCTG-TAMRA-3′) for SIVB670 using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The sensitivity of the quantitative PCR or RT-PCR was three and ten copies for the input SIV DNA and RNA samples, respectively.

Depletion of $CD8^+$T Cells In Vivo

Macaques were first anesthetized and then given an intravenous injection of a chimeric CD8 monoclonal antibody (cMT-807; Centocor Research & Development, Malvern, PA, USA) at 5 mg/kg on days 0, 4, and 7 (Schmitz et al., 1999). Peripheral blood samples (5 ml) were taken from each animal at day 0 and at various time points after antibody injections.

Statistical Analysis

Unpaired data from different groups of animals, and paired data obtained before and after immunization were compared by using the t, Mann-Whitney, and Wilcoxon tests, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, three tables, and Extended Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.11.016.

LICENSING INFORMATION

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