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Long-Term Control of Simian Immunodeficiency Virus (SIV) in Cynomolgus Macaques Not Associated with Efficient SIV-Specific CD8$^+$ T-Cell Responses

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ABSTRACT

The spontaneous control of human and simian immunodeficiency viruses (HIV/SIV) is typically associated with specific major histocompatibility complex (MHC) class I alleles and efficient CD8$^+$ T-cell responses, but many controllers maintain viral control despite a nonprotective MHC background and weak CD8$^+$ T-cell responses. Therefore, the contribution of this response to maintaining long-term viral control remains unclear. To address this question, we transiently depleted CD8$^+$ T cells from five SIV-infected cynomolgus macaques with long-term viral control and weak CD8$^+$ T-cell responses. Among them, only one carried the protective MHC allele H6. After depletion, four of five controllers experienced a transient rebound of viremia. The return to undetectable viremia was accompanied by only modest expansion of SIV-specific CD8$^+$ T cells that lacked efficient SIV suppression capacity *ex vivo*. In contrast, the depletion was associated with homeostatic activation/expansion of CD4$^+$ T cells that correlated with viral rebound. In one macaque, viremia remained undetectable despite efficient CD8$^+$ cell depletion and inducible SIV replication from its CD4$^+$ T cells *in vitro*. Altogether, our results suggest that CD8$^+$ T cells are not unique contributors to the long-term maintenance of low viremia in this SIV controller model and that other mechanisms, such as weak viral reservoirs or control of activation, may be important players in control.

IMPORTANCE

Spontaneous control of HIV-1 to undetectable levels is associated with efficient anti-HIV CD8$^+$ T-cell responses. However, in some cases, this response fades over time, although viral control is maintained, and many HIV controllers (weak responders) have very low frequencies of HIV-specific CD8$^+$ T cells. In these cases, the importance of CD8 T cells in the maintenance of HIV-1 control is questionable. We developed a nonhuman primate model of durable SIV control with an immune profile resembling that of weak responders. Transient depletion of CD8$^+$ cells induced a rise in the viral load. However, viremia was correlated with CD4$^+$ T-cell activation subsequent to CD8$^+$ cell depletion. Regain of viral control to predepletion levels was not associated with restoration of the anti-SIV capacities of CD8$^+$ T cells. Our results suggest that CD8$^+$ T cells may not be involved in maintenance of viral control in weak responders and highlight the fact that additional mechanisms should not be underestimated.

A rare subset of human immunodeficiency virus type 1 (HIV-1)-infected patients called HIV controllers (HIC) are naturally able to maintain durable, tight control of the infection in the absence of therapy (1, 2). These cases are often considered examples in the search for a functional HIV cure (3). Most HIC are infected by replication-competent viruses (4–6), indicating that host mechanisms actively restrain HIV-1 infection. An efficient HIV-specific CD8$^+$ T-cell response is thought to play a decisive role in natural control. Some HLA class I molecules (especially HLA-B*5701 and -B*2705) are associated with lower viral loads (VL) (7) and are overrepresented in HIC (8, 9). Many HIC (strong responders [SR]) possess high frequencies of HIV-specific CD8$^+$ T cells that are highly functional in response to HIV antigens (9–11). These HIV-specific CD8$^+$ T cells can suppress *ex vivo* HIV infection of autologous CD4$^+$ T cells (9, 12), probably due to their ability to upregulate the cytotoxic-granule content (11, 13, 14).

However, HIC exhibit immunological heterogeneity; many of them (weak responders [WR]) are able to maintain viremia at undetectable levels despite displaying weak HIV-specific CD8$^+$ T-cell responses (12, 15, 16). Differences in T-cell responses between SR and WR cannot be explained by their expression of HLA class I alleles because they are overrepresented to the same extent in both groups (17). This raises the question of the real contribution of CD8$^+$ T-cell responses to the maintenance of long-term viral control in these patients. In WR, it is possible that highly reactive HIV-specific memory CD8$^+$ T cells expand and acquire effector functions in response to relapses in viral replication, thereby controlling the virus when necessary. In fact, a recent report showed that CD8$^+$ T cells from WR HIC can gain the capacity to suppress HIV replication after a short period of *in vitro* stimulation with HIV peptides (18). However, cells from antiretroviral-treated patients have also been shown to acquire similar properties following peptide stimulation (19) but cannot prevent viral rebound following treatment interruption.

Cases of spontaneous control of viral replication have been reported in some macaques infected with simian immunodefi-

ciency virus (SIV) (20–22). As in humans, these cases are mostly associated with a favorable genetic background (e.g., Mamu B*08 or B*17 in rhesus macaques [RM] or the H6 haplotype in cynomolgus macaques [CyM]) (20, 23–26). $CD8^+$ T cell-mediated control of infection in RM has been demonstrated through *in vivo* $CD8^+$ cell depletion experiments (21, 27) or by the occurrence of major histocompatibility complex (MHC) escape mutations in viruses from progressor macaques (28). However, these studies have focused mainly on animals carrying protective MHC alleles, and this may be a confounding factor when evaluating the roles of mechanisms other than T-cell responses.

Here, we report a high frequency of spontaneous set point viral control in 6 CyM intrarectally infected with low doses (5 50% animal infectious doses [AID_{50}]) of $SIVmac_{251}$. Five CyM displayed a long-term-controller profile. Four had an MHC haplotype distinct from the H6 haplotype that is usually associated with this phenotype, and they all displayed a strong decrease in $CD8^+$ T-cell antiviral activities over years of viral control. To our knowledge, this is the first report of an animal model that resembles the WR phenotype sometimes observed in HIC. We used this model to explore the contribution of $CD8^+$ T-cell responses in WR by transiently depleting CD8-expressing cells. Next, we performed phenotypic analyses and directly assessed the anti-SIV activity of $CD8^+$ T cells on superinfected autologous $CD4^+$ T cells, a function known to correlate with protection in HIV controllers. As previously reported, $CD8^+$ depletion induced transient viral escape, but unexpectedly, the CD8-mediated anti-HIV immunity was not strongly recalled and no increase in *ex vivo* antiviral activity could be detected at the time of the reestablishment of viral control.

MATERIALS AND METHODS

Ethics statement. Adult CyM (*Macaca fascicularis*) were imported from Mauritius and housed in the facilities of the Commissariat à l'Energie Atomique et aux Energies Alternatives (CEA) (Fontenay-aux-Roses, France). CyMs are used at the CEA in accordance with French national regulations and under the supervision of national veterinary inspectors (CEA permit number A 92-032-02). The CEA complies with the Standards for Human Care and Use of Laboratory Animals of the Office for Laboratory Animal Welfare (OLAW) (USA) under OLAW Assurance number A5826-01. All experimental procedures were conducted according to European guidelines for animal care. This experiment was approved by the ethics committee Comite d'Ethique en Experimentation Animale de la Direction des Sciences du Vivant au CEA under reference number 10-006.

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Animals, infection, and $CD8^+$ depletion. The MHC haplotype was determined as previously described (23). Six animals were intrarectally inoculated with 5 AID_{50} of an uncloned $SIVmac_{251}$ isolate (provided by A. M. Aubertin, Université Louis Pasteur, Strasbourg, France). These animals were followed for up to 6 years postinfection (p.i.). For $CD8^+$ depletion, animals were intravenously treated with a single dose of the anti-human CD8 monoclonal antibody CM-T807 (50 mg/kg of body weight) provided by the National Institutes of Health nonhuman primate reagent resources. These animals were compared to another group of 11 animals infected intrarectally with 50 AID_{50} of the same isolate.

Sample collection and processing. Blood sampling (days −15, −8, 3, 7, 10, 13, 17, 21, 28, 35, 42, 65, and 177), bronchoalveolar lavages (BAL) (days −15, 14, 44, and 177), lymph node (LN) biopsies (days −15, 13, and 35, 42, or 177, depending on the kinetics of the reconstitution of $CD8^+$ T cells), and rectal biopsies (RB) (days −20, 15, and 35, 43, or 177, depending on the kinetics of the reconstitution of $CD8^+$ T cells) were performed after ketamine anesthesia. Blood samples were collected in BD Vacutainer Plus Plastic K3 EDTA tubes (BD Biosciences, France). Tissue samples were collected in phosphate-buffered saline (PBS) or snap-frozen in liquid nitrogen for storage at −80°C.

Plasma was isolated from EDTA blood samples by centrifugation for 10 min at 1,500 × *g* and cryopreserved. Whole blood, peripheral blood leukocytes (PBLs), peripheral blood mononuclear cells (PBMCs), BAL fluid, LN, RB cell suspensions, and purified $CD4^+$ and $CD8^+$ T cells were used for experiments. Peripheral LN cells were obtained using a GentleMACS dissociator (Miltenyi Biotech). Cell suspensions from RB were obtained by a protocol used for humans (29) that was adapted in house for macaques. Briefly, several 1-mm^2 punches of mucosa were collected and digested for 45 min with collagenase II (Sigma-Aldrich), mechanically disrupted with a syringe equipped with an 18-gauge blunt-end needle, and passaged through a 70-μm-pore-size cell strainer. Finally, cell suspensions were isolated using a 30%-70% Percoll gradient. BAL fluid was passed through a 100-μm-pore-size cell strainer and washed with PBS to obtain the final cell suspension.

$CD4^+$ and $CD8^+$ T cells were purified from cell suspensions with antibody-coated magnetic beads in a Robosep instrument (Stemcell Technologies). $CD4^+$ T cells were obtained with a custom positive nonhuman primate $CD4^+$ T-cell selection kit, and untouched $CD8^+$ T cells were obtained subsequently with a custom negative nonhuman primate $CD8^+$ T-cell selection kit (Stemcell Technologies).

T-cell phenotypic characterizations by flow cytometry. Analyses were performed on whole blood, PBLs, or cell suspensions. A list of the antibodies used is provided in Table S1 in the supplemental material. Naive cells were defined as $CD95^-$ $CD28^+$, central memory (CM) cells as $CD95^+$ $CD28^+$ $CCR5^-$ $CCR7^+$, transitional memory (TM) cells as $CD95^+$ $CD28^+$ $CCR5^+$, and effector memory (EM) cells as $CD95^+$ $CD28^-$. Antibodies were added to 50 μl of blood or 2×10^6 cells from tissues and, after 15 min of incubation, red blood cells were lysed with fluorescence-activated cell sorter (FACS) lysing solution (BD Biosciences). Ki67 staining was performed after permeabilization with the IntraStain kit (Dako). The cells were washed and resuspended in CellFix prior to acquisition.

ICS. For intracellular cytokine staining (ICS), cell preparation, culture, stimulation, staining, acquisition, and analysis were performed as described previously (30). Peptide pools consisting of 15-mer overlapping

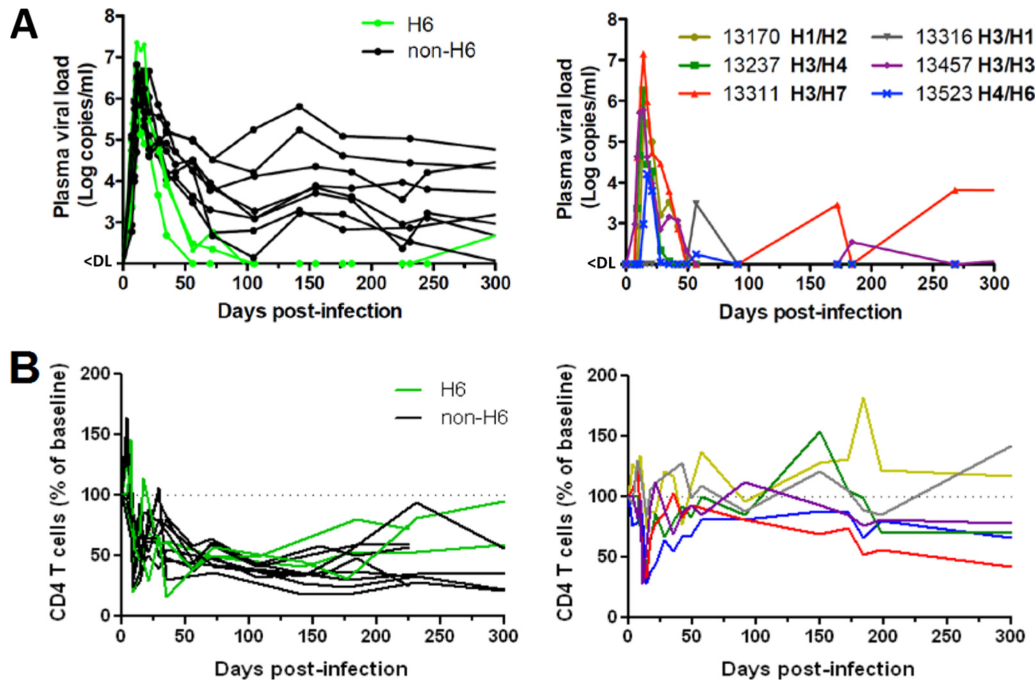

FIG 1 MHC H6 haplotype and low-dose intrarectal infection are both associated with spontaneous control of $SIVmac_{251}$ infection in CyM. (A) Longitudinal evolution of plasma viral loads in macaques exposed intrarectally to 50 AID_{50} ($n = 11$) (left) or 5 AID_{50} ($n = 6$) (right) of $SIVmac_{251}$. In the 50-AID_{50} group, MHC H6-bearing animals are shown in green and non-H6 animals in black. Animals in the 5-AID_{50} group are color coded, and MHC haplotypes are indicated for each animal. The viral-RNA QL and DL were 111 and 37 copies of vRNA/ml, respectively. (B) Longitudinal evolution of $CD4^+$ T-cell blood counts in the 50-AID_{50} group ($n = 11$) (left) and in the 5-AID_{50} group ($n = 6$) (right). $CD4^+$ T-cell counts are expressed as a percentage of the mean preinfection value for each macaque.

peptides (11-amino-acid overlap, 1 μM each, covering the Gag, Vif, Rev, and Nef proteins of $SIVmac_{251}$ [Proteogenix, Strasbourg, France]) were used for stimulation. Gag was covered by two peptide pools, one encompassing p6 to p8 and the other p15 to p27.

Measurement of $CD8^+$ T-cell-mediated SIV suppression. A previously described assay for measuring the capacity of human $CD8^+$ T cells to suppress HIV infection of autologous $CD4^+$ T cells (31) was adapted to the CyM-$SIVmac_{251}$ model. Briefly, purified $CD4^+$ cells were stimulated for 3 days with 10 μg/ml concanavalin A (ConA) in the presence of interleukin 2 (IL-2) (Chiron) at 100 IU/ml. $CD8^+$ T cells were maintained in culture in the absence of mitogens and cytokines. Then, $CD4^+$ T cells (10^5) in 96-well plates were superinfected with $SIVmac_{251}$ (multiplicity of infection [MOI], 10^{-2}) using a spinoculation protocol (32) in the presence or absence of $CD8^+$ T cells (CD8/CD4 ratio, 1:1). After challenge, the cells were washed and cultured for 14 days. The capacity of $CD8^+$ T cells to suppress infection was calculated as the $\log_{10}$ drop in p27 levels registered at the peak of viral replication in $CD4^+$ T cells when $CD8^+$ T cells were present in the culture.

Detection of viral production in culture supernatants. Reverse transcriptase activity was measured by using the Lenti-RT Activity Assay (Cavidi Tech), and p27 protein concentrations were assayed with the Retro-Tek SIV p27 Antigen ELISA (enzyme-linked immunosorbent assay) kit (ZeptoMetrix), both following the manufacturer's instructions.

vRNA quantification in tissues and plasma. Absolute concentrations of plasma viral RNA (vRNA) and tissue vDNA were determined as previously described (33, 34). The SIV Gag primers and probe used were as follows: Forward, GCAGAGGAGGAAATTACCCAGTAC; Reverse, CAA TTTTACCCAGGCATTTAATGTT; and Probe, TGTCCACCTGCCATT AAGCCCGA. The GAPDH (glyceraldehyde-3-phosphate dehydrogenase) primers and probe used were as follows: Forward, GAAGGTGAAG GTCGGAGTC; Reverse, GAAGATGGTGATGGGATTTC; and Probe, CAAGCTTCCCGTTCTCAGCC.

The quantification limit (QL) for vRNA in the classical assay was estimated to be 111 copies/ml, and the detection limit (DL) was estimated to be 37 copies/ml. A more sensitive method was applied for the follow-up of the CD8 depletion phase, as previously described (35). In this case, the QL and DL were 37 and 12.3 copies of vRNA/ml, respectively.

Cytokine quantification. Cytokine concentrations in plasma were assayed with Luminex (23-plex nonhuman primate kit; Merck Millipore) with a MagPix instrument. Plasma IL-7 levels were determined using a Human IL-7 ELISA kit (R&D Systems), as previously described (36).

Western blot analysis. Western blots were performed using a commercial SIV Western Blot Assay kit (ZeptoMetrix) following the manufacturer's instructions.

Statistical analysis. The nonparametric Spearman rank correlation test was used to investigate the relationship between variables. The nonparametric Mann-Whitney test was used to compare data sets between groups, and the paired nonparametric Wilcoxon signed-rank test was used to compare data from the same macaques at different time points. All statistical analyses were performed using GraphPad Prism 5.03 software (GraphPad Software, La Jolla, CA, USA). In the 2-tailed tests, *P* values of 0.05 or lower were considered to be significant.

RESULTS

A high proportion of controllers among cynomolgus macaques exposed intrarectally to 5 AID_{50} of $SIVmac_{251}$. Atraumatic intrarectal exposure of 11 CyM to high doses (50 AID_{50}) of an uncloned $SIVmac_{251}$ isolate resulted in relatively similar peaks of plasma viremia in all animals. The peaks were observed on day 11 p.i., with viral loads ranging from 7×10^5 to 6×10^7 RNA copies/ml (median, 3×10^6 RNA copies/ml) (Fig. 1A). Three CyM bearing the protective H6 haplotype (23, 24) demonstrated control of plasma viremia below 100 copies/ml at the set point (3

months). In contrast, none of the non-H6 macaques controlled their viremia to such a low level at this time (median, 3×10^4 RNA copies/ml), and their $CD4^+$ T-cell counts declined during the first year of infection (Fig. 1B).

In comparison, the exposure of six macaques to 10-fold-lower doses (5 AID_{50}) of the same virus isolate by the same route was associated with diverse peaks of plasma viremia, ranging from 5×10^1 to 1.42×10^7 RNA copies/ml (median, 4.41×10^5 RNA copies/ml) (Fig. 1A). Early spontaneous control below 100 RNA copies/ml at the set point (3 months) was observed in all six macaques, although only one (no. 13523) possessed the H6 haplotype. One macaque (13311) lost control of the infection shortly after, between 3 and 6 months p.i. The remaining five macaques remained below 400 RNA copies/ml for >5 years. One macaque (13316) did not seroconvert and displayed only blips of viremia early during the course of the infection. The $CD4^+$ T-cell count declined during acute infection in four of the five controllers in the 5-AID_{50} group and recovered almost completely at the set point with no major changes afterward (Fig. 1B).

These observations revealed a high proportion of long-term SIV controllers (SIC) in a group of CyM exposed intrarectally to a relatively low dose (5 AID_{50}) of $SIVmac_{251}$ that could not be related solely to a favorable MHC haplotype.

SIV controllers displayed weak $CD8^+$ T-cell activation, cytokine production, and SIV-suppressive capacity during the chronic phase of infection. Because the 5-AID_{50} CyM were not initially dedicated to the analysis of T-cell immunity during primary infection, we could explore the $CD8^+$ T-cell response in the five SIC only after 2 years p.i. We compared the $CD8^+$ T-cell responses to those of the animal that had lost viral control (13311) and six other CyM with uncontrolled viremia (median VL, 5×10^4 copies/ml) that were infected at the same time with a higher dose of the same virus stock. T-cell activation, measured as either CD69 expression or CD38–HLA-DR coexpression, was lower in controllers than in noncontrollers (Fig. 2A). ICS revealed that Gag-specific $CD8^+$ T-cell responses (especially the gamma interferon [IFN-γ]- and MIP-1β-producing cells that constituted the largest proportion of the response) were generally lower in controllers than in noncontrollers (Fig. 2B).

$CD8^+$ T cells from HIC are endowed *ex vivo* with a strong capacity to suppress HIV infection of autologous $CD4^+$ T cells (9, 12). We adapted this technique (31) to assess the capacity of $CD8^+$ T cells from CyM to suppress SIV infection of autologous $CD4^+$ T cells. $CD8^+$ T cells from the five SIC collected on day 634 p.i. showed some capacity to suppress SIV infection, although their capacities were not stronger than those from noncontroller CyM (Fig. 2C). However, the $CD8^+$ T cells from the animal with the H6 haplotype (13523) had the strongest suppressive capacity, even at low effector/target cell ratios (Fig. 2D). The suppressive capacities of the five SIC decreased with time, although the anti-SIV activity of the cells from the H6 animal (13523) faded later (Fig. 2E). The SIV-suppressive capacity of the CyM that lost control shortly after the set point (13311) was relatively stable over time, albeit low. $CD8^+$ T cells from the remaining five CyM displayed weak cytotoxic activity 5.5 years p.i. (Fig. 2E). The virus could be isolated *in vitro* by reactivation of enriched $CD4^+$ T cells from all six macaques. When we evaluated the infectiousness of the viruses from three SICs, we found that they were able to infect $CD4^+$ T cells from uninfected macaques to the same extent as the original $SIVmac_{251}$ isolate (see Fig. S1 in the supplemental material).

In summary, the SIC displayed lower T-cell activation and lower SIV-specific $CD8^+$ responses than viremic animals during the chronic phase despite the persistence of cells infected with replication-competent viruses. The immune profile of these animals resembles that of the HIC WR that we have described previously (12).

***In vivo* depletion of $CD8^+$ cells resulted in transient elevation of viral loads in four controllers.** We assessed the consequences of *in vivo* depletion of $CD8^+$ cells in the five SIC. Animal 13311, which lost control of viremia earlier during infection, was included in this study as a reference control. A single injection of the human monoclonal antibody CM-T807 resulted in profound $CD8^+$ T-cell depletion from the blood (Fig. 3A; see Fig. S2 in the supplemental material); the depletion lasted at least 10 days. The $CD8^+$ T-cell pool progressively recovered in all controllers, with kinetics differing between animals in the following order: 13523, 13457 (days 10 to 13), 13237, 13170 (days 17 to 21), and 13316 (days 65 to 177). The health of the progressor animal (13311) quickly degraded 5 weeks after $CD8^+$ depletion, and the animal died of AIDS hours before scheduled euthanasia and before any detectable $CD8^+$ cell recovery was observed.

Similar kinetics were observed in the peripheral LN (Fig. 3B; see Fig. S3A in the supplemental material), as well as in RB specimens and BAL samples (see Fig. S3B in the supplemental material) from all animals. Due to the limited tissue availability, we could not determine whether the $CD8^+$ cell depletion was incomplete or lasted for a shorter time in tissues from animals 13523 and 13457, which had detectable $CD8^+$ cells in their LN at day 13 after depletion. Other $CD8^+$ cells, including $CD3^-$ $CD8^+$ NK cells (data not shown) and double-positive ($CD4^+$ $CD8^+$) T cells (Fig. 3A), showed amplitudes and kinetics of depletion similar to those of $CD8^+$ T cells. During the depletion, all remaining $CD3^+$ T cells were $CD4^+$ single-positive T cells, and no increase in the numbers of double-negative $CD3^+$ T cells was detected (Fig. 3A).

Upon depletion, four of the five SIC and the viremic CyM experienced a transient increase in their viral-RNA loads in plasma (Fig. 3C) and their levels of cell-associated SIV DNA in lymphoid and mucosal tissues (Fig. 3D). The viremia peaked on day 13 (13523, 13457, and 13237) or 17 (13170) postdepletion. The viremic animal (13311) experienced an earlier peak of viremia (day 7), which then returned to the predepletion level and eventually increased again. Interestingly, the remaining SIC (13316) never lost viral control following depletion, and the viral load in plasma and in tissues remained undetectable throughout the follow-up phase (see below).

The $CD8^+$ T-cell recovery appeared to coincide temporally with the regain of viral control in three controllers (13170, 13523, and 13457) (Fig. 3C), whereas the regain of viral control began despite $CD8^+$ T cells remaining undetectable in blood or tissues in one SIC (13237) (Fig. 3C; see Fig. S2 and S3 in the supplemental material).

To characterize the $CD8^+$ T-cell recovery after depletion, the naive and memory phenotypes and activation statuses of the cells were assessed by flow cytometry. Most $CD8^+$ T cells returning after the depletion phase possessed an EM phenotype (Fig. 4A) and were much more activated during the reconstitution phase than before depletion (Fig. 4B). EM $CD8^+$ T cell activation peaked on day 28 postdepletion, reaching levels higher than that observed in the progressor (13311) at baseline, and progressively faded with time. The macaque bearing the H6 MHC haplotype (13523) re-

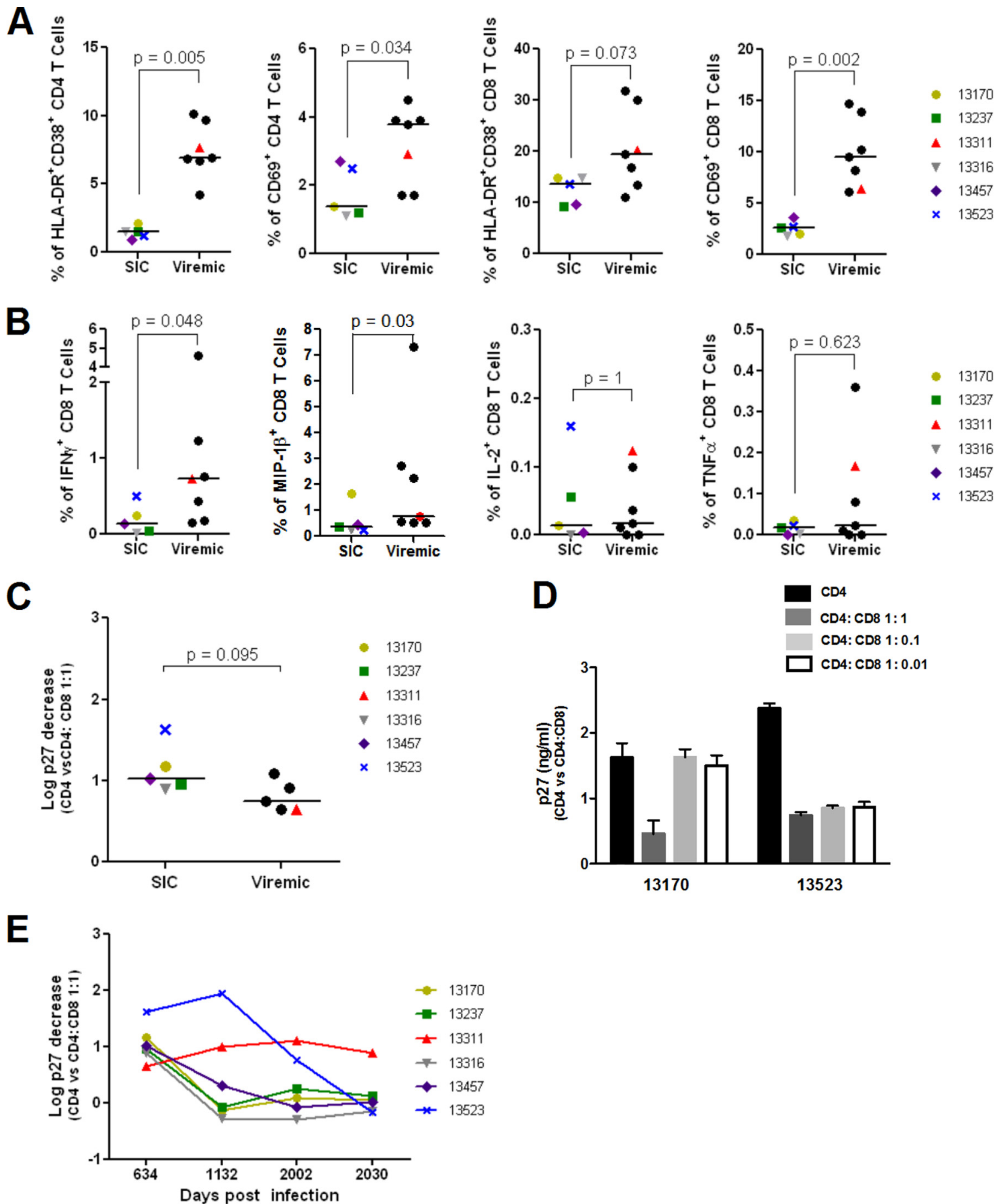

FIG 2 SIV controllers display low T-cell activation and low CD8$^+$ T-cell responses during the chronic phase. Two years p.i., the five long-term controllers of the 5-AID$_{50}$ group were compared to the only viremic animal in the group (13311) and six other noncontroller macaques. (A) The coexpression of HLA-DR and CD38 and the expression of CD69 by CD4$^+$ (left) and CD8$^+$ (right) T cells were assessed to evaluate chronic T-cell activation. (B) SIV Gag-specific CD8$^+$ T-cell responses were assessed by ICS after stimulation with a pool of overlapping SIV Gag p15-p27 peptides. Shown are (from left to right) the percentages of IFN-γ^+, MIP-1β^+, IL-2$^+$, and tumor necrosis factor alpha-positive (TNF-α^+) cells. (C) The SIV-suppressive activity of blood CD8$^+$ T cells from SIC and noncontroller animals was measured on autologous activated CD4$^+$ T cells superinfected *in vitro* with SIVmac$_{251}$. SIV suppression is reported as the log decline in p27 titers in CD4$^+$ T cell supernatants when autologous *ex vivo* unstimulated CD8$^+$ T cells were added at a 1:1 ratio to the culture. (A to C) Each symbol represents one animal, and horizontal lines represent the median value for the group. (D) SIV replication in culture supernatants of CD4$^+$ T cells from SIC 13170 and 13523 in the absence of CD8$^+$ T cells or at various CD4$^+$/CD8$^+$ T-cell ratios. The error bars indicate standard deviations. (E) Longitudinal follow-up of CD8$^+$ T-cell-dependent SIV-suppressive antiviral activity over 3 years in the six macaques exposed to an inoculum of 5 AID$_{50}$ of virus.

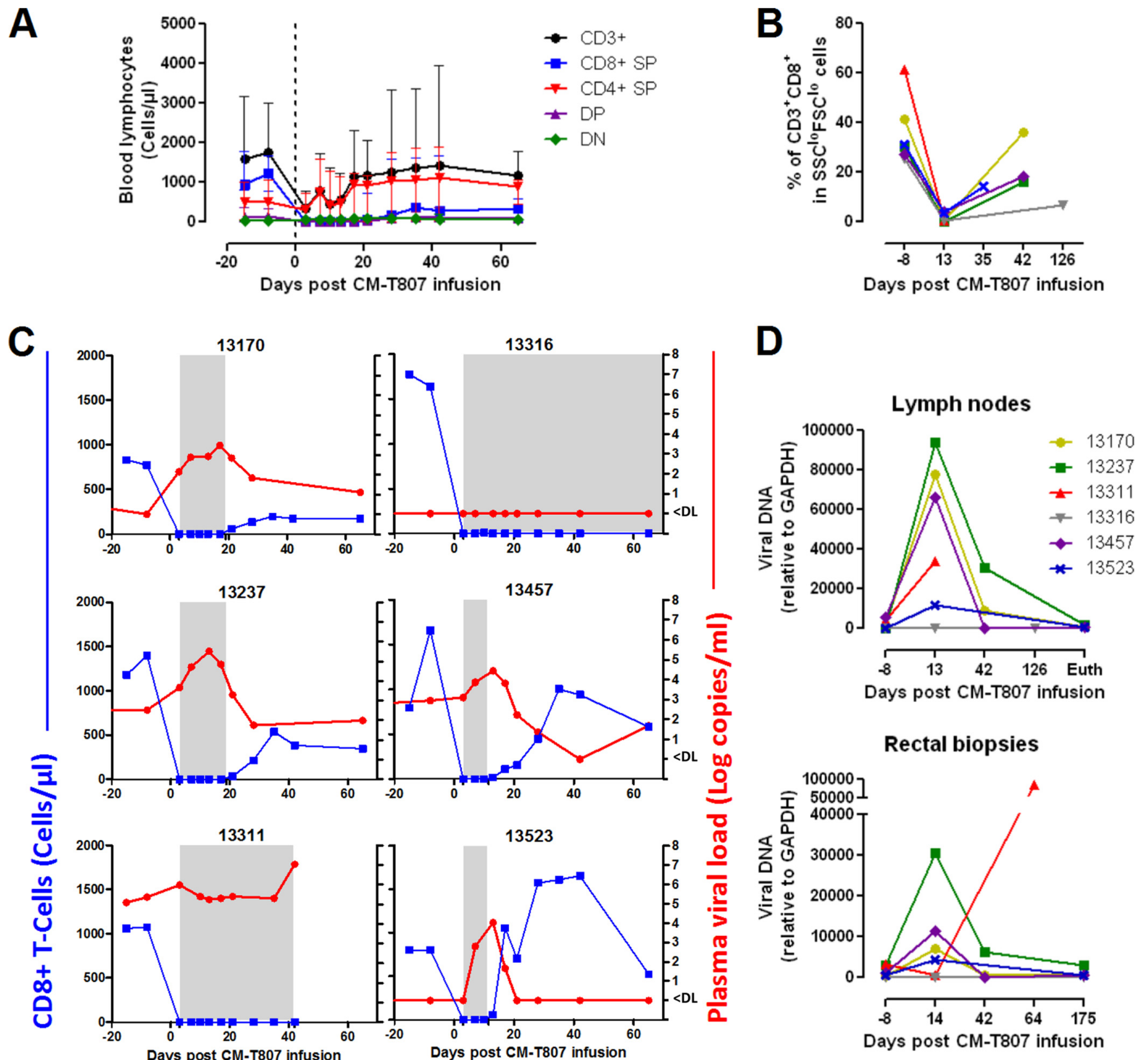

FIG 3 *In vivo* depletion of $CD8^+$ cells after 5 years of chronic infection led to a transient increase in viremia in all but one SIV controller. The consequences of CM-T807 monoclonal antibody (MAb) infusion for T-cell populations and viral loads were monitored by flow cytometry and quantitative PCR (qPCR), respectively. (A) Evolution of circulating T-cell populations, including total $CD3^+$, single-positive ($CD4^+$ or $CD8^+$) (SP), double-positive ($CD4^+$ $CD8^+$) (DP), and double-negative ($CD4^-$ $CD8^-$) (DN) T cells. Median values and ranges are reported. (B) Frequencies of $CD8^+$ T cells in peripheral LN before and after CM-T807 MAb infusion. (C) Temporal association between $CD8^+$ T-cell counts (blue) and viral-RNA loads (red) for each macaque. The time period during which $CD8^+$ T cells were undetectable is shaded in gray. The viral-RNA QL and DL were 37 and 12.3 copies of vRNA/ml, respectively. (D) Cell-associated viral-DNA loads in the peripheral LN (top) and rectal mucosa (bottom).

constituted EM $CD8^+$ T cells the fastest and to the largest extent, but with the lowest activation levels.

Weak expansion of SIV-specific $CD8^+$ T-cell responses and no increase in SIV-suppressive activity were observed during the $CD8^+$ recovery phase. Next, we analyzed the evolution of the SIV-specific $CD8^+$ T-cell response in these animals during the $CD8^+$ cell recovery period. We followed the ability of recovered $CD8^+$ T cells to suppress SIV replication in autologous $CD4^+$ T cells *in vitro*. Activation of $CD8^+$ T cells during reconstitution was not accompanied by an increase in their capacity to suppress SIV infection, which was weak and did not change in the blood of any animal at the time of $CD8^+$ T-cell reconstitution (Fig. 5A). Only cells from macaque 13237 showed slightly higher antiviral activity on day 28. The SIV-suppressive activities of $CD8^+$ T cells isolated from BAL fluid and peripheral LN from SIC were also low at baseline and did not significantly change upon $CD8^+$ T-cell reconstitution (see Fig. S4A in the supplemental material). Total PBMCs could be infected *in vitro* to the same extent as purified

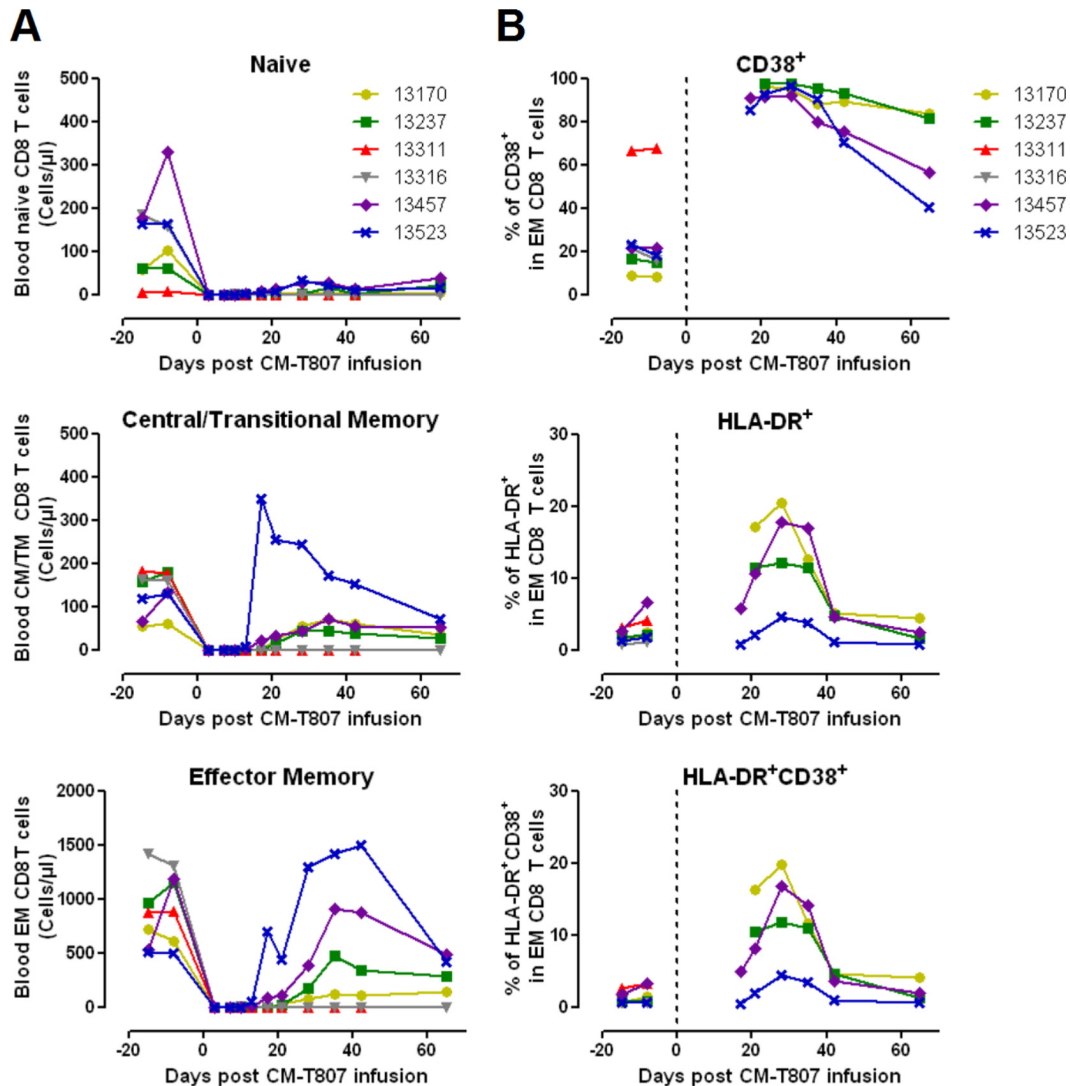

FIG 4 $CD8^+$ T cells recovering after depletion are mostly activated effector memory cells. $CD8^+$ T-cell subpopulations and activation levels (CD38 and HLA-DR expression) in blood were monitored by flow cytometry. Naive, CM, and EM cells were distinguished on the basis of CD28 and CD95 expression levels. (A) Follow-up of absolute counts of naive, CM/TM, and EM $CD8^+$ T cells in blood at various time points. (B) Expression of the activation markers CD38 (top), HLA-DR (middle), or both (bottom) by EM $CD8^+$ T cells. Note that 13311 did not show any reconstitution of $CD8^+$ cells before death and 13316 exhibited extremely late partial reconstitution of $CD8^+$ cells; this precluded any phenotypic analysis of $CD8^+$ T cells in 50 µl of whole blood for these 2 animals during this period.

$CD4^+$ T cells (Fig. 5B; see Fig. S4B in the supplemental material), suggesting the absence of other anti-SIV activities in these cultures.

ICS after stimulation with pools of overlapping peptides covering the SIV Gag, Nef, Rev, and Vif proteins was used to evaluate the frequency of SIV-specific $CD8^+$ T-cell responses in PBMCs after depletion. A transient relative increase in the percentage of cytokine-producing $CD8^+$ T cells was observed in the five SIC upon $CD8^+$ T-cell reconstitution (Fig. 5C), but these increases were translated into an increase in the absolute numbers of circulating SIV-specific $CD8^+$ T cells in only two animals (13170 and 13457) and remained at low amplitude (Fig. 5C and D). Remarkably, the H6 macaque (13523) displayed the smallest relative increase in the frequency of SIV-specific $CD8^+$ T cells. The polyfunctionality of the cells did not increase following the reconstitution of the $CD8^+$ T-cell compartment, consistent with the weak SIV suppression activities of the $CD8^+$ T cells observed for these animals. Furthermore, no trend toward an increase in a particular SIV antigen-specific response was observed (see Fig. S5 in the supplemental material).

Activated $CD8^+$ T cells can produce soluble factors, such as β-chemokines, that are able to block SIV replication. Therefore, we assessed plasma levels of MIP-1α and MIP-1β. There was an increase in the levels of both β-chemokines that peaked a few days postdepletion and decreased before viremia started to decline (see Fig. S6 in the supplemental material).

ICS analysis revealed the expansion of SIV-specific $CD4^+$ T-cell responses in all controllers upon $CD8^+$ depletion; these cells retained better polyfunctionality than those from the viremic macaque (Fig. 5E). Of note, the highest expansion of SIV-specific $CD4^+$ T cells was observed in the H6 animal (13523) (Fig. 5F). In contrast, the progressor macaque (13311) showed a contraction of the SIV-specific $CD4^+$ T-cell response associated with a severe depletion of $CD4^+$ T cells.

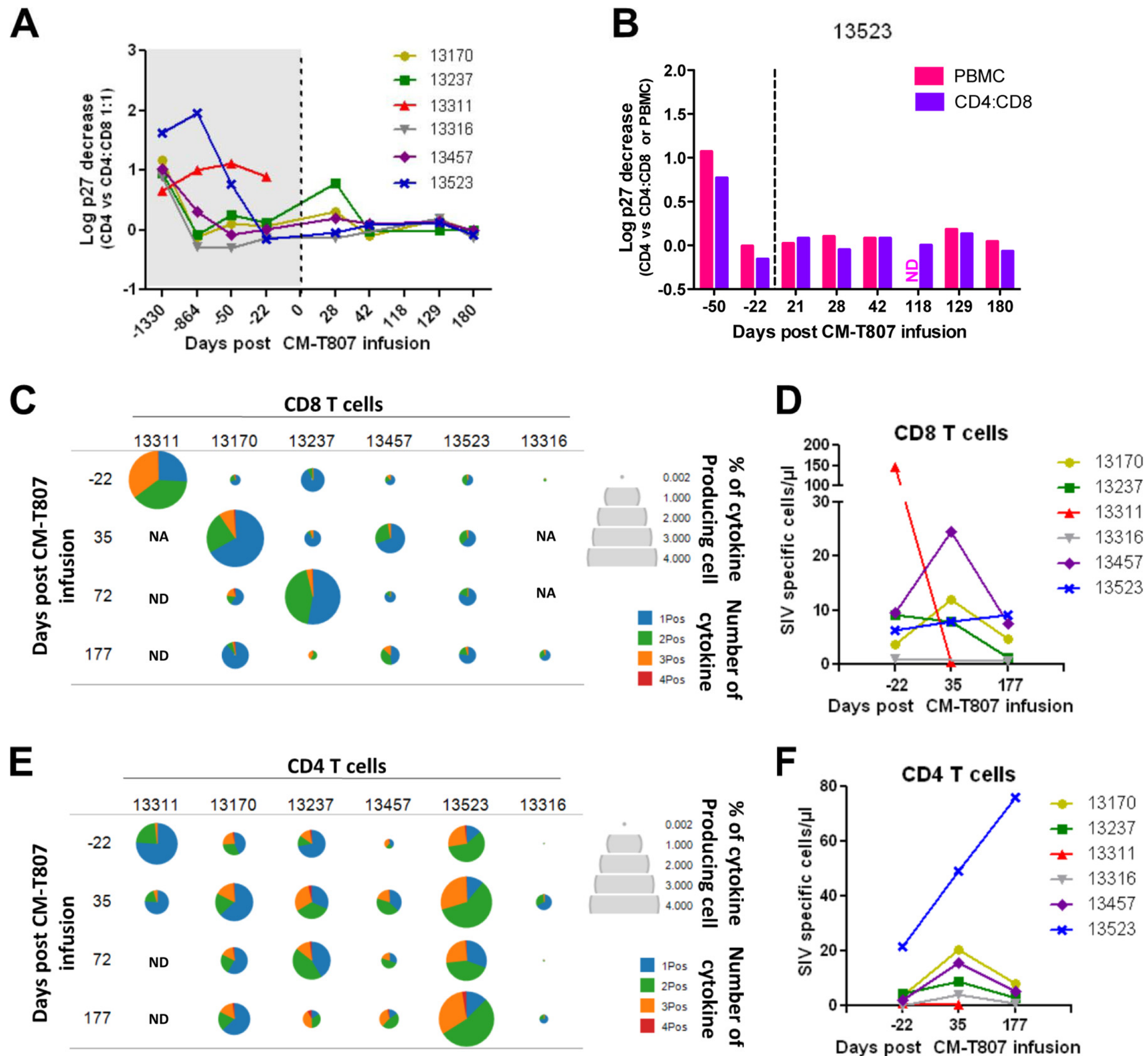

FIG 5 Regain of viral control in SIV controllers upon CD8$^+$ cell reconstitution was not associated with efficient SIV-suppressive antiviral activities. SIV-specific responses were monitored in peripheral blood by functional assays and ICS. (A) Evolution of the capacity of CD8$^+$ T cells to suppress SIVmac$_{251}$ replication in autologous CD4 T cells. Predepletion activities (Fig. 2D) are represented in the gray-shaded area for reference. (B) Suppression of SIV replication in PBMCs and in CD4$^+$-CD8$^+$ cocultures (1:1 ratio) from animal 13523 at various time points in comparison to CD4$^+$ T cells cultured alone. ND, not done. (C and E) Cytokine production by CD8$^+$ (C) and CD4$^+$ (E) T cells assayed by ICS. Cumulative responses after stimulation with pools of overlapping peptides from SIV Gag, Rev, Nef, and Vif are represented. The size of each pie is proportional to the percentage of T cells expressing at least one cytokine, including IFN-γ, TNF-α, MIP1β, and IL-2, and the proportions of cells expressing 1 (1Pos), 2 (2Pos), 3 (3Pos), or 4 (4Pos) cytokines are displayed in each pie. NA, not applicable because the samples did not contain enough CD8$^+$ T cells to be analyzed by flow cytometry. ND, not done because 13311 died 2 months after CD8$^+$ cell depletion. (D and F) Absolute counts of circulating SIV-specific T cells expressing at least one cytokine.

Overall, using a highly effective readout of T-cell functionality (i.e., cytokine production, soluble-suppressive-factor secretion, and direct suppressive ability), we were unable to show evidence of a robust pattern of CD8$^+$ T-cell responses during the return to predepletion viremia levels after viral rebound.

Controllers experienced transient proliferation and activation of transitional memory CD4$^+$ T cells associated with an expansion of effector memory CD4$^+$ T cells and increased viral replication. It was plausible that the increases in the SIV-specific CD4$^+$ T-cell responses were related to the homeostatic expansion of the CD4$^+$ T-cell compartment, which has been recently reported to occur as a consequence of CD8$^+$ cell depletion *in vivo* (37, 38). Thus, we monitored changes in the naive/memory subpopulations and the activation and proliferation of CD4$^+$ T cells by flow cytometry. All SIC experienced a progressive increase in the absolute numbers of CD4$^+$ T cells in blood (Fig. 6A). In particular, there was a strong expansion of CD4$^+$ T_{EM} cells (mean increase, 8.47- ± 5.40-fold). CM and TM CD4$^+$ T cell counts also increased, although to a lesser extent (1.95- ± 1.26- and 2.10- ± 1.08-fold mean increases, respectively). During their expansion phase, CD4$^+$ T cells showed a large increase in the expression of the activation markers CD69 (Fig. 6B), CD38, and HLA-DR (data not shown). Two consecutive peaks of proliferation based on Ki67 expression were observed in the 1st and the 3rd weeks after CD8$^+$ cell depletion (Fig. 6B).

Transient increases in IL-2 (1.95-fold), IL-7 (2.6-fold), and IL-15 (1.8-fold) concentrations in the plasma were observed following CD8$^+$ cell depletion and coincided with the initial peaks of

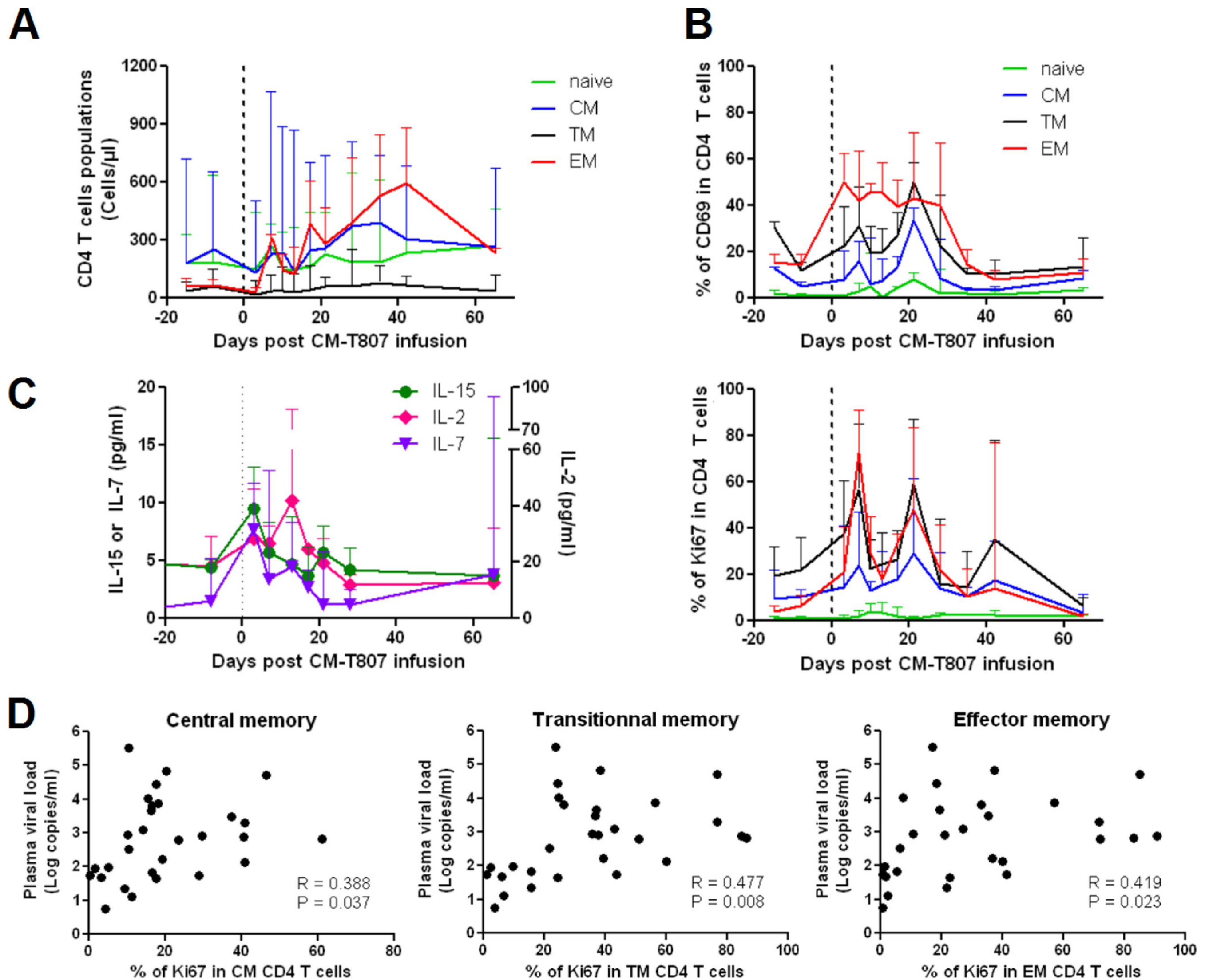

FIG 6 Viral replication in SIV controllers upon CD8$^+$ cell depletion is associated with homeostatic activation levels of the CD4$^+$ T-cell compartment. Flow cytometry and Luminex assays were used to monitor CD4$^+$ T cells and cytokine dynamics in response to CD8$^+$ cell depletion. (A) Absolute blood counts of naive, CM, TM, and EM CD4$^+$ T-cell subpopulations in SIC. (B) Evolution of the activation and proliferation levels of blood CD4$^+$ T-cell subpopulations. Shown are the percentages of activated CD69$^+$ (top) and proliferative Ki67$^+$ (bottom) naive, CM, TM, and EM CD4$^+$ T cells in blood. (C) Plasma IL-2, IL-7, and IL-15 concentrations. (A to C) Median values and ranges are reported. (D) Spearman correlation between Ki67 expression in CM, TM, and EM CD4$^+$ T cells and plasma viral loads after CD8$^+$ cell depletion in SIC. Data pairs for which the viral load was undetectable were excluded.

activation of memory CD4$^+$ T cells (Fig. 6C). The activation and expansion of EM CD4$^+$ T cells coincided with the transient increase in viremia in SIC, and the plasma viral-RNA loads following CD8$^+$ depletion correlated with the percentage of Ki67$^+$ CD4$^+$ memory cells (Fig. 6D). These findings suggest that the transient rebound of viremia in SIC might result from homeostatic CD4$^+$ T-cell activation and proliferation.

An exceptional profile of one SIV controller with persistently undetectable viral loads despite CD8$^+$ cell depletion. Macaque 13316 was the only animal that did not experience any detectable increase in viremia following the depletion of CD8$^+$ cells (Fig. 3C and D). This animal showed the lowest levels of CD4$^+$ T-cell activation and expansion following CD8$^+$ cell depletion (Fig. 7A and data not shown). Moreover, only a few blips of detectable plasma viral loads were detected in the animal during the acute phase (Fig. 1), and the viral load remained below 12 copies/ml for 5 years during the chronic stage. Unlike all of the other animals in the 5-AID$_{50}$ group, macaque 13316 did not seroconvert and showed no detectable reactivity against any SIV proteins before the depletion experiment (Fig. 7B). This macaque had extremely weak T-cell responses upon stimulation with SIV antigens (Fig. 5C and E). The hallmark of persistent infection in this animal included several *in vitro* reactivations of the virus, evidenced by measuring SIVp27 and reverse transcriptase activity from purified CD4$^+$ T cells collected from both blood and LN (Fig. 7C). The virus isolate was able to infect heterologous CD4$^+$ T cells *in vitro*, and the sequencing of a region of *gag* showed a match with the original SIVmac$_{251}$ sequence (data not shown). Interestingly, the detection of viral replication was easier during the first years of control, when SIV could often be reactivated by mitogenic stim-

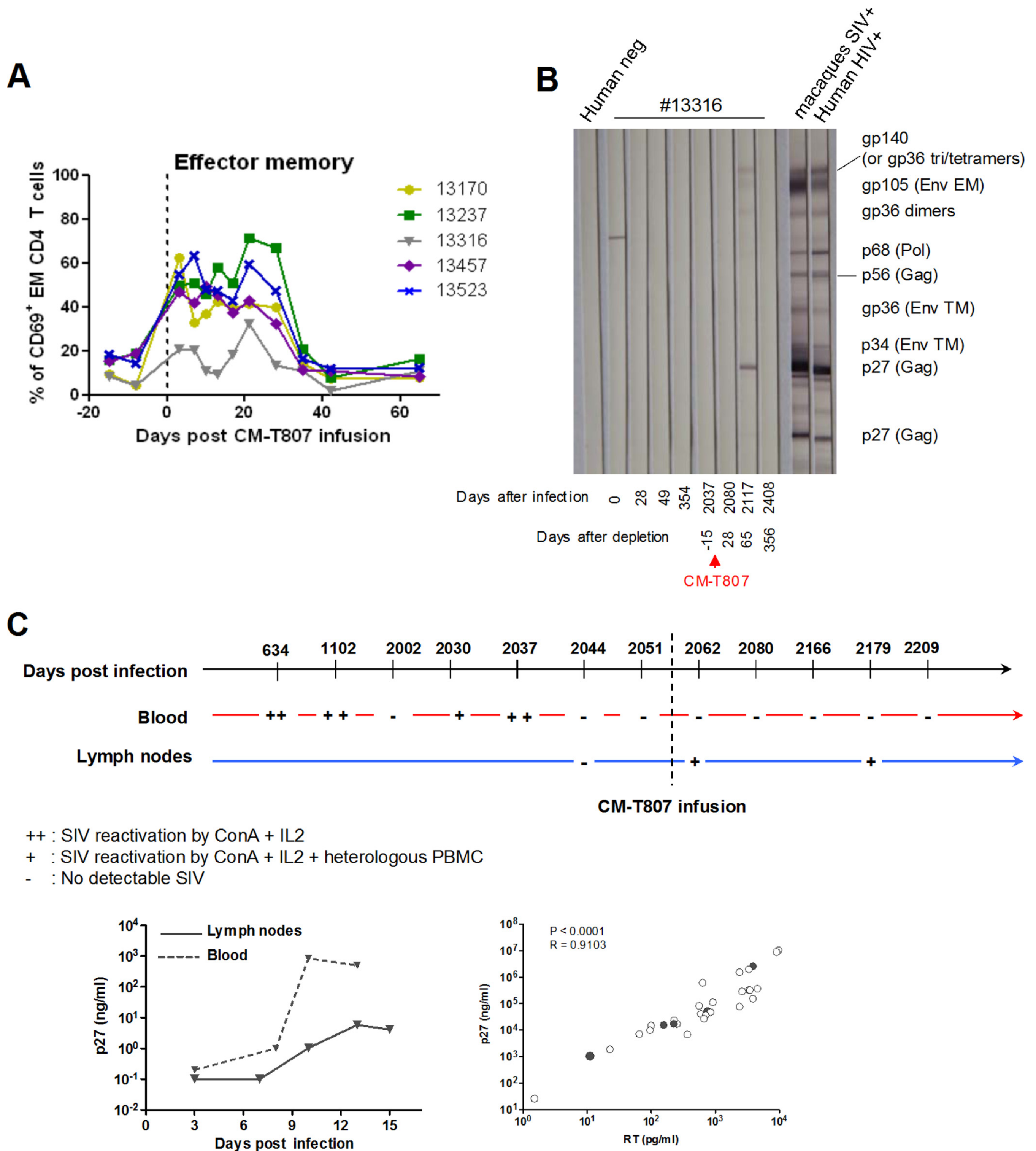

FIG 7 Macaque 13316 displayed an exceptional profile of control with no detectable increase in plasma viral loads but transient seroconversion after $CD8^+$ cell depletion. (A) Frequencies of $CD69^+$ EM $CD4^+$ T cells from SIV controllers during the $CD8^+$ depletion experiment. (B) Western blot SIV reactivity of plasma collected at various time points from macaque 13316, including pre- and postdepletion time points. (C) (Top) Summary of experiments involving the reactivation of viral replication from purified $CD4^+$ T cells of macaque 13316. Samples were obtained from blood, LN, and purified $CD4^+$ T cells. ++, time points at which SIV was isolated upon ConA activation and culture with IL-2; +, time points at which adjunction of heterologous PBMCs was needed for detectable SIV production; −, time points at which SIV could not be detected. (Bottom left) Two representative examples of SIV reactivation upon stimulation of $CD4^+$ T cells isolated from blood or LN from 13316. (Bottom right) Spearman correlation between p27 concentration and reverse transcriptase (RT) activity in various culture supernatants (different time points in the summary) from macaque 13316 (●) and another CyM (○).

ulation of blood $CD4^+$ T cells (Fig. 7C), than at later time points, when it was observed only following stimulation of LN $CD4^+$ T cells. After $CD8^+$ depletion, the viremia remained undetectable, but partial Western blotting reactivity against the Gag and Env proteins was transiently observed (day 65 after depletion) (Fig. 7B). Therefore, macaque 13316 presented an exceptional long-term SIV controller profile that resisted $CD8^+$ cell depletion.

DISCUSSION

In contrast to the rapid-progression macaque models of SIV infection, the $SIVmac_{251}$ infection model of CyM closely resembles HIV infection in humans in terms of viral load, $CD4^+$ T-cell depletion, and rates of progression (39). Thus, it appears to be an optimal model to study host mechanisms able to naturally control infection. Our work provides further evidence that the H6 haplotype in CyM is associated with the control of infection: following intrarectal inoculation of 11 CyM with 50 AID_{50} of $SIVmac_{251}$, the three H6 macaques, but none of the eight non-H6 macaques, were able to control the infection. This association between MHC and the control of infection in CyM seemed to lessen when lower doses of $SIVmac_{251}$ were used for inoculation: intrarectal inoculation of six CyM with 5 AID_{50} of $SIVmac_{251}$ resulted in control of infection in all animals at the set point, despite only one possessing the H6 haplotype. Although one CyM lost control a few months later, the remaining five CyM continued to control SIV viremia for 5 years. This model of CyM controlling infection after intrarectal exposure to low doses of $SIVmac_{251}$ may be useful for investigating the mechanisms leading to the spontaneous control of HIV/SIV viremia independent of known protective MHC haplotypes, as well as those with MHC-associated control (23, 24, 26, 40–42), in the context of pathogenic-virus infection.

Both a low-dose inoculum and infection through the mucosal route in macaques have been associated with a decreased number of transmitted/founder viruses and lower genetic complexity of the infecting virus (43, 44). This restricted viral diversity may facilitate the control of infection by host mechanisms, possibly due to the lack of necessity for broad-spectrum responses. Although we cannot exclude subtle differences in the fitness of the viruses, all low-dose controllers carried replication-competent viruses that are able to infect heterologous $CD4^+$ T cells to the same extent as the original $SIVmac_{251}$. Low-dose SIV exposure in macaques has also been associated with lower plasma proinflammatory cytokine levels during the early phases of infection (45), which may provide an optimal context for the development of adaptive responses.

Unfortunately, we were not able to analyze the immune responses during primary infection in our group of CyM exposed to low-dose SIV. After a couple of years of controlling the infection, they exhibited weaker SIV-specific $CD8^+$ T-cell responses and weaker T-cell activation than noncontroller CyM. We analyzed the *ex vivo* capacities of $CD8^+$ T cells from these macaques to suppress SIV infection of autologous $CD4^+$ T cells using a technique developed for HIV-infected patients to highlight the qualitatively superior HIV-specific $CD8^+$ T-cell response often observed in HIC (9, 12). This activity was not stronger in SIC than in viremic CyM; the exception was macaque 13523, carrying the H6 haplotype, whose $CD8^+$ T cells were able to efficiently suppress SIV infection even at low E/T ratios. Nevertheless, the longitudinal follow-up showed that $CD8^+$ T-cell-mediated SIV suppression waned over time in the SIC. SIV-specific $CD8^+$ T-cell responses were weak and displayed low anti-SIV capacity in blood, BAL fluid, and LN. Only macaque 13311, the animal that lost viral control, maintained almost constant anti-SIV activity *ex vivo*, albeit at a modest level. Although further analyses including the primary phase of infection are needed, our results are compatible with a scenario in which the $CD8^+$ T-cell response contracts once viremia has been efficiently controlled.

Human HIV controllers are usually characterized after several years of control of the infection. During chronic infection, many HIC show high frequencies of robust HIV-specific $CD8^+$ T cells (9–11). However, many others continue to control their viremia despite low frequencies of HIV-specific $CD8^+$ T cells and a weak suppressive capacity in the blood or rectal mucosa (12, 15, 16, 46). In HIC with weak T-cell responses, an efficient memory $CD8^+$ T-cell response may proliferate in response to viral rebound, rapidly acquire effector functions, and thereby control viral relapses (18), but the quiescent T-cell status in many of these patients suggests that these cells are not actively repressing infection (12). After several years of viral control, our SIC with weak T-cell activation and weak SIV-specific $CD8^+$ T-cell responses resembled these HIC; this finding prompted us to explore the role of their weak $CD8^+$ T-cell responses in the long-term maintenance of SIV control.

The *in vivo* depletion of $CD8^+$ cells in SIC macaques has been used to highlight the role of $CD8^+$ T-cell response in the control of SIV infection (21, 27), although the interpretation of such experiments deserves discussion. In our study, we observed a transient rebound of viremia in four of the five SIC, which is consistent with previous reports (21, 27). The viral load in one SIC started to decline before we could detect the recovery of $CD8^+$ T cells in the blood or any of the various tissues tested. In the three other SIC who lost control, recovery of their $CD8^+$ T-cell compartments began earlier and coincided with the drop in viremia. The kinetics of the SIV-specific $CD8^+$ T-cell responses indicates that the frequency of these cells expanded very modestly during the $CD8^+$ T-cell recovery phase compared with the baseline levels and the frequency of SIV-specific $CD8^+$ T cells in the viremic CyM. The only exceptions in which a significant expansion of SIV-specific $CD8^+$ T cells were observed corresponded to late time points in animals 13170 and 13237, when viremia was already back to predepletion levels. No differences in $CD8^+$ T-cell polyfunctionality or capacity to suppress SIV infection were found in pre- and postdepletion blood, LN, and BAL fluid samples, including those collected from the H6-bearing CyM (13523), whose cells had the strongest activities at earlier time points. Intriguingly, $CD8^+$ T cells from this macaque, which reconstituted the $CD8^+$ T-cell compartment faster and more strongly than the other animals, expressed by far the lowest levels of HLA-DR and showed the smallest increase in SIV-specific $CD8^+$ T cells during reconstitution. Overall, our findings suggest that other mechanisms may have contributed to the reestablishment of the control of infection in these macaques.

$CD8^+$ T cells can inhibit HIV/SIV infection through noncytolytic soluble factors (47). Several reports have suggested that these mechanisms may play a major role in the CD8-mediated partial control of SIV infection during primary infection in macaques (48–50). *In vitro* infection of mitogen-activated PBMCs has been widely used to reveal the presence of strong activities of these various soluble antiviral factors. Using this system, we did not observe any significant differences between the levels of

$SIVmac_{251}$ replication in mitogen-activated autologous CD4$^+$ T cells and whole PBMCs. We observed a sharp increase in the plasma MIP-1α and MIP-1β levels a few days after CD8$^+$ depletion, but their peaks preceded the peaks of viremia and their levels subsequently decreased so that they were much lower during the decreasing phase of viremia. Thus, it is unlikely that CD8$^+$ cell-produced soluble factors influenced the viremia during this phase of the study. Although we could not directly analyze the role of NK cells or γδ T cells, our results with PBMCs suggest that no efficient anti-SIV effectors significantly expanded following CD8$^+$ cell depletion and recovery.

In contrast to SIV-specific CD8$^+$ T-cell responses, SIV-specific CD4$^+$ T cells from SIC (which retained better polyfunctionality than those from the viremic macaque) expanded after CD8$^+$ cell depletion, especially in the case of the H6-bearing CyM, 13523. Antigen-specific CD4$^+$ T cells provide help for the generation of functional CD8$^+$ T-cell responses (51). Over the last few years, various reports have suggested that HIV/SIV-specific CD4$^+$ T-cell responses may themselves be endowed with cytotoxic potential (52–54), although it is not clear how this activity is exerted *in vivo* (40). We cannot rule out the possibility that these cells may have contributed to the reestablishment of the control of infection in SIC, although no direct activities were detected in cultures of purified CD4$^+$ T cells from the SIC before or after CD8$^+$ depletion; indeed, these cultures could be infected *in vitro* to levels similar to those of cells from healthy macaques (data not shown).

In agreement with previous reports, the expansion of SIV-specific CD4$^+$ T cells was associated with the activation and expansion of the memory CD4$^+$ T-cell compartment and, in particular, of EM cells (37, 38). Indeed, CD8$^+$ cell depletion was followed by an increase in the number of memory CD4$^+$ T cells upregulating the early activation marker CD69, a subsequent increase in the frequency of Ki67$^+$ proliferating CD4$^+$ T cells, and an expansion in the number of memory cells at later time points. It has been suggested that there is an expansion of EM CD4$^+$ T cells in CD8$^+$ cell-depleted macaques in response to the increased IL-15 concentrations in plasma, but the viral dynamics in primary infection are not affected by this expansion (38). We observed two peaks of CD4$^+$ T-cell activation following CD8$^+$ cell depletion in our group of SIC, reminiscent of an early depletion study in SIV-infected RM (55). These peaks coincided with peaks in plasma levels of IL-15, but also of IL-2 and IL-7. All of these cytokines are regulators of T-cell homeostasis (56). Therefore, it is possible that memory CD4$^+$ T cells were activated in response to transient increases in the levels of these interleukins. CD4$^+$ T-cell activation in SIC may increase the number of target cells for SIV replication, and IL-2, IL-7, and IL-15 have been shown *in vitro* to increase the susceptibility of cells to infection (57, 58) and to reactivate latent provirus from CD4$^+$ T cells (59, 60), including those from HIC with extremely small viral reservoirs (5). We found significant correlations between the levels of plasma viremia and the levels of Ki67$^+$ CD4$^+$ T cells in SIC following CD8$^+$ cell depletion. Our results agree well with the report by Mueller et al. of a correlation between plasma viremia and the number of proliferating CD4$^+$ T cells in CD8$^+$ cell-depleted CyM chronically infected with SHIV89.6P (37). These observations suggest that the homeostatic activation of CD4$^+$ T cells may have played an important role in the loss of the control of viremia in four of our SIC.

Finally, one CyM (13316) represented an exceptional case of control of infection. This macaque did not show a real peak of viremia during acute infection, experienced only two viral blips, and did not seroconvert. Viral RNA and cell-associated DNA were undetectable in either LN or the rectal mucosa at all time points analyzed during chronic infection. Although this profile was evocative of transient or defective infection, the virus could be successfully reactivated *in vitro* from CD4$^+$ T cells purified from this animal at various time points during the follow-up of the study (up to 6 years after infection), confirming that the animal was infected. Viral reactivation from blood samples was easier at earlier time points, and later, the virus could be rescued only from larger quantities of LN cells. These results suggest that the viral reservoir in this CyM was small and shrank further during the study. This type of occult infection is not unknown; a few similar cases have been reported in various macaque models of SIV infection (61, 62) and even among highly HIV-exposed seronegative individuals (63–65). Remarkably, *in vivo* CD8$^+$ cell depletion in this animal lasted for several months, during which SIV replication remained undetectable in either plasma or tissues. Thus, the extreme control of SIV infection in the macaque was independent of the CD8$^+$ T-cell response, further arguing for the existence of additional mechanisms contributing to maintaining control of the infection.

Although it is likely that the CD8$^+$ T-cell response makes an important contribution to establishing natural control of SIV infection, our results suggest that its role may be less important for the maintenance of viral control. Several results from this study support this hypothesis: (i) the anti-SIV CD8$^+$ T-cell responses appear to wane over time, while the viremia remains controlled; (ii) although *in vivo* CD8$^+$ depletion in the SIC was accompanied by a transient loss of control, the viremia was correlated with the activation of CD4$^+$ T cells; (iii) the control of viremia in macaque 13237 started before CD8$^+$ T cells were detected in the blood or tissues; (iv) the regain of viral control was not accompanied by a boost in the SIV-specific CD8$^+$ T-cell response, and 13523 achieved the fastest and deepest control of infection while maintaining extremely low frequencies of SIV-specific CD8$^+$ T cells; and (v) one animal with an extreme phenotype of control maintained an undetectable viremia despite the absence of CD8$^+$ T cells for more than 3 months.

In conclusion, once control of viremia has been established, other mechanisms, perhaps including control of small viral reservoirs, regulation of T-cell activation, or robust specific CD4$^+$ T-cell responses, may overtake the CD8$^+$ T-cell response, at least partially, to maintain control of the infection in the long term. Future longitudinal studies in CyM exposed to a low-dose viral inoculum may provide information about these mechanisms.

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