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Trafficking, Persistence, and Activation State of Adoptively Transferred Allogeneic and Autologous Simian Immunodeficiency Virus-Specific CD8+ T Cell Clones during Acute and Chronic Infection of Rhesus Macaques

Diane L. Bolton,* Jacob T. Minang,† Matthew T. Trivett,† Kaimei Song,* Jennifer J. Tuscher,‡ Yuan Li,† Michael Piatak, Jr.,† David O’Connor,‡ Jeffrey D. Lifson,† Mario Roederer,* and Claes Ohlen†

Despite multiple lines of evidence suggesting their involvement, the precise role of CD8+ T cells in controlling HIV replication remains unclear. To determine whether CD8+ T cells can limit retroviral replication in the absence of other immune responses, we transferred 1–13 × 10^8 allogeneic in vitro expanded SIV-specific CD8+ T cell clones matched for the relevant restricting MHC-I allele into rhesus macaques near the time of i.v. SIV challenge. Additionally, in vitro expanded autologous SIV-specific CD8+ T cell clones were infused 4–9 mo postinfection. Infused cells did not appreciably impact acute or chronic viral replication. The partially MHC-matched allogeneic cells were not detected in the blood or most tissues after 3 d but persisted longer in the lungs as assessed by bronchoalveolar lavage (BAL). Autologous cells transferred i.v. or i.p. were found in BAL and blood samples for up to 8 wk postinfection. Interestingly, despite having a nominally activated phenotype (CD69+HLA-DR+), many of these cells persisted in the BAL without dividing. This suggests that expression of such markers by T cells at mucosal sites may not reflect recent activation, but may instead identify stable resident memory T cells. The lack of impact following transfer of such a large number of functional Ag-specific CD8+ T cells on SIV replication may reflect the magnitude of the immune response required to contain the virus. The Journal of Immunology, 2010, 184: 303–314.

Several lines of evidence suggest that CD8+ T cells are important for limiting AIDS virus replication: certain MHC class I alleles are highly correlated with virus control in both macaques and humans (1, 2); the onset and maintenance of HIV-specific CD8+ T cell responses is associated with reduced viremia (3–5); plasma viremia increases dramatically upon experimental CD8 depletion in the rhesus macaque SIV model (6–8) (although CD8-expressing cell subsets [e.g., NK cells] may be affected by depletion regimens); and the emergence of CD8+ T cell escape mutant viruses in both SIV-infected macaques and HIV-infected humans implies that CD8+ T cells apply selective pressure in vivo (9–12). Given the serious obstacles to develop an HIV vaccine capable of inducing broadly cross-reactive neutralizing Ab responses (13), it is important to define the nature and limits of control of viral replication achievable by cellular immune responses, especially CD8+ cytotoxic T lymphocytes, to guide vaccine design and development efforts.

Adoptive transfer of syngeneic Ag-specific effector T cells in mouse models of disease has been instrumental in demonstrating the importance of CD8+ T cells in tumor suppression and protection against viral infections such as cytomegalovirus and Friend virus (14–16). In part guided by such studies, treatment of human cancers with ex vivo expanded autologous CD8+ T cells specific for tumor Ags have been pursued, with some instances of apparent success (17, 18). However, attempted treatment of HIV infection with similarly expanded autologous HIV-specific CD8+ T cells failed to reduce plasma viremia; the only perceptible effect was a decline in HIV RNA+ CD4+ T cells (19). A major limitation to the long-term success of these human adoptive transfer studies is the poor persistence often observed for in vitro expanded CD8+ T cell clones (20, 21). Thus, efforts have turned to the macaque animal model where in vivo trafficking, persistence, and function are more readily studied. For example, recent results with transfer of autologous CMV-specific CD8+ T cells in Macaca nemestrina suggested that the memory phenotype of the clone from which effector CD8+ T cells are derived may help determine persistence in vivo (22). Such studies highlight the power and potential of nonhuman primate models for examining the role of CD8+ T cells in controlling AIDS viruses.

To test the hypothesis that a pre-existing SIV-specific CD8+ T cell response can curb viral replication, we adoptively transferred allogeneic Mamu A*01- and Mamu B*17-restricted SIV-specific CD8+ T cell clones into rhesus macaques expressing the corresponding
Manu allele(s) before or early in SIV<sub>mac251</sub> infection. Because the recipients and donor were only MHC class I matched at one or two alleles, we refer to these transfers as hemiallogeneic. Employing advances in methods for in vitro expansion of macaque T cell clones (22), CD8<sup>+</sup> T cells were grown to large numbers and maintained cytolytic activity. In addition, endogenous virus-specific CD8<sup>+</sup> T cells isolated from infected monkeys were in vitro expanded for subsequent autologous transfer during chronic infection. We show that i.v. infused autologous effector CD8<sup>+</sup> T cells traffic to the lungs, where they persist for at least 2 mo, but do not necessarily proliferate despite the presence of SIV-infected CD4<sup>+</sup> T cells (as a potential source of Ag-specific stimulation) and displaying an activated phenotype. These bronchoalveolar lavage (BAL)-localized cells showed a nominally activated CD69<sup>+</sup>HLA-DR<sup>+</sup> phenotype, suggesting that this surface phenotype may identify nondividing or even resting cells that have been retained in mucosal sites such as the lungs. Although the hemiallogeneic cells also accumulated in the lungs, they generally did not persist beyond 1 wk. Finally, we found that infusing cells via an i.p. route dramatically altered resulting tissue distribution, such that fewer cells initially populated both the blood and lungs but were maintained at higher levels over time.

Materials and Methods

**Animals and generation of SIV-specific CD8<sup>+</sup> and autologous CD4<sup>+</sup> T cell clones**

SIV-specific CD8<sup>+</sup> T cell clones were generated from PBMCs from an SIV<sub>mac239</sub> chronically infected Indian rhesus macaque, M. mulatta (Manu A01, Baboon 17<sup>+</sup>), as described previously (23). An infected animal was used as the donor because of the large number of Ag-specific cells available for deriving clones. The cells were adoptively transferred either 1 d pre- or 3 d postinfection to four monkeys (501, 506, 228, and 356), matched for Manu A01 and in one case Manu B17, but unmatched for other Manu alleles, thus termed hemiallogeneic. Virus-specific CD8<sup>+</sup> T cell clones were also generated from PBMCs collected from the four recipient monkeys (501, 506, 228, and 356) during chronic infection. The PBMC from two of these monkeys (228 and 356) was presorted into CD8<sup>+</sup> central and effector memory cells based on CD28, CD95, and CCR7 expression (central memory T cell [TCM]: CD28<sup>+</sup>CD95<sup>+</sup>CCR7<sup>+</sup>; effector memory T cell [TEM]: CD28<sup>+</sup>CD95<sup>+</sup>CCR7<sup>+</sup>). PBMC-derived CD8<sup>+</sup> T cells (whole, TCM, or TEM fractions) were stimulated for 1 wk with irradiated autologous PBMC pulsed with overlapping 15-mer peptide pools or accessory (Nef, Env, and Vif) peptide-pulsed wells. Lymphocytes were washed twice and resuspended in complete media. The cells were then restimulated with 1 d pre- or 3 d postinfected autologous PBMCs from the original monkeys or an equivalent number of fresh PBMCs. A cell doubling dilution approach was used. PBMCs were stimulated with pools of 15-mer overlapping peptides spanning SIV Gag and accessory protein sequences in a [51Cr] release assay using peptide matrix IFN-γ ELISPOT as described (27). Assays were repeated with each individual 15-mer or derivative 9-mer, for SIV Gag CM9 and Nef IW9, or 8-mer, for Tat SL8 peptide (all from SynPep, Dublin, CA). Ag specificity and Manu restriction was confirmed by staining with a Gag CM9 (Beckman Coulter), Nef IW9, or Tat SL8 (NIH Tetramer Facility) peptide MHC class I tetramer.

**In vitro functional and phenotypic characterization of CD8<sup>+</sup> T cell clones**

Functional activity of the virus-specific CD8<sup>+</sup> T cell clones was assessed in vitro by measuring intracellular IFN-γ and surface CD107a following stimulation with autologous PBMCs pulsed with SIV Gag CM9, Nef IW9, or Tat SL8 peptides or the relevant peptide pools and SIV<sub>mac239</sub>-infected autologous CD4<sup>+</sup> T cell lines (61). The lytic capacity of the donor CD8<sup>+</sup> T cell clones was also assessed in a [3<sup>Cr</sup>] release assay using peptide-pulsed as well as virus-infected target cells as described previously (23). Briefly, target cells were labeled with 100 Ci sodium chromate (PerkinElmer Life Sciences, Boston, MA) and incubated for 4 h with the virus-specific CD8<sup>+</sup> T cell clones, in triplicate, at various effector-target ratios. Supernatants were transferred into LumaPlates (Packard BioScience, Boston, MA), and radioactivity released was measured using a 1450 Microbeta luminescence/scintillation counter (PerkinElmer Life Sciences). Specific lysis was determined by using the formula: % lysis = 100 × ([mean experimental cpm – mean spontaneous cpm]/mean maximum cpm – mean spontaneous cpm). Spontaneous lysis never exceeded 15% of the maximum release value. The maximum release value was determined from target cells treated with 5% (v/v) Triton X-100 (Sigma-Aldrich, St. Louis, MO).

Surface staining for chemokine receptors, homing markers, and PD-1 was performed on clones using the following Abs: CCR5 (clone 3A4), CCR7 (clone 150503), CCR8 (clone 191704, R&D Systems), CCR9 (clone 112560111, R&D Systems), CD103 (clone 2G5), CD107a (clone CT11), and PD-1 (R&D Systems, catalog number BAF1086), CD3 (clone SP34), CD8 (clone RPA-T8), and CD45 (clone HI30; not cross-reactive with rhesus macaque and thus used to exclude human feeder cells). All chemokine receptor staining was performed at 37°C.

**Virus stocks and infection of CD4<sup>+</sup> T cells**

CD4<sup>+</sup> T cells used as APC were infected with virus produced by transfection of HEK293T cells with SIV<sub>mac239</sub> plasmid using TransIT-293 reagent (Mirus Corporation, Madison, WI) as described (26). CD4<sup>+</sup> T cells were infected as described (26), using the ViraMag Magnetofection reagents (Oz Biosciences, Marseille, France).

**Rhesus macaque infections**

All animals were challenged with pathogenic SIV<sub>mac231</sub> via the saphenous vein with 100 AID<sub>50</sub>. This virus stock was provided by Dr. Norman Letvin of Beth Israel Deaconess Medical Center, Harvard Medical School,
Bostock, MA. Two of the recipient monkeys (501 and 506) were infected 1 d after T cell adoptive transfer, and the other two (228 and 536) were infected 3 d prior to T cell infusion to allow time for virus to establish infection before CD8+ T cell infection.

**CD8+ T cell expansion in vitro, adoptive transfer, and monitoring of persistence in vivo**

SIV Gag CM9- and Tat SL8-specific CD8+ T cell clones were selected for expansion and adoptive transfer to all four recipient monkeys during acute infection from the donor monkey based on in vitro functional responses (IFN-γ/CD107a) to autologous peptide-pulsed (2.5 μg/ml) PBMCs and virus-infected CD4+ T cells as well as lytic capacity in the [51Cr] release assay. An SIV Nef I9 clone was also selected for adoptive transfer to the Mamu+B+17-positive monkey, 501. SIV Gag CM9-, Nef I9- (in the case of 501), and Tat SL8-specific (for 228 and 356 only) CD8+ T cell clones generated from the recipient monkeys after acute infection were selected for autologous infusion during chronic infection using similar criteria. The selected CD8+ T cell clones were expanded in vitro for 6–8 wk through repeated cycles of biweekly stimulation with anti-CD3 mAb (BD Biosciences), irradiated human PBMCs, and the TM EBV-transformed B cell line (B-LCL) as feeder cells, but without anti-CD28 mAb stimulation, to obtain billions of cells of each clone as described (23, 29).

Prior to infusion, CD8+ T cell preparations were stained with a fluorescent cell membrane dye to track tissue distribution and in vivo persistence. PKH26 (red dye; Sigma-Aldrich) and CFSE (Invitrogen, Carlsbad, CA) were used to distinguish i.p and i.v. allogeneic infections. For the autologous infusions during chronic infection the TDM and TCM-derived cells were labeled with PKH26 and CFSE, respectively. PKH26 and CFSE have been shown to segregate evenly during cell division (30, 31). Generally, only a fraction of each population was labeled to minimize manipulation of the clones.

Labeled and unlabeled cells for adoptive transfer to each animal were pooled, washed extensively, resuspended in saline solution supplemented with 2% autologous serum, and infused (1.5 ml/min in 50 ml final volume for i.v or 5 ml/min in final 10 ml volume for i.p.). Clone viability was typically >85% at the time of infusion. To support infused T cell survival and proliferation (32), animals 228 and 356 received s.c. low-dose (104 U/kg/d) IL-2 injections daily for 10 d following all transfers, whereas IL-2 was used as s.c. low-dose (104 U/kg/d) IL-2 injections daily for 10 d following all transfers, whereas IL-2 was administered to 506 and 501 only once shortly after autologous transfer. Blood was collected from the monkeys before and 30 min post-infusion and at multiple points during the first week postinfusion and once a week thereafter. BAL, jejunal biopsies, and LN samples were also collected from the animals 2–4 d postinfusion and BAL once every 2–4 wk thereafter.

The frequency of dye-labeled and SIV Gag CM9, Nef I9 (for 501), and Tat SL8 MHC class I/tetramer–positive CD8+ T cells and the proportion of CD4+ T cells in PBMC, BAL, jejunal, and LN samples collected at the different time points pre- and postinfusion were analyzed by flow cytometry on an LSR II flow cytometer (BD Biosciences) with the additional criteria. The selected CD8+ T cell clones were expanded in vitro, adoptive transfer, and monitoring of persistence in vivo.

**Proviral DNA and viral RNA measurements**

CD4+ T cell-association viral DNA was measured for naive (CD28–/CD95−, T20−, CD28+CD95−), and T20+ (CD28−CD95+) populations isolated by flow cytometry and equal cell pellets were lysed in 25 μl of 10 mM Tris–HCl with 0.1 μg/ml Proteinase K (Roche, Mannheim, Germany) while shaking at 1400 rpm at 56°C for 60 min, followed by protease inactivation at 95°C for 20 min (shaking). Quantitative PCR (qPCR) was performed on a 5-μl template using a PerkinElmer ABI 7900 (PerkinElmer Life Sciences), as previously described (34), with SIV gag primers and probe described previously (8). M. mulatta albumin DNA was measured to normalize gag copies per cell using the following primers: forward, 5′-TCCTGAGA-GAAACCGCAGTATA-3′; reverse, 5′-ATGGTGCTCTGTCACCAAA-3′; and probe, 5′-(FAM)AGAAAGTCCAAAGTCTGCACGGATC (BHQ1)-3′.

Viral RNA from plasma, BAL, LN, or jejunal samples was extracted essentially as described previously (35). To quantify viral replication, a FRET probe-based real-time RT-PCR (TaqMan, Applied Biosystems, Foster City, CA) was used as described (8, 35). All RT-PCR reactions were run on an ABI Prism 7700 Sequence Detection System, and ABI sequence detection software was employed to determine viral RNA copy numbers in test samples (Applied Biosystems).

**Sequencing SIVmac239 regions encoding relevant CD8+ T cell epitopes**

SIVmac239 genomic regions encoding Gag CM9, Nef I9, and Tat SL8 were sequenced from viral RNA isolated from cell-free plasma as described (28). All RT-PCR reaction conditions and primer sequences are described in Minang et al. (61), with the addition of the following I9W epitope primer pair: the mAbs: GAGCCAAAGTCCCTCAA-3′ and 5′-CTTGGGCAAGTGAAGCCTTCTT-3′. Nucleotide sequences derived from both strands of the amplicons were aligned pairwise to the GenBank SIVmac239 sequence (Accession No. M33262.1) (36). Changes in and around the region encoding the epitope resulting in either amino acid replacements or silent mutations were noted.

**Results**

**Functional and phenotypic properties of in vitro expanded SIV-specific CD8+ T cell clones**

To evaluate the impact of CD8+ T cells on viral replication in acute SIV infection, we isolated and expanded SIV-specific cells for infection into rhesus macaques at the time of challenge. CD8+ T cell lines specific for SIV Gag CM9 (Mamu-A+01-restricted), Tat SL8 (Mamu-A+01-restricted), and Nef I9W (Mamu-B+17-restricted) were generated from a chronically infected rhesus macaque as described (23). Individual clones were then isolated and expanded in vitro using biweekly anti-CD3 stimulation and tested for cytolytic activity by [51Cr] release. All tested clones showed marked target cell lysis at a range of effector to target ratios (Fig. 1A) (23). Additional effector functions were measured by flow cytometric analysis of CD107a (a degradation marker) and intracellular IFN-γ expression following stimulation with cognate peptide-pulsed or virus-infected autologous CD4+ T cells (Fig. 1B, 1C). All clones mounted robust responses to cognate peptide, with 37–85% of the cells expressing CD107a, IFN-γ, or both. Thus, the in vitro expanded clones remained SIV-reactive and retained effector function after extensive expansion in vitro.

Clones were also screened for expression of homing and activation markers that might influence their efficacy in vivo. The clones were generally CCR5+, but negative for CCR9, CCR7, and CCR8, consistent with an activated effector memory phenotype (Supplemental Fig. 1A). Expression of the gut homing markers, α4β7 and CD103, as well as the activation/exhaustion marker, PD-1, varied depending on the clone: the I9W-specific clone was α4β7−CD103+, with a small PD-1+ subset, whereas the CM9-specific clone did not express high levels of these markers.

**Adoptive transfer and tracking of transferred cells**

Two Mamu-A+01-positive macaques were i.v. infused with 2–8 × 10^9 cells from each of the CM9- and SL8-specific clones (Fig. 2A). One of the animals, 501 (Mamu-B+17), was also infused with 1 × 10^9 cells of an I9W-specific clone. The recipient animals were matched to the donors only for the Mamu-A+01 and Mamu-B+17 alleles (i.e., hemiallogeneic). One fourth of the infused cells for each clone were labeled with the cell-permeable dye, CFSE, to track the persistence and trafficking of the infused cells in blood and tissues over time. One day following the adoptive transfer, the macaques were infected i.v. with SIVmac251.

The Ag specificity and CFSE fluorescence of the cell mixture was confirmed by flow cytometry prior to infusion (Fig. 3A). Tetramer staining for CM9, SL8, and IW9 indicated that these clones represented 54%, 36%, and 5%, respectively, of the total cell mixture infused into animal 501, proportions consistent with the initial relative number of cells of each clone. CFSE fluorescence within each tetramer-positive population confirmed labeling of ~25% of the cell mixture.

Twenty-four hours after the transfer, infused cells were readily detected in peripheral blood, ranging from 0.4% (I9W) to 2.3%
of the CD8+ T cell compartment (Fig. 3B). The CFSE-labeled fraction of each clone remained at ~25%, suggesting that the labeling did not affect initial cell survival. After 3 d, however, the transferred cells were barely detectable in the periphery, with a 5- to 50-fold reduction from day 1 (Fig. 3C, left). By contrast, over 20% of BAL T cells were CFSE positive at this time, indicating massive localization of the clones to the lung. As only one-fourth of the infused cells were labeled, the finding of 20% CFSE+ cells is consistent with up to 80% of the total T cell population in BAL comprised of infused cells. However, CFSE+ T cells were undetectable in both BAL and peripheral blood by day 9 postinfusion (data not shown), likely due to host rejection or IL-2 withdrawal death. No labeled cells were found in LNs or jejunal biopsies at any point (data not shown), despite expression of the gut homing markers α4β7 and CD103 by some clones (Supplemental Fig. 1). These data suggest that the infused cells localized preferentially to the lungs. Similar results were observed in the periphery of animal 506, although fewer cells were detected (CM9) of the CD8+ T cell compartment (Fig. 3B).

FIGURE 2. Experimental schema for SIV-specific CD8+ T cell adoptive transfer and SIV challenge in rhesus macaques. A and B. The timing (in d) and composition of hemiallogeneic SIV-specific T cell clones infused for each of four animals (identification numbers at left) are indicated relative to the SIV-mac251 challenge (bold arrow). The number of cells, percent of each clone labeled with fluorescent dye, and the route of infusion (i.v., i.p.) are also shown. IL-2 injections for animals in B were performed daily for 10 d. C. Autologous infusions were performed during chronic SIV infection at the indicated months postinfection. When indicated, the CD8+ T cell clone was derived from an Ag-specific cell with either a central (CM, CD62L+) or effector (EM, CD62L-) memory phenotype.
overall, and we did not sample BAL during acute infection. Remarkably, no adverse events were associated with the massive cell infusion, totaling over $10^{10}$ cells, in either animal.

Effect of transferred cells on acute viremia
To determine whether the hemiallogeneic CD8$^+$ T cell clones were able to impact SIV replication during acute infection, we measured plasma viral RNA by quantitative RT-PCR. For both animals, viremia was robust by day 5 postinfection (6 d postinfusion), ranging from $3\times10^5$ to $3\times10^6$ SIV RNA copies/ml, and peaked around day 9 at $5\times10^7$ copies/ml (Fig. 4). Six untreated historical control macaques that received an equivalent SIVmac251 challenge showed similar peak viral loads, suggesting that the infusion did not affect peak viremia. Although viremia appeared to develop with slightly faster kinetics in the two treated animals, overall the adoptive transfer did not have any measurable impact on viremia during the first week of infection.

Due to the short persistence of the transferred cells, we modified the protocol in a subsequent round of experiments in an effort to enhance survival and potential antiviral efficacy of the infused cells. We hypothesized that active viral replication at the time of cell transfer would provide an immediate target for the CD8$^+$ T cell clones, possibly fueling in vivo activation, proliferation, and persistence. Thus, two new animals (228, 356) were infected with SIVmac251 3 d prior to adoptive transfer of virus-specific CD8$^+$ T cell clones (Fig. 2B). In addition, half of the cells were delivered

FIGURE 3. Detection and persistence of CFSE-labeled hemiallogeneic SIV-specific CD8$^+$ T cells following infusion into rhesus macaques. A, Flow cytometric analysis of the expanded CD8$^+$ T cell mixture prior to infusion in animal 501. Twenty-five percent of each clone was labeled with CFSE, and cells were stained with CM9, SL8, and IW9 tetramers. B, Tetramer staining in the peripheral blood 1 d postinfusion, as shown in A. Analysis was performed on CD3$^+$CD8$^+$ lymphocytes. C, Tetramer staining as in B on peripheral blood and CFSE fluorescence of CD3$^+$ T cells in BAL samples 3 d postinfusion. D, Unlabeled (open black symbols) and dye-labeled SL8- and CM9-specific CD3$^+$CD8$^+$ T cells were measured at the indicated days postinfection in the peripheral blood (left panel) and BAL (right panel) of animal 228 by flow cytometry. PKH26- and CFSE-labeled cells were infused i.v. (PKH26, red symbols) and i.p. (CFSE, green symbols), respectively. Open symbols depict the percentage of all tetramer-positive CD8$^+$ T cells, whereas closed symbols depict the percentage of tetramer-positive cells labeled with dye. Circled data points reflect measurements based on a single dye-positive event.
i.v. (PKH-26–labeled) and half i.p. (CFSE-labeled), as the latter may provide a more direct conduit to gastrointestinal sites of SIV replication. To minimize potential cytokine withdrawal death of the clones in vivo, the animals received daily IL-2 injections s.c. for 10 d following the transfer, an approach used clinically. Because CD4+ T cell activation following IL-15 treatment in rhesus macaques does not elevate acute plasma viremia (37), we did not anticipate any effects of IL-2 on viral load.

As with the previous transfers, the labeled SIV-specific cells were not detectable in peripheral blood beyond 3 d (Fig. 3D, 6 d postinfection). The endogenous immune response to SIV was evident by day 10 postinfection, when significant frequencies of SL8-specific CD8+ T cells were observed in peripheral blood (Fig. 3D, black open symbols). Remarkably, some transferred cells persisted in the lungs of one of the animals (228, shown in Fig. 3D) for up to 3 wk, albeit at low frequencies: 0.3% and 0.02% of the total SL8- and CM9-specific CD8+ T cells, respectively, in BAL. To determine whether persistence in this animal was due to a similar genetic background between the donor and recipient, we performed MHC class I sequencing (26). However, there was no evidence of greater MHC allele sharing between animal 228 and the donor than any of the other transfer pairs (data not shown). Thus, the persistence in this case cannot be attributed to a close genetic relationship based on currently available comprehensive MHC class I typing technology.

Despite the protocol modifications, SIV replication did not appear to be affected by the transferred cells. Acute plasma viral load peaked to similar levels as in the first two recipient animals as well as the control animals (Fig. 4). In addition, there was no evidence of immune selection pressure exerted by the infused clones in the two animals we sequenced (501 and 356), as no escape mutations were detected in the targeted virus-specific CD8+ T cell epitopes until 2–4 wk postinfection (data not shown), consistent with the kinetics of pressure exerted by the endogenous CD8+ T cell response (38). Tetramer staining confirmed that the endogenous blood CM9- and SL8-specific responses developed with kinetics typical of macaque responses to SIV infection, peaking 3 to 4 wk postinfection at 1–10% of the CD8+ T cells (39).

**Autologous CD8+ T cell transfer has no impact on chronic SIV replication**

Because hemiallogeneic cells are threatened by host rejection, interpreting the results from this model is complicated. To address the fundamental question of whether transferred SIV-specific CD8+ T cells can limit virus replication in vivo, we thus turned to an autologous transfer model during chronic SIV infection. SIV-specific CD8+ T cell clones were isolated from the infected macaques that received the hemiallogeneic transfer and expanded in vitro (Fig. 2C). Antiviral activity of the autologous CD8+ T cells was confirmed by assessing their capacity to produce IFN-γ in response to cognate SIV peptides or SIV-infected autologous CD4+ T cells (Fig. 5A). The surface expression of homing and activation markers was similar to that observed for the hemiallogeneic transferred clones (Supplemental Fig. 1B). Most clones contained at least a subset of PD-1+ cells, indicating that in vitro expansion did not necessarily result in an exhausted or proapoptotic phenotype (61).

The expanded clones were infused into chronically infected autologous macaques 4–6 mo postchallenge, again with a fraction of each clone infused i.v. and i.p. (Fig. 2C). The combination of clones (specificity, relative cell numbers, memory phenotype derivation), labeling dyes, and infusion routes varied slightly among the transfers administered to each animal due to cell availability and iterative attempts to enhance efficacy. However, plasma viremia did not decrease after any of the five autologous infusions (Fig. 5B). In one case (animal 501; Mamu-B+17), viremia dropped spontaneously by ∼1.5 logs right before the infusion, but no further reduction occurred after the infusion. A brief 2- to 5-fold increase in plasma viremia was observed for most of the animals in the immediate period following the infusion, which then returned to preinfection levels within a week (Fig. 5B, 5C).

To further test whether the transferred cells had any effect on SIV replication through elimination of cells harboring SIV, we measured cell-associated viral DNA by real-time PCR. Peripheral blood CD4+ T cells collected at different time points postinfection were sorted into naive (CD28+CD95−), central memory (CD28+CD95−), and effector memory (CD28−CD95+) populations by flow cytometry. Levels of cell-associated viral DNA in the months preceding the autologous virus-specific CD8+ T cell transfer ranged from undetectable to 2.4 copies per cell in animals 506 and 501 (Fig. 5D and data not shown), with an apparent bias toward infection of TCM and TEM CD4+ populations, as reported previously (40, 41). However, cell-associated viral load did not significantly change following the CD8+ T cell infusion, consistent with unaltered plasma viremia (Fig. 5B). Thus, we were unable to demonstrate any measurable impact of the transferred cells on SIV replication.

To determine whether the CD8+ T cell epitopes targeted by the clones were still present at the time of the autologous infusion, we sequenced plasma virus for two of the animals (501 and 356). The epitopes targeted by one of the two clones infused into each animal had mutated and conferred escape from CD8+ T cell recognition (Net IW9 and Tat TL8 for 501 and 356, respectively, data not shown). Therefore, half of the targeted epitopes in at least two of the animals were no longer present at the time of infusion.
FIGURE 5. Functional activity and in vivo efficacy of expanded SIV-specific cells on chronic viremia. 

A, An expanded CM9-specific CD8+ T cell clone derived from animal 506 postinfection was measured for effector function by intracellular cytokine staining for IFN-γ following stimulation with either peptide-pulsed or SIV-infected CD4+ T cells. The percentage of CD8+ T cells expressing IFN-γ is indicated.

B, Plasma viremia, as measured by SIV RNA copies per ml, is plotted against days postinfection for each animal. Autologous infusions were performed at the indicated time points, with black symbols depicting the first measurement after infusion.

C, The fold change in plasma viremia following CD8+ T cell infusion is plotted for the data in B normalized to viremia at the time of infusion.

D, Cell-associated viral DNA in animal 506 for naive, central memory (CM), and effector memory (EM) T cell subsets. Real-time qPCR was performed on sorted cell populations consisting of naive (CD28+CD95−; black), CM (CD28+CD95+; blue), and EM (CD28−CD95+; red). SIV DNA copies per cell were calculated by dividing the amount of SIV Gag by half of the albumin yield for each sample. All PCR reactions were performed in duplicate, and both values are shown. Dotted lines indicate when autologous SIV-specific clone(s) were infused.
Tracking autologous SIV-specific CD8$^+$ T cells transferred during chronic viremia

To determine the persistence of the autologous transfer and the effect of infusion route on in vivo cell trafficking, CFSE and PKH26 cell labeling was used to distinguish i.v. and i.p. delivery, respectively, in animals 506 and 501. Using animal 501 as an example, i.v.-transferred CM9-specific cells were abundant in both the blood and BAL in the days following the infusion and persisted for at least 2 mo (Fig. 6A, 6B). With 26% of blood CM9-specific cells CFSE$^+$, the transferred cells represented 52% of the blood CM9 population, as only half of the i.v.-infused CM9 clone was CFSE labeled and assuming similar survival of labeled and unlabeled cells during the first 24 h. This represents ~0.3% of the total CD8$^+$ T cell compartment in the blood (0.8% of the CD8$^+$ T cell response was CM9-specific at this time; data not shown). Similarly, with 46% CFSE$^+$ BAL CM9-specific cells 3 d post-transfer, the i.v.-infused cells represent 92% of BAL CM9-specific CD8$^+$ T cells, or 53% of the total CD8$^+$ T cell population in BAL (58% of CD8$^+$ T cells in BAL were SIV Gag CM9-specific; data not shown). For the second set of animals (228 and 356), we detected fewer cells from the infusion of TCM- and TEM-derived autologous clones for unknown reasons.

CD8$^+$ T cell clones infused i.p. (PKH26$^+$) were also readily detected in both the blood and BAL. However, the cells populated these compartments with different kinetics compared with the i.v.-infused cells: labeled cells were not detectable in peripheral blood until the day after the infusion (compared with 30 min for the cells transferred via the i.v. route) and never made up >1% of the total CM9-specific CD8$^+$ T cell population in blood. However, their representation in the blood was more stable over the 2 mo following the infusion, dropping only to 0.1% by day 56, whereas the i.v.-derived cells declined precipitously from 26% to 0.02% during this period. Similarly, considerably fewer cells transferred via the i.p. route homed to the lungs (0.2% of the CM9-specific cells at day 3), although their representation in the BAL slightly increased over time, peaking at 1.1% of CM9-specific CD8$^+$ T cells on day 56, whereas the i.v.-infused cells dropped from 46% to 4.6% (Fig. 6B). Together these data indicate that tissue homing and equilibration of the transferred cells can vary considerably with the route of infusion. In addition, the lack of perceptible effect on chronic viremia was not due to poor persistence of the transferred autologous cells.

Activation status of transferred cells

Because the dye-tracking data indicated that the transferred autologous CD8$^+$ T cell clones were persisting in vivo, we next examined activation markers. All clones were CD69$^+$HLA-DR$^{dim/+}$ at the time of infusion (data not shown). Flow cytometric analysis of BAL CD8$^+$ T cells 5 mo postinfecion (43 d after the autologous infusion) indicated that CD69 is highly expressed by most cells at this site (Fig. 6Aa). This is presumably independent of SIV infection, as similar staining was observed 2 d postchallenge, a time prior to significant viral replication (data not shown). CM9-specific cells expressed slightly elevated MHC class II (HLA-DR) compared with the rest of the CD8$^+$ T cells (Fig. 6Ab), likely due to stimulation by active SIV replication in the lung. The dye-labeled fraction of this population expressed similar CD69 and HLA-DR as the unlabeled fraction (Fig. 6Ac, 6Ad), which was also representative of the preinfusion profile for this and other clones (data not shown). Thus, the activation profile of transferred cells that localized to the lung was similar to that of both the endogenous SIV-specific response and lung-resident CD8$^+$ T cells in general.

We also used the CFSE dye to track the proliferation of transferred clones, as fluorescence is diluted by half with each round of division. The median fluorescence intensity of the CFSE$^+$ CM9-specific cells in the blood and BAL was plotted over time following the autologous cell adoptive transfer into animal 501 (Fig. 6C). These curves were compared with the natural decay of CFSE fluorescence observed for nondividing murine cells, as short-lived labeled cellular proteins turn over (42). Relative to the natural decay rate reference, there was a substantial population of transferred SIV-specific cells that did not proliferate in either the lung or blood. Thus, CD69 and HLA-DR expression by lung-resident T cells does not necessarily indicate actively dividing populations. Rather, these markers may identify memory T cells that preferentially home to or are retained in this site.

Discussion

Adoptive transfer experiments may help elucidate the mechanism and limits of CD8$^+$ T cell control of viral replication, which have important implications for vaccine design. In particular, it has been postulated that naive and even vaccinated animals suffer from insufficient SIV-specific cells arriving at sites of viral replication (43). To determine whether virus-specific CD8$^+$ T cells can control SIV replication when infused in large numbers, we adoptively transferred $10^7$–$10^{10}$ SIV-specific hemiallogeneic CD8$^+$ T cells at or around the time of challenge in four rhesus macaques. Although we did not observe any reduction in acute plasma viremia, the infused CD8$^+$ T cells were generally undetectable in peripheral blood beyond 3 d. It is therefore difficult to conclude whether host rejection of the clones interfered with their antiviral activity or the clones were simply ineffective. To eliminate the prospect of rejection, we modified our protocol and infused autologous SIV-specific CD8$^+$ T cell clones to the same cohort of monkeys during chronic infection. Although a markedly improved persistence was observed for the autologous cells, there was no measurable impact on the plasma viral load or CD4$^+$ T cell-associated SIV DNA. Although we were unable to detect any evidence of virus control by the transferred cells, tracking and phenotypic characterization of the infused cells in vivo revealed some novel immunologic phenomena: a distinct preference for residing in the lungs and continuous expression of activation markers without apparent cell division. Together, these data suggest that an activated T cell phenotype may facilitate recruitment to specific effector/mucosal sites, such as the lungs, and does not necessarily reflect recent Ag stimulation.

The lack of perceptible effect on viral replication by either the hemiallogeneic or autologous CD8$^+$ T cell adoptive transfer may be due to the inability of SIV-specific CD8$^+$ T cells to independently control viremia, limitations of this adoptive transfer model, or a combination of these or related factors. The short persistence of the hemiallogeneic clones suggests that the host rejected them within days of the transfer, as autologous clones expanded and infused in the same manner persisted longer in our chronic infection model and during acute infection, as shown by Minang et al. (61). This may explain the apparent accelerated rate of acute SIV replication observed in the two animals challenged the day after the transfer, as inflammation during a host-versus-graft response might increase the pool of activated CD4$^+$ T cells susceptible to SIV infection at the time of challenge. Rapid clearance of the hemiallogeneic cells may also explain why we did not observe selective pressure exerted on targeted viral epitopes in circulating virus, whereas Minang et al. (61) observed a very early escape mutation following transfer of autologous cells that did persist.
FIGURE 6. In vivo persistence and activation status of SIV-specific CD8+ T cells following autologous transfer. A, CD69 and HLA-DR expression is shown for CD8+ T cells in the lungs (BAL) 43 d postinfusion in animal 501. CD69 and HLA-DR expression by CM9-specific CD8+ T cells are shown in b (dye-negative), c (PKH26+), and d (CFSE+); bulk CD8+ T cells are shown in a.

B, Distribution of infused autologous CD8+ T cells in the blood, BAL, and lymph nodes of animal 501 over time. The percentage of CM9-specific cells labeled with PKH26 (filled symbols; i.p. delivery) and CFSE (open symbols; i.v. delivery) is plotted.

C, Median fluorescent intensity of CFSE-labeled CM9-specific cells in the blood, and BAL is plotted for the indicated days postinfusion. Unlabeled CM9-specific cells (black) are shown as a reference. The expected natural decay rate of CFSE in nondividing cells is plotted in green.
The question remains why there was no measurable effect of the autologous transfer on viremia. It is possible that the transfer procedure or a host-suppressive response impaired the activity of the clones in vivo. However, transferred cells recovered from the lungs 1 mo following transfer in similar experiments performed during acute infection retains effector function upon ex vivo Ag-specific stimulation (61). In addition, several clones expressed little or no PD-1 at the time of infusion. Although our infusions of clones originating from TCM and TEM phenotypes did not show a difference in persistence, as in the study by Berger et al. (22), related studies also failed to detect any difference between TCM and TEM-derived clones in terms of in vivo persistence or antiviral activity (61). Thus, the importance of memory phenotype in determining transferred cell fate is uncertain, and other barriers possibly limited their efficacy. Most likely, by retention in the lungs (or other tissues not sampled), the clones were sequestered from critical sites of virus replication. In addition, some (but not all) of the CD8+ T cell epitopes targeted by the autologous SIV-specific clones were found to have mutated prior to the infusion during chronic viremia, rendering those clones of limited value. It is also possible that soluble factors that suppress HIV-specific CD8+ T cell lytic activity during chronic HIV infection may impact SIV-specific cells as well (44, 45). Alternatively, inflammatory cytokines released by the effector cells may activate latently infected cells or promote new infection of recently activated CD4+ T cells, inadvertently boosting virus production. This could explain the transient spike in plasma viremia observed in the days postinfusion. Notably, autologous HIV-specific CD8+ T cell therapy in humans resulted in a similar elevation in plasma viremia (19).

Although we could not detect an impact on viremia, the in vivo trafficking of the infused clones revealed striking localization properties. When delivered i.v., autologous cells instantaneously populated peripheral blood, where they persisted for at least 2 mo but declined steadily. These cells were highly abundant in the BAL, where they persisted to a greater extent than in the blood, dropping only 10-fold from peak representation compared with a 2000-fold reduction in the blood over 8 wk. Distinct accumulation of the transferred cells in the lungs is consistent with entrapment of i.v.-transferred effector cells as they pass through the constricting vasculature of this organ during pulmonary circulation. Although similar observations have been made for adoptively transferred cells in mice (46, 47), to our knowledge, this, in conjunction with similar findings by Minang et al. (61), is the first documentation of such distribution in primates. Homing to the lungs may be due to expression of adhesion molecules, such as LFA-1, which mediate T cell entry into the airways (48–50). There is also evidence that the morphological rigidity or polarization of effector cells contributes to trapping within pulmonary capillaries (51, 52). Adoptive transfer of bulk, unstimulated PBMCs does not result in accumulation of the transferred cells in the lungs in cynomolgus macaques (53), suggesting that this phenomenon may be dependent on the activated effector phenotype of the transferred cells. Notably, expression of α4β7 and CD103, markers commonly associated with intestinal homing, did not result in detectable localization of clones to the jejunum, as was also observed by Minang et al. (61).

The different localization and tissue equilibration patterns observed for i.p.- and i.v.-infused cells suggest that i.p. transfer largely avoids a massive, early lung entrapment. Rather, the slight, gradual increase in infused cells in BAL combined with the persistent low-level maintenance in blood may reflect slow leakage of peritoneal-deposited cells into circulation, followed by entrapment in the lungs as described above. This would provide a steady supply of i.p.-delivered cells to continuously populate the BAL via the blood, which has been proposed to be a site of recruitment for memory T cells to the lungs (54).

Infused cells that persisted in the lungs displayed an “activated” CD69+HLA-DRdim expression profile; remarkably, a substantial fraction of these cells did not divide in vivo for at least 6 wk while maintaining this phenotype. Thus, expression of either CD69 or HLA-DR, two markers commonly used to identify activated cells in PBMC or in ex vivo analyses, does not necessarily indicate active cell division and should not be equated with concurrent effector functions. Moreover, this phenotype was typical of the resident lung CD8+ T cell population, suggesting that the lung contains a resting or semiresting, long-lived, stable, and non-dividing T lymphocyte population. Similar evidence of non-dividing T cells displaying an effector phenotype in the mouse lung has given rise to the theory that tissue-specific immunoregulatory mechanisms may exist to maintain homeostasis amid high Ag load (55–59). Indeed, this same phenotype for T cells has been seen for gut-associated CD4+ T cells (60). Thus, the hypothesis that the gut is a repository for highly activated dividing T cells that serve as an optimal reservoir of SIV and HIV replication should be re-examined. In any case, the expression of “activation” markers on cells in vivo cannot be used to definitively denote those cells as mitotically active or even necessarily evincing effector functions.

In summary, we found no evidence of independent SIV control by infused CD8+ T cells. The capability of the transferred cells to target virus replication in vivo may have been hampered by several factors, including prompt host-versus-graft rejection for the hemiallogeneic clones infused during acute infection, homing to and confinement within peripheral tissues for many of the clones, viral epitope escape during chronic infection, or simply an insufficiency of cell number. Regardless, we demonstrated the feasibility of expanding massive numbers of Ag-specific CD8+ T cell clones while maintaining a memory phenotype and cytolytic activity. Moreover, infusion of over 1010 cells was well tolerated without any associated adverse events in the animals. We identified persistent transferred cells expressing activation markers that did not proliferate in vivo, suggesting that identification of effector and dividing cells by surface phenotype should be reconsidered. Our findings on T cell trafficking, proliferation, and survival following adoptive transfer will help to design future studies addressing CD8+ T cell control of immunodeficiency virus replication in vivo.

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Disclosures
The authors have no financial conflicts of interest.

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