Adoptive Transfer of Lymphocytes Isolated from Simian Immunodeficiency Virus SIVmac239 Δ nef-Vaccinated Macaques Does Not Affect Acute-Phase Viral Loads but May Reduce Chronic-Phase Viral Loads in Major Histocompatibility Complex-Matched Recipients

Justin M. Greene,^a Jennifer J. Lhost,^a Paul J. Hines,^b Matthew Scarlotta,^a Max Harris,^a Benjamin J. Burwitz,^{b*} Melisa L. Budde,^b Dawn M. Dudley,^a Ngoc Pham,^a Brian Cain,^a Caitlin E. Mac Nair,^b Madelyn K. Weiker,^b Shelby L. O'Connor,^a Thomas C. Friedrich,^{b,c} David H. O'Connor^{a,b}

Department of Pathology and Laboratory Medicine, University of Wisconsin—Madison, Madison, Wisconsin, USA^a; Wisconsin National Primate Research Center, University of Wisconsin—Madison, Madison, Wisconsin, USA^b; Department of Pathobiological Sciences, University of Wisconsin—Madison, Madison, Wisconsin, USA^c

The live attenuated simian immunodeficiency virus (SIV) SIVmac239 Δ nef is the most effective SIV/human immunodeficiency virus (HIV) vaccine in preclinical testing. An understanding of the mechanisms responsible for protection may provide important insights for the development of HIV vaccines. Leveraging the uniquely restricted genetic diversity of Mauritian cynomolgus macaques, we performed adoptive transfers between major histocompatibility complex (MHC)-matched animals to assess the role of cellular immunity in SIVmac239 Δ nef protection. We vaccinated and mock vaccinated donor macaques and then harvested between 1.25×10^9 and 3.0×10^9 mononuclear cells from multiple tissues for transfer into 12 naive recipients, followed by challenge with pathogenic SIVmac239. Fluorescently labeled donor cells were detectable for at least 7 days posttransfer and trafficked to multiple tissues, including lung, lymph nodes, and other mucosal tissues. There was no difference between recipient macaques' peak or postpeak plasma viral loads. A very modest difference in viral loads during the chronic phase between vaccinated animal cell recipients and mock-vaccinated animal cell recipients did not reach significance ($P = 0.12$). Interestingly, the SIVmac239 challenge virus accumulated escape mutations more rapidly in animals that received cells from vaccinated donors. These results may suggest that adoptive transfers influenced the course of infection despite the lack of significant differences in the viral loads among animals that received cells from vaccinated and mock-vaccinated donor animals.

The results of the recent Thai vaccine trial (RV144) demonstrate that success in the human immunodeficiency virus (HIV) vaccine arena is a tantalizing possibility (1–5). In particular, recent discoveries from this vaccine trial have identified several potential correlates of protection which may help guide future vaccine design (2–5). Nevertheless, vaccines will need to offer substantially better protection to be viable, and the correlates of protection are still unclear; therefore, continued advances in basic HIV research may suggest ways to improve upon the 33% reduction in incidence observed in the Thai vaccine trial (6). With this goal in mind, it is important to understand the mechanisms by which effective vaccines can prevent acquisition of immunodeficiency virus infection.

The most effective vaccine tested in macaques is attenuated simian immunodeficiency virus (SIV) (7–9). Attenuated SIVs have repeatedly protected macaques against homologous challenge (10). This apparent sterilizing immunity has been difficult to replicate with other vaccine modalities. To their detriment, attenuated SIVs can become pathogenic in certain macaques and can recombine with pathogenic challenge viruses, preventing their advancement into human clinical trials (9, 11). Through studying the correlates of protection in attenuated SIV vaccines, we may be able to determine which immune mechanisms are involved in protection against infection with pathogenic SIVs, and these lessons may help inform future vaccine design.

In animal models, where the breeding of genetically identical individuals is possible, adoptive transfer of lymphocytes can help identify immune components that protect against various patho-

gens. Previous studies in mice used this technique to determine that all major lymphocyte subsets, including CD4-positive T cells, CD8-positive T cells, and CD19-positive B cells, are critical for protection against Friend virus challenge after vaccination with attenuated Friend virus (12, 13). Similar studies have been nearly impossible in humans and macaques but may help identify which immune cells are sufficient to protect against HIV infection or ameliorate HIV pathogenesis.

Recently, there have been several advancements in adoptive transfer experiments and immunotherapy for HIV and SIV. The therapeutic transfer of autologous CD4 T cells during antiviral therapy or transfer of HIV-specific CD8 T cells led to a nonprogressor status in the recipient animals and reduced the number of infected circulating CD4 T cells, respectively (14, 15). Researchers have expanded SIV-specific CD8 T cells *in vitro* and transferred them into macaques to assess effects on SIV replication (16, 17). These studies found that transferred cells may lead to more rapid escape from relevant CD8 T cell responses but do not have a mea-

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Address correspondence to David H. O'Connor, david.h.oconnor@gmail.com.

* Present address: Benjamin J. Burwitz, Vaccine and Gene Therapy Institute, Oregon Health and Science University, Beaverton, Oregon, USA.

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TABLE 1 MHC-matched MCM animal pairs used in adoptive transfers

Donor animal ID ^a	Donor sex	Donor MHC haplotype	Recipient animal ID	Recipient sex	Recipient MHC haplotype
cy0206	Male	M1/M3	cy0203	Male	M1/M3
cy0210	Male	M1/M3	cy0204	Male	M1/M3
cy0205	Male	M1/M3	cy0208	Male	M1/M3
cy0207	Male	M1/M3	cy0209	Male	M1/M3
cy0211	Male	M1/M3	cy0212	Male	M1/M3
cy0213	Male	M1/M3	cy0214	Male	M1/M3
cy0338	Male	M1/M2	cy0339	Male	M1/M2
cy0341	Male	M1/M2	cy0340	Male	M1/M2
cy0343	Female	M1/M2	cy0342	Female	M1/M2
cy0345	Female	M1/M3	cy0344	Female	M1/M3
cy0346	Female	M1/M3	cy0347	Female	M1/M3
cy0160	Male	M1/M2	cy0391	Male	M1/M2

^a Shading indicates vaccinated macaques.

sureable effect on viral replication (17). Furthermore, many of the transferred cells were trapped in the lungs of the recipient animals. Adoptive transfer studies in mice have also observed this trapping phenomenon (18). None of the preceding studies were able to investigate the correlates of protection using unmanipulated cells *ex vivo*.

Major histocompatibility complex (MHC)-matched macaques that allow for the transfer and persistence of cells between animals provide a powerful tool for dissecting the correlates of immunity in attenuated SIV vaccines (10, 19). We characterized a unique population of cynomolgus macaques from the Indian Ocean island of Mauritius with limited diversity among their MHC class I and class II genes. MHC-matched animals could be readily identified in sufficient numbers for adoptive transfer experiments (20). Cells transferred between MHC-matched Mauritian cynomolgus macaques (MCM) persist for up to 17 days in recipient macaques, while they are undetectable as early as 1 day posttransfer in MHC-mismatched macaques (21, 22). Here we used adoptive transfers to investigate correlates of immune protection in SIVmac239 Δ nef-vaccinated animals. After vaccinating six macaques with SIVmac239 Δ nef and mock vaccinating six control animals, we were able to transfer between 1.25×10^9 and 3.00×10^9 mononuclear cells into MHC-matched recipients. We found that transferred cells trafficked to multiple tissues, including the lungs and intestines. The presence of these donor cells from vaccinated macaques did not reduce acute-phase viral loads but resulted in a small, but insignificant, reduction in chronic-phase viral loads compared to controls and may have affected the kinetics of viral evolution.

MATERIALS AND METHODS

Animals. All animals were cared for by the Wisconsin National Primate Research Center (WNPRC) according to protocols approved by the University of Wisconsin Graduate School Animal Care and Use Committee. Using previously described microsatellite typing, we identified 14 MHC-matched MCM animal pairs for use in adoptive transfers (20). In each pair, we designated one animal a donor and one animal a recipient (Table 1). Eight animal pairs contained the M1/M3 MHC haplotypes, and four animal pairs contained the M1/M2 MHC haplotypes (Table 1). We previously characterized the MHC class I and MHC class II alleles on each of these haplotypes (20, 23–25).

SIVmac239 Δ nef vaccine efficacy study. We are not aware of experiments testing the efficacy of SIVmac239 Δ nef in MCM. We therefore per-

formed a pilot experiment to ensure that live attenuated SIV induced protective immunity in this species. Three animals were vaccinated intravenously with 10 ng p27 SIVmac239 Δ nef, kindly provided by Ronald Desrosiers. SIVmac239 Δ nef-vaccinated macaques were challenged with 1,000 50% tissue culture infective doses (TCID₅₀) of SIVmac239 intrarectally. Recently, we found that intrarectal challenge with 1,000 TCID₅₀ of the same stock of SIVmac239 infected three of three naive MCM.

Adoptive transfer vaccination and challenges. Six animals were vaccinated intravenously with 10 ng p27 SIVmac239 Δ nef. Control animals were either mock vaccinated with phosphate-buffered saline (PBS) or left untreated. After transfer, recipient animals were challenged with 50,000 TCID₅₀ of SIVmac239 intrarectally. This dose was chosen at the outset of these experiments due to a previous report suggesting that a 10-fold-higher challenge dose is required to infect cynomolgus macaques than the dose required to infect rhesus macaques (26).

Viral loads. We assessed SIVmac239 and SIVmac239 Δ nef plasma viral loads using previously described techniques (27, 28). Briefly, the viral RNA (vRNA) was reverse transcribed and then quantified by using a LightCycler 2.0 or LightCycler 480 instrument (Roche, Indianapolis, IN). Serial dilutions of SIV *gag in vitro* transcript were used for an internal standard curve. The limit of detection of this assay was 50 vRNA copies per ml of plasma.

Adoptive transfer. After a 20-week vaccine induction phase, we euthanized donor animals and harvested multiple tissues. We processed spleen, blood, and axillary, mesenteric, mesocolonic, internal iliac, and inguinal lymph nodes into single-cell suspensions. First, we diced the tissues using scalpels and then forced the cells through 100- μ m cell strainers. Cells were then labeled with 1 μ M carboxyfluorescein succinimidyl ester (CFSE) for tracking by flow cytometry. We resuspended cells in 5 to 10 ml of $1 \times$ PBS supplemented with 15 units/ml heparin. Between 1.25×10^9 and 3×10^9 cells were transfused into the saphenous vein by WNPRC staff members.

Flow cytometry. Cells were stained for CD3, CD4, CD8, CD95, CD28, CD19, CD20, CD16, and CD14 to assess memory phenotypes. The antibodies used in these stains were CD3 Alexa Fluor 700 (SP34-2; BD Biosciences), CD3 phycoerythrin (PE) Cy7 (SP34-2; BD Biosciences), CD4 allophycocyanin (APC) (MT466; Miltenyi), CD8 Pacific Blue (RPA-T8; BD Biosciences), CD95 PE Cy5 (DX2; BD Biosciences), CD28 PE (CD28.2; BD Biosciences), CD19 PE Cy7 (J3.119; Beckman Coulter), CD20 APC (2H7; BD Biosciences), CD16 PE (3G8; BD Biosciences), CD14 APC H7 (M5E2; BD Biosciences), and CD14 Alexa Fluor 700 (M5E2; BD Biosciences). Briefly, cells were stained for 30 min at room temperature in $1 \times$ PBS supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT) (fluorescence-activated cell sorter [FACS] buffer). After 30 min, cells were washed with FACS buffer and then fixed with 2% paraformaldehyde (PFA). Data were collected on a BD LSR II instrument (BD Biosciences). Data were analyzed by using FlowJo software version 9.5.3 (TreeStar, Ashland, OR).

SIV sequencing. Viral RNA was isolated from cell-free plasma by using a QIAmp MinElute Virus Spin kit (Qiagen, Valencia, CA). Next, four overlapping PCR amplicons which span the entire SIVmac239 coding sequence were amplified by using the SuperScript III One-Step RT-PCR system with Platinum *Taq* High Fidelity (Invitrogen, Carlsbad, CA). These products were gel purified and fragmented with a Nextera DNA sample prep kit (Epicentre, Madison, WI). Multiplex identifier (MID) tags and adapter sequences for Roche 454 pyrosequencing were added. Purified fragments were pyrosequenced by using the Roche/454 GS Junior system.

Sequencing data analysis. Amino acid alignments were created by using a custom pipeline as previously described (29). FASTQ files generated from the original sff file were converted into FASTA format and translated in all six reading frames. Low-quality sequences were masked. The BLAST-Like Alignment Tool (BLAT) was used to align translated reads to SIVmac239 proteins. Reads aligning to epitopes of interest were taken from the BLAT output table using custom scripts and analyzed for

the presence of variants. We included only reads with high-quality sequence across the entire epitope. Variant sequences present at frequencies of $>1\%$ of reads in at least one animal are displayed in Fig. 6 (29, 30).

To identify reads containing the SIVmac239 Δ nef deletion, sequencing reads were assembled to the reference SIVmac239 sequence (GenBank accession number M33262) (using Geneious Pro version 5.6.5). Portions of reads overlapping the location of the *nef* deletion were extracted and again mapped to the reference *nef* coding sequence. Sequences that aligned to the region of the *nef* deletion were counted as wild-type (WT) SIVmac239 reads. SIVmac239 Δ nef sequences aligned to the reference immediately 5' or 3' of the deleted region.

ELISpot assay. Enzyme-linked immunosorbent spot (ELISpot) assays were performed according to the manufacturer's protocols, as previously described (31). Briefly, cryopreserved peripheral blood mononuclear cells (PBMC) from 4 to 10 weeks postinfection were thawed and resuspended in RPMI 1640 (HyClone, Logan, UT) supplemented with 10% fetal calf serum, 1% antibiotic-antimycotic (HyClone), and 1% L-glutamine (HyClone) (R10 medium) at concentrations of between 7.5×10^4 cells/ml and 2×10^5 cells/ml. Cells were added to precoated monkey gamma interferon (IFN- γ) ELISpot Plus plates (Mabtech Inc., Mariemont, OH) with the relevant peptides. Peptides were added in limiting dilution from 1 mM to 10 nM in 10-fold dilutions. Concanavalin A (10 μ M) was used as a positive control. Samples were made in duplicate and were considered positive when the average numbers of spot-forming cells (SFCs) per 10^6 PBMC were 2 standard deviations higher than the average number of SFCs per 10^6 PBMC in the negative-control wells. The limit of detection was set at 100 SFCs per 10^6 PBMC.

Statistics. Student's *t* test was used to assess the significance of differences between viral load geometric means and the frequency of wild-type SIV sequences using GraphPad Prism (GraphPad Software Inc., La Jolla, CA).

RESULTS

Preliminary trafficking study. Early devastation of the CD4 T cell compartment in gut-associated lymphoid tissue (GALT) is a hallmark of pathogenic HIV and SIV infection. It may be important for CD8 T cells and other lymphocytes to intercept viral replication in these tissues within days of infection to reduce viral loads and prevent or slow progression to AIDS. Therefore, a major goal of our experiments was to traffic donor cells to mucosal tissues like the GALT. In previous adoptive transfer experiments performed by our laboratory, donor cells derived from blood were not detected in the mucosal tissues of recipient animals (22). Here, in a pair of MHC-matched animals, we transferred mononuclear cells from mesocolonic, axillary, mesenteric, inguinal, and internal iliac lymph nodes through the saphenous vein and found that, after 36 h, cells were detectable in nearly every tissue assayed, including intestinal mucosal tissues (Fig. 1a to c). These results demonstrate that, in contrast to our previous studies that transferred PBMC alone, cells are detectable in mucosal tissues when donor cells are isolated from lymph nodes that drain mucosal sites. Recent evidence from a live attenuated SIV correlate analysis found that SIV-specific T cell responses in the lymph nodes predicted viral control, suggesting that these cells might be particularly effective in our own experiments (8).

Vaccination of MCM with SIVmac239 Δ nef. While other attenuated SIVs have proven effective against pathogenic challenge in MCM, we are not aware of any studies using SIVmac239 Δ nef in MCM. We vaccinated three MCM with 10 ng p27 SIVmac239 Δ nef intravenously and challenged them with SIVmac239 intrarectally to assess the protective efficacy of SIVmac239 Δ nef in these animals. SIVmac239 Δ nef replication peaked at levels of between 1.0×10^4 and 1.3×10^6 vRNA

copies/ml plasma (Fig. 2). We waited at least 20 weeks before challenge due to the previously reported direct correlation between time postvaccination and vaccine efficacy (32). After at least 20 weeks, we challenged animals intrarectally with a single dose of 1,000 TCID₅₀ of SIVmac239. This dose previously infected three animals with a single challenge and led to peak viral loads of $>1 \times 10^6$ copies of vRNA/ml plasma (Fig. 2, dotted lines). SIVmac239 Δ nef provided apparent sterilizing immunity for two of three animals. After challenge with SIVmac239, vRNA was undetectable in the plasma for at least 4 weeks postchallenge. We continued to monitor the animals for a year, and one animal had only occasional detectable viral loads, leading us to conclude that this attenuated SIV can protect MCM from SIVmac239 challenge or lead to maintained control of viral replication.

Adoptive transfer tissue collection. Next, we vaccinated six MCM with SIVmac239 Δ nef and mock vaccinated six MCM with 1 $\times$ PBS or no treatment. We euthanized donor macaques at between 20 and 24 weeks postimmunization. At the time of transfer, we harvested multiple tissues from the donor animals, including axillary, inguinal, internal iliac, mesenteric, and mesocolonic lymph nodes; spleen; and blood. We isolated peripheral blood mononuclear cells (PBMC) from blood using Ficoll gradient density centrifugation. The majority of cells in each transfer were isolated from spleen, as this tissue compartment regularly yielded several billion cells (Fig. 3a). Nevertheless, we made a concerted effort to include multiple mucosal draining lymph nodes to increase mucosal trafficking of donor cells. Mesocolonic and mesenteric lymph nodes were generally the second- and third-largest contributors to the transfusate (Fig. 3a). Other tissues contributed to the homogenate at various frequencies. We were able to isolate between 1.5×10^9 and 1×10^{10} cells from tissues before labeling. We transferred bulk mononuclear cells, comprised primarily of CD4 T cells, CD8 T cells, B cells, and NK cells, into recipient animals. NK cells were the smallest component of the transfusate, with other cell types being present at roughly equal frequencies (Fig. 3b).

Cell persistence and trafficking. Donor cells were detectable in the blood for up to 9 days (Fig. 4a). In previous experiments, where we transferred only cells isolated from the blood, we detected cells for up to 17 days posttransfer (22). PBMC composed a much smaller component of the transfusate in the current experiments and may account for the apparent difference in persistence in the periphery. We drew blood within 5 min of transfer and found that up to 27% of the lymphocytes in the blood were donor cells, as detected by CFSE (Fig. 4a). We also found that cells trafficked to multiple tissues. Through bronchoalveolar lavage, we found that transferred cells represented up to 1.0% of lymphocytes isolated from the lung at 7 days posttransfer (Fig. 4b). We also harvested axillary or inguinal lymph nodes at 7 days posttransfer and detected cells at a frequency of up to 2.76% in the lymph node (Fig. 4c). These frequencies were similar to those detected in the blood at 7 days posttransfer.

One animal was euthanized at 16 days posttransfer for reasons unrelated to the adoptive transfer procedure. We collected multiple tissues from this animal to assess the persistence and trafficking of donor cells. We did not detect donor cells at 16 days posttransfer in any tissue (Fig. 4d). Cells were previously detected in the lymph nodes, blood, and lung of this animal at 7 days posttransfer. These results suggest that trafficking from the blood to

FIG 1 Cells isolated from mucosal draining lymph nodes traffic to mucosal tissues after transfer. Multiple mucosal draining lymph nodes were harvested from an MCM and transferred into an MHC-matched MCM. The recipient animal was examined for donor cell presence at 5 min posttransfer (a), cell trafficking to lymphoid tissues at 36 h posttransfer (b), and cell trafficking to mucosal tissues at 36 h posttransfer (c). The percentage of lymphocytes that are donor cells is displayed below each dot plot. IEL, intraepithelial lymphocytes; LPL, lamina propria lymphocytes.

other tissues did not explain the inability to detect cells in blood after 9 days posttransfer and that cells may have been cleared. Nevertheless, we cannot exclude the possibility that cells were present in tissues that we did not examine or lost the CFSE dye. CFSE is frequently used to track cell division because fluorescence intensity of the cells is halved each time the cell divides. In these experiments, we observed a population of cells that may have divided at 1 day posttransfer in both the vaccinated animal cell recipients (VACRs) and the mock-vaccinated animal cell recipients (MVACRs) (data not shown). Due to the small number of CFSE-

positive cells observed at later time points, it became too difficult to discern possible cell division through our CFSE labeling.

Transferred cells did not affect acute-phase viral loads but may have reduced chronic-phase viral loads. All vaccinated donor animals controlled replication of SIVmac239 Δ nef by the time of transfer, except for a single animal, cy0213 (Fig. 5a). We challenged recipient animals with 50,000 TCID₅₀ of SIVmac239 intrarectally within 24 h of transfer. Given the trafficking of cells to mucosal tissues, we expected cells to be present at sites of early viral replication. All animals were infected after a single high-dose

FIG 2 SIVmac239Δnef infection of Mauritian cynomolgus macaques. Quantitative PCR was used to detect copies of vRNA in the plasma of vaccinated macaques. The log copies per ml are plotted on the y axis, and the days postvaccination are plotted on the x axis. The arrow indicates the time of SIVmac239 challenge in the recipient animals. Dashed lines display viral loads from animals previously challenged with the same stock and dose of SIVmac239.

challenge (Fig. 5b and c) and developed peak viral loads of between 1×10^6 and 1×10^8 vRNA copies per ml plasma. Because cells were detected for only 9 days, we expected any functional effect of these transferred cells to occur immediately after challenge, but there were no significant differences in peak viral loads during the acute phase of infection or early set point between VACRs and MVACRs (Fig. 5c and d). After 26 weeks postinfection, we noted slightly reduced viral loads in the VACRs (Fig. 5e).

VACRs had roughly a 0.5-log-lower geometric mean viral load between weeks 26 and 52 ($P = 0.12$) than MVACRs. These differences did not reach statistical significance.

Adoptive transfers may have altered kinetics of SIVmac239 evolution. Since the SIVmac239Δnef vaccine virus is integrated into lymphocytes that comprise the transfusate, we realized that the attenuated virus was likely transferred to recipient animals along with donor cells. Therefore, we examined whether transfer of cells from vaccinated donors led to infection of the recipient. We found that >95% of virus sequences isolated from the plasma of VACRs contained the *nef* deletion characteristic of SIVmac239Δnef at 1 week posttransfer (Fig. 6a). The viral loads at 1 week postinfection were similar between the two VACRs and MVACRs. WT SIVmac239 replaced this virus by 2 weeks posttransfer, demonstrating that SIVmac239 outgrew SIVmac239Δnef after intrarectal challenge. We did not detect SIVmac239Δnef after 2 weeks postchallenge.

Furthermore, because SIVmac239Δnef replicated for 20 to 24 weeks in the donor macaques, it was possible that recipient animals were infected with virus that contained escape mutations in CD8 T cell epitopes shared between SIVmac239 and SIVmac239Δnef (33). We previously reported five CD8 T cell epitopes restricted by MHC class I alleles shared by all animals in this cohort that accumulate mutations under CD8 T cell selection pressure (34, 35). Three of these epitopes are known to escape from CD8 T cell immune responses by 20 weeks postinfection. Therefore, SIVmac239Δnef may have escape mutations in these three epitopes prior to cell transfer. One of the epitopes, Nef₁₀₃₋₁₁₁RM9, is absent from SIVmac239Δnef, but we examined sequences in Pol₅₉₁₋₅₉₈QP8 and Gag₃₈₆₋₃₉₄GW9. We found that one of these two epitopes, Gag₃₈₆₋₃₉₄GW9, contained potential escape mutations in the VACRs at 1 week posttransfer (Fig. 6b). Donor animals had undetectable viral loads at the time of transfer, which precluded plasma virus sequencing in these animals.

We were interested in assessing the effect of these mutations on recognition by CD8 T cells. We used a gamma interferon (IFN-γ) enzyme-linked immunosorbent spot (ELISpot) assay with cryopreserved MVACR and VACR PBMC between 2 and 8 weeks posttransfer to test for Gag₃₈₆₋₃₉₄GW9 variant-specific responses. We included wild-type Gag₃₈₆₋₃₉₄GW9 and three variant epitopes, GW9-P2L, GW9-P2T, and GW9-P5L, and found that only two

FIG 3 Transfusate composition before transfer into recipient animals. (a) Each bar shows the transfusate composition from a different donor animal. The different colors each represent a different tissue that contributed to the overall transfusate. The bar shows the percentage that each tissue contributed to the total number of cells transferred. (b) Each donor animal is plotted along the x axis. The graph displays the percentage of the transfusate that was represented by each lymphocyte type. The remaining percentage of cells (other) was not represented by one of these four populations. Percentages are based on values in the forward-scatter/side-scatter-based lymphocyte gate.

FIG 4 Trafficking and persistence of donor cells after transfer. (a) Percentage of CFSE-positive lymphocytes in the blood longitudinally posttransfer. (b) Percentage of lymphocytes that were CFSE positive in the bronchoalveolar lavage fluid of recipient macaques at 1 week posttransfer (P.T.). (c) Percentage of lymphocytes that were CFSE positive in the lymph node at 1 week posttransfer. ND, not done. (d) Animal cy0347 was necropsied at 16 days posttransfer for reasons unrelated to the transfer or SIV challenge. Multiple tissues were examined for the presence of donor cells at this time point. LN, lymph nodes.

animals met the threshold for positive GW9 responses (Fig. 6c). These animals were animals cy0391 and cy0342, a VACR and an MVACR, respectively. Higher-magnitude responses were detected in animal cy0391. Animal cy0391 exhibited a reduced capacity to produce IFN- γ in response to the variant epitopes, suggesting that they inhibit recognition by CD8 T cells; however, these escape mutations did not completely abrogate recognition by cy0391 CD8 T cells in this assay.

Given the slight viral load differences between VACRs and MVACRs, we were interested in studying whether donor cells were functional *in vivo*. Because VACRs should have vaccine-primed, donor SIV-specific CD8 T cells at the time of challenge, we expected that these donor CD8 T cells would select for variation in CD8 T cell epitopes more rapidly in VACRs than in MVACRs. We deep sequenced virus from several animals at 1, 2, and/or 4 weeks posttransfer to obtain the entire SIV coding sequence. Indeed, we found that in the Gag₃₈₆₋₃₉₄GW9 epitope, the frequency of wild-type sequence in VACRs was significantly lower than the frequency of WT SIVmac239 sequence in MVACRs ($P <$

0.05) at 4 weeks posttransfer (Fig. 6d). In VACRs and MVACRs, we detected only WT SIVmac239 at 2 weeks posttransfer, suggesting that transferred cells might have exerted greater selective pressure in VACRs than the *de novo* responses in the MVACRs (Fig. 6d). Nevertheless, these results are confounded by the fact that animals were likely infected with “preescaped” SIVmac239 Δ nef, which may lead to recombination between SIVmac239 Δ nef and SIVmac239. This recombination could result in more rapid escape kinetics. Thus, these results do not conclusively demonstrate that donor cells were functional or effective posttransfer. Combined, these data indicate that adoptive transfer may have impacted the kinetics of viral evolution in naive recipients.

DISCUSSION

The fields of immunology, pathology, and transplantation have benefited immensely from inbred mouse models and the ability to perform adoptive transfers. The use of genetically identical or syngeneic mice allows for the transfer of cells between mice and the persistence of donor cells in recipient animals. These types of

FIG 5 Plasma viral loads after transfer in recipient animals. (a) Viral loads from vaccinated donor macaques plotted against days after vaccination with SIVmac239 Δ nef. (b) Viral loads from all recipients plotted together. (c) Graph displaying peak acute-phase viral loads from recipient animals. (d) Geometric mean of viral loads between weeks 6 and 26. (e) Geometric mean of viral loads between weeks 26 and 52.

studies have previously been used to identify the correlates of protection from challenge with retroviruses like Friend virus (13). Until recently, similar studies using genetically identical animals have not been possible with HIV because most laboratory mice are not susceptible to HIV.

Investigators have made great strides in validating a humanized mouse model for studying HIV. This model utilizes mice that have immune systems reconstituted with human cells. Humanized mice can be infected with HIV because human CD4 T cell targets are present. These models have successfully demonstrated the protective effects of microbicides (36, 37). Additionally, researchers have observed viral escape from CD8 T cell immune pressure similar to that seen in HIV-infected humans and SIV-infected macaques (38). While these mice continue to be evaluated for the ability to recapitulate features of normal HIV pathogenesis, they will likely complement other approaches and models for the study of HIV. Beyond this mouse model, there is no widely available adoptive transfer model for studying HIV.

Our laboratory has identified a group of macaques with limited MHC diversity, which allows for the temporary persistence of cells transferred between MHC-matched macaques. The existence of MHC-identical MCM made it possible to perform *ex vivo* allogeneic adoptive transfers. This model may also have utility for cell transplants in the nascent field of HIV cure research. Investigators are examining the potential of CCR5 gene-modified T cells as HIV therapies and may benefit from the use of an adoptive transfer model (39, 40). Additionally, investigators are currently looking

into the transfer of epitope-specific cell lines, similar to work that has previously been completed in cancer research (41). Researchers are examining both the ability to culture HIV-specific cells *in vitro* and the ability to genetically modify T cells that carry antigen receptors specific for HIV (17, 42, 42). SIV infection of macaques is currently the best model for HIV, and allogeneic transfer studies may benefit from the use of MCM, where investigators can adjust the degree of MHC matching to increase cell persistence.

We used this model to assess the correlates of protection during attenuated SIV vaccination. We mock vaccinated or vaccinated macaques with SIVmac239 Δ nef. We then harvested tissues from these animals after 20 weeks, transferred them into naive macaques, and challenged recipient animals with the homologous virus SIVmac239. Cells were detectable in recipients for 9 days. While animals were MHC matched, cells were likely cleared due to minor antigen differences and would be expected to persist longer in transfers between genetically identical animals (e.g., monozygotic twins). We hypothesized that a protective effect from these transfers would manifest as a reduction in acute-phase viremia. We did not observe such differences. One explanation for the absence of an effect on viral loads is the low frequency of donor cells in recipient animals. While donor cells represented up to 30% of lymphocytes at 5 min posttransfer, this frequency rapidly decreased by 1 day posttransfer. Additionally, while we did not quantify the number of SIV-specific T cells or B cells before transfer, they were likely an extremely small percentage of the transfusate. We previously reported full-proteome ELISpot results from

FIG 6 VACRs are infected with preescaped SIVmac239ΔNef through adoptive transfer. (a) Virus was sequenced from multiple VACRs at several time points. The percentage of reads from SIVmac239ΔNef and SIVmac239 are displayed in each bar. (b) Virus was sequenced from MVACRs and VACRs at 1, 2, and/or 4 weeks postinfection. Variants of the epitope Gag₃₈₆₋₃₉₄GW9 are shown in each bar. Sequence variants present in >1% of the total sequence reads in at least one animal are displayed in the graph. All other variants were grouped into the “other” category. (c) IFN-γ ELISpot testing of Gag₃₈₆₋₃₉₄GW9 and Gag₃₈₆₋₃₉₄GW9 escape variant epitope recognition by cryopreserved PBMC. (d) Frequency of wild-type SIVmac239 sequence in Gag₃₈₆₋₃₉₄GW9 at 1, 2, and 4 weeks postinfection. Asterisks indicate significant differences ($P < 0.05$) between VACRs and MVACRs at a given time point.

three of the donor animals at 3 and 8 weeks postvaccination, and we found that responses were of minimal magnitude, with no responses above 250 spot-forming cells/million PBMC (43). Thus, the frequency of SIV-specific donor cells in the recipients may have been too low to exact a significant effect on viral replication. The donor T cells that were transferred were a mix of central memory, naive, and effector memory cells, as defined by CD28 and CD95 expression (data not shown), and it may be important to isolate and transfer primarily effector memory cells in future experiments, because these cells have been correlated with protection in other vaccine studies (8, 44). Furthermore, while there is little evidence that passive transfers alone protect against challenge, it is possible that antibodies contribute to the protection provided by SIVmac239ΔNef; therefore, combining passive transfers with these adoptive transfers may have more profound effects on viral control (45).

Of note, when we began infecting MCM, previous studies from

other groups suggested that more virus is required to infect MCM; thus, we used an extremely high dose that was 10-fold higher than a typical high-dose challenge previously used in rhesus macaques (26, 46). However, high-dose challenges do not accurately recapitulate events in heterosexual HIV transmission and may provide an insurmountable obstacle for cellular immunity (46). Investigators recently demonstrated that a high-dose challenge can mask vaccine efficacy compared to a low-dose challenge (47). We have since shown that the use of 1,000 TCID₅₀, instead of 50,000 TCID₅₀, as in this study, consistently infects MCM in a single challenge. Future studies with this “lower-high-dose” challenge may effectively resolve differences between cells adoptively transferred from vaccinated and mock-vaccinated donors. We expect to perform additional transfers using this lower challenge dose to determine whether there are significant viral load differences between VACRs and MVACRs.

After we began this study, we discovered that approximately

50% of animals with the M1 MHC haplotype spontaneously control SIVmac239 replication (31). We do not believe that this significantly confounds the current study because MHC-identical pairs of animals received vaccinated and mock-vaccinated cells, and only one of five MVACR M1 animals controlled viral replication, which may suggest that cell transfers led to increased viral loads, as demonstrated previously (48). In VACRs, the tendency of cell transfers to increase viral load may have been offset by the presence of effective immunity. In other words, SIV-specific responses present in the cells transferred from vaccinated donors may overcome these higher viral loads, resulting in a very modest reduction in chronic-phase viral loads. If transferred cells mediated such an effect, it is difficult to reconcile this phenomenon with their temporary persistence. With these small group sizes, differences in chronic-phase viral load did not reach significance; however, the very slight difference in mean viral loads between VACRs and MVACRs is provocative. We are currently expanding these studies to improve our ability to resolve differences in viral load and are exploring several different mechanisms that might differentiate these two groups.

First, it is possible that donor cells actively suppressed viral replication in the recipients. We expect that the transfer of donor cells, primed by vaccination with SIVmac239 Δ nef, would help recipients control viral replication better than MVACRs who received cells that were not primed by vaccination. The rapid accumulation of escape mutations in the Gag₃₈₆₋₃₉₄GW9 epitope of VACRs compared to MVACRs supports this hypothesis; however, this evidence is confounded by recipient animal infection with preescaped SIVmac239 Δ nef. Because plasma viral loads were undetectable in the donor animals, we believe that the replicating virus at 1 week posttransfer in the recipient animals was derived from provirus or cell-associated virus. Sequencing of provirus from donor cells might indicate whether the mutations that we detected at 1 week posttransfer were present in the donor animal or whether pressure from donor CD8 T cells selected for these escape mutations. Ultimately, if the escape mutations do indicate selective pressure from functional donor cells, these donor cells were unable to significantly reduce viral loads in the recipient animals. Additionally, the rapid growth of SIVmac239 Δ nef may have been a result of immune activation after the transfer. Immune activation may have been particularly acute because, despite MHC class I and class II matching, animals were not genetically identical and were likely mismatched for numerous minor antigens.

Second, infection of recipients with preescaped virus might lead to the generation of CD8 T cells that recognize variant epitopes. If the preescaped SIVmac239 Δ nef induces variant-specific CD8 T cells, then we expect that these variant-specific cells would select for alternative escape mutations at 4 weeks posttransfer. In contrast, we found that two animals sequenced at both 1 and 4 weeks postinfection had the same predominant escape mutations at both time points (Fig. 6b). Additionally, our ELISpot data suggest that animals are occasionally capable of recognizing variant Gag₃₈₆₋₃₉₄GW9 epitopes, but the assay was not sensitive enough to measure whether there were meaningful differences in variant epitope recognition between VACRs and MVACRs.

Finally, a potential explanation for the differences in viral loads between VACRs and MVACRs is that sequence changes in a greater-than-typical number of CD8 T cell epitopes reduced overall viral fitness. We recently reported that escape in two epitopes,

Gag₃₈₆₋₃₉₄GW9 and Rev₅₉₋₆₈SP10, occurs conditionally; escape in Rev₅₉₋₆₈SP10 precludes the simultaneous acquisition of escape variants in Gag₃₈₆₋₃₉₄GW9 (29). It was possible that by infecting animals with preescaped SIV, we favored recombination and escape from multiple CD8 T cell responses simultaneously at the expense of viral fitness. We sequenced viruses from four animals that recognize both Rev₅₉₋₆₈SP10 and Gag₃₈₆₋₃₉₄GW9 at 4 weeks posttransfer. In support of this hypothesis, the cumulative frequency of WT SIVmac239 in these two epitopes was lower in VACRs than in MVACRs; however, these results did not reach significance given the small number of animals (data not shown). In opposition to this hypothesis, recipient animals that do not recognize Rev₅₉₋₆₈SP10 were also capable of controlling viral replication, but we cannot exclude the possibility that these animals exhibited accelerated escape kinetics in an uncharacterized epitope and that rapid accumulation of these escape mutations was ultimately deleterious for the virus. It is nearly impossible to ascertain if these escape mutations arose more rapidly due to recombination or evolved *de novo* in the WT SIVmac239 challenge virus because SIVmac239 Δ nef and SIVmac239 are homologous outside the *nef* deletion. Ultimately, we do not have compelling proof that one of these mechanisms, including effects of transferred cells, priming of variant-specific T cells, or altered kinetics of viral evolution, is leading to reduced chronic-phase viral loads, and it may be a combination of these or unknown mechanisms.

This study represents the first time that adoptive transfers have been used to assess the correlates of protection in SIVmac239 Δ nef. These studies may indicate that the presence of cellular immunity and/or viruses with complex epitope sequences at the time of challenge can lead to differences in chronic-phase viral loads. While the transfer of 1.5×10^9 to 3×10^9 cells was not sufficient to protect against an extremely-high-dose pathogenic challenge with SIVmac239, these results provide evidence that preexisting immunity to SIV, as delivered here through adoptive transfers, might alter the kinetics of viral evolution and could have long-term effects on control of viral replication. Since rapid amplification and replication in GALT and lymph nodes are hallmarks of HIV infection, mechanisms that help the immune response prepare for and adapt to this initial viral insult may be critical to future vaccine design.

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