

Extralymphoid CD8⁺ T Cells Resident in Tissue from Simian Immunodeficiency Virus SIVmac239Δnef-Vaccinated Macaques Suppress SIVmac239 Replication *Ex Vivo*[▽]

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Live-attenuated vaccination with simian immunodeficiency virus (SIV) SIVmac239Δnef is the most successful vaccine product tested to date in macaques. However, the mechanisms that explain the efficacy of this vaccine remain largely unknown. We utilized an *ex vivo* viral suppression assay to assess the quality of the immune response in SIVmac239Δnef-immunized animals. Using major histocompatibility complex-matched Mauritian cynomolgus macaques, we did not detect SIV-specific functional immune responses in the blood by gamma interferon (IFN-γ) enzyme-linked immunospot assay at select time points; however, we found that lung CD8⁺ T cells, unlike blood CD8⁺ T cells, effectively suppress virus replication by up to 80%. These results suggest that SIVmac239Δnef may be an effective vaccine because it elicits functional immunity at mucosal sites. Moreover, these results underscore the limitations of relying on immunological measurements from peripheral blood lymphocytes in studies of protective immunity to HIV/SIV.

Despite over 25 years of intensive research, efforts to develop a successful prophylactic HIV vaccine have failed (6, 39). The extraordinary difficulty of developing an HIV vaccine underscores the fact that the elements comprising an effective immune response directed against HIV are poorly understood. Simian immunodeficiency virus (SIV) infection of Mauritian cynomolgus macaques (MCM) provides the best model for unraveling the correlates of protection against SIV. With SIV infection of MCM, we can select the timing, route, dose, and sequence of the infecting virus. Additionally, the limited genetic diversity of MCM facilitates selection of genetically matched individuals that can be monitored throughout the acute phase of infection and enables more frequent and invasive sampling, especially of mucosal sites.

Macaques infected with live-attenuated SIV, like SIVmac239Δnef, exhibit robust protection from pathogenic SIV infection (8, 10, 20, 25, 29, 35, 40, 44, 49, 53). While previous studies have included considerable heterogeneity in the strain of attenuated SIV, challenge strain, and macaque species used, they demonstrate collectively the broad spectrum of attenuated SIVs that effectively protect macaques against pathogenic challenge. Several studies have also shown that attenuated SIV effectively protects cynomolgus macaques against pathogenic challenge using an SIVmac239C8 virus (1–3). Like SIVmac239Δnef, this virus has a deletion in the *nef* gene, but this deletion is a considerably smaller 12-bp deletion

than the 182-bp deletion in SIVmac239Δnef (24). Importantly, there are no studies that establish the protective efficacy of SIVmac239Δnef in MCM. Nevertheless, peak SIVmac239Δnef viral loads in MCM parallel the range established in rhesus macaques, between 3.2×10^3 and 9.4×10^5 (35), but fall below peak loads established in a separate study (8). The differences observed between the rhesus macaque study and our own could be due to differences in challenge dose. Long-term control of SIVmac239Δnef in MCM is also similar to that in rhesus macaques (35). It is critical to understand why live-attenuated SIV vaccines are so effective, with the ultimate goal of using these principals to develop a vaccine that is safe for use in humans.

There are several plausible explanations for why live-attenuated SIV vaccines provide effective protection against challenge with pathogenic SIV. These explanations range from viral interference to a robust vaccine-elicited immune response (19, 43, 45, 47). We currently understand several aspects of live-attenuated vaccination with SIVmac239Δnef. First, there is an inverse relationship between the degree of attenuation and the level of protection (21). This relationship suggests that vigorous viral replication is important for the generation of an effective anti-SIV immune response. Second, the greater the sequence diversity between the vaccine strain and the challenge strain, the weaker is the protection provided by the vaccine (53). This demonstrates that an adaptive immune response that recognizes similar epitopes or virus features between the vaccine and challenge strains is necessary for protection in this model. Finally, vaccination with live-attenuated SIV requires a 15- to 20-week induction phase to achieve protection in the majority of animals (8). Thus, there is a direct

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relationship between the time postvaccination and the degree of protection between 0 and 20 weeks postvaccination. This temporal relationship suggests that a fully developed memory response is required to protect against pathogenic SIV challenge. Together, these observations argue that SIV-specific CD8⁺ T-lymphocyte responses might be important in protection as these responses would be less useful in the setting of heterologous virus challenge and require both robust viral replication and time to develop. Such responses, however, can be weak to nonexistent in the blood of vaccinated animals and frequently do not correlate with disease progression, leading some investigators to question their importance in protective immunity (25, 29, 45, 46). We hypothesize that continued replication of live-attenuated SIV in the mucosal tissues may lead to effective, compartmentalized memory T-cell responses that are important in controlling pathogenic SIV challenge.

It is possible that prophylactic mucosal immunity is required to prevent viral replication soon after SIV infection and to minimize the destruction of mucosal immune cells that occurs within the first 3 weeks of SIV infection (9, 22, 26, 30). Initial depletion of effector memory CD4⁺ T cells in the gut-associated lymphoid tissue (GALT) combined with continuous viral replication leads to prolonged immune activation, eventual depletion of central memory CD4⁺ T cells, and the development of AIDS. An effective mucosal immune response elicited by live-attenuated SIV vaccination may prevent the initial CD4⁺ T-cell depletion from the gut. Several recent studies confirm a critical protective role for CD8⁺ T cells in the genital tract after vaccination with SHIV89.6, demonstrating that a mucosal immune response is capable of protecting against or ameliorating SIV infection (14–16, 48). Another study has also demonstrated the presence of high-frequency, polyfunctional T-cell responses in the mucosal tissues of elite controllers, i.e., individuals who maintain plasma viral loads below 75 copies/ml, compared to blood from the same individual, tissues of noncontrollers, and antiretroviral drug-treated patients. This study also provides a correlation between mucosal CD8⁺ T-cell responses and HIV control (12).

While studying gut mucosal tissues is clearly an important part of understanding HIV/SIV pathology, there are several challenges to this undertaking. First, accessing gut tissues requires invasive sampling procedures, which are primarily limited to biopsy or time-of-death studies. Biopsies are often limited in number throughout the life span of an animal, while routine necropsy is cost-prohibitive for macaque studies. Second, these tissues are nonsterile. The digestive tract is teeming with floras that contaminate experiments requiring long-term cell culture. Finally, biopsies of mucosal tissues yield very few cells. These low cell numbers make *ex vivo* experiments very difficult or impossible to perform. Investigators have developed techniques to assess gut mucosal lymphocyte function by expanding these cells *in vitro* under sterile conditions with antibiotics and then using them in enzyme-linked immunospot assays (ELISPOT) or intracellular cytokine secretion (ICS) assays (18, 42). However, these experiments still suffer from two problems: (i) cells are altered *in vitro*, which may change their functional capacity; and, (ii) these experiments still rely on indirect measures of CD8⁺ T-cell function. These difficulties have limited research on mucosal CD8⁺ T-cell immunity during SIV infection.

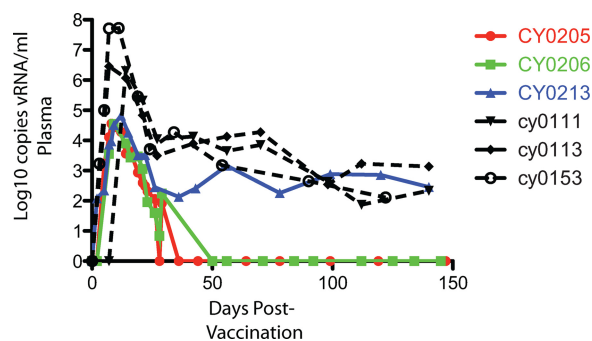


FIG. 1. Vaccination with SIVmac239 Δ nef. Animals were infected intravenously with 10 ng of p27 SIVmac239 Δ nef. Virus was measured in the plasma of vaccinated animals three times a week for the first 4 weeks and every 2 weeks thereafter until week 20. Vaccinated animals are in color and plotted against wild-type SIVmac239-infected animals represented by dashed black lines. All animals in the figure are MHC-I matched. Animal cy0153 was infected as part of an adoptive transfer experiment.

In light of these challenges, we decided to focus on the lung mucosal tissue, using CD8⁺ T cells isolated from bronchoalveolar lavage fluid (BAL). BAL samples lung mucosa where there are a large number of resident lymphocytes that encounter respiratory pathogens. Furthermore, BAL provides a minimally invasive sampling of a mucosal tissue that can be performed frequently, and BAL harbors effector T cells similar to GALT (32). These factors make the lung an ideal site for sampling mucosal CD8⁺ T cells.

We modified an *ex vivo* viral suppression assay that tests the ability of CD8⁺ T cells to prevent viral replication in MCM (7, 27, 28, 36, 51, 54, 55). Using this approach, we compared the suppressive capacity of CD8⁺ T cells isolated from lung and blood, and we found that CD8⁺ T cells from the lung are more effective at suppressing viral replication than CD8⁺ T cells from the blood. This assay does not manipulate lung lymphocytes *in vitro* and provides a direct measure of CD8⁺ T-cell function. Furthermore, our data support the idea that CD8⁺ T cells in blood and mucosal tissue are not functionally equivalent, that blood lymphocytes are not a perfect surrogate for mucosal lymphocytes, and that mucosal T cells attenuate SIV replication to a greater extent than blood T cells.

MATERIALS AND METHODS

Animal care and infections. Animals were cared for by the Wisconsin National Primate Research Center (WNPRC) according to protocols approved by the University of Wisconsin Research Animal Resources Center. All animals were infected with either molecularly cloned 50,000 50% tissue culture infective doses (TCID₅₀) of SIVmac239 Nef-open virus intrarectally (23) or 10 ng of p27 SIVmac239 Δ nef intravenously (10), kindly provided by Ronald Desrosiers. Several SIVmac239-infected animals were infected as part of adoptive transfer experiments.

Microsatellite analysis of MCM. Animals were haplotyped using our previously described panel of microsatellite markers spanning the major histocompatibility complex (MHC) region (52). These haplotypes were used to infer the MHC genotypes of the animals for purchasing MHC-matched animals.

Cell preparations. Blood was drawn from animals into EDTA tubes at the WNPRC and layered over Ficoll-Paque Plus (GE Healthcare Bioscience, Uppsala, Sweden); peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation. BAL was collected by primate center staff. Briefly, saline solution was flushed into the bronchus and aspirated until fluid was no longer recovered. BAL was filtered over a 70- μ m-pore-size cell strainer and then spun

TABLE 1. IFN-γ ELISPOT analysis of T-cell responses in the blood postvaccination^a

Peptide ^b	T-cell response (no. of SFU/1 × 10 ⁶ cells) in the indicated animal at:					
	3 wks postvaccination			8 wks postvaccination		
	CY0205	CY0206	CY0213 ^c	CY0205	CY0206	CY0213
ConA	3,881	3,826	ND	4,080	9,991	1,094
Pol 241-291			ND		98.3	
Vif 41-91	83		ND			
Env 841-879	83.3		ND			
Env 321-371	153.3		ND			
Rev 40-90		118	ND			
Gag 361-411		208	ND			
Pol 641-691		199.2	ND			
Pol 681-727		104.2	ND		103.3	
Pol 757-807					98.3	

^a Three animals were vaccinated with SIVmac239Δnef, and T-cell responses were measured using an IFN-γ ELISPOT assay and stimulation with peptides spanning the entire viral proteome. Positive responses are reported for pools of 10 overlapping 15-mer peptides. Concanavalin A (ConA) was used as a positive control.

^b Residues are given for the indicated peptide. ConA, concanavalin A.
^c Sufficient blood was not available from animal CY0213 to perform ELISPOT analysis 3 weeks after vaccination. ND, not done.

and resuspended in complete medium. Effector CD8⁺ T cells were isolated using an anti-CD8β-phycoerythrin (PE) monoclonal antibody (Beckman Coulter, Fullerton, CA) and anti-PE beads with LS columns (Miltenyi, Auburn, CA). Targets were prepared by depleting CD8⁺ cells using the nonhuman primate CD8 cell-positive selection kit from Miltenyi.

CD8-depleted target cells were incubated in T-25 flasks at 37°C for 1 day at 1.5 × 10⁶ cells/ml with concanavalin A at 5 μg/ml. Cells were washed after 24 h and placed in a new flask in the same volume. After 4 days, cells were washed and plated at roughly 2 × 10⁶/ml in 48-well plates.

Virus was prepared by layering 1 ml of 9 × 10⁹ TCID₅₀ of SIVmac239 over 100 μl of 20% sucrose and spinning at 14,000 rpm for 60 min. The supernatant was removed, and virus was resuspended in complete medium. Twenty-five μl of magnetic beads was added to the resuspended virus, and 30 μl of the virus-bead mix was added to each well of CD8-depleted cells. Plates were placed in the incubator for 20 min on a magnet to ensure sufficient infection of cells.

CD8⁺ T-cell viral suppression assay (VSA). Infected targets were plated in 96-well plates at 25,000 cells/well. Effectors were plated according to the appropriate effector/target (E/T) ratio. After 4 days in culture, cells were initially surface stained with anti-CD8-Pacific Blue, anti-CD4-allophycocyanin (APC), and anti-CD3-Alexa Fluor 700 (BD Biosciences, San Jose, CA) and then stained for intracellular Gag-p27. Anti-Gag p27 antibody 55-2F12 (NIH AIDS Research and Reference Reagent Program) conjugated to fluorescein isothiocyanate (FITC) was combined with bulk permeabilization reagent (Invitrogen, Carlsbad, CA), and cells were incubated at room temperature for 15 min before being washed and fixed with 2.0% paraformaldehyde. Plates were then run on an LSRII flow cytometer (BD) using a high-throughput system and analyzed with FlowJo software, version 8.8.6 (TreeStar, Ashland, OR). All values were normalized by dividing the average percentage of p27-positive (p27⁺) cells in each well by the average percentage of p27⁺ cells in the control wells with no effectors.

Assays performed using vaccinated animals utilized different but MHC-matched animals for effectors and targets. In assays testing effector cells from SIVmac239-infected animals, all VSAs used an autologous system. Six of the naïve animal assays were performed in an allogeneic MHC-matched system, and three were autologous.

Tetramer analysis. Peptide-major histocompatibility complex class I tetramer analysis was performed as previously described (4). Briefly, either freshly isolated PBMC and BAL or thawed PBMC were stained with Gag₃₈₆₋₃₉₄-GW9/Mafa-A1*063, QP8/Mafa-A4*0101, and HL9/Mafa-B*5101 conjugated to either PE or APC. Cells were incubated at 37°C in complete medium for 1 h and then surface stained with anti-CD4-peridinin chlorophyll protein (PerCP), anti-CD8-Pacific Blue, and anti-CD3 Alexa Fluor 700. Cells were subsequently washed twice and fixed for analysis by flow cytometry using an LSRII instrument (BD). We tested each vaccinated animal at two separate time points in the PBMC and two vaccinated animals at a single time point in the BAL.

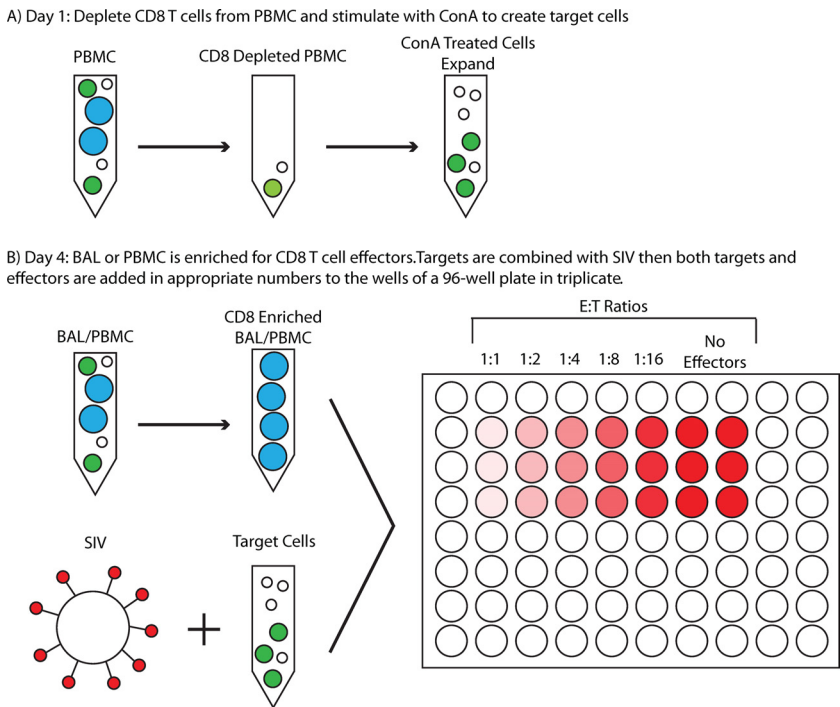


FIG. 2. Viral suppression assay schematic. (A) Lymphocytes were separated from the blood using Ficoll density centrifugation, and CD8⁺ T cells were magnetically separated from the lymphocyte preparation. The CD8-depleted cells were then activated with concanavalin A to create targets. (B) The target cells were combined with SIVmac239 using a previously described magnetofection system. Effector cells were obtained by magnetically enriching for CD8⁺ cells. The targets and effectors were then combined in a 96-well plate, which was stained after 4 days for Gag-p27 and other surface markers.

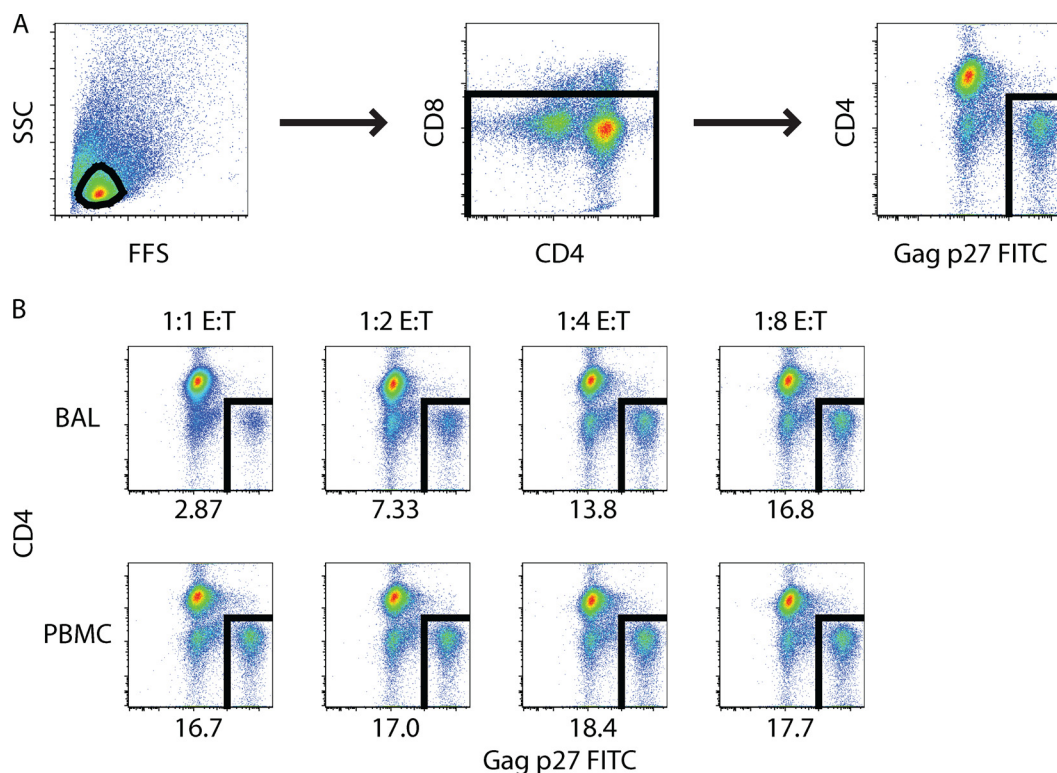


FIG. 3. CD8⁺ T cells from BAL suppress viral replication at several E/T ratios. (A) The gating strategy used throughout the analysis to determine the number of p27⁺ target cells is shown. Cells were first gated for lymphocytes based on their light scatter properties; then CD8⁺ cells were selected. The p27⁺ cells were then quantified as a percentage of the target cells. FFS, forward scatter; SSC, side scatter. (B) Data from a representative assay are shown. The percentages of p27⁺ cells gated as drawn on the right of each dot plot are shown at each E/T ratio.

IFN- γ ELISPOT assays. PBMC were isolated by Ficoll-Hypaque gradient centrifugation. A total of 1×10^5 cells were incubated in duplicate overnight with pools of overlapping 15-mer peptides spanning the entire SIV proteome in gamma interferon (IFN- γ) ELISPOT plates (Mabtech, Columbus, OH). Medium alone was used as a negative control; concanavalin A was used as a positive control. Plates were developed according to the manufacturer's instructions. Spots were imaged and counted objectively with an ELISPOT reader (AID, Strasberg, Germany). The mean number of spot-forming units (SFU) in background wells (medium alone) was subtracted from the mean of the experimental wells. Responses were considered positive if the mean of the sample and background wells differed by more than two standard deviations.

Plasma virus analysis. Plasma virus concentration was determined using previously described methods (52). Briefly, viral RNA was reverse transcribed and then quantified using a SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen, Carlsbad, CA) on a LightCycler 2.0 (Roche, Indianapolis, IN). The final reaction mixtures (20 μ l) contained 0.2 mM deoxynucleoside triphosphates, 3 mM MgSO₄, 0.015% bovine serum albumin, 150 ng of random hexamers (Promega, Madison, WI), 0.8 μ l of SuperScript III reverse transcriptase and Platinum Taq DNA polymerase in a single enzyme mix, 600 nM amplification primers (5'-GTCTGCGTCATCTGGTGCATTC-3' and 5'-CACTAGCTGTCTCTGCACTATGTGTTT-3'), and 100 nM probe (5'-6FAM-CTTCTCAGTGTGTTTCACTTTCTCTCTCTGCG-BHQ1-3', where 6FAM is 6-carboxyfluorescein and BHQ1 is Black Hole Quencher 1). The reverse transcriptase reaction was performed at 37°C for 15 min and then at 50°C for 30 min. An activation temperature of 95°C for 2 min was followed by 50 amplification cycles of 95°C for 2 min and 62°C for 1 min, with ramp times set to 3° per s. Serial dilutions of an SIV Gag *in vitro* transcript were used to generate a standard curve for each run. Copy numbers were determined by interpolation onto the standard curve with the LightCycler software, version 4.0.

Statistics. Parametric *t* tests were performed using GraphPad Prism, version 5.0a for Macintosh (GraphPad Software, San Diego, CA [www.graphpad.com]). Paired *t* tests were used for analyses comparing measurements within animals, and unpaired *t* tests were used for comparisons of measurements between different animals. Error bars on graphs represent the standard error of the mean.

RESULTS

Live-attenuated vaccination does not induce high-frequency functional T-cell responses in the blood. We vaccinated three MHC-matched Mauritian cynomolgus macaques with SIVmac239 Δ nef. Plasma viral loads of SIVmac239 Δ nef peaked between 1×10^4 and 1×10^5 copies of viral RNA (vRNA)/ml of plasma and were controlled to undetectable levels within 4 to 8 weeks in two of three animals (Fig. 1). Animal CY0213 had a higher acute-phase viral load that remained detectable until 20 weeks postvaccination. In Fig. 1, the dashed lines in black represent animals infected with wild-type SIVmac239. All animals in the figure are MHC class I (MHC-I) matched, demonstrating that SIVmac230 Δ nef infection is acutely attenuated in the MCM.

To explore the mechanisms responsible for controlling SIVmac239 Δ nef replication, we performed a full SIV proteome IFN- γ ELISPOT assay using peptide pools of 15-mers overlapping by 11 amino acids. However, high-frequency SIV-specific T-cell responses were not detected in the blood (Table 1). Animals mounted three to four responses by 3 weeks postvaccination. Only one animal, CY0206, mounted a response against a single pool that was detected at both 3 and 8 weeks postvaccination. Although the three macaques were MHC matched, we did not detect responses against the same peptide pools by IFN- γ ELISPOT assay. The low reproducibility and lack of high-frequency immune responses in the blood of our vaccinated animals are consistent with recently published data using rhesus macaques in which often transient T-cell re-

sponses were of similarly low frequency in the vaccine phase 1 month prior to challenge (35) and also concur with a second study using a different attenuated SIV in the rhesus macaque model (29). The low-level replication of SIVmac239Δnef may ultimately preclude the generation of high-frequency peripheral immune responses. It is also possible that transient responses were mounted between the time points studied but were not detected due to our sampling interval. In this light, we next explored whether we could detect functional lymphocytes at other anatomic sites using avant-garde techniques.

Development and application of a novel viral suppression assay. To compare CD8⁺ T-cell responses in blood and an easily accessible mucosal tissue, we elected to sample CD8⁺ T cells from the lung. Resident lung lymphocytes were obtained using BAL by flushing the lungs with saline. We repeatedly recovered approximately 10-fold fewer lymphocytes from BAL than from blood during our lymphocyte isolations. The low cell recovery in BAL precludes widespread peptide-specific evaluations of CD8⁺ T-cell activity and is minimally sufficient for very focused ELISPOT, intracellular cytokine secretion, or tetramer analyses, which typically use 100,000 to 500,000 cells per test. Therefore, we examined the functional capacity of CD8⁺ lymphocytes isolated from these tissues. Specifically, we modified an *ex vivo* viral suppression assay that requires only 150,000 CD8⁺ effector cells (Fig. 2).

The gating strategy used to identify SIV-infected cells on the basis of intracellular Gag p27 levels is shown in Fig. 3A. We first gated lymphocytes based on forward and side scatter characteristics and then selected CD8-depleted cells to assess the percentage of targets that were p27⁺. We tested four to six E/T ratios for the ability of CD8⁺ T cells from both PBMC and BAL to suppress viral replication (Fig. 3B). The representative data shown in Fig. 3B display an example of the inverse relationship between the number of CD8⁺ effector cells in BAL and the percentage of p27⁺ cells.

CD8⁺ T cells from lung suppress viral replication rapidly after live-attenuated vaccination. BAL CD8⁺ T cells in the SIVmac239Δnef-vaccinated macaques rapidly suppressed viral replication (Fig. 4) in contrast to CD8⁺ T cells from PBMC. We observed suppression by 8 days postvaccination in animal CY0206 (Fig. 4A), at 3 weeks postvaccination in animal CY0205 (Fig. 4B), and at 3 weeks postvaccination in animal CY0213 (Fig. 4C). BAL CD8⁺ T cells suppressed viral replication by up to 80% compared to only 0 to 10% for the corresponding cells in PBMC. The response in the BAL and PBMC of animal CY0205, the only animal that was sampled weekly, remained consistent at time points after 4 weeks postvaccination (Fig. 5).

At the 1:1 and 1:2 E/T ratios, we found that PBMC CD8⁺ T cells did not suppress viral replication either before or after vaccination (Fig. 6). In stark contrast, BAL CD8⁺ T cells suppressed viral replication postvaccination at both the 1:1 E/T ($P = 0.0071$) and 1:2 E/T ($P = 0.0047$) ratios at the time of greatest suppression. Furthermore, BAL CD8⁺ T cells postvaccination suppressed viral replication to a greater extent than BAL CD8⁺ T cells prevaccination at both the 1:1 E/T ($P = 0.016$) and 1:2 E/T ($P = 0.028$) ratios. Baseline VSAs were performed prevaccination because target and effector cells were from different, albeit MHC-matched, animals. We did not observe any background viral suppression due to potential

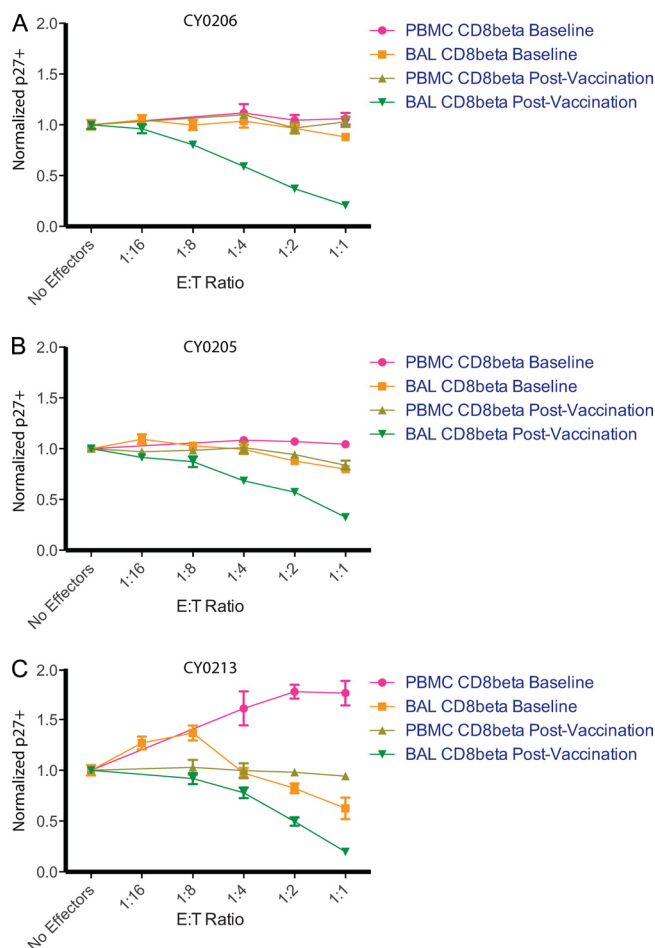


FIG. 4. CD8⁺ T cells from SIVmac239Δnef-vaccinated animals suppress viral replication early after infection. CD8⁺ T cells isolated from BAL and PBMC in SIVmac239Δnef-vaccinated macaques were compared in viral suppression assays both before and after vaccination. All values were normalized by dividing the average percentage of p27⁺ cells in each well by the percentage of p27⁺ cells in the control wells with no effectors. (A) Eight days postvaccination in animal CY0206. (B) Three weeks postvaccination in animal CY0205. (C) Three weeks postvaccination in animal CY0213.

differences in minor antigens although we noted that PBMC effector cells in animal CY0213 appeared to stimulate infection in the baseline assay (Fig. 4). PBMC in this animal did suppress viral replication compared to baseline levels but did not suppress viral replication compared to control wells within the same assay. These results suggest that SIVmac239Δnef may be generating a localized immune response at a mucosal site. Importantly, these results demonstrate that immune responses that are absent or undetectable in blood may be detectable in other tissues.

Differences in the antiviral efficacy of mucosal and blood CD8⁺ T cells from SIVmac239-infected cynomolgus macaques. We looked retrospectively at viral suppression in wild-type SIVmac239-infected MCM. These macaques represented a heterogeneous population of MCM with various immunogenic backgrounds, plasma viral loads, and durations of infection. We observed minimal to modest suppression of viral replication by PBMC and BAL CD8⁺ T cells from uninfected animals. However, in SIV-infected animals, BAL CD8⁺ T cells

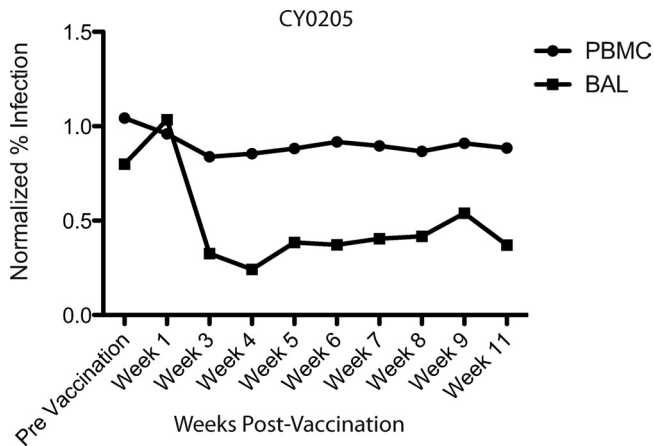


FIG. 5. Consistent suppression of viral replication by BAL-derived CD8⁺ T cells. Viral suppression by CD8⁺ T cells from BAL and PBMC was measured weekly for animal CY0205. The graph shows measurements of p27⁺ cells at the 1:1 E/T ratio; all values were normalized by dividing the average percentage of p27⁺ cells in the experimental wells by the percentage of p27⁺ cells in the control wells with no effectors.

suppressed viral replication far more efficiently than PMBC CD8⁺ T cells (Fig. 7). Moreover, we found that this difference was preserved in E/T ratios as low as 1:4, below which very few effector cells are present (data now shown).

At the 1:1 and 1:2 E/T ratios, BAL CD8⁺ T cells from SIV-infected animals exhibited the most potent suppression of viral replication (Fig. 7A and B). BAL CD8⁺ T cells from infected animals suppressed viral replication significantly better than BAL CD8⁺ T cells from uninfected animals at both E/T ratios ($P < 0.0001$); they also exhibited more potent suppression than PBMC CD8⁺ T cells from infected animals at both E/T ratios ($P = 0.002$). Of note, BAL CD8⁺ T cells from uninfected animals also had a weak tendency to suppress viral replication compared to the corresponding PBMC CD8⁺ T cells at both the 1:1 E/T ($P = 0.005$) and 1:2 E/T ($P = 0.025$) ratios. This weak suppression could result from the naturally activated phenotype of CD8⁺ T cells isolated from extralymphoid tissues, where effector memory cells predominate. These

cells are likely already primed against other pathogens and may be responding and secreting cytokines in response to other antigens present in the lung, thereby leading to non-SIV-specific suppression. Finally, PBMC CD8⁺ T cells from infected animals suppressed more effectively than PBMC CD8⁺ T cells from uninfected animals at both the 1:1 E/T ratio ($P = 0.0003$) and 1:2 E/T ratio ($P = 0.004$); this suggests that SIV-suppressive CD8⁺ T cells are systemic after wild-type SIVmac239 infection. The animals were sampled at different time points postchallenge, thereby making it difficult to assess the relationship between viral suppression and viral loads. However, based on viral loads taken at the time of the viral suppression assay, there did not appear to be any correlation between viral load and viral suppression in PBMC or BAL (data not shown).

Phenotype and specificity of CD8⁺ T cells from PBMC and BAL. At the completion of the 4-day assay, there were generally fewer CD8⁺ T cells in wells with BAL effectors than in wells with PBMC effectors (Fig. 7C). These differences may be due to the activated phenotype of CD8⁺ T cells isolated from mucosal tissues, which typically harbor short-lived effector T cells that may not survive for long periods in culture. We assessed the percentage of CD8⁺ T effector memory cells in both the BAL and PBMC at the time point of greatest suppression in each vaccinated animal. Very few central memory T cells, defined on the basis of CD28 expression within the CD3⁺ CD8⁺ CD95⁺ T-cell population, were present in the lung. However, it is unclear whether this would explain the observed differences in suppression (Fig. 8). It may be that the lung lymphocytes are less robust and that the process of isolation and enrichment leads to increased cell death. Also, the *in vitro* tissue culture microenvironment might be sufficiently different from that in the lung to compromise the expansion of BAL CD8⁺ T cells in comparison to CD8⁺ T cells derived from PBMC. Furthermore, PBMC CD8⁺ T cells could expand more vigorously *in vitro* due to the greater central memory component, which is associated with an increased proliferative capacity. Overall, however, these differences emphasize the ability of lung CD8⁺ T cells to suppress viral replication. Thus, despite the lower numbers of CD8⁺ T cells present in the BAL wells, these cells still exhibited a very strong antiviral effect.

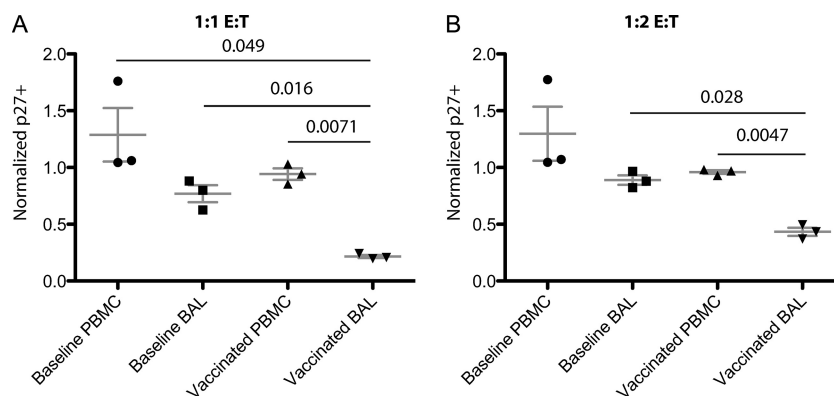


FIG. 6. CD8⁺ T cells isolated from the lung suppress viral replication postvaccination. Measurements represent the time of greatest suppression from the three vaccinated animals postvaccination. These postvaccination time points were 8 days for CY0206, 4 weeks for CY0205, and 3 weeks for CY0213. Significant P values are indicated above the horizontal lines. E/T ratios are shown above the panels. All values were normalized by dividing the average percentage of p27⁺ cells in experimental wells by the percentage of p27⁺ cells in the control wells with no effectors.

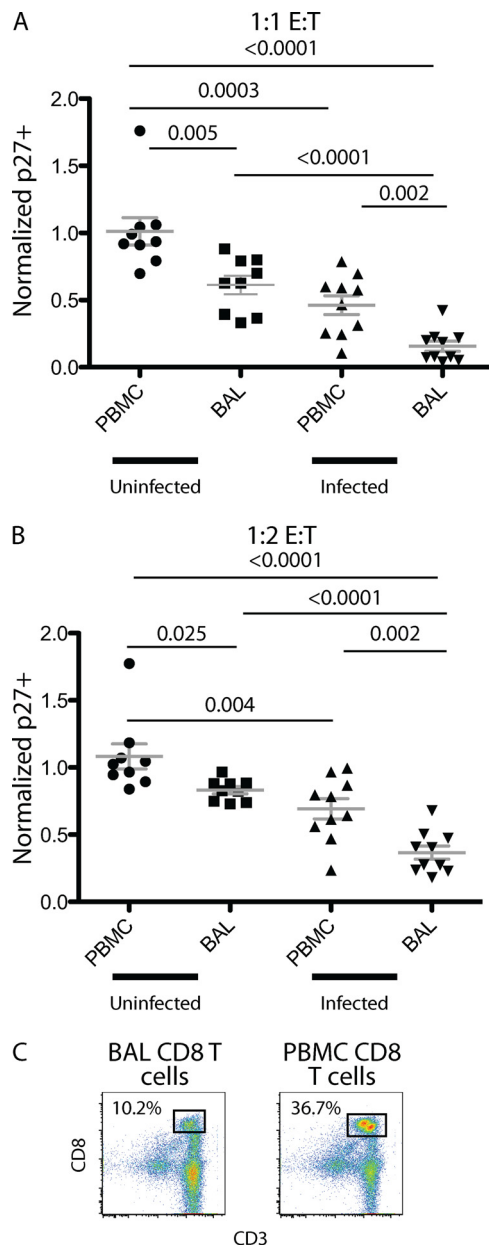


FIG. 7. CD8⁺ T cells from BAL suppress viral replication to a greater extent than CD8⁺ T cells from PBMC. (A and B) Several infected and uninfected animals were used to assess viral suppression at different E/T ratios, as indicated above the panels. Significant *P* values are indicated above the horizontal lines. All values were normalized by dividing the average percentage of p27⁺ cells in experimental wells by the percentage of p27⁺ cells in the control wells with no effectors. (C) The percentage of lymphocytes that were CD8⁺ T cells was measured in the wells at the end of the assay. Higher frequencies of CD8⁺ T cells were present in PBMC samples at the end of the assay.

Finally, we performed tetramer analysis of PBMC and BAL samples from vaccinated animals to assess the physical frequency of SIV-specific responses in both tissues. We tested acute and early chronic-phase PBMC samples with three tetramers (data not shown). Extremely modest cognate CD8⁺ T-cell populations were observed with nearly background lev-

els, as expected in a control, uninfected animal. A single high-frequency response, specific for the Gag₃₈₆₋₃₉₄-GW9/Mafa-A1*063 epitope, was detected in animal CY0213 (Fig. 9). Notably, the frequency of CD8⁺ T cells detected with the Gag₃₈₆₋₃₉₄-GW9/Mafa-A1*063 tetramer in BAL was almost 6.5-fold higher than in PBMC at the same time point (Fig. 9). Thus, despite the modest responses, these results indicate that the increased suppression by BAL CD8⁺ T cells may be due to a higher frequency of SIV-specific T cells in the mucosal tissues after SIVmac239Δnef vaccination.

DISCUSSION

An understanding of the correlates of protection in HIV/SIV infection remains elusive. By comprehending the elements of an effective immune response against the virus, it may be possible to develop technologies that engender these same responses in vaccinated individuals. Here, we modified an *ex vivo* viral suppression assay that directly tests the ability of CD8⁺ T cells to suppress SIV replication. These assays are similar to those described previously (7, 27, 28, 54, 55) and to assays reported recently for the investigation of Gag-specific CD8⁺ T cells in HIV controllers (37). However, in this study, we tested the ability of CD8⁺ T cells derived from both blood and lung to suppress viral replication.

We found that cells isolated from the blood of macaques vaccinated with live-attenuated SIV were not effective at suppressing viral replication. While other investigators have reported vigorous CD8⁺ T-cell responses in the blood of SIVmac239Δnef-vaccinated macaques, recent studies have demonstrated that indirect measurements of CD8⁺ T-cell function may not represent whether a cell is truly functional (7, 51). The weak responses we detected by IFN-γ ELISPOT assays in the blood correlate well with the lack of viral suppression that we observed. Furthermore, we also measured very low frequencies of SIV-specific responses in the blood by tetramer analysis. Additional studies have also reported the use of viral suppression assays to demonstrate the importance of CD8⁺ T-cell function after vaccination with SIVmac239Δnef, SIVmac239Δ3, and simian-human immunodeficiency virus (SHIV) in rhesus macaques (13, 50) and SIVmac251 challenge in cynomolgus macaques (11). In contrast to our experiments, these assays reported viral suppression after vaccination by PBMC CD8⁺ T cells. These differences could potentially be explained by the use of higher E/T ratios, different readouts between assays, differences in the duration of the assay, and, most importantly, the presence of CD8⁺ natural killer cells. Ultimately, using a combination of approaches, we measured very few high-frequency CD8⁺ T-cell responses in the blood of vaccinated macaques and were unable to measure viral suppression by these cells.

We found that CD8⁺ T cells isolated from the lung of vaccinated macaques were extremely effective at suppressing viral replication by 8 to 28 days postvaccination. This BAL CD8⁺ T cell-mediated suppression was maintained at nearly all time points tested postvaccination; in parallel, we were never able to detect strong suppression by PBMC CD8⁺ T cells from the same animals. In contrast to our vaccine results, we found that CD8⁺ T cells isolated from animals that were infected with SIVmac239 could suppress viral

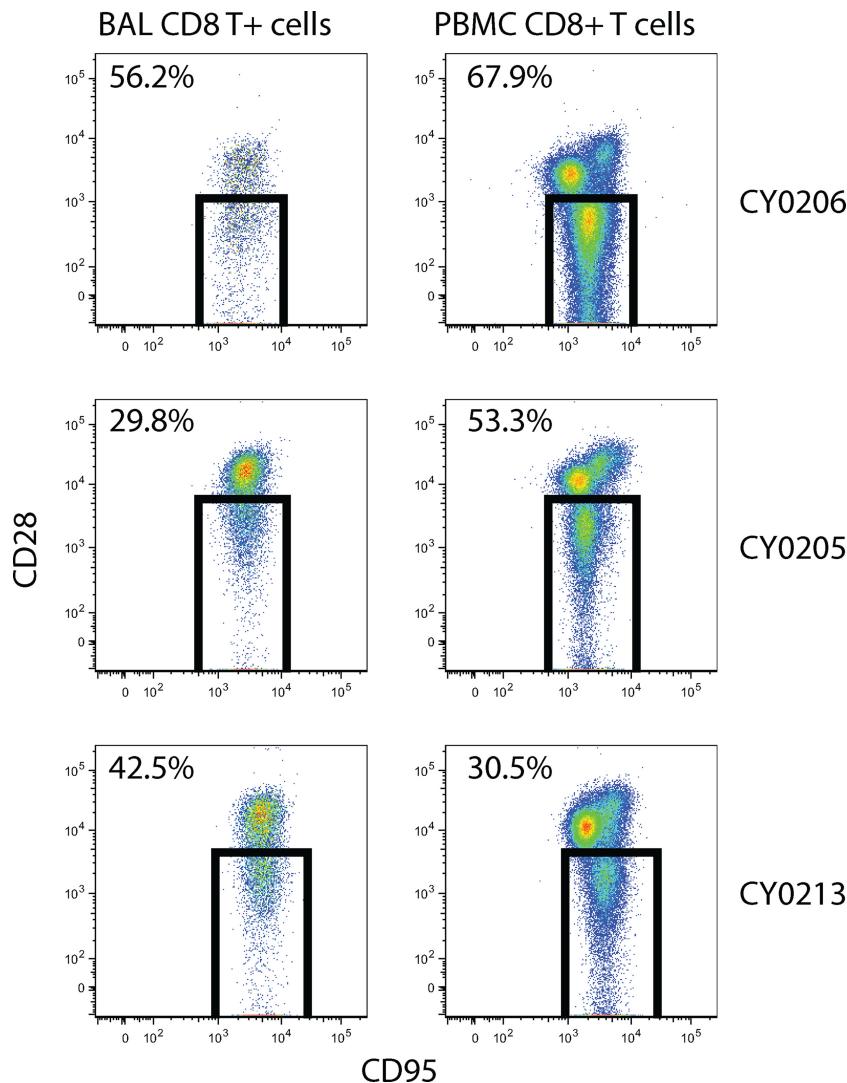


FIG. 8. CD8⁺ T cell effector memory populations from lung and blood. Cells from animals CY0206, CY0205, and CY0213 were stained with anti-CD28-APC and anti-CD95-PerCP-Cy5.5 to define memory status. Gating is based on lymphocytes and CD8⁺ T cells. The percentage of effector memory CD8⁺ CD28⁻ CD95⁺ cells is shown in each case.

replication regardless of tissue type. However, BAL CD8⁺ T cells were more effective at suppressing viral replication than CD8⁺ T cells isolated from the PBMC of the same animals. Despite the increased suppression by BAL, the lung is depleted of effector CD4⁺ T cells during SIV infection, and animals that progress rapidly to AIDS face severe depletion of BAL CD4⁺ T cells (32, 33). Thus, while it is likely due to a variety of different factors, it remains unclear what leads to the development of these effective CD8⁺ T cells in the lung.

Recent studies have demonstrated an increased level of SIV-specific T cells present in BAL after vaccination with an SIV protein-encoding vector based on the rhesus cytomegalovirus (17). Thus, the previously reported T-cell effector memory localization in extralymphoid tissues such as BAL may explain the increased suppression of SIV replication by BAL CD8⁺ T cells (34). However, in our studies, the disparity in viral suppression between BAL and PBMC did not appear to stem from

consistent differences in the distribution of CD8⁺ T-cell effector memory populations between PBMC and BAL as defined by CD28 and CD95 expression. Indeed, while the populations did appear different between the two sites, they did not display consistent patterns that might explain the differences in virus suppression.

Several studies have also addressed the differences between CD8⁺ T-cell responses in blood and mucosal tissues. A study using *in vitro* expanded CD8⁺ T cells from the mucosa and PBMC in chronically HIV-infected individuals showed similarity in the specificity and magnitude of CD8⁺ T-cell responses in both tissues (18). Several other studies have also used different methods to show that responses are generally similar between gut and mucosal tissues including T-cell receptor sequencing and tetramer staining (31, 38, 41). These findings suggest that the differences in the levels of viral suppression between the two tissues may be related to the quality of the response and not cell frequency. In agreement with this idea, it

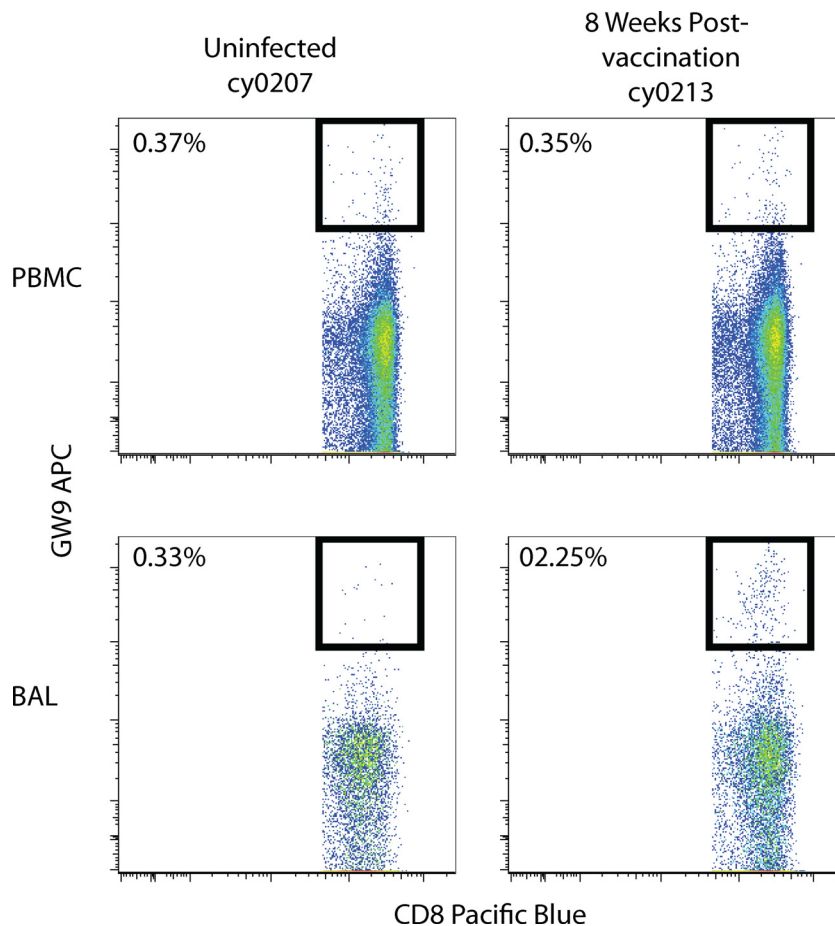


FIG. 9. Gag₃₈₆₋₃₉₄-GW9/*Mafa*-A1*063 tetramer analysis of PBMC and BAL from uninfected animal CY0207 and animal CY0213 at 8 weeks postvaccination. Plots depict the percentages of CD3⁺ CD4⁻ CD8⁺ tetramer-positive cells. The Gag₃₈₆₋₃₉₄-GW9/*Mafa*-A1*063 tetramer was the only one of three that detected a positive response above background. GW9 APC, Gag₃₈₆₋₃₉₄-GW9/*Mafa*-A1*063 conjugated to APC.

was recently reported that there are higher frequencies and greater functionality of HIV-specific cells in BAL than in blood (5). We also measured SIV-specific responses at higher frequencies in BAL than blood by tetramer analysis. Although this response was detected at a very low frequency and although two other CD8⁺ T-cell responses were undetectable by tetramer analysis, the higher frequency of SIV-specific CD8⁺ T cells in BAL may help explain the effective viral suppression mediated by BAL CD8⁺ T cells that we consistently observed. While it would be expected that we detect the BAL tetramer-positive cells by ELISPOT assay in PBMC at low levels, we did not observe positive IFN- γ ELISPOT assay responses using pools of 15-mers overlapping this region. It is possible that these responses were missed because pools of 15-mers are not as sensitive as using the minimal optimal peptide. Additionally, it is plausible that the tetramer-positive cells we detected either are not secreting IFN- γ or are secreting other cytokines that we did not study. Ultimately, the lack of detectable responses in the PBMC matches well with the lack of responses detected by tetramer analysis in the PBMC. SIVmac239 Δ nef may replicate primarily in the mucosal tissues, generating responses that are at very low frequency and difficult to detect in the PBMC. Importantly, these results demonstrate that measure-

ments of immunity in blood during vaccination may be underestimating the magnitude of the elicited immune response.

The early detection of CD8⁺ T-cell responses in the lung initially seems incongruent with previous reports that protection requires a 15- to 20-week induction phase. However, it is entirely possible that these early responses need to develop into a long-lived memory response to be effective after wild-type SIVmac239 challenge. It may also be the case that continued replication and adaptation of SIVmac239 Δ nef with the immune response leads to CD8⁺ T-cell responses against both the infecting strain and potential escape mutations. This co-evolution of host immunity and virus may help to explain the effectiveness of SIVmac239 Δ nef and the reason for the lengthy induction phase. Nevertheless, the ability of mucosal CD8⁺ T cells to suppress viral replication early after vaccination contrasts with the long induction phase required for protection against pathogenic challenge. Still, the generation of immune responses in the lung is concurrent with the rapid decline of SIVmac239 Δ nef plasma loads. This early suppression may help to explain the rapid and better control of acute-phase SIVmac239 Δ nef plasma loads, which are consistently lower than acute-phase wild-type SIVmac239 plasma loads (data not shown).

Ultimately, these results further cement the differences in

immune responses that exist in different anatomical compartments of an HIV/SIV-infected individual. Future work must assess the ability of this assay to define *in vivo* disease outcome. While researchers frequently rely on easily accessible blood draws to obtain immune readouts, this tissue is not a perfect proxy for accurate assessments of the immune response against the virus, and the lung may not be the perfect proxy for GALT. The present study demonstrates that CD8⁺ T cells from mucosal tissues are more efficient at preventing viral replication *ex vivo* and provides a unique method for assessing the suppressive capacity of such cells in macaques either infected with or vaccinated against SIV.

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