Robust Vaccine-Elicited Cellular Immune Responses in Breast Milk following Systemic Simian Immunodeficiency Virus DNA Prime and Live Virus Vector Boost Vaccination of Lactating Rhesus Monkeys

Andrew B. Wilks, Elizabeth C. Christian, Michael S. Seaman, Piya Sircar, Angela Carville, Carmen E. Gomez, Mariano Esteban, Giuseppe Pantaleo, Dan H. Barouch, Norman L. Letvin and Sallie R. Permar

*J Immunol* 2010; 185:7097-7106; Prepublished online 1 November 2010;
doi: 10.4049/jimmunol.1002751
http://www.jimmunol.org/content/185/11/7097
Robust Vaccine-Elicited Cellular Immune Responses in Breast Milk following Systemic Simian Immunodeficiency Virus DNA Prime and Live Virus Vector Boost Vaccination of Lactating Rhesus Monkeys

Andrew B. Wilks,* Elizabeth C. Christian,* Michael S. Seaman,*,† Piya Sircar,* Angela Carville,‡ Carmen E. Gomez,§ Mariano Esteban,§ Giuseppe Pantaleo,‡‡ Dan H. Barouch,† Norman L. Letvin,* and Sallie R. Permar*,#

Breast milk transmission of HIV remains an important mode of infant HIV acquisition. Enhancement of mucosal HIV-specific immune responses in milk of HIV-infected mothers through vaccination may reduce milk virus load or protect against virus transmission in the infant gastrointestinal tract. However, the ability of HIV/SIV strategies to induce virus-specific immune responses in milk has not been studied. In this study, five uninfected, hormone-induced lactating, Mamu A*01* female rhesus monkey were systemically primed and boosted with rDNA and the attenuated poxvirus vector, NYVAC, containing the SIVmac239 matching immunogens. The vaccine-elicited immunodominant epitope-specific CD8+ T lymphocyte response in milk was of similar HIV.

The Journal of Immunology

Therefore, systemic DNA prime and virus vector boost of lactating rhesus monkeys elicits potent virus-specific cellular and transient increases in the proportion of activated or CCR5-expressing CD4+ T lymphocytes in milk occurred after vaccination. whereas an SIV envelope-specific IgA response was only detected in one vaccinated monkey. Importantly, only limited and each virus vector boost. Finally, SIV envelope-specific IgG responses were detected in milk of all monkeys after vaccination, whereas an SIV envelope-specific IgA response was only detected in one vaccinated monkey. Importantly, only limited and transient increases in the proportion of activated or CCR5-expressing CD4+ T lymphocytes in milk occurred after vaccination. Therefore, systemic DNA prime and virus vector boost of lactating rhesus monkeys elicits potent virus-specific cellular and humoral immune responses in milk and may warrant further investigation as a strategy to impede breast milk transmission of HIV. The Journal of Immunology, 2010, 185: 7097–7106.

H IV transmission via breast milk accounts for nearly half of the 350,000 new infant infections occurring annually in developing regions with high HIV prevalence (1). Although infant or maternal antiretroviral prophylaxis can reduce the incidence of breast milk HIV transmission (2–6), effective implementation of these long-term prophylaxis regimens will be complicated by infant toxicity, compliance, and inadequate health care infrastructure. Therefore, investigations of immunologic interventions, such as maternal or infant vaccines, to reduce postnatal transmission of HIV remain crucial for elimination of this mode of HIV transmission. Interestingly, in the absence of antiretroviral prophylaxis, only 10% of breastfeeding infants born to HIV-infected mothers will become infected postnatally (7). This low rate of HIV acquisition, despite daily low-dose exposure for up to 2 y, raises the possibility that protective immune responses in breast milk may prevent HIV acquisition in the majority of breastfeeding infants.

The risk for HIV transmission via breastfeeding has been associated with the level of HIV RNA and cell-associated HIV DNA in milk (8–12), in addition to maternal disease progression (8, 13, 14). Therefore, the reduction of cell-free and cell-associated virus load through enhancement of virus-specific immune responses in milk may result in reduced rates of HIV transmission via breast milk. HIV/SIV-specific CD8+ T lymphocyte responses are known to be critical for containment of systemic virus load (15) and have been identified in human and rhesus monkey milk (16, 17). Furthermore, recent studies of HIV/SIV virus evolution in milk suggest that the virus may replicate locally in the breast milk compartment (18–20). Therefore, enhancement of the local HIV-specific CD8+ T lymphocyte responses in breast milk may improve containment of virus replication in the breast milk compartment and result in a decrease in breast milk virus load. In addition, passive postnatal administration of broadly HIV-neutralizing Ig protected infant macaques from HIV acquisition by oral exposure (21). Therefore, induction of potent, functional HIV-specific Ab responses in breast
milk through maternal vaccination may play a role in blocking HIV acquisition in the infant gastrointestinal tract.

In this study, we aimed to establish the ability of a well-studied, systemic HIV/SIV vaccine prime-boost strategy to induce virus-specific cellular and humoral immune responses in breast milk of lactating monkeys. Nonhuman primates have proved to be an instrumental model for evaluating immunogenicity and efficacy of candidate HIV vaccines. Furthermore, we established a protocol for hormone-induction of lactation to investigate cellular and humoral immune responses in breast milk, because the breast milk produced by the hormone induction is immunologically similar to that of naturally lactating monkeys (17). It has long been noted that rDNA priming prior to virus vector boost enhances vaccine-elicited cellular immune responses (22); therefore, the monkeys in this study were primed with rDNA containing SIV gag, pol, and envelope genes. The monkeys were then boosted with matching SIV immunogens delivered via the attenuated poxvirus vector, NYVAC, a vector that induces strong polynuclear CD4+ and CD8+ T lymphocyte responses (23), and replication-incompetent adenovirus vector serotype 5 (rAd5), a vector that induces potent cellular and humoral immune responses (24–27). Adenovirus and poxvirus vectors were reported to induce mucosal CD8+ T lymphocyte responses in the gastrointestinal tract (28, 29), making these vectors excellent candidates for eliciting immune responses in breast milk.

Although induction of humoral immune responses in milk following systemic vaccination with polysaccharide, protein, or whole-cell vaccines and elicitation of cellular immune responses in milk following live-attenuated virus vaccination were reported (30–37), it has not been established whether any candidate HIV vaccine can elicit humoral and/or cellular immune responses in breast milk. Therefore, our study aimed to characterize systemic cellular and humoral immune responses in breast milk following SIV vaccination. This detailed characterization of cellular and humoral immune responses in milk following systemic vaccination has implications for mother-to-child transmission of HIV and other neonatal pathogens transmitted via breast milk, such as human T lymphotropic virus-1 and CMV. This study of breast milk immune responses elicited by a leading candidate HIV vaccine strategy will form the basis of future investigations of maternal vaccination to induce virus-specific immune responses that are protective against virus transmission via breastfeeding.

Materials and Methods

Generation of vaccine vectors

DNA plasmids containing the SiMVmac239 gag-pol, and envelope genes, were generated by cloning the virus cDNA into the pVR1012 vector, as previously described (38, 39). Replication-incompetent, E1/E3-deleted rAd5 vectors expressing the SiMVmac239 Gag and Envelope and SiE660 Pol were previously described (40). Finally, SiMVmac239 gene-expressing NYVAC vectors were generated by the following protocol: plasmid transfer vectors pCyaA20-SIVgag-pol and gp140 were constructed for insertion of the SiMVmac239 gag-pol and envelope genes into the TK locus of the NYVAC genome. After the desired recombinant plasmids were isolated by screening for expression of β-galactosidase activity, BSC40 cells were infected with NYVAC virus (provided by Sanofi-Aventis, Paris, France) at a multiplicity of 0.05 PFU/cell and transfected with 10 μg DNA of the plasmid transfer vector using Lipofectamine reagent (Invitrogen, Carlsbad, CA). After 72 h, cells were harvested, sonicated, and used for recombinant virus screening. Recombinant NYVAC viruses containing the SIV genes and transiently coexpressing the β-gal marker gene were selected by four consecutive rounds of plaque purification in BSC40 cells stained with 5-bromo-4-chloro-3-indolyl β-galactoside (300 μg/ml). Next, recombinant NYVAC viruses containing the SIV genes and having the β-gal marker gene deleted through recombination at the TK site were isolated by three additional consecutive rounds of plaque purification, screening for nonstaining viral foci in BSC40 cells. Isolated plaques were grown and analyzed for the presence and expression of SIV gene products and for the absence of NYVAC-WT contamination by PCR and Western blot with SIV protein-specific mAbs (provided by Project EVA, European Union). Virus stocks were then generated in CEF cells and purified by two 45% sucrose gradient purifications.

Animals and vaccination

Lactation was pharmacologically induced in five female Mamu-A*01+ rhesus monkeys using depot medroxyprogesterone and estradiol injections and an oral dopamine antagonist, as previously described (17). Once all five monkeys were lactating, the animals were vaccinated i.m. with 5 mg each rDNA plasmid containing the SiMVmac239 gag-pol and envelope genes on weeks 0, 4, and 8. The monkeys were boosted i.m. and intradermally at week 28 with 109 PFU each of the NYVAC vectors containing the SiMVmac239 gag-pol and envelope genes. The monkeys were boosted again at week 38 by i.m. administration of 1010 viral particles of each of the rAd5 vectors containing the SiMVmac239 gag and envelope genes and SIV En660 pol gene. Milk (30–1000 μl) was collected two times per week throughout the vaccination schedule, and blood was collected weekly. Milk was separated into cellular, supernatant, and fat fractions by centrifugation, as previously described (17). Pinch biopsies from the colon and vaginal tract obtained at week 40 were digested with collagenase, and mononuclear cells were isolated on a Percoll gradient within 6 h, as previously described (41). Animals were maintained according to the “Guide for the Care and Use of Laboratory Animals.”

Phenotyping, tetramer staining, and intracellular cytokine staining of mucosal biopsies, breast milk cells, and PBMCs

The vaccine-elicited CD8+ T lymphocyte response was measured by staining milk, colon, and vaginal cells and PBMCs with immunodominant Mamu-A*01-restricted Env p54-alkaline phosphatase and Gag p11C-Q-Dot 655 labeled tetramers. Lymphocyte phenotype was assessed by anti-CD45-FITC (SP34.2), anti-CD3-Pacific Blue (SP34.2), anti-CD4-Pacific Blue (SP34.2), anti-CD25-PerCP-Cy5.5 (L200), and anti-CD8-PE-Cy7 (RPA-T8; all from BD Biosciences, San Jose, CA) mAb staining. Naive and memory lymphocyte subsets were defined by anti-CD95-allophycocyanin (DX2; BD Biosciences) and anti-CD28-PerCP-Cy5.5 (28.2; Beckman Coulter, Fullerton, CA) staining (naive: CD28+, CD95−; effecter memory: CD28+, CD95+ central memory: CD28−, CD95+). The peak response was defined as the highest proportion of tetramer-staining CD8+ T lymphocytes measured after each vaccination (between 2 and 4 wk following each immunization for each monkey).

Activation status of breast milk CD4+ T lymphocytes after vaccination was assessed by mAb staining with anti-CD3-Pacific Blue (SP34.2), anti-CD4-Pacific Blue (SP34.2), anti-CCR5-PerCP-Cy5.5, and anti-CD25-Pecy7 (M-A251), and anti-HLA-DR-alkaline phosphatase-Cy7 (L243) (BD Biosciences, San Jose, CA) mAbs. Isolated plaques were grown and analyzed for the presence and expression of SIV gene products and for the absence of NYVAC-WT contamination by PCR and Western blot with SIV protein-specific mAbs (provided by Project EVA, European Union). Virus stocks were then generated in CEF cells and purified by two 45% sucrose gradient purifications. Data were collected on the LSRII flow cytometer (BD Biosciences) with FACSDiva software and analyzed with FlowJo Software.

Absolute lymphocyte and SIV-specific CD8+ T lymphocyte count

Automated complete cell counts of breast milk are skewed by the presence of fat droplets; therefore, they cannot be used to quantitate lymphocyte number. To calculate absolute lymphocytes per milliliter of milk and blood in a similar fashion, the absolute number of CD45+ CD3+ lymphocytes quantitated by flow-cytometric analysis of each sample type was normalized to the ratio of fluorospheres added to the sample and quantitated by flow cytometry at the end of each staining procedure. To calculate the absolute lymphocyte count in blood, 100 μl EDTA-anticoagulated blood was mixed with 100 μl Flow-Count Fluorospheres (Beckman Coulter; 942 beads/μl) and stained with anti-CD45-FITC (DO58-1283) and anti-CD3-alkaline phosphatase-Cy7 (SP34.2; both from BD Biosciences). The RBCs were lysed and fixed using a TO-Prep machine (Beckman Coulter), and the remaining blood cells and fluorospheres were washed, pelleted, and fixed. To calculate the absolute lymphocyte count in breast milk, 100 μl Flow-Count Fluorospheres were added to the total breast milk cell pellet. Breast
milk cells were stained with the phenotyping Abs listed above. Sample data were collected within 4 h of fixation on the LSRII instrument (BD Biosciences) with FACSDiva software and analyzed with FlowJo software. After flow-cytometric analysis and quantitation of the fluorospheres that remained at the end of the procedure, the number of CD45+CD3+ lymphocyte events was normalized by the ratio of the number of fluorospheres added to the sample/the number of fluorospheres quantitated by flow cytometry. Finally, the normalized number of CD45+CD3+ lymphocytes was divided by the volume of the original sample. Absolute CD3+, CD8+, Gag p11c-specific CD8+, and Env p45-specific CD8+ T lymphocytes per milliliter were calculated by multiplying the percentage of the population by the normalized absolute CD45+CD3+ lymphocyte count. The peak response was defined as the highest absolute number of tetramer-staining CD8+ T lymphocytes measured after each vaccination (between 2 and 4 wk following each immunization for each monkey).

Quantification of SIV envelope-binding IgG, IgA, and neutralizing titer in milk and plasma

SIV Envelope-binding IgG and IgA were measured by incubation of serial 3-fold dilutions of plasma and milk supernatant in duplicate in a 96-well plate coated with rSIVmac239 gp130 (ImmunoDiagnostics, Woburn, MA). After blocking with PBS with 5% nonfat dried milk and 10% FBS, SIV Envelope-binding Ab was detected by an HRP-conjugated, polyclonal goat anti-monkey IgG (Alpha Diagnostics, Owings Mills, MD) or anti-monkey IgA (R&D Systems, Minneapolis, MN). The addition of ABTS-2 peroxidase substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was measured at 450 nm. SIV envelope-specific Ab titer was calculated as the inverse of the lowest dilution of plasma or milk supernatant that had an average OD that was 2-fold greater than that of the PBS negative control.

To measure neutralizing Ab titer in blood and milk, a stock of molecularly cloned T cell line-adapted (TCLA) SIVmac239 Envelope-pseudotyped virus was prepared by transfection in 293T cells and titrated in TZM.bl cells as previously described (43). Neutralization was measured by reduction in luciferase reporter gene expression after a single round of infection in TZM.bl cells, as previously described (43). Briefly, 200 tissue culture infectious dose 50% of virus was incubated with 3-fold serial dilutions of plasma or milk supernatant in duplicate for 1 h at 37°C in 96-well flat-bottom culture plates. TZM.bl cells were added (1 × 104 well in 100 µl volume) in 10% DMEM growth medium containing DEAE-Dextran (Sigma-Aldrich, St. Louis, MO) at a final concentration of 11 μg/ml. Assay controls included replicate wells of TZM.bl cells alone (cell control) and TZM.bl cells with virus (virus control). Following a 48-h incubation at 37°C, 150 μl assay medium was removed from each well, and 100 μl Bright-Glo luciferase reagent (Promega, Madison, WI) was added. The cells were allowed to lyse for 2 min, then 150 μl the cell lysate was transferred to a 96-well black solid plate, and luminescence was measured by a 50% reduction in relative luminescence units compared with the virus control wells after subtraction of cell control relative luminescence units. The neutralizing titer in milk and plasma was calculated as the inverse of the lowest dilution of plasma or milk supernatant that had an average OD that was 2-fold greater than that of the PBS negative control.

The vaccine-elicited neutralizing Ab titer in blood and milk, a stock of molecularly cloned T cell line-adapted (TCLA) SIVmac239 Envelope-pseudotyped virus was prepared by transfection in 293T cells and titrated in TZM.bl cells as previously described (43). Neutralization was measured by reduction in luciferase reporter gene expression after a single round of infection in TZM.bl cells, as previously described (43). Briefly, 200 tissue culture infectious dose 50% of virus was incubated with 3-fold serial dilutions of plasma or milk supernatant in duplicate for 1 h at 37°C in 96-well flat-bottom culture plates. TZM.bl cells were added (1 × 104 well in 100 µl volume) in 10% DMEM growth medium containing DEAE-Dextran (Sigma-Aldrich, St. Louis, MO) at a final concentration of 11 μg/ml. Assay controls included replicate wells of TZM.bl cells alone (cell control) and TZM.bl cells with virus (virus control). Following a 48-h incubation at 37°C, 150 μl assay medium was removed from each well, and 100 μl Bright-Glo luciferase reagent (Promega, Madison, WI) was added. The cells were allowed to lyse for 2 min, then 150 μl the cell lysate was transferred to a 96-well black solid plate, and luminescence was measured. The ID50 titer was calculated as the inverse of the lowest dilution of plasma or milk supernatant that had an average OD that was 2-fold greater than that of the PBS negative control.

Statistical analysis

All comparisons of the magnitude of the vaccine-elicited cellular or humoral immune responses in blood, milk, and other mucosal compartments were performed using the paired, nonparametric Wilcoxon signed-rank test with Prism software (GraphPad, San Diego, CA). The lowest obtainable p value using this paired, nonparametric test with n = 5 animals is p = 0.06.

Results

SIV vaccination and assessment of changes in lymphocyte number and phenotype in milk of lactating rhesus monkeys

Five Mamu A*01†* hormone-induced lactating female rhesus monkeys received priming immunizations i.m. with 5 mg of rDNA plasmids with SIVmac239 gag-pol and env gene inserts on weeks 0, 4, and 8. Animals were boosted i.m. and intradermally at week 28 with 108 PFU of recombinant attenuated pox vector, NYVAC, containing SIVmac239 gag-pol and env genes. Finally, animals were repeat boosted i.m. at week 38 with 1010 viral particles of recombinant adenovirus serotype 5 containing SIV gag, pol, and envelope. The vaccine-elicited cellular and humoral immune responses were assessed in blood and breast milk following each immunization. Animals underwent colon and vaginal biopsies 2 wk following the final immunization to compare the magnitude of the cellular immune responses in breast milk with these mucosal compartments (Fig. 1).

We first investigated the changes in lymphocyte number and relative proportions of CD4+ and CD8+ T memory and naive lymphocytes in milk and blood weekly following systemic vaccination. The lymphocyte number and the proportion of CD8+ effector memory T lymphocytes increased greatly following SIV infection (17), but changes in lymphocyte number and phenotype in milk following maternal vaccination have not been studied. The absolute number of lymphocytes in breast milk before vaccination (median, 4300 CD3+ T cells/ml; range, 1,400–41,800) did not trend toward a statistically significant difference at the peak of the response after each immunization (data not shown). Furthermore, the proportion of CD4+ (median, 64.3%; range: 30–72.4%) or CD8+ (median, 32.5%; range: 25.8–72.8%) T lymphocytes in milk prior to vaccination did not change following each systemic immunization (data not shown). Finally, the proportion of effector memory (median, 89.3%; range: 84.6–96.2%), central memory (median, 10.7%; range: 3.7–15.4%), or naive (median, 0%; range: 0–1.8%) CD4+ T lymphocytes and effector memory (median, 41.9%; range: 33.3–70.2%), central memory (median, 58.1%; range: 30.3–66.7%), or naive (median, 0%; range 0%) CD8+ T lymphocytes did not significantly change in milk following each systemic immunization (data not shown). Accordingly, there were no changes in the number and proportion of memory and naive CD4+ and CD8+ T lymphocytes in blood following vaccination (data not shown).

Robust CD8+ T lymphocyte responses in breast milk of SIV-vaccinated, lactating rhesus monkeys

The vaccine-elicited CD8+ T lymphocyte response specific for the immunodominant Mamu A*01-restricted epitope, Gag p11C, and the subdominant Mamu A*01-restricted epitope, Env p54, were assessed weekly following each immunization. The peak proportion of CD8+ T lymphocytes that were specific for the Gag p11C epitope was greater in breast milk (median, 2.9%; range: 1.8–3.7%) compared with blood (median, 0.41%; range: 0.21–0.87%) following systemic rDNA immunization in all animals (p = 0.06). Upon systemic boosting with the SIV NYVAC live viral vectors,
the peak proportion of Gag p11C-specific CD8\(^+\) T lymphocytes in breast milk (median, 1.6%; range: 1.3–3.7\%) was similar in magnitude to that in blood (median, 1.1%; range: 0.7–2.0\%) \((p = 0.19)\). Finally, the median and range of the peak proportion of Gag p11C-specific CD8\(^+\) T lymphocytes were higher in milk (median, 5.4%; range: 2.5–9.1\%) than in blood (median, 3.5%; range: 2.0–5.1\%) following the rAd5 boost, but the responses in each compartment were not different enough to approach statistical significance \((p = 0.31)\). The median and range of the peak proportion of Gag p11C-specific CD8\(^+\) T lymphocytes were higher in milk (median, 5.4%; range: 2.5–9.1\%) than in blood (median, 3.5%; range: 2.0–5.1\%) following the rAd5 boost, but the responses in each compartment were not different enough to approach statistical significance \((p = 0.31)\). The peak median proportion and absolute number of vaccine-elicited, virus-specific CD8\(^+\) T cells after each prime and boost are summarized in Table I.

**Strong vaccine-elicited, polyfunctional CD8\(^+\) and CD4\(^+\) T lymphocyte responses in breast milk**

We were able to demonstrate SIV Gag-specific IFN-\(\gamma\), TNF-\(\alpha\), and IL-2 production in CD4\(^+\) and CD8\(^+\) T lymphocytes in breast milk by intracellular cytokine staining 3 wk following rDNA priming, as well as NYVAC and rAd5 boost in two vaccinated monkeys with adequate lymphocyte number (CD3\(^+\) T lymphocyte number \(> 1000\) per condition) (Fig. 3). The proportion of Gag-specific cytokine-producing CD4\(^+\) T lymphocytes in milk was similar to that in blood following rDNA prime. However, the proportion of Gag-specific, IFN-\(\gamma\)-producing CD4\(^+\) T lymphocytes was \(5\)-fold higher in milk than in blood in both animals following NYVAC immunization. In addition, the proportions of Gag-specific TNF-\(\alpha\) and IL-2-producing CD4\(^+\) T lymphocytes were \(5\)-fold higher in milk than in blood in one animal and were similar or greater in magnitude in the other animal following NYVAC. Finally, the proportion of Gag-specific IFN-\(\gamma\), TNF-\(\alpha\), and IL-2–producing CD4\(^+\) T lymphocytes was \(4\)-fold higher in milk than in blood in both animals following rAd5 vaccination. As expected, the magnitude of the vaccine-elicited CD4\(^+\) T lymphocyte responses was

---

**FIGURE 2.** Robust vaccine-elicited CD8\(^+\) T lymphocyte responses appear in breast milk following rDNA, pox vector, and recombinant adenovirus vector immunization. Peak Gag p11C-specific CD8\(^+\) T lymphocyte proportion in blood (A) and milk (B) following DNA prime and NYVAC and rAd5 boost. Peak absolute number of Gag p11C-specific CD8\(^+\) T lymphocytes/ml in blood (C) and milk (D) following each immunization. Peak Env p54-specific CD8\(^+\) T lymphocyte proportion in blood (E) and milk (F) following DNA prime and NYVAC and rAd5 boost. Peak absolute number of Env p54-specific CD8\(^+\) T lymphocytes/ml in blood (G) and milk (H) following each immunization.
higher in blood and milk following NYVAC boost compared with those elicited by rAd5 boost, because poxvirus vectors are known to elicit strong CD4+ T lymphocyte responses (44) (Fig. 4A, 4B).

The proportions of Gag-specific cytokine-producing CD8+ T lymphocytes in milk were similar or higher than that in blood after rDNA priming and NYVAC and rAd5 boost. Of note, the proportions of Gag-specific TNF-α– and IL-2–producing CD8+ T lymphocytes in milk were ≥1 log higher than that in blood in both monkeys following rAd5 boost. Furthermore, the proportion of Gag-specific IFN-γ–producing CD8+ T lymphocytes was 1-log higher in milk of one animal than that in blood following rAd5 boost. As expected, the proportions of Gag-specific TNF-α– and IL-2–producing CD8+ T lymphocytes in milk were higher in both monkeys following rAd5 boost than those following rDNA prime and NYVAC boost, because rAd5 vectors are known to induce strong mucosal and systemic CD8+ T lymphocyte responses (23, 24, 40) (Fig. 4C, 4D).

We then compared the polyfunctional cytokine profile of vaccine-elicited breast milk lymphocytes to that in blood after each vaccine vector boost. Interestingly, a distinct polyfunctional cytokine-production profile of the vaccine-elicited T lymphocyte was elicited in milk compared with that in blood (Fig. 4E–H). Although Gag-specific IFN-γ–only-producing cells were a predominant population in blood and milk CD4+ and CD8+ T lymphocyte subsets, the TNF-α– and IFN-γ–producing cells were predominant only in milk vaccine-elicited CD4+ and CD8+ T lymphocytes. Furthermore, the TNF only-producing cells and IL-2, TNF-, and IFN-producing populations accounted for a higher proportion of cytokine-producing cells in milk CD4+ and CD8+ T lymphocytes than in the blood (Fig. 4E–H). This same pattern was found after NYVAC and rAd5 vector boost. This difference in the polyfunctional cytokine profile of vaccine-elicited T lymphocytes in milk and blood likely reflects the regulated trafficking of activated lymphocytes into the breast milk compartment or local proliferation of memory lymphocytes in milk.

The magnitude of the vaccine-elicited CD8+ T lymphocyte immune responses in breast milk is greater than that in the colon and similar to that in the vaginal tract

We compared the proportion of Gag p11C-specific CD8+ T lymphocytes in blood, milk, colon, and vaginal tract 2 wk following the rAd5 boost. The median proportion of vaccine-elicited CD8+ T lymphocytes specific for the immunodominant Gag p11C epitope was higher than that in colon (median, 5.4%; range: 2.5–9.1%) was higher than that in colon (median, 2.2%; range: 0.7–2.9%) and trended toward a statistically significantly different (p = 0.12). In contrast, the median proportion of vaccine-elicited CD8+ T lymphocytes specific for Gag p11C epitope was similar in the milk and the vaginal tract (median, 3.1%; range: 0.8–8.6%) (p = 0.44) (Fig. 5). Therefore, the cellular immune responses elicited by systemic DNA prime-virus vector boost in milk were of similar or greater magnitude compared with that elicited in other mucosal compartments.

Low-magnitude SIV envelope-specific IgG responses are elicited in breast milk by systemic rDNA prime and virus vector boost

Vaccine-elicited neutralizing Ab responses were detected in milk against a TCLA SIVmac239 in a pseudovirus TZM-bl–neutralization assay after NYVAC and rAd5 boost (Fig. 6B). However, this neutralizing Ab response in milk (median ID50, 107; range: 63–966) was ≥2 logs lower than that in plasma (median ID50, 10,442; range: 7,350–21,870) (p = 0.06) (Fig. 6A). An SIV envelope-binding IgG response was detected in milk after NYVAC (median titer, 3; range: 3–100) and rAd5 (median titer, 10; range: 3–100) boost, but the titer was 2–3 logs lower than that in plasma (post-NYVAC median titer, 10,000; range: 3,000–30,000; post-rAd5 median titer, 10,000; range: 10,000–30,000) (p = 0.06) (Fig. 6C, 6D). Furthermore, an SIV Envelope-binding IgA response was only detected in milk of one animal following NYVAC boost and in no animal after rAd5 boost (Fig. 6F). This limited detection of SIV Envelope-binding IgA

### Table I. Median vaccine-elicited cellular and humoral immune responses in blood and milk of systemically vaccinated, lactating rhesus monkeys

<table>
<thead>
<tr>
<th>Immune Parameter</th>
<th>Vaccination</th>
<th>Blood</th>
<th>Milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gag p11C-specific CD8+ T cells (%)</td>
<td>Prevaccination</td>
<td>0.01</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>rDNA</td>
<td>0.4</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>NYVAC</td>
<td>1.1</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>rAd5</td>
<td>3.5</td>
<td>7.3</td>
</tr>
<tr>
<td>Gag p11C-specific CD8+ T cells (cells/ml)</td>
<td>Prevaccination</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>rDNA</td>
<td>1,600</td>
<td>391</td>
</tr>
<tr>
<td></td>
<td>NYVAC</td>
<td>10,900</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>rAd5</td>
<td>18,700</td>
<td>873</td>
</tr>
<tr>
<td>Env p54-specific CD8+ T cells (%)</td>
<td>Prevaccination</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>rDNA</td>
<td>0.2</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>NYVAC</td>
<td>0.24</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>rAd5</td>
<td>0.1</td>
<td>2.3</td>
</tr>
<tr>
<td>Env p54-specific CD8+ T cells (cell/ml)</td>
<td>Prevaccination</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>rDNA</td>
<td>749</td>
<td>119</td>
</tr>
<tr>
<td></td>
<td>NYVAC</td>
<td>1,727</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>rAd5</td>
<td>985</td>
<td>266</td>
</tr>
<tr>
<td>TCLA SIV-neutralizing Ab (ID50 titer)</td>
<td>Prevaccination</td>
<td>13</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>rDNA</td>
<td>1,047</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>NYVAC</td>
<td>1,995</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>rAd5</td>
<td>10,422</td>
<td>107</td>
</tr>
<tr>
<td>Anti-SIV gp130 IgG (titer)</td>
<td>Prevaccination</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>rDNA</td>
<td>0</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>NYVAC</td>
<td>3</td>
<td>10,000</td>
</tr>
<tr>
<td></td>
<td>rAd5</td>
<td>10</td>
<td>10,000</td>
</tr>
<tr>
<td>Anti-SIV gp130 IgA (titer)</td>
<td>Prevaccination</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>rDNA</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>NYVAC</td>
<td>65</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>rAd5</td>
<td>30</td>
<td>0</td>
</tr>
</tbody>
</table>
A response in milk is in contrast to the systemic SIV Envelope-binding IgA response that was detected in all animals after NYVAC (median titer, 30; range: 30–300) and rAd5 (median titer, 30; range: 30–100) boost (Fig. 6E). Therefore, the vaccine-elicited neutralizing Ab response in milk is likely attributable to the SIV envelope-specific IgG in milk rather than SIV envelope-specific secretory IgA. The median SIV envelope-binding and -neutralizing Ab responses after each vaccine vector are summarized in Table I.

FIGURE 4. The vaccine-elicited CD4+ and CD8+ T lymphocyte responses in milk are of higher magnitude and display a distinct polyfunctional cytokine profile compared with that in blood following NYVAC and rAd5 boost. The proportion of blood (A) and milk (B) CD4+ T lymphocytes and blood (C) and milk (D) CD8+ T lymphocytes producing IFN-γ, TNF-α, and IL-2 3 wk following the third rDNA prime and NYVAC and rAd5 boost in two monkeys (294 and 402) with adequate breast milk cell number for this analysis. The polyfunctional cytokine profile of vaccine-elicited Gag-specific CD4+ T lymphocytes in blood (E) and milk (F) and CD8+ T lymphocytes in blood (G) and milk (H) 3 wk following rAd5 boost in the two monkeys (294 and 402) with adequate breast milk cell number for the analysis.
Limited and transient activation of CD4+ T lymphocytes in milk following systemic rDNA prime and live vector virus boost of uninfected monkeys

Because activation of CD4+ T lymphocytes following vaccination has been raised as a concern for enhancing virus transmission (45, 46), we investigated the activation status of the CD4+ T lymphocytes in milk by assessing their IL-2R (CD25), MHC class II molecule HLA-DR, and the HIV coreceptor CCR5 expression throughout the immunization schedule. There was a 2–10% increase in the proportion of CD4+ T lymphocytes in milk expressing CCR5 following the second and third systemic rDNA prime, but this increase lasted ≤2 wk. Because the median number of CD4+ T lymphocytes in milk during the vaccination schedule was 2752 cells/ml, the maximum absolute increase in CCR5-expressing CD4+ T lymphocytes following DNA vaccination is 275 cells/ml. There was no apparent increase in CCR5 expression following NYV AC boost; however, there was a 1–3% increase in the proportion of CCR5-expressing cells lasting ≤2 wk following rAd5 boost (Fig. 7A), representing a maximum absolute increase in CCR5-expressing CD4+ T lymphocytes of 82 cells/ml. Similarly, the proportion of milk CD4+ T lymphocytes expressing CD25 increased by 1–5% following the second and third rDNA prime, but again this increase lasted ≤2 wk. This increased proportion of CD25-expressing CD4+ T lymphocytes following DNA priming represents a maximum absolute increase of 137 CD25-expressing CD4+ T cells/ml. There was a minimal increase in the proportion of CD25-expressing CD4+ T lymphocytes following NYVAC and rAd5 boost (Fig. 7B). Similarly, the proportion of CD4+ T lymphocytes expressing HLA-DR increased 5–10% following the second and third rDNA prime, but this increase lasted <2 wk and represents a maximum absolute increase of 275 HLA-DR–expressing CD4+ T lymphocytes/ml. Finally, there was a small increase in the proportion of CD4+ T lymphocytes expressing HLA-DR in only one of five monkeys following NYVAC boost and in two of five monkeys following rAd5 boost that lasted <2 wk (Fig. 7C). Furthermore, there was no significant change in the mean fluorescence intensity of the CCR5-, CD25-, or HLA-DR–expressing cells after vaccination to indicate a higher level of activation-molecule expression (data not shown). Therefore, the activation of breast milk CD4+ T lymphocytes after systemic vaccination composed of rDNA prime and live virus vector boost was minimal and self-limited.

Discussion

In this study, we established that systemic rDNA priming and virus vector boost can elicit robust immunogen-specific, polyfunctional CD4+ and CD8+ T lymphocyte responses in breast milk.
ingly, the polyfunctional cytokine-production profile of immunogen-specific breast milk lymphocytes was distinct from that in the blood. This finding likely reflects the regulated trafficking of vaccine-elicited memory lymphocytes into this mucosal compartment or local proliferation of this immunogen-specific lymphocyte population within the breast milk compartment. These vaccine-elicited, virus-specific breast milk lymphocytes may be active in the maternal breast milk compartment and could enhance containment of viruses that are shed in breast milk, such as HIV and CMV. The vaccine-elicited breast milk lymphocytes could also be active in the infant gastrointestinal tract, and these responses could play a role in the prevention of mucosally transmitted neonatal pathogens.

We previously described a robust virus-specific CD8\(^+\) T lymphocyte response in breast milk during acute SIV infection that was two to three times higher than that in blood (17). Furthermore, studies of HIV/SIV evolution in breast milk suggested that the breast milk virus population is at least partially populated by virus produced by locally infected cells, reflected by the frequent occurrence of groups of genetically identical viruses in breast milk that are absent or less common in plasma (18–20). Moreover, we reported that breast milk virus quasispecies escape MHC class I-restricted immunodominant CTL responses by mutation of the restricted epitopes at a faster or similar rate to that of the blood virus population (17). Therefore, virus replicating in the breast milk compartment is likely evolving in response to immune pressure from virus-specific CD8\(^+\) T lymphocytes. Collectively, these studies suggest that virus-specific CD8\(^+\) T lymphocytes act on locally replicating virus in the breast milk compartment. Thus, enhancement of the virus-specific CD8\(^+\) T lymphocyte responses in breast milk through maternal vaccination may contribute to improved control of virus replication in the breast milk compartment. Reduction of breast milk virus load may result in a reduction of infant virus transmission.

In this study, we noted high-magnitude Gag-specific CD4\(^+\) T lymphocyte responses in milk and blood following NYVAC boost, consistent with the robust CD4\(^+\) T lymphocyte responses elicited by poxvirus vectors (44). High-magnitude CD8\(^+\) T lymphocyte responses and neutralizing Ab responses in milk and blood were generated following rAd5 boost, consistent with previous reports of vaccine-elicited rAd5 responses (24–27). However, the enhanced CD8\(^+\) T lymphocyte and neutralizing Ab responses following rAd5 boost may be an additive effect of a second boost administered over a short time interval. Therefore, elicitation of durable, high-magnitude breast milk virus-specific CD8\(^+\) T lymphocyte responses for containment of breast milk virus replication and neutralizing Ab responses with the ability to block virus transmission in the infant gastrointestinal tract may require more than one vector boost immunization.

The humoral immune responses elicited in breast milk by this vaccine regimen were low tier and consisted of only IgG responses. Although this limited virus-specific IgA response mirrors the HIV/SIV-specific Ab profile in breast milk of chronically infected hosts (17, 47–49), the predominant breast milk Ab isotype is secretory IgA. Induction of an SIV Envelope-specific IgA response in breast milk may play a role in blocking virus transmission in the infant gastrointestinal tract. The vaccine regimen investigated in this study targets cellular immune responses and, therefore, is not expected to generate potent mucosal Ab responses. It is possible that an Ab-based vaccine regimen, such as a virus vector prime and recombinant protein boost similar to the regimen that elicited a potentially protective Ab response in the recent Thai trial (50), may be more likely to elicit a mucosal IgA response in breast milk (51). Furthermore, using a mucosal route of immunization may be more effective at inducing a mucosal IgA response in breast milk (51–53). Finally, vaccine-induced activation of CD4\(^+\) T lymphocytes has been suggested as a hypothesis for the cause of increased HIV infections in the vaccine arm of the Step (HIV Vaccine Trials Network) trial evaluating a DNA prime/recombinant adenovirus vector boost HIV vaccine strategy (45, 46). Therefore, we sought to assess the extent of the activation of CD4\(^+\) T lymphocytes in milk following systemic vaccination in uninfected monkeys. Activated CD4\(^+\) T lymphocytes in milk of HIV-infected women may be more apt at producing virus and interacting with the breastfeeding infant’s gastrointestinal tract. We found minimal and transient increases in the proportion of CD4\(^+\) T lymphocytes in milk expressing molecules of activation and the HIV/SIV coreceptor CCR5 following vaccination in these uninfected, lactating monkeys. A similar pattern of limited and transient activation occurred in systemic and colonic CD4\(^+\) T lymphocytes following similar vaccine regimens (41). It is unlikely that these transient and minimal increases in the proportion of activated CD4\(^+\) T lymphocytes following vaccination would be clinically significant in HIV-infected lactating women, because breast milk lymphocytes are already highly activated in this setting (54).

The demonstration of vaccine-elicited cellular and humoral immune responses following systemic administration of a candidate HIV/SIV vaccine regimen provides a platform for further development of maternal vaccine strategies that may impede breast milk transmission of HIV. Although antiretroviral prophylaxis will be a mainstay of prevention of HIV transmission via breastfeeding, vaccination could provide a safe and durable adjunctive mechanism of preventing breast milk transmission of HIV. Vaccine strategies are less reliant on patient compliance and health care infrastructure than are drug interventions. Furthermore, an effective maternal vaccine administered after delivery would avoid fetal and infant toxicities. Therefore, it is important to continue to evaluate vaccine regimens well-suited for induction of mucosal responses in breast milk and assess the efficacy of these interventions in protection against HIV/SIV transmission via breastfeeding in nonhuman primate models (55) and clinical studies.

Acknowledgments
We thank Gary Nabel and the Vaccine Research Center (National Institutes of Health, Bethesda, MD) for provision of the SIVmac 239 DNA vaccine constructs. Furthermore, we thank Michelle Lifton, Keith Reimann, and James Whitney for advice and technical assistance.

Disclosures
The authors have no financial conflicts of interest.

References


