Potent Simian Immunodeficiency Virus-Specific Cellular Immune Responses in the Breast Milk of Simian Immunodeficiency Virus-Infected, Lactating Rhesus Monkeys


*J Immunol* 2008; 181:3643-3650; doi: 10.4049/jimmunol.181.5.3643
http://www.jimmunol.org/content/181/5/3643

**References**
This article cites 55 articles, 23 of which you can access for free at:
http://www.jimmunol.org/content/181/5/3643.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Potent Simian Immunodeficiency Virus-Specific Cellular Immune Responses in the Breast Milk of Simian Immunodeficiency Virus-Infected, Lactating Rhesus Monkeys

Sallie R. Permar,2† Helen H. Kang,* Angela Carville,‡ Keith G. Mansfield,§ Rebecca S. Gelman,§ Srinivas S. Rao,¶ James B. Whitney,* and Norman L. Letvin*

Breast milk transmission of HIV is a leading cause of infant HIV/AIDS in the developing world. Remarkably, only a small minority of breastfeeding infants born to HIV-infected mothers contract HIV via breast milk exposure, raising the possibility that immune factors in the breast milk confer protection to the infants who remain uninfected. To model HIV-specific immunity in breast milk, lactation was pharmacologically induced in Mamu-A*01+ female rhesus monkeys. The composition of lymphocyte subsets in hormone-induced lactation breast milk was found to be similar to that in natural lactation breast milk. Hormone-induced lactating monkeys were inoculated i.v. with SIVmac251 and CD8+ T lymphocytes specific for two immunodominant SIV epitopes, Gag p11C and Tat TL8, and SIV viral load were monitored in peripheral blood and breast milk during acute infection. The breast milk viral load was 1–2 logs lower than plasma viral load through peak and set point of viremia. Surprisingly, whereas the kinetics of the SIV-specific cellular immunity in breast milk mirrored that of the blood, the peak magnitude of the SIV-specific CD8+ T lymphocyte response in breast milk was more than twice as high as the cellular immune response in the blood. Furthermore, the appearance of the SIV-specific CD8+ T lymphocyte response in breast milk was associated with a reduction in breast milk viral load, and this response remained higher than that in the blood after viral set point. This robust viral-specific cellular immune response in breast milk may contribute to control of breast milk virus replication. The Journal of Immunology, 2008, 181: 3643–3650.

The benefits of breastfeeding, including optimal nutrition and protection against gastrointestinal and respiratory infections, are well established and significantly improve infant morbidity and mortality in the developing world (1, 2). Moreover, poor access to clean water in the developing world limits the safety of infant replacement feeding. However, HIV is vertically transmitted via breast milk, and mother-to-child transmission via breast milk remains a significant mode of HIV transmission in the world. Nearly 800,000 new infant HIV infections occur each year, and it is estimated that one-third of these infections are attributable to breastfeeding (3).

Risk factors for transmission of HIV via breast milk include duration of breastfeeding (4–7), advanced maternal HIV disease (8–10), and breast abnormalities, such as breast abscess, mastitis, and cracked nipples (6, 11, 12). Moreover, the level of breast milk viral RNA and number of infected breast milk cells, in addition to plasma viral load, are associated with a high risk of HIV transmission to infants (9, 13–15). Mucosally transmitted virus is exposed to distinct immune responses specific to the mucosal compartment (16–19). Virus in genital tract, semen, and breast milk appears genetically divergent from that in the peripheral blood (20–23), indicating that local immune responses shape the evolution of compartmentalized virus.

Because late mother-to-child transmission of HIV occurs in a small minority of breastfeeding infants born to HIV-infected mothers, the majority of infants remain protected from transmission despite ongoing low-dose exposure to the virus, raising the possibility that HIV-specific cellular or humoral immunity in the breast milk may protect infants from HIV transmission. However, limited information exists regarding maternal breast milk compartment-specific immunity and risk of breast milk transmission. Low-titer HIV-specific IgA and IgM Abs in breast milk have been associated with transmission in some (24), but not all studies (25). HIV-specific CD8+ T cells have been demonstrated in the breast milk of HIV-infected women (26). Yet, little is known about the kinetics, magnitude, or function of virus-specific cellular immunity in breast milk during acute and chronic infection. Moreover, the characteristics of breast milk cellular immunity compared with systemic cellular immunity have not been defined.

The SIV/rhesus monkey model is ideal to study viral-specific mucosal cellular immunity, because immunodominant epitopes and viral evolution are well defined in this model. SIV inoculation of lactating rhesus monkeys allows for investigation of mucosal virus-specific immunity and viral replication in a compartment with a direct impact on risk of infection for the developing infant. In the present study, we describe a pharmacologic induction of lactation model in rhesus monkeys. We then define the kinetics and magnitude of viral-specific cellular immunity and viral replication.
during acute SIV infection in these monkeys. The understanding of cellular immune function in the breast milk and its effect on local viral replication that will come from studies in this nonhuman primate model should provide a framework for developing immunologic interventions to prevent breast milk transmission of HIV.

Materials and Methods

Animals, hormone treatment, and viruses

Four nonpregnant, female rhesus monkeys were administered increasing doses of depot medroxyprogesterone and estradiol by the i.m. route for 2 mo to induce mammary gland maturation. The dopamine antagonist, haloperidol, was administered orally after 2 mo of hormone treatment to raise serum prolactin levels. All four monkeys began lactating within 6 wk of haloperidol treatment, and breast milk was collected by manual massage under ketamine sedation two to three times weekly. Ten units of oxytocin were administered by the i.m. route immediately before sampling. Dose, route, and frequency of estradiol, medroxyprogesterone, and haloperidol were optimized to achieve serum levels of estradiol, progesterone, and prolactin similar to those reported during pregnancy and the postpartum period in rhesus monkeys (27, 28). The optimal dosing schedule was as follows: 2 mg/kg estradiol cypionate i.m. bimonthly, 5 mg/kg medroxyprogesterone acetate weekly, 0.15 mg/kg haloperidol by mouth twice daily.

Mammary gland biopsies were performed on each animal more than 3 mo after the initiation of lactation. Tissue samples were sectioned and stained with H&E. Breast milk was also manually collected once from each of 19 naturally lactating female breeder rhesus monkeys that were between 2 and 36 wk postpartum. Breast milk samples with visible blood contamination or less than 100 CD3+ cells were not included in the cellular analyses (n = 5).

Four female Mamu-A*01+ rhesus monkeys were selected for the study after PCR-based MHC typing. Following induction of lactation by hormone treatment, the monkeys were i.v. inoculated with SIVmac251. Blood and breast milk samples were collected three times per week during the first 4 wk after inoculation and then twice weekly until day 126 after inoculation. The monkeys were maintained in accordance with the guidelines of the Committee on Animals for Harvard Medical School and the “Guide for the Care and Use of Laboratory Animals” (National Research Council, National Academc Press, Washington, D.C., 1996).

Breast milk leukocyte differentials

LW/NL-X stain was prepared, as previously reported (29). Briefly, 500 g of methylene blue chloride (EMD Chemicals), 56 ml of 95% ethyl alcohol (Pharmacol), and 40 ml of xylene (Fisher Scientific) were mixed and left standing for 24 h at 4°C, and then 4 ml of glacial acetic acid (Fisher) was added. A total of 10 μl of fresh whole breast milk was spread on a 1-cm circle on a glass cell count slide (Bellco Glass) and allowed to air dry. The slide was fixed with methanol and treated with blocking agent (5% BSA). The slide was then incubated with anti-CD3 Alexa700 (RPA-T8; BD Biosciences), anti-CD28 PerCP-Cy5.5 (SP34.2; BD Biosciences), anti-CD95 FITC (DX2; BD Biosciences), and anti-CD28 PE-Cy7 (B27; BD Biosciences), and anti-IL-2 allophycocyanin (MQ-17H12; BD Biosciences). PBMC and breast milk cells from SIV-infected animals were also stained with soluble Mamu-A*01 PE- or allophycocyanin-labeled tetrameric complexes containing Tat TL8 (TTPESNL) or Gag p11C, as previously described (31). Data were collected on the LSRII instrument (BD Biosciences) with FACSDiva software and analyzed with FlowJo software.

Ab assays

IgM and IgG levels were measured in duplicate using monkey-specific ELISA kits (Alpha Diagnostics) and standards per protocol. Breast milk supernatant was diluted between 1/10 and 1/12,000 for the assays. The IgA and secretory IgA (sIgA) levels were measured by coating a 96-well poly- styrene plate with anti-IgA Ab (Rockland), blocked with PBS-0.25% non- fat dry milk and 0.05% Tween 20. Plasma was diluted 1/20,000, and breast milk supernatant was diluted between 1/50 and 1/1,000 (for IgA) or 1/100 and 1/102,400 (for sIgA) and incubated for 2 h. Ab was detected after 1-h incubation with an HRP-conjugated anti-IgA Ab (Alpha Diagnostics) or an HRP-conjugated anti-sIgA (Nordic) and addition of ABTS-2 peroxidase substrate system (Kirkegaard & Perry Laboratories). A standard curve for the IgA assay was created using standards from a monkey-specific IgA ELISA kit (Alpha Diagnostics), and sIgA titer was determined by serial 2-fold dilutions with a cutoff of twice OD reading of the PBS-negative control. Both assays were read at 410 nm.

Plasma and breast milk SIV quantitative RT-PCR

Total breast milk supernatant was centrifuged at 16,100 × g for 1.5 h, and the viral pellet was stored in RNA later (Ambion). RNA from plasma and the homogenized viral pellet was isolated using the QIAamp viral RNA kit (Qiagen), according to protocol, with the final product resuspended in 60 μl of RNase-free water. A total of 25 μl of the RNA suspension was used in a reverse-transcriptase reaction containing SuperScript III RT enzyme (Invitrogen) and the Gag-specific primer 5′-GCAAATCTGATCTGT CAGCGGCTC-3′, according to manufacturer’s protocol. A quantity amounting to 10 μl of the resulting cDNA was used in a real-time PCR using the TaqMan EZ RT-PCR kit (Applied Biosystems), as well as a Gag-specific labeled probe (5′-FAM-CTTCTCTAAGGTTGTTACACACT TCTCTCTGGC-TAMRA) and flanking primers (5′-GTCCTGG CACATTGTGGTCCATC-3′ and 5′-CATGATAGGGTCCTGCAACT CTTGTG-3′). Reactions were performed in duplicate on the 7000 ABI PRISM Sequence Detector (Applied Biosystems) at 95°C for 10 min, then 50 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 30 s. An RNA standard was transcribed from a plasmid containing the SIV Gag gene using the Megascipt T7 kit (Ambion), quantitated by OD, and serially diluted to generate a standard curve. The sensitivity of the assay was 600 copies. Preliminary experiments demonstrated that the quantitation of breast milk viral load correlated well to known amount of viral RNA added to breast milk supernatant from SIV-uninfected monkeys (data not shown). For breast milk samples obtained after SIV inoculation that demonstrated detectable levels of viral RNA, samples were also tested for the presence of viral RNA by performing quantitative real-time PCR with primers and probes for the Gag and Nef genes.

Ig classes and lymphocyte subsets or subset ratios in paired blood and breast milk of naturally lactating monkeys were compared using the exact Wilcoxon rank sum test. Two-sided p value interpretation of significance was adjusted for multiple comparisons using Holm’s method. Wilcoxon rank sum test was used for comparisons of SIV viral load and SIV-specific CD8+ T lymphocytes in blood and breast milk, and Spearman’s correlation was used to compare intracellular cytokine production and breast milk viral load. Analyses were performed using Stata and StatXact software, and graphs were made using Prism software.

Statistical analyses

Ig classes and lymphocyte subsets or subset ratios in paired blood and breast milk of naturally lactating monkeys were compared using the exact Wilcoxon sign rank. The same comparisons between natural lactation (NL) and hormone-induced lactation (HIL) monkey breast milk were performed using the exact Wilcoxon rank sum test. Two-sided p value interpretation of significance was adjusted for multiple comparisons using Holm’s method. Wilcoxon rank sign test was used for comparisons of SIV viral load and SIV-specific CD8+ T lymphocytes in blood and breast milk, and Spearman’s correlation was used to compare intracellular cytokine production and breast milk viral load. Analyses were performed using Stata and StatXact software, and graphs were made using Prism software.

Results

Comparison of lymphocyte subsets in breast milk and peripheral blood of rhesus monkeys reveals skewing of breast milk lymphocytes toward a memory phenotype

T lymphocyte subsets in breast milk were compared with those in peripheral blood using paired samples from 14 healthy, normally lactating rhesus monkeys. Although the percentage of CD4+ and CD8+ T lymphocytes was similar between blood and breast milk,
indicated a higher percentage of CD4$^+$ H9262/1,971 compared with peripheral blood (Fig. 2). As anticipated, IgA was the major Ig class in breast milk.

Table I. The frequencies of naive and memory CD4$^+$ and CD8$^+$ T lymphocytes

<table>
<thead>
<tr>
<th>Lymphocyte Subset</th>
<th>Median Range</th>
<th>2-Sided p Value$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4$^+$ naive ratio$^a$</td>
<td>0.07 0.01-0.23</td>
<td>0.001</td>
</tr>
<tr>
<td>CD4$^+$ effector memory ratio$^a$</td>
<td>0.12 0.07-0.36</td>
<td>0.01</td>
</tr>
<tr>
<td>CD8$^+$ naive ratio$^a$</td>
<td>0.02 0-0.25</td>
<td>0.0004</td>
</tr>
<tr>
<td>CD8$^+$ effector memory ratio$^a$</td>
<td>0.75 0.52-0.88</td>
<td>1.00</td>
</tr>
</tbody>
</table>

$^a$Although breast milk and peripheral blood have similar percentages of CD4$^+$ and CD8$^+$ T lymphocytes, breast milk has a significantly lower percentage of naive CD4$^+$ and CD8$^+$ T lymphocytes and a higher percentage of effector memory CD4$^+$ T lymphocytes.

$^b$Value of p was determined using the exact Wilcoxon sign rank test for paired values, and the bolded p values remained significant after adjusting for multiple comparisons.

Although breast milk and peripheral blood have similar percentages of CD4$^+$ CD95$^{-}$ CD28$^+$ and CD8$^+$ CD95$^{-}$ CD28$^+$ (p = 0.0004) subsets. Accordingly, the median percentages of CM and EM lymphocytes were higher in the breast milk than the blood in both CD4$^+$ (79.8% vs 52.2% CM; 10.8% vs 2.3% EM; Fig. 1A) and CD8$^+$ (70.9% vs 42.9% EM; 23.6% vs 12.4% CM; Fig. 1B) T lymphocyte subsets. Breast milk also demonstrated a significantly higher CD4$^+$ EM ratio (percentage of EM/percentage of EM + percentage of CM) (Table I; p = 0.001) than peripheral blood, indicating a higher percentage of CD4$^+$ effector cells in the memory lymphocyte subset in the breast milk when compared with peripheral blood. In contrast, the CD8$^+$ EM ratio was similar in breast milk and peripheral blood (Table I).

Cellular and humoral immunologic constituents in breast milk during HIL and NL are similar

To develop a nonhuman primate model of HIV/SIV immunity in breast milk that circumvents reliance on breeder monkeys and monkey breeding cycles, four nonpregnant, female rhesus monkeys underwent HIL. All four animals began lactating after 2 mo of estrogen and medroxyprogesterone injections and 4–6 wk of dopamine antagonist therapy. Histologic evidence of mammary gland development was achieved within 12 wk of hormone treatment in all animals (data not shown). The distribution of the HIL breast milk cells (n = 4) – 2 mo after initiation of lactation was more similar to the reported cellular content of early milk (32–36) than that of mature milk, with elevated macrophage/monocyte (median 44%, range 42–78%) and neutrophil (median 39%, range 11–40%) content. However, the neutrophil and monocyte/macrophage content of the HIL breast milk was similar, whereas early human milk is expected to have a substantial monocyte/macrophage predominance (36).

Comparisons of lymphocyte subsets in breast milk of hormone-induced, lactating monkeys 12 wk after initiation of lactation (n = 4) and naturally lactating monkeys between 2 and 36 wk postpartum (n = 14) were performed to support the use of the hormone-induced, lactating female monkeys for studies

FIGURE 1. Rhesus monkey breast milk lymphocytes are skewed more than PBLs toward a memory phenotype; however, the distribution of breast milk lymphocyte phenotypes is similar in hormone-induced and naturally lactating monkeys. CD4$^+$ (A) and CD8$^+$ (B) T lymphocytes with a CM (CD95$^+$, CD28$^+$), EM (CD95$^+$, CD28$^+$), and naive (CD95$^-$, CD28$^-$) phenotype were quantitated in breast milk (open symbols) and the peripheral blood (closed symbols). CD4$^+$ (A) and CD8$^+$ (B) T lymphocytes with CM, EM, and naive phenotype in breast milk of rhesus monkeys during HIL (open symbols) and NL (closed symbols). The solid line indicates the median value.
of adaptive immunity. A similar skewing toward a memory phenotype was seen in HIL and NL breast milk in both the CD4 (medians of 84.7 vs 79.8% CM and 10.5 vs 10.8% EM; Fig. 1A) and CD8 (medians of 36.6 vs 23.6% CM and 57.3 vs 70.9% EM; Fig. 1B) lymphocyte populations. Accordingly, the HIL and NL breast milk EM ratios in CD4 (median of 0.119 vs 0.109; p = 0.65) and CD8 (median of 0.747 vs 0.597; p = 0.19) were comparable. Although the median naive ratio of CD4 T lymphocytes trended toward significantly different in HIL and NL breast milk (0.004 vs 0.72; p = 0.034), none of the comparisons of lymphocyte subsets and naive or EM lymphocyte ratios in HIL and NL breast milk remained significant after adjustment for multiple comparisons.

In comparing the Ab classes between hormone-induced, lactating monkeys 12 wk after initiation of lactation and naturally lactating monkeys between 8 and 12 wk postpartum, the median concentration of IgM was found to be similar in HIL and NL breast milk (6.8 vs 14.1 µg/ml; Fig. 2A). In contrast, the median IgG (257.4 and 48.6 µg/ml; p = 0.001; Fig. 2B) and IgA concentration (807.5 vs 195.9 µg/ml; p = 0.026; Fig. 2C) were consistently higher in the HIL breast milk. As expected, the median titer of sIgA was ~1 log higher in HIL breast milk than in NL breast milk (HIL breast milk median titer, 9,600; range, 3,200–102,400 vs NL breast milk median titer, 400; range, 200–1600; p = 0.0075; data not shown), suggesting that the increased IgA content in HIL breast milk is due to mucosally derived sIgA rather than IgA translocated from serum. Although the higher IgA concentration in HIL breast milk did not remain significant after adjustment for multiple comparisons, the IgG concentration remained significantly higher in HIL breast milk compared with NL breast milk. This finding may be explained by the relative immaturity of HL milk compared with NL milk and a higher Ab content that might be expected in early milk compared with mature milk (37, 38).

Functional Gag-specific CD8 T lymphocyte cellular immunity in breast milk of chronically SIV-infected rhesus monkeys

To confirm that Ag-specific cellular immunity could be monitored in breast milk of rhesus monkeys, breast milk lymphocytes from two chronically SIV-infected, lactating Mamu A*01 rhesus monkeys were stained with tetrameric complexes specific for the immunodominant SIV epitopes Gag p11C and Tat TL8. CD8 T lymphocytes specific for both immunodominant epitopes were demonstrated in the breast milk (data not shown). Furthermore, a functional CD8 T lymphocyte immune response consisting of minimal IL-2, but significant TNF-α and IFN-γ intracellular cytokine production after Gag p11C stimulation was also demonstrated in breast milk lymphocytes of chronically SIV-infected monkeys using standard intracellular cytokine-staining techniques (30) (Fig. 3). These data further validate that hormone induction of lactation in rhesus monkeys provides an excellent model for studying Ag-specific cellular immunity in breast milk during acute and chronic SIV infection.
SIV viral load is 1–2 logs lower in breast milk than plasma at both peak and set point of viremia

Although plasma viral load peaked as expected on day 10 after SIV inoculation (Fig. 4A), breast milk viral load peaked slightly later, between days 14 and 21 (Fig. 4B). The peak of viral load in the breast milk (median, $8.3 \times 10^5$; range, $3.8 \times 10^5$–$1.0 \times 10^7$) was 1–2 logs lower than the peak of viral load in the plasma (median, $8.2 \times 10^7$; range, $1.8 \times 10^7$–$1.9 \times 10^8$). This differential was maintained after viral set point (defined as day 50 after inoculation) with breast milk viral load (median, $2.4 \times 10^4$; range, $4.0 \times 10^3$–$1.0 \times 10^5$) remaining 1–2 logs lower than plasma viral load (median, $1.8 \times 10^6$; range, $4.7 \times 10^4$–$2.7 \times 10^6$). The breast milk viral load trended toward significantly lower than plasma viral load at both peak and set point in this study of only four animals (both $p = 0.12$).

SIV-specific CD$^8^+$ T lymphocyte response is significantly higher in breast milk than blood during acute SIV infection

Although the kinetics of the CD$^8^+$ T lymphocyte response specific for the Mamu A*01+ immunodominant epitopes Tat TL8 and Gag p11C in breast milk paralleled the blood, the magnitude of the SIV-specific cellular response was considerably higher in breast milk (Fig. 5, A–H). The percentage of CD$^8^+$ T lymphocytes in breast milk specific for Tat TL8 (median, 30.4%; range, 28.3–37.5%), a response that is important for early control of viremia, was ~2–3 times higher than in blood in all animals at the peak of the response (median, 14.4%; range, 11.1–17.3%) (Fig. 5, A–D). Additionally, the percentage of CD$^8^+$ T lymphocytes specific for Gag p11C, a response that is important for long-term control of SIV viremia, was also ~2–3 times higher in breast milk (median, 37.2%; range, 27.6–44.3%) than in blood (median, 15.4%; range,
indicating a functional role for CD8\(^+\) T lymphocytes in breast milk during acute SIV infection approached a similar magnitude as the absolute number in peripheral blood at the peak of the cellular immune response. In breast milk, the absolute number of Tat TL8-specific CD8\(^+\) T lymphocytes at the peak of the Ag-specific cellular immune response ranged from 2 to 289 cells/\(\mu l\) breast milk (median = 27 cells/\(\mu l\)), whereas the absolute number of Tat TL8 CD8\(^+\) T lymphocytes in peripheral blood varied between 39 and 450 cells/\(\mu l\) blood (median = 156 cells/\(\mu l\)). Likewise, the absolute number of Gag p11C-specific CD8\(^+\) T lymphocytes ranged from 3 to 316 cells/\(\mu l\) breast milk (median = 27 cells/\(\mu l\)), and the absolute number of Gag p11C-specific CD8\(^+\) T lymphocytes in peripheral blood fell between 49 and 430 cells/\(\mu l\) blood (median = 197 cells/\(\mu l\)) at the peak of the Gag p11C-specific cellular immune response.

**Emergence of CD8\(^+\) T lymphocyte response in breast milk is associated with decline in breast milk viral load**

Importantly, the appearance of the Tat TL8-specific CD8\(^+\) T lymphocyte response in breast milk occurred near the time of the initial decline in breast milk viral load after the peak of viral replication (Fig. 5, I–L). Although the initial decline in breast milk viral load could be explained by a simultaneous decline in plasma viral load, sustained lower breast milk viral load after viral set point was concurrent with persistent higher percentages of Gag p11C-specific CD8\(^+\) T lymphocytes in breast milk than in plasma (Fig. 5, M–P). The percentage of CD8\(^+\) T lymphocytes in breast milk that produced TNF-\(\alpha\) and IFN-\(\gamma\) after stimulation with Gag p11C on day 115 after SIV (Fig. 3) inoculation trended toward an inverse association with breast milk viral load on day 112 in all monkeys that continued lactating (\(p = 0.33, n = 3\); data not shown), further indicating a functional role for CD8\(^+\) T lymphocytes in control of breast milk viral replication.

**Discussion**

In these experiments, we have introduced a novel nonhuman primate model for studying breast milk immunity during HIV/SIV infection. The advantages of pharmacologic induction of lactation in rhesus monkeys include independence from both primates breeding cycles and care of infant monkeys, and ease of sample collection. Lymphocyte subsets were similar in HIL and NL breast milk, indicating that Ag-specific cellular immune response in each type of breast milk should be comparable. Not surprisingly, the HIL breast milk displayed characteristics similar to colostrum or early milk with higher Ig (37, 38), macrophage/monocyte, and neutrophil content (32–35) than mature milk, because the artificial lactation protocol most likely induces breast milk that is not fully mature. However, human colostrum has a macrophage/monocyte predominance that was not observed in the HIL breast milk, suggesting that the HIL breast milk may be on the continuum between early milk and mature milk. Because breast milk viral load (41) and rates of HIV transmission are higher in early lactation (42), the apparent immaturity of the HIL breast milk is advantageous for modeling the virologic and immunologic factors contributing to this period of high risk for the breastfeeding infant.

The lactation model is a valuable tool to investigate Ag-specific mucosal immunity, because lymphocytes and Ab can be collected in the fluid phase at multiple time points and immune responses can be evaluated simultaneously in the mucosal compartment and the blood. In the lactation-induction SIV/rhesus monkey model, we were able to quantitate epitope-specific T lymphocytes, as well as characterize functional, ex vivo stimulated cellular responses without the high background activation that often limits the ability to monitor cellular immune responses. Although blood contamination of manually collected breast milk is a concern, our data are consistent with the detection of breast milk-specific humoral and cellular immune responses, because the defined phenotype of the lymphocytes and the Ab content of the rhesus monkey breast milk were distinct from the blood and consistent with previous descriptions of human breast milk immune responses (26, 35, 37, 38, 43).

Although HIV-specific CD8\(^+\) T lymphocytes have previously been demonstrated in breast milk of HIV-infected women (44), the kinetics and function of this population of cells have not yet been evaluated. This study has documented a robust SIV-specific cellular immunity in breast milk that parallels the kinetics of the CD8\(^+\) T lymphocyte response in the blood, but is 2–3 times higher at the time of both peak and set point of viremia. The robust SIV-specific cellular response in the breast milk was not related to high Ag burden, because the viral load in the breast milk remained at least 1 log lower than that in the blood throughout acute infection.

The high frequency of SIV-specific T lymphocytes in breast milk measured during acute and chronic SIV infection was not predicted. The SIV-specific CD8\(^+\) T lymphocyte response reported in the gastrointestinal and genital tracts during acute infection is less than or equal to the CD8\(^+\) T lymphocyte response in the blood (18, 19, 45, 46), despite intense viral replication in those mucosal compartments. The robust mucosal CD8\(^+\) T lymphocyte response in breast milk provides further evidence that the mucosal compartments are immunologically distinct (16–18). Moreover, the high levels of cellular immunity in breast milk during acute infection may contribute to prevention of viral transmission from mothers to infants during breastfeeding.

It is well established that the SIV-specific CD8\(^+\) T lymphocyte response is essential for the control of viral replication in the peripheral blood (47, 48). Although control of viral replication in mucosal compartments is most likely affected by both anatomic and immunologic factors, the breast milk SIV-specific CD8\(^+\) T lymphocytes most likely play a key role in the containment of breast milk viral load because of several supporting observations. First, the breast milk viral load remained appreciably less than the viral load in plasma throughout acute and chronic SIV infection in association with a particularly high SIV-specific CD8\(^+\) T lymphocyte response in the breast milk. Second, the initial containment of breast milk viral load occurred near the time of the emergence of the Tat TL8-specific CD8\(^+\) T lymphocyte response in the breast milk, although this association may be explained by the concurrent reduction in plasma viral load. Finally, maintenance of low breast milk viral load after viral set point was coincident with a sustained high-level Gag p11C-specific CD8\(^+\) T lymphocyte response in breast milk.

The potency of the breast milk SIV-specific cellular immune response during acute SIV infection may contribute to containing mother-to-child transmission of the virus. Because high breast milk HIV RNA viral load is associated with infant breast milk transmission (9, 15, 49), cellular immune containment of viral shedding in breast milk could contribute to a reduction in risk of transmission via breastfeeding. HIV-specific cytotoxic CD8\(^+\) T lymphocytes in breast milk are expected to reduce breast milk viral load by eliminating local cellular reservoirs of virus, including...
macrophage/dendritic cells and activated CD4+ T lymphocytes. These cell types are in high concentration in early milk (34) and most likely play an important role in the transmission of HIV via breastfeeding. Additionally, the infant is exposed to a high frequency of HIV-specific maternal CD8+ T lymphocytes that may be absorbed by the infant gastrointestinal tract, because animal studies have demonstrated the absorption of maternal lymphocytes into the gastrointestinal mucosa (50, 51), as well as the bloodstream (52), of suckling neonates. Furthermore, maternal lymphocytes transmitted in breast milk may play an active role in the developing neonatal immune system, because evidence of acquisition of maternal mitogen and Ag-specific cellular responses via breastfeeding exists in both experimental animal (53) and human (34, 54, 55) studies. Further investigation of the high frequency viral-specific CD8+ T lymphocytes in breast milk is certainly warranted. Through these studies, maternal intervention to enhance this breast milk immune response may be a viable strategy for prevention of mother-to-child transmission via breastfeeding.

Acknowledgments
We thank Adam Buzby, Kevin Carlson, Saran Bao, and Michelle Lifton for their technical assistance. We also thank Corrine Welt, Dan Barouch, and Barton Haynes for their generous advice and assistance.

Disclosures
The authors have no financial conflict of interest.

References


