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Breast Milk of HIV-Positive Mothers Has Potent and Species-Specific *In Vivo* HIV-Inhibitory Activity

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ABSTRACT

Despite the nutritional and health benefits of breast milk, breast milk can serve as a vector for mother-to-child HIV transmission. Most HIV-infected infants acquire HIV through breastfeeding. Paradoxically, most infants breastfed by HIV-positive women do not become infected. This is potentially attributed to anti-HIV factors in breast milk. Breast milk of HIV-negative women can inhibit HIV infection. However, the HIV-inhibitory activity of breast milk from HIV-positive mothers has not been evaluated. In addition, while significant differences in breast milk composition between transmitting and nontransmitting HIV-positive mothers have been correlated with transmission risk, the HIV-inhibitory activity of their breast milk has not been compared. This knowledge may significantly impact the design of prevention approaches in resource-limited settings that do not deny infants of HIV-positive women the health benefits of breast milk. Here, we utilized bone marrow/liver/thymus humanized mice to evaluate the *in vivo* HIV-inhibitory activity of breast milk obtained from HIV-positive transmitting and nontransmitting mothers. We also assessed the species specificity and biochemical characteristics of milk's *in vivo* HIV-inhibitory activity and its ability to inhibit other modes of HIV infection. Our results demonstrate that breast milk of HIV-positive mothers has potent HIV-inhibitory activity and indicate that breast milk can prevent multiple routes of infection. Most importantly, this activity is unique to human milk. Our results also suggest multiple factors in breast milk may contribute to its HIV-inhibitory activity. Collectively, our results support current recommendations that HIV-positive mothers in resource-limited settings exclusively breastfeed in combination with antiretroviral therapy.

IMPORTANCE

Approximately 240,000 children become infected with HIV annually, the majority via breastfeeding. Despite daily exposure to virus in breast milk, most infants breastfed by HIV-positive women do not acquire HIV. The low risk of breastfeeding-associated HIV transmission is likely due to antiviral factors in breast milk. It is well documented that breast milk of HIV-negative women can inhibit HIV infection. Here, we demonstrate, for the first time, that breast milk of HIV-positive mothers (nontransmitters and transmitters) inhibits HIV transmission. We also demonstrate that breast milk can prevent multiple routes of HIV acquisition and that this activity is unique to human milk. Collectively, our results support current guidelines which recommend that HIV-positive women in resource-limited settings exclusively breastfeed in combination with infant or maternal antiretroviral therapy.

Breast milk provides newborn infants with all of their daily nutritional and fluid requirements. It is also a rich source of hormones, antimicrobial factors, and immune components that decrease the susceptibility of breastfed infants to infectious diseases (1). For these reasons, the World Health Organization (WHO) recommends that mothers exclusively breastfeed their infants for the first 6 months of life (2). However, despite the nutritional and health benefits of breastfeeding, in some instances, breast milk can serve as a vector for mother-to-child transmission of infectious pathogens (3).

Infants born to HIV-positive women can acquire HIV orally by ingesting their mother's breast milk which may contain virus particles (cell-free HIV) and/or virus-infected cells (cell-associated HIV). An estimated 240,000 children become infected with HIV annually, mostly via breastfeeding (4). In developed countries, HIV-positive mothers abstain from breastfeeding and substitute formula for breast milk. In resource-limited regions, HIV-positive mothers breastfeed their infants because the health benefits of breast milk outweigh the risk of HIV transmission. In this setting, the WHO recommends that HIV-positive mothers exclusively breastfeed their infants for the first 6 months of life in combina-

Received 3 July 2015 **Accepted** 10 August 2015

Accepted manuscript posted online 19 August 2015

Citation Wahl A, Baker C, Spagnuolo RA, Stamper LW, Fouda GG, Permar SR, Hinde K, Kuhn L, Bode L, Aldrovandi GM, Garcia JV. 2015. Breast milk of HIV-positive mothers has potent and species-specific *in vivo* HIV-inhibitory activity. J Virol 89:10868–10878. doi:10.1128/JVI.01702-15.

Editor: G. Silvestri

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/JVI.01702-15.

tion with infant or maternal antiretroviral therapy (ART) (5). Although these strategies significantly reduce HIV transmission, the success of ART relies on adherence, which is limited by cost, access, and social factors. It is estimated that only 67% of HIV-positive pregnant women in low- and middle-income countries receive ART to prevent mother-to-child transmission (4). Therefore, novel approaches that effectively prevent breastfeeding HIV transmission without denying infants the health benefits of breast milk are urgently needed.

Surprisingly, in the absence of ART, the majority of infants breastfed by HIV-positive women do not acquire HIV (~85%) despite ingesting breast milk daily for several months to years (~250 liters per year) (6). Higher levels of alpha-defensins, IL-15, erythropoietin, oligosaccharides, long-chain polyunsaturated fatty acids (LCPUFAs) and antibody-dependent cell-mediated cytotoxicity in breast milk have been correlated with a decreased risk of breastfeeding HIV transmission (7–12). Breast milk also contains several factors that inhibit HIV infection *in vitro* (13–22). For example, Fouda et al. recently demonstrated that tenascin-C (TNC), a novel high-molecular-weight protein in breast milk, can inhibit HIV infection *in vitro* by capturing and binding to HIV virions via the chemokine coreceptor binding site on the HIV envelope protein (23). While these observations suggested that breast milk limits HIV transmission, until recently it was difficult to directly assess the effect of breast milk on oral HIV transmission. *In vivo* HIV studies are severely limited by the strict species tropism of the virus.

For this purpose, we developed a novel humanized mouse model of oral HIV transmission utilizing bone marrow/liver/thymus (BLT) humanized mice. We demonstrated that systemic reconstitution of BLT mice with human hematopoietic cells renders them susceptible to oral HIV transmission. Oral HIV transmission in BLT mice results in the presence of virus in plasma and saliva, virus dissemination, and systemic $CD4^+$ T cell depletion, recapitulating the human condition (24). We then used BLT mice to demonstrate that whole breast milk obtained from HIV-negative women can prevent oral HIV transmission (24). The *in vivo* HIV-inhibitory activity of breast milk observed is due to intrinsic antiviral factors as the breast milk of HIV-negative women does not contain any kind of HIV-specific antibodies or immune cells.

Although we demonstrated a protective effect of breast milk on oral HIV transmission, it remained unknown whether the breast milk of HIV-positive women could inhibit HIV infection *in vivo*. In addition, while significant differences in breast milk composition have been correlated with transmission risk, the HIV-inhibitory activity of breast milk obtained from transmitting and nontransmitting HIV-positive mothers had not been directly compared. This knowledge could have significant implications in regard to the design of novel approaches to prevent HIV transmission through breastfeeding. Therefore, we evaluated the *in vivo* HIV-inhibitory activity of breast milk obtained from HIV-positive transmitting and nontransmitting mothers. Our results demonstrate that the breast milk of HIV-positive transmitting and nontransmitting mothers can inhibit HIV infection *in vivo*. Next, we assessed the specificity of breast milk's *in vivo* HIV-inhibitory activity and its ability to provide sterilizing protection from HIV acquisition to gain a better understanding of the factors in breast milk that inhibit HIV infection *in vivo*. First, we established that the HIV-inhibitory activity of milk is restricted to humans. Next, we characterized properties of breast milk's *in vivo* HIV-inhibitory activity. Finally, we demonstrated that human breast milk contains natural and potent inhibitors that efficiently inhibit vaginal HIV transmission and provide sterilizing protection from an intravenous HIV exposure.

MATERIALS AND METHODS

Generation of humanized BLT mice. Humanized BLT mice were generated as previously described by transplanting human liver-derived $CD34^+$ hematopoietic stem cells intravenously into 6- to 8-week old NOD.Cg-Prkdc^{scid} ll2rg^{tm1Wjl}/SzJ (NSG; The Jackson Laboratory, Bar Harbor, ME) or NOD.Cg-Prkdc^{scid} ll2rg^{tm1Wjl} Tg(HLA-A2.1)1Enge/SzJ (NSG-A2; The Jackson Laboratory, Bar Harbor, ME) mice previously implanted (under the kidney capsule) with a piece of autologous human liver sandwiched between two pieces of human thymus (Advanced Bioscience Resources, Alameda, CA) (25–27). Systemic reconstitution with human immune cells was monitored in peripheral blood (PB) by flow cytometry, as we previously described (25–27). Mice were maintained under specific-pathogen-free conditions by the Division of Laboratory Animal Medicine according to protocols approved by the Institutional Animal Care and Use Committee at the University of North Carolina–Chapel Hill.

HIV exposure of humanized BLT mice. Stocks of HIV-1_{JR-CSF} were prepared and titers were determined on TZM-bl cells as previously described (25, 26) to calculate the number of tissue culture infectious units (TCIU) per ml. Oral HIV exposures were performed as previously described (24). Briefly, anesthetized BLT mice were exposed orally to HIV by distilling virus (1.4×10^6 to 1.8×10^6 TCIU) resuspended in 20 μl of vehicle (RPMI medium or phosphate-buffered saline [PBS]), vehicle supplemented with human milk oligosaccharides, tenascin-C, or lactoferrin (AbD Serotec, Kidlington, United Kingdom) or milk (human, cow, goat, camel, rhesus macaques, and African green monkeys) directly into the oral cavity. Vaginal exposures were also performed essentially as previously described (25, 26), except that 3.5×10^5 TCIU HIV was mixed by pipetting with either 5 μl of vehicle (RPMI medium) or 8 μl of whole human breast milk prior to vaginal exposure. To assess the efficacy of a topical application of breast milk on vaginal HIV transmission, 20 μl of breast milk was pipetted into the vagina of anesthetized BLT mice 15 min prior to or 4 h before and after vaginal administration of virus. Intravenous exposures of BLT mice to HIV were performed via tail vein injection (200 μl, total volume) following incubation of virus with whole human breast milk (20 μl, total volume) for 10 min at room temperature and subsequent dilution with RPMI medium (1:10). As a positive control for HIV infection, BLT mice were also exposed intravenously to HIV resuspended in 200 μl of RPMI medium.

Human and nonhuman milk. We utilized whole breast milk obtained from HIV-positive women enrolled in the Zambian Exclusive Breastfeeding (ZEB) study at 1 month postpartum to evaluate the *in vivo* HIV-inhibitory of breast milk from HIV-positive nontransmitting and transmitting mothers. Details of the ZEB study are described elsewhere (28). Briefly, HIV-infected women were recruited from two antenatal care clinics in Lusaka, Zambia, from May 2001 to September 2004 and were monitored through delivery and up to 2 years postpartum with their infants. Single-dose nevirapine was administered to women at the onset of labor as prophylaxis to prevent mother-to-child transmission. The study was approved by the investigators' institutional review boards, and all women consented in writing to participate in the study. All women were counseled to exclusively breastfeed for the first 4 months or longer. HIV infection of infants was monitored in peripheral blood by PCR at birth, monthly to 6 months of age, and then every 3 months thereafter until 2 years of age. All positive tests were confirmed by testing two or more samples. HIV infection of infants was considered to have occurred through breastfeeding if the infant first tested positive by PCR after 42 days of birth with an earlier negative result.

Whole breast milk was obtained from HIV-negative women (Innovative Research, Novi, MI) to evaluate the HIV-inhibitory activity of heat-inactivated, skim, and proteinase K-treated breast milk and to assess the

effect of breast milk on vaginal and intravenous HIV acquisition. Whole breast milk was heat inactivated for 30 min in a 62.5°C water bath (with agitation every 10 min) and subsequently cooled in an ice bath prior to exposure. The skim fraction of breast milk was isolated after centrifugation (1,000 × *g* for 10 min) and aspiration of the lipid layer. Breast milk was incubated with active or inactivated (incubated 90°C for 20 min) proteinase K (Roche, Indianapolis, IN) at 50 μg/ml at 55°C for 1 h, followed by 90°C for 20 min. Untreated whole breast milk and whole breast milk treated with active or inactive proteinase K was loaded onto a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel, followed by Coomassie blue staining to visualize proteins.

Whole rhesus macaque (*Macaca mulatta*) milk was collected at 1 month postpartum from animals housed at the California National Primate Research Center according to protocols approved by the Institutional Animal Care and Use Committee during an ongoing study. Whole African green monkey milk (pooled from five donors) was collected from animals housed and maintained by the Duke University Medical Center Division of Laboratory Animal Resources Vivarium as a part of an ongoing study according to protocols approved by the Institutional Animal Care and Use Committee. Whole camel milk (pooled from six donors) was obtained from the Camel Milk Association (Marion, MI). Human milk oligosaccharides (HMOs) were isolated and purified from the milk of 36 HIV-negative women as previously described (29). Briefly, after removal of the lipid layer of breast milk by centrifugation, proteins were precipitated from the aqueous phase of breast milk by the addition of ice-cold ethanol and subsequent centrifugation. Ethanol was removed from the HMO-containing supernatant by roto-evaporation, and lactose and salts were removed by gel filtration chromatography. The milk was donated to the Bode laboratory at the University of California, San Diego, after approval by the university's institutional review board. Breast milk from HIV-negative women was enriched for TNC, a high-molecular-weight protein, by high-pressure liquid chromatography (HPLC) high-molecular-weight protein fractionation. Briefly, breast milk was delipidized by centrifugation at 4°C. Proteins in the aqueous fraction were fractionated by size exclusion using a Superose 6 column (GE Healthcare, Pittsburgh, PA) on an HPLC apparatus (Waters, Milford, MA). The high-molecular-weight fractions were concentrated using Amicon 30K filter columns (EMD Millipore, Billerica, MA) following strong anion exchange by HPLC. TNC was quantified in the high-molecular-weight breast milk fractions using a TNC enzyme-linked immunosorbent assay, and purity was determined by SDS-PAGE and Western blot analysis (30). The TNC-enriched high-molecular-weight fractions of breast milk were utilized for oral HIV challenges.

Analysis of HIV transmission in humanized BLT mice. HIV transmission was determined by the presence of viral RNA in plasma (viral load analysis) essentially as we have previously described (25, 26). The presence of HIV DNA in tissues and peripheral blood cells collected from BLT mice at necropsy was determined by real-time PCR analysis of DNA extracted from mononuclear cells as previously described (25, 26). As a control for the presence of DNA extracted from human cells, all samples were tested for the presence of human gamma globin DNA by real-time PCR.

Statistical analysis. Statistical analyses were performed in Prism, version 5 (GraphPad, La Jolla, CA). We used a log-rank Mantel-Cox test to compare the rates of transmission between groups of BLT mice after oral, vaginal, and intravenous HIV exposure.

RESULTS

***In vivo* HIV-inhibitory activity of breast milk from HIV-positive transmitting and nontransmitting mothers.** We utilized BLT humanized mice to evaluate the *in vivo* HIV-inhibitory activity of breast milk obtained from HIV-positive mothers. BLT mice were exposed to a single oral dose of HIV resuspended in whole breast milk collected at 1 month postpartum from HIV-positive mothers enrolled in the Zambian Exclusive Breastfeeding (ZEB) study (28). We evaluated breast milk samples collected from four mothers that did not transmit HIV to their infants (nontransmitters) and four mothers that transmitted HIV to their infants through breastfeeding (transmitters). Breastfeeding HIV transmission was considered to have occurred in the ZEB study if the infant first tested positive by PCR after 42 days of birth with an earlier negative result. As a positive control for oral HIV transmission, BLT mice were exposed to HIV in vehicle. After oral HIV exposure, we monitored levels of HIV-RNA longitudinally in plasma with real-time PCR. HIV transmission was defined by the presence or absence of detectable HIV-RNA in plasma. Lack of HIV infection was confirmed at necropsy by the absence of detectable HIV-DNA in peripheral blood (PB) and tissues harvested from BLT mice.

We observed 100% transmission in BLT mice exposed orally to HIV in vehicle (Fig. 1 and see Table S1 in the supplemental material). However, HIV transmission was significantly reduced in BLT mice exposed orally to HIV resuspended in breast milk from HIV-positive transmitters ($P = 0.0145$; 2/8 mice infected) and nontransmitters ($P = 0.0077$; 2/8 mice infected) (Fig. 1 and see Table S1 in the supplemental material). Furthermore, although we readily detected HIV-DNA in the PB and tissues analyzed from BLT mice exposed orally to HIV in vehicle, we did not detect any HIV-DNA in the PB or tissues collected from aviremic BLT mice exposed orally to HIV in breast milk of HIV-positive mothers (see Table S1 in the supplemental material). Breast milk obtained from HIV-positive transmitting and nontransmitting mothers were equally likely to inhibit oral HIV transmission ($P = 0.9676$). We observed oral HIV transmission in the presence of breast milk obtained from two transmitting and two nontransmitting mothers (Fig. 1; see also Table S1 in the supplemental material). Sequencing of HIV-RNA isolated from the plasma of viremic mice exposed to HIV in breast milk confirmed that the transmitting virus was the input virus (HIV-$1_{JR\text{-}CSF}$) and not maternal virus that may be present in the breast milk samples. Overall, our results demonstrate that breast milk of HIV-positive mothers can prevent HIV infection *in vivo* and that the breast milk of HIV-positive transmitting mothers does not possess less potent *in vivo* HIV-inhibitory activity than the breast milk of HIV-positive nontransmitting mothers.

Species specificity of HIV-inhibitory activity of milk. We determined whether this HIV-inhibitory effect was specific to human breast milk by evaluating the protective features of the milk of other taxa. Although mammalian milk primarily consists of water, fats, proteins, and carbohydrates, across the mammalian class, milk composition varies substantially as a function of phylogeny, nutritional ecology, and developmental priorities (31–37). For this purpose, we obtained unpasteurized whole milk from species that are closely related to humans (African green monkeys [AGMs] and rhesus macaques [RMs]) and from more distantly related species that are abundant sources of dietary milk (cow, goat, and camel). To assess the *in vivo* HIV-inhibitory activity of milk collected from these species, we exposed groups of BLT mice orally to HIV resuspended in vehicle (positive control), cow, goat, camel, RM, or AGM milk as we described for human breast milk above except that we reduced the rate of oral HIV transmission to better appreciate whether the milk of the species tested inhibits, enhances, or has no effect on HIV transmission.

Our results revealed that oral transmission of HIV was not significantly inhibited (or enhanced) by cow, goat, camel, RM, or AGM milk (Fig. 2 and see Table S2 in the supplemental material).

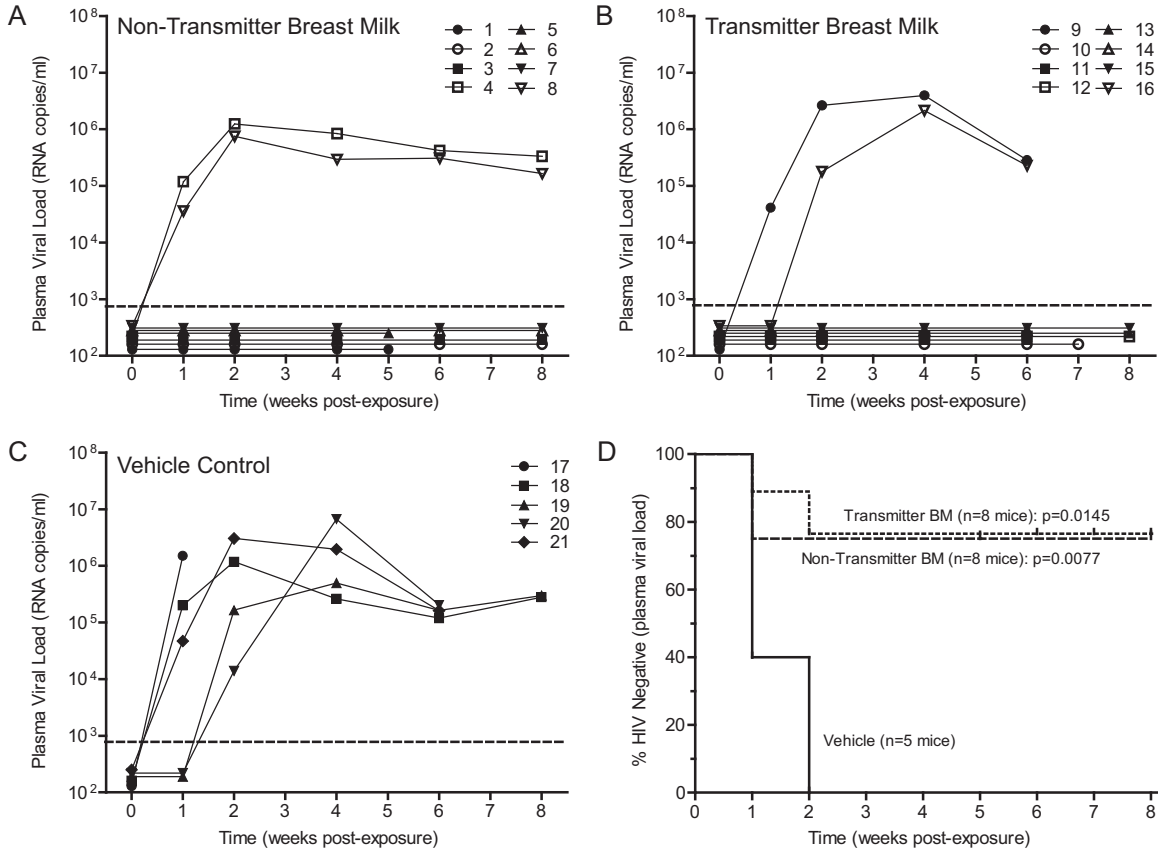

FIG 1 Breast milk of HIV-positive nontransmitting and transmitting mothers inhibits HIV infection *in vivo*. Groups of BLT mice were exposed orally to HIV resuspended in breast milk (BM) obtained 1 month postpartum from HIV-positive nontransmitting (4 donors, 2 mice per donor) (A) and transmitting (4 donors, 2 mice per donor) (B) mothers. (C) As a positive control for oral HIV transmission, BLT mice were exposed to HIV in vehicle ($n = 5$). After oral exposure, HIV-RNA was measured longitudinally in plasma with real-time PCR, and the assay limit of detection is indicated with a dashed line (750 copies/ml of plasma). The plasma viral load for each mouse is indicated. For panels A and B, data for BLT mice exposed to virus in the presence of BM from the same donor are depicted by the same shape. (D) A Kaplan-Meier plot depicts the percentage of BLT mice in each exposure group that did not become infected with HIV. HIV transmission was defined by the presence of HIV-RNA in plasma. A log-rank Mantel-Cox test was used to compare HIV transmission between BLT mice exposed orally to HIV in vehicle and in BM (*P* values are indicated).

HIV-RNA was readily detected in the plasma of BLT mice exposed orally to virus in vehicle (66% transmission) and nonhuman milk (transmission rates: 75% cow milk, $P = 0.8784$; 50% goat milk, $P = 0.5522$; 50% camel milk, $P = 0.5522$; 50% RM milk, $P = 0.4380$; and 50% AGM milk, $P = 0.4380$) (Fig. 2). These results are in stark contrast to our studies with human breast milk in which we observed significantly reduced oral HIV transmission in the presence of breast milk from HIV-negative (24) (Fig. 2) and HIV-positive (Fig. 1) women. Collectively, these results reveal that the HIV-inhibitory activity of milk is species specific and that the ability of human breast milk to inhibit HIV infection *in vivo* is not due to a nonspecific inhibitory property of milk.

Nature of breast milk's HIV-inhibitory activity. We characterized the nature of breast milk's HIV-inhibitory activity by evaluating the effect of heat-inactivation and lipid removal on breast milk inhibition of HIV transmission. In addition, we evaluated the *in vivo* HIV-inhibitory activity of individual breast milk components that have been shown to inhibit HIV infection *in vitro*. Several immune components of breast milk are significantly reduced by Holder pasteurization (heating to 62.5°C for 30 min) (38). Therefore, we exposed BLT mice orally to HIV resuspended in heat-inactivated (62.5°C, 30 min) whole breast milk from HIV-negative women. We observed that heat-inactivated breast milk significantly reduced oral HIV transmission in BLT mice compared to vehicle ($P = 0.0143$; 0/4 mice infected) (Fig. 3A and see Table S3 in the supplemental material). Heat-inactivated whole breast milk inhibited oral HIV transmission as efficiently as untreated whole breast milk (24). After oral exposure, we did not detect any HIV-RNA in the plasma of BLT mice exposed to virus in heat-inactivated breast milk at any time point analyzed. Also, at necropsy, we did not detect any HIV-DNA in their PB or tissues (Fig. 3A and see Table S3 in the supplemental material). These results reveal that the *in vivo* HIV-inhibitory activity of human breast milk is not sensitive to heat inactivation.

Next, we determined if the skim fraction of human breast milk is sufficient to inhibit oral transmission of HIV. The majority of the 260+ proteins and carbohydrates in breast milk reside in the skim fraction (31). We isolated the skim fraction after centrifuging whole breast milk from HIV-negative women (1,000 × *g* for 10 min) and aspirating off the cream (lipid layer). In contrast to BLT mice exposed orally to virus in vehicle, HIV-RNA was detected in the plasma of only one animal exposed to virus in the presence of skim breast milk (Fig. 3A and see Table S3 in the supplemental material). Skim breast milk significantly reduced oral HIV trans-

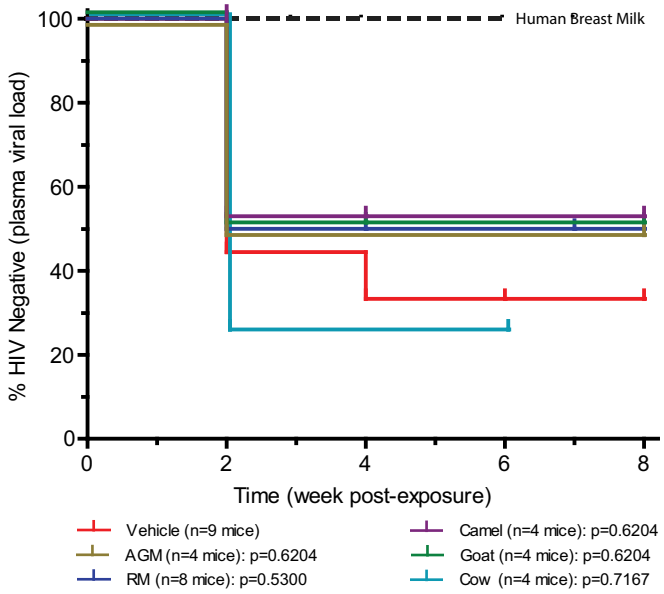

FIG 2 The milk of other species does not inhibit HIV transmission. BLT mice were exposed orally to HIV in the presence of cow ($n = 4$, light blue line), goat ($n = 4$, green line), camel ($n = 4$, purple line), rhesus macaque (RM), ($n = 8$, blue line), or African green monkey (AGM) ($n = 4$, brown line) milk. As a positive control, BLT mice were exposed orally to HIV in vehicle ($n = 11$, red line). Infection was monitored longitudinally in plasma with real-time PCR. HIV transmission was defined by the presence of HIV-RNA in plasma. A Kaplan-Meier plot depicts the percentage of BLT mice in each exposure group that did not become infected with HIV. Oral HIV transmission in the presence of human breast milk (BM) from HIV-negative women (5 donors; $n = 18$ mice) is illustrated with a dashed black line (24). A log-rank Mantel-Cox test was used to compare HIV transmission between BLT mice exposed orally to HIV in vehicle and in milk (P values are indicated).

mission *in vivo* ($P = 0.0452$; 1/5 mice infected). Furthermore, real-time PCR analysis did not reveal any HIV-DNA in the PB or tissues of BLT mice that did not have detectable viremia, confirming a complete lack of infection (see Table S3 in the supplemental material).

Next, we proceeded to evaluate the contribution of milk proteins to breast milk's *in vivo* HIV-inhibitory activity. First, we evaluated the *in vivo* HIV-inhibitory activity of individual breast milk proteins that have been shown to inhibit HIV infection *in vitro*. To evaluate the *in vivo* HIV-inhibitory activity of TNC, we exposed BLT mice orally to HIV in vehicle or vehicle supplemented with TNC-enriched breast milk (78 to 168 μg of TNC/ml). Subsequently, we observed HIV-RNA in the plasma of 7 of 8 BLT mice (87% transmission) exposed orally to HIV in the presence of TNC (Fig. 3B). These results indicate that TNC does not inhibit oral HIV transmission at physiological concentrations ($P = 0.7075$). We also observed that breast milk purified lactoferrin (LF), a well-characterized component of breast milk that has been shown to inhibit HIV infection *in vitro*, does not inhibit oral HIV transmission in BLT mice at maximal concentrations (7 mg/ml) observed in human colostrum (39) (4/4 mice infected; $P = 0.1088$) (Fig. 3B).

To further establish the role of milk proteins in breast milk inhibition *in vivo*, we then treated whole breast milk with 50 μg of active or inactive proteinase K (PK)/ml. Proteolytic digestion of breast milk proteins by PK was confirmed by SDS-PAGE and Coomassie blue staining (see Fig. S1 in the supplemental material). Oral exposure of BLT mice to HIV resuspended in breast milk treated with active PK and inactive PK revealed that proteolytic digestion of breast milk proteins does not significantly alter the ability of breast milk to inhibit HIV infection *in vivo*. We did not detect HIV-RNA in the plasma of 8/9 BLT mice exposed orally to HIV in the presence of breast milk treated with active PK ($P = 0.001$) and 6/7 BLT mice orally exposed to HIV in breast milk treated with inactive PK ($P = 0.0034$). In addition, we did not detect HIV-DNA in the tissues of plasma viremia negative animals (Fig. 3C and see Table S4 in the supplemental material). Collectively, these results indicate the *in vivo* HIV-inhibitory activity of milk is not protein dependent.

We then proceeded to evaluate the ability of human milk oligosaccharides (HMOs) to inhibit HIV infection *in vivo*. Increased concentrations of HMOs in breast milk have been associated with decreased HIV transmission during breastfeeding (11). In addition, in comparison to the milks of the other species evaluated here, including RM, the oligosaccharides present in human breast milk are more abundant and diverse (32, 36, 40, 41). Furthermore, HMOs are not affected by Holder pasteurization and are present in the skim fraction of breast milk (38). HMOs could potentially reduce HIV transmission by inhibiting DC-SIGN mediated transfer of HIV from dendritic cells to $CD4^+$ T cells (42). To evaluate the effect of HMOs on oral HIV transmission, we orally exposed BLT mice to HIV in vehicle supplemented with 20 mg/ml HMOs (the average concentration of HMOs present in human colostrum) (36) purified from the breast milk of HIV-negative women (29). Surprisingly, we observed 75% transmission in BLT mice exposed orally to HIV in the presence of HMOs (3/4 mice infected) (Fig. 3D). These results indicate that although increased concentrations of HMOs in breast milk are correlated with decreased breastfeeding HIV transmission, HMOs alone do not inhibit oral HIV transmission. Altogether, these results suggest that that multiple components of breast milk (i.e., lipids, proteins, and/or carbohydrates) likely work in concert to inhibit oral HIV transmission during breastfeeding.

Effect of breast milk on other modes of HIV acquisition. In the future, the HIV-inhibitory factors in breast milk or molecules that mimic their activity could potentially be used to prevent other routes of HIV acquisition. Therefore, we determined if breast milk could inhibit other modes of HIV infection *in vivo* or if the inhibitory effect of breast milk is specific to oral transmission.

Previously, we demonstrated that the female reproductive tract of BLT mice is reconstituted with human target cells for HIV infection ($CD4^+$ T cells, dendritic cells, and macrophages), rendering them susceptible to vaginal HIV transmission (25, 27). In addition, we previously validated BLT mice for the *in vivo* evaluation of topical HIV preventive strategies (25, 26). Therefore, we used BLT mice to determine whether human breast milk can inhibit vaginal transmission of HIV, the most relevant mucosal route of HIV acquisition globally (5).

First, we confirmed the susceptibility of BLT mice to HIV transmission following a single vaginal exposure to HIV in vehicle. Transmission was monitored in plasma as described above. Under these conditions, we observed 100% transmission (5/5 mice infected) (Fig. 4 and see Table S5 in the supplemental material). Importantly, when we exposed BLT mice vaginally to HIV mixed with human breast milk immediately prior to exposure, no mouse became infected (0/4 mice infected), as determined by the absence

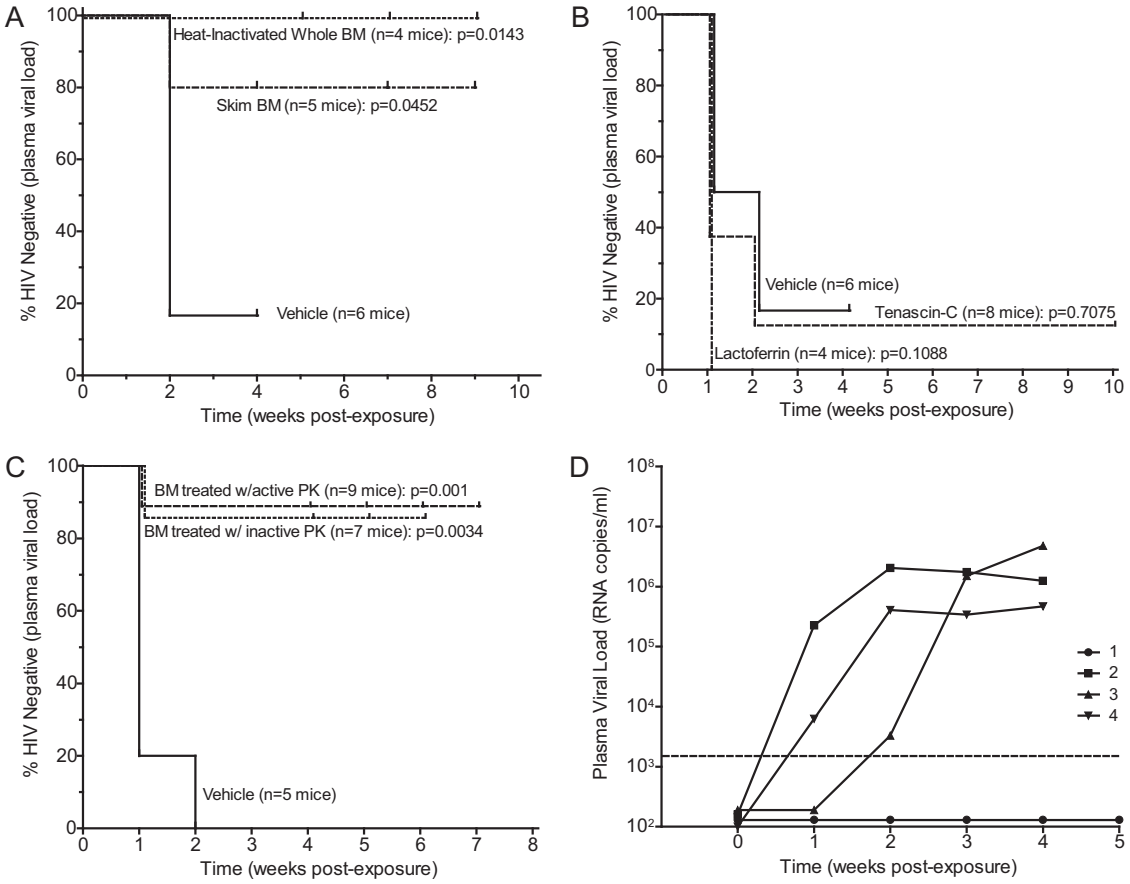

FIG 3 Nature of breast milk's HIV-inhibitory activity. (A) BLT mice were exposed orally to HIV in the presence of heat-inactivated whole breast milk (BM) ($n = 4$), the skim fraction of BM ($n = 5$), or vehicle ($n = 6$, positive control). (B) BLT mice were orally exposed to HIV in vehicle ($n = 6$, positive control) or in vehicle supplemented with tenascin-C (78 to 168 μg/ml)-enriched BM ($n = 8$) or lactoferrin (7 mg/ml; $n = 4$). (C) BLT mice were exposed orally to HIV in vehicle ($n = 5$, positive control) or in the presence of BM treated with active ($n = 9$) or inactive ($n = 7$) proteinase K. HIV infection was monitored longitudinally in plasma with real-time PCR. Kaplan-Meier plots depict the percentage of BLT mice in each exposure group that did not become infected with HIV. HIV infection was defined by the presence of HIV-RNA in plasma. A log-rank Mantel-Cox test (P values are indicated) was used to compare HIV transmission between BLT mice exposed orally to virus in vehicle and in BM. (D) BLT mice ($n = 4$) were exposed orally to vehicle supplemented with human milk oligosaccharides (20 mg/ml). HIV infection was monitored longitudinally in plasma with real-time PCR; the limit of detection is indicated with a dashed line.

of HIV-RNA in the plasma of any mouse at any time point post-exposure (Fig. 4). Lack of HIV transmission was confirmed at necropsy by the absence of HIV-DNA in PB and harvested tissues (see Table S5 in the supplemental material). These results demonstrate that breast milk can prevent vaginal HIV transmission, the most common mode of mucosal HIV acquisition, when virus is mixed with human breast milk ($P = 0.0081$).

Having demonstrated that human breast milk can prevent vaginal infection, we proceeded to determine whether breast milk can inhibit vaginal HIV transmission when it is applied directly into the vagina prior to exposure, mimicking topical application of a microbicide. When we applied breast milk topically to the vagina 15 min prior to vaginal HIV exposure, we observed a significant reduction in transmission ($P = 0.0086$; 2/6 mice infected) (Fig. 4 and see Table S5 in the supplemental material). CAPRISA004, the first successful clinical microbicide trial in humans, demonstrated a significant reduction in vaginal HIV transmission when the microbicide tenofovir was applied vaginally once before and once after sexual intercourse within a 24-h period (43). Previously, we also demonstrated that topical application of a 1% tenofovir gel, administered up to 12 h before and within 12 h after vaginal HIV exposure, dramatically reduced HIV transmission in BLT mice (1/8 mice infected) (15). Therefore, we next assessed the effect of two topical applications of breast milk, administered once before and once after vaginal HIV exposure, on vaginal HIV transmission. When breast milk was applied topically 4 h before and after vaginal HIV exposure, we observed a 50% reduction in vaginal HIV transmission ($P = 0.1332$; 2/4 mice infected) (Fig. 4 and see Table S5 in the supplemental material). Lack of vaginal HIV transmission was confirmed in BLT mice that received breast milk topically at necropsy by the absence of HIV-DNA in PB and tissues (see Table S5 in the supplemental material). Collectively, these results indicate that breast milk can significantly reduce vaginal HIV transmission when virus is mixed with breast milk or when breast milk is applied topically immediately prior to exposure (Fig. 4).

The fact that breast milk is not as effective at preventing vaginal HIV transmission when it is applied topically 4 h before and after exposure suggests that inhibitory factors in breast milk may act directly on the virus to prevent mucosal transmission. Therefore, we next wanted to determine whether breast milk can prevent HIV infection when virus is incubated with breast milk prior to

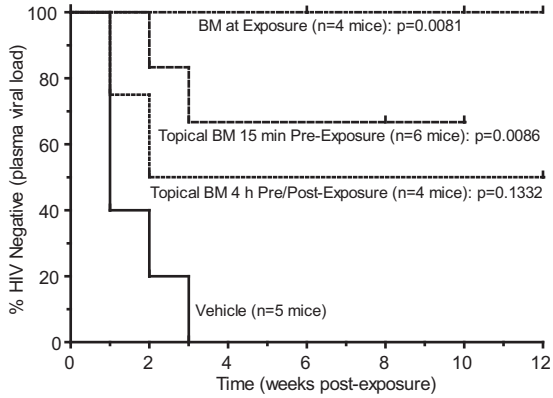

FIG 4 Human breast milk can inhibit vaginal HIV transmission. The inhibitory effect of breast milk on vaginal HIV transmission was examined by exposing groups of BLT mice vaginally to HIV in vehicle ($n = 5$, positive control) or breast milk ($n = 4$). The effect of topically applied breast milk (BM) administered to BLT mice 15 min prior to exposure ($n = 6$) or 4 h pre- and postexposure ($n = 4$) on vaginal HIV transmission is shown. Infection was monitored in plasma by measuring viral load. A Kaplan-Meier plot depicts the percentage of BLT mice in each exposure group that did not become infected with HIV following vaginal HIV exposure in the presence or absence of BM (as determined by the absence of HIV-RNA in plasma). A log-rank Mantel-Cox test (*P* values are indicated) was used to compare HIV transmission between BLT mice that were exposed vaginally to HIV in vehicle (no BM) to those that received HIV vaginally in BM (BM at exposure) or in vehicle following topical vaginal application of BM (BM 15 min preexposure and BM 4 h pre/postexposure).

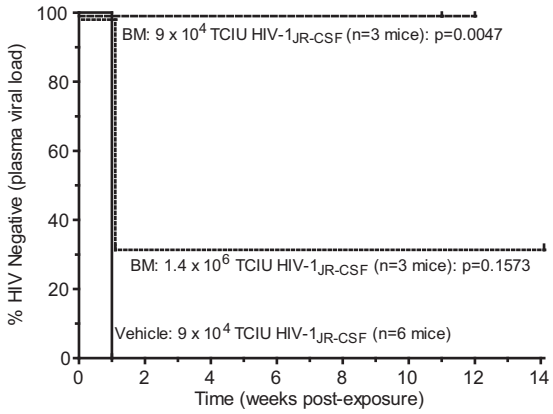

FIG 5 Human breast milk prevents intravenous HIV infection. BLT mice were exposed intravenously to 9×10^4 TCIU HIV mixed with breast milk (BM) ($n = 3$) or vehicle ($n = 6$, positive control) for 10 min prior to exposure. BLT mice ($n = 3$) were also exposed to a high intravenous dose of HIV (1.4×10^6 TCIU) pretreated with BM. A Kaplan-Meier plot depicts the percentage of BLT mice in each group that did not become infected following intravenous exposure to HIV in the presence or absence of BM. HIV infection was defined by the presence of HIV-RNA in plasma. A log-rank Mantel-Cox test (*P* values are indicated) was used to compare HIV transmission between BLT mice exposed intravenous to HIV in whole BM versus vehicle.

intravenous injection (bypassing the mucosa). HIV is most effectively transmitted in humans following an intravenous exposure. For this purpose, we incubated HIV with whole breast milk for 10 min then diluted the virus-breast milk mixture with vehicle (1:10) prior to intravenous injection into BLT mice. BLT mice exposed intravenously to HIV in vehicle served as a positive control for HIV infection. Surprisingly, while we readily detected HIV-RNA in the plasma of all BLT mice exposed intravenously to HIV in vehicle at 1 week postexposure (6/6 mice infected), we did not detect any HIV-RNA in the plasma of any BLT mouse injected intravenously with HIV pretreated with breast milk at any time point postexposure (0/3 mice infected) (Fig. 5 and see Table S6 in the supplemental material). In addition, at necropsy (11 to 12 weeks postexposure), we did not detect any HIV-DNA in the PB or tissues of BLT mice exposed intravenously to HIV pretreated with breast milk (see Table S6 in the supplemental material). However, when we increased the dose of virus pretreated with breast milk 15-fold and intravenously injected BLT mice, we observed increased HIV infection (2/3 mice infected) (Fig. 5 and see Table S6 in the supplemental material). These results suggest that HIV-inhibitory factor(s) in breast milk may act directly on the virus and that this activity can be saturated by increased amounts of virus.

DISCUSSION

Although up to one-half of HIV infections in children can be attributed to breastfeeding, only a small percentage of infants (~15%) breastfed by HIV-positive women become infected with HIV despite daily exposure to virus in breast milk for several months to years (44). Previously, our laboratory and others demonstrated that the low risk of acquiring HIV during breastfeeding (0.00028 per day of breastfeeding) (45) is likely due to the innate HIV-inhibitory activity of breast milk; the breast milk of HIV-negative women, which contains no HIV-specific antibodies or immune cells, can inhibit HIV infection in cell culture and prevent oral HIV transmission *in vivo* (24, 46–48). Here, we demonstrated that breast milk of HIV-positive mothers (transmitters and nontransmitters) significantly inhibits HIV transmission. We also demonstrated that breast milk can prevent multiple routes of HIV acquisition and that this activity is unique to human milk. In addition, our results indicate that breast milk possesses multiple factors that contribute to breast milk inhibition of HIV.

The ability of breast milk obtained from HIV-negative women to inhibit HIV infection *in vitro* and *in vivo* has been established; however, to our knowledge, the HIV-inhibitory activity of breast milk obtained from HIV-positive women had not been evaluated. In addition, although differences in breast milk composition between transmitting and nontransmitting HIV-positive mothers have been correlated with an increased risk for breastfeeding HIV transmission, it is not known if changes in the composition of breast milk alters its *in vivo* HIV-inhibitory activity. This information could be critical for the design of novel approaches to prevent HIV transmission through breastfeeding that do not deny infants in resource-limited settings the health benefits of breast milk. Therefore, we evaluated the ability of breast milk obtained from HIV-positive transmitting and nontransmitting mothers to inhibit oral HIV transmission in BLT mice. Our results revealed that breast milk of HIV-positive mothers can significantly inhibit HIV infection *in vivo*. These results are consistent with the observation that most infants breastfed by HIV-positive women do not acquire HIV during breastfeeding.

Our data also revealed no significant difference in the *in vivo* HIV-inhibitory activity of breast milk obtained from HIV-positive nontransmitting and transmitting mothers. The breast milk samples evaluated in our study were obtained from HIV-positive mothers at 1 month postpartum. It is highly unlikely that our results are due to residual nevirapine in breast milk samples, as the

level of nevirapine in breast milk falls below the limit of detection by 17 days postadministration (49). One explanation for our result is that HIV transmission in the presence of breast milk is influenced by transient changes in breast milk composition. The composition of breast milk, including the concentration of previously characterized anti-HIV factors in breast milk, changes during lactation (50, 51). In addition, while the risk of transmission remains throughout the lactation period, early lactation and weaning are associated with an increased risk of transmission. In the future, it will be important to evaluate the *in vivo* HIV-inhibitory activity of breast milk obtained from HIV-positive mothers at multiple time points postpartum. Another potential explanation for our observation is that cell-associated HIV is transmitted more frequently than cell-free HIV and is less susceptible to breast milk inhibition, especially at earlier stages of lactation. Longitudinal analyses of cell-free and cell-associated viral loads in breast milk of HIV-positive nontransmitting and transmitting mothers indicated that the level of cell-associated HIV in breast milk was predictive of breastfeeding transmission during early lactation (52, 53). Further *in vivo* analysis comparing the ability of breast milk from HIV-positive mothers to inhibit oral transmission of cell-free and cell-associated HIV transmission will be needed to address this issue. Breastfeeding HIV transmission has been correlated with viral, maternal, and infant risk factors (54). Viral characteristics associated with breastfeeding HIV transmission include: replicative fitness, the length of the variable loop, number of N-linked glycosylation sites in Env, and subtype (55–58). In the future, it will be important to determine if breast milk inhibits all virus isolates equally, and, in particular, as they become available, transmitted/founder viruses that were acquired via breastfeeding. It should also be noted that mice do not have tonsils, a potential site of HIV transmission following an oral exposure. The presence of human HIV target cells has been demonstrated in the nasal-associated lymphoid tissue, the functional equivalent of tonsils, in BLT mice (24).

Defining the mechanism by which breast milk inhibits HIV infection *in vivo* will aid the development of novel approaches to prevent HIV transmission. Although several components of breast milk have been shown to inhibit HIV infection *in vitro*, their contribution to breast milk inhibition *in vivo* has not been examined. Collectively, our data indicate that the *in vivo* HIV-inhibitory activity of milk is specific to humans and that multiple factors in breast milk may act to collectively inhibit oral HIV transmission.

We defined the species specificity of milk's *in vivo* HIV-inhibitory activity by evaluating oral HIV transmission in the presence of whole milk obtained from species that are closely related to humans (AGMs and RMs) and from species that are distantly related to humans but are more abundant sources of milk (cows, goats, and camels). Although the composition of AGM milk has not been well studied, the composition of RM milk closely resembles that of human breast milk, in comparison to the milk of the other species tested (cows, goats, and camels). More importantly, RM milk possesses several of the innate factors, and at concentrations similar or slightly reduced to those observed in human breast milk, that have been reported to either inhibit HIV *in vitro* (i.e., LF and lysozyme) or are associated with a decreased risk for HIV transmission (i.e., LCPUFAs) (59–61). Despite these similarities, we observed no significant decrease in HIV transmission when we exposed BLT mice orally to HIV in the presence of RM milk (50% transmission in RM milk versus 66% transmission in vehicle). We also observed no significant decrease in oral HIV transmission when mice were exposed to virus in AGM, cow, goat, or camel milk. Interestingly, while breastfeeding SIV transmission has been documented in RMs, AGMs rarely transmit SIV postnatal (62, 63). Although we did not evaluate the ability of AGM and RM milk to inhibit SIV infection, our data suggest that the lack of postnatal SIV transmission in AGMs is likely not due to differences in milk composition between the two nonhuman primates. Taken together, our results indicate that the *in vivo* HIV-inhibitory activity of milk is specific to humans and that the ability of breast milk to inhibit HIV transmission is not due to a nonspecific property of milk.

To aid the identification of the factor(s) in breast milk that inhibit HIV infection *in vivo*, we performed a series of experiments to better understand the nature of breast milk's *in vivo* HIV-inhibitory activity. Our results demonstrating the potent ability of heat-inactivated breast milk and the skim breast milk to inhibit oral HIV transmission suggested that the inhibitory factor(s) in breast milk may be a heat-stable protein or carbohydrate. To examine the contribution of milk proteins to breast milk's *in vivo* HIV-inhibitory activity, we exposed BLT mice orally to HIV resuspended in proteinase K-treated breast milk. Surprisingly, we observed decreased oral HIV transmission in the presence of proteinase K-treated breast milk, indicating that breast milk proteins are not essential for breast milk inhibition of HIV infection *in vivo*. Our results also demonstrated that the previously characterized anti-HIV breast milk proteins TNC and LF were not able to significantly inhibit HIV infection *in vivo* when evaluated individually at physiologic concentrations. These data indicate that while breast milk factors may display HIV-inhibitory activity *in vitro*, they may not be potent enough at physiological concentrations in breast milk to significantly inhibit HIV infection *in vivo*. We then proceeded to determine whether HMOs isolated from the breast milk of HIV-negative women can inhibit oral HIV transmission. While increased concentrations of HMOs in breast milk are correlated with a decreased risk for HIV transmission during breastfeeding (5), the results of our study indicate that bulk HMOs do not inhibit HIV infection *in vivo*. We observed no significant decrease in HIV transmission when we exposed BLT mice orally to HIV in vehicle containing a high concentration of purified HMOs. Increased concentrations of non-3′-sialylated HMOs have also been associated with a decreased breastfeeding transmission. In the future it may be important to evaluate the efficacy of non-3′-sialylated HMOs alone to inhibit oral HIV transmission. Although we did not evaluate the ability of the lipid fraction to inhibit oral HIV transmission, it is possible that lipids contribute to breast milk's *in vivo* HIV-inhibitory activity, given that this activity was retained after proteolytic digest of milk proteins. The observation that the *in vivo* anti-HIV activity of breast milk is maintained in the skim fraction of milk and after heat-inactivation and proteolytic digest of milk indicates that there is redundancy in breast milk's *in vivo* HIV-inhibitory activity. Altogether, our data suggest that breast milk contains multiple factors (lipids, proteins, and/or carbohydrates) that together inhibit oral HIV transmission and suggest that breast milk can inhibit HIV transmission even if the concentration of one or more anti-HIV factors in breast milk is reduced. In addition, the HIV-inhibitory activity of breast milk of HIV-positive mothers may be further enhanced by the presence of HIV-specific antibodies and immune cells.

HIV is most frequently transmitted vaginally to women who live in resource-limited settings, such as sub-Saharan Africa, and lack access to acceptable and/or affordable prophylaxis, such as condoms and microbicides (64). In the absence of an effective vaccine that provides protection from HIV infection, there is an urgent need to develop and implement novel prevention strategies. Non-ART-based prevention modalities have been evaluated in humans in the past. However, none were efficacious and in some instances they increased transmission (65). Our laboratory and others have shown that human breast milk has a strong inhibitory effect on HIV infection *in vitro* and *in vivo*. Therefore, we hypothesized that breast milk could also prevent other modes of mucosal HIV transmission. Our data reveal that whole human breast milk can significantly inhibit vaginal HIV transmission when breast milk is mixed with virus before exposure and when it is applied topically to the vagina 15 min prior to exposure. Although we did not observe statistically significant protection when breast milk was applied topically 4 h before and after vaginal HIV exposure, HIV transmission was reduced by 50%. In comparison, during CAPRISA004, HIV transmission was reduced by 39% when a 1% tenofovir gel was applied topically up to 12 h before and after vaginal HIV exposure (43). Collectively, our results suggest that the anti-HIV factors in milk or molecules that mimic their activity have the potential for use as a microbicide component. We did observe that the protective effect of breast milk on vaginal HIV transmission decreased when breast milk was applied topically prior to exposure in comparison to vaginal exposures performed with virus resuspended in breast milk. In addition, vaginal transmission was increased as the time between the vaginal exposure and the topical application of breast milk increased. These results suggest that the protective factors in breast milk may act directly on the virus and not the target cell. Indeed, we observed 100% protection when we bypassed the mucosa and exposed BLT mice intravenously to HIV pretreated with breast milk for 10 min. However, the protective effect of breast milk could be saturated by increasing the intravenous HIV inoculum 15-fold.

Overall, the results of our experiments indicate that breast milk contains powerful *in vivo* HIV-inhibitory activity that can prevent multiple routes of HIV acquisition. Our data here suggest that the HIV-inhibitory activity of breast milk may be attributed to multiple factors that act directly on the virus. Regardless of their potential use as topical HIV preventatives, identification of the substances present in milk capable of inhibiting mucosal HIV transmission may aid the development of novel approaches to prevent mother-to-child transmission. Natural approaches to prevent vertical or horizontal HIV transmission might be especially useful in resource-limited settings where they are most needed.

ACKNOWLEDGMENTS

This study was supported in part by National Institutes of Health grant AI073146-05 (J.V.G.), by UNC Center for AIDS Research P30 AI50410 (J.V.G.), by an NIAID institutional training grant (5T32AI007273-27) (A.W.), and by an amfAR Mathilde Krim Fellowship in Basic Biomedical Research (108684-55-RKMT) (A.W.). RM samples were made available via the ARMMS program (Archive of RM Milk Samples) supported by NSF BCS-0921978 (K.H.).

We thank current and previous laboratory members for their technical and intellectual contributions to this work.

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