Tenascin-C is an innate broad-spectrum, HIV-1–neutralizing protein in breast milk


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Achieving an AIDS-free generation will require elimination of postnatal transmission of HIV-1 while maintaining the nutritional and immunologic benefits of breastfeeding for infants in developing regions. Maternal/infant antiretroviral prophylaxis can reduce postnatal HIV-1 transmission, yet toxicities and the development of drug-resistant viral strains may limit the effectiveness of this strategy. Interestingly, in the absence of antiretroviral prophylaxis, greater than 90% of infants exposed to HIV-1 via breastfeeding remain uninfected, despite daily mucosal exposure to the virus for up to 2 y. Moreover, milk of uninfected women inherently neutralizes HIV-1 and prevents virus transmission in animal models, yet the factor(s) responsible for this anti-HIV activity is not well-defined. In this report, we identify a primary HIV-1–neutralizing protein in breast milk, Tenascin-C (TNC). TNC is an extracellular matrix protein important in fetal development and wound healing, yet its antimicrobial properties have not previously been established. Purified TNC captured and neutralized mult-clade chronic and transmitted/founder HIV-1 variants, and depletion of TNC abolished the HIV-1–neutralizing activity of milk. TNC bound the HIV-1 Env envelope protein at a site that is induced upon engagement of its primary receptor, CD4, and is blocked by V3 loop–(198 and F39F) and chemokine coreceptor binding site–directed (178) monoclonal antibodies. Our results demonstrate the ability of an innate mucosal host protein found in milk to neutralize HIV-1 via binding to the chemokine coreceptor site, potentially explaining why the majority of HIV-1–exposed breastfed infants are protected against mucosal HIV-1 transmission.

Prevention of HIV-1 transmission via breastfeeding is central to improving infant HIV-free survival in regions of high HIV-1 prevalence. Antiretroviral prophylaxis administered to the infant and/or mother can significantly reduce postnatal HIV-1 transmission (1–3). However, issues of maternal/infant toxicities, adherence, and the development of antiretroviral drug-resistant viruses limit the effectiveness of this prevention strategy (4, 5). Thus, novel strategies to prevent HIV-1 transmission via breastfeeding are vital to eliminating infant HIV-1 infection. Interestingly, despite chronic, daily exposure to the virus for up to 2 y of life, greater than 90% of HIV-1–exposed, breastfed infants will escape infection (6). This low rate of HIV-1 transmission via this mode of transmission suggests that innate or adaptive immune responses in breast milk may protect the majority of infants against virus acquisition. Establishing the mechanism by which the majority of HIV-1–exposed, breastfed infants are naturally protected against HIV-1 acquisition will inform novel strategies to eliminate infant HIV-1 infection.

Breast milk from uninfected individuals is inherently inhibitory of HIV-1 replication (7–9). Moreover, it was recently established that milk of uninfected women abrogates oral HIV-1 transmission in humanized mice (10). Several antiviral glycoproteins contained in milk have been reported to inhibit HIV-1 replication, including lactoferrin (11, 12), mucin-1 (13), and secretory leukocyte protease inhibitor (14, 15). In addition, a recent study reported an association between the prevalence of certain oligosaccharides in milk and the risk of infant HIV-1 acquisition (16), potentially explained by the ability of oligosaccharides to prevent HIV-1 virion interaction with dendritic cells (17, 18). However, a previous report of the HIV-1–inhibitory properties of mucosal fluids noted that the majority of the HIV-neutralizing activity of milk was solely contained in the high molecular mass protein fraction (>500 kDa), which would not be accounted for by previously identified HIV-1–neutralizing factors in breast milk (7). Thus, the primary, high molecular mass HIV-1–neutralizing factor in breast milk remains to be identified, and identification of this factor may inform immunologic strategies to prevent postnatal HIV-1 transmission.

Results

Identification of a High Molecular Mass Innate HIV-1–Neutralizing Protein in Breast Milk. To identify the high molecular mass HIV-1–neutralizing protein in milk, we screened mature milk samples from uninfected women (collected between 2 wk and 7 mo postpartum) for neutralizing activity against a panel of multiclade chronic and transmitted/founder (T/F) pseudoviruses or infectious molecular clones in an HIV-1 neutralization assay in TZM-bl reporter cells (19, 20). The 50% inhibitory dilution (ID50) of milk samples against this panel of HIV-1 strains ranged from 3 to 42 (Fig. S1). We next fractionated a more potently neutralizing milk sample (milk 10; Fig. S1) by size-exclusion chromatography. Consistent with a previous report (7), all of the detectable HIV-1 neutralization activity was contained in the high molecular mass (>500 kDa) fraction [50% inhibitory concentration (IC50)], against the chronic, neutralization tier 2 HIV-1 protein.

Significance

Achieving an AIDS-free generation will require elimination of breast milk transmission of HIV-1, as breastfeeding is a cornerstone of infant survival in developing regions. Antiretroviral prophylaxis considerably reduces postnatal HIV-1 transmission, yet its efficacy is limited by access, adherence, toxicities, and resistance of maternal HIV-1 strains. Alternative, safe strategies of impeding postnatal HIV-1 transmission will be required to eliminate infant HIV-1 infection. In this paper, we identify an innate HIV-neutralizing protein in breast milk, Tenascin-C, which captures and neutralizes HIV-1 virions via binding to the chemokine coreceptor binding site on the HIV-1 envelope. This protein has the potential to be developed as a prevention strategy for postnatal and other modes of HIV-1 transmission.
variant C.Du156, 407 μg/mL (Fig. S2). We further fractionated the active milk fraction by ion-exchange chromatography, narrowing the detectable HIV-1 neutralization activity to a single protein fraction (peak 3, IC₅₀ against C.Du156, 382 μg/mL; Fig. L4). Reduction SDS-PAGE of this fraction revealed a single unique 250-kDa band that was not visualized in the nonneutralizing fractions (Fig. L4). Ultra-performance liquid chromatography-tandem mass spectroscopy (LC/MS/MS) of this protein band and comparison of the results with a human protein database revealed 76 unique peptides with >90% likelihood match to the extracellular matrix protein Tenascin-C (TNC). The identity of the protein as TNC was confirmed by Western blot analysis using an anti-TNC mAb (Fig. 1B).

HIV-1 Neutralizing Activity of TNC. TNC plays a role in fetal brain and mammary gland development, as well as wound healing (21, 22), but no antimicrobial property of this hexameric extracellular matrix protein has previously been described. Depletion of TNC from mature milk samples of two uninfected women using an anti-TNC monoclonal IgG (81C6) (23) resulted in severe reduction of the HIV-1 neutralization activity of the breast milk samples to background levels of the assay (Fig. 1B) (19). Moreover, both TNC purified from a glioma cell line (Millipore) and recombinant TNC (produced in BHK cells) demonstrated dose-dependent HIV-neutralizing activity. Importantly, TNC also neutralized HIV-1 in primary human peripheral blood mononuclear cells (PBMCs) (Fig. 2). To rule out TNC-induced cell toxicity accounting for the neutralizing activity, TNC was incubated with TZM-bl cells in the absence of virus, and luciferase expression was measured after 48 h of incubation. There was no detectable virus neutralization when TZM-bl target cells were preincubated with TNC before virus inoculation. Purified TNC demonstrated broad-spectrum HIV-1 neutralization activity against multiclade chronic and T/F HIV-1 Envelope (Env) variants isolated from both adults and postnatally infected infants in comparison of the results with a human protein database revealed the neutralizing activity. In fact, TNC produced in various cell types appears to have distinct N-link glycosylation patterns, based on PNGase treatment and Western blot analysis of the resulting deglycosylated forms (Fig. S3). Finally, quantitation of TNC based on relative abundance measured by unbiased mass spectrometry of 10 mature human milk samples revealed a milk TNC concentration range of 2.2–671 μg/mL, spanning the measured IC₅₀ of TNC against HIV-1 variants (Fig. S1).

TNC Captures HIV-1 Virions, Blocks Virus–Epithelial Cell Binding, and Binds to the HIV-1 Env Protein in a Charge-Dependent Manner. As TNC is a large, multimeric protein, the predicted mechanism of its HIV-1–neutralizing activity is inhibition of virus entry. In fact, purified TNC was able to capture virions with chronic and T/F HIV-1 Env expressed, including those transmitted via breast-feeding, at a significantly higher potency than lactoferrin and albumin, yet at a similar potency to that of a broadly HIV-1–neutralizing monoclonal antibody that is protective against infant HIV-1 acquisition (2G12) (26) (Fig. 3A). TNC is a large, multimeric protein, yet at a similar potency to that of a broadly HIV-1–neutralizing monoclonal antibody that is protective against infant HIV-1 acquisition (2G12) (26). Moreover, no cell toxicity was observed in the TNC wells by microscopic examination. The HIV-1–neutralizing activity of TNC is directed against the virus and not the target cells, as there was no detectable virus neutralization when TZM-bl target cells were preincubated with TNC before virus inoculation. Purified TNC demonstrated broad-spectrum HIV-1 neutralization activity against multiclade chronic and T/F HIV-1 Envelope (Env) variants isolated from both adults and postnatally infected infants in the TZM-bl reporter cell assay (IC₅₀ range, 82–158 μg/mL; Fig. S1) in a dose-dependent manner (Fig. 2A). TNC also neutralized the T/F HIV-1 CH40 variant in PBMCs (IC₅₀ of CH40, 27 μg/mL; IC₅₀ of CH40, 71 μg/mL) more potent than the activity measured in the TZM-bl assay (Fig. 2B). However, the opposite was true with T/F HIV-1 variant CH77 (Fig. 2C), indicating that the neutralizing potency of TNC in PBMCs may be dependent on virus-specific factors. The neutralizing potency of TNC against HIV-1 in TNC-bl reporter cells was higher than that of other breast milk proteins previously shown to neutralize HIV-1, including lactoferrin (11, 24) and mucin-1 (13) (IC₅₀ >300 μg/mL; Fig. S1). TNC neutralized both CCR5 (such as the T/F strains) and CXCR4 (B.MN) co-receptor-tropic HIV-1 variants (Fig. S1). Consistent with the broad activity of innate antimicrobial proteins, TNC also displayed neutralizing activity against the mouse retrovirus murine leukemia virus (MLV; IC₅₀ 109 μg/mL), yet no activity was detected against the simian immunodeficiency virus strain mac251 (>180 μg/mL; Fig. S1). Interestingly, recombinant TNC produced in BHK cells (25), but not CHO or HEK293T cells, recapitulated the HIV-1–neutralizing activity of purified TNC in the TZM-bl neutralization assay, indicating that cell type-specific posttranslational modifications may be important for the neutralizing activity. In fact, TNC produced in various cell types appears to have distinct N-link glycosylation patterns, based on PNGase treatment and Western blot analysis of the resulting deglycosylated forms (Fig. S3). Finally, quantitation of TNC based on relative abundance measured by unbiased mass spectrometry of 10 mature human milk samples revealed a milk TNC concentration range of 2.2–671 μg/mL, spanning the measured IC₅₀ of TNC against HIV-1 variants (Fig. S1).
(clade B), ConS gp140 (group M consensus), and the T/F HIV-1 variants Env C.1086 gp120/140 (clade C) and C.CH505 gp140 trimer (28) (Fig. S4). The dissociation constant ($K_d$) of HIV-1 Env B.MN gp120 binding to TNC-S ($K_d$ 54.8 nM) was equal to that of TNC-L ($K_d$ 58.2 nM) (Fig. S5).

**TNC Binds to a CD4-Inducible Epitope on the V3 Loop of the HIV-1 Env Protein in a Region Overlapping the Chemokine Coreceptor Binding Site.** To map the neutralizing epitope of the HIV-1 Env that is bound by TNC, we first compared the kinetics of TNC binding to HIV-1 Env gp120 and gp140 proteins. TNC-S and TNC-L binding to T/F HIV-1 C.1086 and CH505 gp140 proteins demonstrated a slower off-rate (binding to TNC-S, $K_d$ 1.55 × 10$^{-3}$ and 1.56 × 10$^{-3}$ s$^{-1}$, respectively) than that of their matched gp120 (binding to TNC-S, $K_d$ 9.1 × 10$^{-3}$ and 4.5 × 10$^{-3}$ s$^{-1}$, respectively) (Fig. S4). Thus, the gp120 epitope bound by TNC is partially dependent on the conformation of the gp41–120 complex. We then incubated HIV-1 B.MN gp120 with a panel of mAbs directed against defined HIV-1 Env epitopes and determined their ability to block TNC–Env interaction (Fig. 4A), including anti-gp120 conformational C1 (A32, 16H3) (29, 30), anti-V3 loop (1Bb, F39E) (29), anti-V2 linear (CH50) (31), anti-V2 conformational (697D) (32), anti-CD4 binding site (CH31), and a negative-control anti-RSV mAb (Synagis). TNC binding to HIV-1 Env gp120 was potently blocked by anti–HIV-1 Env mAbs 1Bb and F39E (84.3% and 87.7%, respectively), both directed against the V3 loop of the Env protein (Fig. 4B). Interestingly, TNC–Env binding was enhanced by Env preincubation with mAb A32 (Fig. 4A), an anti-C1 mAb that induces a conformational change that opens the coreceptor binding site (33) similar to that induced by CD4 receptor engagement. We therefore tested the hypothesis that TNC bound to an epitope of the HIV-1 Env protein whose accessibility is enhanced by CD4 receptor binding. In fact, preincubation of some HIV-1 Env gp120 and gp140 proteins with soluble CD4, including B.MN gp120 and the T/F HIV-1 variant C.1086 gp120 and gp140 proteins, enhanced TNC binding (Fig. 4B), and this increased signal was not due to soluble CD4 binding to the TNC chip (Fig. S6). Moreover, the CD4-inducible (CD4i) mAb 17B, which has a binding site that overlaps that of the chemokine coreceptor (34, 35), blocked TNC binding to the CD4-captured gp120 protein (Fig. 4C). Finally, binding of TNC to CD4-bound gp120 was substantially reduced by increasing the NaCl concentration from 137 to 250 mM (Fig. 4D), indicating that TNC–Env binding is dominated by electrostatic interactions. The strong influence of charge on TNC binding to Env are consistent with the reported electrostatic complementarity of the gp120 V3 loop and chemokine receptors (36–38).

Thus, TNC neutralizes HIV-1 via interaction with the chemokine coreceptor binding site on the HIV-1 Env.

Finally, we investigated the antiviral function of TNC directed against HIV-1 virions in the pre–CD4-bound conformation compared with the post–CD4-bound open conformation. In fact, the ability of TNC to capture HIV-1 B.MN virions was increased ~1.5-fold when the virions were preincubated with soluble CD4 (Fig. S7A). However, no increased neutralizing activity was observed when HIV-1 virions were incubated with TZM-bl cells for 10 min on ice before addition of TNC, allowing CD4 interaction but preventing cell–virus fusion, compared with the incubation of TNC and virions before the addition of TZM-bl cells (Fig. S7B). Similarly, we did not detect any increased neutralizing activity in the presence of the nonneutralizing, anti-C1 mAb A32, which induces CD4i conformational change (Fig. S7C). Therefore, although TNC has a higher affinity for CD4-bound gp120 than gp120 alone, it is able to mediate its neutralizing activity against HIV-1 virions without prior gp120 engagement of CD4. As low-level weakly or nonneutralizing HIV Env-specific antibodies are present in breast milk (8) of HIV-infected women, we investigated whether TNC antagonizes the effect of an HIV-neutralizing IgG mAb isolated from colostrum and directed against the same CD4i region of gp120 (39). In fact, TNC did not antagonize the neutralizing effect of CH50 against a tier 1 clade-matched HIV-1 strain (MW965) across a range of mAb concentrations (Fig. S7D), despite being directed against the same region of gp120. Therefore, TNC likely acts in concert with HIV-neutralizing antibodies also present in breast milk.

**Discussion**

Despite substantial mucosal virus exposure of nursing infants born to HIV-infected mothers, only a small minority (<10%) acquire HIV-1 via this route. Defining the immune mechanisms responsible for the protection of the overwhelming majority of HIV-1-exposed, breastfed infants may guide strategies to eliminate perinatal and other modes of mucosal HIV-1 transmission. We have identified an innate breast milk protein that neutralizes chronic and T/F HIV-1 variants at its in vivo concentration, TNC. TNC is an extracellular matrix protein known to be important in fetal development and wound healing, but antimicrobial activity has not previously been described for this protein. The presence of...
Fig. 4. TNC binds to HIV-1 gp120 at a CD4-inducible epitope that overlaps the chemokine coreceptor binding site via a charge–charge interaction. (A) Interaction of TNC and B.MN gp120 is potently blocked by preincubation of the Env protein with anti-V3 loop mAbs 19B and F393F. Percent blocking is in parentheses. (B) Binding to both the C clade T/F HIV-1 C.1086 Env gp120 and gp140 proteins is enhanced by preincubation of Env proteins with soluble CD4. (C) TNC binding to B.MN gp120 captured by soluble CD4 is abolished by prebinding of mAb 17B, an mAb directed against the chemokine coreceptor binding site. Approximately 1,200 response units (RU) of 17B mAb bound to B.MN gp120 are captured on the immobilized CD4 surface. (D) Binding of CD4-captured TNC to MN gp120 (100 µg/mL) is abrogated in 250 mM NaCl buffer. All data are representative of at least two experiments. In Fig. S2, the percent blocking was determined by SPR. Percent blocking is in parentheses. (E) The electrostatic interaction of TNC with gp120 may be similar to that of previously described V3 loop-derived peptides (44), cell-associated heparan sulfate (48), polyanions including dextran sulfate (49), heparan sulfate (48), polyanions including dextran sulfate (49), and tyrosine-sulfated antibodies (41). However, whether TNC interacts with the same conserved sulfotyrosine-binding pocket remains to be determined. Because charge plays a dominant role in TNC binding to Env, its inhibitory effect may be expected to be enhanced against viruses harboring gp120 with increased net charge, as in the case of CXCR4-tropic viruses and CCR5-tropic variants with enhanced net charge (50, 51). However, the stronger binding of BMN gp120 to TNC by SPR compared with other HIV-1 Env variants did not predict the potency of TNC neutralization of this variant, as the CXCR4-tropic MN variant was less potently neutralized than the other HIV-1 variants tested (Fig. S1). Similarly, binding affinity to HIV-1 Env does not always predict the HIV-1 neutralization potency of anti–HIV-1 Env mAbs (28). Because both net charge and V3 loop flexibility can be important for coreceptor binding (50, 52), the flexibility of the V3 loop on monomeric gp120 in the CD4-bound state may not reflect the conformational states of the trimeric spike of the Env (53).

As both the short and long forms of TNC bound to HIV-1 Env, the Env binding site on TNC is likely outside the splice region within the FN-III domain of TNC. Outside this splice region, there are 8 FN-III domains that demonstrate distinct binding to cellular receptors, 14 epidermal-like growth factor domains, and a terminal fibrinogen knob (22, 25), which has been implicated to play a role in regulating the tissue damage response via signaling through TLR-4 (54). It is interesting that human TNC isolated from two different sources, breast milk and a glomia cell line, and recombinant TNC produced in BHK cells neutralized HIV-1, but not all recombinant forms of the protein were able to neutralize the virus. Thus, the ability of TNC to bind to the HIV-1 Env in a charge-dependent manner may be affected by chemical differences introduced posttranslationally, such as the distinct glycosylation we detected in these various TNC products.

In our studies, TNC mediated the majority of the HIV-1–neutralizing activity in milk of an uninfected individual, consistent with a previous report that isolated the HIV-1–neutralizing activity of breast milk to the high molecular mass fraction (7). TNC is likely acting in concert with other anti-HIV factors in breast milk, such as lactoferrin, as its neutralizing potency is consistent across distinct HIV-1 variants, unlike that of whole breast milk (Fig. S1). Given its broad-spectrum HIV-1–binding and –neutralizing activity, this innate, mucosal HIV-1 inhibitor could theoretically be developed as an HIV-1 prophylactic agent that could be orally administered to infants before breastfeeding, similar to oral rehydration salts that are
rutinely administered to infants in developing regions. As an existing component of breast milk, TNC has a unique safety advantage for clinical development as a mucosal prophylaxis agent. Moreover, use of this protein as an oral infant HIV-1 prophylactic agent would not introduce a new antigen to the infant gastrointestinal tract, which has been hypothesized to explain the increased rate of postnatal HIV-1 transmission in the setting of mixed infant feeding (55). Thus, TNC holds promise for development as a safe, HIV-1-neutralizing host protein that can be used for reducing mucosal HIV-1 transmission.

Materials and Methods

Size-Exclusion and Strong Anion-Exchange Chromatography. Milk samples from HIV-1-uninfected women between 2 and 7 mo after delivery were delipidized by centrifugation at 21,000 × g, filtered, and concentrated 10× before loading on a protein liquid chromatography size-exclusion column (Superdex 200 10/300GL; GE Healthcare). Proteins were further fractionated using a 1 M NaCl elution gradient (Source 150 4.6/100 PE; GE Healthcare).

TNC Depletion and HIV-1 Neutralization. Ten milligrams of anti-TNC IgG1 mAb 81C6 (SB) was coupled to 600 μg cyanogen bromide-activated Sepharose beads (GE Healthcare), incubated with delipidized/filtered milk overnight, and then centrifuged to remove the protein-bound beads. For virus neutralization assays, purified protein and milk samples were incubated with 293T cell-produced HIV-1 infectious molecular clones (CH40, CH77, CH58, CM235), or HIV-1 Env pseudoviruses (all other variants) were tested for neutralization potency in TZM-bl target cells (19, 20). Additionally, an HIV-1 infectious molecular clone (NL-Luc.TZA.CH040.excto) (59) produced in human PBMCs was incubated with purified TNC and tested for neutralization in activated human PBMCs (60, 61). For determining the interaction between TNC and breast milk HIV-neutralizing antibodies, HIV MWN965 virions were incubated for 1 h with either the colostrum HIV-neutralizing mAb CH08 (39), TNC (final concentration 175 μg/mL), or CH08 and TNC, and then TZM-bl cells were added. For determining whether Env–CD4 engagement enhanced the neutralization activity of TNC, TZM-bl cells were incubated with HIV virions (HIV DU156.12) on ice for 10 min, and then TNC was added (final concentration 350 μg/mL). Neutralization was measured after 48 h as a reduction in RLUs compared with the virus-only control. To rule out the possibility of endotoxin-mediated neutralization in the PBMC assay, endotoxin was measured in the purified TNC lots and the amount of endotoxin detected (≤0.3 ng/mL; Limulus Amebocyte Lysate Pyrogen Plus Kit; Lonza) was found to be 10-fold lower than that which mediates 80% HIV-1 neutralization in the PBMC neutralization assay (62).

HIV-1 Env Binding. Recombinant and purified TNC proteins (25) were immobilized by amine coupling to SPR sensor chip CM5 (65) and washed overnight in pH 7.4 buffer. HIV-1 Env proteins (66) were injected at 100 μg/mL over immobilized surfaces and binding was measured with a BIACore 3000 (GE Healthcare). Non-specific binding was subtracted over a surface immobilized with anti-RSV IgG (Synagis). Binding activity of immobilized TNC was confirmed by injecting anti-TNC mAb (clone 2D2H5; Abcam). MN gp120 was incubated with saturating concentrations of anti-gp120 mAbs for 1 h and injected over a TNC-immobilized surface. Percent blocking was calculated as follows: (response with gp120 in buffer – response with gp120 in buffer)/response with gp120 in buffer) * 100. Mabs included: a negative control anti-RSV (Synagis), anti-gp120 conformational C1 (A32, 16H3), anti-V3 loop (19b, F39F, 17B), anti-v2 linear (CH58), anti-v2 conformational (69TD), and anti-CD4 binding site (CH31). For CD4 induction of HIV-1 Env binding gp120 or gp140 proteins were preincubated with soluble CD4 (sCD4) at a 1:1 molar ratio and binding to TNC was measured as above. The effect of sCD4 on binding was determined for gp120 bound to TNC along with sCD4. The effect of sCD4 on binding was determined for gp120 bound to TNC along with sCD4.

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