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(54) Title: HIV-1 NEUTRALIZING FACTOR

(57) Abstract: The present invention relates, in general, to HIV- 1 and, in particular, to a method inhibiting HIV- 1 transmission (e.g., postnatal transmission) to a mammalian subject (e.g., a human infant) and to compounds and compositions suitable for use in such a method.

HIV-1 NEUTRALIZING FACTOR

[1] This application claims the benefit of U.S. Serial No.: 61/752,872 filed January 15, 2013 and U.S. Serial No. No.: 61/893,426 filed October 21, 2013, the entire contents of each application are herein incorporated by reference.

[2] This invention was made with government support under Grant Nos. K08AI087992, U19AI067854, and 5R21AI106494 - 02 awarded by the National Institutes of Health. The government has certain rights in the invention.

TECHNICAL FIELD

[3] The present invention relates, in general, to HIV-1 and, in particular, to a method inhibiting HIV-1 transmission (e.g., postnatal transmission) to a mammalian subject (e.g., a human infant) and to compounds and compositions suitable for use in such a method.

BACKGROUND

[4] Prevention of HIV-1 transmission via breastfeeding is central to improving infant HIV-free survival in developing regions with high HIV-1 prevalence. Fortunately, it has been established that antiretroviral prophylaxis administered throughout the period of breastfeeding to the infant and/or mother can significantly reduce postnatal HIV-1 transmission. However, issues of antiretroviral resistance, infant/maternal toxicities, and adherence limit the effectiveness of this prevention strategy. Thus, novel strategies to prevent HIV-1 transmission via breastfeeding are vital to eliminating infant HIV-1 infection.

[5] Interestingly, despite chronic, daily exposure to HIV-1, approximately 90 percent of breastfeeding infants born to HIV-1-infected mothers will escape infection. The inherent low rate of infant HIV-1 transmission via breastfeeding suggests that innate or adaptive immune responses in breast milk may protect the great majority of infants against virus acquisition. Breast milk, even from uninfected individuals, is inherently inhibitory of HIV-1 replication (Fouda et al, J. Virol. 85(18):9555 (2011), Kazmi et al, Clin. Vaccine Immunol. 13:1111 (2006)). Moreover, it was recently established that breast milk of uninfected women abrogates oral HIV-1 transmission in humanized mice (Wahl et al, PLoS Pathog. 8:e1002732 (2012)). There are several antiviral glycoproteins contained in breast milk previously reported to have anti-HIV-1

properties, including lactoferrin (Harmsen et al, J. Infect. Dis. 172:380 (1995), Moriuchi and Moriuchi, J. Immunol. 166:4231 (2001)) and secretory leukocyte protease inhibitor (SLPI) (Farquhar et al, J. Infect. Dis. 186:1173 (2002), Hocini et al, Clin. Diagn. Lab Immunol. 7:515 (2000), McNeely et al, J. Clin. Invest. 96:456 (1995)). Moreover, a recent study reported an association between the prevalence of certain oligosaccharides in milk and the risk of infant HIV acquisition (Bode et al, Am. J. Clin. Nutr. 96:831 (2012)). However, a previous report of the HIV-1-inhibitory properties of mucosal fluids noted that the majority of the anti-HIV-1 activity of breast milk was solely contained in the high molecular weight protein fraction of breast milk (Kazmi et al, Clin. Vaccine Immunol. 13:1111 (2006)). The present invention results, at least in part, from studies designed to establish the mechanism by which the majority of HIV-1-exposed, breastfed infants are naturally protected against HIV-1 acquisition. These studies have resulted in the identification of Tenasin-C (TNC) as an HIV-1 Env binding protein in breast milk that neutralizes HIV-1 at its *in vivo* concentration. The invention provides, at least in part, a prophylactic agent, safe for infant consumption, and a method of using same to reduce the risk of HIV-1 acquisition.

SUMMARY OF THE INVENTION

[6] In general, the present invention relates to HIV-1. More specifically, the invention relates to a methods inhibiting HIV-1 transmission (e.g. postnatal transmission) to a mammalian subject (e.g., a human infant). The invention further relates to compounds and compositions suitable for use in such methods. The invention provides an HIV-neutralizing protein in breast milk, TNC, isolated and recombinant TNC and fragments thereof. The presence of TNC in breast milk may help to explain why the majority of breastfed infant remain uninfected. We have mapped TNC's HIV Env epitope specificity, and defined its antiviral function. Compositions comprising TNC or fragments thereof can be used a prophylactic agent that could be safely employed to prevent HIV-1 transmission.

[7] In certain aspects the invention provides compositions comprising recombinant or substantially purified TNC for use as a prophylactic to prevent or treat HIV-1 infection. In certain embodiments the compositions comprise fragments of TNC, for example but not limited to TNC-S, fragments comprising or consisting essentially of FNIII 1-8 domains, or any combination thereof. In certain embodiments, the compositions comprise fragments of TNC, for

example but not limited to fragments comprising or consisting essentially of FNIII 1-8 domains, and fbg. In certain embodiments the fragments are multimerized. In other aspects the invention provides prophylactic methods of preventing HIV-1 infection using the compositions comprising TNC or fragments thereof as described herein.

[8] Objects and advantages of the present invention will be clear from the description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

[9] **Figures 1A-1D**. TNC is a major HIV-1-neutralizing protein in breast milk which captures HIV-1 virions and interacts with a CD4-inducible epitope of the HIV-1 Env. Fig. 1A) Anion exchange chromatography of the high molecular weight fraction of breast milk of uninfected women using a gradient of 1M NaCl (green line) revealed three distinct protein peaks (blue lines). The IC_{50} (μg/ml) of each protein peak against a neutralization tier 2 chronic HIV-1 variant, Du156, is reported in Table 1. The unique band on reduced Coomassie gel in the neutralizing protein fraction (peak three) was identified as TNC by LC/MS. Mass spectrometry analysis of the smaller protein bands in peak 3 confirmed that these proteins matched the identity of those contained in the inactive fractions (heavy chain of immunoglobulin and lactoferrin). Fig. 1B) Breast milk of three uninfected women was depleted of TNC by incubation with agarose beads covalently linked to anti-TNC IgG1 mAb (81C6) and control polyclonal human IgG1. The depletion was specific to TNC, confirmed by anti-TNC western blot and Coomassie gel. Depletion of TNC severely reduced neutralization activity against HIV-1 Du156 compared to nondepleted and control IgG-treated milk samples below the 50% neutralization cut off in the TZM-bl neutralization assay. Line graphs represent the mean percent virus neutralization and standard deviation of two assays performed in duplicate. Fig. 1C) Purified TNC captures chronic clade B (BAL), chronic clade C (Du156), clade B transmitted/founder (T/F) (CH40) and clade C infant transmitted/founder (BF1677) HIV-1 virions with similar potency to that of the protective anti-HIV-1 Env mAb 2G12. Asterix indicates significantly lower ($p < 0.05$) in virion capture compared to TNC, using a Mann-Whitney U test (Prism 5 software) Fig. 1D) Recombinant TNC-L binds with higher affinity to a chronic clade B gp120 Env protein (MN gp120) than a consensus gp140 Env protein (ConS gp140) or a transmitted/founder CH0505 gp140 Env trimer. The interaction of TNC and MN gp120 is most potently blocked by preincubation with mAbs

directed against the V3 loop of the HIV Env protein (19B and F393F). TNC binding to the T/F HIV-1 1086C Env gp120 and gp140 protein is enhanced by preincubation with soluble CD4.

[10] **Figure 2.** The structure and functions of a novel HIV-1 inhibitor isolated from breast milk, the extracellular matrix protein TNC. (See also Fig. 1 of Aukhil et al, J. Biol. Sci. 268(4):2542-2553 (1993)).

[11] **Figure 3.** TNC-L neutralizes chronic HIV-1 variant Du156 in a dose-dependent fashion. HIV-1 Du156 replication in TZM-b1 cells is reduced in the presence of increasing concentrations of purified TNC.

[12] **Figure 4.** TNC is a primary HIV-1-neutralizing protein in breast milk. A) Anion exchange chromatography with a linear gradient of 1M NaCl (green line) of the high molecular weight fraction of milk revealed three distinct protein peaks (blue lines). The IC_{50} of protein peaks against HIV-1 *env* variant C.Du156 is reported. The unique 250kDa band on reducing SDS-PAGE gel in the neutralizing protein fraction (peak three, black arrow) was identified as TNC by high resolution liquid chromatography-tandem mass spectometry (LC/MS/MS). Analysis of the smaller protein bands in peak 3 (blue arrows) confirmed matching identity with those contained in inactive fractions (lactoferrin and heavy chain of immunoglobulin). B) Milk of two uninfected women was depleted of TNC by mAb 81C6 and control polyclonal human IgG immunoprecipitation, confirmed by western blot and SDS-PAGE gel. Depletion of TNC reduced neutralization activity of the milk samples against HIV-1 C.Du156 Env pseudovirus compared to control IgG-treated milk samples. Line graphs indicate mean percent virus neutralization and standard deviation of two assays performed in duplicate.

[13] **Figure 5.** TNC neutralizes HIV-1 in a dose-dependent manner in both TZM-bl reporter cells and primary PBMCs. A) Percent neutralization of HIV-1 pseudovirus Du156 in TZM-bl reporter cells with increasing concentrations of TNC. B and C) Percent neutralization of Clade B T/F HIV-1 variant CH040 (B) and CH77 (C) Renilla luciferase (LucR)-repoter HIV-1 infectious molecular clones in TZM-bls (squares) and primary PBMCs (circles) with increasing concentration of TNC. Assays were performed in duplicate.

[14] **Figure 6.** TNC captures HIV-1 virions and blocks HIV-1 interaction with colonic epithelial cells. A) Purified TNC captures HIV-1 virions expressing Env from a chronic clade B (B.BAL), chronic clade C (C.Du156), clade B transmitted/founder (T/F, B.CH40) and clade C infant T/F (C.BF1677) strains with similar potency to anti-HIV-1 Env mAb 2G12 (positive

control). Bars indicate the mean percent virus capture with lines indicating the standard deviation. Asterix indicates significantly lower ($p < 0.05$) virion capture compared to TNC, using a two-tailed Mann-Whitney U test (Prism 5 software) to compare the results of two assays performed in triplicate. B) Purified TNC inhibits infectious T/F C.1086 HIV-1 virus binding to a monolayer of HT29 colonic epithelial cells in a dose-dependent manner, detected by addition of TZM-bl reporter cells following TNC-virion incubation/washing of monolayer and assessment of the reduction in relative light units compared to virus-only control. The broadly-neutralizing antibody VRC01 was used as a positive control, and the anti-RSV mAb, Synagis, used as a negative control. Results are displayed as mean percent virus inhibition of virion epithelial cell binding compared to virus only wells of two experiments performed in quadruplicate, with lines indicating the standard deviation.

[15] **Figure 7.** 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. The Env and mAbs were incubated at 1:2 molar ratio (Env:mAb) and then flowed over TNC-S immobilized surface. Percent blocking is in parentheses. B) Binding to both the C clade T/F HIV-1 C.1086 Env gp120 and gp140 protein 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, a mAb directed against the chemokine coreceptor binding site. Approximately 1200 response units (RU) of 17B mAb bound to B.MN gp120 captured on immobilized CD4 surface. D) Binding of CD4-captured TNC to MN gp120 (100μg/mL) is abrogated in 250 mM NaCl buffer. All data is representative of at least two experiments.

[16] **Figure 8.** Broad-spectrum HIV-1-neutralizing activity of breast milk of uninfected women and purified milk proteins. Neutralization activity of breast milk of uninfected women and breast milk proteins including TNC, lactoferrin, and mucin-1 in the TZM-bl reporter cell assay, reported as inhibitory dilution 50% (ID_{50}) or inhibitory concentration 50% (IC_{50}), respectively.

[17] **Figure 9.** Size-exclusion fractionation of breast milk reveals that HIV-1–neutralizing activity is solely contained in the high molecular mass fraction. Breast milk fractions (peaks 1–4) were tested for neutralization against the chronic clade C HIV-1 variant C.Du156 (tier 2 neutralization sensitivity) in the TZM-bl neutralization assay. Only the highest molecular mass

fraction (peak 1, >500 kDa) had detectable neutralizing activity, with an inhibitory concentration 50% (IC50) of 407 μg/mL. The IC50 of each size-fractionated protein peak is listed in the table.

[18] **Figure 10.** Distinct N-linked glycosylation pattern of Tenascin-C (TNC) produced in different cell lines. TNC purified from breast milk, purified from a glioma cell line (Millipore), and recombinantly produced by HEK293T cells have distinct banding patterns on an anti-TNC Western blot after deglycosylation with 100 U/μL PGNase (Right).

[19] **Figure 11.** Spliced short (TNC-S, Fig. 11A) and long (TNC-L, Fig. 11B) isoforms of TNC bind to HIV-1 Envelope (Env) gp120 and gp140 proteins. Recombinant TNC-L and TNC-S were covalently coupled to the surface plasmon resonance (SPR) chip, and HIV-1 Env gp120 and gp140 were flowed over the chip. TNC isoforms bound to both clade B and C and consensus Env gp120 and gp140 proteins, including the purified gp140 trimer of the transmitted/founder (T/F) virus CH0505. TNC-S and TNC-L bound with similar affinity to B.MN gp120 (54.8 and 58.2 nM, respectively).

[20] **Figure 12.** Binding of TNC to Env gp140 has a slower off-rate than the binding of TNC to Env gp120. Recombinant TNC-L (Fig. 12B) and TNC-S (Fig. 12A) were covalently coupled to the SPR chip, and HIV-1 Env gp120 and gp140 were flowed over the chip. TNC isoforms bound to both gp120 and gp140 proteins of the T/F HIV-1 variants B. CH0505 and C.1086, but the binding to gp140 proteins had a slower off-rate.

[21] **Figure 13.** CD4 binding to TNC does not account for the increased binding of soluble CD4-preincubated gp120 than gp120 alone to TNC. Recombinant TNC-S was covalently coupled to the SPR chip, and HIV-1 B.MN gp120 was flowed over the chip both before and after soluble CD4 preincubation. As a control, soluble CD4 alone was flowed over the TNC-S chip and no binding was detected.

[22] **Figure 14.** Env CD4 triggering mildly increases TNC HIV-1 virion capture but not neutralization, and TNC does not antagonize the neutralizing activity of an antigp120 CD4-inducible mAb isolated from colostrum. (A) Preincubation of HIV-1 B.MN virions with soluble

CD4 increases the efficiency of TNC virion capture by ~1.5-fold. (B) Preincubation of HIV-1

virions (B.Du156) with TZM-bl cells for 10 min on ice to allow virion–CD4 interaction before addition of TNC does not enhance the neutralizing potency of TNC. Graphs represent data from two assays performed in duplicate; lines indicate SD. (C) Preincubation of T/F HIV C.1086 virions with 100 μg/mL anti-C1 Env mAb A32, which is known to induce a conformational change of the HIV-1 Env similar to that of CD4 binding, for 1 h before the addition of TNC (200 μg/mL) and TZM-bl cells does not enhance TNC neutralization. (D) TNC does not antagonize the neutralizing potency of the CD4- inducible, colostrum mAb CH08 against HIV-1 C.MW965 at a range of concentrations performed in duplicate.

[23] **Figure 15.** Anion Exchange after Size Exclusion Fractionation of semen and cervico-vaginal lavage (CVL) samples using methods as described in Example 4.

[24] **Figure 16.** TNC Purification from Semen and CVL using Size Exclusion and Anion Exchange using methods as described in Example 4.

[25] **Figure 17** shows neutralization of DU156 virus by TNC from semen and SVL fractions.

[26] **Figure 18** is an illustration of truncated TNC proteins and electron micrograph of native TNC.

[27] **Figure 19** shows relative binding of truncated TNC proteins to MN gp120 Env protein.

[28] **Figure 20** shows high affinity of FNIII regions of TNC for gp120.

[29] **Figure 21** shows high binding strength of TNC FNIII1-8 domains and fbg knob to gp120 Env protein measured by surface plasmon resonance.

[30] **Figure 22** shows that TNC FNIII 1-8 domain captures whole HIV-1 virions.

[31] **Figure 23** is a schematic of the interaction of TNC, the HIV-1 Env gp120 protein, and the HIV-1 cellular receptors.

DETAILED DESCRIPTION OF THE INVENTION

[32] HIV-1 transmission via breastfeeding accounts for nearly half of the 350,000 new infant HIV infections occurring annually. Formula feeding is not a viable strategy to reduce HIV transmission via breastfeeding in areas of high HIV-1 prevalence, as non-breastfed infants have high rates of mortality due to respiratory and diarrheal diseases in resource-poor regions. Thus, alternative prevention strategies are required to eliminate infant HIV-1 acquisition via breastfeeding. As introduction of solid foods or formula prior to six months of age is associated with an increased risk of infant HIV acquisition via breastfeeding, oral strategies to reduce

postnatal HIV acquisition should avoid introduction of new protein antigens to the neonatal gut that are not contained in milk. The identification of potent natural inhibitors of HIV-1 in breast milk makes possible the development of a natural breastfeeding supplement that can boost innate mucosal anti-HIV immunity in the neonatal gut. As described in the Examples that follow, detailed fractionation of breast milk proteins from an uninfected individual and assessment of the HIV-inhibitory activity of each distinct protein fraction has resulted in the isolation of a potent HIV-inhibitory protein - that protein is the extracellular matrix protein, TNC. This protein can be used as a low-cost natural breastfeeding supplement to provide prophylaxis against infant postnatal HIV acquisition.

[33] The present invention thus relates, in one embodiment, to a method of inhibiting (e.g., preventing of reducing the risk of) HIV-1 transmission. In a preferred embodiment, the invention relates to a method of inhibiting HIV-1 transmission via breastfeeding. In accordance with this embodiment, transmission of HIV-1 from, for example, an infected woman (e.g., a mother) to a breastfed infant (e.g., human infant) can be effected by administering to the infant TNC, or a portion or derivative thereof having the HIV-1 neutralizing activity of TNC.

[34] While the invention is described in detail with reference to the prevention of HIV-1 transmission from an infected mother to a breast fed infant, TNC, or portion or derivative thereof having HIV-1 neutralizing activity, can also be used as a topical microbicide to protect against homosexual or heterosexual viral transmission.

[35] In the methods of the invention, TNC, or portion or derivative thereof having HIV-1 neutralizing activity, is administered to the individual. The TNC, or portion or derivative thereof, is administered in a form that, when administered, inhibits transmission of the virus via virion capture (e.g., by binding to the V3 loop of HIV-1 Env protein. The complete amino acid sequence and full length cDNA sequence of human TNC is given in Nies et al (J. Biol. Chem. 266(5):2818-2823 (1991) – see also Siri et al, Nucl. Acids Res. 19:525-531 (1991)). Aukhil et al (J. Biol. Chem. 268(4):2542-2553 (1993)) describes the domains (e.g., EGF, FN-III and fibrinogen-like domains) of TNC. Portions of TNC suitable for use in the invention can include, for example, the FN-III fibrinogen-like domain, or portion thereof that binds to the HIV-1 Env. Additional non-limiting examples of TNC fragments are described in Figures 18-22.

[36] TNC, or portion or derivative thereof having HIV-1 neutralizing activity, is obtainable from a variety of sources. The protein can be isolated from natural sources (for example, from

breast milk (e.g., from human breast milk)). It can also be produced recombinantly in prokaryotic (e.g., E. coli) or eukaryotic cells, e.g., in BHK cells. Production of TNC in BHK cells appears to yield a product having posttranslational modifications important for neutralizing activity. TNC, or portion or derivative thereof, can also be produced chemically using standard techniques.

[37] Preferred ranges for the neutralizing activity of the TNC (or portions or derivatives) include an IC_{50} of 0.1 μg/ml to 1000 μg/ml, preferably <10 μg/ml.

[38] TNC (or portions or derivatives thereof) can be administered alone, or in compositions or medicaments comprising the TNC (or portions or derivatives thereof) and a physiologically acceptable carrier or excipient. The carrier and/or composition can be sterile. The formulation should suit the mode of administration.

[39] Suitable pharmaceutically acceptable carriers include but are not limited to water, salt solutions (e.g., NaCl), saline, buffered saline. The pharmaceutical preparations can, if desired, be mixed with auxiliary agents, e.g., preservatives, stabilizers, emulsifiers, salts, buffers, coloring, flavoring and/or aromatic substances and the like which do not deleteriously react with the active compounds.

[40] The composition or medicament can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. Oral formulations can include standard carriers, for example, lactose, magnesium carbonate starch, magnesium stearate, mannitol, polyvinyl pyrollidone. Formulations suitable for use in protecting against sexual transmission can take the form of gels, films or suppositories. The composition or medicament can be formulated in accordance with the routine procedures.

[41] TNC, or portion or derivative thereof, for use in inhibiting viral transmission to a breastfed infant can be distributed as a powder with instructions for dissolution in a solvent (e.g., breast milk or water) prior to oral administration.

[42] In accordance with the invention, the TNC, or portion or derivative thereof, can be administered in conjunction with other active agent(s), including, for example, other anti-retroviral agent(s), including, for example, lactoferrin or SLPI.

[43] TNC, or portion or derivative thereof having HIV-1 neutralizing activity, is administered in an effective amount (i.e., a dosage amount that is sufficient to inhibit/prevent HIV-1 transmission). Optimal dosing regimens can be readily determined by one skilled in the art. In

the case of administration to breast-fed infants, about 10 μg/ml to about 1000 μg/ml can be administered, for example, with each breast feeding.

[44] The invention additionally pertains to pharmaceutical compositions comprising TNC, or portion or derivative thereof, as described herein, in a container (e.g., bottle) with a label containing instructions for administration of the composition for inhibition of viral transmission.

[45] The invention also relates to the use of TNC, or portion or derivative thereof having HIV-1 neutralizing activity, in the manufacture of a medicament for use in inhibiting HIV-1 transmission.

[46] It will be appreciated that the inhibition of HIV-1 transmission is useful in the fields of both human use and veterinary use. Thus, the subject to be treated can be a human or non-human mammal, preferably, a human (e.g., a human infant).

[47] While the invention is described in detail with reference to the inhibition of HIV-1 transmission, TNC, or portion or derivation thereof, can be used to inhibit transmission of other viruses (e.g., other retroviruses), as well as to inhibit bacterial infection.

[48] Certain aspects of the invention can be described in greater detail in the non-limiting Examples that follows.

EXAMPLE 1

[49] Experimental Details

[50] *Size exclusion and strong anion exchange chromatography*

[51] Milk samples collected from HIV-1 uninfected women between 1 week and 7 months after delivery were delipidized by centrifugation, filtered, and concentrated 10X before loading on a fast protein liquid chromatography size exclusion column (Superdex 200 10/300GL, GE Healthcare). The high molecular weight proteins were further fractionated using a 1M NaCl elution gradient (SOURCE 15Q 4.6/100 PE).

[52] *Mass spectrometry*

[53] Protein bands were processed (URL: www-dot-genome-dot-duke-dot-edu/cores/proteomics/sample-preparation) and analyzed using Data Dependent Acquisition (DDA) on a nanoscale capillary LC/MS/MS system (nanoAcquity LC and SYNAPT G2 HDMS, Waters Corp). Data was processed using a Mascot pipeline (Demon, Distiller, and Server 2.2 Matrix Sciences) and searched against a forward/reverse UniProt_human database. The reversed

form of this database was concatenated with the forward form to allow control of False Positive Discovery Rate (FDR) as calculated by the Scaffold (Proteome Software) implementation of Peptide/Protein Prophet (Keller et al, OMICS 6:207 (2002)). For quantitation of TNC concentration in milk, an internal standard of yeast alcohol dehydrogenase (Waters Corp) was added as a surrogate standard. Samples were heated at 60°C for 2 hours and analyzed using ion-mobility assisted Data Independent Acquisition (maDIA) on a nanoscale capillary LC/MS/MS system. Approximate mole amount was calculated using the Top 3 method (Silva et al, Anal. Sci. 22:861 (2006)).

[54] *TNC depletion and HIV-1 neutralization*

[55] 10 mg of the anti-TNC IgG mAb 81C6 was coupled to 600 mg cyanogen bromide-activated separhose 4B beads (GE Healthcare) and incubated with delipidized/filtered milk overnight, then centrifuged at 2000g for 5 min to remove the protein-bound beads. Purified TNC and milk samples were incubated with HIV-1 Env pseudoviruses or infectious molecular clones produced by 293T cells and tested for neutralization potency in TZM-bl target cells, as described (Li et al, J. Virol. 79:10108 (2005)).

[56] *HIV-1 virion capture*

[57] 0.3μg of each protein was coated in a 96 well plate and blocked with 5% bovine serum albumin (Sigma). HIV-1 Env pseudovirions (10-20 ng of Gag p24) were incubated in the well for two hours at 37°C and unbound virions were removed by washing four times with PBS. The bound virions were lysed and quantitated by p24 ELISA (PerkinElmer). The percent virus capture: (Gag p24 amount of bound virions)/(Gag p24 amount added to the well).

[58] HIV-1 Env surface plasmon resonance (SPR)

[59] TNC proteins (Aukhil et al, J. Biol. Chem. 268:2542 (1993), Ohashi et al, J. Biol. Chem. 279:6534 (2004)) were directly immobilized by amine coupling to SPR sensor chip CM5 (Alam et al, J. Immunol. 178:4424 (2007)), with the modification of washing in pH 7.4 buffer overnight. HIV-1 Env gp120/140 proteins (Liao et al, Virology 353:268 (2006)) were injected at 100 μg/mL over TNC immobilized surfaces for 2 min in PBS, pH 7.4 and binding was measured with a BIAcore 3000 (GE Healthcare). Non-specific binding was subtracted over a control surface immobilized with an anti-RSV IgG mAb (Synagis). TNC conformation was confirmed by injecting anti-TNC antibody (clone T2H5, Abcam). MN gp120 was incubated with saturating concentrations of anti-gp120 mAbs for 1 hour and injected over TNC-S immobilized surface.

Percent blocking: (Response with gp120 in buffer – Response with gp120 bound to mAb)/ Response with gp120 in buffer. MAbs included: anti-gp120 conformational C1 (A32), anti-V3 loop (19b, F39F), anti-V2 linear (CH58), anti-V2 conformational (697D) and anti-CD4 binding site (CH31).

[60] Recombinant TNC expression

[61] Recombinant TNC was expressed in BHK cells with the pNUT vector, and purified with a combination of ammonium sulfate precipitation, gel filtration and anion exchange chromatography (Aukhil et al, J. Biol. Chem. 268:2542 (1993)). TNC was also expressed in CHO cells from the pEE14 expression vector, or in HEK cells by transient expression in serum-free media adapted from recombinant fibronectin expression Ohashi et al, J. Biol. Chem. 286:39188 (2011)) and purified with ammonium sulfate precipitation and gel filtration chromatography

[62] Results

[63] To identify the high molecular weight HIV-1-neutralizing protein in milk, uninfected milk samples were screened for neutralizing activity against a panel of multiclade chronic and transmitted/founder (T/F) viruses (inhibitory dose 50% (ID_{50}) range: 3 – 42, Table 1) and a potently neutralizing sample (milk #10) was selected for fractionation by size-exclusion chromatography. As expected, all of the detectable HIV-1-neutralization activity was contained in the high molecular weight (MW; >500 kDa) fraction (inhibitory concentration (IC_{50}): 660 μg/ml). The high-MW, active milk fraction was further fractionated by ion exchange chromatography, narrowing the detectable HIV-1-neutralization activity to a single protein fraction (peak 3, Fig. 1A). A reduced SDS-PAGE gel of this fraction revealed a single unique 250kD band (Fig. 1A). Ultra performance liquid chromatography-mass spectroscopy of this unique protein band and comparison of the results to a human protein database revealed 76 unique peptides that had a >90% likelihood match to the extracellular matrix protein, TNC. The identity of the protein was confirmed by western blot (Fig 1B).

[64] Depletion of TNC from milk samples of two uninfected women using anti-TNC monoclonal IgG (81C6, generously provided by Dr. Darell Bigner) (Murphy-Ullrich et al, J. Cell Biol. 115:1127 (1991)) resulted in a clear reduction of HIV-1 neutralization activity (Fig. 1B) (Li et al, J. Virol. 79:10108 (2005)). Moreover, purified TNC (Millipore) demonstrated broad spectrum, dose-dependent neutralizing activity against chronic and T/F HIV-1 variants isolated

from adults and postnatally-infected infants, without any evidence of cytotoxicity (IC_{50} range: 82 – 158 μg/ml, Table 1 and Fig. 3). Moreover, the neutralizing activity was directed against the virus and did not solely block at the level of the target cells, as the neutralizing activity was only apparent with virus preincubation and not target cell preincubation (data not shown). Interestingly, recombinant TNC produced by the Erickson lab in BHK cells (Aukhil et al, J. Biol. Chem. 268:2542 (1993)), but not CHO or HEK293T cells (data not shown), recapitulated the HIV-1 neutralizing activity of purified TNC, indicating that posttranslational modifications may be important for the neutralizing activity. 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, a concentration range spanning the measured HIV-1 IC_{50} of the protein (Table 1).

[65] As TNC is a large, multimeric protein (Taylor et al, J. Cell Biochem. 41:71 (1989)), the likely mechanism of its HIV-1-neutralizing activity is inhibition of virus entry. In fact, purified TNC was able to capture chronic and T/F HIV-1 virions, including those transmitted via breastfeeding at a similar potency to that of a broadly-HIV-1 neutralizing monoclonal antibody (mAb) that is protective against infant HIV-1 acquisition (2G12) (Fig 1C). Both the short (TNC-S) and the long (TNC-L) recombinant isoforms of the protein and the purified protein bound to HIV-1 Env gp120 and gp140 proteins by surface plasmon resonance (SPR), including HIV-1 MN gp120, Con S gp140, the T/F Env 1086C gp120/140, and the T/F Env CH0505 gp140 trimer (Fig. 1D, only TNC-L shown). Moreover, TNC binding to HIV-1 MN gp120 was potently blocked by the anti-HIV-1 Env mAbs 19B and F39F (84.3% and 87.7%, respectively), both directed against the V3 loop of the HIV-1 Env (Fig. 1D). Interestingly, TNC binding to some HIV-1 Envs, including HIV-1 T/F 1086C gp120 and gp140 was enhanced by sCD4 preincubation, indicating that the TNC interacts with a CD4-inducible (CD4i) epitope. Thus, an innate breast milk protein has been identified that binds to a CD4i epitope on the HIV-1 Env and neutralizes HIV-1 at its *in vivo* concentration.

EXAMPLE 2

[66] TNC plays a role in fetal brain and mammary gland development, as well as wound healing; and is highly expressed in certain breast and brain tumors. TNC interacts with integrins (cell-adhesion molecules) and other extracellular matrix proteins with its epidermal-like growth

factor repeat region and fibronectin (FN)-type domains (Midwood and Orend, J. Cell Commun. Signal 3:287-310 (2009), Orend and Chiquet-Ehrismann, Cancer Lett. 244:143-163 (2006)) (Fig. 2). Its anti-infective properties have not previously been investigated. The studies described herein establish that this novel HIV-1 inhibitor has activity against a broad range of chronic and transmitted/founder (T/F) HIV-1 viruses, including variants transmitted via breastfeeding. Moreover, depletion of TNC from milk severely reduces its inherent neutralizing activity.

[67] As described above, to identify the protein(s) responsible for the activity, an uninfected milk sample with potent anti-HIV-1 activity (milk #10, Table 1) was fractionated by size-exclusion chromatography. The HIV-1-neutralization activity was found to be contained in the high molecular weight (MW) fraction (>250kD). The high-MW, active milk fraction was further fractionated by ion exchange chromatography, narrowing the detectable HIV-1-neutralization activity to a single protein fraction. A reduced Coomassie gel of this fraction revealed a single unique band at 250kD. Ultra performance mass spectroscopy of this unique protein band and comparison of the results to a human protein database revealed 76 unique peptides that had a >90% likelihood match to the extracellular matrix protein, TNC. The identity of the protein was also confirmed by western blot with an anti-TNC polyclonal antibody. Mass spectrometry analysis of the smaller protein bands in peak 3 confirmed that these proteins (heavy chain of immunoglobulin and lactoferrin) were carried over from inactive fractions, peak 1 and 2. Importantly, depletion of TNC using heparin-coated beads, resulted in significantly reduced neutralization activity of breast milk that did not reach 50% neutralization of virus, confirming TNC is a primary mediator of the innate HIV-1-neutralizing activity of breast milk.

[68] Further confirming the HIV-1-neutralizing activity of TNC, a commercially-available purified TNC preparation (Millipore) demonstrated broad spectrum neutralizing activity against chronic and T/F HIV-1 variants isolated from adults and postnatally-infected infants, with an inhibitory concentration 50% (IC_{50}) range of 82 – 122 μg/ml (Table 1). Moreover, TNC neutralized HIV-1 with greater potency than lactoferrin, a predominant breast milk protein also noted to have HIV-1 inhibitory activity (Moriuchi and Moriuchi, J. Immunol. 166:4231-4236 (2001)) (IC_{50} against HIV-1 Du156: 77μg/ml for TNC and 250μg/ml for lactoferrin). Remarkably, TNC-L neutralized chronic HIV-1 variant Du156 in a dose dependent fashion (Fig. 3). Furthermore, the neutralizing activity of breast milk from two additional uninfected individuals was also isolated to the protein peak containing TNC. Quantitation of TNC based on

relative abundance determined by unbiased mass spectrometry of 10 mature human milk samples (collected >1 week after delivery) revealed a milk TNC concentration range of 2.2 – 671 μg/ml.

[69] As TNC is a large, multimeric protein, the mechanism of its HIV-1-neutralizing activity is likely inhibiting virus entry. To test this theory, SPR was employed to determine whether each of the natural isoforms of TNC: TNC-long (TNC-L), the isoform found in breast milk, and TNC-short (TNC-S), an alternative splice protein lacking the FNIIIA-D domains and only found in cartilage (Fig. 2), was able to bind to HIV-1 Env proteins. Confirming the theory that TNC interacts with HIV-1 Env to mediate its inhibitory activity, TNC-L and TNC-S bound to both HIV-1 Env gp120 and gp140 proteins in solution indicating that TNC binds to an HIV-1 Env epitope in the gp120 protein, and not the gp41 protein. Moreover, the ability of both TNC isoforms to bind HIV-1 Env indicates that the TNC binding site is not within the FNIIIA-D region of TNC which is absent in the TNC-S protein. Finally, TNC was able to capture both chronic and T/F HIV-1 virions. Thus, the HIV-1-neutralizing activity of TNC is likely mediated by the ability of TNC to bind to the HIV-1 Env protein and capture infectious virions, preventing HIV-1 infection of target cells.

[70] To further define the region of TNC-L that mediates the HIV-1-neutralization and Env-binding, truncation proteins of the TNC (Aukhil et al, J. Biol. Chem. 268:2542-2553 (1993)) will be produced and purified. These recombinant proteins have previously been cloned and produced in either mammalian cell lines (BHK or CHO) or bacterial expression system, thus the protein constructs and protocols for protein production and purification are available. Both HIV-1 neutralization assays in TZM-bl cells and HIV-1 Env binding assays by SPR will be performed with each of the TNC truncation proteins to determine which domains are required for HIV-1 neutralization. In addition, polyclonal antibodies against TNCfbg, TNCfn1-5, TNCfn6-8, and TNCfnA-D domains will be utilized to perform both neutralization and binding inhibition assays. The ability of each recombinant TNC truncation protein to capture HIV-1 virions will be determined in a virus capture assay. Moreover, anti-TNC polyclonal antibodies can be used to inhibit the virus capture, confirming the SPR binding and neutralization-inhibition assays. Once the active site is narrowed to a small region of TNC, single, double, or triple domains within the active region will be cloned and recombinantly produced for binding, neutralization, and virus capture assays to further narrow and identify the domains of TNC that are required for HIV-1 neutralization.

[71] TNC is well-known to bind to epithelial cells via interactions with integrins (Midwood et al, J. Cell Commun. Signal 3:287-310 (2009), Orend and Chiquet-Ehrismann, Cancer Lett. 244:143-163 (2006)) and syndecan (Salmivirta et al, J. Biol. Chem. 266:7733-7739 (1991)). Thus, TNC may have the ability to interrupt HIV-1 Env binding to mucosal epithelial cells and block mucosal HIV-1 transmission, including transmission via breastfeeding. Therefore, potential anti-HIV-1 functions of TNC will be assessed that may be important in blocking mucosal transmission in the neonatal gastrointestinal tract. For HIV-1 virions to infect CD4-expressing target cells in the infant gut, they must first bind to and transcytose the columnar epithelial cell layer. The interaction of the HIV-1 Env with epithelial cells may be dependent on the putative HIV-1 epithelial cell attachment factor, Galactosyl ceramide (GalCer) (Fantini et al, Aids 8:1347-1348 (1994), Yahi et al, Aids 6:335-336 (1992)). Thus, an investigation will be made of the ability of TNC to interrupt the virus' ability to attach and interact with mucosal epithelial cells, events required for establishing infection.

[72] First, the ability of TNC to inhibit the binding and internalization of HIV-1 virions will be determined by a monolayer of HT-29 colonic epithelial cells in an established assay (Fouda et al, Retrovirology 10(1):3 (2013) Epub ahead of print), Mantis et al, J. Immunol. 179:3144-3152 (2007)). HIV-1 virions (25ng of Gag p24) will be incubated with TNC or an irrelevant protein (such as albumin), then added to polarized HT29 monolayers in a 96-well plate. To assess the efficiency of HIV-1 virion binding in the presence of TNC, the monolayers will be washed, the cells lysed, and the amount of HIV-1 Gag p24 that was bound or internalized by epithelial cells quantitated. To assess the efficiency of HIV-1 virion internalization in the presence and absence of TNC, the cells will be washed with a low concentration of trypsin (Mantis et al, J. Immunol. 179:3144-3152 (2007)) after incubation with the TNC-virion complexes, removing the HIV-1 virions bound to the surface of the epithelial cells. The cells will then be washed and lysed prior to quantitation of the amount of HIV-1 internalized by the epithelial cells using a Gag p24 ELISA. These epithelial cell assays will determine whether TNC reduces the efficiency of HIV-1 binding to epithelial cells, a required step for mucosal transmission. Moreover, a determination will be made of the ability of TNC to interrupt HIV-1 gp140 binding to the putative epithelial cell attachment fator, GalCer, via Biolayer Inferometry (BLI) with liposomes containing GalCer (Alam et al, J. Immunol. 178:4424-4435 (2007)). ConS gp140 monomers and oligomers will be incubated with TNC prior to dipping the biosensor loaded with GalCer

liposomes into the well containing the TNC/gp140 mixture. Interaction of the gp140 Env proteins with Galcer in the presence and absence of TNC will be measured by BLI (Fouda et al, Retrovirology 10(1):3 (2013) Epub ahead of print)). As TNC interacts with both epithelial cell surface proteins and HIV-1 Env, and is ingested with the breast milk-associated virus, it is possible that it plays a role in interrupting key initial virion-host interactions required for postnatal HIV-1 transmission.

[73] It is expected that the anti-HIV-1 activity of TNC can be narrowed to a single or small number of functionally-distinct domains, informing both the mechanism of TNC HIV-1 inhibition and the minimum protein requirement for the neutralizing activity. In addition to testing neutralization and binding activity of small TNC segments, larger segments with deletion of small EGF or FN-III domains (Fig. 2) can also be screened to maintain the conformation of the protein. Methods for production of TNC truncation proteins have been previously optimized in various cell types (including CHO, HEK, and BHK) (Aukhil et al, J. Biol. Chem. 268:2542-2553 (1993)). As differences in the ability of recombinant TNC proteins to mediate the neutralizing activity of the purified protein have been noted, the TNC proteins will be produced in each of these cell lines. If the recombinant proteins cannot recapitulate the neutralizing activity of TNC, existing polyclonal antibodies raised against defined regions of TNC will be used to map the active site through antibody inhibition of neutralization, SPR binding, and virus capture.

[74] Despite recent major advances in isolation of HIV-1-neutralizing mAbs Bonsignori et al, J. Virol. 85(19):9998 (2011), Scheid et al, Nature 458:636-640 (2009), Zhou et al, Science 329:811-817 (2010)), a relatively small number of broadly-neutralizing epitopes have been defined on the HIV-1 Env protein. Moreover, many of the defined HIV-1 neutralizing epitopes appear to be restricted from access to most antibodies (such as the CD4 binding site) or difficult to elicit antibodies against (such as gp41 MPER). Thus, defining novel neutralizing epitopes on the HIV-1 Env is critical to the advancement of HIV-1 vaccine development. Mapping the neutralizing epitope on this innate HIV-1-neutralizing factor opens the door to defining a novel target on the HIV-1 Env for immunogen development.

[75] To narrow the region of the HIV-1 Env that is bound by TNC, a panel of HIV-1 Env-binding mAbs with distinct specificity will be screened for their ability to block TNC binding to the HIV-1 Env via SPR. As it was previously established that HIV-1 ConS gp140 and MN

gp120 can bind to TNC (Fig. 5), these Env constructs will be incubated with a panel of anti-HIV-1 gp120 antibodies prior to flowing over TNC bound to the SPR chip. The anti-Env mAbs that will be used in the TNC-Env blocking assay include the following: 1) anti-V2 (CH58, CH59); 2) anti-conformational V2 (697D); 3) anti-V3 (19B, F39F); anti-C1 (16H3, CH57); 4) anti-V2,V3 quaternary (PG9, PG16, CH01-04); 5) anti-N-linked glycans of the outer gp120 domain (2G12); 6) anti-CD4bs (1b12, VRC01, VRC03, VRC-CH31, sCD4); 7) CD4-inducible epitope (A32); and 8) polyclonal antibodies generated against HIV-1 Env V1, V2, V3, C1, and C2. If carbohydrate groups are suspected to be important for the Env-TNC interaction (carbohydrate-dependent Env-specific mAb such as 2G12 or PG16 interrupt binding), the TNC binding experiments will be repeated with gp140 treated with mannosidases (Calarese et al, Proc. Natl. Acad. Sci. USA 102:13372-133727 (2005)) to confirm a carbohydrate-dependent interaction of TNC and Env. The results of the antibody blocking assays will be confirmed in the TNC-virion capture assay by incubating the virus with anti-Env mAbs prior to the capture assay (Fig. 6). Nonspecific polyclonal IgG and the anti-RSV mAb, Synagis, will be used as negative controls in these blocking assays. Screening for anti-Env mAb blocking of Env-TNC interaction will narrow the region of the Env that is bound by TNC. Using the results of the Env-specific mAb-blocking assays, overlapping peptides will be designed that span the region that is implicated to bind to TNC in the antibody-blocking assays. The strength of TNC binding to the Env peptides will be assessed in both SPR and ELISA-based peptide binding arrays, as previously described (Friedman et al, PLoS One 7:e37648 (2012)), narrowing the binding region to the minimum number of amino acids. Once the active domain of TNC is defined, the binding strength of the active TNC domain(s) will be determined, TNC binding to Env captured in various conformations on an antibody-coated surface assessed, and a comparison made of its ability to bind to oligomeric gp140 trimers and monomeric gp140 by SPR.

[76] In addition to mapping the TNC binding epitope on HIV-1 Env, neutralization mapping studies will be performed utilizing panels of single-round HIV-1 YU2 gp120 mutants. An assessment will be made of the ability of TNC or the defined active domain to neutralize an established panel of HIV-1 Env pseudoviruses containing point mutations in portions of the stem and outer region of the V1, V2 and V3 loops via neutralization assays in Cf2Th cells (Madani et al, Structure 16:1689-1701 (2008), Si et al, Proc. Natl. Acad. Sci. USA 101:5036-5041 (2004)).

This panel of Env pseudovirus mutants will provide functional mapping of the Env neutralization epitope of TNC, establishing the neutralizing Env epitope.

[77] The neutralizing epitope of TNC may be conformationally-dependent, and thus peptide-binding assays may not fully assess the TNC binding. In this case, available Env region protein constructs (V1, V2, V3, C1) will be utilized in TNC binding SPR experiments. In addition, site-directed mutagenesis will be used to mutate/delete specific regions of Env (Madani et al, J. Virol. 78:3742-3752 (2004)) that are implicated to bind to TNC by the antibody-blocking assays, and the relevant pseudoviruses and gp120 proteins produced.

[78] To investigate the ability of TNC in breast milk to protect against postnatal HIV-1 acquisition, a determination will be made of the association between the endogenous concentration of TNC in milk of HIV-1-infected women and the risk of infant HIV-1 acquisition. A TNC ELISA based on an established protocol (Lightner et al, J. Cell Biol. 108:2483-2493 (1989)) and a quantitated recombinant TNC preparation as a standard will be used to determine the concentration of TNC in milk. This assay will be validated for milk by measuring the TNC concentration in milk of 30 uninfected subjects and comparing to the TNC concentration determined by unbiased mass spectrometry. Using the validated ELISA, the concentration of TNC in milk of HIV-1-infected, lactating Malawian women enrolled in the placebo arm of the BAN study will be measured (Chasela et al, N. Engl. J. Med. 362:2271-2281 (2010)). In this placebo arm, 668 breastfeeding mother-infant pairs received single-dose nevirapine around delivery and infants received seven days of zidovudine/lamivudine and rapidly weaned at 6 months of age, resulting in a 5.7% postnatal transmission rate. To assess the impact of the milk TNC concentration on the risk of postnatal HIV-1 acquisition, a case-control study design and a student's t test will be employed to compare the breast milk TNC level in postnatal-transmitting mothers at the time point prior to transmission and in nontransmitting mothers matched for age, CD4 count, and time point. As a secondary analysis, Spearman's rank correlation will be used to determine if the concentration of TNC in breast milk correlates with breast milk virus RNA load, indicating that TNC is associated with containment of virus replication in the mammary gland.

EXAMPLE 3

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

[80] 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-1transmission, yet toxicities and the development of drug-resistant viral strains may limit the effectiveness of this strategy. 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 two years. 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 are 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 multiclade chronic and transmitted/founder (T/F) HIV-1 variants and depletion of TNC abolished the HIV-1-neutralizing activity of milk. TNC bound the HIV-1 Envelope protein at a site on gp120 that is induced upon engagement of its primary receptor, CD4, and is blocked by V3 loop (19B and F39F) and chemokine coreceptor binding site-directed (17B) 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.

[81] Obtaining the goal of an AIDS-free generation will require elimination of breast milk transmission of HIV-1, as breastfeeding is a corner stone of infant survival in developing regions. Antiretroviral prophylaxis considerably reduces postnatal HIV-1 transmission, yet is 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 a novel, 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 novel HIV-1 prevention strategy for postnatal and other modes of HIV-1 transmission.

[82] **Introduction**

[83] 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. Despite chronic, daily exposure to the virus for up to two years of life, greater than 90 percent 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.

[84] 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 weight 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 weight 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.

[85] **Results**

[86] *Identification of a high molecular weight innate HIV-1-neutralizing protein in breast milk*

[87] To identify the high molecular weight HIV-1-neutralizing protein in milk, we screened mature milk samples from uninfected women (collected between two weeks and seven months 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 (ID_{50}) of milk samples against this panel of HIV-1 strains ranged from 3 – 42 (Fig. 8). We next

fractionated a more potently-neutralizing milk sample (milk #10, Fig. 8) by size-exclusion chromatography. Consistent with a previous report (7), all of the detectable HIV-1-neutralization activity was contained in the high molecular weight (>500 kDa) fraction (50% inhibitory concentration, IC_{50}, against the chronic, neutralization tier 2 HIV-1 variant C. Du156: 407 μg/ml) (Fig. 9). 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, IC_{50} against C.Du156: 382 μg/ml, Fig. 4A). A reducing SDS-PAGE gel of this fraction revealed a single unique 250kDa band that was not visualized in the nonneutralizing fractions (Fig. 4A). Ultra performance liquid chromatography-tandem mass spectroscopy (LC/MS/MS) of this protein band and comparison of the results to 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 4B).

[88] *HIV-1 neutralizing activity of TNC*

[89] 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. 4B) (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 PBMCs (Fig. 5). 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 hours of incubation. There was no reduction of relative luciferase units (RLU) at the highest tested concentration of TNC (150 μg/ml) compared to the cell control (mean RLU+/- range: 391 +/- 10 vs 362 +/- 26, respectively). 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 prior to virus inoculation. Purified TNC demonstrated broad-spectrum HIV-1 neutralizing activity against multiclade chronic and T/F HIV-1 Env variants isolated from both adults and

postnatally-infected infants in the TZM-bl reporter cell assay (IC_{50} range: 82 – 158 μg/ml, Fig. 8) in a dose-dependent manner (Fig. 5A). TNC also neutralized the T/F HIV-1 CH40 variant in PBMCs (IC_{50} of CH40: 27 μg/ml; IC_{80}: 71 μg/ml) more potently than the activity measured in the TZM-bl assay (Fig. 5B). However, the opposite was true with T/F HIV-1 variant CH77 (Fig. 5C), 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 TZM-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) (IC_{50} >300 μg/ml, Fig. 8). TNC neutralized both CCR5 (such as the T/F strains) and CXCR4 (B.MN) coreceptor-tropic HIV-1 variants (Fig. 8). Consistent with the broad activity of innate antimicrobial proteins, TNC also displayed neutralizing activity against the mouse retrovirus, murine leukemia virus (MLV, IC_{50}: 109 μg/ml), yet, no activity was detected against the simian immunodeficiency virus strain mac251 (>180μg/ml, Fig. 8). 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. 10). Finally, quantitation of TNC based on relative abundance measured by unbiased mass spectrometry of ten mature human milk samples revealed a milk TNC concentration range of 2.2 – 671 μg/ml, spanning the measured IC_{50} of TNC against HIV-1 variants (Fig. 8).

[90] *TNC captures HIV-1 virions, blocks virus-epithelial cell binding, and binds to the HIV-1 Env protein in a charge-dependent manner*

[91] 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 breastfeeding, at a significantly higher potency than lactoferrin and albumin, yet a similar potency to that of a broadly-HIV-1 neutralizing monoclonal antibody (mAb) that is protective against infant HIV-1 acquisition (2G12) (26) (Fig. 6A). As this extracellular matrix protein binds HIV-1 virions and is known to interact with a number of cellular receptors (25), we hypothesized that TNC would be able to block infectious virus from binding to mucosal epithelial cells, representing a potential

additional role of TNC in impeding mucosal HIV-1 transmission to infants. Confirming this hypothesis, TNC was able to block up to 66% infectious virus binding to a monolayer of colonic columnar epithelial cells in a dose-dependent manner (Fig. 6B).

[92] To define the region of TNC that interacts with the HIV-1 virion, we utilized surface plasmon resonance (SPR) to detect binding of the HIV-1 Env protein to both the long (TNC-L) and alternatively-spliced short (TNC-S) isoforms of TNC (25, 27). The conformation of immobilized TNC was confirmed by intact binding to an anti-TNC mAb (T2H5, Abcam). Both TNC-L and TNC-S, as well as purified TNC, bound to multiclade HIV-1 Env gp120 and gp140 proteins, including chronic HIV-1 variant B.MN gp120 (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. 10). 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. 12).

[93] *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*

[94] 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 CH0505 gp140 proteins demonstrated a slower off rate (binding to TNC-S: $k_d = 1.55x10^{-3}$ and $1.56x10^{-3}$ s^{-1}, respectively) than that of their matched gp120 (binding to TNC-S: $k_d = 9.4 x10^{-3}$ and $4.5x10^{-3}s^{-1}$, respectively)(Fig. 11). Thus, the gp120 epitope bound by TNC is partially-dependent on the conformation of the gp41:gp120 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. 7A), including: anti-gp120 conformational C1 (A32, 16H3)(29, 30), anti-V3 loop (19b, F39F)(29), anti-V2 linear (CH58)(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 19B and F39F (84.3% and 87.7%, respectively), both directed against the V3 loop of the Env protein (Fig. 7B). TNC-Env binding was enhanced by Env preincubation with mAb A32 (Fig. 7A), an anti-C1 mAb that induces a conformational change which 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. 7B), and this increased signal was not due to soluble CD4 binding to the TNC chip (Fig. 13). 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. 7C). Finally, the binding of TNC to CD4-bound gp120 was substantially reduced by increasing the NaCl concentration from 137 to 250 mM (Fig. 7D), indicating that the TNC-Env binding is dominated by electrostatic interactions. The strong influence of charge and CD4i and V3 mAb-blocking of 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.

[95] Finally, we investigated the antiviral function of TNC directed against HIV-1 virions in the preCD4-bound conformation compared to the post CD4-bound, open conformation. In fact, the ability of TNC to capture HIV-1 B.MN virions was increased approximately 1.5 fold when the virions were preincubated with soluble CD4 (Fig14). However, no increased neutralizing activity was observed when HIV-1 virions were incubated with the TZM-bl cells for 10 minutes on ice prior to addition of TNC, allowing CD4 interaction but preventing cell-virion fusion, compared to the incubation of TNC and virions before the addition of TZM-bl cells (Fig. 14B). Similarly, we did not detect any increased neutralizing activity in the presence of the nonneutralizing, anti-C1 mAb A32 which induces the CD4i conformational change (Fig14). Therefore, while 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 the gp120 (39). In fact, TNC did not antagonize the neutralizing effect of CH08 against a tier 1 clade-matched HIV-1 strain (MW965) across a range of mAb concentrations (Fig.14), despite being directed against the same region of gp120. Therefore, TNC likely acts in concert with HIV-neutralizing antibodies also present in breast milk.

[96] **Discussion**

[97] 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 postnatal 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 this innate antimicrobial protein in milk may contribute to the relatively low rate of HIV-1 transmission via breastfeeding.

[98] TNC appears to mediate its HIV-1-neutralizing activity via capturing HIV-1 virions and binding to the HIV-1 Env proteins of chronic and T/F HIV-1 variants. As a hexameric protein, the multivalency of TNC may contribute to its ability to capture and neutralize the virus. TNC-HIV-1 Env binding mapped to the V3 loop of the gp120 protein, was enhanced in the presence of soluble CD4, and was blocked from binding to HIV-1 Env by the CD4i anti-coreceptor binding site mAb 17B. Thus, this protein is likely exerting its HIV-1 neutralizing activity via blocking chemokine coreceptor contact on the HIV-1 Env protein. Importantly, TNC is able to bind to gp120 and capture virions in the absence of the CD4 molecule (Figs. 7B and 14A). In an environment where there is limited presence of CD4-expressing cells, such as the breast milk/infant gut interface, TNC interaction with Env may be sufficiently avid to compete with Env binding to the chemokine receptor. Chemokine coreceptor binding to Env requires CD4 triggering, as studies using reconstituted CCR5 showed no detectable binding to Env gp120 and only bound in the presence of CD4 (40). Therefore, TNC could exert its effect on HIV-1 in the absence of Env-CD4 engagement by capturing the virion via binding the V3 loop and subsequently preventing coreceptor binding.

[99] The V3 loop of the HIV-1 Env is a highly flexible and positively charged domain, characteristics important to coreceptor binding (41), and coreceptor tropism is determined by the net charge in this region of the Env (42). Indeed, we found that TNC binding to Env is dominated by electrostatic interactions. In fact, a positively-charged, heparin-binding domain has been described within the chemokine coreceptor binding site (43) and this region is likely mediating the TNC-Env binding. The charge-dependent interaction of TNC with the HIV-1 Env V3 loop may also explain the nonspecific activity that we detected against the nonhuman

retrovirus, MLV. The interaction of V3 loop with the HIV-1 coreceptor CCR5 has been proposed to involve sulfated tyrosines in the N-terminal extracellular domain of CCR5 (44-47). The electrostatic interaction of TNC to Env gp120 may be similar to that of previously-described V3 loop derived peptides (44), cell-associated 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. Since 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 B.MN gp120 to TNC by SPR compared to 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. 8). Similarly, binding affinity to HIV-1 Env does not always predict the HIV-1 neutralization potency of anti-HIV-1 Env mAbs (28). Since both net charge and V3 loop flexibility can be important for co-receptor 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).

[100] As both the short and long form of TNC bound to HIV-1 Env, the Env binding site on TNC is likely outside of the splice region within the FN-III domain of TNC. Outside of this splice region, there are eight FN-III domains that demonstrate distinct binding to cellular receptors, fourteen 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). Human TNC isolated from two different sources, breast milk and a glioma cell line, and recombinant TNC produced in BHK cells neutralized HIV-1 were able to neutralize the virus. Under the conditions tested, 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 detected in these various TNC products.

[101] 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 weight fraction (7). In fact, the presence of TNC in breast milk may explain the natural protection of the majority of HIV-1-exposed, breastfed

infants. However, 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. 8). Given its broad-spectrum HIV-1-binding and neutralizing activity, this innate, mucosal HIV-1-inhibitor could theoretically be developed as an infant HIV-1 prophylactic agent which could be orally administered to infants prior to breastfeeding, similar to oral rehydration salts which are routinely 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). Furthermore, use of this innate mucosal host protein as a prophylactic agent may avoid the issue of antiretroviral-resistant virus strains that complicate maternal/infant antiretroviral prophylaxis regimens. Thus, TNC holds promise for development as a safe, HIV-1-neutralizing host mucosal protein that can be employed for reducing mucosal HIV-1 transmission.

[102] **Material and Methods**

[103] *Size exclusion and strong anion exchange chromatography*

[104] Milk samples from HIV-1 uninfected women between two week and seven months after delivery were delipidized by centrifugation, filtered, and concentrated 10X before loading on a protein liquid chromatography size exclusion column (Superdex 200 10/300GL, GE Healthcare). Proteins were further fractionated using a 1M NaCl elution gradient (SOURCE 15Q 4.6/100 PE).

[105] *Mass spectrometry*

[106] Protein bands were processed (http://www.genome.duke.edu/cores/proteomics/sample-preparation) and analyzed using Data Dependent Acquisition (DDA) on a nanoscale capillary LC/MS/MS system (nanoAcquity LC and SYNAPT G2 HDMS, Waters Corp). Data was processed with Mascot pipeline (Demon, Distiller, and Server 2.2 Matrix Sciences) and searched against a forward/reverse UniProt_human database (56). For quantitation of TNC concentration in milk, an internal standard of yeast alcohol dehydrogenase (Waters Corp) was added as a surrogate standard. Samples were analyzed using ion-mobility assisted Data Independent Acquisition (maDIA) on a nanoscale capillary LC/MS/MS system. Approximate mole amount was calculated using the Top 3 method (57).

[107] *TNC depletion and HIV-1 neutralization*

[108] Ten mg of anti-TNC IgG1 mAb 81C6 (58) was coupled to 600mg cyanogen bromide-activated separhose beads (GE Healthcare) and incubated with delipidized/filtered milk overnight, 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-LucR.T2A-CH040.ecto)(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 MW965 virions were incubated for 1 hour with either the colostrum HIV-neutralizing mAb CH08{Friedman, 2012 #47}, TNC (final concentration 175 μg/ml) or CH08 and TNC, then TZM-bl cells were added. For determining whether Env CD4 engagement enhanced the neutralizing activity of TNC, TZM-bl cells were incubated with HIV virions (HIV DU156.12) on ice for 10 minutes, then TNC was added (final concentration 350 μg/ml). Neutralization was measured after 48 hours as a reduction in luciferase activity as compared to 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 ($\leq$0.3 ng/ml, Limulus Amebocyte Lysate Pyrogent Plus kit, Lonza) was found to be 10 fold lower than that which mediates 80% HIV-1 neutralization in the PBMC neutralization assay (62).

[109] *HIV-1 virion capture and inhibition of epithelial cell binding*

[110] To assess the ability of TNC to capture HIV-1 virions, 0.3μg of purified breast milk proteins TNC (Millipore), lactorferrin (Sigma), or mucin-1 (Abcam) or 2G12 mAb (NIH AIDS Reagent Program) were coated on a 96-well plate and blocked with 5% bovine serum albumin (Sigma). HIV-1 Env pseudovirions (10-20 ng of Gag p24) were incubated in the well for two hours at 37°C and unbound virions were removed by washing. The bound virions were quantitated by p24 ELISA (PerkinElmer). Percent virus capture was calculated by: (p24 amount of bound virions)/(p24 amount added to the well).

[111] To determine the ability of TNC to impede infectious virus binding to colonic epithelial cells, a modified previously-reported protocol was used (63). Colonic HT-29 cells (ATCC) were grown to confluence on a 96 well flat bottom plate in Modified McCoy's 5a Medium

supplemented with 10% fetal bovine serum (FBS) and antibiotics. The HT29 cells washed once with serum-free media and treated with100µl of 50µg/ml mitomycin C for one hour to prevent further division, followed by two washes. Then 3.6 x 10^6 $TCID_{50}$ of HIV-1 C.1086 Env-IMC-LucR infectious molecular clone diluted in serum-free media was incubated together with increasing concentrations of purified TNC for one hour at 37° C, then added in quadruplicate to the colonic epithelial cell monolayer. The plates were then incubated at 37°C for four hours to allow for virion-epithelial cell binding. To determine the amount of virus bound to the epithelial cell monolayer, the monolayers were washed twice with PBS to remove free virus, and 1 x 10^4 TZM-bl reporter cells were added to each well of the virus-bound monolayer. After 48hrs, luciferase reagent (Bright-Glo, Promega) was added to the well and the relative luminescence units (RLU) were measured, representing amount of infectious virus bound to the monolayer. Percent inhibition was calculated by dividing the RLU of each well by the median RLU of epithelial-bound virus that was not preincubated with TNC. Results from two independent assays performed in quadruplicate were averaged. The anti-RSV mAb Synagis was used as a negative control, while the broadly-neutralizing anti-HIV-1 CD4 binding site mAb VRC01 (64) was used as a positive control.

[112] *Recombinant TNC expression*

[113] Recombinant TNC was expressed in BHK cells with the pNUT vector, and purified with a combination of ammonium sulfate precipitation, gel filtration and anion exchange chromatography (25). TNC was also expressed in CHO cells from the pEE14 expression vector, or in HEK cells by transient expression in serum-free media adapted from recombinant fibronectin expression and purified with ammonium sulfate precipitation and gel filtration chromatography.

[114] *HIV-1 Env binding*

[115] 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 mAb (Synagis). Binding activity of immobilized TNC was confirmed by injecting anti-TNC mAb (clone T2H5, Abcam). MN gp120 was incubated with saturating concentrations of anti-gp120 mAbs for 1 hour and injected over TNC immobilized surface. Percent blocking

was calculated by: (response with gp120 in buffer – response with gp120 bound to mAb)/ response with gp120 in buffer. 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 (697D) and anti-CD4 binding site (CH31). For CD4 induction of HIV-1 Env binding, gp120 or gp140 protein was pre-incubated with sCD4 at 1:1 molar ratio and binding to TNC measured as above. The effect of salt on TNC binding to CD4-captured Env gp120 was assessed by increasing salt concentration in the gp120 sample buffer to 250mM NaCl and either using PBS (pH7.4) or PBS (pH 7.4) with 250mM NaCl as running buffer. Binding of MN gp120 to immobilized TNC was measured as described above.

[116] **References for Example 3:**

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63. Fouda GG, *et al.* (2013) Postnatally-transmitted HIV-1 Envelope variants have similar neutralization-sensitivity and function to that of nontransmitted breast milk variants. *Retrovirology* 10:3.
64. Wu X, *et al.* (2010) Rational design of envelope identifies broadly neutralizing human monoclonal antibodies to HIV-1. *Science* 329(5993):856-861.
65. Alam SM, *et al.* (2007) The role of antibody polyspecificity and lipid reactivity in binding of broadly neutralizing anti-HIV-1 envelope human monoclonal antibodies 2F5 and 4E10 to glycoprotein 41 membrane proximal envelope epitopes. *J Immunol* 178(7):4424-4435.
66. Liao HX, *et al.* (2006) A group M consensus envelope glycoprotein induces antibodies that neutralize subsets of subtype B and C HIV-1 primary viruses. *Virology* 353(2):268-282.

[117] **EXAMPLE 4: TNC from semen and CVL samples.**

[118] Figures 15-17 show isolation and analysis of TNC from semen and CVL samples.

[119] **EXAMPLE 5:** Defining the HIV-Neutralizing Domain of Tenascin C. Figures 18-23.

[120] Infant HIV-1 transmission through breastfeeding accounts for nearly half of the 330,000 infant infections occurring annually. About 90% of infants chronically exposed to HIV-1 through breastfeeding are naturally-protected against the infection. Tenascin-C (TNC),binds the V3 loop of the HIV Envelope and mediates HIV neutralization. Provided herein are methods to identify the HIV neutralizing domain of TNC, a step in developing this innate antiviral protein as anoral prophylaxis agent that could prevent or reduce infant infection of HIV-1 acquisition.

[121] Methods:

[122] Recombinant TNC-L and TNC-S were expressed in HEK cells with the pEE14 vector and purified with ammonium sulfate precipitation and gel filtration chromatography. TNC truncation proteins were bacterially expressed with the pET11b vector and purified by ammonium sulfate precipitation and anion exchange chromatography. ELISA was used to assess the ability of truncated TNC proteins to bind to HIV-1 B.MN Envelope (Env) gp120. Surface Plasmon Resonance (Biacore 4000) was used to measure the binding kinetics of truncated TNC proteins to HIV-1 B.MN Env gp120. Plate based TNC virus capture assay utilizing p24 ELISA to detect captured HIV-1 B. MN virion was used to assess the ability of truncated TNC proteins to capture whole virions.

[123] Figure 18 is an illustration of truncated TNC proteins and electron micrograph of native TNC. Truncated proteins used to identify the HIV Env binding domain of TNC. In certain embodiments, the truncated protein includes FNIII1-8domains and fbg. Figure 19 shows relative binding of truncated TNC proteins to MN gp120 Env protein. Truncated TNC proteins

were coated on the plate and incubated with MN gp120 Env protein at 40 μg/ml. After washing, binding was measured by anti-Env mAb 16H3 by ELISA, reported in OD450.

[124] Figures 20 and 21 show the Gp120 binding domain of TNC. Figure 20 shows high affinity of FNIII regions of TNC for gp120. Gp120 Binding ELISA was used to determine the effective binding concentration 50% (EC50) of each truncated TNC protein coated on the plate to MN gp120 Env protein in solution. Anti-Env mAb 16H3 as used for detection. Figure 21 shows high binding strength of TNC FNIII1-8 domains and fbg knob to gp120 Env protein measured by surface plasmon resonance. Truncated TNC proteins were amine coupled to CM5 SPR chip, immobilized between 500-5500 RU. HIV-1 B.MN gp120 Env protein was flowed over the chip at 50 μg/ml.

[125] Figures 22 and 23 show TNC virion interaction. Figure 22 shows that TNC FNIII 1-8 domain captures whole HIV-1 virions. Truncated TNC proteins were coated on the plate and whole virions were incubated in the well. After washing, captured virions were measured using p24 ELISA. Mann Whitney U test was used to compare virus capture of each TNC truncation protein. Figure 23 is a schematic of the interaction of TNC, the HIV-1 Env gp120 protein, and the HIV-1 cellular receptors.

[126] To assess the binding of gp120 to TNC domains by SPR, truncated TNC proteins were amine coupled to CM5 SPR chip and HIV-1 B.MN gp120 Env protein was flowed over the chip. Surface plasmon resonance results showed that the FNIII1-8 domains and the fbg knob had high binding strength to gp120 Env protein. Truncated TNC proteins were also coated onto a plate and incubated with whole virions and captured virions were measured using p24 ELISA. Results showed that the FNIII1-8 domain captured whole virions compared to other truncated proteins. Finally, a gp120 Env binding assay was developed by coating truncated TNC proteins and incubated with gp120 Env protein and detected with anti-Env mAb 16H3. This gp120 binding assay showed that the FNIII regions of TNC have high affinity for gp120 Env. These assays suggest that the FNIII1-8 domains of TNC are possible binding sites to gp120 and that there could also be multiple binding sites.

[127] In certain embodiments, ELISA and SPR data suggests that the FNIII1-8 domains are binding sites for gp120. In other embodiments, virus capture assay shows that HIV-1 virions bind to the FNIII1-8 of TNC. In certain embodiments, different TNC domains, implicated by

each assay, suggest that there are multiple binding sites or combinations of TNC domains that bind virions.

[128] Defining the HIV-1 Envelope (Env) neutralizing epitope that is bound by TNC

[129] We also have worked to define the neutralizing epitope of TNC, employing panels of anti-HIV-1 Env mAbs, Env peptides, and Env mutants to identify the HIV-1 Env epitope that interacts with TNC, potentially revealing a unique neutralizing epitope of HIV-1 which can inform vaccine immunogen design. We have amine coupled a gp70-scaffolded and linear V3 peptide to an SPR chip and found TNC binds to this antigen, confirming the V3 specificity of TNC's Env binding. Also, we have obtained linear, overlapping V3 peptides to be able to map TNC binding to a specific V3 amino acid sequence.

[130] Establishing the effect of endogenous TNC in breast milk on the risk of infant HIV acquisition in a cohort of HIV-infected lactating women

[131] We also aim to determine the concentration and function of TNC in milk of a large cohort of postnatal-transmitting and nontransmitting HIV-1-infected women and determine if the endogenous TNC concentration in milk correlates with the risk of postnatal HIV-1 transmission.

[132] To measure the concentration of TNC in breast milk, we developed a "sandwich" ELISA where we coated with an anti-TNC antibody to capture the TNC in the breast milk and detected with T2H5, and anti-TNC detection antibody. We have seen that the amount of TNC is donor dependent and varies between women. However, TNC amount is consistently higher in milk donated immediately after birth and rapidly decreases with time postpartum. Neutralizing activity in breast milk was measured using a TZM-bl cell based assay. We are currently assessing the correlation between TNC concentration in breast milk and neutralizing activity as well as the amount and function of TNC in women who transmit and do not transmit to their infants. To measure the function of TNC to block virion CCR5-binding, we are currently developing a cell based binding assay which uses cells that express CCR5, but do not express CD4. In this assay, we incubate a monolayer of cells with CD4-Ig to open the CD4 binding site, and then incubate with TNC. Then we added HIV-1 to allow the virus to bind to the assay and unbound virus are washed out and bound virus are quantified using p24 ELISA.

* * *

All documents and other information sources cited herein are hereby incorporated in their entirety by reference.

Table _ 1: HIV-1 neutralizing activity of breast milk of uninfected and HIV-infected women and purified TNC

	clade B T/F			chronic clade C		clade C infant T/F			chronic clade AE	nonhuman retroviruses	
	CH40 (tier 2)	CH77 (tier 2)	CH058 (tier 2)	MW965 (tier 1)	DU156.12 (tier 2)	BF1677 (tier 2)	BF329 (tier 2)	BF942 (tier 2)	CM235 (tier 2)	SVA.MLV	SIV mac251
HIV- milk											
1*	<3	10	3	7	4	5	5	7	3	<3	3
2			9	9	5	8	8	9	8	<3	10
3				10	8	<3	3	4	6	<3	8
4						3	3	4	4	<3	6
5	9			7	9	4	4	5	2	<3	<3
6	6	5	5	6	9	<3	<3	<3	<3	<3	<3
7	<3	10	9	3	8	<3	3	<3	<3	<3	<3
8	5		5	3	3	<3	3	<3	<3	<3	<3
9	6	9	5	7		<3	5	<3	3	3	<3
10	5		6	3		3	3	<3	<3	8	<3
Purified protein											
TNC#	122	118	116	120	110		110	100	113		>180
lactoferrin	>250		>250	>250	>250	>250	>250			>250	>250

ID_{50} 3-5 or IC_{50} 150-180
ID_{50} 5-10 or IC_{50} 110-150
ID_{50} >10 or IC_{50} <110
Not done

* = numbers indicate inhibitory dilution 50% (ID_{50})
# = inhibitory concentration 50% (IC_{50}, µg/ml)

What is claimed is:

1. An anti-microbial composition comprising recombinant Tenacin (TNC) or a fragment thereof.
2. The composition of claim 1, comprising a TNC fragment described in Figure 18.
3. A method to prevent HIV-1virus infection comprising administering to a subject in need thereof the composition of claim 1 or 2 in an amount sufficient such that the virus entry into the subject's cell is blocked.
4. A method to prevent HIV-1 virus entry in a cell comprising contacting a cell with the composition of claim 1 or 2 whereby TNC or the fragment thereof binds to the CD4 coreceptor site on the HIV-1 envelope protein and blocks the virus entry into the cell.
5. The method of claim 4, wherein the cell is an epithelial cell.
6. The method of claim 3 to 5 wherein the composition is administered orally or at mucosal sites.

Figure 1

A

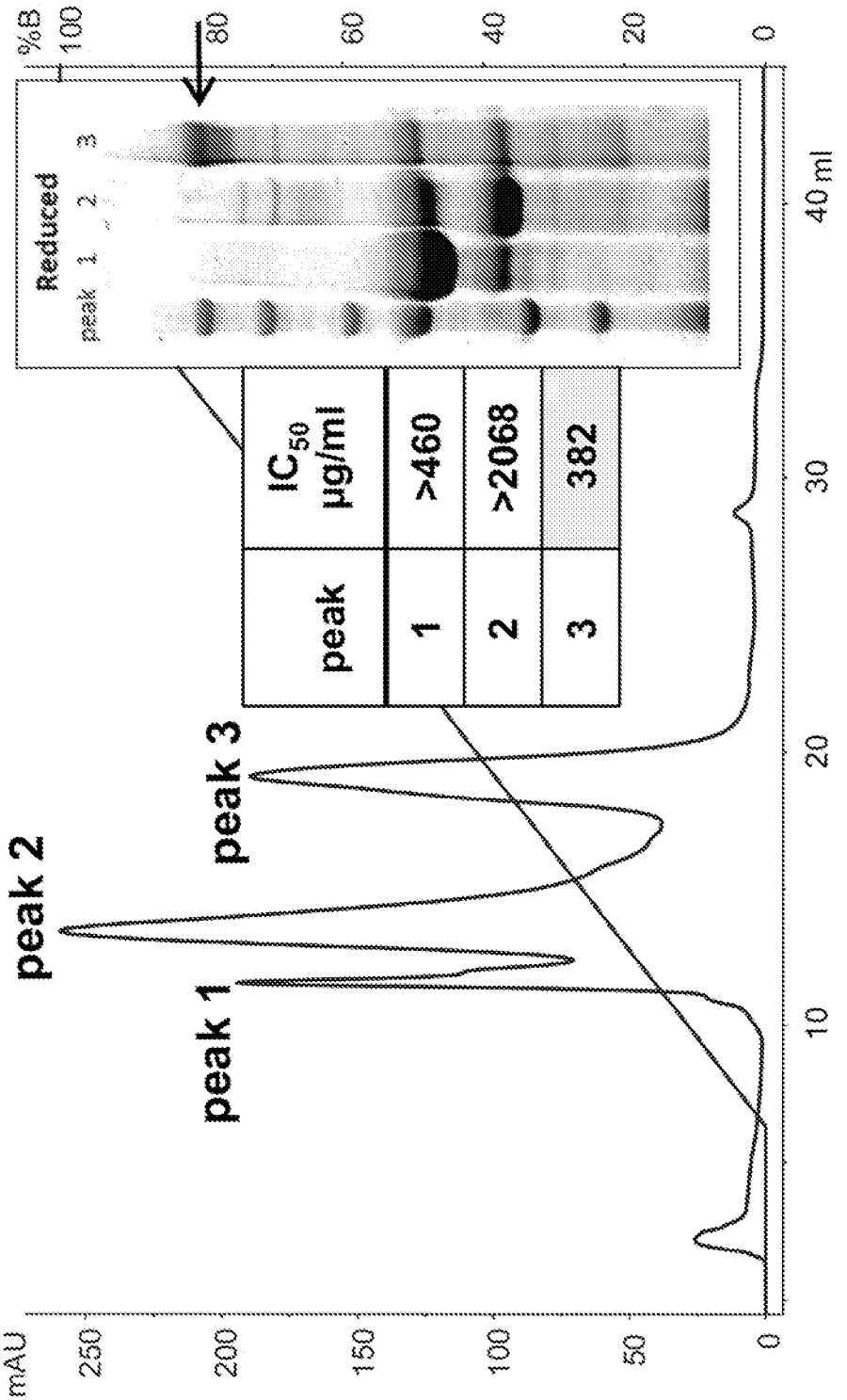

%B
100
80
60
40
20
0
Reduced
peak 1 2 3
mAU
250
200
150
100
50
0
peak 2
peak 3
peak 1
10
20
30
40 ml
peak
IC50 µg/ml
1
>460
2
>2068
3
382

Figure 1

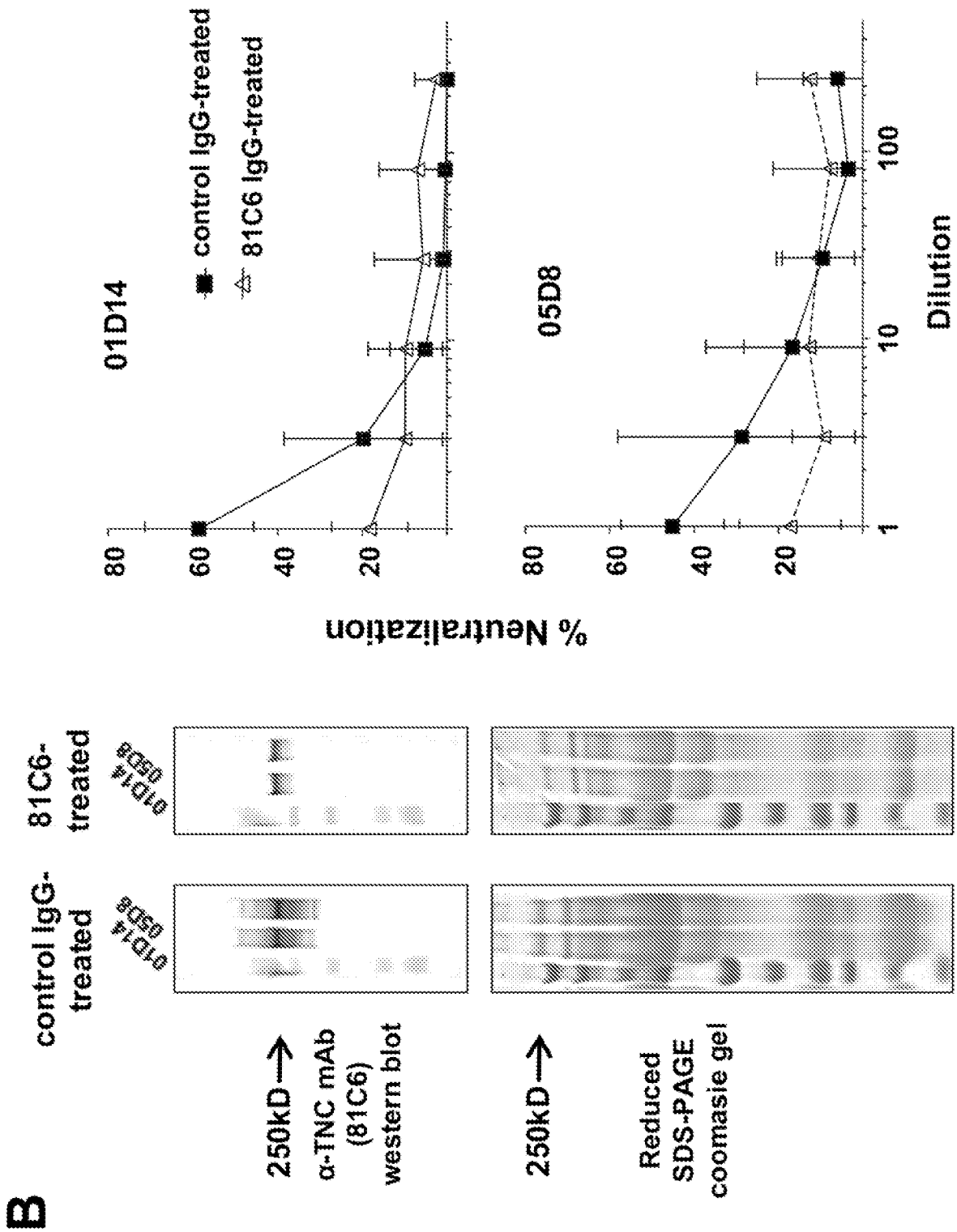

% Neutralization
01D14
05D8
control IgG-treated
81C6 IgG-treated
Dilution
1
10
100
20
40
60
80
B
control IgG-treated
81C6-treated
01D14
05D8
250kD
α-TNC mAb (81C6) western blot
Reduced SDS-PAGE coomasie gel

Figure 1

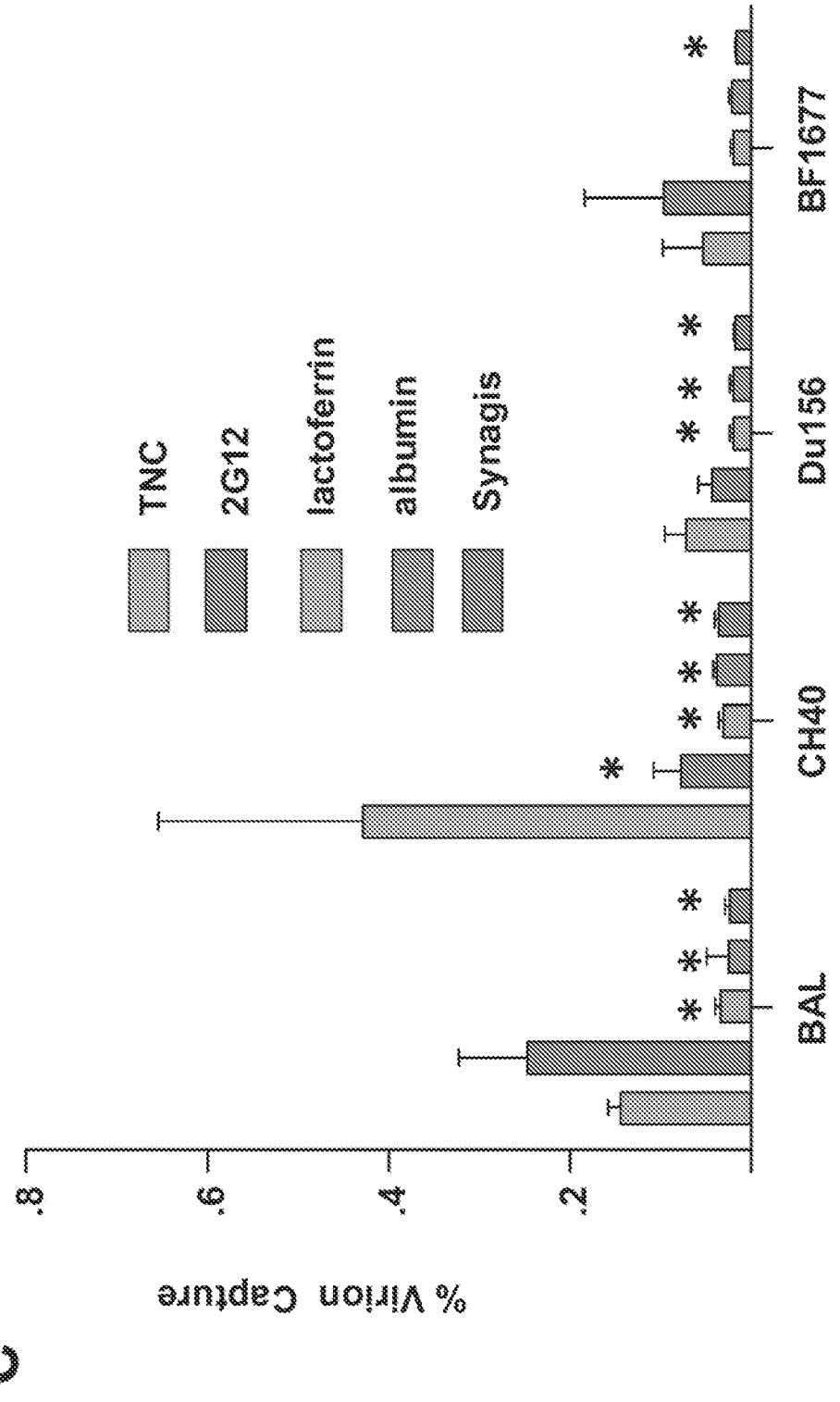

C
% Virion Capture
.8
.6
.4
.2
TNC
2G12
lactoferrin
albumin
Synagis
BAL
CH40
Du156
BF1677
*

Figure 1

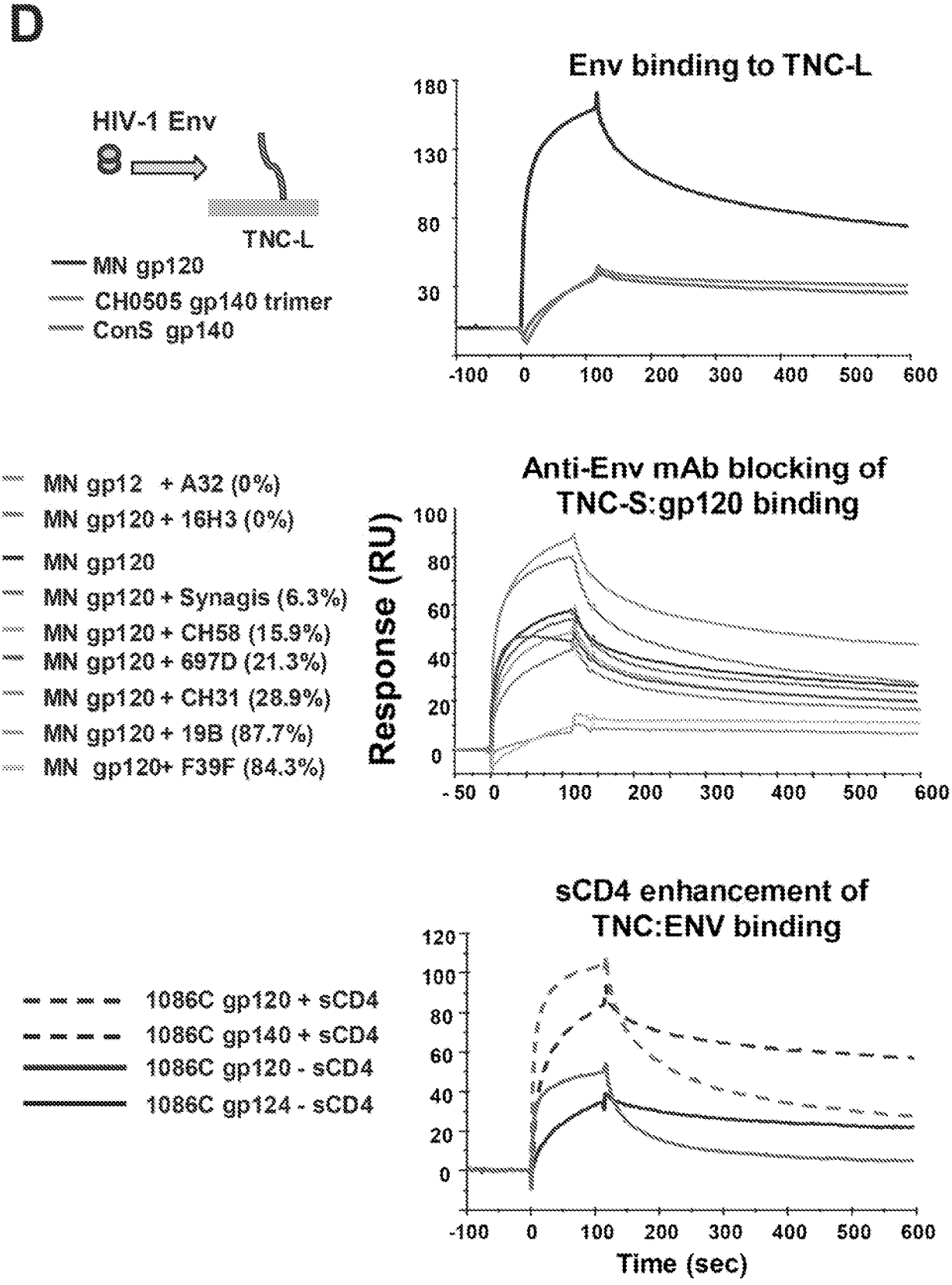
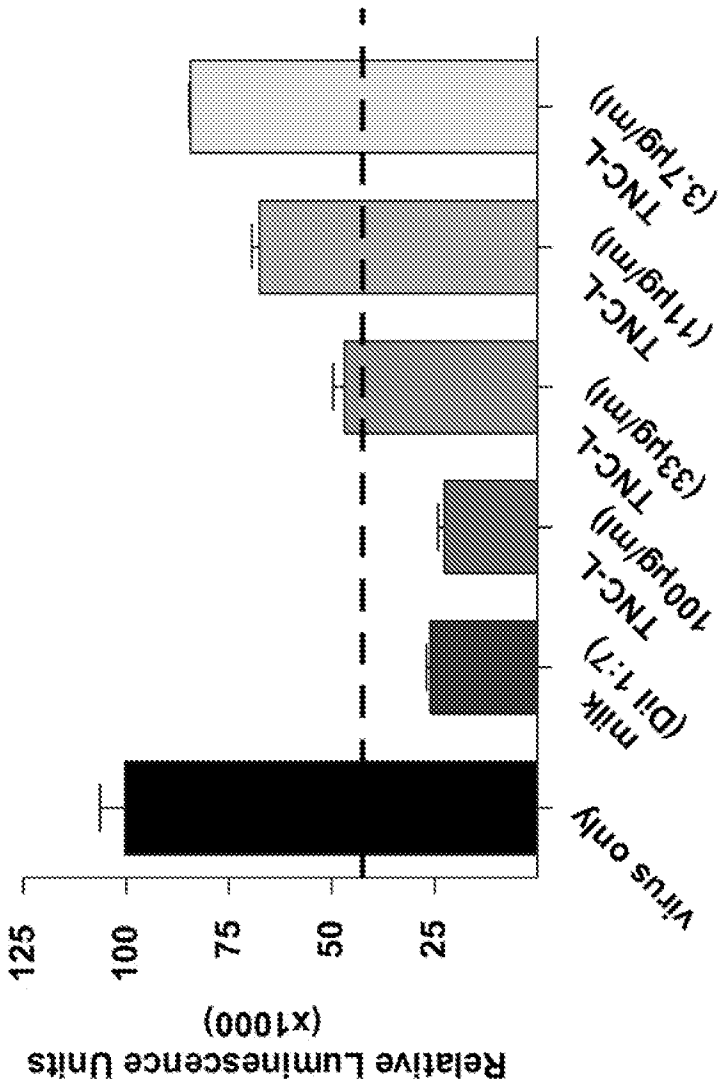

D
HIV-1 Env
TNC-L
MN gp120
CH0505 gp140 trimer
ConS gp140
Env binding to TNC-L
180
130
80
30
-100
0
100
200
300
400
500
600
MN gp12 + A32 (0%)
MN gp120 + 16H3 (0%)
MN gp120
MN gp120 + Synagis (6.3%)
MN gp120 + CH58 (15.9%)
MN gp120 + 697D (21.3%)
MN gp120 + CH31 (28.9%)
MN gp120 + 19B (87.7%)
MN gp120+ F39F (84.3%)
Anti-Env mAb blocking of
TNC-S:gp120 binding
Response (RU)
100
80
60
40
20
0
- 50
0
100
200
300
400
500
600
1086C gp120 + sCD4
1086C gp140 + sCD4
1086C gp120 - sCD4
1086C gp124 - sCD4
sCD4 enhancement of
TNC:ENV binding
120
100
80
60
40
20
0
-100
0
100
200
300
400
500
600
Time (sec)

Figure 2

The structure and functions of a novel HIV-1 inhibitor isolated from breast milk, the extracellular matrix protein Tenascin C (TNC)

Figure 3



TNC-L neutralizes chronic HIV-1 variant Du156 in a dose dependent fashion
HIV-1 Du156 replication in TZM-bl cells is reduced in the presence of increasing concentrations of purified TNC.

Figure 4A

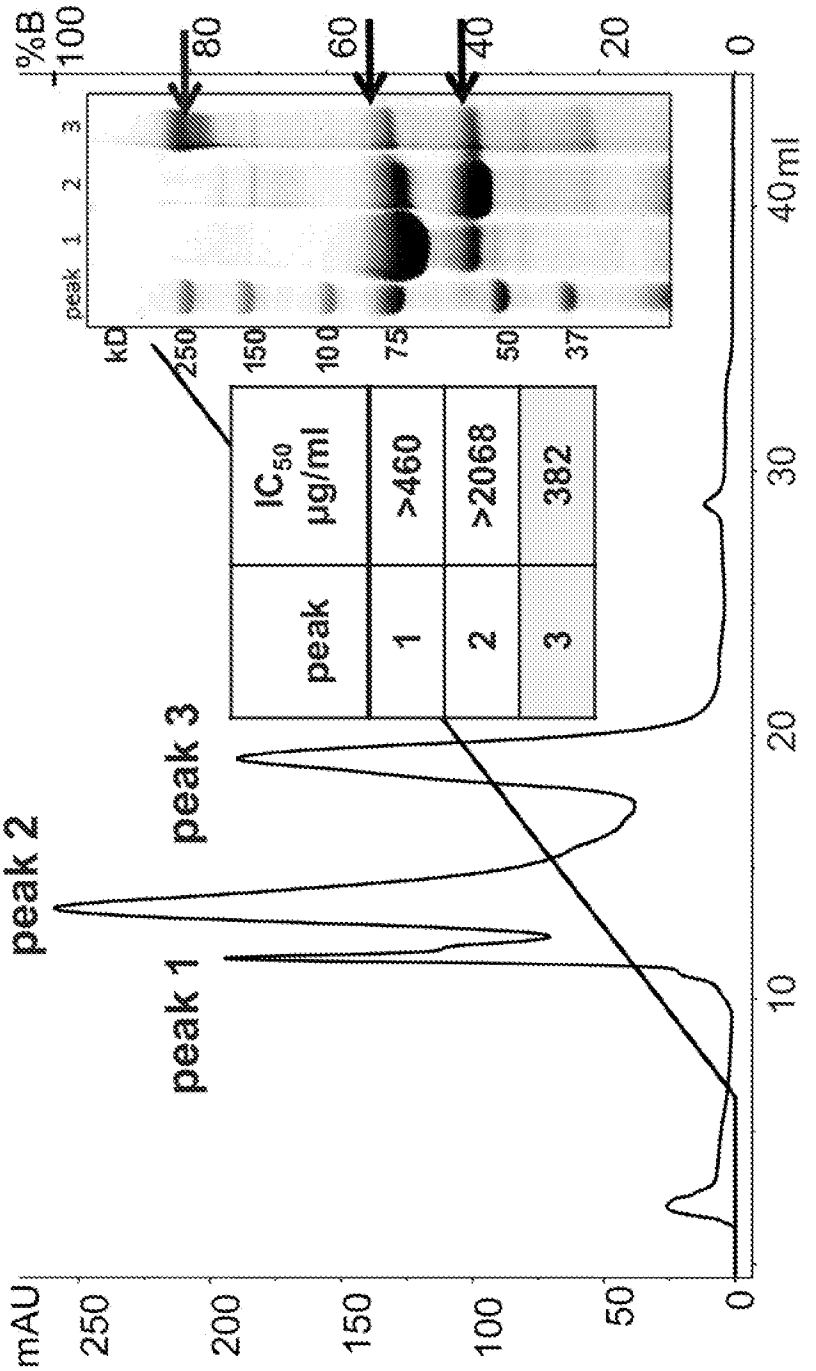

mAU
250
200
150
100
50
0
peak 2
peak 1
peak 3
10
20
30
40ml
%B
100
80
60
40
20
0
peak
1
2
3
kD
250
150
100
75
50
37
peak
IC50 µg/ml
1
>460
2
>2068
3
382

Figure 4B

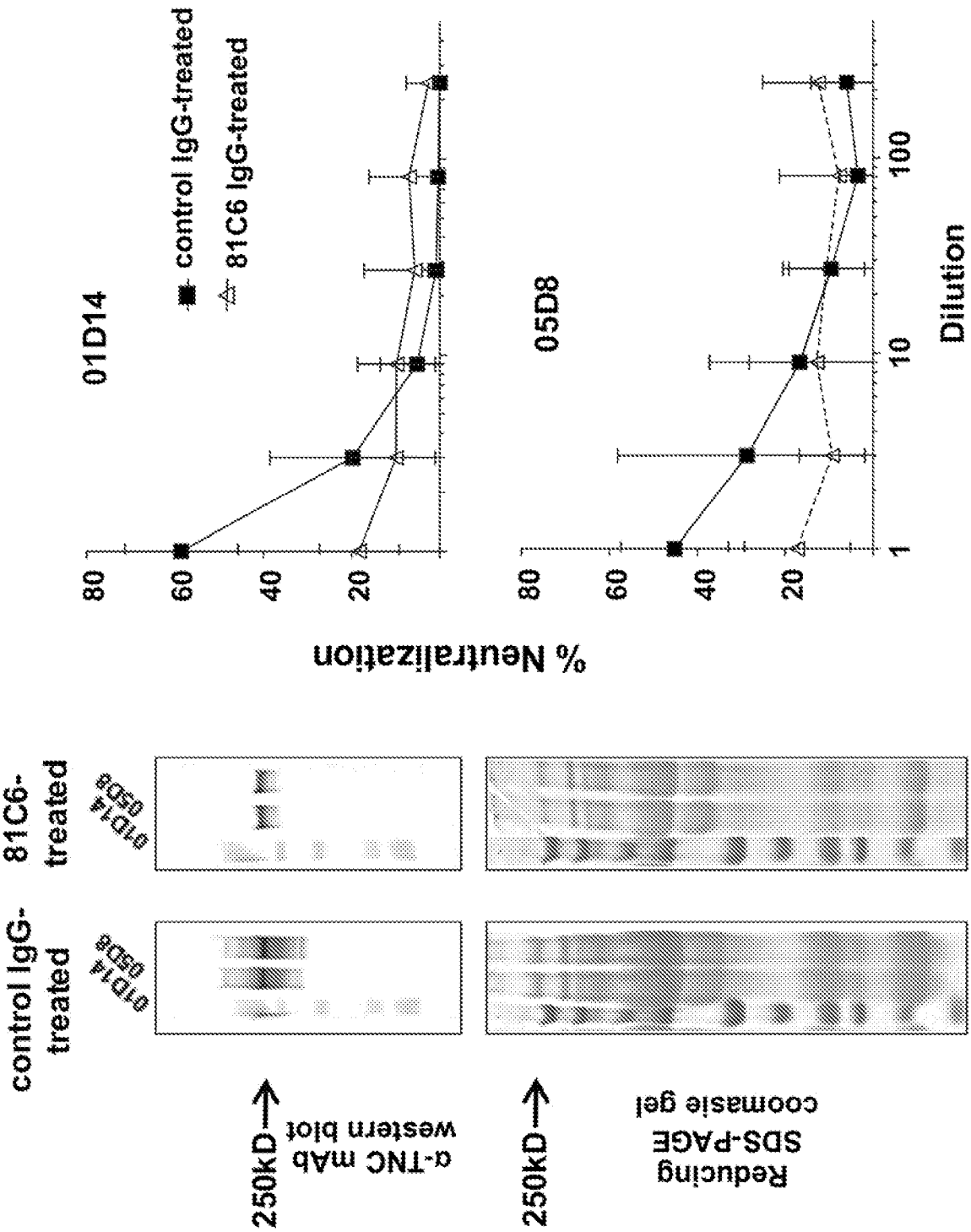

01D14
05D8
control IgG-treated
81C6 IgG-treated
% Neutralization
80
60
40
20
1
10
100
Dilution
control IgG-treated
81C6-treated
250kD
α-TNC mAb western blot
Reducing SDS-PAGE coomasie gel

Figure 5

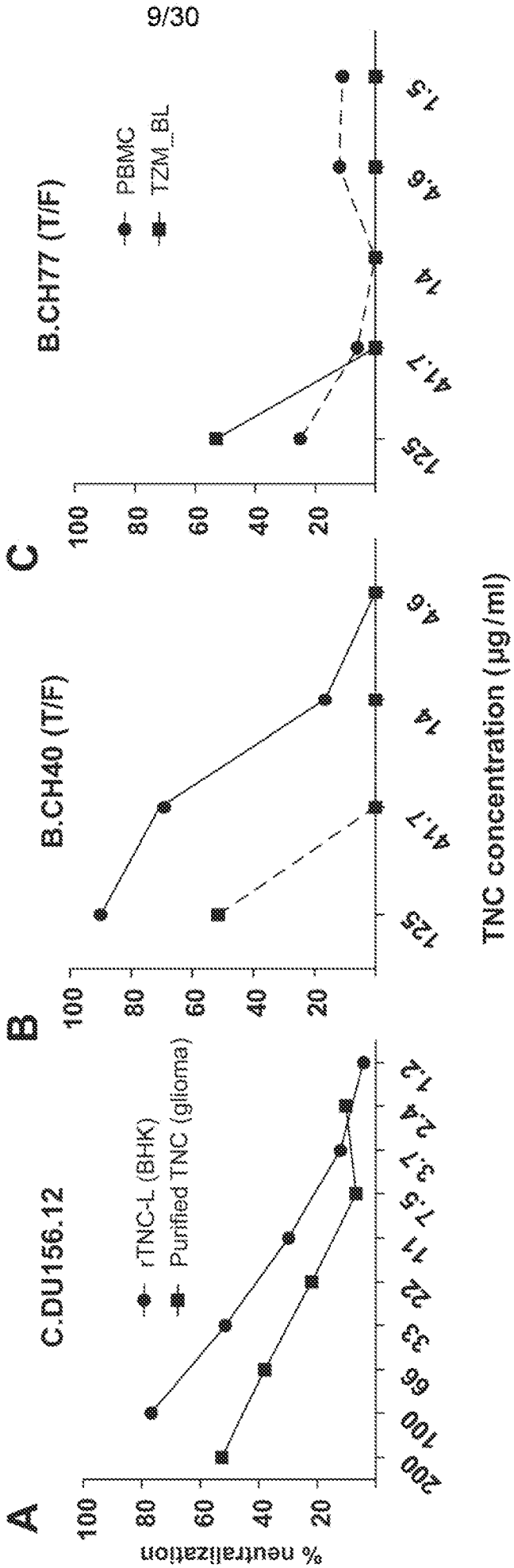

A
C.DU156.12
% neutralization
rTNC-L (BHK)
Purified TNC (glioma)
B
B.CH40 (T/F)
C
B.CH77 (T/F)
PBMC
TZM_BL
TNC concentration (µg/ml)

Figure 6

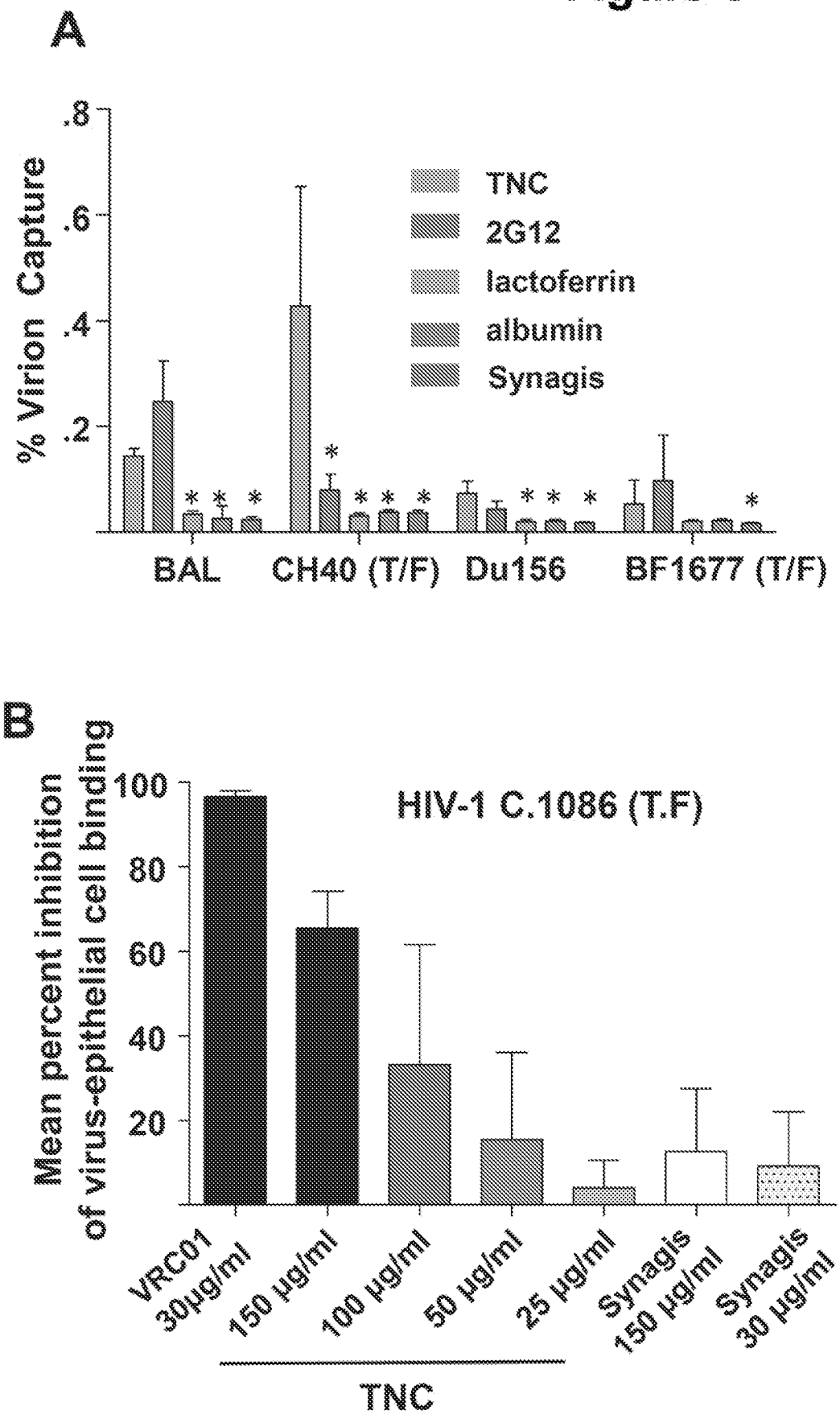

A
% Virion Capture
.8
.6
.4
.2
TNC
2G12
lactoferrin
albumin
Synagis
BAL
CH40 (T/F)
Du156
BF1677 (T/F)
B
Mean percent inhibition of virus-epithelial cell binding
100
80
60
40
20
HIV-1 C.1086 (T.F)
VRC01 30µg/ml
150 µg/ml
100 µg/ml
50 µg/ml
25 µg/ml
Synagis 150 µg/ml
Synagis 30 µg/ml
TNC

Figure 7

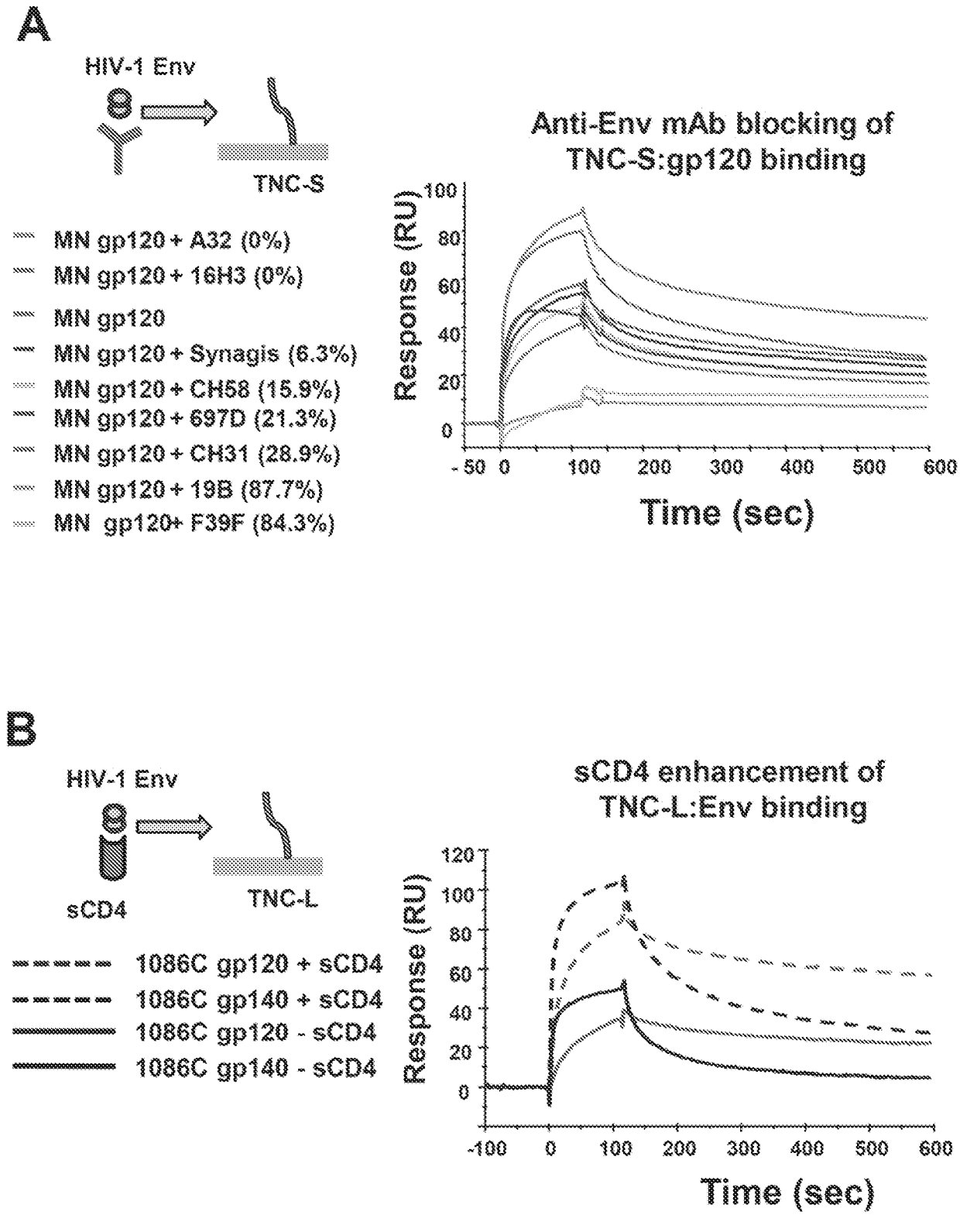

A
HIV-1 Env
TNC-S
MN gp120 + A32 (0%)
MN gp120 + 16H3 (0%)
MN gp120
MN gp120 + Synagis (6.3%)
MN gp120 + CH58 (15.9%)
MN gp120 + 697D (21.3%)
MN gp120 + CH31 (28.9%)
MN gp120 + 19B (87.7%)
MN gp120+ F39F (84.3%)
Anti-Env mAb blocking of TNC-S:gp120 binding
Response (RU)
100
80
60
40
20
0
- 50
0
100
200
300
400
500
600
Time (sec)
B
HIV-1 Env
sCD4
TNC-L
1086C gp120 + sCD4
1086C gp140 + sCD4
1086C gp120 - sCD4
1086C gp140 - sCD4
sCD4 enhancement of TNC-L:Env binding
Response (RU)
120
100
80
60
40
20
0
-100
0
100
200
300
400
500
600
Time (sec)

Figure 7

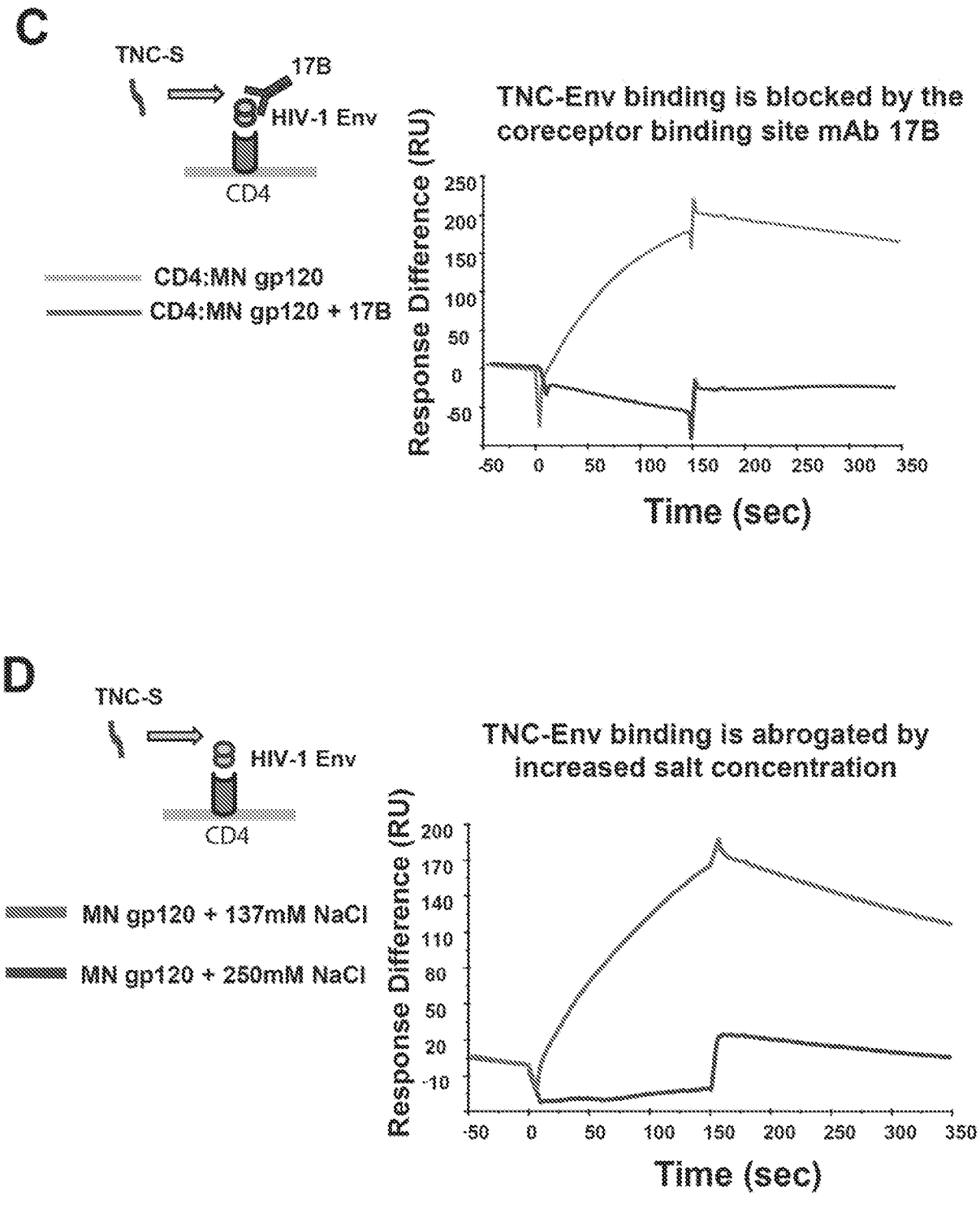

C
TNC-S
17B
HIV-1 Env
CD4
CD4:MN gp120
CD4:MN gp120 + 17B
TNC-Env binding is blocked by the coreceptor binding site mAb 17B
Response Difference (RU)
250
200
150
100
50
0
-50
-50 0 50 100 150 200 250 300 350
Time (sec)
D
TNC-S
HIV-1 Env
CD4
MN gp120 + 137mM NaCl
MN gp120 + 250mM NaCl
TNC-Env binding is abrogated by increased salt concentration
Response Difference (RU)
200
170
140
110
80
50
20
-10
-50 0 50 100 150 200 250 300 350
Time (sec)

Figure 8

	chronic clade B		clade B T/F			chronic clade C		clade C infant T/F			clade C T/F	chronic clade AE	nonhuman retroviruses	
	MN tier 1A X4	YU2 tier 1B R5	CH40 tier 2 R5	CH77 tier 2 R5	CH058 tier 2 R5	MW965 tier 1 R5	DU156.12 tier 2 R5	BF1677 tier 2 R5	BF329 tier 2 R5	BF042 tier 2 R5	1086C tier 2 R5	CM235 tier 2 R5	SVA MLV	SIV mac251
HIV- milk														
1*	< 3	3	<3	10	3	7	4	5	5	7		3	<3	3
2	< 3	3	15	26	9	9	5	8	8	9		8	<3	10
3	3	3	25	27	14	10	8	<3	3	4		6	<3	8
4	< 3	5	17	30	13	32	33	3	3	4		4	<3	6
5	< 3	<3	9	15	12	7	9	4	4	5		2	<3	<3
6	< 3	< 3	6	5	5	6	9	<3	<3	<3		<3	<3	<3
7	< 3	3	<3	10	9	3	8	<3	3	<3		<3	<3	<3
8	< 3	3	5	15	5	3	3	<3	3	<3		<3	<3	<3
9	< 3	<3	6	9	5	7	24	<3	5	<3		3	3	<3
10			5	15	6	3	20	3	3	<3		<3	6	<3
Purified protein														
TNC*	130	158	122	116	116	120	110	108	110	100	92	113	109	>180
lactoferrin	>300		>300		>300	>300	>300	>300	>300				>300	
Mucin-1	>300		>300		>300	>300	>300	>300	>300				>300	

ID_{50} 3-5 or IC_{50} 150-180
ID_{50} 5-10 or IC_{50} 110-150
ID_{50} >10 or IC_{50} <110
Not done

* = numbers indicate inhibitory dilution 50% (ID_{50})
# = inhibitory concentration 50% (IC_{50}, µg/ml)

Figure 9

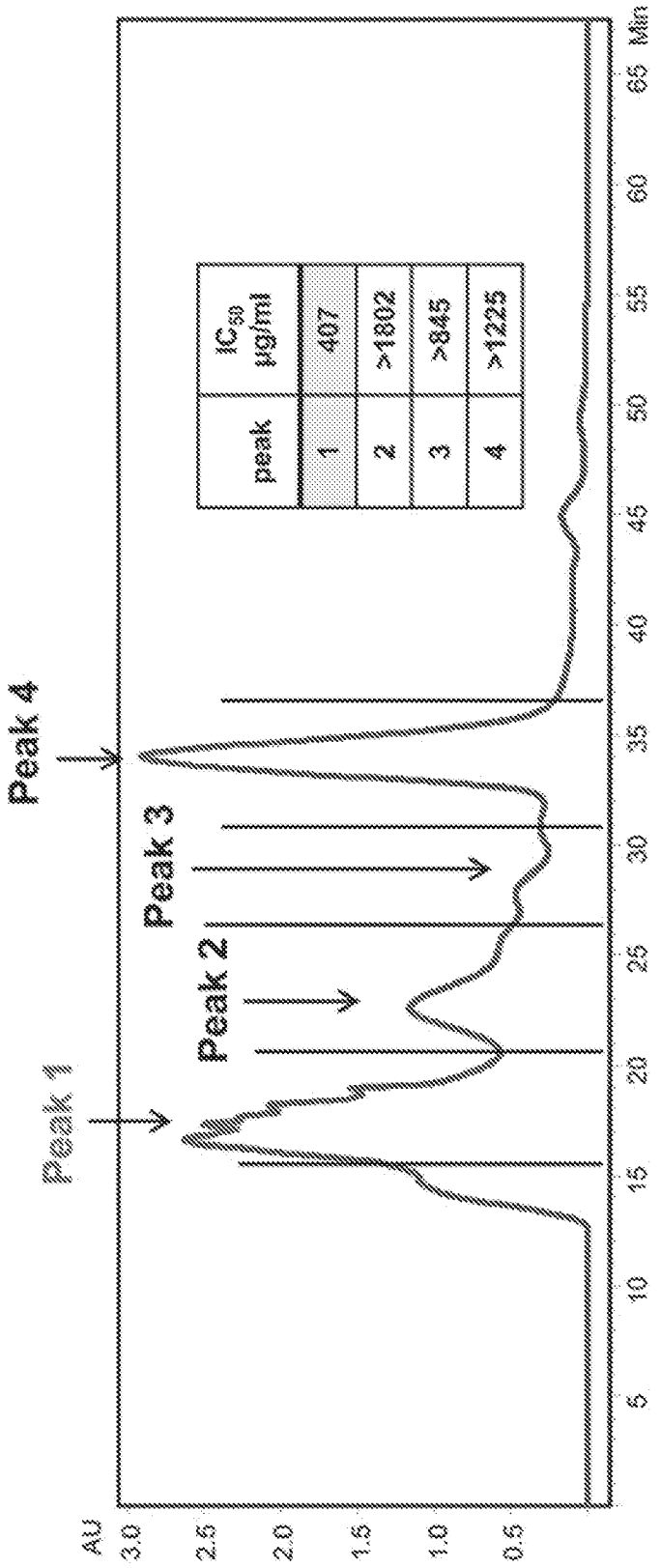

Peak 4
Peak 3
Peak 2
Peak 1
AU
3.0
2.5
2.0
1.5
1.0
0.5
5
10
15
20
25
30
35
40
45
50
55
60
65
Min
peak
IC50 µg/ml
1
407
2
>1802
3
>845
4
>1225

Figure 10

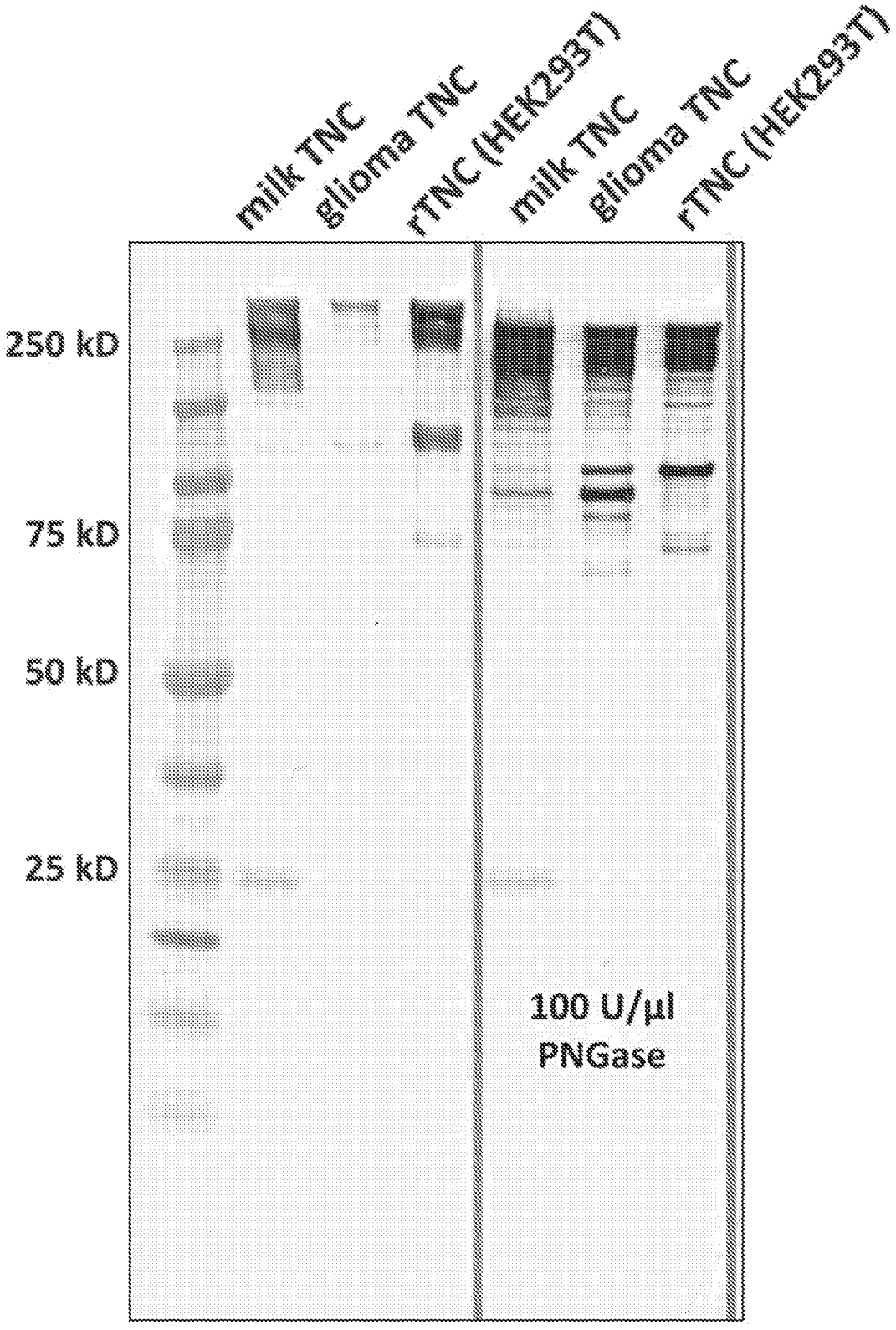

milk TNC
glioma TNC
rTNC (HEK293T)
milk TNC
glioma TNC
rTNC (HEK293T)
250 kD
75 kD
50 kD
25 kD
100 U/µl
PNGase

Figure 11A

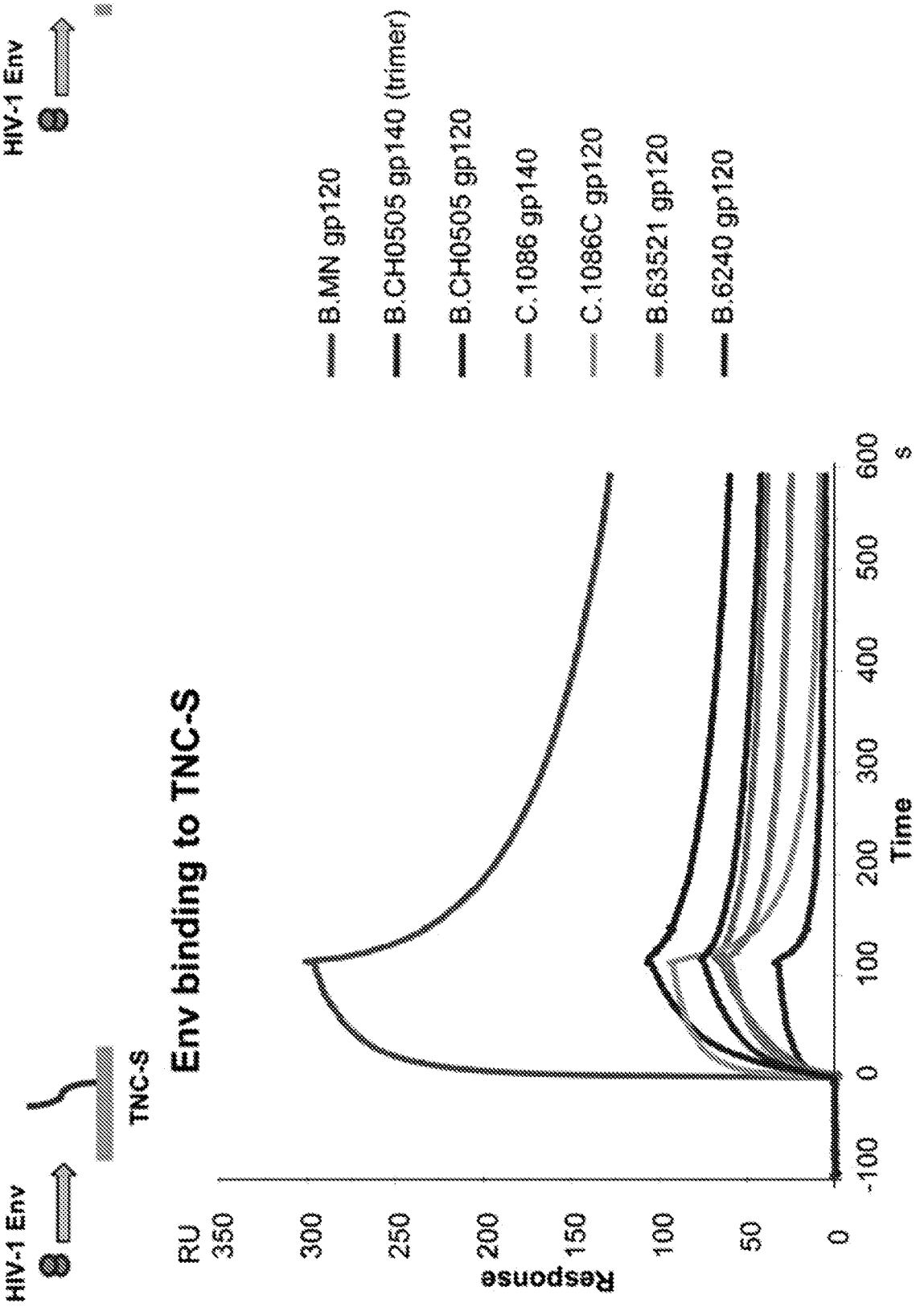
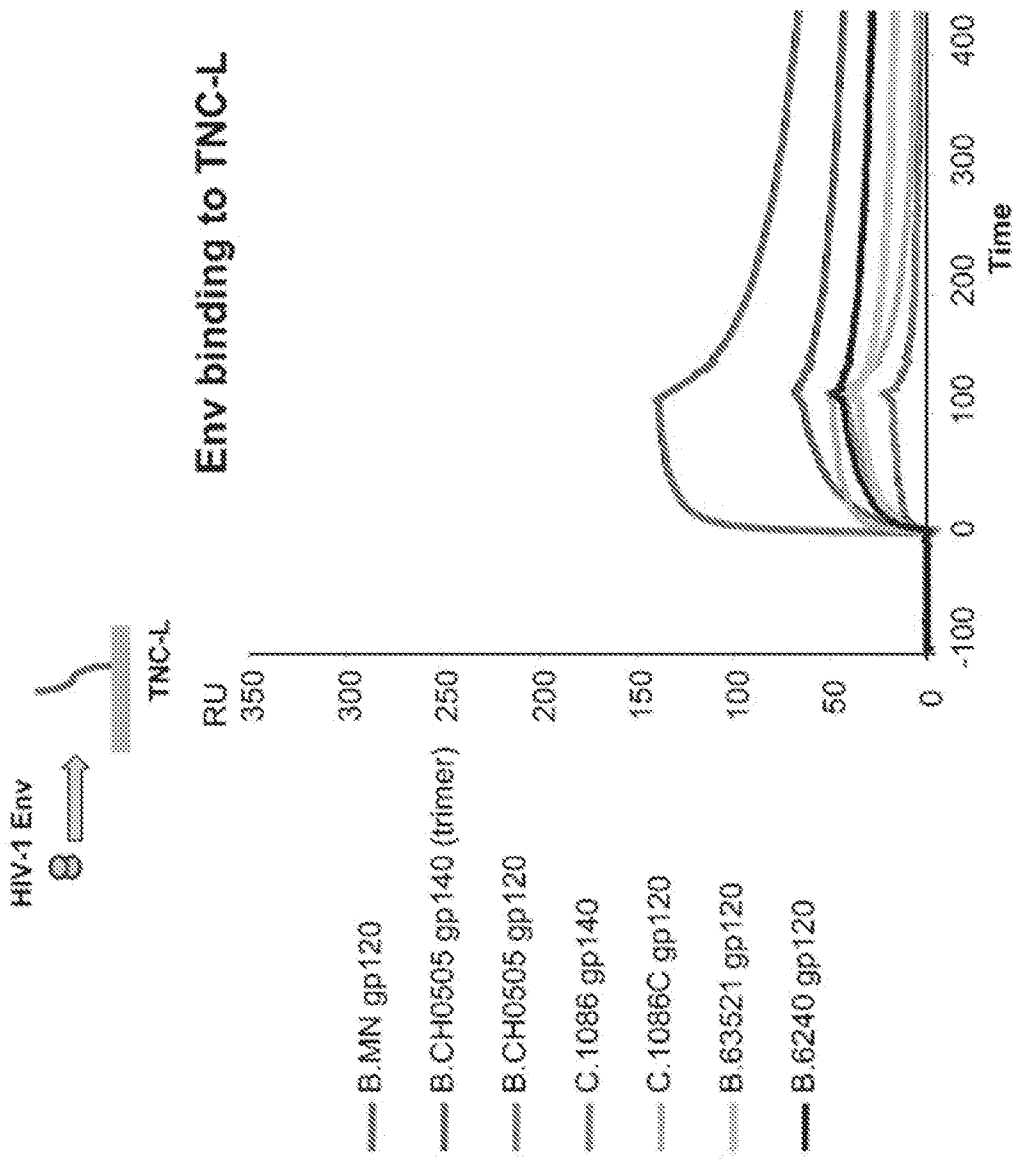
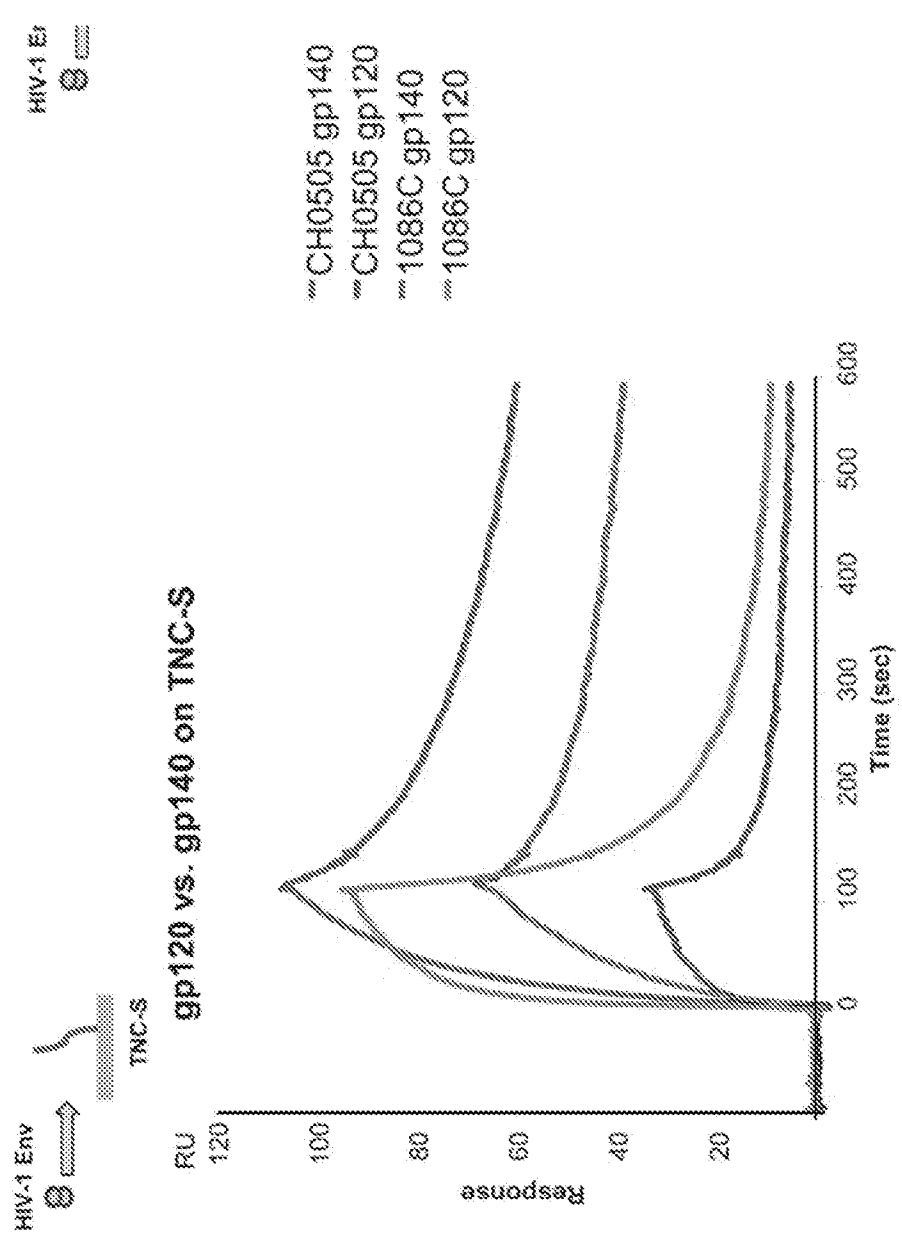

HIV-1 Env
TNC-S
HIV-1 Env
Env binding to TNC-S
B.MN gp120
B.CH0505 gp140 (trimer)
B.CH0505 gp120
C.1086 gp140
C.1086C gp120
B.63521 gp120
B.6240 gp120
RU
350
300
250
200
150
100
50
0
Response
-100
0
100
200
300
400
500
600
Time
s

Figure 11B

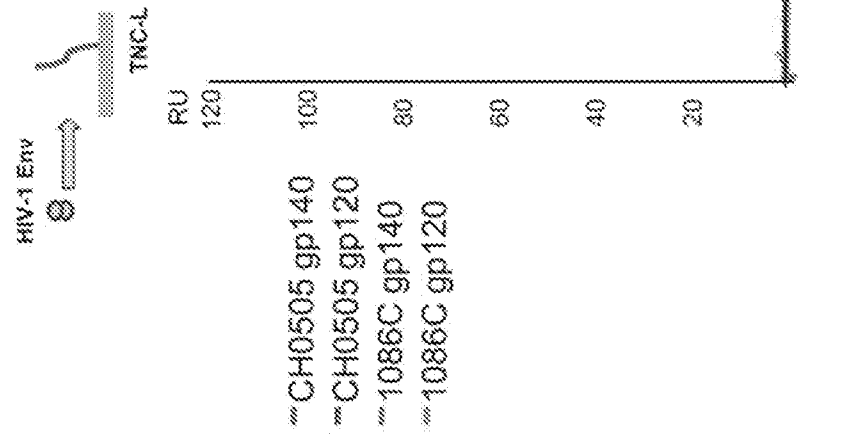

HIV-1 Env
TNC-L
Env binding to TNC-L
RU
350
300
250
200
150
100
50
0
-100
0
100
200
300
400
Time
B.MN gp120
B.CH0505 gp140 (trimer)
B.CH0505 gp120
C.1086 gp140
C.1086C gp120
B.63521 gp120
B.6240 gp120



Env	Dissociation rate constant (k_d, $s^{-1} \times 10^{-3}$)
CH0505 gp120	4.5
CH0505 gp140	1.56
1086 gp120	9.1
1086 gp140	1.55

Figure 12A

Figure 12B



Env	Dissociation rate constant (k_d, s^{-1} x10^{-3})
CH0505 gp120	5.97
CH0505 gp140	1.1
1086 gp120	7.1
1086 gp140	1.2

Figure 13

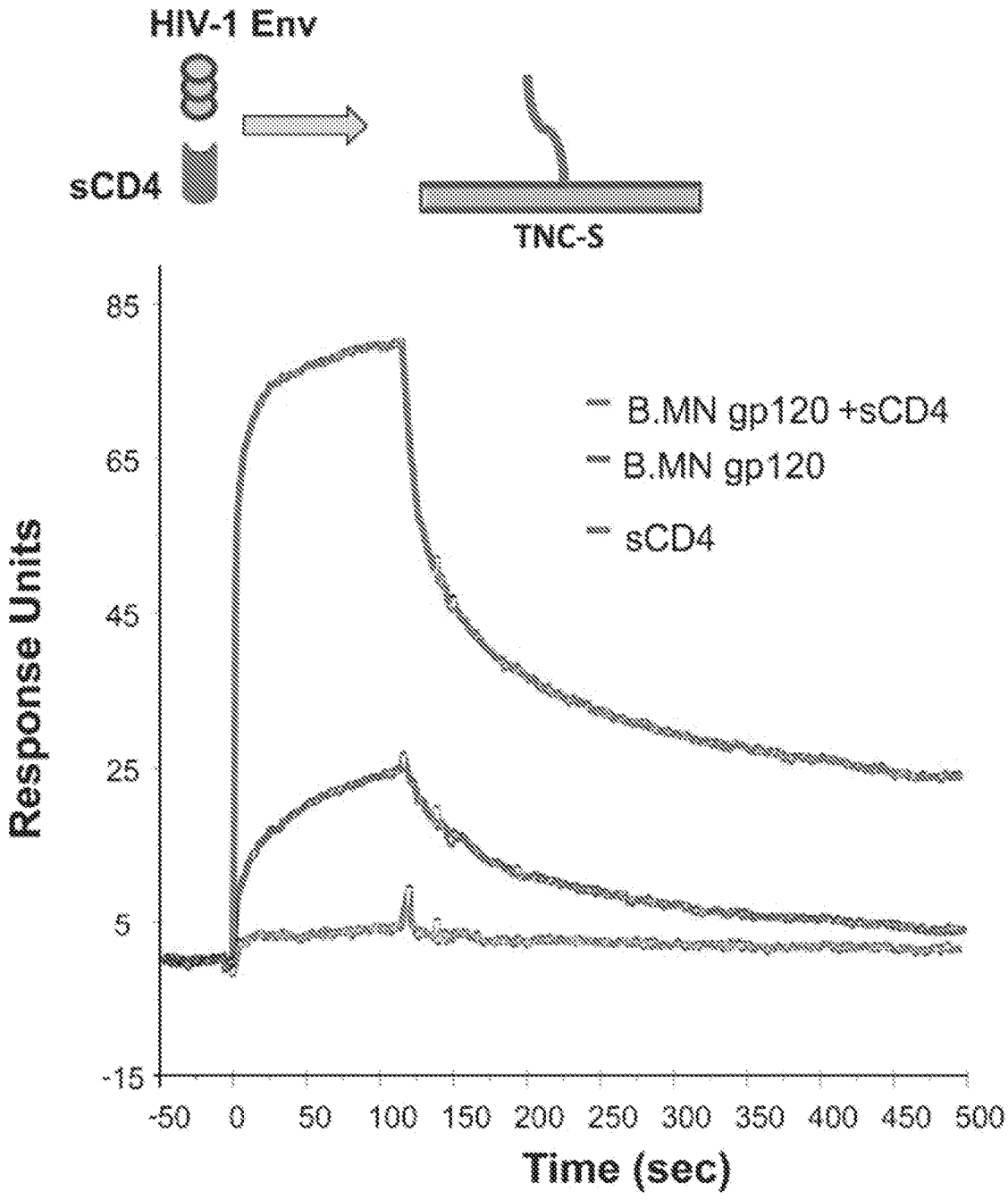

HIV-1 Env
sCD4
TNC-S
B.MN gp120 +sCD4
B.MN gp120
sCD4
Response Units
85
65
45
25
5
-15
-50
0
50
100
150
200
250
300
350
400
450
500
Time (sec)

Figure 14

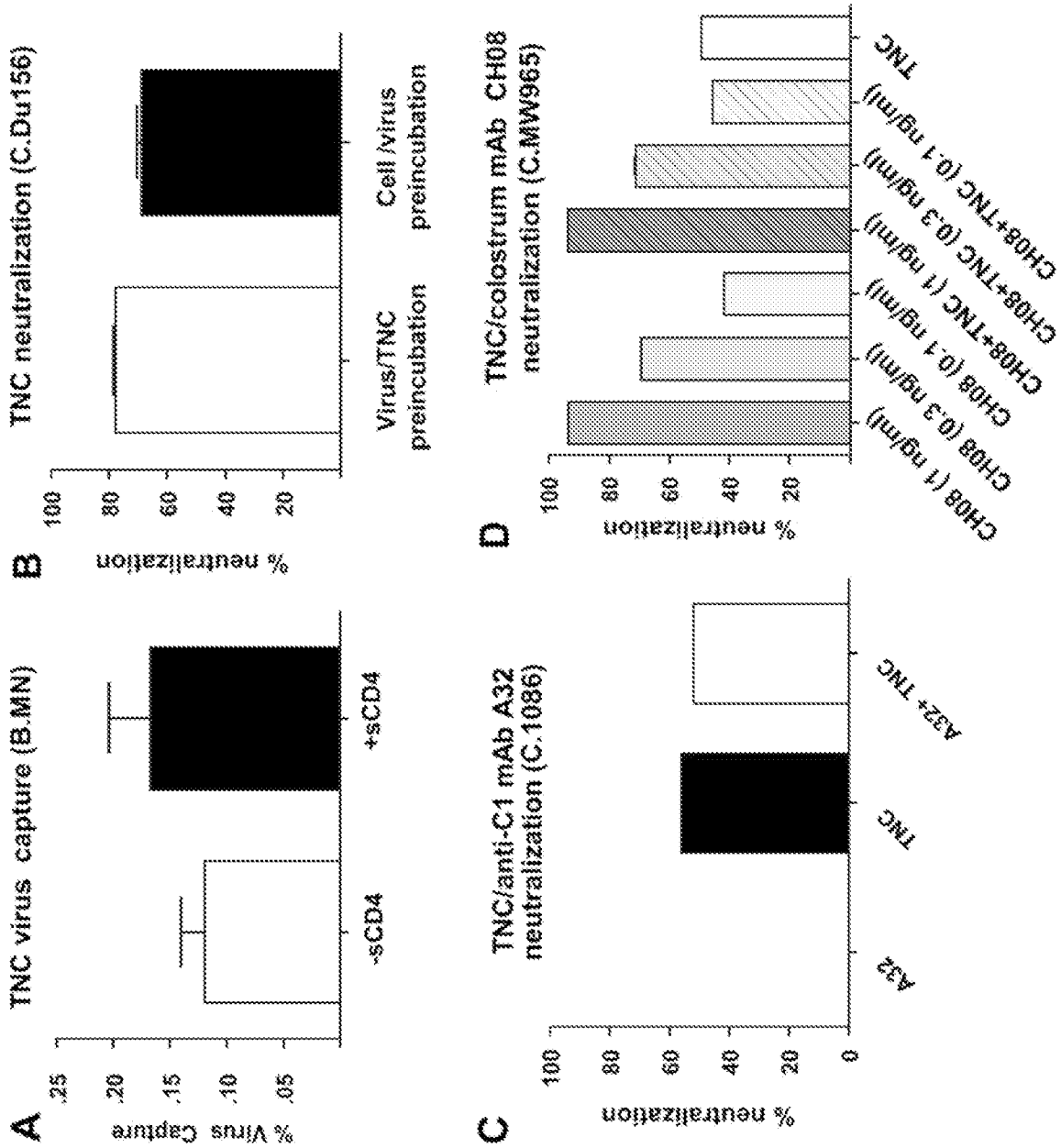

A
TNC virus capture (B.MN)
% Virus Capture
.25
.20
.15
.10
.05
-sCD4
+sCD4
B
TNC neutralization (C.Du156)
% neutralization
100
80
60
40
20
Virus/TNC preincubation
Cell /virus preincubation
C
TNC/anti-C1 mAb A32 neutralization (C.1086)
% neutralization
100
80
60
40
20
0
A32
TNC
A32+ TNC
D
TNC/colostrum mAb CH08 neutralization (C.MW965)
% neutralization
100
80
60
40
20
CH08 (1 ng/ml)
CH08 (0.3 ng/ml)
CH08 (0.1 ng/ml)
CH08+TNC (1 ng/ml)
CH08+TNC (0.3 ng/ml)
CH08+TNC (0.1 ng/ml)
TNC

Figure 15

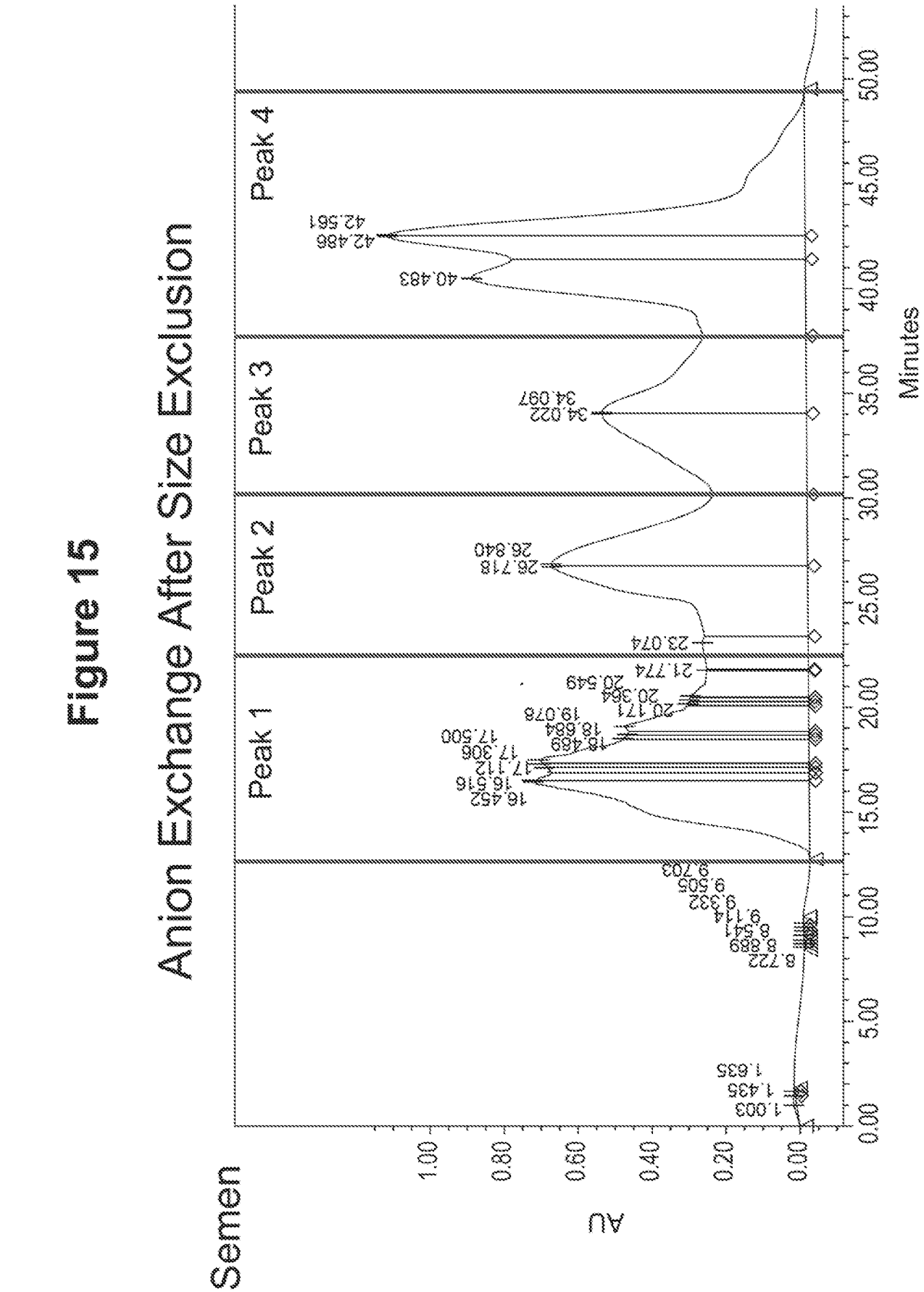

Anion Exchange After Size Exclusion
Semen
AU
Minutes
Peak 1
Peak 2
Peak 3
Peak 4
1.003
1.435
1.635
8.722
8.889
9.541
9.114
9.332
9.505
9.703
16.452
16.516
17.112
17.306
17.500
18.489
18.684
19.078
20.171
20.364
20.549
21.774
23.074
26.718
26.840
34.022
34.097
40.483
42.486
42.561

Figure 15 (Cont'd)

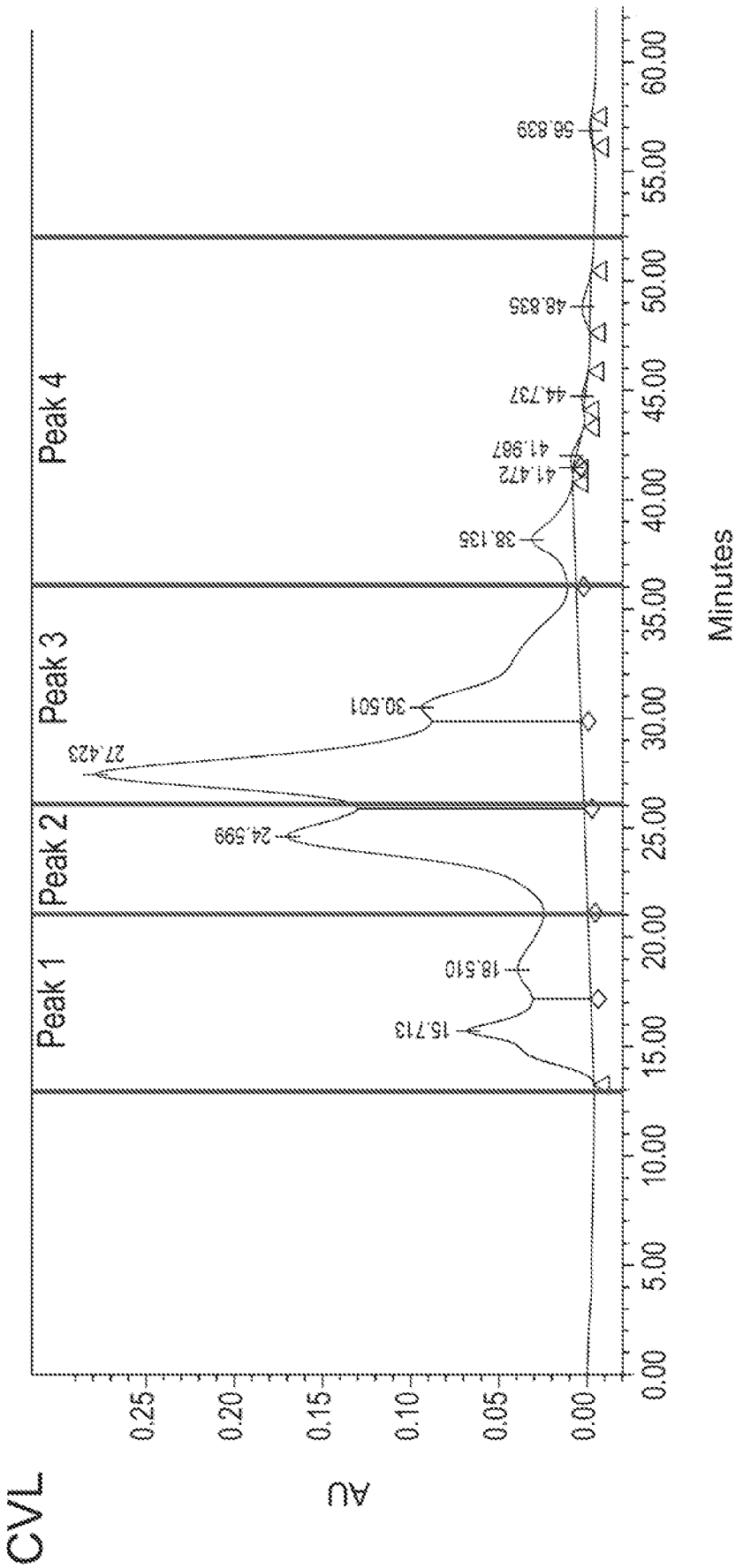
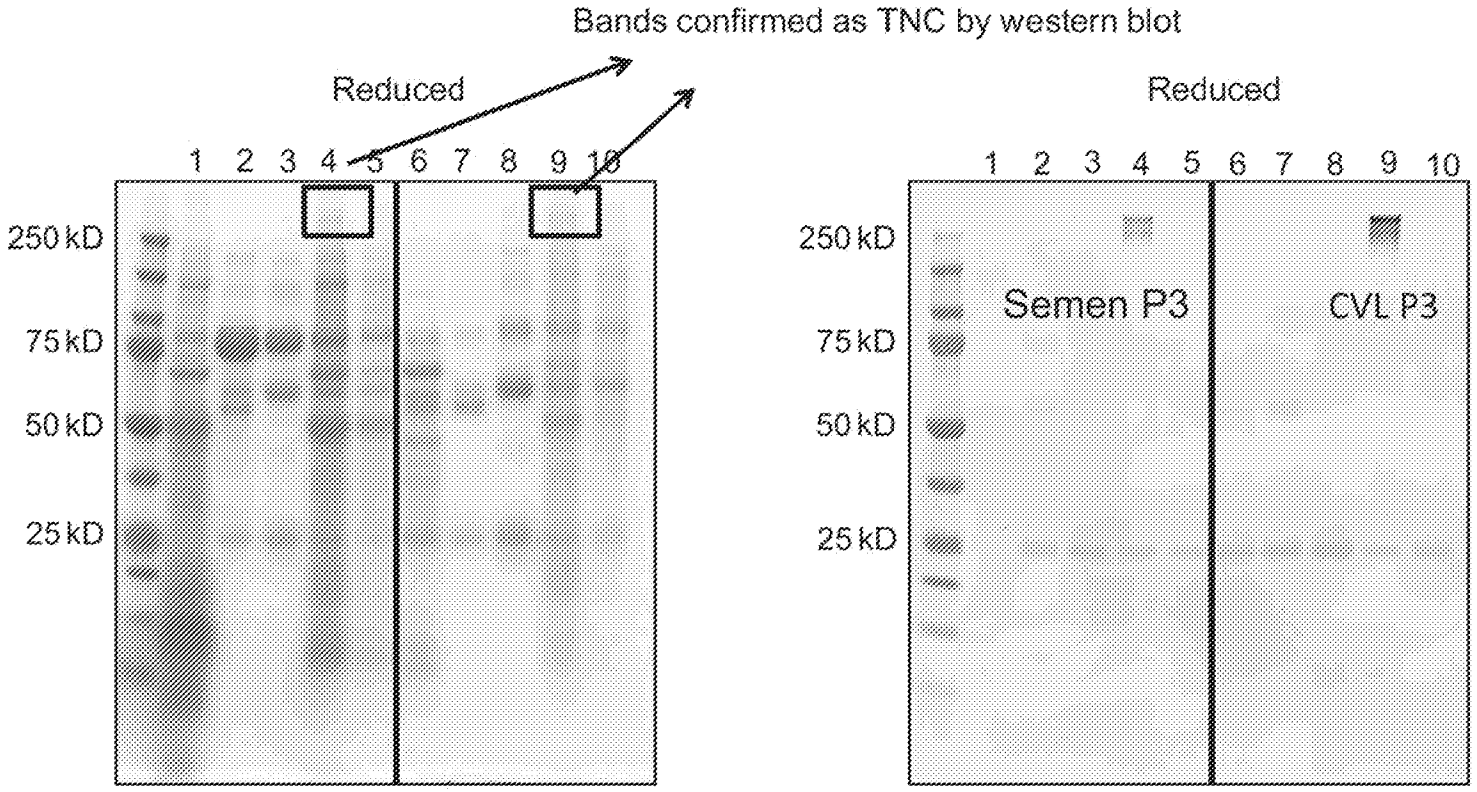


Figure 16

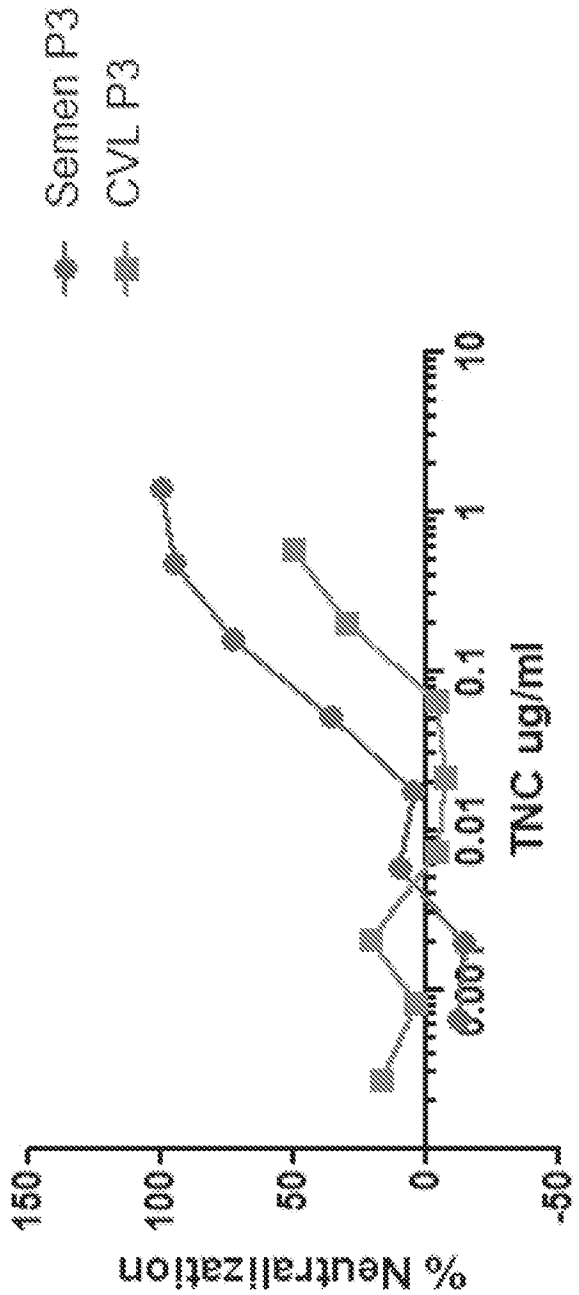
TNC Purification from Semen and CVL using Size Exclusion and Anion Exchange



1. Semen 1:10
2. Peak 1
3. Peak 2
4. Peak 3
5. Peak 4
6. CVL 1:10
7. Peak 1
8. Peak 2
9. Peak 3
10. Peak 4

Primary: mouse anti-human TNC, 1 ug/ml; clone 81C6
Secondary: goat anti-mouseIgG – AP: 1:5000

Figure 17



Sample	TNC (ug/ml)	ID_{50}	IC_{50} (ug/ml)
Semen P3	6.87083	89	0.0772
CVL P3	11.372875	20	0.568644

Figure 18

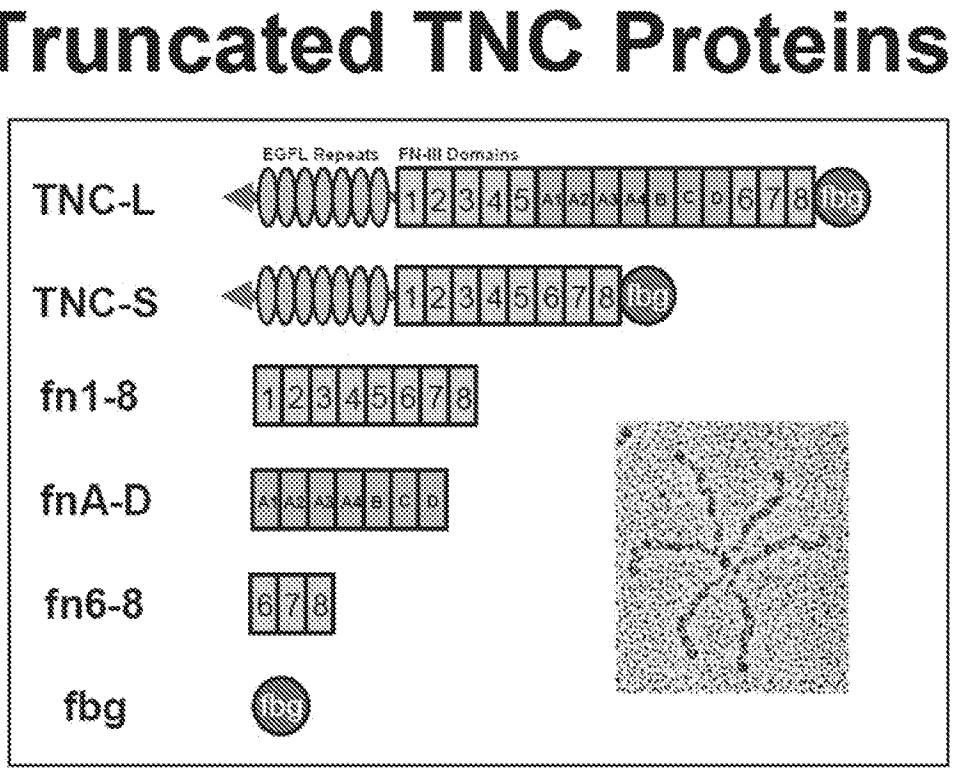
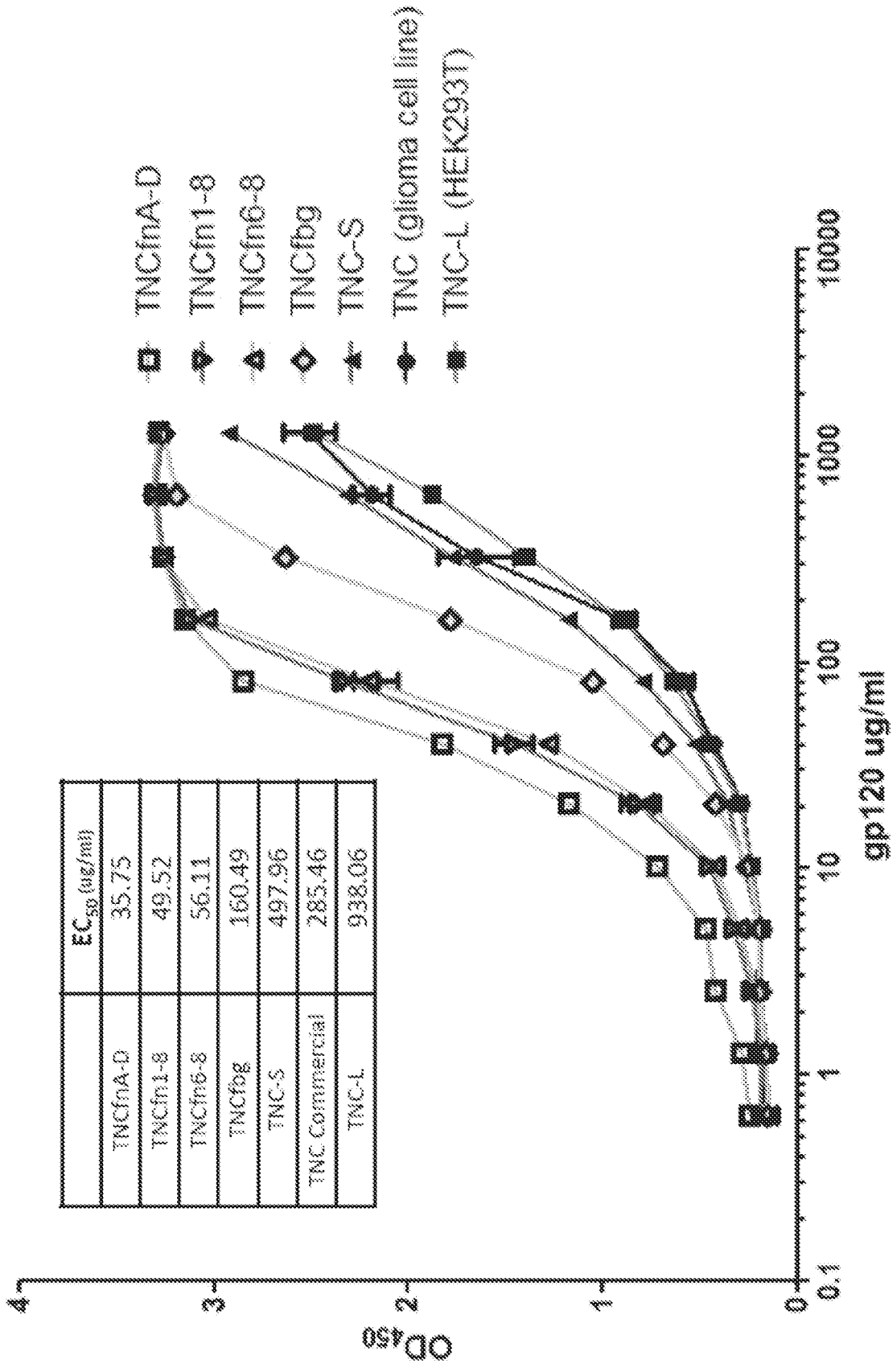


Figure 19

	Reactivity
TNC-L	++
TNC-S	++
TNCfn1-8	+++
TNCfnA-D	++++
TNCfn6-8	+++
TNCfbg	++
TNCfn6-8fbg	+
TNC-S(Δfbg)	+
TNC(Δfn-fbg)	++

$OD_{450} < 0.5$	+
$0.5<OD_{450}<1.0$	++
$1.0<OD_{450}<1.5$	+++
$OD_{450}>1.5$	++++

Figure 20



	EC_{50} (ug/ml)
TNCfnA-D	35.75
TNCfn1-8	49.52
TNCfn6-8	56.11
TNCfbg	160.49
TNC-S	497.96
TNC Commercial	285.46
TNC-L	938.06

Figure 21

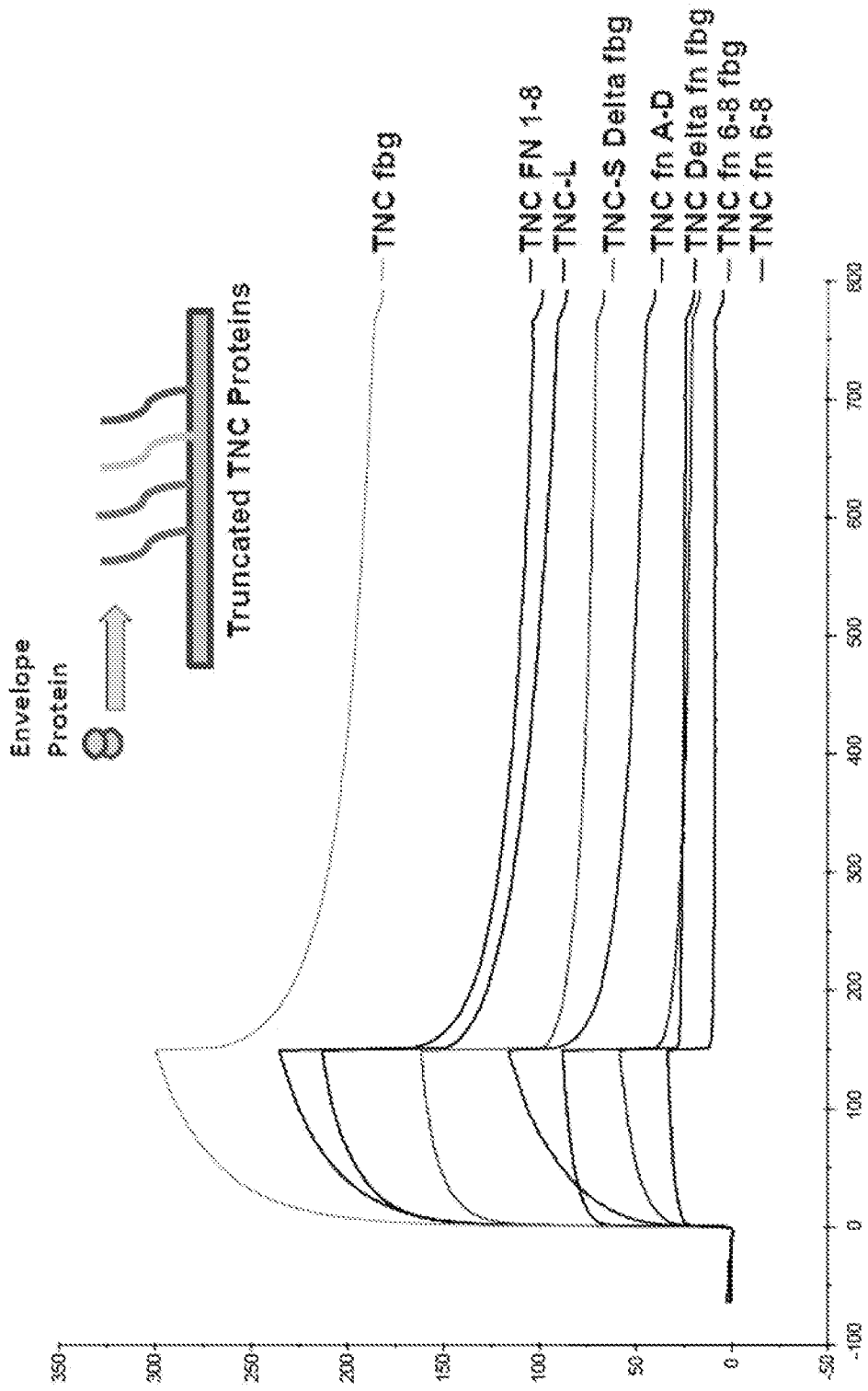

Envelope Protein
Truncated TNC Proteins
TNC fbg
TNC FN 1-8
TNC-L
TNC-S Delta fbg
TNC fn A-D
TNC Delta fn fbg
TNC fn 6-8 fbg
TNC fn 6-8
350
300
250
200
150
100
50
0
-50
-100
0
100
200
300
400
500
600
700
800

Figure 22

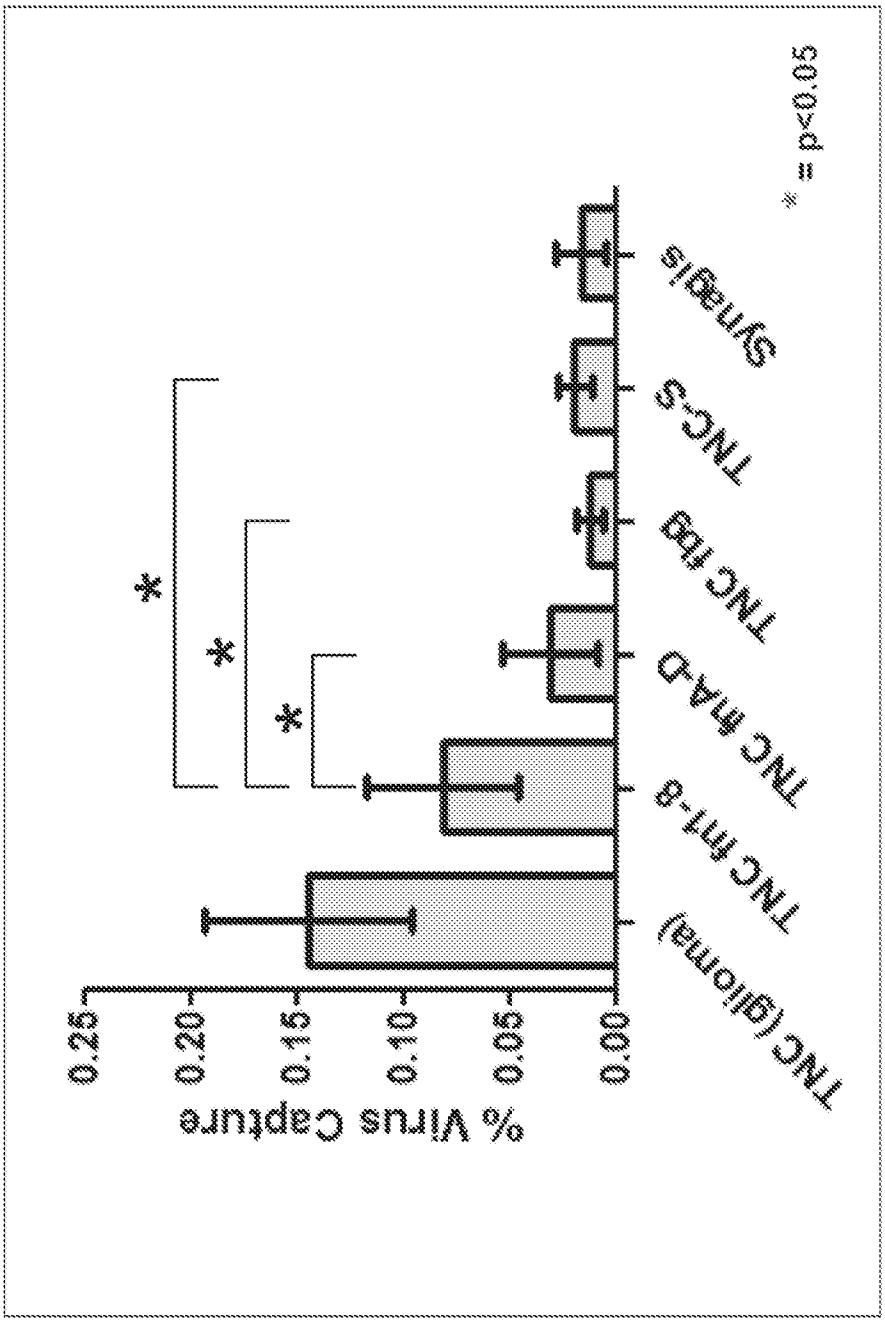
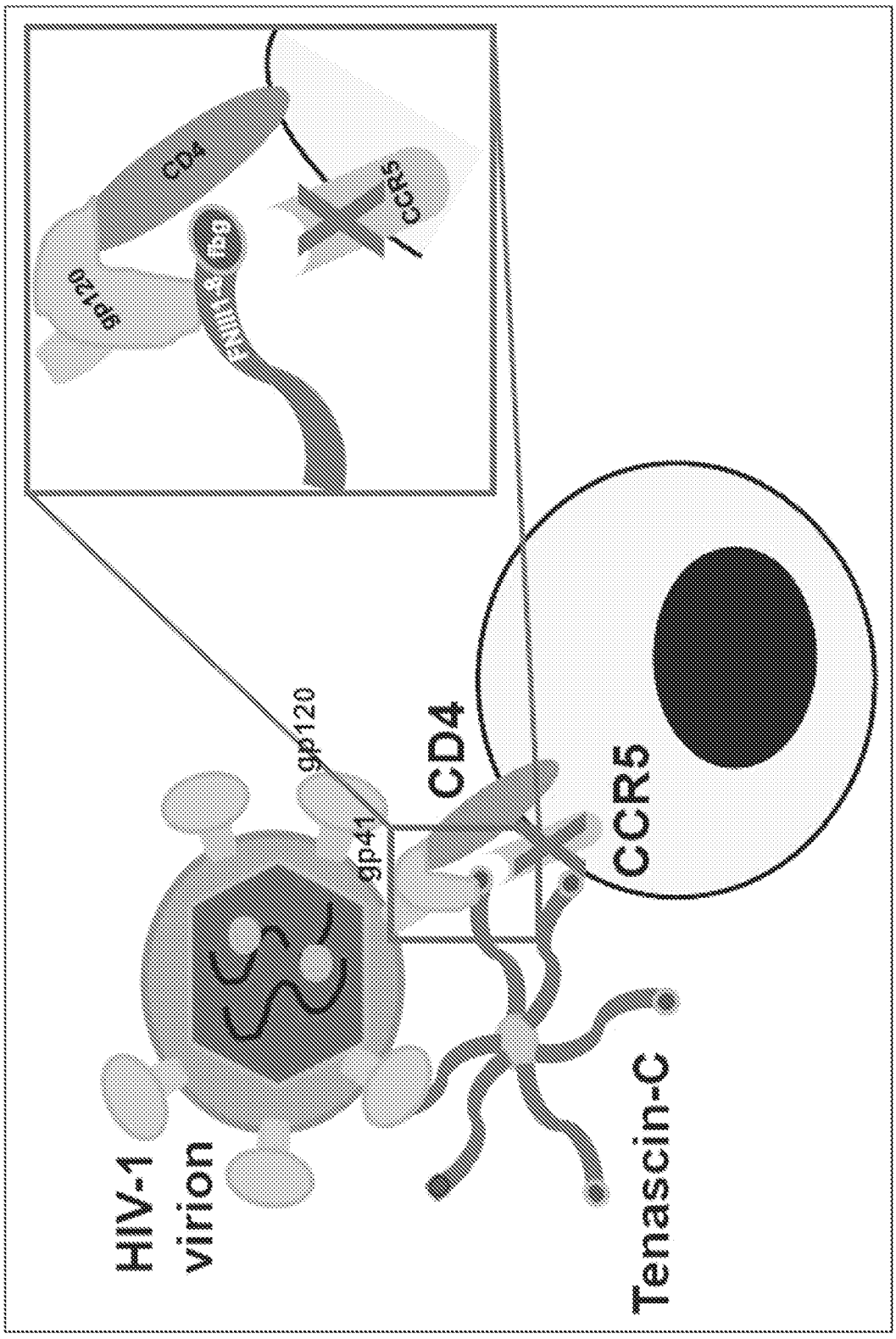

TNC Virion Interaction
% Virus Capture
0.25
0.20
0.15
0.10
0.05
0.00
TNC (glioma)
TNC fn1-8
TNC fnA-D
TNC fnB
TNC-S
Synaptic
* = p<0.05
*
*
*

Figure 23


HIV-1
virion
gp120
gp41
CD4
CCR5
Tenascin-C
CD4
gp120
gp41
CCR5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/011659

A. CLASSIFICATION OF SUBJECT MATTER

C07K 14/435(2006.01)i, A61P 31/18(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K 14/435; A61K 39/395; C12Q 1/02; A61K 49/00; A61K 51/00; A61P 31/18; A61K 39/12; A61K 38/17; A61K 38/16

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: tenacin, tenascin, HIV-1, CD4, neutralizing protein

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2011-0159012 A (PESHEVA, PENKA) 30 June 2011 See claim 1.	1,2
A	US 2010-0310592 A1 (MERIZZI, GIANFRANCO) 09 December 2010 See claims 11, 14 and 15.	1,2
A	US 2010-0297003 A1 (DE SANTIS, RITA et al.) 25 November 2010 See claim 1.	1,2
A	US 2007-0191265 A1 (WAKAMIYA, NOBUTAKA et al.) 16 August 2007 See abstract.	1,2
PX	FOUDA, GENEVIEVE G. et al., `Tenascin-C is an innate broad-spectrum, HIV-1-neutralizing protein in breast milk`, Proc. Natl. Acad. Sci. U. S. A., 21 October 2013, Vol. 110, No. 45, pp. 18220-18225 See pages 18221 and 18223.	1,2

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 April 2014 (25.04.2014)	**25 April 2014 (25.04.2014)**

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/011659

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 3-6
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 3-6 pertain to methods for treatment of the human body by therapy, and thus relate to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 6
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2014/011659

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2011-0159012 A1	30/06/2011	None	
US 2010-0310592 A1	09/12/2010	CA 2711930 A1	23/07/2009
		CN 101969998 A	09/02/2011
		EP 2244732 A1	03/11/2010
		IT TO20080027 A1	16/07/2009
		RU 2010133961 A	27/02/2012
		WO 2009-090512 A1	23/07/2009
US 2010-0297003 A1	25/11/2010	AR 047819 A1	22/02/2006
		AU 2005-217237 A1	09/09/2005
		AU 217237 B2	22/12/2011
		BR PI0508082 A	17/07/2007
		CA 2558718 A1	09/09/2005
		CN 1946740 A	11/04/2007
		CN 1946740 B	05/09/2012
		EP 1718678 A2	08/11/2006
		IT RM20040105 A1	27/05/2004
		JP 2008-506355 A	06/03/2008
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		WO 2005-082938 A3	15/12/2005
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		AU 246119 B2	21/02/2008
		CA 2472438 A1	08/01/2004
		CN 1620307 A	25/05/2005
		CN 1876833 A	13/12/2006
		CN 1876833 B	11/08/2010
		EP 1518557 A1	30/03/2005
		EP 1518557 A4	03/12/2008
		US 2005-0123899 A1	09/06/2005
		US 2007-0184477 A1	09/08/2007
		US 2007-0190652 A1	16/08/2007
		US 2009-0048165 A1	19/02/2009
		WO 2004-002511 A1	08/01/2004