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Bile salt-stimulated lipase from human milk binds DC-SIGN and inhibits human immunodeficiency virus type 1 transfer to CD4+ T cells

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ABSTRACT

A wide-range of pathogens including HIV-1, Hepatitis C, Ebola, Cytomegalovirus, Dengue, Mycobacterium, Leishmania, and Candida albicans can interact with DC-specific ICAM3-grabbing non-integrin (DC-SIGN), expressed on DCs and a subset of B cells. More specifically the interaction of the gp120 envelope protein of HIV-1 with DC-SIGN can facilitate the transfer of virus to CD4+ T-lymphocytes in trans and enhance infection. We have previously identified that a multimeric LeX component in human milk binds to DC-SIGN, preventing HIV-1 from interacting with this receptor. Biochemical analysis reveals that the compound is heat resistant, trypsin sensitive and larger than 100kDa in size, indicating a specific glycoprotein as the inhibitory compound. By testing human milk from three different mothers we found the level of DC-SIGN binding and viral inhibition to vary between samples. Using SDS-PAGE, western blot and MALDI analysis we identified bile salt stimulated lipase (BSSL), a LeX containing glycoprotein found in human milk, to be the major variant protein between the samples. BSSL isolated from human milk bound to DC-SIGN and inhibited the transfer of HIV-1 to CD4+ T-lymphocytes. Two BSSL isoforms isolated from the same human milk sample showed differences in DC-SIGN binding illustrating that alterations in the BSSL forms explain the differences observed. These results indicate that variations in BSSL lead to alterations in LeX expression by the protein, which subsequently alters the DC-SIGN binding capacity and the inhibitory effect on HIV-1 transfer. Identifying the specific molecular interaction between the different forms may aide in the future design of antimicrobial agents.
INTRODUCTION

Dendritic cells (DCs) express, among other C-type lectins, the DC specific ICAM-3 grabbing non-integrin (DC-SIGN) receptor (Mitchell et al., 2001; Weis et al., 1998). DC-SIGN has been shown to interact with a wide array of pathogens including hepatitis C, Ebola, cytomegalovirus, Dengue virus, mycobacterium, Leishmania, Helicobacter pylori (Geijtenbeek & van Kooyk, 2003). The gp120 envelope glycoprotein of HIV-1, HIV-2 and SIV (Pohlmann et al., 2001; Yu Kimata et al., 2002; Geijtenbeek et al., 2000b; Geijtenbeek et al., 2000c; Geijtenbeek et al., 2000a; Wu et al., 2004) can interact with DC-SIGN and has been implicated to play an important role in HIV-1 transmission and disease progression through infection or propogation of viral replication in CD4+ T-lymphocytes establishment of infection (Geijtenbeek et al., 2000b; Geijtenbeek et al., 2000c; Hu et al., 2000; Pope et al., 1995; Reece et al., 1998; Rowland-Jones, 1999). DC-SIGN is also expressed by a subset of B-cells in the tonsils and blood, these cells also transfer HIV-1 to CD4+ T-lymphocytes in culture, indicating a role for B cells in HIV-1 transmission and disease progression (Rappocciolo et al., 2006).

We previously identified that Lewis X (Le\(^x\)), 3-fucosyl-N-acetyllactoseamine, in human milk can inhibit DC-SIGN dependent transfer of HIV-1 to CD4+ T-lymphocytes by binding to DC-SIGN and blocking the viral interaction with the receptor (Naarding et al., 2005). We also demonstrated that the sugar epitope in milk is likely part of a larger molecule, either as an oligosaccharide or protein associated. The sugar epitope has been identified in many bodily fluids, including saliva, blood and human milk and has also been shown to be present in a number of pathogens (Bergman et al., 2004) and pathogen extracts (van Die et al., 2003).

Bile salt stimulated lipase (BSSL) is a Le\(^x\) carrying glycoprotein (McKillop et al., 2004) secreted by the pancreas, which is activated by bile salts in the intestine. BSSL is also expressed by the mammary gland and present in human milk (Blackberg et al., 1987) at a concentration of 100-200 \(\mu\)g/ml (Stromqvist et al., 1995). Neonates normally only secrete small amounts of colipase-dependent pancreatic lipase into the duodenum (Hernell & Blackberg, 1994) so gastric lipase as well as BSSL in human milk markedly enhance fat digestion in the newborn (Bernback et al., 1990). Both pancreatic and human milk BSSL were shown to be identical at the amino acid level (Nilsson et al., 1990; Kumar et al., 1992), while substantially varying in their carbohydrate content (Sugo et al., 1993; Mas et al., 1993) with Le\(^x\) being present at the C-terminus of BSSL (McKillop et al., 2004; Landberg et al., 1997). BSSL is also present in testis and adrenals (Lee et al., 1997) and blood plasma of humans (Lombardo et al., 1993). BSSL is produced by stimulated macrophages (Li & Hui, 1997) and eosinophils (Holtsberg et al., 1995) and its activity has been detected in endothelial cells (Li & Hui, 1998), aortic homogenate (Shamir et al., 1996) and human placenta (Chen & Morin, 1971).

Here we demonstrate that the compound in human milk that binds DC-SIGN and inhibits HIV-1 transfer is heat resistant, trypsin sensitive and over 100 kDa in size. Human milk
samples from different mothers were shown to possess varying levels of inhibitory activity associated with altered Le\(^e\) expression and DC-SIGN binding of a specific protein. MALDI analysis led to the identification of BSSL as the variant protein. We illustrate that BSSL isolated from human milk can bind DC-SIGN and inhibit HIV-1 transfer to CD4\(^+\) T-lymphocytes and that binding can be alleviated with an antibody against Le\(^e\), demonstrating the significance of the Le\(^e\) epitope. We have identified BSSL as a major glycoprotein in human milk that has the capacity to bind DC-SIGN and have shown differences in its binding capacity between different mothers.

**RESULTS**

**Biochemical analysis of the DC-SIGN binding compound in human milk.** In order to determine whether the previously identified inhibitory activity of human milk is protein associated or not, we trypsin treated milk S3 and tested this in the DC-SIGN-Fc binding ELISA (Figure 1A). Human milk incubated with medium showed DC-SIGN specific binding.

![Figure 1](image_url)

**Figure 1.** Biochemical analysis of DC-SIGN binding component in human milk. (A) The DC-SIGN-Fc binding capacity of the trypsin treated human milk (HM) (1/40 end concentration) was measured by DC-SIGN-Fc binding ELISA. The DC-SIGN-Fc binding background level was obtained by preincubation with AZN-D1 (DC-SIGN specific blocking antibody) and EGTA. * represents \(P < 0.01\) compared to the binding of the human milk incubated with RPMI. (B) Raji-DC-SIGN cells were incubated with the trypsin treated human milk (HM) or controls in the presence of HIV-1 before washing and addition of CD4\(^+\) T-lymphocytes. As a control Raji or Raji-DC-SIGN cells were incubated with PBS and virus before addition of CD4\(^+\) T-lymphocytes. CA-p24 production was measured at day 7 by standard ELISA. * represents \(P < 0.01\) inhibition compared to Raji-DC-SIGN (C) Human milk (HM) (1:2 dilution) was heated at 99°C for 10 min before determination of the DC-SIGN-Fc binding capacity. AZN-D1 and EGTA were used as controls to show DC-SIGN-Fc specific binding. * represents \(P < 0.001\) when comparing heat treated milk to a non-treated sample. Standard deviations are depicted in all graphs.
whereas the trypsin treated milk showed a significant reduction in binding to DC-SIGN ($P < 0.01$), although not all activity was lost. This result was confirmed in the DC-SIGN-mediated HIV-1 transfer assay, demonstrating a reduced inhibition of HIV-1 infection with the trypsin treated human milk in comparison to the untreated sample (Figure 1B). We could not alleviate the activity entirely, highlighting that the inhibitory activity is robust. These results reiterate that the inhibitory compound in human milk is protein or protein associated.

We next tested whether heating of the human milk S3 could alleviate DC-SIGN binding or HIV-1 inhibitory activity. Heating the milk to 99°C induced no loss of DC-SIGN-Fc binding in either the ELISA assay (Figure 1C) or in the gp120 bead adhesion assay with either Raji-DC-SIGN cells or iDC. Heating the milk sample also did not diminish inhibition in the HIV-1 viral transfer assay (data not shown), indicating that the protein does not loose its antiviral function when the native structure is lost. Interestingly, significant enhancement ($p < 0.001$) of DC-SIGN binding was observed in the binding ELISA (Figure 1C) as well as increased inhibition in the Raji-DC-SIGN transfer assay (data not shown) for the heated human milk sample and has been reproducible in four separate experiments.

The >100kDa fraction contains the active compound in human milk. To gain an indication as to the size of the protein responsible for the inhibitory activity we performed size fractionation of human milk. S3 was fractionated and the obtained fractions were tested in the DC-SIGN-Fc binding ELISA (Figure 2A). Specific binding to DC-SIGN was only observed with the >100 kDa fraction, however, non-specific binding was seen with the 30-100 kDa fraction. We also performed a gp120 bead adhesion assay for the same fractions and observed that only the >100 kDa fraction is able to inhibit interaction of DC-SIGN with gp120 (Figure 2B), indicating that the binding observed with the 30-100 kDa fraction is indeed non-specific (Figure 2A). The gp120-Raji-DC-SIGN interaction could be inhibited with AZN-D1, mannan and EGTA illustrating the DC-SIGN specificity of the binding. Incubation of iDC and with the different fractions before addition of fluorescent gp120 beads (data not shown) also confirmed that the >100 kDa contains the inhibitory compound(s). In the Raji-DC-SIGN culture assay both the unfractionated milk and the >100 kDa fraction of the human milk show significant ($P < 0.01$) inhibition compared to the PBS control (Figure 2C). The reduced transmission observed with the 30-100 kDa fraction is not statistically significant and unlikely due to factors binding DC-SIGN since the fraction was negative in the gp120 bead adhesion assay (Figure 2B).

Variation in binding activity between human milk samples from three different mothers. To determine whether variability in the DC-SIGN binding capacity of human milk exists between mothers, we tested milk samples from three individuals (S1, S2 and S3). The three samples were tested in the DC-SIGN-Fc ELISA (Figure 3A) and the DC-SIGN transfer culture assay (Figure 3B). In the DC-SIGN-Fc ELISA both S1 and S2 demonstrate low to no
binding differences compared to the relevant AZN-D1 and EGTA controls, whereas S3 shows increased binding ($P < 0.01$) in comparison to AZN-D1 and EGTA (Figure 3A). The DC-SIGN-Fc ELISA results were confirmed by the DC-SIGN transfer culture assay (Figure 3B), which showed a significant loss of inhibition at a dilution of 1:256 for S1 and S2 in comparison to S3, which still showed significant inhibition ($P < 0.02$) of HIV-1 transfer at a dilution of 1:2048. These results demonstrate that the inhibitory activity is significantly different between mothers.

**Identification of BSSL as an inhibitory glycoprotein.** Since we previously identified that Le$^x$ is involved with the inhibitory activity (Naarding et al., 2005) of milk. We performed western-blot analysis with both $\alpha$Le$^x$ Ab as well as the DC-SIGN-Fc product to try to identify the factor. We performed western-blot staining of human milks S1, S2 and S3 with a Le$^x$ specific antibody (Figure 4A). Upon comparison of the different samples we observed a band above 100 kDa in S3, which was not detected in S1 or S2, for equal total protein amount, indicating a difference in Le$^x$ expression. The Le$^x$ staining result also demonstrates that the lower molecular weight proteins are efficiently glycosylated to contain Le$^x$ epitopes.

**Figure 2.** The inhibitory component of human milk is present in the >100kDa fraction of human milk. (A) DC-SIGN-Fc binding of the different size fractionation was determined for the human milk fractions (1:100) in the DC-SIGN-Fc binding ELISA with AZN-D1 and EGTA controlling for DC-SIGN binding specificity. * represents $P < 0.01$ compared to the relevant control. Standard deviations are depicted. (B) Raji-DC-SIGN cells were incubated with the different size fractions before washing and addition of fluorescent gp120 beads. To control for DC-SIGN specific binding the cells were also incubated with EGTA, mannan and AZN-D1. (C) The Raji-DC-SIGN cells were incubated with the different human milk size fractions (1/4) and virus before addition of CD4$^+$ T-lymphocytes. As a control Raji or Raji-DC-SIGN cells were incubated with PBS and virus before addition of CD4$^+$ T-lymphocytes. CA-p24 production was measured at day 7 by standard ELISA. * represents $P < 0.01$ compared to the PBS control. Standard deviations are depicted.
with no major quantitative difference between the three mothers, suggesting that these lower Le^a associated molecular weight proteins are not involved in the observed inhibitory activity, which is in accordance with the size fractionation experiment showing the factor as >100 kDa. In the DC-SIGN-Fc stained western-blot (Figure 4B) the S3 sample contains a clear band at a similar molecular weight, but much less pronounced than in S1 and S2. We also observe that the equivalent band of S1 and S2 runs higher in the gel than the one from S3. These results are in accordance with the differences in inhibitory activity observed between the samples. The Coomassie stained SDS-PAGE gel (Figure 4C) also shows a band

Figure 3. Differences in DC-SIGN binding capacity of human milk samples from three mothers (S1-S3). (A) The DC-SIGN-Fc binding capacity was measured for three different human milk samples (1:200). Pre-incubation of DC-SIGN-Fc with AZN-D1 and EGTA controlled for DC-SIGN specific binding. * represents P < 0.01 compared to normal DC-SIGN-Fc binding. (B) Different dilutions of the milk samples (S1-S3) were tested in the Raji-DC-SIGN transfer culture assay. To control for infection, Raji or Raji DC-SIGN cells were incubated with PBS and virus before addition of CD4^+ T-lymphocytes. CA-p24 production was determined on day 7 by standard ELISA. * represents P < 0.02 compared to the PBS control. Standard deviations are depicted in both graphs.

Figure 4. Western blot and Coomassie staining of three human milk samples with different DC-SIGN binding capacity. (A) western blot of human milk S1, S2 and S3 stained with Le^a specific antibody. (B) western blot stained with DC-SIGN-Fc. (C) Coomassie stained SDS-PAGE gel.
at the corresponding molecular weight and demonstrates even more clearly that the bands in human milk S1 and S2 run higher than the band of interest in S3.

We subsequently extracted the protein from the appropriate SDS-PAGE bands of S1, S2 and S3. The peptide mass fingerprint analysis of the selected protein bands identified it as human bile salt stimulated lipase (AAA63211) with 20 peptides out of 35 matching (at 30 ppm or below). The sequence coverage was 32 % and the Probability based MOWSE score 199 (with protein scores greater than 76 considered significant). The identification of BSSL is in accordance with the observation that the protein expresses Le\(^\text{a}\) (McKillop et al., 2004).

**BSSL can inhibit DC-SIGN binding and DC-SIGN mediated transfer of HIV-1 to CD4\(^+\) lymphocytes.** To confirm that BSSL can serve as an inhibitory compound in human milk we isolated BSSL from S4. The isolated BSSL was tested in the DC-SIGN transfer culture assay (Figure 5A) and showed significant inhibition at 30 \(\mu\)g/ml and 1.2 \(\mu\)g/ml \((P < 0.05)\). The addition of BSSL (30 \(\mu\)g/ml) to Raji-DC-SIGN cells did not affect cell counts or viabilities as tested by trypan blue exclusion (data not shown), suggesting that the protein is not toxic to the cells. The DC-SIGN-Fc ELISA (Figure 5B) showed binding at concentrations of 30 \(\mu\)g/ml, 3 \(\mu\)g/ml and 0.3 \(\mu\)g/ml BSSL \((P < 0.01)\) illustrating that BSSL can indeed bind to DC-SIGN and inhibit DC-SIGN mediated transfer of HIV-1 to CD4\(^+\) T-lymphocytes. In order to show that BSSL has the same characteristics as the human milk inhibitory factor we performed trypsinization and heat treatment of purified BSSL and determined it to be heat resistant and trypsin sensitive (data not shown), in accordance with the results obtained with human

**Figure 5.** BSSL binds DC-SIGN and prevents transfer of HIV-1 to CD4\(^+\) lymphocytes. (A) Raji-DC-SIGN cells were incubated with different dilutions of BSSL isolated from human milk (S4) and virus before addition of CD4\(^+\) T-lymphocytes. As a control Raji and Raji-DC-SIGN cells were incubated with PBS instead of BSSL. CA-p24 production was determined on day 7 by standard ELISA. * represents \(P < 0.05\) compared to the PBS control. (B) DC-SIGN-Fc binding capacity was determined by ELISA for different dilutions of BSSL isolated from human milk (S4). To control for DC-SIGN specificity DC-SIGN-Fc was pre-incubated with AZN-D1 and EGTA to allow comparison to the relevant binding without inhibitor. * represents \(P < 0.01\). Standard deviations are depicted in both graphs.
milk. Interestingly, as with the human milk sample the BSSL binding is not fully abrogated by trypsin treatment suggesting again a robust activity against the binding of HIV-1 to DC-SIGN.

**BSSL binding to DC-SIGN can be blocked with Le^x antibodies.** To confirm our hypothesis that Le^x is (or part of) the active component of the glycoprotein BSSL, we pre-incubated BSSL with Le^x IgM before addition of DC-SIGN-Fc in the DC-SIGN-Fc binding ELISA. The DC-SIGN binding capacity of BSSL could be blocked by pre-incubation with the Le^x specific Ab (Figure 6), confirming that the Le^x expressed by BSSL is crucial for DC-SIGN binding.

**Two isoforms of BSSL from the same human milk sample show a difference in DC-SIGN binding capacity.** To further analyze the correlation between the size of BSSL and the DC-SIGN binding capacity we isolated two isoforms, variant in size, from the same mother. The larger isoform (132 kDa) demonstrated a decrease in DC-SIGN binding compared to the smaller form (102 kDa) of BSSL (Figure 7), indicating that the differences in binding between the mothers is most likely due to the difference in the BSSL isoforms. Since the two bands are isolated from a single milk sample from the same mother then the different binding patterns are unlikely to be due to differences in Le^x secretor status or difference in the activity of fucosyltransferases.

**DISCUSSION**

DC-SIGN binding differences between milk from different mothers were observed and analyzed by Western blot and MALDI technology, which enabled us to identify BSSL as a
DC-SIGN binding glycoprotein found in human milk that can efficiently block the transfer of HIV-1 to CD4+ lymphocytes. We had previously identified that a Le\(^x\) saccharide containing human milk compound could bind to DC-SIGN and thereby prevent the interaction of the receptor with the gp120 molecule of HIV-1 (Naarding et al., 2005). We demonstrate here that the inhibitory activity of human milk resides within the >100 kDa size fraction, even though Le\(^x\) motifs are also linked to proteins of lower molecular weight, corresponding to our previous observation that not all Le\(^x\) containing compounds could mimic the inhibitory effect of human milk (Naarding et al., 2005). We show that the inhibitory activity of purified milk BSSL shares properties with whole milk (Naarding et al., 2005) in being trypsin sensitive and heat resistant. We also demonstrate that as with whole milk pre-treatment of purified native BSSL with an antibody against Le\(^x\) interferes with both the binding activity and the block of viral transfer. Collectively, these results indicate that BSSL in human milk is one of the main or the only glycoprotein(s) binding to DC-SIGN providing the viral inhibitory activity of human milk.

BSSL is expressed in human milk at a concentration of 100-200 µg/ml, in our experiments 30 µg/ml of BSSL showed inhibition of viral transfer and high binding to DC-SIGN. The observed BSSL concentration corresponds to 1/3-1/6 dilution of the human milk even though we previously showed complete inhibition of viral transfer at a 1/128 dilution (Naarding et al., 2005). This discrepancy could be the result of the presence of an additional inhibitory factor in milk, even though we observe complete correlation between the DC-SIGN binding capacity and the Le\(^x\) and DC-SIGN-Fc staining of the BSSL band in the western blot analysis. More likely the DC-SIGN binding activity of BSSL is diminished by the purification from human milk. No pro-enzyme of BSSL exists, and BSSL is always present in its active form in human milk. Breakdown products of the BSSL protein could be present in human milk but we demonstrated no DC-SIGN binding activity in the smaller size fractionations suggesting that the larger protein is required to provide for the inhibition.

Variations in DC-SIGN binding and viral transfer inhibition capacities were observed in milk from different mothers in which variations in BSSL protein size and Le\(^x\) expression patterns were apparent. Differences were also identified between two isoforms of BSSL isolated from the same milk sample. The BSSL gene is located on chromosome 9 and contains 11 exons. Exon 11 has on average 16 repeat domains of 11 amino acids (amino acids 536-711) at its C-terminus containing a high number of O-glycosylation sites whilst the N-terminus contains only one potential N-glycosylation site (Asn 187) (Baba et al., 1991; Reue et al., 1991). Variant isoforms of BSSL have been shown to differ in the number of repeats at the C-terminus of the molecule (Stromqvist et al., 1997; Lindquist et al., 2002) which has been shown to be the domain with a high expression of Le\(^x\) (McKillop et al., 2004), likely explaining for the observed correlation between Le\(^x\) expression and BSSL size. In accordance with our observation it has been shown that the expression of Le\(^x\) can vary between mothers (Landberg et al., 1997).
BSSL has been characterized for its lipase activity which is lost after heating at 50°C (Hernell, 1975). Here we show that the DC-SIGN binding capacity is not lost after boiling indicating that enzymatic function and tertiary structure of the protein are not required for DC-SIGN binding or inhibition of viral transfer. It has been postulated that the high proline content in the C terminal region prevents it from folding into a compact secondary structure resulting in a flexible, open configuration (Reue et al., 1991), which could explain for the heat resistant property of the repeat section. We are currently in the process of analyzing further the enhancing effect to inhibition upon heating of the milk and determining whether heating of the weak binding S1 and S2 samples provides for a similar enhancement.

Interestingly BSSL is found in the blood plasma of a number of mammals, including humans (Lombardo et al., 1993) and has been postulated to be derived from the pancreas (Bruneau et al., 2003) and has also been shown to be produced by stimulated macrophages and human eosinophils (Holtsberg et al., 1995) and is present in testis and adrenals (Lee et al., 1997) endothelial cells (Li & Hui, 1998), aortic homogenate (Shamir et al., 1996) and human placenta (Chen & Morin, 1971). Given the wide-array of anatomical sites and cell-types producing BSSL it will be interesting to determine whether expression of BSSL can influence microbial transmission, dissemination and pathogenesis of a number of infectious agents. Differential expression of BSSL or indeed its variant isoforms may prove to have significant implications for HIV-1 pathogenesis in general by relating to the overall viral loads obtained in individuals after infection. This type of association will be identified through screening HIV-1 infected cohorts for the presence of the variant BSSL isoforms or the expression levels in plasma. Another immunomodulatory molecule Mac-1 has been shown to interact with DC-SIGN (van Gisbergen et al., 2005). Mac-1 is expressed on neutrophils and therefore identifying natural proteins that can block DC-SIGN may have implications for modulating immune responses involving this cell type.

Fucosyltransferases are responsible for the complex sugar additions and modifications of proteins. Mutations in fucosyltransferase genes have been previously identified (Kelly et al., 1995) and could result in the variant expression of Le^a on BSSL and other proteins. Individuals can be divided into secretors or non-secretors for specific sugar epitopes based on expression patterns of such proteins. Our results show that lower molecular weight proteins are equally stained in western blots with the Le^a Ab using milk from different mothers suggesting that the difference observed for BSSL is not due to alterations in such fucosyltransferases and that the Le^a glycosylation machinery is functional for all mothers tested.

Here we identify a Le^a expressing glycoprotein, BSSL, which binds to DC-SIGN and can prevent its interaction with HIV-1, leading to the blocking of HIV-1 trans infection of CD4^+ T-lymphocytes. It remains to be determined whether BSSL is the only DC-SIGN binding glycoprotein present in human milk or whether other glycoproteins can provide binding and inhibitory function. Linking variant BSSL isoforms or levels of expression with viral transmission rates via breastfeeding may provide evidence for DC-SIGN involvement in
the mucosal transmission of HIV-1. Interestingly, a molecule in cervicovaginal lavage has also been identified to bind to DC-SIGN but is as yet unidentified (Jendrysik et al., 2005). Elucidating the precise molecular interactions of the different BSSL forms, with different inhibitory activities, may lead to the development of a DC-SIGN binding molecule that can be incorporated into future antimicrobial or immunomodulatory agents.

**MATERIALS AND METHODS**

**Cells.** The Raji and Raji-DC-SIGN cell lines were obtained and cultured as previously described (Geijtenbeek et al., 2000b; Naarding et al., 2005). Peripheral Blood Mononuclear Cells (PBMCs) were isolated from three buffy coats by standard Ficol-Hypaque density centrifugation, pooled and frozen in multiple vials. After thawing, PBMCs were activated with phytohemagglutinin (2 µg/ml) and cultured in RPMI medium containing 10% FCS, penicillin (100 units/ml) and streptomycin (100 units/ml) with recombinant interleukin-2 (100 units/ml). On day 3 the cells underwent CD4+ enrichment by incubation with CD8 immunomagnetic beads (Dynal, Breda, The Netherlands) and negatively selected according to the manufacturers instructions and cultured with IL-2 (100 units/ml). Dendritic cells for use in the gp120 bead adhesion assay were generated from PBMCs with cells layered on a standard Percoll gradient (Pharmacia, Uppsala, Sweden). The light fraction with predominantly monocytes was collected, washed, and seeded in 24-well or 6-well culture plates at a density of 5 x 10^5 cells or 2.5 x 10^6 per well, respectively. After 60 min at 37°C the adherent cells were cultured to obtain immature DCs in Iscove’s modified Dulbecco’s medium (IMDM) with gentamicin (86 µg/ml) and 10 % fetal clone serum (Hyclone, Logan, Utah) and supplemented with granulocyte-macrophage colony-stimulating factor (GM-CSF) (500 units/ml) and Interleukin-4 (IL-4) (250 units/ml).

**Viruses.** Replication competent HIV-1 stocks were generated by the passage of viruses through CD4+ lymphocytes with Tissue Culture Infectious Dose (TCID_{50}/ml) determined by limiting dilution on CD4+ enriched lymphocytes (Pollakis et al., 2001). Subtype B molecular cloned viruses LAI (X4) was used as the virus in all experiments.

**Biochemical analyses of human milk.** Human milk was incubated with trypsin-EDTA (1X) (Invitrogen, Breda, The Netherlands) or RPMI (Invitrogen, Breda, The Netherlands) for 3 hours while shaking at 37°C, after which the trypsin was inactivated by heating at 95°C for 10 min.

Human milk was fractionated with the use of microcon centrifugal Filter devices (Millipore, Amsterdam, The Netherlands), sizes 3,000; 10,000; 30,000 and 100,000 NMWL. First the milk was loaded onto the 3,000 NMWL filter device, the retained fraction was loaded to the next filter, whilst compensating for lost volume with PBS. The standard manufacturers protocol was followed to obtain the different fractions.
Human milk and BSSL. Human milk samples were collected from three mothers (S1, S2 and S3) in Amsterdam, the Netherlands. S1 and S3 were collected after 6 months of lactation and sample S2 was taken at an unknown timepoint. Internal review board approval was not required since the samplings were to be discarded material. The milk was centrifuged at 400xg for 10 min followed by centrifugation at 530xg for 10 min to remove lipid and cells. The human milk samples were sterilized by sequential filtration through 0.45 µm and 0.2 µm syringe filters (Schleicher & Schuell, Amsterdam, The Netherlands). Milk samples were also collected from two additional mothers in Umeå, Sweden, from which BSSL was isolated (S4 and S5).

BSSL was isolated from human milk as previously described (Blackberg & Hernell, 1981) but with using a second heparin-Sepharose chromatography rather than affi-Gel blue sepharose for final purification. Collected fractions were analyzed for BSSL by lipase activity, SDS-PAGE and immunoblotting (Blackberg & Hernell, 1981).

**DC-SIGN-Fc binding ELISA.** The DC-SIGN-Fc chimera contained the extracellular portion of DC-SIGN (amino acids 64 to 404) fused at the C-terminus to a human immunoglobulin (Ig) G1 Fc fragment which has been previously described (Geijtenbeek et al., 2002). Human milk or BSSL were diluted in 0.2 M NaHCO₃, coated on ELISA plates (maxisorb plate; Nunc, Amsterdam, The Netherlands) and incubated overnight at 4°C or 2 hours at 37°C. This was followed by blocking with TSM (20 mM Tris, 150 mM NaCl, 1 mM CaCl₂, 2 mM MgCl₂) containing 1% BSA for 30 min at 37°C before addition of soluble DC-SIGN-Fc (5 µg/ml) for 2 hours at RT, the binding was determined by incubation of a peroxidase labeled anti-IgG1 antibody for 30 min at RT. DC-SIGN-Fc binding specificity was determined by pre-incubation of the DC-SIGN-Fc with either 50 µg/ml DC-SIGN specific mouse antibody AZN-D1 (Geijtenbeek et al., 2000c) or 10 mM EGTA (Sigma-Aldrich, Zwijndrecht, The Netherlands) for 20 min before addition of the DC-SIGN-Fc to the coated human milk. Due to inter-assay variation large differences can be observed in the OD values but each independent experiment is performed with the relevant controls to demonstrate binding specificity.

**DC-SIGN mediated HIV-1 transfer assay.** The assay was performed as previously described (Naarding et al., 2005). The Raji and Raji-DC-SIGN cells were plated at a concentration of 2 x 10⁴ cells/well in a 96 well format. Dilutions of human milk or BSSL were made in PBS containing 10% FCS and spiked with 3.7 log TCID₅₀/ml of the appropriate virus before addition to the Raji-DC-SIGN cells. As controls PBS containing 10% FCS was spiked with the same TCID₅₀/ml of virus before addition to Raji or Raji-DC-SIGN cells. After a two hour incubation the culture was washed with PBS before addition of CD4⁺ enriched T-lymphocytes at a concentration of 1 x 10⁵ cells/well. CA-p24 values were determined on day 7 using a standard ELISA protocol (Moore et al., 1990). In short 96 wells plates were coated with a sheep anti-p24 specific antibody (Biochrom ag, Berlin, Germany), after which the culture supernatant was added. As a conjugate a mouse anti-HIV-1-p24
alkaline phosphatase conjugate antibody (Aalto bio Reagens Ltd., Dublin Ireland) was used. Development was performed by Lumi-phos plus (Lumigen inc., Southfield, MI, USA) according to the manufactures instructions and measured on the Lumistar Galaxy (BMG labtechnologies GmbH, Offenburg, Germany). CA-p24 levels were determined by a standard curve present on each plate (e-coli expressed recombinant HIV-1-p24, (Aalto bio Reagens Ltd., Dublin, Ireland).

**gpi20 bead adhesion assay.** Beads were prepared as previously described (Geijtenbeek et al., 2000c), in short, streptavidin was covalently coupled to Carboxylate-modified TransFluoSpheres (488/645 nm, 1.0 μm; Molecular Probes). The streptavidin-beads were incubated with biotinylated F(ab')2 fragment goat-anti-human IgG (6 μg/ml; Jackson Immunoresearch, West Grove, PA) and subsequently incubated overnight with gpi20-Fc chimera. Fifty thousand cells, Raji-DC-SIGN cells were pre-incubated with human milk or fraction thereof, 20 μg/ml AZN-D1 (DC-SIGN specific mouse antibody) (Geijtenbeek et al., 2000c), 5 mM EGTA (Sigma-Aldrich, Zwijndrecht, The Netherlands) or 5 mM mannan (Sigma-Aldrich, Zwijndrecht, The Netherlands) for 30 min at RT. The ligand-coated beads (20 beads/cell) were added to the pre-incubated cells and incubated for 30 min at 37°C after which the cells were washed with TSM containing 0.5% BSA. After washing, the cells were resuspended in 100 μl TSM-BSA buffer and the adhesion was measured by flow cytometry (BD Biosciences).

**Western blots and Coomassie staining.** The concentration of the human milk samples was standardized to 30 μg/ml and separated on 8% SDS-PAGE gels (BioRad, Veenendaal, The Netherlands). The gel was transferred to polyvinylene difluoride membranes (Millipore, Amsterda, The Netherlands), and stained with a mouse anti-human LeA Ab, C3D-1 (Santa Cruz) (0.2 μg/ml) and a mix of two different goat anti mouse IgG antibodies (Biorad, Veenendaal, The Netherlands, 0.07 μg/ml and Biosource, Breda, The Netherlands, 1/10,000) or the membrane was stained with DC-SIGN-Fc (600 μg/ml) and a goat anti human IgG (Jackson Immunoresearch, West Grove, PA). Visualization was performed using enhanced chemiluminescence (Amersham Biosciences, Inc., Diegem, Belgium). For the Coomassie staining 60 μg of each human milk sample was loaded on an 8% SDS-PAGE. The gel was stained with 50% methanol, 2% acetic acid and 0.25% Coomassie after which the gel was destained with 30% methanol and 2% acetic acid, the gel was stored in water with 1% acetic acid at 4°C for further analyzes.

**MALDI protein identification.** The protein bands of interest were cut from the gel after staining. For mass spectrometry analysis the gel slices were S-alkylated with iodoacetamide and vacuum dried using a speedvac. The in-gel digestion with trypsin (Roche Molecular Biochemicals, Almere, The Netherlands, sequencing grade) and extraction of the peptides after the overnight incubation were done according to Shevchenko et al. (Shevchenko et al., 1996). The collected eluates were dried overnight in a speedvac. The peptides...
were redissolved in 6 µl of a solution containing 1% formic acid and 60% acetonitrile. The peptide solutions were mixed 1:1 (v/v) with a solution containing 52 mM α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) in 49% ethanol / 49% acetonitril / 2% TFA and 1 mM Ammoniumacetate. Prior to dissolving, the α-cyano-4-hydroxycinnamic acid was washed briefly with acetone. The mixture was spotted on a target plate and allowed to dry at room temperature. Reflectron MALDI-TOF spectra were acquired on a M@LDI (Micromass Wythenshawe, UK). The resulting peptide spectra were used to search with MassLynx ProteinProbe (Micromass Wythenshawe, UK) in a Fasta database or the sequence databases of the Mascot search engine (http://www.matrixscience.com).

Statistics. All statistical comparisons were performed using ANOVA. $P < 0.01$, $P < 0.02$ and $P < 0.05$ were considered statistically significant.

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