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Stax, M.J.

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Chapter 3

Binding of human breast milk component bile-salt stimulated lipase (BSSL) to pathogen receptor DC-SIGN varies according to BSSL gene polymorphism

Martijn J Stax¹, Michael WT Tanck², Marloes A Naarding¹, Olle Hernell³, Robert Lyle⁴, Per Brandtzaeg³, Merete Eggesbo⁶, Georgios Pollakis⁴ and William A Paxton¹

¹Lab of Exp Virol, Dept of Med Microbiol, Center for Infection and Immunity Amsterdam (CINIMA), ²Dept Clin Epidem Biostat and Bioinf, Academic Medical Center, Amsterdam, Netherlands, ³Dept of Clinical Sciences, Pediatrics, Umeå University, SE - 901 87 Umeå, Sweden, ⁴Ulleval Univ Hosp, Dept Med Genet, N-0407 Oslo, ⁵LIIPAT,Ctr Immune Regulat, Univ of Oslo, and Dept of Pathology, Oslo Univ Hosp, Rikshospitalet, N-0027 Oslo, Norway, and ⁶Norwegian Inst Publ Hlth, Dept Genes & Environm, Div Epidemiol, N-0403 Oslo, Norway

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ABSTRACT

Dendritic cells bind an array of antigens and DC-SIGN has been postulated to act as a receptor for mucosal pathogen transmission. We have previously described that bile-salt stimulated lipase (BSSL) from human breast milk potently binds DC-SIGN and blocks DC-SIGN mediated trans-infection of CD4+ T-lymphocytes with HIV-1. Here we describe that DC-SIGN binding properties are highly variable for milks derived from different mothers and between mothers from different geographical regions. We found that differences in DC-SIGN binding are correlated with a genetic polymorphism in BSSL which is related to the number of 11 amino acid repeats at the C-terminus of the protein. The observed variation in DC-SIGN binding properties among milk samples may have implications for the risk of mucosal transmission of pathogens during breastfeeding.
INTRODUCTION

Human breast milk contains a large array of pathogens, antigens and host factors that interact with esophageal and gut mucosa of the breastfed infant (Newburg, 2009; Brandtzaeg, 2010). There is accumulating evidence that breast milk protects the newborn against infections as indicated by the decreased morbidity and mortality due to diarrhea among children that receive breastfeeding (Newburg, 2009; Nduati et al., 2000). Furthermore, only 4-12% of human immunodeficiency virus type-1 (HIV-1) positive mothers transmit HIV-1 to their child during breastfeeding in spite of frequent exposure of the child to HIV-1 positive breast milk for up to several years (Coovadia & Kindra, 2008). If we would have a better understanding of how human breast milk influences mucosal infections, we would be able to develop therapeutics aimed at prevention.

The anti-microbial activity of human breast milk is, at least in part, the result of secretory antibodies and prebiotic factors. Glycans in breast milk have been associated with protection against transmission of specific mucosal pathogens (Newburg, 2009; Ruvoen-Clouet et al., 2006). Furthermore, blood group antigen genes involved in post translational protein modification and resulting in differentiated glycan fingerprints have been linked to pathogen driven selection in humans (Fumagalli et al., 2009). This stresses the relevance of glycans in the continuous competition with rapidly evolving pathogens where one glycan type may protect against certain pathogens whilst enhance infection with other pathogens. Although innate immune molecules present in breast milk can contribute to protection against infection, additional immune responses need to be activated in the child for further effector and memory purposes. Antigen presenting cells such as dendritic cells (DCs) that capture invading pathogens through pathogen coat sugars regulate such processes.

Pathogen receptor DC-specific intercellular adhesion molecule-3 grabbing non integrin (DC-SIGN) is highly expressed in mucosal tissues by DCs present in the submucosa. DC-SIGN binds a wide range of pathogens such as HIV-1, Helicobacter pylori and Candida albicans (Geijtenbeek et al., 2000; Appelmelk et al., 2003; Cambi et al., 2003; de Jong et al., 2008). Such interaction between pathogen and DC-SIGN expressing cells could occur at damaged epithelia. Normally, invading pathogens captured by DC-SIGN are degraded and the processed antigens are subsequently presented to the appropriate T-cells. Although the capture and presentation of invading pathogens is essential for inducing adaptive immune responses, some pathogens escape from full degradation and in fact hijack this system to enhance infection (Geijtenbeek et al., 2000; de Witte L. et al., 2006). The biological relevance of DC-SIGN in transmission of HIV-1 and Mycobacterium tuberculosis is supported by linkages between DC-SIGN polymorphisms and risk of infection (Liu et al., 2004; Barreiro et al., 2006). We previously reported that bile-salt stimulated lipase (BSSL) from human breast milk strongly binds to DC-SIGN and interferes with DC-SIGN mediated viral transmission in vitro (Naarding et al., 2005; Naarding et al.,
BSSL polymorphism associates with binding variation

2006). Interestingly, BSSL has also been associated with protection against Norwalk virus infection, which is a major cause of gastroenteritis (Ruvoen-Clouet et al., 2006; Lindesmith et al., 2003). These studies indicated two possible anti-microbial mechanisms for BSSL, either through binding to DC-SIGN (HIV-1) (Naarding et al., 2006) or binding to the virus particle (Norwalk virus)(Ruvoen-Clouet et al., 2006).

BSSL is a lipase that is expressed in human blood and breast milk which aids breastfed infants with the digestion of milk triglycerides (Li & Hui, 1997; Blackberg & Hernell, 1981). The glycoprotein contains a mucin-like repeated 11 amino acid motif at the C-terminal tail that is abundantly modified by O-linked glycosylation. The repeated motif, encoded by exon 11 of the gene, protects the glycoprotein from proteolysis but does not play a role in the enzymatic activity of the lipase (Lindquist et al., 2002; Hernell & Blackberg, 1994). Furthermore, the O-glycosylated repeated motif expresses Lewis a and Lewis x sugars known to interact with DC-SIGN (Wang et al., 1995; Guo et al., 2004). We previously described that BSSL binds to DC-SIGN via Lewis x sugars. Furthermore we suggested that variation in BSSL protein size among mothers may relate to variation in DC-SIGN blocking capacities (Naarding et al., 2006).

The aim of this study was to characterize the DC-SIGN binding properties of breast milk derived from healthy breastfeeding mothers. Furthermore, we compared BSSL size variations with DC-SIGN binding capacity of breast milks. Our results reveal that the DC-SIGN binding capacity of breast milk is highly variable among mothers as well as among groups of mothers from differing geographical regions. We were able to correlate allelic polymorphisms in the BSSL gene with the DC-SIGN binding phenotype of breast milk. The observed variation in DC-SIGN binding properties of different milks may have implications for the risk of mucosal pathogen transmission during breastfeeding.

RESULTS

BSSL protein size is linked to DC-SIGN binding capacity of the corresponding human breast milk. We have previously identified BSSL from human milk as a strong DC-SIGN binding glycoprotein with variably sized BSSL isoforms differing in their capacity to bind DC-SIGN (Naarding et al., 2006). We hypothesized that the DC-SIGN binding capacity of BSSL and human breast milk is correlated with the BSSL protein size. To test this hypothesis, we determined the BSSL protein size and DC-SIGN binding capacity of human breast milk for 17 mothers from the Netherlands. BSSL average protein size was estimated for breast milk separated by SDS-PAGE (Figure 1A). The DC-SIGN binding capacity of milk was detected by Western blot (Figure 1B) and quantified for all milk samples by ELISA (Figure 1C). DC-SIGN binding capacity was highly variable (Figure 1B+C), independent of BSSL protein levels in the milks (Figure 1A). We calculated the relative DC-SIGN binding capacity with the strongest DC-SIGN binding milk arbitrarily set to 100%. We then compared BSSL protein sizes of weak DC-SIGN binding milk (<20%) to strong DC-SIGN binding milk (>20%).
Figure 1: DC-SIGN binding is highly variable and correlates with BSSL protein size. (A) Example of SDS-PAGE separation of breast milk from 7 mothers showing the bile-salt stimulated lipase (BSSL) of variable sizes. (B) Western blot stained with DC-SIGN of the same 7 milks as depicted in Figure A. (C) Breast milks with smaller BSSL protein have stronger DC-SIGN binding capacity than breast milks with larger BSSL protein. Molecular weights (MW) of BSSL protein was compared in milks with strong DC-SIGN binding capacity versus milks with weak DC-SIGN binding capacity. Median protein sizes in the weak and in the strong DC-SIGN binding groups are indicated by a horizontal line.

Figure 1C shows that the BSSL protein size was significantly smaller (p=0.020) in the strong DC-SIGN binding group than in the weak DC-SIGN binding group.

**DC-SIGN binding capacity of human breast milk is highly variant within and between different cohorts.** After identifying variation in DC-SIGN binding capacity of breast milk derived from a group of mothers from the Netherlands, we investigated the level of variation within and between different cohorts. We therefore tested DC-SIGN binding of human breast milk derived from the Netherlands (n=78), Sweden (n=21), Norway (n=146) and Egypt (n=24) using ELISA in triplicate. Figure 2A shows the DC-SIGN binding of all breast milk samples with the milk having the strongest DC-SIGN binding set to 100%. These results show that DC-SIGN affinity of human breast milk is highly variable among mothers. We confirmed the variation in DC-SIGN binding properties in all four cohorts (Figure 2B). In addition, significant differences were apparent between the cohorts. The breast milk

Figure 2: DC-SIGN affinity of breast milk is highly variable between mothers within cohorts and between different cohorts. (A) DC-SIGN binding capacity (y-axis) of milk derived from different mothers (x-axis) is highly variable. DC-SIGN affinity for milks from all cohorts was measured in triplicate in a DC-SIGN binding ELISA. Data are shown as mean values of triplicates ± standard error of the means (error bars). (B) DC-SIGN binding capacity is variable between different cohorts. Median DC-SIGN affinity is indicated by a horizontal line for each cohort.
samples from Sweden bound significantly stronger to DC-SIGN than the milks from the other cohorts (Figure 2B). Breast milk samples from the Netherlands had a marginally stronger (p=0.061) DC-SIGN binding than the tested samples in the Norwegian cohort.

The size of BSSL exon 11 is highly polymorphic. BSSL protein size variation may be related to variation in the variable number of tandem repeats (VNTR) domain encoded by BSSL exon 11. BSSL binds to DC-SIGN through Lewis type sugars present in this VNTR domain that is located at the C-terminus of the protein (Naarding et al., 2006; Wang et al., 1995). We established a PCR to determine the exact size of the VNTR domain (Figure 3A). The domain is highly variable in the number of repeats in the populations

![Figure 3: BSSL exon 11 is highly polymorphic in number of repeats.](image)

(A) Typical agarose gel analysis of BSSL exon 11 PCR genotyping including 3 marker lanes and 7 genotyped DNA samples. (B) Allelic variation in number of repeats is highly variable in all tested cohorts. (C) BSSL genotype distribution is highly variable in all cohorts with most mothers having at least 1 allele with 16 repeats.
we tested, with repeat numbers ranging from 12 to 18 and alleles with 16 repeats being most common (Figure 3B). The allele frequency distribution in the Egyptian cohort differs significantly from the Caucasian cohorts (p<0.001). Additionally, the allele frequency distribution in the Norwegian mothers differs significantly from that in Dutch mothers (p=0.001) and marginally (p=0.074) from the Swedish mothers. When looking at the genotype distribution in Figure 3C we observed that 79% of all mothers (82% in Caucasian cohorts) had at least 1 allele with 16 repeats.

**DC-SIGN binding capacity correlates to BSSL genotype.** Differences in BSSL protein size are linked to the DC-SIGN binding properties of milk (Figure 1C). In addition, we hypothesized that variation in DC-SIGN binding may be caused by size differences in the VNTR domain of the BSSL gene. To test this possibility we compared VNTR domain sizes with DC-SIGN binding potency of the corresponding milks. We arbitrarily defined 12 to 15 repeats as low (L) and 16 to 18 repeats as high (H) repeat number. Mothers have either two low (LL), one allele with low and one with high (LH) or two alleles with high repeat numbers (HH). The BSSL repeat number has a significant (p=0.018) effect on the DC-SIGN binding capacity for all cohorts (Figure 4). In the Caucasian cohorts we found that mothers with the LH genotype had significantly stronger DC-SIGN binding milk (p=0.016) than mothers with the HH genotype. These LH mothers also had marginally significant stronger DC-SIGN binding milk (p=0.064) than mothers with the LL genotype.

The LL genotype reaches low frequencies, as the majority of individuals carry at least 1 allele with 16 repeats (Figure 3C). We selected the large group of mothers with at least one 16 repeat allele and tested the effect of size variation in the second allele on DC-SIGN binding. We split the mothers into two groups: a group combining the 16 repeat allele with an L allele (16+L) and a group combining the 16 repeat allele with another H allele (16+H).
The results (Figure 5) revealed that mothers with the 16+L genotype have significantly stronger DC-SIGN binding milk than mothers with the 16+H genotype ($p=0.009$ for all cohorts; $p=0.016$ for Caucasians). When split into exact number of repeats, we found that particularly genotypes 13/16 and 15/16 tended to translate into higher affinity for DC-SIGN although this difference was not statistically significant (Figure 6).

**DISCUSSION**

In this study we demonstrate that the DC-SIGN binding capacity of human breast milk from different mothers is highly variable. We confirmed this finding in 4 independent cohorts and report that geographical variation exists in the DC-SIGN binding capacity. BSSL genes have either a high number of repeats (H=16 to 18) or a low number of repeats (L=12 to 15) in the VNTR domain. We observed that the combination of an L allele with an H allele (LH) correlates with strong DC-SIGN binding of breast milk. In addition, for mothers with at least one BSSL allele of 16 repeats we report that strong DC-SIGN binding is correlated with a small repeat number (16+L) in the second allele.

The observed variation in DC-SIGN binding capacity may in theory be related to diverse factors such as the BSSL expression level or polymorphisms in the BSSL gene. Analysis of DC-SIGN binding variation in breast milk samples with similar BSSL expression levels demonstrates that factors other than BSSL protein expression levels determine the DC-SIGN binding efficiency. Although we showed that BSSL polymorphisms are correlated with DC-SIGN binding we do not exclude that in case of breast milk samples with significantly deviating BSSL expression levels DC-SIGN binding can be influenced. We previously reported that BSSL is the major DC-SIGN binding component in human breast milk, but mucin 1 - mainly present in milk lipid vesicles - also binds to DC-SIGN (Saeland et al., 2009). However, we measured DC-SIGN binding of the aqueous phase of breast
milk, which contains the BSSL glycoprotein and excludes the lipids (Saeland et al., 2009; Naarding et al., 2006). We therefore believe that BSSL and not mucin 1 is the main source of the DC-SIGN binding we measured.

DC-SIGN forms tetramers with four binding pockets that have higher affinity for glycoproteins such as HIV gp120 than monomeric DC-SIGN (Bernhard et al., 2004). It therefore seems likely that glycoproteins carrying multiple DC-SIGN interacting sugars will have a higher affinity for DC-SIGN than glycoproteins with less of such glycans. Since BSSL is polymorphic in the number of potential O-glycosylation sites due to variation in the VNTR domain this might translate into increased affinity of long BSSL forms for DC-SIGN. In contrast, we initially found that breast milks with small BSSL proteins have a stronger DC-SIGN binding capacity than breast milk with large BSSL proteins. But our genotyping studies showed that the LH genotype is associated with stronger DC-SIGN binding than the LL and the HH genotype. This suggests that the combination of a low with a high repeat molecule provides the combination with optimal DC-SIGN binding properties. Therefore the number of glycans could play a role, but additionally other factors likely influence the DC-SIGN binding properties of BSSL such as the three dimensional structure of the protein. BSSL proteins form dimers between either two equally or differently sized BSSL proteins (McKillop et al., 1998). We propose that the quaternary structure of the BSSL dimer complex plays an additional role in determining the DC-SIGN binding properties by influencing the way BSSL sugars are presented to DC-SIGN. The combination of a high and low repeat BSSL protein is possibly presenting the best combination of a high number of DC-SIGN interacting glycans with an optimal three dimensional structure for binding to DC-SIGN.

Not all O-linked BSSL glycans carry DC-SIGN binding Lewis sugars (Wang et al., 1995) and the number of Lewis sugars per BSSL molecule may be influenced by variable activity of glycosidases (Nishihara et al., 1994; Lindesmith et al., 2003). The activity of such enzymes...
may add an additional level of complexity to the observed link between BSSL genotypes and DC-SIGN binding capacity of breast milk. This may explain the different behavior of LL genotyped breast milks with regard to DC-SIGN affinity in the Norwegian population versus Dutch population. However, care should be taken because of the relatively low number of LL genotyped mothers (n=9) in the Dutch cohort and the high level of variation in DC-SIGN binding observed in this group. Cloning and expression of recombinant BSSL forms in cell lines should aid in further clarifying observed differences in DC-SIGN binding between BSSL proteins with variably sized repeat motifs.

Pathogen binding to DC-SIGN results in uptake by DCs and subsequent antigen presentation to T-cells but this mechanism appears to have been hijacked by some pathogens to promote their transmission (Geijtenbeek et al., 2000; de Witte L. et al., 2006). In addition to antigen presentation, pathogen binding by DC-SIGN triggers signal transduction resulting in modulation of DC immune activation status. DC-SIGN binding of Lewis type or high mannose glycans results into two separate routes of signaling depending on the glycan bound (Gringhuis et al., 2009). Human milk, semen and cervicovaginal secretions contain factors that interfere with the interaction between DC-SIGN and the pathogen in vitro (Naarding et al., 2005; Stax et al., 2009; Jendrysik et al., 2005). We therefore suggest that the DC-SIGN blocking activity of human secretions such as milk may, at least in part, explain why the risk of HIV-1 transmission via breastfeeding or sex is relatively low. Furthermore, the interaction between breast milk and DC-SIGN may influence the immune activation levels of DCs and other immune cells during breastfeeding. We observed a high level of variation in the DC-SIGN blocking properties of breast milk from different mothers. For the child this variation could result in differential immune activation levels and differences in the risk of infection, depending on the breastfeeding mother.

Although blocking the interaction between pathogen and DC-SIGN may be beneficial against DC-SIGN mediated transmission, receptor availability may still be necessary for inducing effective adaptive immune responses against other pathogens. Such conflicting roles for DC-SIGN may explain why the observed variation in DC-SIGN binding properties of human breast milk may be beneficial at the population level. Exposure to pathogens may result in selective pressures in the direction of either strong or weak DC-SIGN blocking depending on the type of circulating pathogens dominating a specific population. We therefore speculate that the observed geographical variation in DC-SIGN binding may be a result of local selective pressures exerted over an evolutionary timescale. Furthermore, we suggest that variation in DC-SIGN blocking properties of mucosal secretions might be a general theme in natural protection against rapidly evolving mucosal pathogens. As part of innate immunity, the identified BSSL size variation may represent one of multiple strategies for a population to have variable levels of protection against mucosal pathogens. Our study may help develop BSSL derived therapeutic molecules for mucosal application against pathogenic infections for individuals with low natural protection levels.
MATERIALS AND METHODS

Processing of human breast milk. Human breast milk was collected from 245 healthy Caucasian mothers from The Netherlands, Sweden and Norway and 24 healthy Egyptian mothers. All sample collections were in accordance with internal ethics review boards. The milk was centrifuged twice at 530 x g for 30 min at 4 °C to remove lipid and cells. The cells were reconstituted in L6 lysis buffer and the DNA was purified as previously described (Boom et al., 1990).

BSSL protein size estimation. Processed human breast milk was separated using 8% SDS-PAGE gels (Bio-Rad, Veenendaal, The Netherlands). Gels were stained with Coomassie stain and the average BSSL protein size was estimated. Relative protein size values were calculated with the mother with the smallest protein size set to 100%.

DC-SIGN binding ELISA. ELISA plates were coated with human milk 100 fold diluted in 0.2 M NaHCO₃ buffer. Plates were blocked with 5% BSA and subsequently incubated with a recombinant human DC-SIGN-Fc chimera (R&D systems) in TSM buffer (20 mM TRIS, 150 mM NaCl, 1 mM CaCl₂ and 2 mM MgCl₂) containing 5% BSA as previously described (Stax et al., 2009; Naarding et al., 2006). Peroxidase labeled anti human Ig-Fc antibodies (Jackson Immunology) were used to quantify the bound DC-SIGN-Fc. Non specific binding of DC-SIGN-Fc was determined for each individual sample by pre-incubating the calcium dependent DC-SIGN-Fc for 20 min with 20mM EGTA (Sigma-Aldrich).

Genotyping PCR. Lidberg and colleagues previously identified the carboxyl ester lipase-like gene (CELL, genbank accession number M94580) with high homology to the BSSL gene (Lidberg et al., 1992). CELL contains a VNTR domain resembling the exon 11 VNTR domain of BSSL although Lidberg and colleagues reported the CELL VNTR to contain 7 repeats less than BSSL. Interference of CELL during the PCR amplification of BSSL was prevented by designing primers that indisputably only amplify BSSL exon 11 and not the CELL VNTR. Primers (forward primer: ACCAACTTCTGGCTACTGGACCTC, reverse primer: TGATACCAAGGCTCATGGGACGCTAAAAC) contained a FAM label for detection. After initial denaturation for 4' at 94° C the following program was run for 35 cycles: 30'' 94° C, 3' 60° C, 1' 72° C followed by an extended elongation for 7' at 72° C. The PCR product was mixed with the Genescan™ – 1200 LIZ® Standard (Applied Biosystems; catalog#: 4379950) and the ABI 3100 capillary sequencer (Applied Biosystems) in Genescan mode was used for PCR product size determination. Data was analyzed using Genemapper software (Applied Biosystems).

Statistical analysis. BSSL protein sizes are presented as individual observations and average values of triplicate DC-SIGN binding capacity measurements were plotted. Median values were indicated in the figures. BSSL protein size of the weak and strong DC-SIGN binding capacity groups was compared using a Mann-Whitney test. Differences in DC-SIGN binding capacity between cohorts were analyzed using the Kruskal-Wallis test, with subsequent pairwise Mann-Whitney tests in case of an overall significant difference.
Allele frequencies were compared between the cohorts using a Fisher-Exact test. The effect of the genotype on DC-SIGN binding capacity was analyzed using a non-parametric ANOVA (rank transformed values), with adjustment for cohort and the cohort*genotype interaction. All analyses were carried out using SPSS (release 17, SPSS Inc.) and p-value < 0.05 were considered statistically significant.

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