Mucin 6 in seminal plasma binds DC-SIGN and potently blocks dendritic cell mediated transfer of HIV-1 to CD4⁺ T-lymphocytes

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Many viruses transmitted via the genital or oral mucosa have the potential to interact with dendritic cell-specific intercellular adhesion molecule-3 grabbing non integrin (DC-SIGN) expressed on immature dendritic cells (iDCs) that lie below the mucosal surface. These cells have been postulated to capture and disseminate human immunodeficiency virus type-1 (HIV-1) to CD4⁺ lymphocytes, potentially through breaches in the mucosal lining. We have previously described that BSSL (bile salt-stimulated lipase) in human milk can bind DC-SIGN and block transfer. Here we demonstrate that seminal plasma has similar DC-SIGN blocking properties as BSSL in human milk. Using comparative SDS-PAGE and Western blotting combined with mass spectrometry we identified mucin 6 as the DC-SIGN binding component in seminal plasma. Additionally, we demonstrate that purified mucin 6 binds DC-SIGN and successfully inhibits viral transfer. Mucin 6 in seminal plasma may therefore interfere with the sexual transmission of HIV-1 and other DC-SIGN co-opting viruses.

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Introduction

Millions of newly diagnosed infections with human immunodeficiency virus-1 (HIV-1) occur yearly, which could be prevented if a good vaccine or microbicides were available. Approximately 80% of new infections with HIV-1 are established through sexual intercourse (Shattock and Moore, 2003). However risk of transmission is relatively low with an estimated high number of sexual exposures required for transmission to occur. Estimates for per-sexual contact infectivity range from 1:200 for receptive anal intercourse to 1:2000 for receptive vaginal intercourse (O’Brien et al., 1994; Varghese et al., 1998). Bodily fluids such as semen and cervical vaginal lavage will undoubtedly contribute to the cellular environment at the exposed mucosa during sexual intercourse. It is therefore important to identify possible bodily fluid components affecting initial interactions of HIV-1 with potential target cells. Such substances, in addition to known factors, including virus load (Butler et al., 2008), will likely contribute to host dependent risk of HIV-1 transmission.

During sexual transmission of HIV-1 the virus first has to cross the mucosal epithelial layer of the recipient to gain access to susceptible cells. Cell types that have been postulated to act as primary targets for transmitted HIV-1 are CD4⁺ T-lymphocytes, Langerhans cells (LCs) and dendritic cells (DCs) (Haase, 2005; de Witte et al., 2007). These cell types all can be infected with HIV-1 but, in addition, DCs have been shown, in vitro, to transfer HIV-1 to CD4⁺ T-lymphocytes (Geijtenbeek et al., 2000a) without the necessity of infection. DCs are antigen presenting cells that express several C-type lectins which are binding receptors for HIV-1. The major C-type lectin on DCs that has been shown to interact with HIV-1 is DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3 grabbing non integrin). DC-SIGN binds to carbohydrates containing oligo mannose and Lewis sugars with terminal fucoses of both "self" glycans and glycans of pathogens (Van Liempt et al., 2004; Van Liempt et al., 2006; Guo et al., 2004; van Kooyk and Geijtenbeek, 2003). It recognizes a wide range of pathogens such as Helicobacter pylori, Schistosoma mansoni, Mycobacterium tuberculosis and many viruses (e.g. HIV-1, hepatitis C virus, herpes simplex virus) (Geijtenbeek et al., 2000b; Geijtenbeek and van Kooyk, 2003; Poumbourios and Drummer, 2007; de Jong et al., 2008; Jameson et al., 2002; Soilleux et al., 2002; Rappocciolo et al., 2006).

DC-SIGN is used by antigen presenting cells to capture pathogens or pathogen antigens which are subsequently processed and presented to T-lymphocytes. Several pathogens transmitted via genital or oral mucosa interact with DC-SIGN expressed on immature dendritic cells at the mucosa (Halary et al., 2002; de Jong et al., 2008; Poumbourios and Drummer, 2007). Although capture of pathogens by antigen presenting cells via DC-SIGN is necessary for antigen...
presentation, it can also be used by pathogens to enhance host infection. Specifically, immature dendritic cells have been postulated to capture and disseminate HIV-1 via DC-SIGN following exposure. This would then, probably, be occurring at damaged epithelia, where the cell types expressing DC-SIGN come into contact with HIV-1 and its carrier fluid (Geijtenbeek et al., 2000a). The higher incidence of HIV-1 transmissions in the context of secondary infections that result in a damaged mucosal epithelium supports the hypothesis that lesions in the epithelia provide access for HIV-1 (Celentano et al., 1996; Corbett et al., 2002; Kaul et al., 2000, 2008).

A number of bodily secretions including human seminal, seminal plasma (SP) and cervical vaginal lavage (CVL) contain components influencing the interaction of HIV-1 with CD4+ T-lymphocytes and DCs (Naarding et al., 2005, 2006; Jendryszik et al., 2005; Sabatte et al., 2007; Munch et al., 2007). We have previously shown that bile salt-stimulated lipase (BSSL) in human milk is able to bind to DC-SIGN and block subsequent HIV-1 transfer to CD4+ T-lymphocytes via DCs (Naarding et al., 2006). We hypothesized that seminal plasma (SP) contains one or more components that have DC blocking properties similar to BSSL from human milk. In this study we show that SP, indeed, has DC blocking properties and we show that the DC-SIGN binding factor in SP is over 460 kDa. Using different analyses, including mass spectrometry, we identified mucin 6 as the DC-SIGN binding component in SP. Additionally we tested SPs from different donors and demonstrated that the level of DC-SIGN blocking shows significant differences between SPs from different donors.

Results

Dialyzed seminal plasma is not cytotoxic to Raji DC-SIGN cells

We initially tested the effect of seminal plasma (SP) on transfer of HIV-1 to CD4+ T-cells by Raji DC-SIGN cells. In this experiment we observed a high percentage (>90%) of cell death in cell cultures treated with SP. We hypothesized that the cytotoxicity of SP is caused by its basic pH and that the cytotoxicity could be alleviated by dialysis against PBS. To test this hypothesis, we cultured Raji DC-SIGN cells in the presence of different dilutions of SP or dialyzed SP (DSP). In Fig. 1 we show a decrease in viability for cells cultured with 32× diluted SP. More concentrated solutions of SP induced substantial cell death with over 80% dead cells. These results are in line with previous observations (O’Connor et al., 1995). In contrast to SP, DSP was not cytotoxic for the cells at the tested dilutions. No cytotoxicity of DSP was observed either with immature monocyte derived dendritic cells (IMDDC) or CD4+ T-cells. Therefore we have used DSP in our assays in order to exclude interference of cytotoxicity of SP during our analyses.

Identification of DC-SIGN blocking component(s)

After observing that DSP blocks DC-SIGN mediated transfer of HIV-1 to CD4+ T-lymphocytes, we aimed to identify the component or components present in DSP that are responsible for the observed DC-SIGN binding and blockage.

We showed that DSP blocks DC-SIGN and blocks both Raji DC-SIGN and IMDDC mediated transfer of HIV-1 to CD4+ T-lymphocytes. We next tested the DC-SIGN binding capacity and the ability to block HIV-1 transfer to CD4+ T-lymphocytes by IMDDCs of DSPs derived from different donors. We compared DC-SIGN binding of DSP derived from eight different donors in a DC-SIGN binding ELISA as well as from different donors. We compared DC-SIGN binding of DSP derived from different donors in a DC-SIGN binding ELISA as well as from different donors. We compared DC-SIGN binding of DSP derived from different donors in a DC-SIGN binding ELISA as well as from different donors. We compared DC-SIGN binding of DSP derived from different donors in a DC-SIGN binding ELISA as well as from different donors. We compared DC-SIGN binding of DSP derived from different donors in a DC-SIGN binding ELISA as well as from different donors. We compared DC-SIGN binding of DSP derived from different donors in a DC-SIGN binding ELISA as well as from different donors.}

![Fig. 1.](image)

Dialyzed seminal plasma is not cytotoxic to Raji DC-SIGN cells
components in DSP that provide the DC-SIGN blocking properties. The first step was to determine the size range in which the DC-SIGN blocking component(s) are present. Therefore we separated DSP into fractions containing components with a molecular weight lower than 30 kDa, molecular weight between 30 and 100 kDa and a fraction with components larger than 100 kDa. DC-SIGN affinity was measured in the DC-SIGN binding ELISA and non-fractionated DSP was used as an input control for the fractionation. Fig. 4 shows the DC-SIGN binding of DSP strongly binds DC-SIGN and blocks DC-SIGN mediated transfer of HIV-1 to CD4+ T-lymphocytes. (A) DC-SIGN affinity of DSP (donor 1) was tested in a DC-SIGN ELISA. The binding signal (measured at 450 nm) for DC-SIGN-Fc pre-incubated with TSM was significantly stronger than the binding signal with EGTA inactivated DC-SIGN-Fc (negative control). (B) The HIV-1 transfer blocking properties of DSP (donor 1) were tested with Raji DC-SIGN cells pre-incubated with RPMI (control (Ctrl)) or different dilutions of DSP. Transfer was blocked for 80% with 10,000 fold diluted DSP. (C) Additionally, the DC-SIGN blocking properties of DSP (donor 1) were tested in a co-culture of Raji or Raji DC-SIGN cells with CD4+ T-lymphocytes in the presence of HIV-1 mixed with RPMI or different concentrations of DSP. Both an R5 (NSI-18) and an X4 virus (LAI) were tested. (D and E) The blocking properties of DSP for HIV-1 transfer to CD4+ T-lymphocytes via IMDDCs was tested with IMDDCs incubated with RPMI (control (Ctrl)), 1000 fold diluted DSP (donor 10) or DC-SIGN blocking antibody AZN-D1. Blocking properties were tested for both a CCR5 (NSI-18, D) and a CXCR4 (LAI, E) using virus. Data from all figures are shown as mean values of triplicates ± standard error of the means (error bars).
fractionated and non-fractionated DSP corrected for non specific binding (negative control). The DC-SIGN binding capacity of the fraction with components larger than 100 kDa had a binding capacity similar to that of non-fractionated DSP. In contrast, the fraction with components between 30 kDa and 100 kDa, and the fraction with components smaller than 30 kDa had no significant DC-SIGN affinity. These results demonstrate that the component or components in DSP binding to DC-SIGN are larger than 100 kDa.

The DC-SIGN binding component was identified by separating DSP using denaturing SDS-PAGE and analyzing the protein positive for DC-SIGN staining (Western blot) with MALDI-TOF. DSPs from donors 1, 2 and 3 were separated using SDS-PAGE and blotted on a PVDF membrane. The PVDF membrane was stained with DC-SIGN-Fc and the SDS-PAGE gel with a Colloidal Coomassie stain (Fig. 5). The DC-SIGN staining showed a single strong signal for a component larger than 460 kDa (Fig. 5). The protein in the Coomassie stained gel corresponding to the DC-SIGN signal on the PVDF membrane was extracted from gel and subsequently analyzed using MALDI-TOF. Additionally, protein bands negative for DC-SIGN binding were extracted and analyzed (negative controls). The DC-SIGN binding protein band was selected for peptide mass fingerprint analysis. The acquired peptide spectrum from the digested SDS-PAGE band was searched against non-redundant sequence databases (MSDB and SwissProt) using the online MASCOT search engine without search restrictions. In both cases the top score represented Human Mucin 6.

MSDB: Q6W4X9_HUMAN MOWSE Score: 117 with 26 matching peptides. SwissProt: Muc6_Human MOWSE Score: 99 with 29
Fractions 2, 5, 8, 14, and 38 (indicated with asterisk) were digested with trypsin and analyzed with LC/MS/MS. The vast majority of DC-SIGN binding activity was found in fractions containing proteins with molecular weight higher than 700 kDa, which corresponds to the observed activity on gel. The strong binding fraction 2, 5, and 8 were shown to contain mucin 6, a component responsible for the DC-SIGN binding activity found on gel. The strong binding fraction 2, 5, 8, 14, and 38 were digested with trypsin and analyzed with LC/MS/MS. The majority of the DC-SIGN binding was found in the fractions containing molecular weights higher than 700 kDa corresponding to the observation of DC-SIGN binding on Western blot.

In this study we demonstrate that, in vitro, a SP component strongly inhibits DC-SIGN mediated infection of CD4+ T-lymphocytes in-trans. Furthermore, we showed that the viral transfer blocking properties of DSPs derived from different donors vary significantly.

**Discussion**

After identifying the DC-SIGN blocking component in DSP as mucin 6, we aimed to test the DC-SIGN blocking properties of mucin 6 from DSP in a transfer assay. For this we fractionated DSP using a superose 6 10/300 GL column with an FPLC. Elution of protein from the fractionated DSP was monitored by measuring the optical density at 280 nm (upper, boxed graph in Fig. 6). The collected fractions were tested for DC-SIGN binding in a DC-SIGN ELISA (Fig. 6, bar graph below) and fractions 2, 5, 8, 14, and 38 were digested with trypsin and analyzed with LC/MS/MS. The vast majority of DC-SIGN binding activity was found in fractions containing proteins with molecular weight higher than 700 kDa, which corresponds to the observed molecular weight of DC-SIGN binding activity found on gel. The strong DC-SIGN binding fractions 2, 5, and 8 were shown to contain mucin 6, confirming our previous identification of mucin 6 as the DC-SIGN binding protein in the SDS-PAGE gel. Although the main component in fraction 14, with intermediate DC-SIGN affinity, was identified as clusterin, mucin 6 derived peptides were also detected in this fraction. A non-DC-SIGN binding fraction (number 38) contained serum albumin as well as clusterin peptides, demonstrating that clusterin is not the DC-SIGN binding component. Additionally, in fraction 38 no mucin 6 derived peptides were found. In conclusion, all fractions having DC-SIGN binding activity contained mucin 6 peptides. Fractions without binding affinity did not contain any mucin 6 derived peptides. These results further confirm our initial identification of mucin 6, from the SDS-PAGE gel extracted DC-SIGN binding protein (Fig. 5), as the DC-SIGN blocking component in seminal plasma.

The capacity of mucin 6 to block DC-SIGN mediated transfer of HIV-1 to CD4+ T-lymphocytes was tested with fractions derived from the superose purification of DSP. We incubated Raji and Raji DC-SIGN cells with fraction 5, containing mucin 6, or fraction 38, without mucin 6. Raji and Raji DC-SIGN cells incubated with non-fractionated DSP or RPMI were included as controls. Fig. 7 shows that fraction 5, containing mucin 6, was able to fully block transfer of HIV-1 by Raji DC-SIGN cells while fraction 38 without mucin 6 did not inhibit the transfer. In addition to identifying the glycoprotein responsible for the DC-SIGN blocking properties of DSP, we characterized which sugars are providing mucin 6 with its DC-SIGN binding capacity. DC-SIGN is known to bind to carbohydrates containing oligo mannose or Lewis sugars with terminal fucose. We therefore used α1-2,3,6-mannosidase and α1-3,4-fucosidase to deglycosylate SP. The DC-SIGN binding capacity of deglycosylated SP was then tested for DC-SIGN affinity in a DC-SIGN binding ELISA. Our results (Fig. S2A) show a non significant decrease in DC-SIGN binding of mannosidase treated SP versus mock treated SP. Upon fucosidase treatment, however, we observed a significant (p < 0.01) decrease of 50% in DC-SIGN binding capacity of treated SP versus mock treated SP. These results suggest that Lewis sugars are the main groups providing mucin 6 with its DC-SIGN binding capacity. We then used an ELISA assay to identify if Lewis sugars are indeed present in the SP sample tested. Our results show (Fig. S2B) that Lewis x and Lewis y are indeed present in SP, confirming previous observations of Chalabi et al. (2002). Taken together, our results strongly suggest that mucin 6 is the (major) component in DSP that provides DSP with the capacity to block DC-SIGN mediated transfer of HIV-1 to CD4+ T-lymphocytes.
The component in seminal plasma that binds DC-SIGN and inhibits DC-SIGN mediated in-trans infection of CD4+ T-lymphocytes was identified as mucin 6 (MUC6, gastric mucin-6; mapped to chromosome 11p15.5 (Toribara et al., 1993). The basis for this identification is threefold: 1) the protein in the gel at the position of DC-SIGN binding was unequivocally identified as mucin 6 by peptide mass fingerprint analysis, 2) the bulk of the binding activity was in gel filtration fractions containing proteins over 700 kDa with a strong correlation between amount of binding activity and the abundance of mucin 6 derived peptides, and 3) mucin 6 is known to express the Lewis y sugars (Nordman et al., 2002) that can bind to DC-SIGN (and we showed that removal of DC-SIGN binding Lewis fucoses from SP greatly decreases the DC-SIGN affinity of SP).

Previously we reported that BSSL in human milk binds DC-SIGN and blocks DC-SIGN mediated HIV-1 transfer (28). When we observed that DSP also has DC-SIGN binding and blocking properties, we hypothesized that the component responsible is BSSL. However, using SDS-PAGE and Western blotting we noticed that the major DC-SIGN binding component in DSP is larger than 460 kDa. This implied that the responsible factor is not BSSL, which is 100–120 kDa in size. Using MALDI-TOF analysis we subsequently identified mucin 6 as the major DC-SIGN binding component of DSP. The protein in the SDS-PAGE gel corresponding with DC-SIGN binding activity proved to be mucin 6 with a significant MOWSE score in our peptide mass fingerprint analysis while no other proteins could be identified.

Although we are only looking at very high molecular weight proteins of over 700 kDa, it could still be possible that less abundant proteins with very high DC-SIGN binding activity co-migrate with mucin 6 in a SDS-PAGE gel. However, it is highly unlikely that a molecule at such low concentrations (MALDI-TOF analysis did not identify any protein other than mucin 6) would provide this level of strong inhibitory activity. Ultimately, expression of mucin 6 or depletion of the molecule from SP will confirm mucin 6 as the inhibitory factor. But these are complex experiments to perform given the size of the molecule, the extent/complexity of glycosylation required for binding to occur and the lack of materials available. We therefore purified mucin 6 from DSP with the aid of gel filtration and analyzed binding affinity and protein content (with tryptic digestion followed by LC-MS/MS) of the resulting fractions in tandem. Using this approach we showed that the fractions containing mucin 6 have strong DC-SIGN binding activity, confirming our previous identification of mucin 6 as the (major) DC-SIGN binding component in DSP. Additionally we showed in a HIV-1 transfer assay with Raji DC-SIGN cells that the mucin 6 containing fraction is able to block transfer of HIV-1 via DC-SIGN. Collectively, given the evidence available we are convinced that the binding and inhibitory activity of SP can be contributed to mucin 6.

Although the DC-SIGN blocking component in DSP (mucin 6) is not identical to the DC-SIGN blocking component in human milk (BSSL), the two factors do share the properties that are likely to provide effective DC-SIGN binding: both factors are large glycoproteins and they have the same type of highly O-glycosylated tandem repeats which form the scaffold for DC-SIGN binding glycans (Landberg et al., 2000; McKillop et al., 2004; Wang et al., 1995; Andrianifahanana et al., 2006; Linden et al., 2008). Additionally, both glycoproteins express Lewis sugars that can bind to DC-SIGN (Van Liempt et al., 2006; Guo et al., 2004; Nordman et al., 2002; Van Liempt et al., 2004).

Mucin 6 is a member of the mucin family which consists of large cell membrane associated and secreted glycoproteins (Linden et al., 2008). These glycoproteins are widely expressed in most epithelia where the highly glycosylated proteins form a mucus layer that protects epithelial cells from the exterior environment (Linden et al., 2008) and proteolysis (Toribara et al., 1993; Jentoft, 1990). Several members of the mucin family have also been described to be present in secreted fluids such as saliva, CVL and human milk (Habte et al., 2006, 2007).

We observed that DSPs from different donors have differences in DC-SIGN affinity and viral transfer blocking capacity. This observation may, in part, be explained by donor dependent differences in mucin 6 expression levels or differences in post translational modifications (Ruveno-Clouet et al., 2006). Mucin 6 contains a large tandem repeat domain which is highly O-glycosylated and the size of this domain has been shown to vary considerably between individuals (Rousseau et al., 2004; Vinall et al., 1998). Interestingly, Nguyen et al. (2006) found that short mucin 6 alleles are associated with H. pylori infection. It may therefore be interesting to test if the size of the mucin 6 alleles also affects DC-SIGN binding of the glycoprotein.

Although our results suggest that the risk of HIV-1 transfer via DC-SIGN is greatly reduced in the presence of semen, transmission of seminal HIV-1 particles via DC-SIGN may still occur at low levels. We observed significant differences in DC-SIGN affinity (up to 2.5 fold differences in ELISA signal intensity) between DSPs derived from the small number of donors that we tested. Moreover, it is not unlikely that we will find larger differences in DC-SIGN affinity when the number of screened donors is increased. This indicates that semen of some individuals will have a reduced protective effect for DC-SIGN compared to the semen of others. This variation in DC-SIGN affinity of seminal plasmas from different individuals may translate into differences between individuals in the risk of transmitting HIV-1 or other DC-SIGN using pathogens.

In addition to semen and mother milk, also cervical vaginal lavage (CVL) has the capacity to block DC-SIGN mediated transfer of HIV-1 to CD4+ T-lymphocytes (Jendryšik et al., 2005). The DC-SIGN blocking component of CVL has not been identified yet but mucin 6 is present in CVL (Andersch-Bjorkman et al., 2007). Based on our observations of blocking properties of mucin 6, we hypothesize that mucin 6 is providing CVL with the described DC-SIGN blocking capacity. With the observation of DC-SIGN binding and blocking properties of semen, mother milk and CVL, we see a general theme emerging. Whether mucin 6 is the blocking compound in CVL or not, in all secretions we find naturally occurring DC-SIGN binding molecules competing with pathogens for binding to antigen presenting cells (APCs). This could reflect the fact that, while binding to APCs via DC-SIGN can be important for subsequent antigen presentation, it can also be used by a pathogen to infect hosts more efficiently. This equilibrium could then be under extra control by the production of competing DC-SIGN binding molecules. Production of these DC-SIGN binding molecules may have evolved to protect individuals and their offspring against continuous challenge by pathogens.

Langerhans cells and dendritic cells are among the first cells to be encountered by HIV-1. Both cell types are preferentially infected by CCR5 using virus (Reece et al., 1998), the virus type known to establish initial infection. With our study we show that mucin 6 in SP blocks DC-SIGN mediated transfer of HIV-1 to CD4+ T-cells. This finding suggests that, in the presence of semen, HIV-1 capture by DC-SIGN is not likely to play an important role during the initial establishment of infection with HIV-1. But DC-SIGN is not required for infection of DCs and thus it remains possible that DCs are directly infected, leading to the establishment of a new infection with HIV-1. On the other hand we cannot conclude that DC-SIGN plays a role when semen is not present or when DC-SIGN blocking by semen is not 100%. Another remaining question regards the effect of semen on other C-type lectins. Langerhans cells express the C-type lectin langerin (de Witte et al., 2007) which is able to bind HIV-1. In contrast to the sequel upon DC-SIGN binding, virus captured by langerin is degraded (in the Birbeck granules) before it can be transmitted to CD4+ T-cells (de Witte et al., 2006). Langerhans cells can be productively infected when langerin is blocked and therefore it may be of interest to test whether SP influences infection of Langerhans cells. The third cell type that may serve as initial target for HIV-1 during the establishment of new infections is the CD4+ T-lymphocyte. Infection levels of CD4+ T-lymphocytes, in vitro, are low, but the infection levels dramatically
increase when these CD4+ cells are cultured in the presence of DC-SIGN expressing cells. Additionally, Munch et al. (2007) have shown that semen derived amyloid fibrils enhance infection of CD4+ T-lymphocytes by direct CD4+ cell attachment and indirect via DC-SIGN expressing cells. Additionally, Munch et al. (2007) have shown which effect will be dominant in vivo. What is clear is that, regardless of which mechanism is dominant during transmission, the low rate of HIV-1 transmissions per-sex act suggests that the establishment of new infections is not very efficient.

With several antiviral properties described for mucins to date, these glycoproteins are components of the innate immune response (Habte et al., 2006, 2008; Nguyen et al., 2006). The identification of mucin 6 as a glycoprotein that, like BSSS, is able to prevent viral hijacking via DC-SIGN provides further insight regarding the innate immune system. Knowledge gained from understanding the molecular interactions of mucin 6 and BSSS with DC-SIGN can be used in the development of microbicides that can help prevent HIV-1 or other pathogen transmissions. The reason being that in the absence of semen or in situations where the seminal blocking of DC-SIGN is relatively low, additional DC-SIGN blocking may be beneficial in reducing the likelihood of transmission. Furthermore, molecules like mucin 6 and BSSS may be used to specifically target therapeutic agents or vaccine vehicles to DCs via DC-SIGN. Follow up studies are needed to gain more insight into the molecular structure and glycan organization of mucin 6 and BSSS, optimal for DC-SIGN binding. For the development of these molecules as microbicides, additional research is also required to determine the minimal sizes of these glycoproteins still providing optimal DC-SIGN binding.

Materials and methods

Cells

The Raji and Raji DC-SIGN cells were obtained and cultured as previously described (Geijtenbeek et al., 2000a; Naarding et al., 2005, 2006). Peripheral blood mononuclear cells (PBMCs) were isolated from three buffy coats using ficoll–hypaque density centrifugation and pooled before freezing. The cells were cultured in RPMI containing 10% FCS, recombinant IL-2 (100 U/ml), penicillin (100 U/ml) and streptomycin (100 U/ml) and were activated with phytohemagglutinin (3 μg/ml). At day 3 the PBMCs were enriched for CD4 positive cells by negative selection using CD8 immunomagnetic beads (Dynal Biotech). CD4 positive cells were cultured in RPMI containing 10% FCS, recombinant IL-2 (100 U/ml), penicillin (100 U/ml) and streptomycin (100 U/ml).

Immature monocyte derived dendritic cells (IMDDCs) were prepared as previously described (Sallusto and Lanzavecchia, 1994). Human blood monocytes were isolated from buffy coats by use of a ficoll gradient and a subsequent CD14 selection step using a MACS system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Purified monocytes were differentiated into IMDDCs in the presence of interleukin-4 (IL-4) and granulocyte-macrophage colony-stimulating factor (500 and 800 U/ml, respectively; Serching-Plough, Brussels, Belgium) and used on day 6. The phenotype of the IMDDCs was measured by following intracellular CA-p24 expression. Co-culture of DC-SIGN expressing cells and CD4+ T-lymphocytes was performed as previously described (van Montfort et al., 2007). In short, 1×107 immature monocytes derived dendritic cells (IMDDCs) were incubated for 30 min with 1000 fold diluted DSP from various donors, or with 20 μg/ml DC-SIGN blocking antibody at 37 °C. Virus (1.000 TCID50/ml end concentration) was captured by pre-treated IMDDCs for 2 h at 37 °C. Unbound virus was removed by washing IMDDCs three times with medium and cells were co-cultured with 2.0×104 CD4+ enriched T-lymphocytes. Raji and Raji DC-SIGN cells incubated with RPMI without DSP before adding virus were used as controls. Replication of transferred HIV-1 was measured at day 7 using a standardized capsid-p24 ELISA protocol (Naarding et al., 2006).

Transfer by DCs: Transfer of HIV-1 to CD4+ enriched T-lymphocytes by dendritic cells was performed as previously described (van Montfort et al., 2007). In short, 1×107 immature monocytes derived dendritic cells (IMDDCs) were incubated for 30 min with 1000 fold diluted DSP from various donors, or with 20 μg/ml DC-SIGN blocking antibody at 37 °C. Virus (1.000 TCID50/ml end concentration) was captured by pre-treated IMDDCs for 2 h at 37 °C. Unbound virus was removed by washing IMDDCs three times with medium and cells were co-cultured with 2.0×104 CD4+ T-lymphocytes in a 96 wells plate. Medium was removed after 48 h and cells were cultured in fresh RPMI, containing rIL-2 (2 mg/ml) and indinavir (1 μM; NIBSC) for 3 days. Transmission was determined as the amount of infected CD4+ T-lymphocytes measured by following intracellular CA-p24 expression by FACS flow cytometry per 1.0×107 CD3+ T-lymphocytes.

Virus

Replication-competent HIV-1 stocks were generated by the passage of viruses through CD4+ T-lymphocytes as previously described (Naarding et al., 2005, 2006). Subtype B LAI (X4) and NSI-18 (R5) were used.

Seminal plasma

Semen was centrifuged for 15 min at 14000 rpm. The supernatant (seminal plasma) was dialyzed in 4.0.5× PBS over night at 4 °C using a 3.5K Slide-A-Lyzer® Dialysis Cassette (Pierce). The buffer was replaced by fresh 0.5× PBS the next morning and left for another 4 h. The seminal plasma was collected and centrifuged again for 15 min at 14,000 rpm before storage at −20 °C. Semen samples were donated by healthy volunteers and used after informed consent. For determining the protein concentration of DSP, the DSP samples were diluted 1:10 in PBS and the optical density at 280 nm was measured.

Seminal plasma cytotoxicity test

Dilutions of filter sterile SP or filter sterile dialyzed SP (DSP) were made in RPMI supplemented with penicillin (100 U/ml) and streptomycin (100 U/ml). Raji DC-SIGN cells were incubated for 2 h after addition of the SP or DSP dilutions. The cells were washed with PBS and cultured. Cell viability was determined at day 2 using classical trypan blue staining. The cytotoxicity was determined by calculating the ratio of non viable versus viable cells.

DC-SIGN mediated transfer of HIV-1 in presence of DSP

DSP dilutions were made in RPMI. Raji or Raji DC-SIGN cells (5×106 cells/well) were pre-incubated with dilutions of DSP before addition of 250 TCID50/ml HIV-1 isolate NSI-18. After 60 minute incubation, the cells were washed and co-cultured with 2×104 CD4+ enriched T-lymphocytes. Raji and Raji DC-SIGN cells incubated with RPMI without DSP before adding virus were used as controls. Replication of transferred HIV-1 was measured at day 7 using a standardized capsid-p24 ELISA protocol (Naarding et al., 2006).

Transmission by DCs: Transfer of HIV-1 to CD4+ enriched T-lymphocytes by dendritic cells was performed as previously described (van Montfort et al., 2007). In short, 1×107 immature monocytes derived dendritic cells (IMDDCs) were incubated for 30 min with 1000 fold diluted DSP from various donors, or with 20 μg/ml DC-SIGN blocking antibody at 37 °C. Virus (1.000 TCID50/ml end concentration) was captured by pre-treated IMDDCs for 2 h at 37 °C. Unbound virus was removed by washing IMDDCs three times with medium and cells were co-cultured with 2.0×104 CD4+ T-lymphocytes in a 96 wells plate. Medium was removed after 48 h and cells were cultured in fresh RPMI, containing rIL-2 (2 mg/ml) and indinavir (1 μM; NIBSC) for 3 days. Transmission was determined as the amount of infected CD4+ T-lymphocytes measured by following intracellular CA-p24 expression by FACS flow cytometry per 1.0×107 CD3+ T-lymphocytes.
Fluorescent-activated cell sorting analysis of infected cells

Infection was measured using fluorescent-activated cell sorting (FACS) analysis (van Montfort et al., 2007). In short, two days after infection 1 mM Indinavir was added to cell cultures. Cells were fixed 4 days after infection in 3.7% formaldehyde and permeabilized in 0.1% saponin [Riedel-de-Haën, S060905] and 1% BSA/50 mM NaH4Cl2 in PBS. Immunostaining was performed with 1:100 anti-human CD3 APC [BD Pharmingen cat:555335]/1:200 anti P24-FITC [Coulter Clone. Cat 6604665]. The fraction of infected cells was determined using FACS.

DC-SIGN binding ELISA

ELISA plates were coated with 1000 fold diluted in 0.2 M NaHCO3 buffer. For the fractionated DSP a 250 fold dilution was coated. Plates were incubated with a recombiant 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 (Naarding et al., 2005, 2006). Peroxidase labeled anti-human 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 20 mM EGTA (Sigma-Aldrich).

Deglycosylation of seminal plasma

ELISA plates were coated with SP 1000 fold diluted in 0.2 M NaHCO3 buffer. Coated wells were treated with α-1,2-3,8-mannosidase (Prozyme) or α-1,3,4-fucosidase (Calbiochem) according to the protocol of the manufacturer. Incubations were performed overnight at 37 °C. After deglycosylation, the wells were washed with TSM buffer and a DC-SIGN ELISA was performed to determine the DC-SIGN binding.

Lewis sugar ELISA

ELISA plates were coated with SP 1000 fold diluted in 0.2 M NaHCO3 buffer. Coated wells were incubated with mouse anti Lewis b mAb (T218) (Calbiochem), mouse anti Lewis x (VWR International) or mouse anti Lewis y mAb (F3) (Calbiochem) 40 fold diluted in TSM containing 5% BSA or with TSM/BSA only as a negative control. Anti mouse IgG-HRP antibodies were used to detect bound anti Lewis antibodies.

Fractionating seminal plasma using centrifugal filter devices

DSP was fractionated using YM-30 and YM-100 Microcon® centrifugal filter devices (Millipore) and as directed in instructions to users.

SDS-PAGE and Western blot analysis of DSP

DSP was separated using 3–8% NuPage TRIS/acetate gels (Invitrogen). Gels were stained with a Colloidal Coomassie stain (Fermentas) and destained with deionized water. For immunoblotting the gels were blotted on polyvinylidene difluoride membranes (PVDF-F; Millipore) and stained with DC-SIGN-Fc (R&D systems, Inc.), and goat anti-human IgG1 antibody (Jackson Immunoresearch, West Grove, PA). Visualization was performed using Odyssey infrared imaging system (LI-COR Biosciences, USA).

Size fractionation of DSP with sepharose

DSP was size fractionated using a superose 6 10/300 GL column (GE Healthcare) on an Äkta FPLC (GE Healthcare). PBS was used for column washing and elution.

Identification of DC-SIGN binding components using mass spectrometry

Protein bands excised from gel were analyzed using Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF, M@LDI, Micromass Wythenshawe, UK) and LC-ESI-MS/MS (QSTAR-XL, Applied Biosystems/MDS Sciex, Toronto, Canada). In summary, stained protein bands were cut from the gel. For mass spectrometry analysis gel slices were S-alkylated with iodoacetamide and vacuum dried. The in-gel digestion with trypsin (Roche Molecular Biochemicals, sequencing grade) and extraction of the peptides upon overnight incubation were done according to Shevchenko et al. (1996). Dried 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-hydroxyxinnamic acid (Sigma-Aldrich Chemie BV) in 49% ethanol/49% acetonitril/2% TFA and 1 mM Ammoniumacetate. Prior to dissolving, the α-cyano-4-hydroxyxinnamic acid was washed briefly with acetone. Reflectron MALDI-TOF spectra were acquired on a M@LDI (Micromass Wythenshawe, UK). The resulting peptide spectra were used to search the MSPDB databases of the Mascot search engine (http://www.matrixscience.com). Peptide mixtures and tryptic digests of fractions of interest (see size fractionation of DSP) were also analyzed. For LC-ESI-MS/MS analysis, peptide separation was performed using a nanoscale reversed-phase vented column system fitted onto an Agilent 1100 series HPLC system, essentially as described in van Breeumen et al. (2008). Sample was loaded onto a 2 cm × 100 μm ID C18 trapping column (Nanoseparations, Bithoven, The Netherlands) and trapped peptides were separated with a 63 cm × 50 μm ID C18 reversed phase analytical column (Nanoseparations, Bithoven, The Netherlands) using a linear gradient of 8–30% solvent B for 95 min (flow rate of 125 nl/min). The column was interfaced to a QSTAR-XL mass spectrometer for online electrospray ionization-mass spectrometry (ESI-MS) via a liquid junction with nebulizer using an uncoated fused-silica emitter (New Objective, Cambridge, MA, USA) operating around 4.7 kV (ID, 20 μm, tip ID 10 μm). Survey scans were acquired from m/z 300–1200 and the three most intense ions were automatically selected for tandem MS. Acquired peptide spectra were searched against a non-redundant protein sequence database (SwissProt/TREMBL) using the online Mascot search engine.

Statistical analysis

Statistical analysis was performed using two tailed unpaired t-tests. p values <0.05 were considered statistically significant.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jvirol.2009.06.011.

References


Geijtenbeek, T.B., van Koooy, Y. 2003. Pathogens target DC-SIGN to influence their fate. DC-SIGN functions as a pathogen receptor with broad specificity. APMS 111, 698–714.


