Characterization of DC-SIGN binding glycoproteins and the role in HIV-1 infection
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CHAPTER 6
General discussion
GENERAL DISCUSSION

Twenty-nine years after the first recognition of AIDS, the disease still represents a major health problem throughout the world. Despite enormous progress in understanding HIV-1 since its discovery, no definite answer to the AIDS epidemic has been formulated to date. Millions are newly infected each year with HIV-1, so new ways of prevention and treatment are still urgently required. We therefore need to increase our understanding of host factors that influence HIV-1 transmission and disease progression. Knowledge about such host factors and polymorphisms in their genes observed in individuals with natural resistance against HIV-1 acquisition or pathogenesis can be useful for the development of natural anti-HIV-1 compounds or new therapies.

Several strategies may be used to limit HIV-1 transmissions among which are condom use and male circumcision (Weller & Davis, 2002; Chersich & Rees, 2008). Reality is that the HIV-1 epidemic is still not under control. Although use of condoms is the most effective way of protection against sexual HIV-1 transmission and their use should be promoted, condoms are often not used due to social or cultural circumstances (Weber et al., 2005; Chersich & Rees, 2008). This stresses the necessity to develop other means to prevent the spread of HIV-1. A vaccine represents the most obvious solution, but despite enormous efforts for many years, there is still no prospect for an effective vaccine against HIV-1 any time soon.

Microbicides would provide an alternative or synergistic addition to vaccination. Microbicides are agents that are topically applied in the vagina or rectum in order to prevent sexual transmission of HIV-1 or other sexually transmittable diseases (STI). Microbicides are aimed at either destroying the virus particle, blocking HIV-1 binding to its receptors or preventing reverse transcription in infected cells. A forth-proposed type of microbicide aims to prevent inflammatory responses against invading HIV-1 particles in order to avoid the attraction of HIV-1 target cells to the infected area and subsequent amplification of the infection (Li et al., 2009).

Although prevention of HIV-1 infection has priority, new treatment options are still required since present therapy is unable to completely clear HIV-1 from an infected individual and HIV-1 ultimately escapes from their effects. A potential target for microbicides or therapy is the HIV-1 receptor DC-SIGN, which was proposed to play a role during HIV-1 transmission and disease course (Geijtenbeek et al., 2000; Liu et al., 2004; Koizumi et al., 2007). Functions described for DC-SIGN include roles in pathogen recognition, cell migration and immunological synapse formation. In vitro data demonstrate that HIV-1 can utilize DC-SIGN to enhance infection and modulate immune activation and genetic screening of HIV-1 progression cohorts suggest a role for DC-SIGN in HIV-1 transmission and disease course (Geijtenbeek et al., 2000; Arrighi et al., 2004; Gringhuis et al., 2009; Gringhuis et al., 2010; Liu et al., 2004; Koizumi et al., 2007).

The neck region of DC-SIGN between the C-terminal domain and the transmembrane domain is typically formed by 7 repeats of 23 amino acids. It plays a crucial role in...
oligomerization and support of the carbohydrate-recognition domain and is believed to influence the pathogen-binding properties of DC-SIGN (Barreiro et al., 2005). This region seems to be under strong selective forces with a very low polymorphism frequency. Liu and colleagues observed that 3 out of 94 exposed seronegatives carried a heterozygous repeat deletion in the neck region while no heterozygotes were found in a group of 316 seropositive individuals (Liu et al., 2004). Koizumi and colleagues investigated the effect of a single nucleotide polymorphism (SNP) in the DC-SIGN promoter region at position -139 within a cohort of 102 seropositive individuals. The -139C SNP appeared in progressors with significantly higher allelic frequency (0.333) than slow progressors (0.204) (Koizumi et al., 2007). These studies suggest a role for DC-SIGN polymorphisms in HIV-1 transmission and disease course but it is not unlikely that DC-SIGN function is modulated at an additional level by DC-SIGN ligands.

In this thesis we focused mainly on the interaction between host glycoproteins and DC-SIGN but, in addition, host molecules that directly bind to HIV-1 also influence HIV-1 infectivity. The DC-SIGN blocking properties of human semen and milk described in chapters 2, 3, 5 and by Naarding et. al. (Naarding et al., 2005) suggest that availability of DC-SIGN for HIV-1 may well be limited during HIV-1 transmission in the presence of these bodily fluids. Furthermore, we observed donor dependent variation in DC-SIGN binding capacity of both semen and milk. This observation suggests that DC-SIGN availability may be variable depending on the semen or milk “donor”. Presence of such DC-SIGN blocking compounds in bodily secretions and individual variation in DC-SIGN blocking may have resulted from selective pressures in co-evolution between human and pathogen.

Variation in DC-SIGN binding capacity of milk and semen may be determined by several factors. These factors include BSSL or mucin 6 expression levels, activity of glycosidases adding the required Lewis sugars and structural organization of the DC-SIGN binding glycoproteins. Both BSSL and mucin 6 exist of multiply repeated Lewis sugar carrying motifs that are highly variable in the number of repeats. Such variation in the number of repeats influences both the number of Lewis motifs linked to the protein and its structural organization. In chapter 3 we studied the relation between the number of repeats in the BSSL gene and DC-SIGN binding capacity of milk and observed a link between the two. We hypothesize that not only variable DC-SIGN binding properties of BSSL but also those of mucin 6 are related to variation in repeat numbers. Furthermore, since in addition to milk BSSL is also expressed in blood, we investigated the role of BSSL repeats in HIV-1 transmission, disease course and host immunology (chapter 4). Our results suggest that indeed BSSL genotype is associated to HIV-1 disease progression and host immune cell levels.

**IMPLICATIONS FOR HIV-1 TRANSMISSION**

In this thesis we describe the identification and characterization of glycoproteins from milk (BSSL) and semen (mucin 6) that have strong DC-SIGN binding and HIV-1 transfer-blocking
properties. Although the identified DC-SIGN binding glycoproteins are not identical they do share properties likely of importance for DC-SIGN binding and blocking. Both are large glycoproteins (>100 kDa) that carry multiply repeated regions with high densities of O-linked glycans also known as mucin (-type) repeats (Ruvoen-Clouet et al., 2006). Furthermore, both glycoproteins carry Lewis type sugars known to bind DC-SIGN (Landberg et al., 2000; McKillop et al., 2004; Wang et al., 1995; Andrianifahanana et al., 2006; Linden et al., 2008). Although more glycoproteins carry Lewis type glycans, not all glycoproteins have DC-SIGN binding properties, which is possibly related to the number of Lewis glycans present and structural organization of the glycoprotein. Interestingly we observed variation in DC-SIGN binding capacity of milk linked to differently sized BSSL suggesting a structural constraint to the binding properties.

DC-SIGN forms tetramers in the cell membrane and it is therefore likely that for efficient DC-SIGN binding, a glycoprotein carrying multiple Lewis type sugars with optimal three dimensional organization is required (van Liempt et al., 2006). It seems logical that large glycoproteins such as mucin 6 and BSSL carrying multiply repeated glycosylated mucin type motifs would fit to this requirement. Additional significance of the large sizes of BSSL and mucin 6 may be that due to the size, interaction between DC-SIGN and other potential ligands may be sterically hindered.

Taken together, our research suggests that BSSL from milk and mucin 6 from semen may interfere with variable potency with the interaction between DC-SIGN and pathogen during breastfeeding or sexual intercourse (chapters 2, 3 & 5). The fact that semen and milk as well as cervical vaginal secretions (Jendrysik et al., 2005) exhibit strong DC-SIGN blocking activities may have implications for the availability of DC-SIGN as HIV-1 receptor during transmission. It seems reasonable to conclude from our research that DC-SIGN does not play a (major) role as an attachment receptor for HIV-1 during transmission when semen or milk is present. However, even in situations with high levels of DC-SIGN blocking, HIV-1 binding to DC-SIGN may still occur at low levels. Furthermore, we observed strong donor dependent differences in DC-SIGN blocking activities of milk and semen, suggesting that DC-SIGN function in transmission may vary depending on the presence and activity of DC-SIGN blockers.

It seems likely that DC-SIGN at least in part is blocked during HIV-1 transmission, but the intensity of this interference remains to be determined. Studies of the identified BSSL marker (chapter 3) in MTCT cohorts with mothers known to transmit HIV-1 to their child via breastfeeding may help address the question as to whether DC-SIGN usage is significant with regard to HIV-1 transmission. Collaborations have been setup for future testing of this hypothesis in a cohort of HIV-1 positive mothers from South Africa and should provide an answer to this question.

DC-SIGN does not only play a role in pathogen capture for antigen presentation but is also involved in immune modulation via direct signaling. Gringhuis and colleagues observed that pathogens expressing mannose- or fucose type sugars induced DC-SIGN
mediated enhancement or suppression of proinflammatory responses (Gringhuis et al., 2009). We demonstrated that both BSSL and mucin 6 bind DC-SIGN mainly through Lewis type sugars (chapters 2 & 5). This may suggest that BSSL and mucin 6 induce suppression of proinflammatory immune responses as might be expected from bodily fluids such as milk and semen. Whereas the aim of breast milk is to provide the infant with essential nutrients such as amino acids and sugars, immune responses against milk would not be beneficial to the child. Similarly, in the case of semen, immune responses towards semen would not increase the chance of successfully producing (healthy) offspring. Indeed several studies report that both milk and semen suppress recipient immune responses (Chalabi et al., 2002; Kelly & Critchley, 1997; Robertson & Sharkey, 2001; Robertson et al., 2009; Walker, 2010). It is not unlikely that BSSL and mucin 6 contribute to the suppression of inflammation by suppressing DC-SIGN mediated immune responses, either through immune modulation or interfering with pathogen capture or both. We have hypothesized that this dampening may be a way to reduce over-immune activation in newborns. Infants are suddenly exposed to a large array of antigens in human milk soon after delivery, which could lead to a “cytokine rush” or overstimulation of immune responses.

CO-EVOLUTION UNDER PATHOGEN PRESSURE

There is increasing evidence of blood group antigen involvement in susceptibility to pathogen infection. The high rate of polymorphisms in blood group antigen related genes such as those encoding glycans and the high prevalence of these polymorphisms suggest the existence of evolutionary pressure by different pathogen challenges (Moulds et al., 1996; Schaeffer et al., 2001; Ruiz-Palacios et al., 2003; Fumagalli et al., 2009). Pathogens interact with host glycans in several ways: glycans can serve as attachment receptors for infection, decoy receptors or host glycoproteins can compete with pathogens for pathogen receptor binding. Decoy receptors are secreted host molecules that mimic host receptors and bind to pathogens and in this way neutralize infectivity. In particular the FUT2 and FUT 3 gene, encoding fucosyltransferases 2 and 3 involved in the synthesis of DC-SIGN binding Lewis type sugars have been associated to pathogen infection (Newburg, 2009; Ruvoen-Clouet et al., 2006; Boren et al., 1993; Sheinfeld et al., 1989).

FUT2 was linked to HIV-1 transmission and disease progression although controversial reports were published with respect to the effect of FUT2 in transmission (Sheinfeld et al., 1989; Blackwell et al., 1991; Ali et al., 2000; Puissant et al., 2005; Kindberg et al., 2006). Blackwell and Ali reported a higher incidence of HIV-1 infection in individuals with a functional FUT2 gene, so-called secretors (Ali et al., 2000; Blackwell et al., 1991). Ali and colleagues found this relation in commercial sex workers but not in non-commercial sex workers and the number of participants of the Blackwell study were limited (n=54). In contrast, Puissant and colleagues reported a decreased frequency of secretor positivity in HIV-1 infected patients when compared to controls although this was limited to individuals
with blood group A (Puissant et al., 2005). The latter study included 968 HIV-1 positive and 32,032 control individuals visiting a French hospital with no information on inclusion of commercial sex workers.

Possible differences between outcomes of the FUT2 studies on HIV-1 transmission may relate to the number of individuals included and the method of Lewis type determination. Furthermore, differences in number of participants enrolled in the studies with commercial sex workers in one publication and non-commercial sex workers in the two other studies as well as different ethnicity may have contributed to the different observations. Taken together it is possible that the FUT2 gene influences HIV-1 transmission directly by producing HIV-1 inhibitory glycans or possibly indirect by decreasing risk of co-infections. In addition, factors not included in the studies may have played a role among which are polymorphisms in glycoproteins carrying fucoses such as those identified for BSSL (chapter 3). It will be interesting to include BSSL polymorphisms in studies on the relation between FUT2 and HIV-1 disease progression or add information on FUT2 polymorphisms to our own cohort study.

We demonstrated that fucose carrying glycoproteins in semen and milk block the interaction between HIV-1 and DC-SIGN but another example of glycan involvement in disease is the infection with Norwalk virus. Norwalk virus infects through binding 2-fucosylated structures expressed on epithelial cells. Hosts with functional FUT2 genes, so-called secretors, express 2-fucose glycans and can be infected whereas non-secretor individuals lacking functional FUT2 genes cannot be infected (Hutson et al., 2005; Lindesmith et al., 2003). BSSL in the milk from secretor mothers acts as a decoy receptor by binding to Norwalk virus and inhibiting infection of the child whereas BSSL from non-secretor mothers is unable to prevent Norwalk virus infection (Ruvoen-Clouet et al., 2006). It seems that expression of glycosidases may have opposing effects with functional FUT2 genes in the child rendering the child at risk to infection but functionality in the mother providing protection to the child. Additionally, activity of genes involved in glycosylation such as FUT2 and FUT3 likely also influence the DC-SIGN binding capacity of BSSL, which depends on the expression of Lewis sugars.

It is not unlikely that evolutionary pressure on glycans extends to the composition of glycoproteins that interact with pathogen or pathogen receptors. BSSL and mucin 6 are glycoproteins with significant cost in production due to their size and intensity of glycosylation but are abundantly expressed in bodily fluids. This suggests that these molecules play an important role in these fluids. As suggested before, BSSL and mucin 6 may provide tolerance to their carrier fluid but in addition these glycoproteins possibly limit infection by DC-SIGN using pathogens. These functions are highly relevant for survival and procreation.

Although pathogen capture by DC-SIGN induces immune responses necessary to fight infection, some pathogens are believed to abuse this mechanism to establish infection. Restricted availability of DC-SIGN for pathogen capture due to BSSL and mucin 6 may
then allow the scavenger function of DC-SIGN but prevent over-exposure to pathogens. This would result in decreased risk of DC-SIGN mediated infection as well as prevent the induction of too strong immune responses. The necessity to prevent a potentially hazardous interaction between pathogen and DC-SIGN depends on the presence of DC-SIGN abusing pathogens in a population. Exposure to pathogens may result in selective pressures in the direction of either strong or weak DC-SIGN blocking, which is dependent on the type of circulating pathogens dominating a specific population. This may be reflected by the observed differences in the DC-SIGN binding capacity of milk derived from different geographical regions and high degree of variation in the BSSL gene as described in chapter 3. Furthermore, the observed variation in DC-SIGN binding capacity of both milk and semen within a population could reflect the different pathogen pressures over time within a population.

With its dual role in innate immunity it is not unlikely that evolution of BSSL and possibly also mucin 6 variants is not only driven by DC-SIGN binding of pathogens. The role of BSSL as a decoy receptor and related glycosylation patterns may result in additional pathogen pressures. Therefore it seems likely that constant exposure to different pathogens may result in opposing selective pressures and thus in maintenance of high levels of variation in genes involved in host-pathogen interaction.

**BSSL IN DISEASE PROGRESSION**

DC-SIGN was not only proposed to play a role in transmission but also during disease course. With the presence of BSSL in blood, the possibility exists that BSSL interferes with the interaction between HIV-1 and DC-SIGN in HIV-1 infected individuals. In addition to DC-SIGN, BSSL binds to CXCR4 and is therefore likely to interfere with the interaction of SDF1 and HIV-1 with CXCR4. In chapter 3 we linked DC-SIGN binding capacity of milk to BSSL genotype and therefore we hypothesized that this genotype may also influence the interaction between BSSL and DC-SIGN or CXCR4 in HIV-1 infected individuals.

Chapter 4 describes the identification of a link between BSSL genotype and HIV-1 disease progression and emergence of X4 variants. For our analyses we included several co-factors known to affect HIV-1 disease progression (CCR5-Δ32, HLA-B57, virus load - and CD4 setpoint) to correct for possible dependencies of observed BSSL effects on these factors. Although inclusion of co-variates in analyses results in decreased statistical power due to the fact that not for all samples co-variante data is available, inclusion of co-variates increased statistical significances in most cases.

Although a minimal but not statistically significant relation between BSSL genotype and CD4 setpoint was observed, BSSL genotype was associated to CD4 cell numbers prior to infection. We demonstrated that a particular BSSL (HH) genotype is correlated to elevated numbers of CD4 cells in uninfected individuals whereas the same genotype was linked to slow disease progression in HIV-1 infected men. Whether increased CD4
cell numbers are related to interactions between BSSL and DC-SIGN or CXCR4 remains to be investigated but it indicates a link exists between BSSL genotype and immune cell homeostasis.

The BSSL genotype that correlated to high CD4 cell numbers in blood was also found more frequently in uninfected individuals with higher risk behavior (HRSN), although no statistical significance was found in the latter. These observations seem contradictory since increased CD4 cell numbers would suggest that there are potentially more targets for HIV-1 available. However, it could be that increased CD4 cell numbers in blood reflect a decreased cell number at the mucosa. Furthermore, it could be imagined that BSSL binding to CXCR4, involved in the regulation of T-lymphocyte migration, proliferation and differentiation (Moser & Loetscher, 2001; Wu & Yoder, 2009), modulates the interaction between SDF1 and CXCR4. Such modulation could potentially lead to a decreased sensitivity of CXCR4 expressing cells for SDF1 homing signals directing these cells to the lymphoid tissues or mucosa. Future research should elucidate if a relation between BSSL and T-lymphocyte migration indeed exists.

CONCLUDING REMARKS

Our research demonstrates that semen and milk contain glycoproteins that potently block the interaction between HIV-1 and DC-SIGN suggesting that milk and semen influence HIV-1 transmission. This raises the question as to whether DC-SIGN acts as an HIV-1 attachment receptor for transmission or whether such an interaction is prevented by BSSL or mucin 6. The identified polymorphism in BSSL that associates with DC-SIGN binding capacity of milk provides us with a tool to study DC-SIGN mediated transmission via breastfeeding by HIV-1 infected mothers. Obviously DCs expressing DC-SIGN lie below the mucosal surface but breaches in the protective epithelial layer are common. Breaches in the epithelial layer were especially more common in the years before usage of anti-bacterial drugs due to a higher incidence of (then untreated) mucosal infections.

Since DC-SIGN binding of milk was found to be highly variable, the risk of DC-SIGN mediated transmission may also vary depending on the breastfeeding mother. Studying the effect of BSSL polymorphisms on HIV-1 transmission by breastfeeding mothers could provide (indirect) evidence for a role of DC-SIGN in transmission. If an association between BSSL genotype and HIV-1 transmission is found, the BSSL genotype could serve as a marker to predict the risk of HIV-1 transmission via breastfeeding. Furthermore, given their possible immune modulating roles, it may be interesting to study the relation between DC-SIGN binding capacity of milk and semen and the development of allergy in children (milk) and male fertility (semen). Ultimately, our study may help develop BSSL derived therapeutic molecules for mucosal application against DC-SIGN related pathogenic infections for individuals with low natural protection levels.
Our research not only suggests that DC-SIGN exposure to HIV-1 may be limited by semen and milk but also stresses the importance to include these fluids in vaccine or microbicide testing. Most vaccines and microbicides are tested in rhesus macaques before proceeding to clinical trials and therefore influence of semen and milk on SIV transmission should be studied in this model. Such testing will enhance our understanding of HIV-1/SIV transmission and enable optimization of vaccine and microbicide testing in rhesus macaques and hopefully speed up development of effective therapeutics.

In addition to the observed correlation between BSSL genotype and DC-SIGN binding of milk we observed a similar link with HIV-1 disease progression. Furthermore, the analysis of HRSN and seropositive individuals suggested a possible correlation between BSSL genotype and protection against HIV-1 infection. Although the mechanisms behind this observation need to be further investigated, the identification of BSSL as a marker for HIV-1 disease course and possibly risk of infection provide a new target for therapy development. Taking into account the high frequency of BSSL polymorphisms, BSSL may be a promising candidate for therapeutics development with impact on a relatively high number of individuals.

**REFERENCE LIST**


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