C-type lectins L-SIGN and DC-SIGN

functions in infection and homeostasis

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Contents

Abbreviations 9

Chapter 1 11
General introduction

Chapter 2 27
L-SIGN participation in tumour metastasis. *A novel carbohydrate ligand for L-SIGN.*

Chapter 3 41
Hepatitis C virus targets DC-SIGN and L-SIGN to escape lysosomal degradation.

Chapter 4 59
HCV immune complex formation favours antigen presentation after uptake by DC-SIGN and L-SIGN.

Chapter 5 75
Potency of HIV-1 envelope glycoprotein gp120 antibodies to inhibit the interaction of DC-SIGN with HIV-1 gp120.

Chapter 6 93
Lewis^{x} component in Human Milk binds DC-SIGN and inhibits HIV-1 transfer to CD4^{+} T lymphocytes.

Chapter 7 113
Interactions of DC-SIGN with Mac-1 and CEACAM1 regulate contact between dendritic cells and neutrophils.

Chapter 7 addendum 129
L-SIGN mediates adhesion and rolling of neutrophils.

Chapter 8 135
C-type lectins are involved in monocytes/Kupffer cell migration in the liver.

Chapter 9 147
General discussion

Summary 167

Samenvatting voor niet ingewijden 171

Dankwoord 177

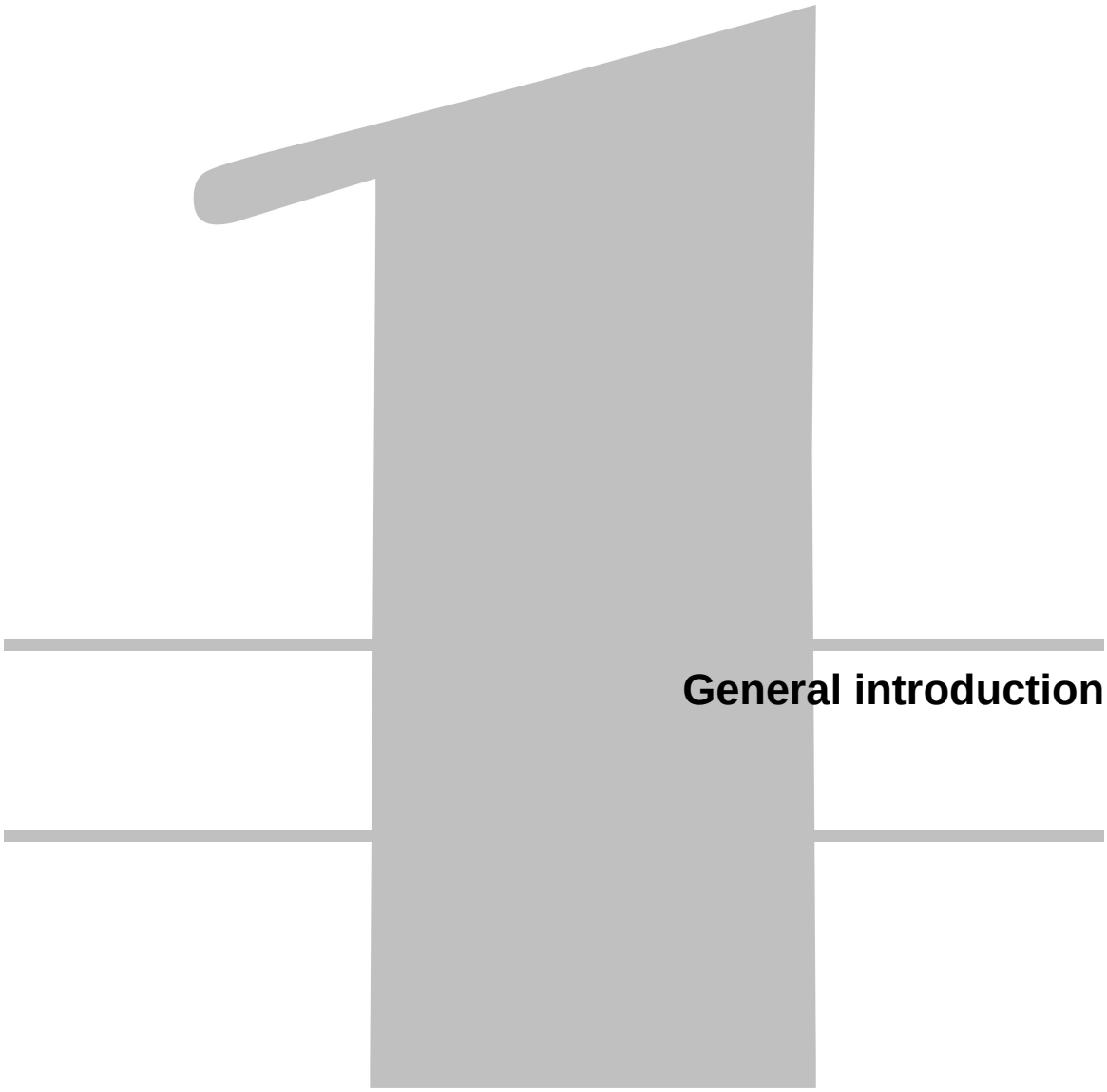
Curriculum Vitae 181

List of publications 182

Colour figures 183

Abbreviations

AAL	*Aleuria aurantia* lectin
Alum	aluminium
APC	antigen presenting cell
ASGP-R	asialoglycoprotein receptor
BSA	bovine serum albumin
BSSL	Bile salt stimulated lipase
CD	cluster of differentiation
CD4bs	CD4 binding site
CD4i	CD4 induced
cDNA	complementary DNA
CEA	carcinoembryonic antigen
CEACAM1	CEA related cell adhesion molecule 1
CHO	Chinese hamster ovary cells
CLR	C-type lectin receptor
CMV	cytomegalo virus
CRD	carbohydrate recognition domain
DC	dendritic cell
DC-SIGN	DC-specific ICAM3-grabbing non-integrin
EEA-1	early endosome antigen 1
ELISA	Enzyme-Linked Immuno Sorbent Assay
FasL	Fas-ligand
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FMLP	formyl-methionylleucylphenylalanine
Fut	fucosyltransferase
GalNAc	N-acetylgalactosamine
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GNA	*Galanthus nivalis* lectin
gp	glycoprotein
GT	glycosyltransferase
HCV	hepatitis C virus
HIV-1	human immunodeficiency virus 1
HLA	human leukocyte antigen
HRP	horseradish peroxidase
IC	immune complex
ICAM	intercellular adhesion molecule
iDC	immature DC;
IFN	interferon
IL	interleukin
ITIM	immunoreceptor tyrosine-based inhibitory motif
KC	Kupffer cell
LAMP	lysosome-associated membrane protein
LDL-R	low density lipoprotein receptor
Le	Lewis
LFA	leukocyte functional antigen-1
LNFP	lacto-*N*-fucopentaose
LPS	lipopolysaccharide
LSEC	liver sinusoidal endothelial cell
LSECtin	liver and lymph node sinusoidal endothelial cell C-type lectin
L-SIGN	liver and lymph node-specific ICAM3-grabbing non-integrin
LSP-1	leukocyte-specific protein-1
LTA	*Lotus tetragonolobus* lectin
LV	lentviral
MAA	*Maackia amurensis* agglutinin
ManLAM	Mannosylated lipoarabinomannan
mDC	mature DC
MGL	macrophage galactose type C-type lectin
MHC	major histocompability complex
MIP	macrophage inflammatory protein
MR	mannose receptor
mRNA	messenger RNA
MTCT	mother-to-child transmission
NPA	*Narcissus Pseudonarcissus* Lectin
PAA	polyacrylamide
PAMP	pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PE	phycoerythrin
PMN	polymorphonuclear
PPR	pattern recognition receptors
R5	CCR5 coreceptor using HIV-1 isolate
RNA	ribonuclease acid
RT-PCR	real time PCR
sCD4	soluble CD4
siRNA	small interfering RNA
SIV	simian immunodeficiency virus
SLPI	secretory leukocyte protease inhibitor
SNA	*Sambucus nigra* agglutinin
SR-B1	scavenger receptor class B type I
ST	sialyl transferase
TCID	tissue culture infectious dose;
Th	helper T
TLR	Toll-like receptor
TNF	tumour necrosis factor
UEA-1	*Ulex europaeus* agglutinin 1
VLP	virus like particle
wt	wild type
X4	CXCR4 coreceptor using HIV-1 isolate.



General introduction

Introduction

Antigen presenting cells (APCs) are specialized cells that capture antigens and process them for antigen presentation on MHC class II molecules to T and B lymphocytes. Macrophages, dendritic cells (DCs) and B cells are the main APCs for antigen presentation to T cells and follicular DCs for presentation to B cells. In this way an antigen specific response is generated, modulated by expression of costimulatory molecules and production of cytokines. Endothelial cells can also present antigens to T cells, although endothelial cells need to be activated to express MHC class II. In contrast, in the liver a specialized endothelial cell population is localized, liver sinusoidal endothelial cells (LSECs) that constitutively express MHC II and possess a great endocytic capacity and lysosomal activity [1]. Therefore these LSECs can act as professional APC.

Dendritic cells

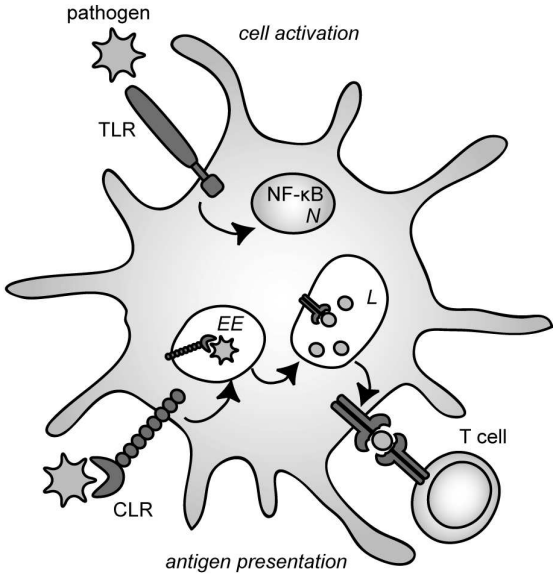
DCs function as a bridge between innate and adaptive immunity. Immature DCs are positioned at strategic positions in the body, where they act as sentinels of the immune system constantly screening the environment. DCs function in pathogen recognition, however unlike innate immune cells, they do not function primarily in the destruction of pathogens, but moreover they present pathogen-derived antigens to T cells and induce antigen-specific immune responses [2]. DCs not only present antigens to T cells but also direct the differentiation of $CD8^+$ T cells to cytotoxic T cells and $CD4^+$ T cells to T helper type 1, type 2, or regulatory T cells [3-5].
Pathogens possess pathogen associated molecular patterns (PAMPs) that are recognized by pattern recognition receptors (PPR). These PAMPs are highly conserved and pathogen specific. DCs express several PPRs like Fcγ receptors, complement receptors, scavenger receptors, C-type lectin receptors (CLRs) and several members of the Toll-like receptor (TLR) family [6-11]. The TLR family consists, up to now, of 11 different TLRs, with each a specific recognition pattern. TLR4 for example binds lipopolysaccharide (LPS), a cell-wall component of gram-negative bacteria [12]. TLRs act upon interaction with microbial ligands by a cascade of inflammatory signals resulting in DC activation and migration to lymph nodes.
Besides TLRs, DCs also express a wide variety of CLRs that recognize pathogens through specific carbohydrate moieties. CLRs are composed of one or more carbohydrate recognition domains (CRDs) and calcium is involved in the interaction with ligands [13;14]. In contrast to TLRs that respond by activation of DCs, CLRs internalize pathogens for antigen processing and presentation (Fig. 1.1). Among the CLRs expressed on DCs are the mannose receptor (MR), DEC-205, Dectin-1 and -2, DCIR, DLEC, CLEC, langerin, MGL and DC-SIGN [11]. They are expressed on specific DC subsets, langerin for example is only expressed on Langerhans cells [15], and their expression is tightly regulated and dependent on the activation state of the DC. DC-SIGN for example is highly expressed on monocyte-derived DCs, but expression is down-regulated upon maturation [16]. Besides pathogens, CLRs recognize also endogenous ligands, mediating cell-cell interactions and soluble ligand binding, resulting in tolerance as was shown for DEC-205 [17]. The combined stimulation of CLR and TLR results in fine tuning and modulation of the TLR mediated response. The simultaneously activation of Dectin-1 and

TLR2 by *Saccharomyces cerevisiae* derived zymosan results in the synergistic production of pro-inflammatory mediators by DCs [18]. On the other hand, the combined binding of the *Mycobacterium tuberculosis* component ManLAM by TLR4 and DC-SIGN results in interleukin-10 (IL-10) production and inhibition of DC activation [19].
The CLR family can be divided in type I and type II CLRs, depending on the number of CRDs present in the extracellular domain. Type I CLRs, like MR and DEC205 have multiple CRDs, whereas type I CLRs, like DC-SIGN and MGL have just one CRD. Furthermore, CLRs can be divided on basis of the amino acid sequence of the CRD; CLRs with EPN based CRD recognizing mainly mannose structures and CLRs with QPD based CRD recognizing galactose structures [20].

DC-SIGN receptor family

In human three members of the DC-SIGN family have been identified: dendritic cell-specific ICAM-grabbing non-integrin (DC-SIGN, CD209), liver and lymph node specific SIGN (L-SIGN, CD209R, CD299), and liver and lymph node sinusoidal endothelial cell C-type lectin (LSECtin). They are located on chromosome 19p13.3 in close proximity to the CD23 gene [21;22].
DC-SIGN is expressed on DCs [16;23], and L- SIGN and LSECtin are expressed on liver sinusoidal endothelial cells and on lymph node sinusoidal endothelial cells [22;24;25].
L-SIGN shares 77% amino acids with DC-SIGN, with the highest homology in the CRD. However, although DC-SIGN has 7 complete and one incomplete neck repeats, there are 7 alleles for L-SIGN identified, resulting in 3 to 9 neck repeats [25]. The neck repeats function in multimerisation of the receptors. Indeed it has been shown that DC-SIGN is forming tetramers when expressed on the cell surface [26;27]. In the cytoplasmic tail of DC-SIGN there are three internalisation motifs: a tyrosine based motif, a di-leucine motif and a triacidic cluster. L-SIGN shares the di-leucine motif and the triacidic cluster but is lacking the tyrosine motif. These motifs are associated with internalisation and targeting to late endosomes/lysosomes and indeed it has been demonstrated that DC-SIGN can take up ligands and target these to

Figure 1.1



Interactions of pathogens with TLRs or CLRs on DCs have different outcomes.
Upon binding of a pathogen to a TLR, a signalling cascade is started resulting in NF-κB translocation with subsequent DC maturation. Upon interaction of a pathogen with a CLR, the complex is internalized into the lysosomal pathway, degraded and presented on MHC II to $CD4^+$ T cells. *N*=nucleus, *EE*=early endosome, *L*=lysosome.

lysosomes for antigen presentation [28;29].
DC-SIGN recognizes mannose structures as expected by the presence of an EPN based CRD. However, DC-SIGN binding is not only based on primary mannose groups but also depends on the protein backbone, which organizes carbohydrate structures in a distinct three-dimensional structure [30]. Besides the specificity for mannose structures, DC-SIGN also binds fucose-containing carbohydrates such Lewis^a (Le^a), Le^b, Le^x, and Le^y [26;31-33].
L-SIGN has the highest degree of homology in the CRD compared to DC-SIGN, and therefore the binding characteristics are very similar. L-SIGN binds mannose structures and the fucose containing sugars Le^a, Le^b, and Le^y [34]. However, whereas DC-SIGN recognizes Le^x antigen, L-SIGN lacks the capacity to bind this particular fucose containing carbohydrate structure. This is based on the difference in one amino acid in the CRD: DC-SIGN has a valine on position 351 (Val351), whereas L-SIGN has on this position a serine molecule (Ser363) [30;35]. Le^x binding capacity is restored by replacing Ser363 in L-SIGN with a valine residue, which results in the formation of a hydrophobic pocket [35].

Murine DC-SIGN homologues.
In mice several DC-SIGN homologues are described, but only mDC-SIGN and mSIGNR1 are expressed on APCs. mDC-SIGN is expressed on CD8a$^-$ and CD8a$^+$ DCs [36] and on plasmacytoid pre-DCs that differentiate into CD8$^+$ CD205$^-$ DCs [37], but this C-type lectin is non-functional [38]. mSIGNR1 is expressed on a macrophage population in lymph nodes, in spleen on marginal zone macrophages [39;40], in liver on sinusoidal endothelial cells [39], and on peritoneal macrophages [41]. That mSIGNR1 is expressed on LSECs similar as human L-SIGN makes it a suitable candidate for investigating the *in vivo* role of L-SIGN.

Cellular ligands for DC-SIGN

Immature DCs migrate from the blood to peripheral tissue across the endothelial cell layer. Before diapedesis through the endothelial cell layer, DCs should tether and role over the endothelial cells. DC-SIGN is mediating DC adherence and rolling over endothelial cells by binding to intercellular adhesion molecule-2 (ICAM-2) expressed on endothelial cells [42]. The DC-SIGN-ICAM-2 interaction is strong enough to resist the shear stress of the blood flow [42]. Besides in the process of migration of immature DCs to peripheral tissues, DC-SIGN-ICAM-2 mediated rolling could also be involved in the homing of DCs to secondary lymphoid organs. Binding of DC-SIGN to ICAM-2 is endothelial cell specific, as for instance ICAM-2 on platelets is not recognized due to different glycosylation [43]. Moreover, glycosylation of ICAM-2 can differ between endothelial cells, dependent on activation state or localization, thereby defining specific sites for DC migration.
After antigen uptake, DCs interact with T cells to present antigen on MHC class II. This interaction is initiated by DC-SIGN-ICAM-3 interactions, followed by ICAM-1-leukocyte functional antigen-1 (LFA-1) interactions [16]. Moreover, the interaction of DC-SIGN on DCs with ICAM-3 on T cells promotes the recruitment of LFA-1 to the immunological synapse at the DC-T cell interface [44]. Eventually this is strengthening the interaction between DC and T cell and thereby facilitating T-cell receptor interactions with MHC II-peptide complexes.

Neutrophils (PMNs) and DCs reside in different compartments during homeostasis. However, during inflammation neutrophils and DCs accumulate both at the site of infection. Neutrophils can transfer antigens to DCs [45] and therefore should interact with DCs. DCs facilitate this interaction by DC-SIGN mediated binding to Mac-1 and CEACAM1 on the neutrophil membrane [46;47] (Fig. 1.2).

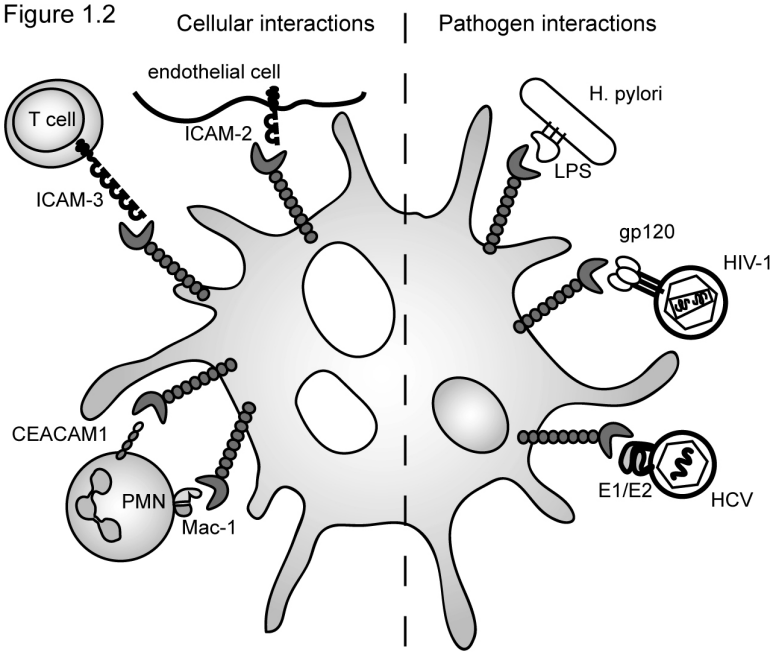
DC-SIGN/L-SIGN-pathogen interactions

DC-SIGN was originally identified as a human immunodeficiency virus 1 (HIV-1) gp120 binding receptor [48]. DC-SIGN plays a key-role in the dissemination of HIV-1 by DCs through HIV-1 gp120 binding [49]. Other studies have demonstrated that DC-SIGN also functions as a receptor for other viruses, such as cytomegalovirus (CMV) [50], and Ebola virus [51], bacteria, including *Mycobacterium tuberculosis* [19], and helminths such as *Schistosoma mansoni* egg antigens [32]. Most pathogens use DC-SIGN for their own benefit; viruses, such as HIV-1 [49] and hepatitis C virus (HCV) [52], use the interaction with DC-SIGN for viral dissemination by circumventing lysosomal degradation and transmission to other cells, and bacteria, such as *Mycobacterium tuberculosis* [19] and *Helicobacter pylori* [53] modulate DC mediated immune responses by binding to DC-SIGN (Fig. 1.2).

DC(-SIGN) and HIV-1.

T cells are the main targets of HIV-1, and the virus enters these cells via interaction of gp120 envelope glycoprotein on HIV-1 and CD4 and a chemokine coreceptor (CCR5 or CXCR4) on the T cell, which results in membrane fusion. However, DCs are thought to be the first cells to encounter the virus upon infection [54]. Adults are mainly infected as a result of sexual transmission; whereas mother-to-child transmission is the main cause of infection among

Figure 1.2



Examples of cellular and pathogen ligands for DC-SIGN. DCs interact via DC-SIGN with ICAM-2 on endothelial cells, ICAM-3 on T cells and CEACAM1 and Mac-1 expressed on PMNs (left side). DC-SIGN on DCs mediates interaction with particular bacteria via binding of Le^x structures on LPS, and with viruses via binding of highly glycosylated envelope proteins.

children. DC-SIGN$^+$ DCs are present in both genital and intestinal mucosa [23]. In cervix and vagina, DC-SIGN expressing cells are located in the subepithelial lamina propria underneath the squamous epithelial layer [23;49], the rectal mucosa is rich in DC-SIGN$^+$ DCs [23], and in the intestines, DC-SIGN expressing cells are located in the Peyer's patches [23]. Thus DC-SIGN$^+$ DCs are present at all sites susceptible of HIV-1 infection.
DCs themselves are poorly infected by HIV-1, but the presence of DCs greatly enhances infection of T cells [55;56]. DC-SIGN on DCs recognizes gp120 via high mannose residues on the glycoprotein [57-59]. Upon binding of DC-SIGN and HIV-1, DCs internalise the virus into CD81$^+$ vesicles [60], where it can be retained for several days [49]. DCs then travel to lymph nodes and upon CD4$^+$ T cell encounter, HIV-1 is re-localised in a so-called 'infectious synapse' at the DC-T cell interface [61]. In the infectious synapse, which resembles the immunological synapse, CD4 is present at high concentrations and hereby T cell-infection is enhanced. In the immunological synapse, DC-SIGN binding to ICAM-3 enhances LFA-1 redistribution to the synapse, strengthening the interaction between DC and T cell [16]. A similar process might be involved in the interaction of DC and T cell upon viral transmission in the infectious synapse. The presence of DC-SIGN is necessary for enhancement of T-cell infection, since knockdown of DC-SIGN expression with siRNA abolishes the T-cell infection *in trans* [62].
However, upon internalisation in DCs, HIV-1 can also partially be degraded [49] and presented to T cells. Cross presentation of HIV-1 antigens to CD8$^+$ T cells on MHC class I requires membrane fusion [63;64], whereas MHC class II restricted presentation to CD4$^+$ T cells occurs without membrane fusion in acidic endosomal vesicles [65]. Both mechanisms of presentation are DC-SIGN dependent.

L-SIGN can, similar to DC-SIGN, mediate uptake and transmission of HIV-1 [24;25]. L-SIGN positive cells are located in liver and lymph node in close proximity to CD4$^+$/CCR5$^+$ cells, Kupffer cells (KCs) and CD4$^+$ T cells respectively, to which virus can be transmitted. Homozygosity for 7 neck repeats in L-SIGN has shown to result in an increased susceptibility for HIV-1 [66] demonstrating that L-SIGN is indeed involved in HIV-1 infections.

DC-SIGN/L-SIGN and HCV.
HCV is an enveloped plus stranded RNA virus [67] that is mainly infecting hepatocytes, but in the recent years more and more evidence for infection of other cells arises. In the liver, positive signals of replicative intermediate RNA were detected in different subsets of sinusoidal cells (LSECs, KCs, and Stellate cells), suggesting that HCV replication may also occur in these cell types [68;69]. An indication for an extrahepatic virus reservoir is the rapid reinfection of the liver in chronic HCV patients after liver transplantation, and indeed, HCV can replicate in monocytes and B cells [70]. Infection with HCV results in chronic and persistent infection in 70% of the patients [71]. The virus can persist because of immunomodulation by directly affecting immune cell function and by a high mutation rate.
Several HCV receptors have been described: CD81 [72], and the CD36 family member SR-B1 [73] are both binding HCV envelope glycoprotein E2. In addition, the hepatocyte specific asialoglycoprotein receptor (ASGP-R) [74] and LDL-R [75] have shown to interact with HCV.
Both DC-SIGN and L-SIGN bind HCV envelope glycoproteins [76-78]. Therefore, HCV might directly target DCs to modulate immune responses. Indeed it has been shown that the DC number is lower in chronic HCV infected patients than in healthy donors [79]. Although DCs present HCV antigens to T cells [80], the T cells might be not properly stimulated, because DC

maturation and their immune stimulatory functions are impaired in HCV positive patients [81;82].
HCV might not only target DC-SIGN and L-SIGN for dissemination by immunomodulation through DCs, but also by transmission to adjacent hepatocytes upon binding by L-SIGN on LSECs [52].

The Liver

The liver functions in digestion, detoxification of blood born substances, and in production of plasma proteins. It receives oxygen rich blood from the hepatic artery and 75% of the blood supply comes from the hepatic portal vein. This blood is coming from the gastro-intestinal tract and is rich in food antigens, environmental toxins and bacterial products like LPS. Although most of these antigens are non-self, an immune response to food-borne antigens is rarely induced, and clearance of e.g. LPS takes place without signs of inflammation [87;88].

The liver is composed of parenchymal and non-parenchymal cells. The parenchymal cells, the hepatocytes, are the main participants in the metabolic function of the liver. The liver sinusoid contains the most of the non-parenchymal cells. Among these non-parenchymal cells are the liver resident macrophages (Kupffer cells), the stellate or Ito cells that function in fat and vitamin A storage and production of extracellular matrix components, pitt cells, a liver natural killer cell like population, and sinusoidal endothelial cells. Between the endothelial cell layer and the hepatocytes the space of Disse is situated. Lymph is collected in the space of Disse to subsequently flow into lymph vessels (Fig. 1.3).

Endothelial cells of the liver sinusoids are different from endothelial cells residing in other tissues. These liver sinusoidal endothelial cells (LSECs) are fenestrated and lack a basement membrane [89]. With the fenestrae, dynamic structures clustered in sieve plates, LSECs regulate by varying the size and amount of their pores the flow of substances from the bloodstream into the space of Disse, and thus subsequently to the hepatocytes [90;91]. Furthermore LSECs regulate uptake by hepatocytes of material by transcytosis from the luminal to the abluminal side of the endothelial cell layer [92], and LSECs can change the glycosylation of substances and thereby further facilitating uptake by hepatocytes [93]).

Because the small size of only 5 to 7 µm and the low and intermittent blood flow in the sinusoids, passing blood cells might interact with LSECs independent of selectin-adressin interaction [94]. Normally adhesion of leukocytes to the endothelial cell layer is a two-step process starting with P and E selectin mediated tethering and rolling of leukocytes over endothelial cells and then ICAM1-LFA1 mediated adhesion. In liver sinusoids blood flow is low and therefore adhesion of leukocytes to LSECs takes place without prior rolling [94].

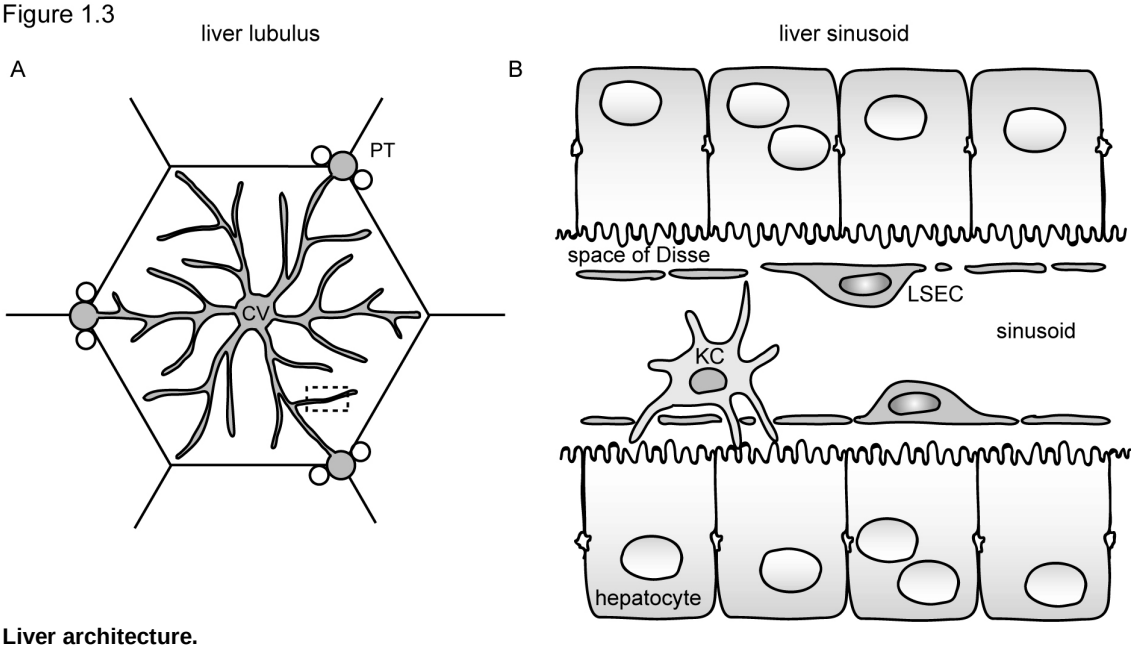
Tolerance induction.
The liver is capable of promoting tolerance, as was demonstrated already in 1967 by the possibility to induce antigen-specific tolerance by administration of the antigen via the portal vein [95;96]. Furthermore, allogenic liver transplants can be accepted without immunosuppression [97], and the acceptance of other transplants is increased when simultaneously a liver from the same donor is transplanted [98]. Several cell populations have been implicated in liver specific tolerance induction. For example, removal of KCs from the liver abrogates tolerance induction [99;100]. Also LSECs are capable of promoting tolerance. Adoptive transfer of antigen challenged LSECs results in tolerance to the antigen in the recipient [101]. Both murine and human LSECs constitutively express MHC class II on their surface [102;103], and mouse LSECs also express the costimulatory molecules

CD40, CD80 and CD86 [104;105]. Strikingly, human LSECs express CD40 but lack expression of CD80 and CD86 [106]. LSEC presentation on MHC II to $CD4^+$ leads to IL-10/IL-4 expressing T cells with regulatory function [107]. Overall, the $CD4^+$ Th1/Th2 balance in the liver seems to be preferentially resulting in Th2 responses; the Th1 cytokine interferon (IFN) γ is suppressed [108] and $CD4^+$ Th1 cells become non-functional in the liver [109]. LSECs also cross-present antigens to $CD8^+$ T cells, however the $CD8^+$ T cell are not cytotoxic but tolerant for the antigen [101]. Furthermore there are indications for liver specific selective retention of activated $CD8^+$ T cells [110], where they undergo apoptosis and are cleared from the blood [111]. However this tolerogenic milieu in the liver could also promote the survival of hepatotropic pathogens, such as hepatitis B and C virus.

Clearance of redundant cells.

Beside a role in processing food metabolites, the liver also functions in the clearance of redundant cells. T cells are cleared from the blood when they pass the liver, where both KCs and LSECs are able to bind apoptotic cells [112]. KCs recognize apoptotic T cells through phosphatidyl serine that is normally located in the inner membrane, but upon apoptosis is translocated to the outer membrane. LSECs express Galactin-1 that can induce apoptosis of T cells and facilitates binding of apoptotic T cells [113]. Furthermore, activated $CD8^+$ T cells have an increased expression of LFA-1 and in combination with the constitutive expression of ICAM-1 on LSECs this could result in an increased trapping of especially activated $CD8^+$ T cell in the liver. LSECs can phagocytose subsequently the apoptotic cells [112;114]. The liver is also implicated in the clearance of redundant PMNs. Although PMNs are also cleared at other sites, matured and activated PMNs are predominantly cleared in the liver where KCs participate in the phagocytosis of apoptotic PMNs [115;116].

Figure 1.3



Liver architecture.

(A) The liver parenchyma is divided by connective tissue into liver lobules. These hexagonal structures of plates of hepatocytes radiating from the central vein (CV). At the corners of the lobulus, the portal triads (PT), composed of a bile duct, hepatic artery and portal vein branches are situated. (B) The hepatocytes are adjoined at two or more sides to the sinusoids. These sinusoids are lined with fenestrated endothelial cells (LSEC). In between the hepatocytes and the endothelial cell layer, the space of Disse is situated. Kupffer cells (KC) penetrate the endothelial cell layer to reach the hepatocytes through the space of Disse.

Thesis Outline

In the research described in this thesis the functional role of L-SIGN and DC-SIGN in the immune system is investigated. L-SIGN and DC-SIGN have comparable binding characteristics, with specificity for fucose and high mannose containing carbohydrates. However, there are also differences in binding characteristics; whereas DC-SIGN is binding Le^x containing ligands, L-SIGN is not. In **chapter 2** a novel L-SIGN specific carbohydrate ligand, highly expressed on the well-differentiated hepatoma cell line HUH7, is described. This ligand contains sialic acids, of which the expression is lost in metastatic hepatocellular carcinoma cells. Therefore these hepatocellular carcinoma cells could loosen the interaction with surrounding cells and metastasise to remote sites. On the other hand expression of L-SIGN binding carbohydrates is increased upon malignant transformation of colon epithelial cells (**chapter 2**). This could be involved in the frequent metastasis of colon carcinomas to the liver.
DC-SIGN and L-SIGN bind several pathogens such as HIV-1 and HCV virus. HCV envelope glycoproteins E1 and E2 are both recognized by DC-SIGN and L-SIGN, and upon internalisation HCV virus like particles are retained in early endosomes in antigen-presenting cells (**chapter 3** [83]), promoting viral dissemination. Additionally, after DC-SIGN or L-SIGN mediated uptake of HCV immune complexes, antigens were presented to $CD4^+$ T cells. Furthermore degradation of HCV is enhanced in the presence of HCV specific antibodies (**chapter 4**).
DC-SIGN and L-SIGN also interact with the HIV-1 envelope glycoprotein gp120. DC-SIGN positive DCs are located at sites of infection and capture of HIV-1 by DC-SIGN on DCs enhances viral transmission of HIV-1 to $CD4^+$ T cells. In addition, DC-SIGN binding to gp120 induces conformational changes that enhance gp120 binding to CD4 (**chapter 5** [84]). Therefore blocking HIV-1-DC-SIGN interactions could decrease transmission of virus. We investigated blocking with a panel of anti-gp120 antibodies and demonstrate that although the V3 loop is not essential for DC-SIGN binding, antibodies against the V3 loop of gp120 reduce DC-SIGN binding, suggesting that these antibodies sterically hinder DC-SIGN binding to gp120 (**chapter 5** [84]).
Furthermore DC-SIGN-gp120 interactions can be blocked by a component present in human milk. The blocking milk component contains Le^x that binds to DC-SIGN and thereby blocks HIV-1 gp120 binding (**chapter 6** [85]). The milk component doe not block L-SIGN-gp120 interactions as Le^x can not be bound by L-SIGN.

Besides the recognition of pathogens, DC-SIGN and L-SIGN also mediate interactions with immune cells. In addition to interactions with T cells via ICAM-3 binding [16], DC-SIGN and L-SIGN are mediating interactions with neutrophils. DC-SIGN is recognizing both neutrophil Mac-1 and CEACAM1. This interaction modulates both DC and neutrophil responses (**chapter 7** [46;86]). L-SIGN recognizes only Mac-1 on neutrophils, and by binding to L-SIGN, neutrophils facilitate rolling over L-SIGN expressing endothelium (**chapter 7** addendum).
Additionally, L-SIGN can mediate binding to monocytes (**chapter 8**). Monocytes are macrophage precursor cells and in mouse the interaction with mSIGNR1 is involved in homeostasis of the resident liver macrophages, the Kupffer cells. mSIGNR1 deficient mice have less KCs than wild type mice (**chapter 8**).

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L-SIGN participation in tumour metastasis in the liver

A novel carbohydrate ligand for L-SIGN

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Abstract

The C-type lectin receptors (CLRs) DC-SIGN and L-SIGN recognize pathogens and self-ligands through recognition of specific carbohydrate moieties on glycoconjugates. DC-SIGN has been shown to function in both pattern recognition and immune regulation. Due to a high degree of homology between the carbohydrate recognition domains of DC-SIGN and L-SIGN, both CLRs have affinity for high mannose structures and the fucose containing Lewis^{a}, Lewis^{b}, and Lewis^{y} antigens, with the exception of Lewis^{x}, which is only bound by DC-SIGN.

DC-SIGN, expressed on dendritic cells, mediates interactions with colon carcinoma cells, which express high levels of Lewis^{y}-and Lewis^{x}-decorated CEA and CEACAM1. Here we show that, similar to DC-SIGN, L-SIGN found on liver sinusoidal endothelial cells (LSECs) recognizes tumour derived CEA, probably because of the presence of Lewis^{y} on CEA, and therefore could participate in colon cancer liver metastasis.

We furthermore demonstrate that L-SIGN recognizes a novel L-SIGN specific carbohydrate ligand on hepatoma cells. Notably, the ligand is neuraminidase-sensitive suggesting that L-SIGN recognizes a sialic acid containing glycan structure that is not recognized by DC-SIGN. Hepatocytes express high amounts of sialylated glycans, which are abrogated in metastasizing hepatocellular carcinoma cells. We therefore think that L-SIGN may have a dual function implicated both in the maintenance of normal liver structure by binding sialic acid structures expressed on healthy hepatocytes as well as the formation of liver metastasis by recognizing Lewis^{y} expressing tumour associated CEA.

Introduction

C-type lectin receptors (CLRs) mediate important interactions in homeostasis and pathogen recognition. CLRs contain one or more carbohydrate recognition domains (CRDs) that are able to interact with carbohydrate moieties expressed on pathogens and self-antigens. CLRs with an EPN motif in the binding domain mainly recognize mannose and fucose structures, whereas CLRs with a QPD motif recognize galactose containing glycan structures [1]. Mutagenesis of the EPN motif of MBP-A into QPD changes the mannose specificity into galactose specificity [1], confirming the importance of these motifs in defining the carbohydrate specificity.

The dendritic cell specific ICAM-3 grabbing non-integrin (DC-SIGN) binds several pathogens and self-antigens [2;3]. DC-SIGN is a CLR that has one CRD containing an EPN motif, and therefore recognizes high-mannose glycans. The DC-SIGN carbohydrate specificity is well documented and DC-SIGN has been demonstrated to recognize, besides high mannose structures, also non-sialylated fucose-containing Lewis-type antigens, such as Lewis^a (Le^a), Lewis^b (Le^b), Lewis^x (Le^x), and Lewis^y (Le^y) [4-7] when present both on pathogens and self-antigens.

L-SIGN, the DC-SIGN homologue, is not expressed on DCs but on liver sinusoidal endothelial cells (LSECs) [8;9], which are specialized endothelial cells with scavenger and antigen presenting functions [10;11]. L-SIGN shares 77% amino acid sequence identity with DC-SIGN, with the highest degree of homology in the CRD. The high degree of homology between the CRDs of DC-SIGN and L-SIGN is reflected in their ligand specificity. L-SIGN, similar to DC-SIGN, recognizes high-mannose glycans [4;12], and binds high-mannose-containing ligands such as HIV-1 and HCV envelope proteins [9;13]. Furthermore, L-SIGN also recognizes Le^y, Le^a, and Le^b antigens [12]. In contrast to DC-SIGN, L-SIGN does not bind Le^x. The lack of Le^x binding by L-SIGN is due to one amino acid replacement in the CRD of L-SIGN. Whereas DC-SIGN has a valine at position 351 (Val351), L-SIGN has at this same position a serine molecule (Ser363) [14;15]. Le^x binding capacity is restored by replacing Ser363 in L-SIGN with a valine residue, thereby creating a hydrophobic pocket [15]. Thus, although L-SIGN and DC-SIGN are quite similar, there are differences in expression and binding specificity pattern, and no ligand unique for L-SIGN only has been described so far.

Both DC-SIGN and L-SIGN function as pathogen recognition receptors. DC-SIGN was first identified as a receptor for the highly glycosylated HIV-1 gp120 envelope protein [16]. By now a plethora of pathogenic, self and altered self-ligands that are bound by both DC-SIGN and L-SIGN have been identified. Specificity of DC-SIGN and L-SIGN for these ligands is based on the glycan profile of these ligands. Often when the ligands express mannose-rich structures or Le^y, Le^a, or Le^b antigens, both DC-SIGN and L-SIGN recognize the ligand. When only Le^x is expressed, the ligand is exclusively recognized by DC-SIGN, and not by L-SIGN.

Glycosylation is a highly dynamic and heterogeneous phenomenon, varying not only within tissues or even cell subsets, but also in different (patho)physiological conditions within the same cell type. Oncogenic transformation often results in a perturbation of the glycosylation machinery [17], which has severe effects on the metastatic properties of the tumour. In the case of colon epithelial cells, malignant transformation results in an increased expression of Le^x and Le^y epitopes [18]. As a consequence DC-SIGN discriminates between healthy colon tissue and colon carcinoma tissue. DC-SIGN specifically binds to carcinoembryonic antigen

(CEA) and CEA related cell adhesion molecule 1 (CEACAM1) from primary colon carcinoma and from colon carcinoma cell lines [19].
Colon carcinomas often metastasize in the liver where L-SIGN is strategically expressed on LSECs. We therefore investigated whether L-SIGN is involved in binding of colon carcinoma derived CEA. We demonstrate that L-SIGN, similar to DC-SIGN, recognizes colon carcinoma derived CEA and therefore could function in colon carcinoma metastasis to the liver. Furthermore, we investigated the interaction of DC-SIGN and L-SIGN with a hepatocyte derived tumour, often metastasising in remote sites in the liver. Surprisingly, the hepatoma cell line expresses a ligand containing sialic acid residues exclusively recognized by L-SIGN. Sialylated glycans are abundantly expressed on normal hepatocytes, and loss of sialic acid structures on the cell surface during oncogenesis is associated with bad prognosis. Therefore, L-SIGN could participate in maintenance of normal liver structure.

Materials and methods

Antibodies.
The following antibodies were used: col-1 (anti-CEA (Becton Dickinson, San Jose, CA), DC-SIGN- and L-SIGN-specific antibody AZN-D2 [16], anti-stalk 4 (anti-L-SIGN/DC-SIGN), avidin-Alexa Fluor 488 (Molecular Probes, Eugene, OR), anti-mouse-biotin (Jackson Immunoresearch, Cambridgeshire, UK), and anti-human Fc-FITC (Jackson Immunoresearch, Cambridgeshire, UK).

Cells.
Raji-1 and K562 transfectants expressing wild-type DC-SIGN or L-SIGN were generated by transfection with 10 µg pRc/CMV-DC-SIGN or pRc/CMV-L-SIGN plasmid by electroporation as previously described [9;16]. HUH7 (human hepatoma cell line [20]) and SW948 (human colorectal adenocarcinoma cell line, ATCC number CCL-237) were cultured in DMEM and RPMI respectively supplemented with penicillin/streptomycin and 10% FCS. Cells were subcultured regularly.

Cell-cell adhesion assay.
To examine cellular interactions of DC-SIGN and L-SIGN with colon carcinoma SW948 and hepatoma HUH7 cells, SW948 and HUH7 cells were grown to confluent cultures in flat-bottom 96 well plates. Raji-1 cells transfected with DC-SIGN or L-SIGN were labelled with the green fluorescent dye Calcein-AM (1 µM, Molecular Probes, Eugene, OR) for 15 minutes at 37°C and incubated with SW948 and HUH7 cells in TSM (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$) 0.5% BSA for 2 hours at 37°C. To determine specific adhesion the Raji transfectants were preincubated with anti-DC-SIGN and anti-L-SIGN antibodies or isotype control antibodies (20 µg/ml) for 30 minutes at 37°C. Unbound cells were washed away, and remaining cells were lysed in lysis buffer (50 mM Tris-HCl pH 8.0, 0.1% SDS). Cell-cell adhesion was measured by fluorimetry of cell lysates at 488 nm (Fluostar Galaxy, BMG Labtechnologies Inc., Durham, NC). Maximal adhesion (100%) was calculated by subtraction of background fluorescence from the fluorescence without washing. Binding of cells in the samples was calculated by subtraction of the background fluorescence from the measured fluorescence divided by the 100% adhesion value.

Soluble DC-SIGN and L-SIGN binding assay.
The DC-SIGN-Fc chimera [21] contained the extracellular portion of DC-SIGN (amino acid residues 64-404) fused at the C-terminus to a human IgG1-Fc fragment. The L-SIGN-Fc chimera was composed of the extracellular portion of L-SIGN fused to human IgG1-Fc fragment with a triple glutamine linker. DC-SIGN-Fc and L-SIGN-Fc were produced in Chinese hamster ovary K1 (CHO) cells and purified with a protA column. To determine DC-SIGN and L-SIGN binding to SW948 and HUH7 cells, cells were

Table 2.1.
DC-SIGN and L-SIGN binding to normal colon and colon carcinoma derived CEA[a]

	K562-DC-SIGN		K562-L-SIGN	
	normal	tumour	normal	tumour
patient 1	+[b]	++	-	+++
patient 2	++	++	+	++
patient 3	++	+++	++	+++
patient 4	++	++	-	++
patient 5	+	++	-	+
patient 6	-	++	-	+

[a] Binding of K562-DC-SIGN and L-SIGN cells to beads coated with CEA derived from normal colon or colon carcinoma material is shown. One representative experiment out of three is shown.
[b] -, 0-5% binding; +, 5-10% binding; ++, 10-20% binding; +++, 20% or more binding.

harvested and incubated with 10 µg/ml DC-SIGN or L-SIGN-Fc in TSM/BSA for 45 minutes at 37°C. Cells were subsequently stained with FITC labelled anti-human-Fc antibodies and binding was analysed by flow cytometry (FACScan, Becton Dickinson, Oxnard, CA). Binding was expressed as mean fluorescence intensity of the cells. Specificity of binding was determined by preincubating DC-SIGN-Fc and L-SIGN-Fc with EGTA (10 mM).

Fluorescent bead adhesion assay.
Carboxylated TransFluorSpheres (488/645 nm, 1.0 µm; Molecular Probes, Eugene, OR) were coated with normal colon tissue or colon carcinoma derived CEA or DC-SIGN- or L-SIGN-Fc [16]. In short, streptavidin was covalently coupled onto TransFluoSpheres as described by manufacturer. Streptavidin-coated beads were incubated with biotinylated goat anti-mouse or goat anti-human Fc $F(ab')_2$ fragments (10 µg/ml, Jackson ImmunoResearch) in PBS 0.5% BSA for 2 hours at 37°C, and washed. Subsequently, the beads were incubated overnight at 4°C with mouse anti-CEA antibodies, washed, and incubated with lysates from colon material or DC-SIGN- or L-SIGN-Fc (2 µg/ml) respectively for 48 hours.
The fluorescent bead adhesion assay was performed as previously described [22]. In the experiments investigating DC-SIGN and L-SIGN binding to CEA, mock transfected K562 cells or K652 cells transfected with DC-SIGN or L-SIGN were preincubated for 30 min at 37℃ with inhibitors (mannan (1 mg/ml), or EGTA (10 mM)). Next, CEA-coated fluorescent beads (20 beads/cell) were added to the cells for 45 min at 37℃, washed and analyzed by flow cyt ometry (FACScan, Becton Dickinson, Oxnard, CA), by measuring the percentage of cells that had bound fluorescent beads.
In the experiments investigating DC-SIGN- and L-SIGN-Fc coated beads adhesion to SW948 and HUH7 cells the beads were preincubated with inhibitors (anti-DC-SIGN/L-SIGN antibodies (20 µg/ml), or EDTA (10 mM)) for 30 min at 37℃. Subsequently the cells were added to the beads and incubated for 45 min at 37℃, washed and analyzed by flow cytometry (FAC Scan, Becton Dickinson, Oxnard, CA), by measuring the percentage of cells that had bound fluorescent beads.

Real time-PCR.
RT-PCR was performed as previously described [23]. In short, SW948 and HUH7 cells were lysed, and mRNA was isolated using an mRNA capture kit (Roche, Switzerland/Boehringer Mannheim GmbH, Germany). Subsequently cDNA was generated with a Reverse Transcription System kit (Promega, USA) according to the manufacturer's instructions. Briefly, cell lysates were incubated with biotin-labelled oligo$(dT)_{20}$ for 5 minutes at 37°C and 50 µl was incubated in streptavidin coated tubes for 5

minutes at 37°C. After washing, 30 µl of reverse transcription mixture (5mM $MgCl_2$, 1x reverse transcripture buffer, 1 mM dNTP, 0.4 U recombinant RNasin ribonuclease inhibitor, 0.4 U AMV reverse transcriptase, 0.5 µg random hexamers) was added and incubated for 10 minutes at room temperature and 45 minutes at 42°C. Samples were heated for 5 minutes at 99°C to inactivate AMV reverse transcriptase and to isolate cDNA. Samples were stored at -20°C until analysis.
Gene expression of the selected genes was quantified by real time PCR with the SYBR Green method using an ABI 7900 HT sequence detection system (Applied Biosystems, USA). Primers were synthesized by Invitrogen (Invitrogen, Belgium) and have been previously described [23]. In short, reactions were set on a 96 well plate by mixing 4 µl of SYBR Green Master Mix (Applied Biosystems, USA) with 2 µl of an oligonucleotide solution containing 5 nmol/µl of both oligonucleotides and 2 µl of a cDNA solution corresponding to 1/100 of the cDNA synthesis product. The thermal profile for all the reactions was 2 min at 50°C, followed by 10 min at 95°C and then 40 cycles of 15 sec at 95°C and 1 min at 60°C. The housekeeping gene GAPDH was used as endogenous reference [24]. The relative abundance was calculated according to the ΔCt method [25]: $100x2^{(Ct\ GAPDH-Ct\ glycosyltransferase)}$.

Lectin staining.
To evaluate the expression of carbohydrate structures on SW948 and HUH7 cells, cells were harvested and incubated with a panel of biotinylated lectins (10 µg/ml in TSM/BSA, Vector laboratories, Peterborough, UK). The lectins used are *Ulex europaeus* agglutinin 1 (UEA-1), recognizing α-1,2-linked fucose residues, *Lotus tetragonolobus* lectin (LTA), recognizing α-1,3/4-fucosylated oligosaccharides, *Aleuria aurantia* lectin (AAL), binding preferentially to Fucα1,6GlcNAc (core fucose) or to Fucα1,3/4Galβ1,4/3GlcNAc (Lewis-type) structures, *Narcissus Pseudonarcissus* Lectin (NPA), recognizing α-linked mannose, preferentially α-1,6 linked polymannose structures, *Galanthus nivalis* lectin (GNA), which preferentially binds α-1,3 linked mannose structures, *Sambucus nigra* lectin (SNA), which preferentially binds to α-2,6 linked sialic acid to terminal galactose or GalNAc, and *Maackia amurensis* agglutinin (MAA), recognizing α-2,3-sialylated structures.
Lectin binding was detected by staining with avidin-Alexa Fluor 488 and measured by flow cytometry analysis (FACScan, Becton Dickinson, Oxnard, CA). Binding was expressed as mean fluorescence intensity of the cells.

Neuraminidase treatment
Sialic acids were removed from cell surface glycans by incubating intact cells prior to flow cytometry in 100 µl solution in 50 mM sodium acetate, 154 mM sodium chloride, 9 mM calcium chloride, 25 mg/l human serum albumin (pH 5.5) containing 10 mU of *Vibrio cholerae* neuraminidase (Roche, Switzerland) for 60 minutes at 37°C. After washing, efficiency of neuraminidase treatment was determined by staining the cells with SNA and MAA as described above.

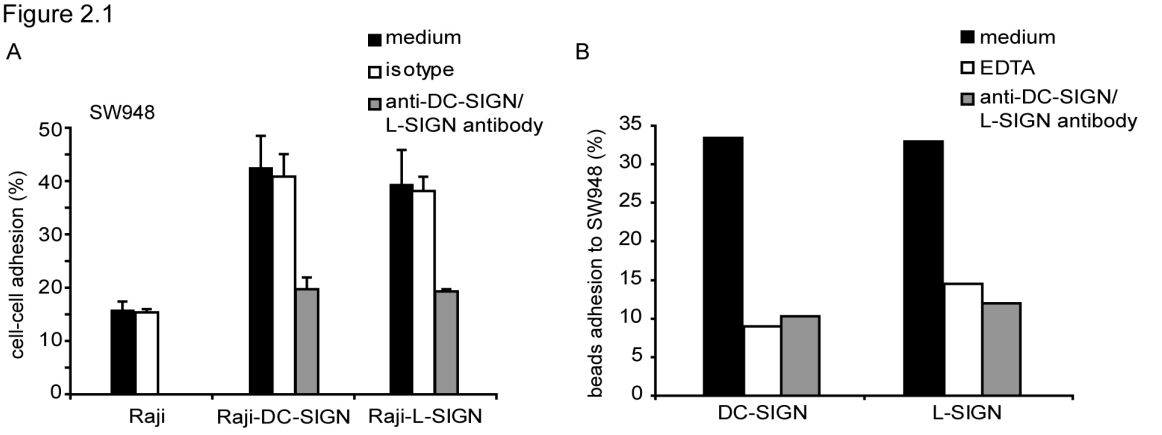
Results

L-SIGN and DC-SIGN bind to the colon carcinoma cell line SW948.
DC-SIGN has been demonstrated to bind the colon carcinoma cell line SW948 [19]. In order to study whether L-SIGN can play a role in interactions with colon carcinoma cells, and thereby function in liver metastasis formation, we investigated whether L-SIGN interacts with SW948 cells. For this a cell-cell based adhesion assay was developed in which both Raji cells transfected with DC-SIGN or L-SIGN were investigated for their potency to bind a monolayer of SW948 cells. Both DC-SIGN and L-SIGN transfected cells strongly bound to SW948 cells (40% binding, Fig. 2.1A). This binding was specific since mock-transfected cells did not interact with SW948 and anti-DC-SIGN/L-SIGN antibodies could block the interaction to background levels (Fig. 2.1A).
To further confirm L-SIGN binding to SW948, fluorescent beads were coated with DC-SIGN-Fc or L-SIGN-Fc chimeras, incubated with SW948 cells, and binding was measured by flow

cytometry. Both DC-SIGN-Fc and L-SIGN-Fc coated beads interacted strongly with SW984 cells (Fig. 2.1B). This binding was specific since specific antibodies against DC-SIGN and L-SIGN as well as EDTA blocked the interaction (Fig. 2.1B).

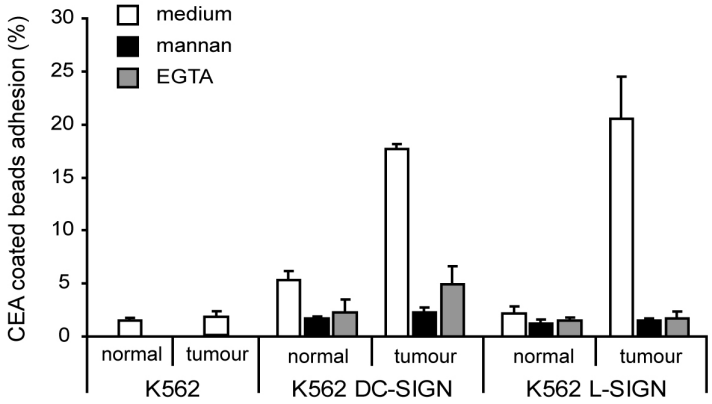
L-SIGN binds primary colon carcinoma CEA more strongly than normal colon CEA.
DCs interact with colon carcinoma cells through DC-SIGN binding of tumour CEA(CAM1). Tumour CEA(CAM1) carries higher levels of the carbohydrates $Le^{x/y}$ than normal colon tissue, resulting in DC-SIGN recognition [19]. Since colon carcinomas frequently metastasize in liver sinusoids and the colon carcinoma cell line SW948 binds L-SIGN (Fig. 2.1), we investigated whether L-SIGN, a C-type lectin highly related to DC-SIGN and expressed on LSECs, interacts with tumour CEA. Therefore, normal colon tissues and tumour tissues were lysed and CEA was captured from the lysate with a CEA specific antibody coated on fluorescent beads. K562 cells transfected with DC-SIGN or L-SIGN and mock-transfected K562 cells were incubated with CEA coated beads and adhesion was measured by flow cytometry. Clearly both K562-DC-SIGN and K562-L-SIGN cells were able to bind more strongly to beads coated with tumour-derived CEA than to beads coated with CEA from healthy colon tissue (Table 2.1). This binding could be blocked by preincubating the cells with EGTA or mannan (Fig. 2.2). Mock-transfected K562 cells did not bind to CEA from either normal or tumour material (Fig. 2.2).
This indicates that L-SIGN, similar to DC-SIGN, binds more strongly to tumour derived CEA than to CEA derived from healthy colon tissue. This interaction is probably due to the increased Le^{y} expression on tumour-derived CEA since L-SIGN does not bind Le^{x} residues [15;19]. This demonstrates that L-SIGN binds to primary colon carcinoma cells and colon carcinoma cell lines. Therefore, L-SIGN could be involved in liver metastasis of colon carcinomas.

Figure 2.1



Binding of DC-SIGN and L-SIGN to the colon carcinoma cell line SW948.
(A) Cellular binding of DC-SIGN and L-SIGN transfected cells to colon carcimoma SW948 cells. SW948 cells were cultured in 96 well plates and fluorescently labelled Raji cells transfected with DC-SIGN or L-SIGN were incubated on the cells. Binding after several washes was measured using fluorimetry. Binding specificity was confirmed using DC-SIGN/L-SIGN specific antibodies (20 µg/ml, grey bars), or isotype controls (white bars). Error bars represent standard deviations (n=4). One representative experiment out of three is shown. (B) Binding of DC-SIGN- and L-SIGN-Fc coated beads to SW948 cells. DC-SIGN- and L-SIGN-Fc were coupled to fluorescent beads and incubated with SW948 cells. Adhesion was measured by flow cytometry. Specificity was determined by blocking with EDTA (10 mM, white bars) or DC-SIGN/L-SIGN specific antibodies (20 µg/ml, grey bars). One representative experiment out of three is shown.

Figure 2.2

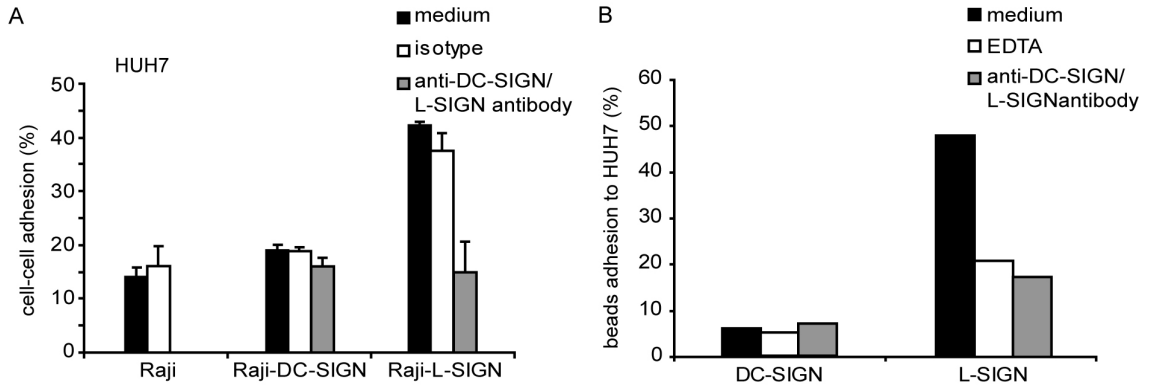


DC-SIGN and L-SIGN bind more strongly to colon carcinoma derived CEA than to normal colon CEA.
CEA isolated from colon carcinoma or non-neoplastic lysates was captured on fluorescent beads. The CEA coated beads were incubated with DC-SIGN, L-SIGN or mock transfected K562 cells. Adhesion was measured by flow cytometry. Specificity was determined by blocking with mannan (1 mg/ml, black bars) or EGTA (10 mM, grey bars). Error bars represent standard deviations (n=3). One representative patient out of six is shown.

L-SIGN exclusively binds hepatoma cell line HUH7.
Besides metastasis of colon carcinoma in the liver, also tumours from hepatic origin frequently form intrahepatic metastasis. Therefore, we investigated the binding of L-SIGN and DC-SIGN to the hepatoma cell line HUH7. A cell-cell adhesion assay was performed similar as with SW948 cells. Fluorescently labelled Raji cells transfected with DC-SIGN or L-SIGN were added on a monolayer of HUH7 cells and binding was measured using fluorimetry. Strikingly, only Raji-L-SIGN cells were binding to HUH7 cells. No binding of Raji-DC-SIGN or mock-transfected Raji cells was observed (Fig. 2.3A). The binding of Raji-L-SIGN cells could be specifically blocked by an L-SIGN specific antibody (Fig. 2.3A). Next, we further investigated adhesion of DC-SIGN-Fc and L-SIGN-Fc coated beads to HUH7. In this assay, L-SIGN-Fc coated beads bound strongly to HUH7 cells and DC-SIGN-Fc coated beads did not bind the cells (Fig. 2.3B), supporting the data from the cell-cell adhesion assay. This indicates that L-SIGN recognizes a ligand expressed on the hepatocyte cell line that is not recognized by DC-SIGN, demonstrating that this ligand is exclusive for L-SIGN.

HUH7 cells express high levels of sialic acid.
These data suggest that there is a difference in glycosylation between the colon carcinoma cell line SW948 and the hepatoma cell line HUH7 resulting in the differential binding of DC-SIGN and L-SIGN, with both DC-SIGN and L-SIGN binding to SW948 but only L-SIGN binding to HUH7. This is of special interest since no carbohydrate ligand is yet identified that is exclusively bound by L-SIGN and not by DC-SIGN. In order to identify which L-SIGN specific glycan structure is expressed on HUH7 cells, we compared the expression of glycosylation-related genes in SW948 cells and HUH7 cells. The expression levels of a set of glycosyltransferases (GTs) involved in the synthesis of Lewis-type, sialylated, and galactosylated structures in SW948 and HUH7 cells was assayed by quantitative real-time PCR. The most prominent difference in GT expression is reflected in the high expression of fucosyltransferases (Fut) 1, -2, -4 and -10 in SW948 cells, whereas HUH7 cells express only Fut10 (Fig. 2.4A). Fut1, -2, and -4 are necessary for the generation of fucosylated carbohydrates such as Le^x and Le^y, while Fut10 has been suggested to be involved in this process, although has not been demonstrated yet [26]. In contrast, HUH7 cells expressed high levels of sialyltransferases, especially of ST6Gal1 and ST3Gal6 that were not expressed by SW948 cells (Fig. 2.4A). ST3Gal4 is expressed in both SW948 and HUH7 at moderate levels. These data predict a higher expression of fucosylated glycans on SW948 cells while HUH7

Figure 2.3



L-SIGN binds specific to the hepatoma cell line HUH7.

(A) Cellular interactions between L-SIGN transfected cells and hepatoma HUH7 cells occur but not between DC-SIGN transfected cells and HUH7 cells. HUH7 cells were cultured in 96 wells plate to confluent monolayers and fluorescently labelled Raji cells transfected with DC-SIGN or L-SIGN were incubated on the cells. Binding after several washes was measured using fluorimetry. Binding specificity was confirmed using DC-SIGN/L-SIGN specific antibodies (20 µg/ml, grey bars), or isotype controls (white bars). Error bars represent standard deviations (n=4). One representative experiment out of three is shown. (B) L-SIGN-Fc coated beads, but not DC-SIGN-Fc coated beads bind to HUH7 hepatoma cells. DC-SIGN- and L-SIGN-Fc were coupled to fluorescent beads and incubated with HUH7 cells. Adhesion was measured by flow cytometry and represented as percentage cells positive for beads binding. Specificity was determined by blocking with EDTA (10 mM, white bars) or DC-SIGN/L-SIGN specific antibodies (20 µg/ml, grey bars). One representative experiment out of three is shown.

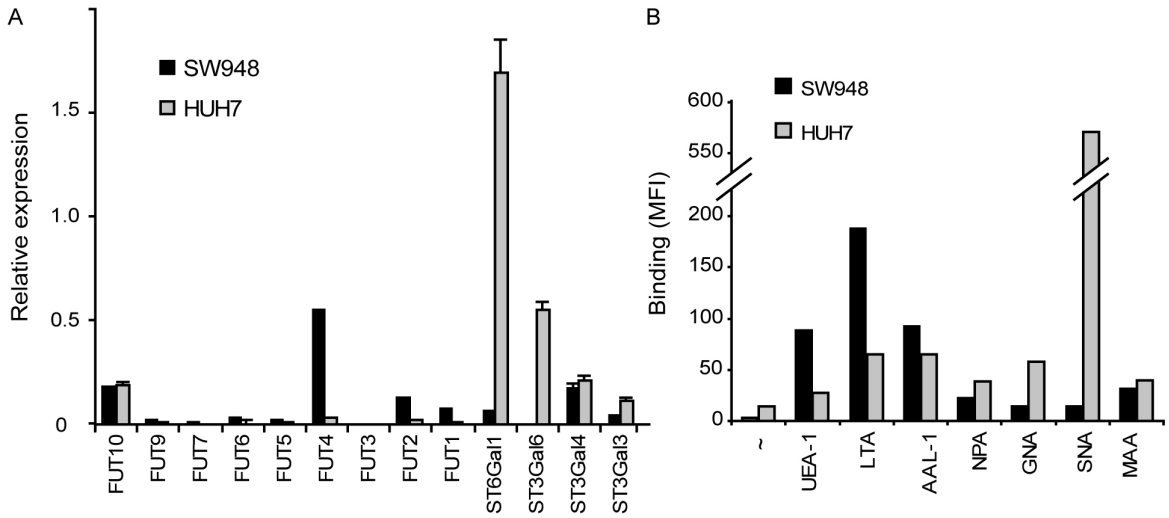
glycans are expected to be highly sialylated. This modification is known to sterically inhibit DC-SIGN binding [5;27].

To confirm actual glycan expression on the cells, SW948 and HUH7 cells were stained with a panel of lectins with known glycan specificities (Fig. 2.4B). Indeed, SW948 cells were strongly bound by α-L-Fucose-specific lectins, *Ulex europaeus* agglutinin 1 (UEA-1), Lotus tetragonolobus lectin (LTA), and *Aleuria aurantia* lectin (AAL). SW948 cells were not stained by sambucus nigra lectin (SNA), which binds preferentially to sialic acid attached to terminal galactose in α-2,6 linkage (Fig. 2.4B, black bars). In contrast, the hepatoma cell line HUH7 was strongly bound by SNA, confirming a high expression of α-2,6 linked sialylated carbohydrates (Fig. 2.4B, grey bars). The α-L-Fucose binding lectins UEA-1, LTA, and AAL showed only low binding to HUH7 cells. Thus, SW948 are rich in fucosylated glycan structures whereas HUH7 cells express only low levels of fucosylated carbohydrates and high levels of sialylated glycan structures.

Neuraminidase abrogates L-SIGN-HUH7 interactions.

Because sialic acids present on glycan structures may mask DC-SIGN binding sites [27], and our GTs and glycan profiles indicate that they may serve as L-SIGN ligands, we investigated the effect of neuraminidase treatment on DC-SIGN and L-SIGN binding. Neuraminidase cleaves terminal sialic acid residues from carbohydrate moieties on proteins, resulting in terminal galactose, GalNAc, or fucose. The panel of lectins (Fig. 2.4B) was used to identify which changes in carbohydrate structures were generated by neuraminidase treatment of HUH7 cells. As expected, SNA binding to neuraminidase treated cells was reduced indicating that the neuraminidase treatment was effective (Fig. 2.5A). The fucose recognizing lectins UEA-1, LTA, and AAL did not show increased binding to neuraminidase treated HUH7 cells compared to control HUH7 cells (Fig. 2.5A).

Figure 2.4



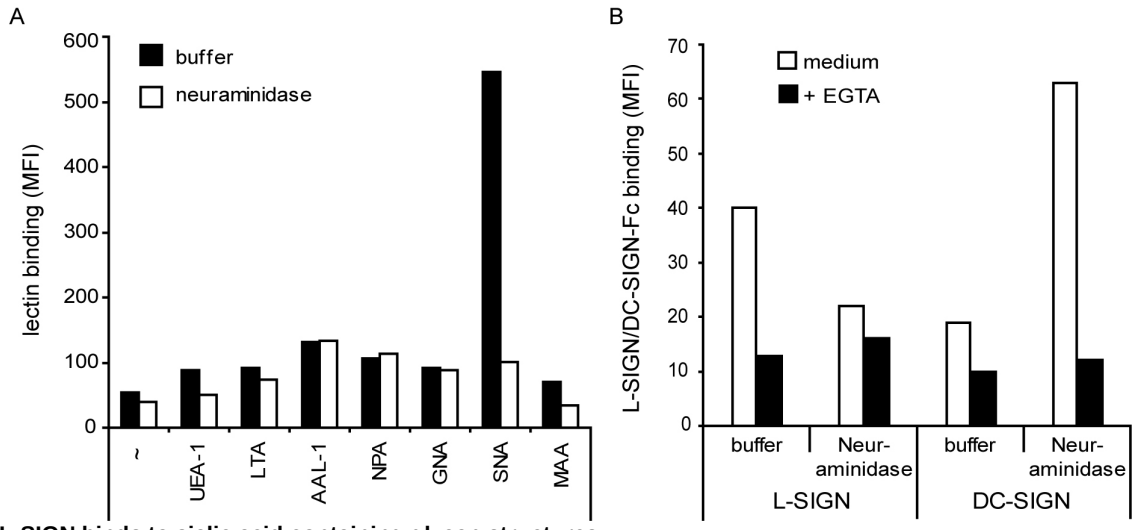
Glycosylation profile of HUH7 and SW948 cells.
(A) Glycosyltransferases expressed in HUH7 and SW948. Total mRNA was isolated from SW948 (black bars) and HUH7 (grey bars) cell lysates. After reverse transcription to cDNA, relative expression compared to expression of the household gene GAPDH was determined by real time PCR. Expression levels of fucosyl transferases (FUT) 1 to 7, Fut9 and Fut10, sialyl transferase (ST) 6Gal1, ST3Gal3, ST3Gal4 and ST3Gal6 were determined. (B) Glycan expression pattern of SW948 and HUH7 cells. SW984 (black bars) and HUH7 (grey bars) were stained with the biotinylated fucose specific UEA-1, LTA, and AAL, mannose specific NPA, and GNA, and sialic acid specific SNA, and MAA lectins. Lectin binding was determined by counterstaining with fluorescent-labelled avidin and represented as mean fluorescence intensity of the cells. One representative experiment out of three is shown.

L-SIGN- and DC-SIGN-Fc binding to neuraminidase treated HUH7 was compared to the binding of untreated cells. L-SIGN-Fc binding to HUH7 cells after neuraminidase treatment was greatly decreased (Fig. 2.5B). In contrast, DC-SIGN binding to neuraminidase treated cells was increased compared to untreated cells (Fig. 2.5B). Thus removal of sialic acid residues from the glycan structures present on HUH7 eliminates the binding epitope of L-SIGN, whereas the removal of sialic acid unmasks the DC-SIGN binding epitopes.
These data suggest that the hepatoma cell line expresses a ligand which contains sialic acid residues that is exclusively recognized by L-SIGN and not by DC-SIGN.

Discussion

We demonstrate here that L-SIGN, similar to DC-SIGN, is involved in tumour antigen recognition and that L-SIGN also recognizes a novel glycan structure yet to be characterized and distinct from DC-SIGN on the well differentiated hepatoma cell line HUH7. Earlier studies have demonstrated that DC-SIGN on DCs recognizes colon carcinoma derived CEA and CEACAM1 [19]. Malignant transformation of colon epithelial cells may change the glycosylation machinery, resulting in higher expression of Lewis type antigens [18;28]. It has been demonstrated that binding of DC-SIGN to the colon carcinoma cell line SW948 is partially dependent on Le^x and Le^y expression, since α1,3/4-Fucosidase treatment, which disrupts Lewis antigen structures, reduces DC-SIGN binding to SW948 derived CEA and CEACAM1 [19]. Here we demonstrate that L-SIGN, similar to DC-SIGN, binds to the colon carcinoma cell line SW948. Furthermore, we demonstrate that L-SIGN binds more strongly to tumour derived

Figure 2.5



L-SIGN binds to sialic acid containing glycan structures.
(A) Glycan expression on HUH7 cells after neuraminidase treatment. HUH7 cells were incubated with buffer (black bars) or with neuraminidase (white bars) in order to remove sialic acid residues from carbohydrate structures on the cell surface, and binding of a selected panel of lectins was investigated similar as in Figure 4B. One representative experiment out of two is shown. (B) DC-SIGN- and L-SIGN-Fc binding to HUH7 cells after neuraminidase treatment. HUH7 cells were incubated with buffer or with neuraminidase and subsequently incubated with DC-SIGN- or L-SIGN-Fc. Cells were counterstained with fluorescent labelled anti-human Fc antibodies and binding is represented as mean fluorescent intensity of the cells. Binding specificity was confirmed by blocking with EGTA (10 mM, black bars). One representative experiment out of two is shown.

CEA than to CEA from healthy tissue. Colon carcinomas often form metastasis in the liver, and L-SIGN expressed on liver sinusoidal endothelial cells could be involved in binding and dissemination of tumour cells. Binding to L-SIGN may facilitate the arrest of metastasizing cells in the liver sinusoid which subsequently proliferate to secondary tumours. Furthermore, the intrinsic tolerogenic capacity of LSECs [10;29] may hamper the induction of adequate immune responses to the metastasizing tumour cells. It has been postulated that DC-SIGN binding of tumour antigens to DC-SIGN expressing cells without an additional activation stimulus might also result in tolerance induction [30]. Indeed it was demonstrated that *in vivo* DCs in close proximity to CEA expressing tumours have an immature phenotype [19] and therefore lack the capacity to induce a proper T-cell response to the tumour. Therefore the binding of colon carcinoma cells to both L-SIGN and DC-SIGN might be beneficial for the tumour, firstly by preventing an appropriate T-cell response by binding to both DC-SIGN and L-SIGN on APCs, and secondly facilitating the arrest and dissemination of tumour cells in the liver due to capture by L-SIGN on LSECs in the liver sinusoid.

We observed that next to its interaction with colon carcinoma cell line SW948 and colon carcinoma tissue of patients, L-SIGN is also binding the hepatoma cell line HUH7. Strikingly, DC-SIGN does not interact with these cells, indicating the expression of an L-SIGN specific ligand on these cells. We observerd that HUH7 cells have different GTs and glycan expression levels compared to SW948 colon carcinoma cells. The most striking difference is the high expression of sialyltransferases ST6Gal1 and ST3Gal6 in HUH7, which are absent in SW948 cells. Sialic acid residues can mask DC-SIGN ligands, and indeed DC-SIGN did not bind HUH7 cells. However, DC-SIGN binding could be restored by removal of sialic acids by neuraminidase treatment of the hepatoma cell line which is consistent with its capacity for non-sialic acids. In contrast, L-SIGN binding to HUH7 was abrogated when sialic acid residues

were removed by neuraminidase treatment of the cells, suggesting that L-SIGN in contrast to DC-SIGN is recognizing sialic acid containing carbohydrates.
Normal non-neoplastic hepatocytes express high levels of α-2,6-sialylated glycoconjugates, and binding of the plant lectin SNA, recognizing α-2,6 linked sialic acid, is mainly confined to the sinusoidal side of hepatocytes [31]. In hepatocellular carcinomas this SNA binding pattern to the membrane is disrupted, and instead SNA stains dense aggregates in the cytoplasm of neoplastic hepatocytes [31]. The HUH7 cell line used in this study is derived from a well-differentiated hepatoma with similar characteristics as healthy hepatocytes [20]. Since L-SIGN binds this well-differentiated hepatoma cell line, it will be highly likely that L-SIGN also binds normal hepatocytes. L-SIGN binding to sialylated structures on hepatocytes could therefore be associated with maintenance of normal liver structure, and the loss of sialylated carbohydrate expression on the hepatocyte surface upon malignant transformation could therefore result in loss of normal hepatic organisation and spread of tumour cells.

The carbohydrate recognition pattern of DC-SIGN has extensively been studied, and DC-SIGN has demonstrated to have specificity for high mannose and non-sialylated fucose-containing Lewis antigens such as Le^x, Le^y, Le^a, and Le^b. Sialylation of these Lewis type carbohydrates results in loss of DC-SIGN binding [5;12].
L-SIGN shares 77% amino acid sequence identity with DC-SIGN, with the highest similarity in the CRD and its binding characteristics have demonstrated to be very similar to DC-SIGN [4;12]. However, the difference in just one amino acid in the CRD of L-SIGN results in the inability to bind Le^x [14;15]. L-SIGN has at position 363 a serine molecule (Ser363), whereas DC-SIGN has a valine at this position (Val351). Mutation of the serine into a valine restores the ability of L-SIGN to bind Le^x [15].
Here we demonstrate that, in contrast to DC-SIGN, L-SIGN binds sialic acid residues expressed by hepatic cells. This is the first identification of an L-SIGN specific carbohydrate ligand. Davis et al. reported the differential binding of DC-SIGN and L-SIGN to West Nile virus, indicating an L-SIGN specific binding to West Nile virus produced in human cells which did not interact with DC-SIGN [32]. However, the glycan profile responsible for this difference was not investigated. Using a glycan array the specificity of soluble L-SIGN was demonstrated only to bind high-mannose oligosaccharides [14]. However, studies using beads coated with polyacrylamide (PAA)-linked carbohydrates and L-SIGN expressing cells, demonstrated that L-SIGN also interacts with fucose containing ligands Le^y, Le^a, and Le^b [12]. The different results found in these two assays could be due to the multimerisation of L-SIGN, but could also be due to multivalency of the carbohydrates and the protein backbone. The murine homologue of DC-SIGN and L-SIGN, mSIGNR1 has demonstrated to have also specificity for sialylated glycan structures since mSIGNR1 expressing cells bind sialylated Lewis antigens coupled to a PAA-backbone [12]. In this assay using cells expressing the C-type lectins and PAA-coupled carbohydrates on beads, L-SIGN did not bind sialylated Lewis antigens [12]. However it is possible that for binding, the carbohydrates should be expressed on an appropriate backbone. The fact that L-SIGN could bind to sialic acid structures expressed on hepatic cells, as demonstrated here, and not to sialic acid structures present on the glycan array or as PAA-coupled beads remains to be defined in the near future.

In conclusion this study demonstrates a dual role for L-SIGN on LSECs in the liver. Firstly, L-SIGN may function in the dissemination of colon carcinoma metastases in the liver, through binding and arrest of colon carcinoma cells. On the other hand, L-SIGN may function in

homeostasis control through its interaction with specific sialic acid containing ligands on healthy hepatocytes.

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Hepatitis C virus targets DC-SIGN and L-SIGN to escape lysosomal degradation

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Abstract

Hepatitis C virus (HCV) is a major health problem. However, the mechanism of hepatocyte infection is largely unknown. We demonstrate that the DC-specific C-type lectin DC-SIGN and its liver-expressed homologue L-SIGN/DC-SIGNR are important receptors for the HCV envelope glycoproteins E1 and E2. Mutagenesis analyses demonstrates that both HCV E1 and E2 bind the same binding site on DC-SIGN as the pathogens HIV-1 and mycobacteria, which is distinct from the cellular ligand ICAM-3.

HCV virus-like particles are efficiently captured and internalized by DCs through binding of DC-SIGN. Antibodies against DC-SIGN specifically block the HCV capture by both immature and mature DCs, demonstrating that DC-SIGN is the major receptor on DCs. Interestingly, internalized HCV virus-like particles are targeted to non-lysosomal compartments within immature DCs, where they are protected from lysosomal degradation similarly as was demonstrated for HIV-1. Lewis^x antigen, another ligand of DC-SIGN was internalized to lysosomes, demonstrating that the internalization pathway of DC-SIGN-captured ligands may depend on the structure of the ligand. Our results suggest that HCV may target DC-SIGN to 'hide' within DCs and to facilitate viral dissemination. L-SIGN expressed by THP-1 cells internalizes HCV particles into similar non-lysosomal compartments, suggesting that L-SIGN on LSECs may capture HCV from blood and transmit it to hepatocytes, the primary target for HCV.

We therefore conclude that both DCs and LSECs may act as reservoirs for HCV and that the C-type lectins DC-SIGN and L-SIGN, as important HCV receptors, may represent a molecular target for clinical intervention in HCV infection.

Introduction

Hepatitis C virus (HCV) is the causal agent of hepatitis C, which is a major health problem affecting 170 million people worldwide [1]. Approximately 90% of patients develop chronic hepatitis [2], of which 20-30% progress to liver cirrhosis and end-stage liver disease [3;4]. HCV is an enveloped positive-stranded RNA virus [5] that belongs to the Flaviviridae family. The genome encodes for a single polyprotein [6;7] and a combination of host and viral peptidases processes the polyprotein into at least nine different structural and non-structural proteins [8-10]. The HCV envelope is formed by two heavily N-glycosylated type I transmembrane envelope glycoproteins E1 (31 kD) and E2 (70 kD) [11-13], that are expressed as heterodimers on the virus membrane [13].

A characteristic feature of HCV is the high incidence of persistent infection and chronic hepatitis with a strong risk for the development of hepatocellular carcinoma, although some patients exhibit acute self-limited infection [14]. This high incidence of chronicity suggests that the virus has developed efficient mechanisms to escape host immune responses. Indeed, cellular immune responses are weak in chronically infected patients [15-17], although the reason for this poor reaction remains unclear.

HCV mainly infects hepatocytes but also peripheral blood mononuclear cells (PBMCs). However, the precise mechanisms of early HCV infection are largely unknown, especially how HCV infects hepatocytes in liver. Attempts to elucidate these early events have been hampered by the difficulties to obtain sufficient amounts of free virions from either plasma of infected individuals or *in vitro* systems for virus propagation. Nevertheless, it is generally accepted that HCV envelope glycoproteins E1 and E2, as with other enveloped viruses, may play a major role in virus binding and entry into target cells. Indeed, several putative HCV receptors have been identified that interact with the HCV envelope glycoproteins, such as CD81 [18], the scavenger receptor class B type I (SR-B1) [19], and the asialoglycoprotein receptor (ASGP-R) [20]. Recently, it was demonstrated that the C-type lectins DC-SIGN and L-SIGN/DC-SIGNR may be involved in HCV binding through their interaction with the HCV envelope glycoprotein E2 [21-23].

DC-SIGN is specifically expressed on dendritic cells (DCs) [24;25], and plays a key-role in the dissemination of HIV-1 by DCs through HIV-1 gp120 binding [26]. Recent studies have demonstrated that DC-SIGN also functions as a receptor for other pathogens, including cytomegalovirus (CMV) [27], Ebola virus [28], and *Mycobacterium tuberculosis* [29]. It is becoming clear that other pathogens besides HIV-1 target DC-SIGN to promote their survival and, similarly, HCV binding to DC-SIGN may not only promote HCV dissemination but may also modulate DC function necessary for establishing chronic infections. Indeed, it has been shown that chronic HCV infection impairs DC maturation as well as their immune stimulatory function [30;31]. Thus, DCs may be a target for HCV to escape immune surveillance and knowledge about the interaction of DCs with HCV is essential to fully understand and combat HCV infections.

The liver homologue of DC-SIGN, L-SIGN is specifically expressed by liver sinusoidal endothelial cells (LSECs) [32;33], a specialized endothelial cell type with antigen presenting cell (APC) function [34]. L-SIGN binds HIV-1 gp120 comparable to DC-SIGN, and may be involved in HIV-1 transmission to T cells [32]. Similarly, L-SIGN expressed by LSECs may

capture HCV from blood and mediate infection of adjacent hepatocytes, the main target cells for HCV.
In order to unravel the interaction of HCV with both C-type lectins DC-SIGN and L-SIGN and their role in virus dissemination, we investigated the interaction of both C-type lectins with virus-like particles (VLPs) consisting of either the HCV glycoproteins E1 or E2 alone, or in a E1/E2 heterocomplex, as a suitable surrogate for native HCV particles. We demonstrate that the C-type lectins DC-SIGN and L-SIGN interact similarly with both glycoproteins E1 and E2 and that the HCV glycoproteins occupy the same binding pocket in DC-SIGN as HIV-1 gp120 and mycobacterial ManLAM. Both immature and mature DCs strongly bind the VLPs of E1 and E2 and this interaction is primarily mediated by DC-SIGN. Moreover, VLPs are rapidly internalized by DC-SIGN on immature DCs and targeted to EEA-1-positive early endosomes, where the VLPs are protected from degradation. In contrast, Lewis^x (Le^x)-antigens are internalized by DC-SIGN and targeted to the lysosomes for destruction. Furthermore, we demonstrate that LSECs efficiently capture HCV particles *in situ* through L-SIGN. L-SIGN rapidly internalizes HCV into early endosomes. Our data suggest that HCV may target DC-SIGN and L-SIGN to hide within DCs and LSECs respectively.

Materials and Methods

Antibodies and proteins.
The following antibodies were used: mouse anti-MR (Clone 19, BD Pharmingen, San Diego, CA, USA), DC-SIGN-specific mouse antibody AZN-D1 [24], DC-SIGN- and L-SIGN-specific antibody AZN-D2 [26], mouse anti-L-SIGN (R&D Systems, Minneapolis, MN, USA), mouse anti-LAMP-1 (H4A3, BD Pharmingen, San Diego, CA, USA), mouse anti-EEA-1 (BD Pharmingen, San Diego, CA, USA), human and mouse anti-HCV E1 (1C4 and 23C12 respectively, Innogenetics, Ghent, Belgium) and mouse anti-E2 antibody (4H6B2, Innogenetics, Ghent, Belgium), goat anti-human IgG1 conjugated with FITC and PO (Jackson Immunoresearch, West Grove, PA, USA), goat anti-mouse conjugated with FITC (Zymed Laboratories Inc., South San Francisco, CA, USA) goat anti-mouse conjugated with Alexa Fluor 488 and 594 and streptavidin Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA).
HIV-1 gp120 was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS (NIAID, NIH, USA: HIV-1 IIIB gp120).

HCV envelope glycoproteins.
HCV envelope glycoproteins E1 and E2 were expressed separately either in Vero cells by infection with recombinant vaccinia virus or in the yeast Hansenula polymorpha. Both glycoproteins were derived from the structural region of a genotype 1b isolate and lacked the C-terminal membrane anchor (E1: aa 192-326 and E2: aa 384-673). A C-terminal his-tag was added for purification purposes, with the exception of the E1 protein produced in Vero cells.
Despite the truncation of the membrane anchor all proteins were retained intracellularly and were thus purified from cell lysates. In brief, the envelope proteins as expressed in Vero cells were extracted by means of Triton X-100 and purified using lentil affinity chromatography resulting in an E1 or E2 enriched protein fraction consisting mainly out of aggregated material. These aggregates were reduced to monomeric proteins by incubation with DTT and Empigen BB. After blocking of the free cysteines with iodoacetamide the monomeric E1 protein was recovered by means of size exclusion chromatography in the presence of Empigen BB. For the E2 protein both a size exclusion chromatography and a nickel metal affinity chromatography were required in order to obtain pure E2 monomers. In case of expression in yeast, the cells were lysed by means of GuHCl and sulfonation. The recovered proteins were purified by nickel metal affinity chromatography in the presence of Empigen BB. Finally, the proteins were reduced by incubation with DTT and free cysteines blocked with iodoacetamide.

Vero cell or yeast cell derived monomeric E1, E2 or equimolar mixtures of E1 and E2 were subjected to an additional size exclusion chromatography in the presence of betain. This step allowed to exchange the detergent Empigen resulting in formation of particles with a size ranging from 10-100 nm.

Cells.
Immature DCs were cultured as described before [35]. In short, human blood monocytes were isolated from buffy coats by a Ficoll and a 52% Percoll gradient, and subsequent an adherence step. Adhered monocytes were differentiated into immature DCs in the presence of IL-4 and GM-CSF (500 and 800 U/ml, respectively; Schering-Plough, Brussels, Belgium). At day 6 the phenotype of the cultured DCs was confirmed by flow cytometric analysis. The DCs expressed high levels of MHC class I and II, CD11b, CD11c and ICAM-1, and low levels of CD80 and CD86. DCs were matured by adding lipopolysaccharide (LPS, 2 µg/ml, *S. typhosa*; Sigma Aldrich, St. Louis, MO) for 2 days. Maturation was verified by analysis of the expression of CD80, CD83 and CD86.
Stable THP-1 and K562 transfectants expressing wild-type L-SIGN, DC-SIGN, or DC-SIGN mutants [36], were generated as previously described [26].

Soluble DC-SIGN-Fc binding ELISA.
The DC-SIGN-Fc chimera [36] contained the extracellular portion of DC-SIGN (amino acid residues 64-404) fused at the C-terminus to a human IgG1-Fc fragment. The soluble DC-SIGN-Fc binding assay was performed as previously described [36]. Soluble ligands were coated on ELISA plates (maxisorb plate NUNC, 0.25 µg/well unless indicated) overnight at 4°C, followed by blocking with 1% BSA for 30 minutes at 37°C. Soluble DC-SIGN-Fc (5 µg/ml) was added for 2 hours at 37°C, and binding was determined by anti-IgG1 ELISA. Specificity was determined (unless indicated) in the presence of 50 µg/ml blocking antibodies, 100 µg/ml mannan or 10 mM EGTA.

Fluorescent bead adhesion assay.
Binding of ligand-coated beads to cells was done as described by Geijtenbeek et al. [24]. In short, streptavidin was covalently coupled to the carboxylate-modified TransFluorSpheres (488/645 nm, 1.0 µm; Molecular Probes, Eugene, OR, USA). The streptavidin-coated beads were incubated with biotinylated $F(ab')_2$ fragment goat anti-mouse IgG (6 µg/ml; Jackson Immunoresearch), followed by an overnight incubation with mouse-anti-HCV E1 or E2 antibody (23C12 or 4H6B2) at 4°C. The beads were washed and incubated with 250 ng/ml purified HCV envelope glycoproteins E1 or E2 overnight at 4°C. The fluorescent beads adhesion assay was performed as described before [37]. In short, 50,000 cells were incubated with beads for 45 minutes at 37°C. Mannan, EGTA and blocking antibodies against DC-SIGN and L-SIGN were used to determine specificity of adhesion. Binding was measured by FACS analysis.

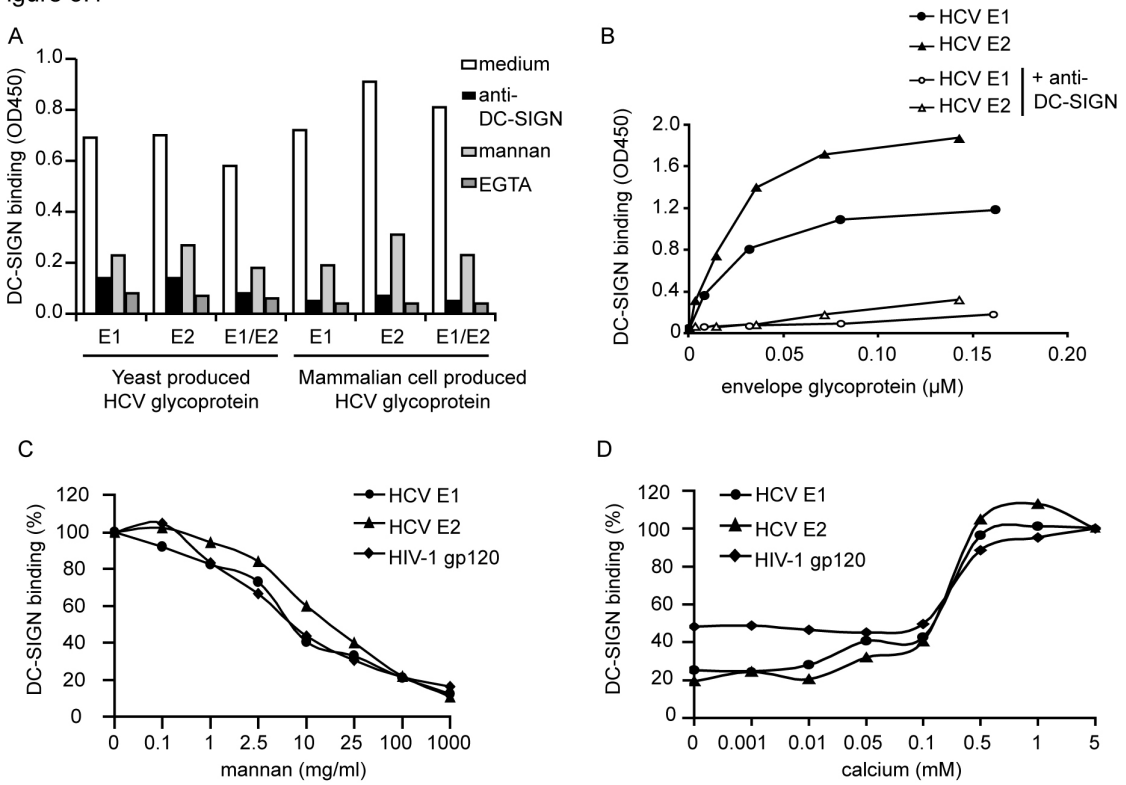
DC activation.
Immature DCs (120,000 cells/ml) were cultured in the presence of IL-4 (500 U/ml; Schering-Plough) and GM-CSF (800 U/ml; Schering-Plough). The effect of HCV on activation was determined by incubating immature DCs (day 6) with HCV-VLPs (12.5 µg/ml) in absence or presence of LPS (10 ng/ml) for 48 h at 37°C. Activation was determined by cell-surface expression of the costimulatory molecules CD83, and CD86 using PE-conjugated antibodies.

Immunofluorescence microscopy.
Cells were incubated 4 hours or overnight at 37°C with HCV E1/E2 (30 µg/ml) or biotinylated Le^x antigen (10 µg/ml). The transferrin receptor was detected by incubating the cells with Alexa Fluor 594-conjugated transferrin (10 µg/ml, Molecular Probes, Eugene, OR, USA) for 15 minutes at 37°C prior to fixation. Labelled cells were fixed in 3% paraformaldehyde in PBS for 15 minutes and permeabilized in PBS/0.1% saponin prior to staining. Cells were stained in PBS/0.5% BSA with antibodies against HCV envelope glycoprotein E1 or E2, and against LAMP-1, EEA-1 or DC-SIGN, and subsequently with FITC- or Alexa Fluor 488/594-conjugated secondary antibodies, or with streptavidin-Alexa Fluor 488. Next, cells were allowed to adhere to poly-L-lysine coated glass slides and mounted in anti-bleach reagent. Fixed slides were either imaged with a ZEISS Axiovert 200 Marianas™ inverted microscope (Intelligent Imaging Innovations, Denver, CO, USA) equipped with a motorized stage (stepper-motor z-axis increments: 0.1 µm), multiple fluorescence as well as brightfield channels, and a cooled CCD camera

[Cooke Sensicam (Cooke, Tonawanda, NY), 1280 x 1024 pixels] with true 16-bit capability at 40X objective, or they were examined with a Nikon Eclipse E800 fluorescence microscope and pictures were captured with a digital NIKON DXM1200 camera at 40X objective. In both cases images were acquired in 3 independent series/sessions. Pictures were analyzed with SlideBook™ 4 digital microscopy software (Intelligent Imaging Innovations, Denver, CO) or Jasc Paint Shop Pro™ software.

Liver section staining.
Human liver tissue was obtained from biopsies of therapeutic surgeon and cryofrozen. 8 µm sections were placed on gelatin-coated slides and stored at -80°C. The sections were incubated with HCV-VLPs (10 µg/ml) for 2 hours and stained with the mouse anti-HCV antibody 23C12 and an Alexa Fluor 594-conjugated secondary antibody or with the human anti-HCV antibody 1C4 and a FITC-conjugated secondary antibody. Binding of HCV envelope glycoprotein was blocked by incubation in the presence of 50 µg/ml AZN-D2. L-SIGN was detected by staining the liver sections with an anti-L-SIGN antibody and Alexa Fluor 594-conjugated secondary antibody. Sections were analyzed with a Nikon Eclipse E800 fluorescence microscope and pictures were captured with a digital NIKON DXM1200 camera at 20X objective.

Figure 3.1



DC-SIGN specifically binds HCV envelope glycoproteins E1 and E2.
(A) DC-SIGN binds both yeast Hansenula- and mammalian cell-produced HCV envelope glycoproteins. DC-SIGN interaction with HCV envelope glycoproteins E1 and E2 (0.25 µg/well), produced by either yeast Hansenula or mammalian cells using recombinant vaccinia virus, was determined in an Fc-based ELISA. Specificity of the binding was confirmed using the DC-SIGN specific blocking antibody AZN-D1 (50 µg/ml), mannan (100 µg/ml) and the calcium chelator EGTA (5 mM). (B) DC-SIGN binds stronger to HCV envelope glycoprotein E2 than to E1. HCV glycoproteins E1 and E2 were titrated (0-16 or 0-14 µM resp.) and DC-SIGN-Fc binding was determined as described above. DC-SIGN-specific antibody AZN-D1 (50 µg/ml) was used to specifically block the interaction. (C) DC-SIGN has a higher affinity for envelope glycoprotein E2 than for envelope glycoproteins E1 and gp120. Mammalian produced glycoprotein E1 and E2 (10 nM) and gp120 (2 nM) were coated and DC-SIGN-Fc binding was determined as described above. Mannan was titrated (0-1000 µg/ml) to block the interaction. Binding is represented as percentage of the maximal binding. (D) DC-SIGN binding to HCV envelope glycoproteins E1 and E2, and HIV-1 envelope gp120 is equally dependent on calcium. Viral envelope glycoproteins E1 and E2 (10 nM) and gp120 (2 nM) were coated and DC-SIGN-Fc binding was measured as described above in the presence of a varying of calcium (0-5 mM). Binding is represented as percentage of maximal binding at 5 mM calcium. Standard deviations are < 2%.

Results

DC-SIGN interacts with HCV envelope glycoproteins E1 and E2.

The interaction of DC-SIGN with HCV envelope glycoproteins was investigated using the DC-SIGN-Fc binding assay [36]. Purified HCV envelope glycoproteins E1 and E2 produced in either mammalian cells or in yeast Hansenula polymorpha are reconstituted into VLPs. Recombinant DC-SIGN-Fc specifically interacted with both mammalian- and yeast-produced HCV envelope glycoproteins E1, E2 and the mixed E1/E2 VLPs. This interaction could be blocked by the DC-SIGN-specific antibody AZN-D1, the calcium chelator EGTA, and the polycarbohydrate mannan (Fig. 3.1A). No difference in binding was observed between yeast and mammalian-produced envelope glycoproteins (Fig. 3.1A). Further experiments were performed with HCV envelope glycoproteins produced in mammalian cells. DC-SIGN bound stronger to glycoprotein E2 than to E1 (Fig. 3.1B), and the binding could be specifically blocked by anti-DC-SIGN antibodies (Fig. 3.1B). The interaction of DC-SIGN to HCV envelope glycoproteins E1 and E2 was further investigated by titration of mannan to block the interaction. Mannan could completely block the interaction of DC-SIGN with both E1 and E2 (Fig. 3.1C), although higher concentrations of mannan were necessary to block HCV E2 binding than to block HCV E1 and HIV-1 gp120 binding to DC-SIGN (Fig. 3.1C). This indicates that DC-SIGN has the same affinity for HCV glycoprotein E1 and HIV-1 gp120, and a higher affinity for HCV glycoprotein E2.

The calcium dependency of the DC-SIGN interaction with the HCV envelope glycoproteins was investigated. The interaction of DC-SIGN with HCV envelope glycoproteins E1, E2 and HIV-1 gp120 was equally dependent on calcium (Fig. 3.1D) with K_i50 values between 0.15 and 0.25 mM. At calcium concentrations lower than 0.1 mM the binding of these three ligands was almost completely abolished. These data demonstrate that DC-SIGN binds both HCV glycoprotein E1 and E2 similarly as HIV-1 gp120.

Table 3.1
Identification of ligand binding site of DC-SIGN for HCV and HIV-1 envelope glycoproteins

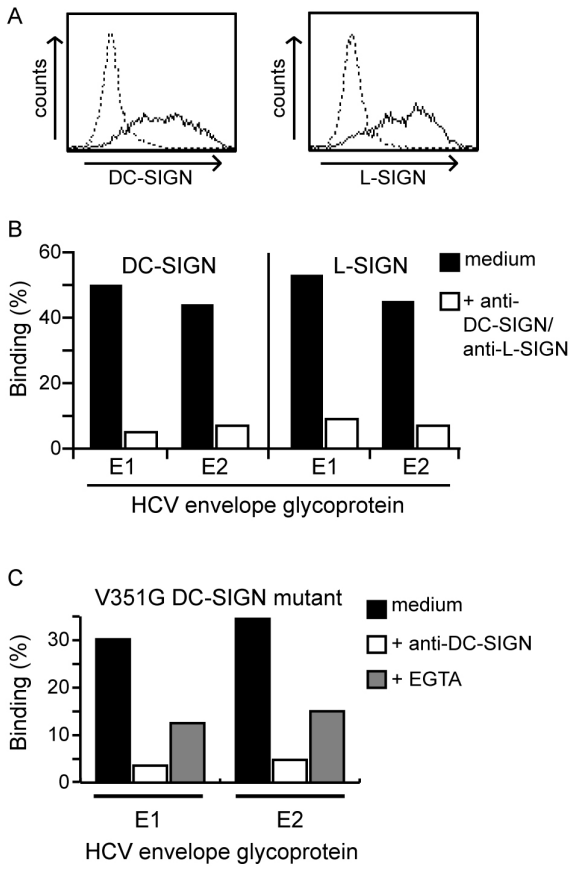
DC-SIGN expressed by K562 transfectant	fluorescent bead adhesion (%)		
	gp120	E1	E2
wild type DCSIGN	45	50	45
E346Q	4	3	7
N349D	0	5	9
N356D	0	5	9
D366A	3	4	5
D320A	7	4	6
E324A	0	4	7
N350A	1	4	8
D355A	2	4	8

Cellular DC-SIGN and L-SIGN bind to HCV envelope glycoproteins.

K562 cells stably expressing DC-SIGN and L-SIGN were used to investigate the binding of cellular DC-SIGN and L-SIGN to HCV envelope glycoproteins. The transfectants expressed similar high levels of DC-SIGN and L-SIGN (Fig. 3.2A). K562 transfectants expressing either DC-SIGN or L-SIGN strongly bound to both HCV envelope glycoproteins E1 and E2 (Fig.3.2B), whereas the mock-transfected K562 cells did not bind the HCV glycoproteins (data not shown). The observed binding of both DC-SIGN and L-SIGN to the HCV glycoproteins was comparable and could be completely blocked by antibodies against DC-SIGN and L-SIGN (Fig. 3.2B).

We used DC-SIGN mutants containing specific mutants in their ligand-binding site in order to investigate the binding site of DC-SIGN for HCV envelope glycoprotein E1 and E2 in more detail. The C-type lectin domain of DC-SIGN contains two calcium binding sites [24], site 1 positioned at the auxiliary site of DC-SIGN and site 2 located near the primary ligand binding site. The Ca^{2+} ion at site 1 coordinates the correct positioning of the loops forming primary binding site [36;38]. To investigate the role of this calcium ion, four mutations were generated. Mutating Asp^{320} (D320A), Glu^{324} (E324A), Asn^{350} (N350A) or Asp^{355} (D355A) into alanine resulted in the loss of calcium binding at site 1 and subsequent loss of binding to HCV envelope glycoproteins (Table 3.1), as was also previously shown for both ICAM-3 and HIV-1 gp120 [36]. Changing Asp^{366} that coordinates calcium binding at site 2 into an alanine residue (D366A), resulted in complete loss of binding to both HCV and HIV-1 glycoproteins (Table 3.1). The amino acid residues that are in close contact with the Ca^{2+} at site 2 (Glu^{347}, Asn^{349}, Glu^{354} and Asn^{365}) form the core of the ligand binding site [36]. Changing Glu^{347} (E347Q) into Gln, or Asn^{349} (N349D) and Asn^{365} (N365D) into Asp, also resulted in complete loss of binding to HCV

Figure 3.2

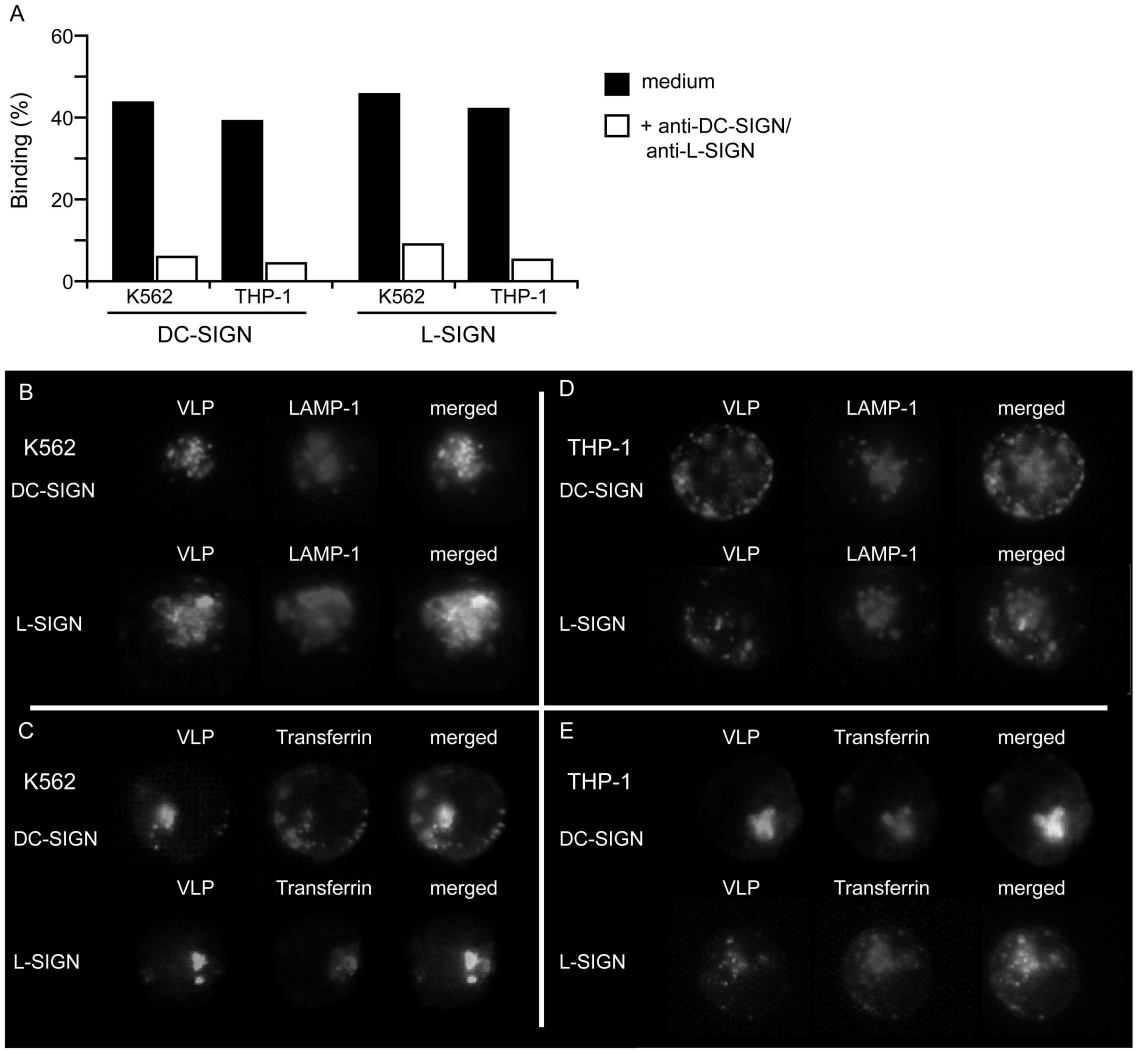


HCV envelope glycoproteins are bound by cellular DC-SIGN and L-SIGN.

(A) K562 transfectants express similar levels of DC-SIGN and L-SIGN. K562 cells were transfected with DC-SIGN or L-SIGN as described in materials and methods. Expression was measured by FACS-staining with the DC-SIGN and L-SIGN specific antibody AZN-D2. Dotted lines represent isotype control, black lines AZN-D2 staining. Mean fluorescence indices were >700. (B) Cellular DC-SIGN and L-SIGN bind to both envelope glycoproteins E1 and E2. HCV envelope glycoprotein binding by K562 cells expressing DC-SIGN or L-SIGN was measured with a fluorescent-coated bead adhesion assay. The L-SIGN and DC-SIGN-specific blocking antibody AZN-D2 (50 µg/ml) was used to determine the specificity of the interaction. (C) The DC-SIGN Val^{351} mutant binds both HCV envelope glycoproteins E1 and E2. K562 transfected with DC-SIGN V351G binding to glycoproteins E1 and E2 coated beads was investigated in the presence or absence of the DC-SIGN specific antibody AZN-D2 (50 µg/ml) and EGTA (10 µM). Standard deviations are < 5%.

envelope glycoproteins E1 and E2 and HIV-1 gp120 (Table 3.1).
Recently, we demonstrated that the binding site of DC-SIGN for its cellular ligand ICAM-3 is distinct from that of its pathogen ligands HIV-1 gp120 [36] and mycobacterial ManLAM [29], since a specific mutation in DC-SIGN (V351G) abrogated ICAM-3, but not HIV-1 gp120 and ManLAM binding. Interestingly, the DC-SIGN V351G mutant also interacts with HCV E1 as well as E2 (Fig. 3.2C), demonstrating that both HIV-1 and HCV bind similarly to DC-SIGN at a distinct site from the cellular ligand ICAM-3.

Figure 3.3



Internalization pathway of DC-SIGN and L-SIGN is dependent on cellular background.
(A) DC-SIGN and L-SIGN expressed by K562 and THP-1 transfectants bind HCV-VLPs similarly. Binding of HCV-E1/E2-VLPs to K562 and THP-1 transfectants with both DC-SIGN and L-SIGN was measured. Interaction was blocked by the DC-SIGN and L-SIGN specific antibody AZN-D2 (50 µg/ml). Standard deviations are <5%. (B-E) DC-SIGN- or L-SIGN-bound HCV is targeted to the early endosomes (transferrin^{+}) in THP-1 transfectants, in contrast to the lysosomal (LAMP-1^{+}) targeting in K562 transfectants. K-562 (B, C) and THP-1 cells (D, E) expressing DC-SIGN or L-SIGN were incubated overnight with HCV-VLPs. HCV was detected using a human anti-HCV antibody and a FITC-labelled secondary antibody. Intracellular targeting was determined by staining the endosomal compartments with a mouse antibody against the lysosomal/late endosomal specific LAMP-1 and Alexa Fluor 594 labelled secondary antibody (B, D), or by co-incubating the cells for 15 minutes with Alexa Fluor 594 labelled transferrin which is specifically transported to the early endosomes (C, E). Cells were analyzed by fluorescence microscopy.

HCV virus-like particles are internalized by DC-SIGN and L-SIGN transfectants.

DC-SIGN can function as an antigen receptor internalizing antigens [39]. Using a DC-SIGN specific antibody as an antigen, it was shown that DC-SIGN rapidly targets the antigen to lysosomal compartments for degradation and subsequent presentation on MHC class II [39]. In order to investigate the internalization and intracellular targeting of HCV-VLPs after capture by both DC-SIGN and L-SIGN, we used stable transfectants of the erythroleukemic cell line K562 and the monocytic cell line THP-1. Both DC-SIGN- and L-SIGN-transfected K562 and THP-1 cells efficiently bound HCV-E1/E2-VLPs (Fig. 3.3A).

Transfectants were incubated overnight with E1/E2-VLPs. Intracellular compartments were subsequently stained with an antibody against LAMP-1, a marker for lysosomal/late endosomal vesicles, and with transferrin, which accumulates in the early endosomes through transferrin receptor uptake [40]. Cells were analyzed by immunofluorescence microscopy. In all transfectants, HCV-VLPs were rapidly internalized by DC-SIGN and L-SIGN and targeted to the endosomal pathway (Fig. 3.3B). Remarkably, our results demonstrate that the internalization pathway of both DC-SIGN and L-SIGN is dependent on the cell type. Both DC-SIGN and L-SIGN expressed by K562 cells target HCV-E1/E2-VLPs to the LAMP-1-positive compartments (Fig. 3.3B), and not to the transferrin-positive early endosomes (Fig. 3.3C). In contrast, HCV-VLPs internalized by DC-SIGN and L-SIGN expressed by THP-1 cells are not targeted to the lysosomes. In these cells HCV-VLPs reside not within LAMP-1 positive compartments (Fig. 3.3D) but in transferrin-positive vesicles (Fig. 3.3E).

The internalization was specific for both DC-SIGN and L-SIGN, since both mock-transfected K562 and THP-1 cells did not internalize HCV-VLPs (data not shown). Thus, both DC-SIGN and L-SIGN efficiently internalize HCV-VLPs, but the internalization pathway is dependent on the cell type, suggesting that cellular origin is important. Therefore we investigated antigen uptake with DC-SIGN on primary cells.

DC-SIGN on dendritic cells targets HCV to early endosomes.

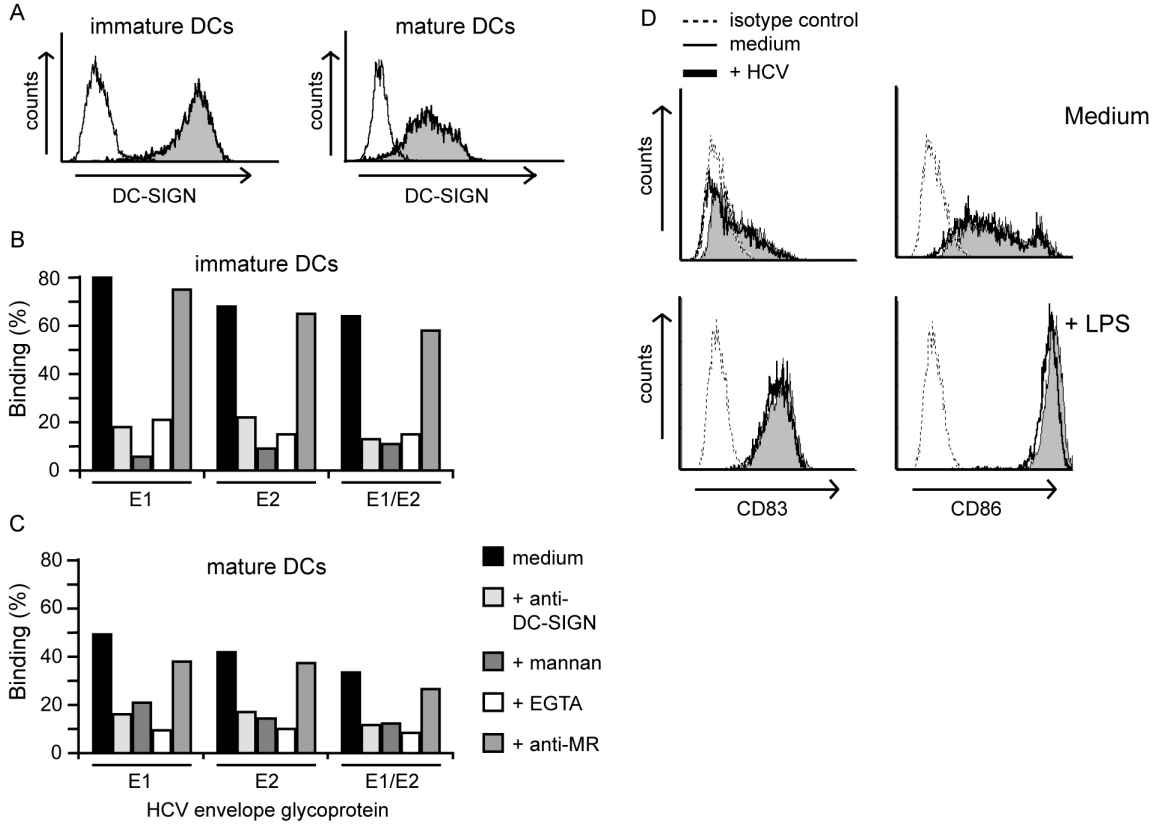
DCs express high levels of various pathogen receptors such as DC-SIGN (Fig. 3.4A) and the mannose receptor (MR) [39]. We investigated the interaction of immature DCs with the different HCV envelope glycoproteins. The contribution of DC-SIGN on immature DCs in the binding to HCV envelope glycoproteins was investigated using specific blocking antibodies. Immature DCs showed a strong binding to HCV E1, E2 and E1/E2-VLPs. This binding could be blocked by a specific anti-DC-SIGN antibody, mannan, and EGTA, but not by an antibody against MR (Fig. 3.4B), demonstrating that DC-SIGN is the major receptor for HCV envelope glycoproteins on immature DCs.

Moreover, mature DCs also captured the different HCV envelope glycoproteins, and this could be blocked by mannan, EGTA and an antibody against DC-SIGN but not by an MR-specific antibody (Fig. 3.4C). This indicates that also on mature DCs, DC-SIGN is the major receptor for HCV envelope glycoproteins. However, this binding was less strong than the binding by immature DCs, due to down regulation of DC-SIGN expression (Fig. 3.4A).

Next, we investigated the effect of HCV on DC activation/maturation. Activation was determined by measuring the expression levels of the maturation markers CD83 and CD86. Immature DCs were incubated with HCV-VLPs for 48 hours. Both CD83 and CD86 expression did not change compared to DCs incubated without HCV (Fig. 3.4D), demonstrating that the HCV-VLPs do not induce maturation. We have demonstrated that mycobacterial lipoarabinomannan binding to DC-SIGN prevents DC activation [29]. In contrast, HCV had no effect on LPS-induced DC activation (Fig. 3.4D). This supports recent data showing that DCs function normally in chronic HCV infected patients [41], suggesting that HCV employs a

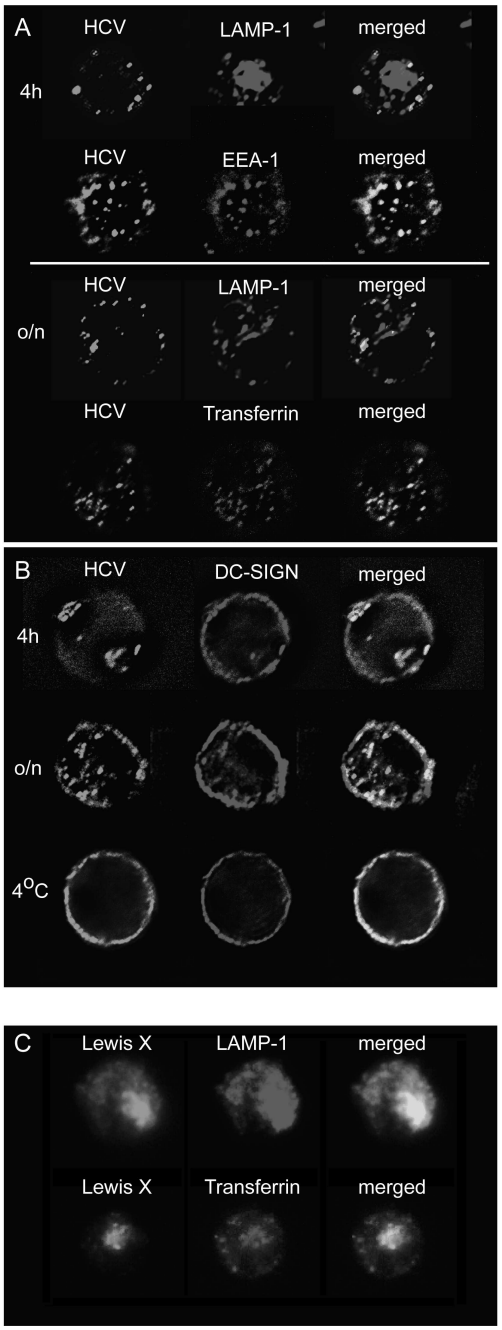
different way to evade DC immunity. Therefore, the fate of HCV-VLPs upon binding by immature DCs was investigated. Immature DCs were incubated with HCV-VLPs for 4h or overnight and co-localization with the lysosomal marker LAMP-1 or early endosomal markers EEA-1 [42] and transferrin was analyzed. After 4 hours, colocalization of the HCV-VLPs was observed with transferrin positive but not with LAMP-1 positive vesicles. Even after 24 hours, HCV was still primarily present in the EEA-1 positive vesicles (Fig. 3.5A), similar as was observed for THP-1 transfectants (Fig. 3.3D and E). HCV-VLP internalization was DC-SIGN dependent, since blocking of DC-SIGN with a DC-SIGN antibody completely abolished internalization of HCV-VLPs (data not shown). Subsequently, DC-SIGN co-localization with HCV-VLPs after incubation was investigated. After incubation 4°C, both HCV-VLPs and DC-SIGN were localized at the cellsurface of the DCs (Fig. 3.5B). When incubated at 37°C for 4 hours to allow internalization, internalized HCV-VLPs co-localized with DC-SIGN (Fig. 3.5B).

Figure 3.4



Dendritic cells strongly bind to HCV glycoprotein E1 and E2 through DC-SIGN.
(A) Immature DCs express high levels of DC-SIGN. LPS-matured DCs express lower levels of DC-SIGN. Monocyte-derived dendritic cells were isolated as described in materials and methods. Expression of DC-SIGN was measured by FACS-staining with the DC-SIGN specific antibody AZN-D2. Open histograms represent isotype control, filled histograms AZN-D2 staining. (B, C) Immature DCs and mature DCs bind strongly to HCV envelope glycoproteins E1 and E2, and mixed HCV E1/E2-VLPs via DC-SIGN. Immature (B) and LPS-matured DCs (C) binding to HCV envelope glycoproteins was determined by a fluorescent bead adhesion assay. Specificity was determined by anti-DC-SIGN antibody AZN-D2 (50 µg/ml), mannan (100 µg/ml), EGTA (10 µM), and anti-mannose receptor antibody (clone 19) (50 µg/ml). Standard deviations are < 5%. (D) HCV-VLPs do not affect DC activation/maturation. Immature DCs were incubated with HCV (12.5 µg/ml) alone, LPS (10 µg/ml) alone, or HCV and LPS together for 48 h and activation was determined by measuring the expression of CD83 and CD86. Dotted lines represent isotype controls, filed histograms with thin lines represent incubations without HCV, open histograms with thick lines represent incubations without or with LPS in combination with HCV-VLPs. Upper panel are incubations without LPS, lower panel are incubations with LPS. One representative experiment out of three is shown.

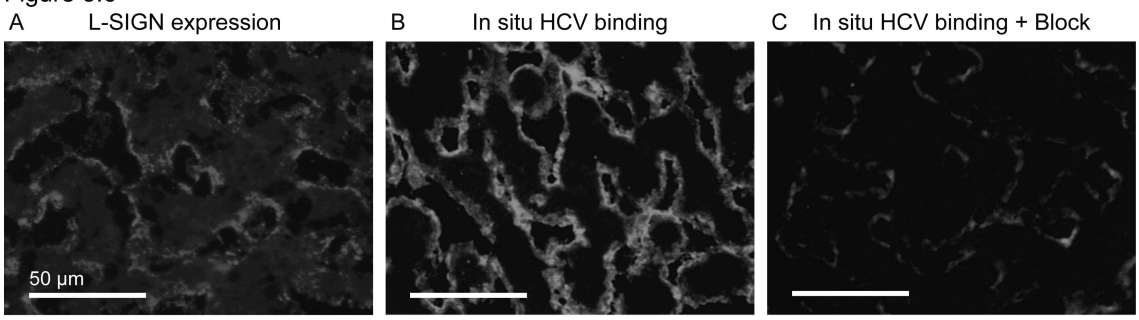
Figure 3.5



DC-SIGN on immature DCs targets HCV-VLPs to early endosomes, but Lewis^x antigen to lysosomes. (A) Immature DCs were incubated with HCV-VLPs (30 µg/ml) for 4 hours or overnight. HCV was detected with a human anti-HCV antibody and a FITC-labelled secondary antibody. Intracellular targeting was determined by staining the endosomal compartments with a mouse antibody against the lysosomal/late endosomal specific LAMP-1 or the early endosome specific marker EEA-1 and an Alexa Fluor 594 labelled secondary antibody, or by co-incubating the cells for 15 minutes with Alexa Fluor 594 labelled transferrin which is specifically transported to the early endosomes. (B) Immature DCs were incubated with HCV-VLPs (30 µg/ml) for 4 hours or overnight at 37 or 4°C. HCV was detected as described for figure 3.5A. Localization of DC-SIGN was determined with the DC-SIGN specific antibody AZN-D2 and an Alexa Fluor 594 labelled secondary antibody. Cells were analyzed on a 3i Marianas^{TM} digital imaging microscopy workstation and SlideBook^{TM} software. (C) DC-SIGN on immature DCs targets its ligand Lewis^x antigen to late endosomes/lysosomes. Immature dendritic cells were incubated with Lewis^x (10 µg/ml) for 4 hours. Intracellular targeting was determined by staining the endosomal compartments with a mouse antibody against the lysosomal/late endosomal specific LAMP-1 or by co-incubating the cells for 15 minutes with Alexa Fluor 594 labelled transferrin which is specifically transported to the early endosomes. Cells were analyzed by fluorescence microscopy.

Even after prolonged incubation (24 hours), DC-SIGN co-localized with HCV-VLPs, suggesting DC-SIGN has, besides a role in internalization, a role in intracellular routing of HCV-VLPs.
Strikingly, the internalization pathway of DC-SIGN on immature DCs is dependent on the ligand, since the carbohydrate blood-group antigen Lewis^x and mycobacterial ManLAM were efficiently captured and internalized by immature DCs through DC-SIGN, and targeted to transferrin negative, LAMP-1-positive lysosomes (Fig. 3.5C and [29]) but not on ligand-binding site as ManLAM is binding the same site as HCV E1 and E2 and gp120. Together, these data suggest that HCV targets DC-SIGN to enter DCs and escape degradation by preventing targeting to the lysosomes.

Figure 3.6



HCV interacts with L-SIGN expressing LSECs *in situ.*
(A) L-SIGN is expressed by human liver sinusoidal endothelial cells, as was determined by staining of liver tissue with an L-SIGN specific antibody. (B) Binding of HCV-VLPs by liver tissue was determined by incubating liver sections with HCV-VLPs (10 µg/ml) for 2 hours at 37°C. HCV binding was detected using a human anti-HCV antibody and FITC labelled secondary antibody. (C) HCV-VLP binding to LSECs is specifically blocked by the L-SIGN-specific antibody AZN-D2. Sections were pre-incubated with AZN-D2 (50 µg/ml) for 30 minutes at room temperature before adding HCV-VLPs as described in A. Sections were analyzed with fluorescence microscopy with a 20X objective. Bars are 50 µM.

HCV interact with LSECs in situ.

In the liver hepatocytes are the main target of HCV and although putative HCV receptors have been identified, it is unclear how HCV infects the hepatocytes. We have shown that cell lines transfected with L-SIGN can interact with HCV envelope glycoproteins (Fig. 3.2). L-SIGN is expressed on LSECs [32;33], the liver sinusoidal endothelial cells that are in close contact with the blood and could capture HCV from the blood and transmit it to the hepatocytes. However, these cells are difficult to isolate. Therefore we used an *in situ* binding assay to investigate which are the major HCV binding cells in the liver.

L-SIGN was stained in liver tissue sections with an anti-L-SIGN antibody, and analyzed by immunofluorescence microscopy (Fig. 3.6A). HCV envelope glycoprotein binding *in situ* was investigated by incubating the liver sections with HCV-VLPs. VLPs were detected with anti-HCV E1 antibody and fluorescent-labelled secondary antibody (Fig. 3.6B). The staining pattern of HCV-VLPs was similar to the L-SIGN staining in the sections, suggesting that the HCV binds to the L-SIGN expressing LSECs. Moreover, the HCV binding to LSEC was specifically blocked by a blocking antibody against L-SIGN (Fig. 3.6C), demonstrating that this binding was mediated by the C-type lectin L-SIGN. Strikingly, HCV-VLPs interacted only with the LSECs and not with hepatocytes that express the putative HCV receptor ASGP-R and are the main target cells for HCV. These data suggest that HCV is captured by L-SIGN that mediates internalization of HCV, and thus LSECs act as a HCV reservoir and may transmit the virus to hepatocytes.

Discussion

Our results demonstrate that HCV is efficiently captured by DCs through DC-SIGN and by LSECs through L-SIGN. The C-type lectins interact with both envelope glycoproteins E1 and E2. Strikingly, internalized HCV-VLPs are targeted by DC-SIGN on immature DCs to early endosomal compartments, where HCV is protected from degradation. Similarly, HCV captured by L-SIGN expressed on THP-1 is targeted to early endosomal compartments. In contrast, another ligand of DC-SIGN, Lewis X blood-group antigen, is targeted to the lysosomes. Thus,

HCV may target DC-SIGN and L-SIGN to use DCs and LSECs respectively as reservoirs to facilitate viral dissemination.

Because of the lack of a suitable cell culture system for *in vitro* propagation of HCV and the unavailability of virions in sufficient quantities, truncated and secreted versions of the HCV envelope glycoproteins have been used as soluble surrogates for native virus particles. Here, we have used VLPs of the HCV envelope glycoproteins E1 and E2. The envelope glycoproteins E1 and E2 were produced in either yeast Hansenula or mammalian cells using a recombinant vaccinia virus, and were assembled into a VLP of noncovalently linked E1 or E2 homomers or E1/E2 heteromers. We have used these purified HCV-VLPs to investigate their interaction with both DC-SIGN and L-SIGN.

Both cellular DC-SIGN and L-SIGN bind strongly to the envelope glycoproteins E1 and E2 alone as well as to the E1/E2 heterodimer particles (Fig. 3.2). The interactions were specifically inhibited by blocking antibodies against DC-SIGN and L-SIGN. Recently, it was shown that both DC-SIGN and L-SIGN interact with the HCV envelope glycoprotein E2 [21-23] and envelope glycoprotein E1 [23]. Here we demonstrate that DC-SIGN bound stronger to envelope glycoprotein E2 than to glycoprotein E1. Lozach et al. [22] demonstrated that the interaction of DC-SIGN with glycoprotein E2 is dependent on N-linked glycosylations. HCV envelope glycoprotein E2 has 11 potential N-linked glycosylation sites where envelope glycoprotein E1 has only 6 sites [13] what can explain the stronger binding of DC-SIGN to HCV envelope glycoprotein E2. Furthermore, we demonstrate that the interaction of DC-SIGN with HCV envelope glycoproteins E1 and E2 and HIV-1 envelope protein gp120 was equally dependent on calcium (Fig. 3.1E), suggesting that these viral envelope glycoproteins interact at the same binding site in DC-SIGN. Indeed site-directed mutagenesis demonstrates that both HCV glycoproteins E1 and E2 bind the primary ligand-binding site in DC-SIGN through coordination with the primary Ca^{2+}-ion at site 2 (Table 3.1). Moreover, HCV envelope glycoproteins E1 and E2, like the pathogenic ligands HIV-1 gp120 and mycobacterial ManLAM, bind to the V351G DC-SIGN mutant, whereas the cellular ligand ICAM-3 does not bind this mutant [29;36]. These observations further suggest that DC-SIGN may distinguish between different types of ligand and may tailor its responses specifically to the ligand it recognizes.

DC-SIGN functions as a more universal pathogen receptor [43] and the pathogens HIV-1 and *M. tuberculosis* target DC-SIGN to escape immune surveillance [26;29;44]. The interaction of the HCV envelope glycoproteins E1 and E2 with both DC-SIGN and L-SIGN, suggests that DC-SIGN and L-SIGN may be involved in the pathogenesis of HCV. Little is known about the dissemination of HCV and the role of the immune system in the pathogenesis of HCV is complex and largely unknown. It has been shown that HCV may affect DCs; during chronic HCV infection DCs are impaired in their immune function [30;31]. In contrast, a recent study demonstrated that monocyte-derived DCs from patients suffering from chronic HCV infection are functionally normal [41]. Here we demonstrate that immature DCs strongly bind to HCV envelope glycoproteins E1, E2 and E1/E2-VLPs through DC-SIGN, since the interactions were completely blocked by antibodies against DC-SIGN (Fig. 3.4B). Mature DCs also captured HCV E1, E2 and E1/E2-VLPs through DC-SIGN, although less efficiently as immature DCs (Fig. 3.4C). This may be due to the lower expression of DC-SIGN on mature DCs than on immature DCs (Fig. 3.4A) [23]. The complete inhibition of the interaction of both immature and mature DCs with HCV particles by antibodies against DC-SIGN demonstrates that DC-SIGN is the primary receptor for HCV on both immature and mature DCs, and not the HCV E2 receptor CD81 that is also expressed by DCs [23].

HCV-VLPs are rapidly internalized upon capture by both DC-SIGN and L-SIGN (Fig. 3.3). Depending on the cell type, the captured HCV-VLPs were targeted differently to intracellular compartments by both C-type lectins. In the cell line THP-1, captured HCV-VLPs were targeted to transferrin-positive non-lysosomal compartments, whereas VLPs captured by the erythroleukemic cell line K562, expressing either DC-SIGN or L-SIGN, were targeted to LAMP-1-positive lysosomal compartments (Fig. 3.3). These results demonstrate that there is a difference in internalization pathway between both cell lines. HCV-VLPs, captured by primary immature DCs through DC-SIGN, were targeted to early endosomal vesicles, where they reside for over 24 hours, similar to the THP-1 transfectants. This indicates that the internalization pathway observed in THP-1 transfectants may represent a more native situation as found in DCs. In addition, it was recently demonstrated that transmission of HIV-1 is also cell-type dependent [45]. DC-SIGN does not facilitate HIV-1 infection of DCs but protects the virus from degradation by internalizing the virus in non-lysosomal compartments [26;44]. The DC-SIGN-bound HIV-1 is efficiently transmitted to recipient $CD4^+$ T cells upon co-culture of DCs with $CD4^+$ T cells, which results in a productive infection of T cells [26]. Like DCs, THP-1 cells expressing DC-SIGN continued to infect T cells with HIV-1 for several days, whereas DC-SIGN expressing 293 cells were infective only for a short period. However, HIV-1 is not retained in early endosomal compartments as HCV, or in lysosomes, but in other, undefined, vesicles [44;45]. But that also HIV-1 is not targeted to the lysosomes and thus probably not being degraded in THP-1 cells and DCs, can explain why in these cells transmission is possible over a prolonged period. The internalization pathway of DC-SIGN-bound ligands is not only dependent on the cell type but also on the ligand, as another carbohydrate-containing ligand of DC-SIGN, $Lewis^x$ antigen, was rapidly internalized and targeted to the lysosomal compartment in both immature DCs and THP-1 transfectants (Fig. 3.5C, data not shown). Altogether, these results support a role for DC-SIGN in the capture of HCV virions by immature DCs similar to HIV-1, and show that THP-1 transfectants are a suitable model for pathogen internalization. A recent study suggests that the THP-1 cells used to study DC-SIGN/L-SIGN function have B-cell like characteristics [46]. Indeed, the THP-1 cells used in this study express CD19 (data not shown) and therefore may have B-cell like characteristics. Interestingly, B cells have a similar antigen-processing/presenting machinery as DCs and our results demonstrate that DC-SIGN expressed by these THP-1 cells behaves similar as on immature DCs. Therefore, THP-1 cells are a suitable model for studying pathogen internalization.

Hepatocytes are productively infected by HCV, and the hepatocyte specific ASGP-R has been proposed as a HCV receptor [20]. However, we demonstrate here that not the hepatocytes but the LSECs bind HCV-VLPs in primary liver tissue (Fig. 3.6), suggesting that LSECs capture HCV from blood and transfers HCV to hepatocytes. Moreover, L-SIGN has been shown to function, similar as DC-SIGN, in HIV-1 transmission [32], and L-SIGN expressed by LSECs, could capture HCV from blood and transfer it to hepatocytes. A similar mechanism was demonstrated for another hepatic virus Hepatitis B virus [47]. This virus enters LSECs and Breiner et al. [47] proposed that the virus travels via endosomal compartments to the hepatocytes that are subsequently infected. Our data further indicate that both LSECs and DCs may efficiently capture and protect the virus and function as HCV reservoir.

Our recent results suggest that mycobacteria target DC-SIGN by secreting ManLAM to downregulate DC-mediated immune responses and thus promote pathogen survival [29]. It is not clear whether viruses are able to suppress DC functions by binding DC-SIGN through a similar mechanism as mycobacteria. The immunosuppressive setting that characterizes infections with CMV and HIV-1, two viruses that interact with DC-SIGN, implies that such a mechanism of immuno-modulation may exist (reviewed in [43]). It was suggested that DCs

isolated from patients suffering from chronic HCV infections have an impaired maturation and immune-stimulatory function [30;31]. However, our data indicate that immature DCs are not affected by HCV-VLPs and furthermore, Longman et al. recently showed that DCs from chronic infected patients are not impaired in their immune function [41]. This suggests that HCV may target DC-SIGN to escape lysosomal degradation in DCs and thereby evade immunity. Although further investigations into the function of DC-SIGN and L-SIGN will be necessary to determine their importance in HCV infections, our results suggest that both DC-SIGN and L-SIGN may be potential targets for designing strategies to combat HCV infections.

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HCV immune complex formation favors antigen presentation after uptake by DC-SIGN and L-SIGN

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Abstract

HCV interacts with human dendritic cells (DCs) and liver sinusoidal endothelial cells (LSECs) through the C-type lectins DC-SIGN and L-SIGN, respectively. These antigen-presenting cells efficiently internalize HCV but the virus is retained in a non-lysosomal compartment, indicating that this prevents efficient antigen presentation and promotes viral transmission. Here we show that HCV virus like particles are also susceptible for degradation by DCs, and degradation is more efficient in the presence of HCV-specific antibodies. We demonstrate that degradation of the HCV-immune complexes leads to MHC class II presentation by DCs. Strikingly, this is dependent on DC-SIGN, suggesting that immune complexes captured by DC-SIGN are internalized into lysosomal compartments for antigen processing. Moreover, induction of DC maturation by HCV-VLPs on an aluminium carrier strongly enhances antigen presentation of immune complexes by DCs. Thus, antibody targeting and maturation-induction directs DC-SIGN-captured ligands into lysosomal compartments and efficient antigen presentation. Moreover, we demonstrate that L-SIGN captures HCV-immune complexes leading to antigen presentation to $CD4^+$ T cells, demonstrating that L-SIGN is an antigen receptor on LSECs. Human LSECs express both L-SIGN and MHC class II molecules and our data suggest that LSECs are important as antigen presentation cells of viral antigens in the liver.

Introduction

Hepatitis C virus (HCV) is a major health problem as about 3 percent of the world population is infected with this virus [1;2]. However, only in the late 80s the virus was cloned [3] and identified as the causative agent of non-A, non-B viral hepatitis. Infection with the hepatitis C virus results in chronic and persistent infection in 70% of the patients [4] and can lead to liver cirrhosis and end-stage liver disease [5;6]. IFN-α ribavirin based therapy is often ineffective and there is no vaccine available yet. Therefore, several vaccines, including dendritic cell based vaccines and recombinant HCV envelope based are under development.

Although viral persistence is high, HCV specific antibodies can be detected in every infected individual. However, the presence of these antibodies does not predict the outcome of the infection [7]. Furthermore, virus specific $CD4^+$ and $CD8^+$ T cells are found in HCV infected patients [8;9]. Nevertheless, these T cells are insufficient to combat infections, which is reflected in the high persistence. There are several reasons for T-cell failure during HCV infection: T-cell maturation can be impaired, or T-cell dysfunction can be caused more directly by super activation due to high antigen exposure (reviewed in [10]). Also the virus could produce T-cell suppressive factors. For example, HCV core protein interacts with the complement receptor gC1qR [11] and thereby inhibits T-cell proliferation.
There are also indications for impairment of dendritic cell (DC) function, which subsequently could result in an impaired T-cell response. DCs are potent T-cell stimulators; they capture antigens and process them for antigen presentation. However, in chronic HCV infection myeloid and plasmacytoid DC count is lower than in healthy individuals [12-15]. Maturation and immune stimulatory functions of these DCs are impaired, especially in inducing Th1 responses [12;13;16-18]. Also monocyte derived DCs of chronic HCV infected individuals are impaired in stimulating T cells [17-19]. In contrast, other studies demonstrated that monocyte-derived DCs [20;21], myeloid, and plasmacytoid DCs [14;22] from patients suffering from chronic HCV infection are functionally normal. Thus, more research is necessary to understand the role of DCs in the establishment of HCV infections.

HCV, a hepacivirus of the Flaviviridae family, is an enveloped positive-stranded RNA virus [3]. The HCV envelope is formed by two heavily N-glycosylated type I transmembrane envelope glycoproteins E1 (31 kD) and E2 (70 kD) [23-25], that are expressed as heterodimers on the virus membrane [25]. Although there are hyper variable loops present in envelope protein E2, most of the glycosylation sites are well conserved as glycosylation is important for proper folding and heterodimerisation of the proteins [26] and for entry of the virus [27].
HCV mainly infects hepatocytes, however it is becoming clear that also other cells, such as DCs, can be infected [28;29]. DCs express the C-type lectin receptor DC-specific ICAM-3 grabbing nonintegrin (DC-SIGN), a receptor that can bind HCV envelope proteins E1 and E2 [30-33]. This C-type lectin is the major HCV receptor on DCs and is involved in internalization of HCV and in retention of the virus in early endosomes [32]. Furthermore, it has been shown that cell lines expressing DC-SIGN can transmit virus to hepatoma cells [34]. Therefore, DCs could function as HCV reservoirs and be involved in transmission of HCV to other cells. HIV-1 is using DC-SIGN on DCs in a similar fashion to facilitate its dissemination to target cells [35].

However, DC-SIGN also functions as an antigen uptake receptor leading to MHC class II presentation [36].
Liver/lymph node SIGN (L-SIGN), the homologue of DC-SIGN specifically expressed on liver sinusoidal endothelial cells (LSECs) [37;38], also interacts with HCV envelope proteins [30-33]. LSECs are specialized endothelial cells with antigen-presenting cell (APC) characteristics [39]. These cells possess scavenger functions and in combination with Kupffer cells, the resident liver macrophages, they are responsible for clearance of various substances from portal blood. Furthermore, LSECs have shown to mediate transcytosis of virus to hepatocytes [40]. Although L-SIGN is expressed on APCs, a role of L-SIGN in antigen uptake for presentation has not been described yet.

We demonstrate here that the HCV receptors DC-SIGN and L-SIGN, expressed on APCs, can internalize HCV-E1-virus like particles (VLPs), resulting in antigen presentation to $CD4^+$ T cells, indicating that besides DCs, also LSECs can present HCV antigens. Furthermore, we demonstrate that HCV is targeted more efficiently to lysosomes for degradation in the presence of HCV specific antibodies. Finally, HCV-E1 on an aluminium carrier (HCV-E1-Alum) induces DC maturation and potentiates antigen presentation by DCs.

Materials and methods

Antibodies and proteins.
The following antibodies were used: AZN-D1 and AZN-D2 (mouse IgG1, anti-DC-SIGN and anti-DC-SIGN and L-SIGN respectively) [41;42]; DCN46.1 (mouse IgG2b, anti-DC-SIGN, BD); L19 (anti-CD18 mouse IgG1) [43], mouse anti-L-SIGN (R&D Systems, Minneapolis, MN, USA), human and mouse anti-HCV E1 (1C4 and 23C12 respectively, Innogenetics, Ghent, Belgium) and mouse anti-E2 antibody (4H6B2, Innogenetics, Ghent, Belgium), mouse anti-CD80-PE, anti-CDD86-PE, and anti-HLA-DR-PE (BD Pharmingen, San Diego, CA, USA), mouse anti-CD83-PE (Beckman Coulter) goat anti-human IgG1 conjugated with FITC and HRP (Jackson Immunoresearch, West Grove, PA, USA), goat anti-mouse conjugated with FITC (Zymed Laboratories Inc., South San Francisco, CA, USA) goat anti-mouse conjugated with Alexa Fluor® 488 and 594 and streptavidin Alexa Fluor® 488 (Molecular Probes, Eugene, OR, USA).

Cells.
Human blood monocytes were isolated as described before [44] from haplotyped buffy coats, obtained from blood bank donors. In short, PBMCs were isolated by a Ficoll gradient and monocytes were isolated from the PBMCs using anti-CD14 microbeads (Miltenyi Biotec, Auburn, CA). Immature DCs were generated by culturing monocytes in RPMI 1640/10% FCS in the presence of IL-4 (500 U/ml; Schering-Plough, Kenilworth, NJ, USA) and GM-CSF (800 U/ml; Schering-Plough) for 5-8 days. At day 6 the phenotype of the cultured DCs was confirmed by flow cytometric analysis. The DCs expressed high levels of MHC class I and II, CD11b, CD11c and ICAM-1, and low levels of CD80 and CD86.
HLA-DR1 typed immortalized B cells (Epstein Barr virus-transformed human B lymphocytes) were generated by lentiviral transduction with LV-DC-SIGN or L-SIGN (kind gift of Vincent Piquet). Transduction efficiency was tested by staining with AZN-D1 and AZN-D2 antibodies.
Hd7, a $CD4^+$ T cell clone that recognizes a peptide derived from mouse IgG1 antibody in HLA-DR0101/DQw1 [45], was maintained in RPMI supplemented with 5% human serum, penicillin, streptomycin, glutamine and 1000 U/ml recombinant human IL-2.

HCV envelope glycoproteins.
HCV E1 envelope glycoprotein was expressed in the yeast Hansenula polymorpha. The glycoprotein is derived from the structural region of a genotype 1b isolate and lacks the C-terminal membrane anchor (aa 192-326).
After lysis of the cells and sulphonation of the protein the monomeric E1 is recovered in the presence of the detergent Empigen® (Albright & Wilson Ltd, UK) from. Finally, the Empigen is exchanged for PBS-betain and the E1 assembles in to particles with a size ranging from 10-100 nm. HCV-E1/E2-VLPs were generated as described before [32].
For formulation on aluminium the E1 is formulated to Alhydrogel® (Superfos Biosector A/S, Denmark) in a ratio of 50 µg protein to 0.13% Al_2O_3.

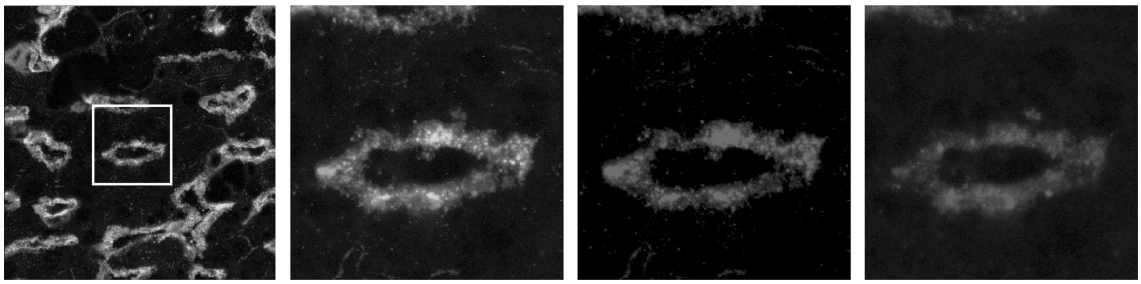
Antigen presentation.
Antigen-presenting cells (DCs or immortalized B cells, 10,000 cells/well) were pre-incubated with serial dilutions of antibodies or HCV-E1-VLP-antibody complexes for 2 hours at 37°C, washed and cocultured with 20,000 Hd7 T cells. After 24h, IFN-γ production was measured using a commercial human IFN-γ ELISA (cytoset, Biosource, Camarillo, CA, USA). Specificity of DC-SIGN and L-SIGN binding was determined by blocking with an anti-DC/L-SIGN mIgG2b antibody or mannan.

Maturation induction.
Immature DCs (day 5) were cultured with the indicated stimuli (10 µg/ml) with or without LPS (10 ng/ml) for 24h at 37°C. Maturation was measured by staining the cells for CD80, CD83 and CD86 expression using PE-conjugated antibodies.

Liver section staining.
Human liver tissue was obtained from biopsies of therapeutic surgeon and cryofrozen. 4 µm sections were placed on gelatine-coated slides and stored at -80°C. Sections were fixed with acetone. Sections were stained with anti-L-SIGN antibody and anti-HLA-DR antibody. As secondary antibodies, rabbit-anti-mouse IgG2b, goat anti rabbit Alexa Fluor® 488, and goat anti mouse IgG1-Alexa Fluor® 594 were used. Microscopical analysis was performed with a Leica TCS SP2 AOBS confocal laser-scanning microscope (Leica Microsystems, Heidelberg, Germany).

HCV degradation.
Dendritic cells were incubated with HCV-E1/E2-VLPs with or without anti-HCV envelope antibodies at room temperature for 45 minutes. The cells were washed and placed at 37°C. At the designated time points, samples were taken and cells were lysed in lysis buffer. HCV content in the samples was determined using a sandwich ELISA. Human anti-HCV-E1 antibodies (1C4, 2 µg/ml) were coated on a NUNC-maxisorb plate, and the plate was incubated with the cell lysates. HCV-E1/E2-VLPs were detected with a mouse anti-E2 antibody (4H6B2). HCV-E1/E2-VLP content was quantified using a standard series dilution.

Figure 4.1



L-SIGN is, like DC-SIGN, expressed on antigen-presenting cells.
L-SIGN and HLA-DR are co expressed on liver sinusoidal endothelial cells. Cryosections of liver tissue were stained with anti-L-SIGN (green) and anti-HLA-DR (red). Sections were analyzed with confocal microscopy. Original magnification 63x.

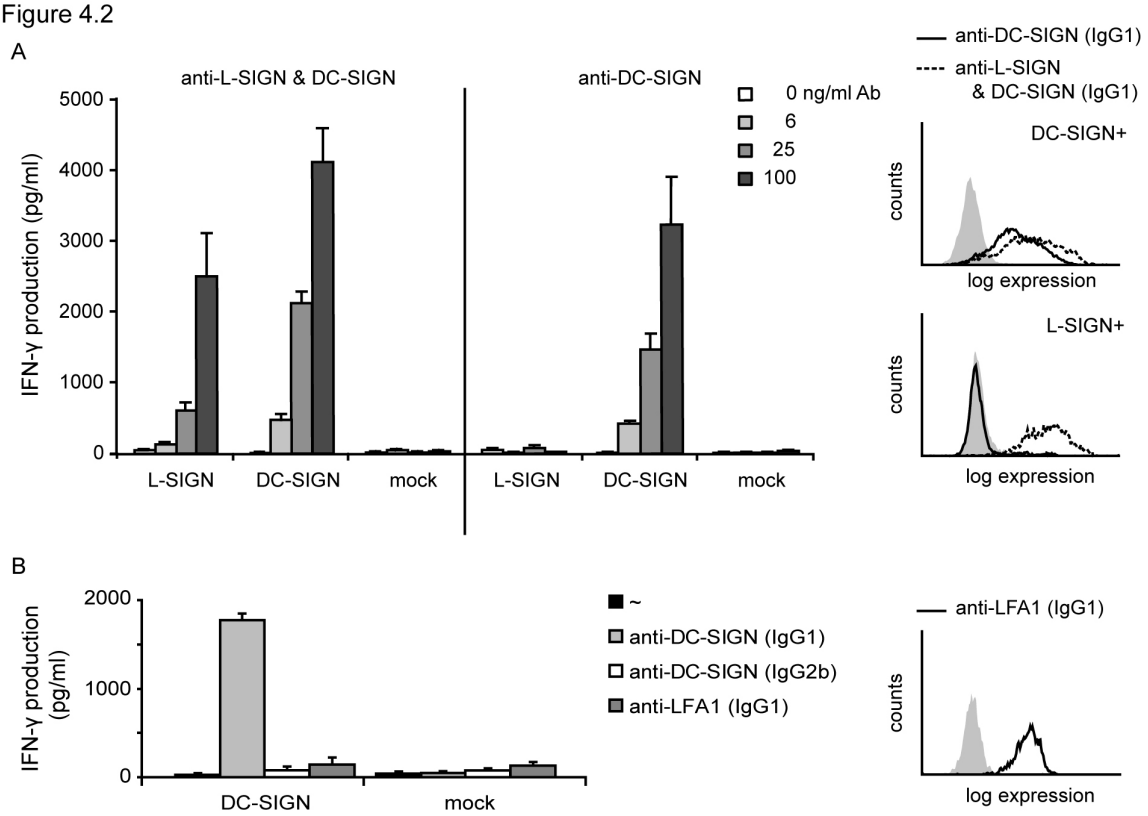
Results

L-SIGN is expressed on antigen-presenting cells.
DC-SIGN is specifically expressed on DCs, and shown to function as an antigen receptor. Its liver specific homologue L-SIGN is expressed on specialized endothelium in liver sinusoids [37;38]. L-SIGN-positive LSEC in human, in contrast to conventional endothelium, constitutively express HLA-DR, needed for antigen presentation to $CD4^+$ T cells (Fig. 4.1).
Human LSEC also express CD40, however they lack CD80 and CD86 [46]. This indicates that in human liver, L-SIGN is expressed on cells with antigen presenting cell characteristics, but lacking some costimulatory molecules, indicating that these cells could present antigen to T cells, but T-cell activation might be erroneous.

L-SIGN can mediate uptake and presentation of antigens to $CD4^+$ T cells.
As L-SIGN is expressed on antigen presenting cells, we investigated antigen uptake and presentation by L-SIGN. In order to investigate antigen presentation of L-SIGN ligands, immortalized B cells transduced with L-SIGN were incubated with anti-L-SIGN antibody (AZN-D2, IgG1) as a ligand and cocultured with the mouse IgG1 specific human $CD4^+$ T-cell clone, Hd7. The antibody to L-SIGN was internalized, processed and presented to T cells, as the T cells were activated, measured by IFN-γ production (Fig. 4.2A). The presentation was an L-SIGN specific process and not mediated by Fc receptors, as an irrelevant antibody (anti-DC-SIGN, IgG1) was not processed and presented to T cells (Fig. 4.2A). Immortalized B cells transduced with DC-SIGN could internalize, process and present both DC-SIGN and DC-SIGN/L-SIGN specific antibodies (Fig. 4.2A and [36]). Mock-transduced B cells could not activate the T cells when incubated with an anti-L-SIGN/DC-SIGN or an anti-DC-SIGN antibody (Fig. 4.2A), confirming specificity. Furthermore, uptake of antibodies by membrane turnover was excluded since the L19 antibody (IgG1) specific for CD18/LFA1, which is highly expressed on the immortalized B cells (Fig. 4.2B, histogram), was not presented to the $CD4^+$ T cells (Fig. 4.2B). Hd7 specificity for mouse IgG1 was further confirmed since DC-$SIGN^+$ cells incubated with DC-SIGN specific mouse IgG2b antibody DCN46.1 did not result in T-cell activation (Fig. 4.2B). This indicates that both DC-SIGN and L-SIGN can function as an antigen uptake and presentation receptor.

L-SIGN and DC-SIGN mediate uptake and presentation of HCV antigens to $CD4^+$ T cells.
We have shown before that both L-SIGN and DC-SIGN are HCV binding receptors due to their high affinity for envelope proteins E1 and E2 and that L-SIGN and DC-SIGN mediated internalization of HCV-VLPs results in retention of the VLPs in non-lysosomal compartments [32]. Our findings that both L-SIGN and DC-SIGN can mediate antigen presentation to $CD4^+$ T cells (Fig. 4.2), initiated our investigation whether also HCV envelope antigens were taken up and presented. Because of the lack of HCV envelope specific T cell clones, we used the mIgG1 specific T-cell clone Hd7. We used B cells transduced with L-SIGN or DC-SIGN and HCV-E1 immune complexes (HCV-E1-IC). HCV-E1-IC were made from HCV-E1 virus like particles using mouse anti-HCV envelope specific IgG1 antibodies. The B cell-line expressing DC-SIGN or L-SIGN were incubated with these HCV-E1-IC and cocultured with the IgG1 specific Hd7. Cells expressing DC-SIGN or L-SIGN presented HCV-E1-IC peptides on HLA-

Figure 4.2

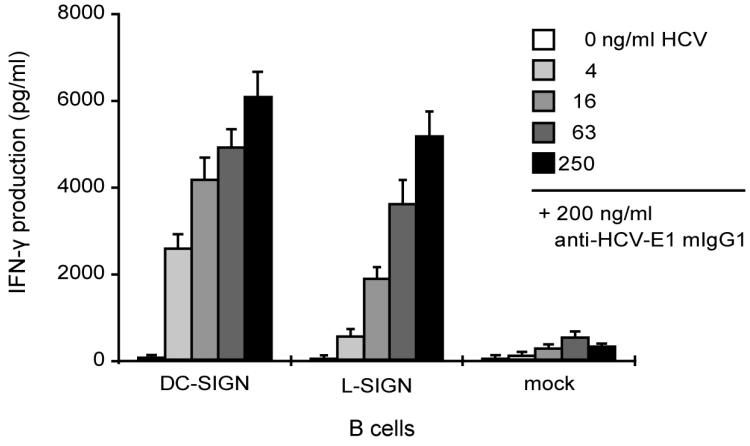


L-SIGN, like DC-SIGN, can function as an antigen receptor.
(A) Antigen presentation of L-SIGN and DC-SIGN specific antibody AZN-D2 (IgG1) and DC-SIGN specific AZN-D1 (IgG1) by B cells expressing L-SIGN or DC-SIGN. Immortalized B cells were transduced with lentiviral vectors containing DC-SIGN or L-SIGN and stained with DC-SIGN specific AZN-D1 (mIgG1, continuous lines in histograms), DC-SIGN & L-SIGN specific AZN-D2 (mIgG1, dashed lines in histograms) to confirm expression. Filled histograms are representing isotype staining. The cells were incubated with the designated antibodies and cocultured with mIgG1 specific Hd7 T cells. After 24 hours, IFN-γ production as a measure for T-cell activation was determined by sandwich ELISA. Error bars represent standard deviations (n=3). (B) Immortalized B cells do not take up and process anti-LFA-1 antibodies, Hd7 does not recognize mouse IgG2b DC-SIGN antibody DCN46.1. Mock-transduced B cells or B cells transduced with DC-SIGN were incubated with AZN-D2 (anti-DC-SIGN-mIgG1, light grey bars), DCN46.1 (anti-DC-SIGN-mIgG2b, white bars), or L19 (anti-LFA-1-mIgG1, dark grey bars), and cocultured with Hd7 T cells. Error bars represent standard deviations (n=3). Expression of LFA1 on immortalized B cells was determined by flow cytometry (black line in histogram).

DR1 in a dose dependent way. The parental, mock transduced, cell line did not present HCV-E1-IC derived antigens to the T cells (Fig. 4.3). Therefore our data demonstrate that, although HCV particles captured by DC-SIGN and L-SIGN are retained in early endosomes [32], part of the captured antigens are degraded for antigen presentation.

DC-SIGN on DCs mediates uptake and presentation of HCV antigens to CD4$^+$ T cells.
As HCV-E1-IC derived antigens are presented by DC-SIGN transfectants, we investigated the presentation of HCV derived antigens by primary DC-SIGN positive cells DCs. We incubated HLA-DR1$^+$ haplotype matched DCs with HCV-E1-IC made from HCV-E1 and mouse IgG1 HCV envelope specific antibodies, and cocultured these with the Hd7 T cells. After 24 hours of incubation IFN-γ production was measured. The DCs incubated with HCV-E1-IC elicited T-cell activation in a dose dependent way as measured by IFN-γ production (Fig. 4.4). Presentation of HCV by DCs has been shown before to be DC-SIGN independent [47]. However here we show that antigen presentation of HCV is DC-SIGN dependent, since it was blocked by a DC-SIGN specific antibody or mannan.

Figure 4.3



L-SIGN and DC-SIGN mediate HCV-E1-IC antigen presentation to CD4$^+$ T cells. Presentation of HCV-E1-IC antigens by B cells expressing L-SIGN or DC-SIGN was determined by incubation of B cells with HCV-E1-IC. HCV-E1-IC were formed by incubating HCV-E1-VLPs with mouse IgG1 anti-HCV antibodies (200 ng/ml). Immortalized B cells transduced L-SIGN or DC-SIGN or mock-transduced B cells were incubated with the HCV-E1-IC and cocultured with Hd7 T cells. Activation of T cells was determined after 24 hours by measuring IFN-γ production with sandwich ELISA. Error bars represent standard deviations (n=3).

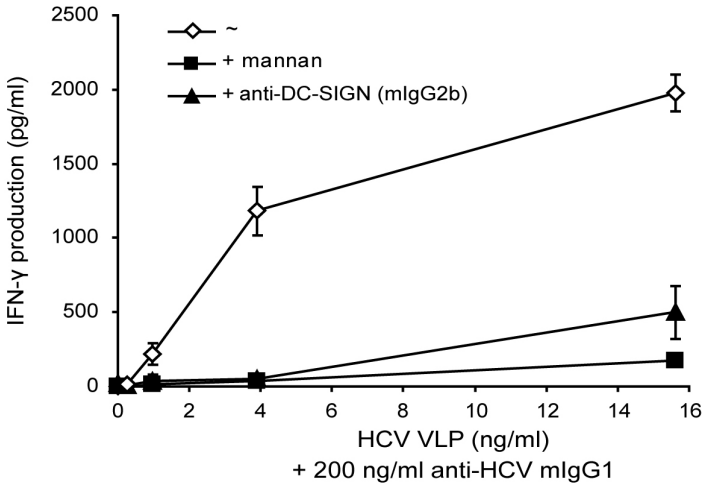
DCs degrade HCV-IC more rapidly than non-complexed HCV-VLPs.

Previously we have shown that hepatitis C virus is retained in early endosomes in dendritic cells [32] and in this way it could circumvent lysosomal degradation and subsequent antigen presentation. However, HCV specific T cells are present in HCV infected people [48], and we demonstrate here that DCs present HCV-E1-IC derived antigens indicating that HCV is degraded by DCs. We therefore quantified the degradation of HCV-VLPs and HCV-immune complexes in DCs. DCs were incubated with HCV-VLPs, either complexed or not with mouse anti-HCV antibodies or human anti-HCV antibodies. After binding of HCV-VLPs at room temperature for 45 minutes, DCs were transferred to 37°C to allow internalization. Samples were taken at different time-points, and after cell lysis, HCV-VLP content was measured using a sandwich ELISA. As shown in figure 4.5, HCV-VLP content in all cells decreased over time. However, in the presence of an anti-HCV antibody the amount of HCV decreased more rapid than without an antibody. Both the mouse and the human antibody could elicit this enhanced degradation (Fig. 4.5). These data suggest that although after 24 hours of incubation a substantial amount of HCV is still intact in DCs, HCV has also been degraded. This degradation is enhanced in the presence of HCV specific antibodies, probably due to the involvement of Fc receptors.

HCV-E1-Alum induces maturation of DCs.

HCV-VLP degradation was enhanced in the presence of HCV specific antibodies, which implies DCs target HCV more efficiently to lysosomes and thereby more efficiently present antigens to T cells. However, upon the first encounter of HCV with DCs in the body, antibodies to HCV are not abundant. Furthermore, in order to stimulate naive T cells, DCs should have a mature phenotype. HCV-VLPs do not induce DC maturation [32] and therefore DCs incubated with HCV-VLPs probably will not be able to provoke a good T cell response by naive T cells. To enhance antibody responses, several vaccination strategies have been investigated. One method of inducing an enhanced antigen specific response is administration of the antigen conjugated to an aluminium-based adjuvant. HCV-E1-Alum has already been used in a clinical trail as a therapeutic vaccine [50]. Therefore we investigated the effect of HCV-E1-Alum on DCs *in vitro*. The interaction of DCs with HCV-E1 did not induce maturation and LPS induced maturation was not enhanced nor blocked by HCV [32]. However, after incubation of immature DCs with HCV-E1-Alum, DCs matured as reflected in an increase of the maturation markers

Figure 4.4



DC-SIGN on DCs mediates HCV-E1-IC Ag presentation to CD4$^+$ T cells.
Presentation of HCV antigens by DCs was determined by incubating DCs with HCV-E1-IC and coculturing with Hd7. Activation of T cells was determined after 24 hours by measuring IFN-γ production (white diamonds). DC-SIGN specificity was determined by blocking DC-SIGN with a DC-SIGN specific antibody DCN46.1 (IgG2b, 5 µg/ml, black triangles) and mannan (200 µg/ml, black squares). Error bars represent standard deviations (n=3).

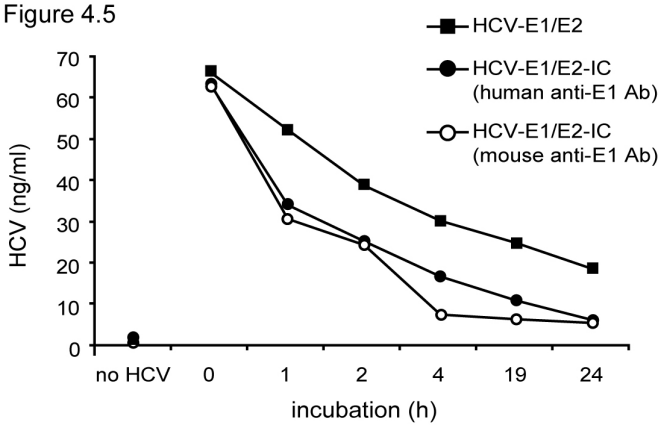
CD80, CD83, and CD86 (Fig. 4.6A). The presence of anti-HCV antibodies alone did not influence HCV-E1-Alum induced maturation (data not shown). This indicates that HCV-E1-Alum is a good candidate to enhance DC based T-cell activation.

HCV-E1-Alum enhances antigen presentation by DCs.
To investigate the effect of the aluminium carrier on antigen presentation, we compared HCV-E1 and HCV-E1-Alum in the antigen presentation assay. Both HCV-E1 and HCV-E1-Alum were allowed to form immune complexes with HCV specific antibodies. These immune complexes were incubated with DCs and Hd7 T cells. Both HCV-E1 and HCV-E1-Alum-IC derived antigens were presented to the CD4$^+$ T cells, however the presentation of HCV-E1-Alum antigens was much more efficient than that of HCV-E1 (Fig. 4.6B). This could be caused by the maturation of DCs induced by aluminium (Fig. 4.6A). In order to investigate the effect of DC maturation on activation of the T cell clone, we performed the antigen presentation assay with immature DCs incubated with HCV-E1, with or without LPS. Antigen presentation during these conditions was compared with antigen presentation of HCV-E1-Alum. The presence of LPS during the assay did not alter activation to T cells compared to DCs incubated only with HCV-E1-IC (Fig. 4.7A). Strikingly, when the expression of maturation markers was measured after 18 hours, all DCs showed a similar degree of maturation (Fig. 4.7B). To investigate in a different way whether enhanced antigen presentation of HCV-E1-Alum is the result of induced maturation, we compared presentation of HCV-E1-Alum-IC with HCV-E1-IC by immortalized B cells. Antigen presentation of HCV-E1-Alum-IC by B cells was not increased when compared to HCV-E1-IC (Fig. 4.7C). This indicates that enhanced presentation of HCV-E1-Alum-IC derived antigens is DC specific and could be caused by differences in maturation state during antigen presentation or by enhanced uptake of HCV-E1-Alum.

Discussion

L-SIGN is expressed on sinusoidal endothelial cells in the liver. These cells constitutively express HLA-DR, but not co-stimulatory molecules like CD80 and CD86 [46]. We demonstrate here that L-SIGN expressed in cell lines is able to internalize ligands for presentation to CD4$^+$

Figure 4.5



HCV is partially degraded in DCs.
DCs were incubated with HCV-E1/E2-VLPs (black squares) or with HCV-E1/E2-VLPs in combination with human (black circles) or mouse anti-HCV (white circles) antibodies as immune complexes. At different time point samples were taken and HCV content in cell lysates was tested using a sandwich ELISA using a monoclonal human anti-HCV-E1 and a mouse monoclonal anti-HCV-E2 antibody. Amount of intact HCV-E1/E2-VLPs was calculated using a standard series of HCV-E1/E2-VLP dilutions. One representative experiment out of three is shown.

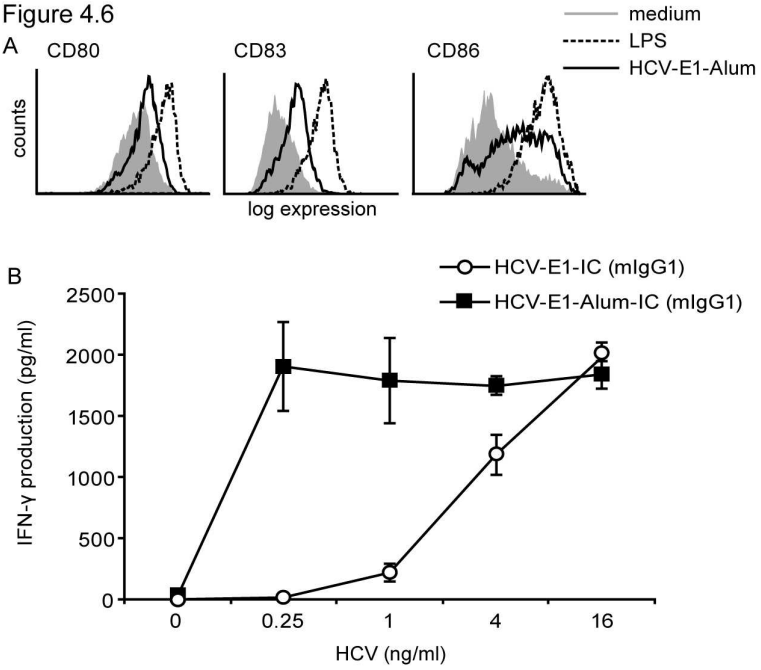
T cells. This presentation was L-SIGN mediated, as irrelevant antibodies were not presented to the T cells. Furthermore, the uptake of the L-SIGN specific antibody was not due to membrane turnover, as anti LFA-1 antibodies bound to the membrane of the B cells were not presented to the T cells. Before it was suggested that L-SIGN lacks the capacity to internalize antigens for antigen presentation, and that L-SIGN functions merely as an adhesion receptor [51]. We demonstrate here that L-SIGN is indeed, like DC-SIGN, functioning as an antigen uptake receptor. Additionally, we demonstrate that L-SIGN can present HCV antigens, indicating that L-SIGN positive LSECs in the liver are important for presentation of antigens from hepatotropic HCV virus.

DCs express the HCV binding receptor DC-SIGN. Upon interaction with DC-SIGN, HCV is internalized, retained in non-lysosomal compartments [32], and could be transmitted to hepatocytes [34]. However, besides protection and transmission, DCs also mediate HCV antigen presentation to $CD4^+$ and $CD8^+$ T cells [47]. In contrast with this previous report, in which a DC-SIGN independent mechanism of HCV uptake and presentation by DCs was described [47], we demonstrate that this uptake and presentation of HCV derived antigen is DC-SIGN specific. In the previous report by Barth *et al.,* the binding of HCV-like particles produced in a baculovirus-insect cell expression system could not be blocked by using anti-DC-SIGN antibodies or mannan [47]. However, this binding study was performed in the absence of calcium, and the lack of calcium has been demonstrated to prevent DC-SIGN function [32]. We clearly demonstrate that both HCV-VLP binding [32] and presentation by DCs is dependent on DC-SIGN.

We previously described that HCV is retained in early endosomes in DCs after uptake via DC-SIGN [32]. Here we quantified HCV-VLP retention in DCs, and demonstrate that after 24 hours of incubation 30% of HCV-VLPs is still intact (Fig. 4.5). This remaining part is probably retained in the early endosomes as previously shown [32]. Furthermore we demonstrate that in the presence of HCV specific antibodies, the degradation of HCV-E1/E2-VLPs in DCs is enhanced to 95% compared to the 70% without antibodies. This suggests that after initial DC-SIGN binding, Fc receptors are participating in targeting of HCV-IC to lysosomes. This implies that DCs can process and present HCV antigens more efficiently when HCV is bound by HCV specific antibodies, and thereby increasing T-cell stimulation by DCs.

DCs are good targets for vaccines and several groups are trying to find a suitable way to target DCs and get a proper immune response. One method to enhance immune responses is the use of aluminium adjuvants. Aluminium adjuvants are composed of aluminium hydroxide or aluminium phosphate gels, and have been used since 1926 [52]. Aluminium adjuvants

Figure 4.6



HCV-E1-Alum potentiates antigen presentation.
(A) DC maturation by HCV-E1-Alum. Immature DCs were incubated with HCV-E1-Alum and after 24 hours maturation was determined by measuring expression levels of costimulatory molecules CD80, CD83 and CD86. Expression levels on DCs incubated with LPS were a positive control (dashed histograms). (B) HCV-E1-Alum is presented more efficiently than HCV-E1 by DC. Presentation of HCV-E1-IC (open circles) and HCV-E1-Alum-IC (filled squares) derived antigens was determined by incubating DCs with HCV-E1-IC or HCV-E1-Alum-IC and coculturing with Hd7 T cells. IFN-γ production as a measure of T cell activation was determined by ELISA after 24 hours. Error bars represent standard deviations (n=3).

preferentially promote humoral immune responses [53] and fail to stimulate cell mediated immune responses [54]. The HCV vaccine composed of recombinant E1 conjugated to an aluminium carrier is a potent antibody and helper T-cell inducing vaccine, which lead to stabilization or reduction of liver fibrosis [50].

We demonstrate here that HCV-E1-Alum can induce DC maturation. Previously it has been shown that mouse DCs are not affected by aluminium adjuvants; mouse DCs did not upregulate maturation markers and also did not display an enhanced antigen presentation [55]. However another study showed that uptake of antigen on aluminium adjuvants by murine DCs is enhanced [56]. We demonstrate here that uptake and presentation of HCV-E1-Alum is greatly enhanced compared to HCV-E1. Although DCs mature upon antigen presentation to T cells, enhanced presentation of HCV-E1-Alum derived antigens could be due to maturation caused by the aluminium, as immortalized B cells were not affected by HCV-E1-Alum. However, enhanced presentation could also be caused by enhanced uptake of HCV when it is associated with the aluminium-containing carrier.

Taken this together, HCV-E1-Alum is a good candidate as HCV vaccine, especially because HCV-E1 does not show the strain-to-strain hypervariability, that is seen in E2 [57]. Promising results have been shown in a clinical trail using the HCV-E1-Alum as a therapeutic vaccine [50], and here we demonstrate that *in vitro* HCV-E1-Alum has a great capacity to enhance DC mediated T-cell activation.

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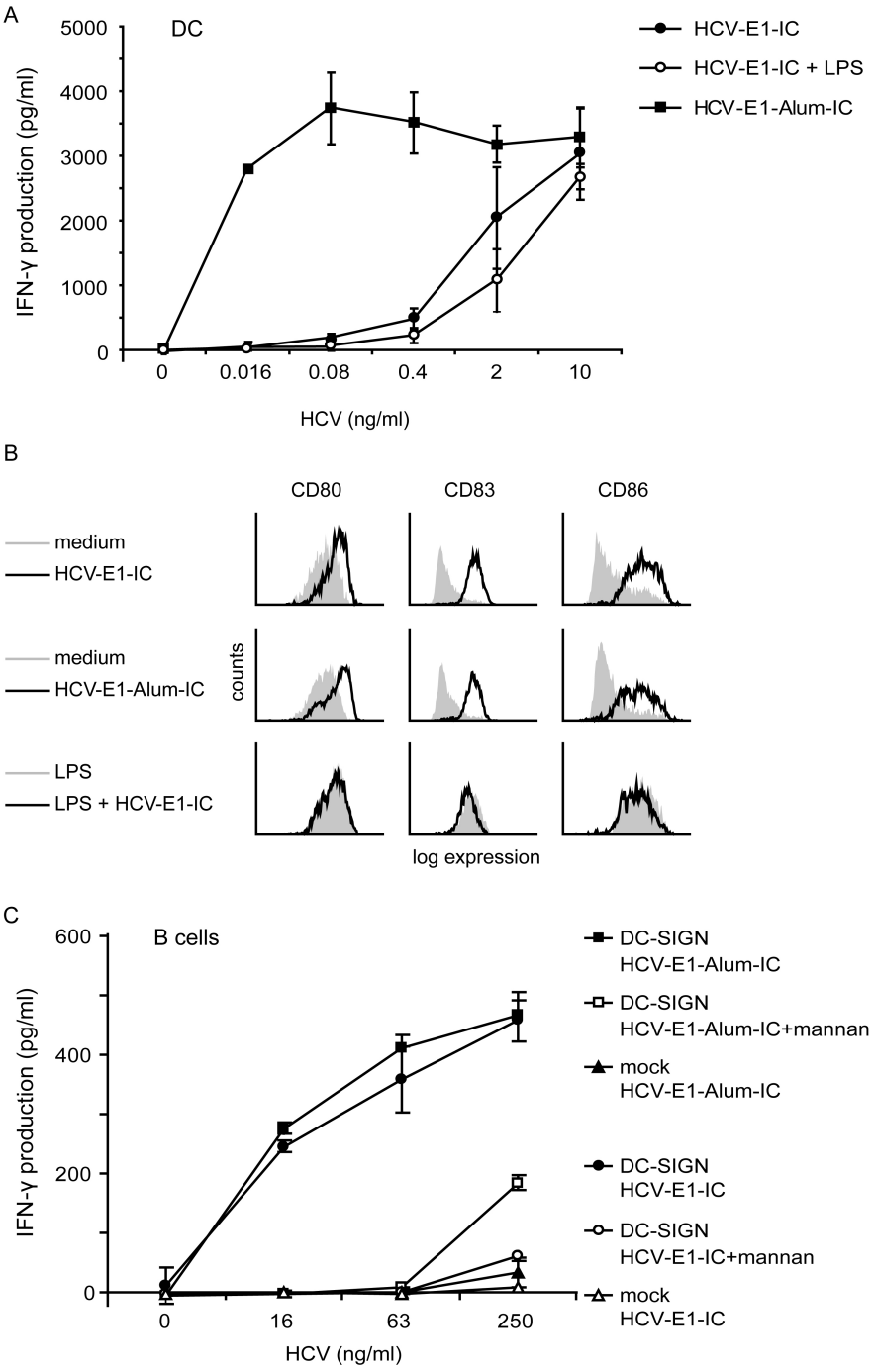
Figure 4.7



Increased antigen presentation of HCV-E1-Alum is not solely due to maturation.
(A) Presentation of HCV-E1-Alum was compared to HCV-E1 in combination with LPS to induce DC maturation. DCs were incubated with HCV-E1-Alum-IC (filled squares), HCV-E1-IC (filled circles), or HCV-E1-IC with LPS (open circles), and cocultured with Hd7 T cells. IFN-γ production as a measure of T cell activation was determined by ELISA after 24 hours. Error bars represent standard deviations (n=3). (B) DC maturation induced upon antigen presentation. DCs were incubated with HCV-E1-Alum, HCV-E1, or HCV-E1 and LPS. Expression of maturation markers CD80, CD83, and CD86, after coculture with Hd7 T cells for 24 hours was measured with flow cytometry. One representative experiment out of three is shown. (C) B cells present HCV-E1 and HCV-E1-Alum-IC in a comparable way. Immortalized B cell transduced with DC-SIGN or mock-transduced B cells were incubated with HCV-E1-IC or with HCV-E1-Alum-IC and cocultured with Hd7 T cells. DC-SIGN specificity was confirmed by blocking with mannan. Error bars represent standard deviations (n=3).

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Potency of HIV-1 envelope glycoprotein gp120 antibodies to inhibit the interaction of DC-SIGN with HIV-1 gp120

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Abstract

The interaction of DC-SIGN with gp120 provides an attractive target for intervention of HIV-1 transmission. Here, we have investigated the potency of gp120 antibodies to inhibit the DC-SIGN-gp120 interaction. We demonstrate that although the V3 loop is not essential for DC-SIGN binding, antibodies against the V3 loop partially inhibit DC-SIGN binding, suggesting that these antibodies sterically hinder DC-SIGN binding to gp120. Polyclonal antibodies raised against non-glycosylated gp120 inhibited both low and high avidity DC-SIGN-gp120 interactions in contrast to polyclonal antibodies raised against glycosylated gp120. Thus, glycans present on gp120 may prevent the generation of antibodies that block the DC-SIGN-gp120 interactions. Moreover, the polyclonal antibodies against gp120 efficiently inhibited HIV-1 capture by both DC-SIGN transfectants and immature dendritic cells. Therefore, non-glycosylated gp120 may be an attractive immunogen to elicit gp120 antibodies that block the binding to DC-SIGN. Furthermore, we demonstrate that DC-SIGN binding to gp120 enhanced CD4 binding, suggesting that DC-SIGN induces conformational changes in gp120, which may provide new targets for neutralizing antibodies.

Introduction

Human immunodeficiency virus type 1 (HIV-1) establishes persistent infections in humans, caused by the destruction of $CD4^+$ T cells, which results in the development of AIDS. Entry of HIV-1 into T cells requires the interaction of the envelope glycoprotein gp120 with the CD4 receptor and chemokine co-receptors, leading to membrane fusion and virus entry. Upon primary infection, dendritic cells (DCs) in mucosal tissues are thought to be the first cells to be encountered by HIV-1. The presence of DCs greatly enhances the efficiency of HIV-1 infection of $CD4^+$ T cells, although DCs themselves are infected poorly [1;2]. Recently, the DC-specific intercellular adhesion molecule-3 (ICAM-3) grabbing nonintegrin (DC-SIGN) has been identified to mediate the initial capture of HIV-1 through its binding of gp120 [3]. After capture, DC-SIGN transmits virus very efficiently to T cells *in trans* [3]. The captured HIV-1 particles are internalized by DCs, and remain in an infectious form in an endocytic compartment for several days [3;4]. Transmission to T cells can take place through an 'infectious synapse', where internalized HIV-1 is concentrated at the interface between DC and T cell [5]. The putative importance of DC-SIGN in initial infection and dissemination suggests that inhibiting the DC-SIGN-gp120 interaction provides an attractive approach for intervention of HIV-1 transmission.

DC-SIGN is a type II transmembrane protein that belongs to the C-type lectin family [6], and specifically binds to a characteristic internal feature of high-mannose carbohydrate structure [7]. In the HIV-1 envelope glycoprotein gp120 five relative conserved (C1-C5) regions and five variable regions (V1-V5) have been identified [8]. Gp120 contains a relatively large number of N-linked carbohydrates and approximately 50% of the mass of gp120 can be attributed to carbohydrate structures [9;10]. Since carbohydrates are clustered together on the outer domain of the gp120 molecule, this region is weakly immunogenic and known as the 'silent' face [8;11]. Furthermore, it has been implicated that the carbohydrate cluster forms a 'glycan shield' preventing neutralizing antibodies from binding through steric inhibition [12]. Differential glycosylation of the envelope glycoproteins affects DC-SIGN binding and enhancement [13]. Even though it is likely that high-mannose structures on gp120 are recognized by DC-SIGN [13-15], protein-protein interactions may also be important in binding [16].

Strategies targeting DC-SIGN could help in combating HIV-1 dissemination by blocking the DC-SIGN-HIV-1 interaction. However, DC-SIGN is involved in important cellular functions [17], and we have demonstrated that using antibodies against DC-SIGN to block HIV transmission will also prevent the cellular functions of DC-SIGN [3;6;18]. Therefore, antibodies against gp120 may provide a powerful alternative to block DC-SIGN-HIV-1 interaction.

Here we have investigated the potency of a panel of monoclonal antibodies with defined epitopes on gp120 to inhibit the DC-SIGN-gp120 interaction. We demonstrate that although the V3 loop is not essential for DC-SIGN binding, antibodies against the V3 loop partially inhibit DC-SIGN binding. To investigate whether carbohydrates on gp120 prevent the generation of blocking antibodies, we compared the potency of polyclonal antibodies raised against either glycosylated gp120 or non-glycosylated gp120 to block the interaction of DC-SIGN with gp120. Interestingly, polyclonal antibodies against non-glycosylated gp120 could specifically block the high avidity DC-SIGN-gp120 interaction without affecting the immunological role of DC-SIGN. In future vaccination strategies, this non-glycosylated gp120 may be used as an immunogen to elicit blocking antibodies for the DC-SIGN-gp120 interaction. Finally, our data indicate that DC-SIGN induces conformational changes in gp120 to enhance CD4 binding, which may

contribute to the DC-SIGN-mediated enhancement of HIV-1 infection. These putative conformational rearrangements in gp120 induced by DC-SIGN may provide new targets for the generation of neutralizing antibodies.

Material and methods

Proteins and plasmids.
The following proteins were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1_{IIIB} gp120, HIV-1_{BaL} gp120, and HIV-1_{CM235} gp120, whereas sCD4 was from dr. Norbert Schuelke. Recombinant HIV-1_{SF2} gp120 [19-22] (lot 33MJE191) expressed in CHO cells and HIV-1_{SF2} gp120 (Env 2-3) [23;24] (lot 11 MGC014), a full-length non-glycosylated polypeptide analogue of gp120 produced in yeast, were obtained from Chiron Corporation, Emeryville, CA. Plasmid encoding the gp120-Fc from the JR-FL isolate was obtained through the NIH. Plasmids encoding the gp120ΔV1/V2 protein from the HIV-1_{YU2} isolate, lacking the 52 N-terminal residues and the V1/V2 variable loops, and the gp120ΔV1/V2ΔV3 (Δ298-329), containing a deletion of the V3 variable loop of gp120, were a generous gift from dr. J. Sodroski, Dana-Farber Cancer Institute, Boston [25]. Plasmids encoding CD4-Fc and ICAM-3-Fc were kindly provided by dr. D.L. Simmons, Institute of Molecular Medicine, Oxford, United Kingdom.

Antibodies.
The following monoclonal antibodies against HIV-1 were obtained through the NIH AIDS Research and Reference Reagent Program: 257-D IV, 268-D IV [26;27], 447-52D [28], 697-D [29], 654-D [30], and 670-D [30] from Dr. Susan Zolla-Pazner F425 A1g8, F425 B4a1, F425 B4e8 [31] and F105 [32;33] from Dr. Marshall Posner and Dr. Lisa Cavacini, 2G12 [34] from Dr. Hermann Katinger, 48d [35;36] from Dr. James Robinson, IgG1b12 [37] from Dr. Dennis Burton and Carlos Barbas. Polyclonal antibodies against HIV-1_{SF2} gp120 and polyclonal antibodies against non-glycosylated HIV-1_{SF2} gp120 were obtained from Chiron Corporation, Emeryville, CA [19-22]. The polyclonal antibodies were pooled bleedings from nine animals, and contained similar amounts of IgG (data not shown). The polyclonal sheep antibody D7324, raised against a synthetic peptide representing the 15 C-terminal amino acids of HIV-1_{IIIB} gp120 was purchased from Aalto Bio Reagents (Dublin, Ireland).
The mouse anti-DC-SIGN antibodies AZN-D1 and AZN-D2 were generated in our laboratory [3;6]. The mouse antibody DC28, directed against the neck domain of the repeat region of DC-SIGN, was a generous gift from dr. J.A. Hoxie, Department of Medicine, University of Pennsylvania, Philadelphia. The mouse anti-CD4 antibody CD4v4 was obtained from Becton Dickinson (Alphen aan de Rijn, The Netherlands). A horseradish peroxidase (HRP) labelled goat-anti mouse IgG antibody (Jackson Immunoresearch, West Grove, PA, USA) was used to detect the above-mentioned antibodies. The HRP-labelled goat-anti human IgG antibody (Jackson Immunoresearch) was used to detect DC-SIGN-Fc in the soluble DC-SIGN-Fc adhesion assays. HRP-labelled mouse-anti goat IgG antibody (Sigma-Aldrich, Zwijndrecht, The Netherlands) was used to detect the polyclonal antibodies against HIV-1_{SF2} gp120 and non-glycosylated HIV-1_{SF2} gp120.

Cells.
Immature DCs were cultured from monocytes in the presence of 500 U/ml IL-4 and 800 U/ml GM-CSF (both from Schering-Plough, Amstelveen, The Netherlands) as previously described [6].

Transient expression of gp120 variants.
Transient transfection of 4 µg of the plasmid encoding either the gp120ΔV1/V2 protein or the gp120ΔV1/V2ΔV3 protein, with 1 µg of the plasmid encoding the HIV-1 Tat protein, or 4 µg of the plasmid encoding $gp120_{JR-FL}$-Fc protein, or CD4-Fc protein or ICAM-3-Fc protein in the 293T cell line were carried out using Lipofectamin (Invitrogen, Carlsbad, CA) according to the manufacturers instructions. At 3 days post transfection, culture supernatants containing the proteins of interest were collected, cleared by centrifugation and stored at -20°C.

Table 5.1 Interference of gp120 antibodies with the DC-SIGN-gp120 BaL interaction[a]

Antibody	Epitope location (gp120 amino acids)[b]	Effect of antibody (50 µg/ml) on DC-SIGN binding to gp120 BaL
697-D	V2 (161-180)	↓[c]
257-D IV	V3 loop (305-309)	↓
268-D IV	V3 loop (310-315)	↓
447-52D	V3 loop (312-315)	↓↓
F425 B4a1	V3 loop	0
F425 B4e8	V3 loop	0
670-D	C5 (498-504)	↓
654-D	CD4bs	↓
F105	CD4bs	0
IgG1b12	CD4bs	0
F425 A1g8	CD4i	0
2G12	carbohydrates	0

[a] The effect of gp120 antibodies on DC-SIGN binding to gp120-coated microtiter wells is shown. The values represent the relative binding of DC-SIGN (0.75 µg/ml) in the presence of the gp120 antibodies (50 µg/ml), compared to a 100% binding of DC-SIGN to gp120 in the absence of the gp120 antibodies. Data obtained from three independent experiments.

[b] gp120 amino acids are numbered according to the sequence of the HXBc2 gp120 glycoprotein, where residue 1 is the methionine at the amino terminus of the signal peptide.

[c] 0, 75 to 110% maximal binding (i.e., no effect); ↓, 50 to 74% of maximal binding; ↓↓, 25 to 49% of maximal binding.

Fluorescent beads adhesion assay.

Carboxylate-modified TransFluorSpheres (488/645 nm, 1.0 µm; Molecular Probes, Eugene, OR) were coated with native gp120, or CD4-Fc, gp120-Fc or ICAM-3-Fc proteins containing medium of the transient transfection in 293T cells. The beads were prepared as previously described [3;16]. DC-SIGN binding characteristics of gp120-Fc-beads from the HIV-1_{JR-FL} strain were similar as observed with native gp120-coated beads. Specificity was determined in the presence of 80 µg/ml anti-DC-SIGN antibody AZN-D2. Binding of CD4-coated beads to immature DCs was carried out in the presence of 5 µg/ml $gp120_{MN}$. The fluorescent beads adhesion assay was performed essentially as described [3;16].

Soluble DC-SIGN-Fc adhesion assay.

All experiments were carried out with a soluble recombinant DC-SIGN-Fc chimera and $gp120_{IIIB}$, unless stated otherwise. DC-SIGN-Fc consists of the extracellular portion of DC-SIGN fused at the COOH terminus to a human IgG1-Fc fragment [16] and is produced in CHO cells. This soluble DC-SIGN-Fc chimera is functional as previously demonstrated [16]. In the DC-SIGN-Fc adhesion assay, flat bottom microtiter-plate wells (Maxisorb; Nunc, Copenhagen, Denmark) were coated overnight at 4℃ with gp120 (0.5 µg/ml) or gp120 antibody D7324 directed to the C5 region (Aalto Bioreagents) at a concentration of 10 µg/ml (500 ng/well) diluted in 0.2 M $NaHCO_3$ buffer pH 9.2, followed by blocking with 1% bovine serum albumin for 30 min at 37°C. If D7324 was used, gp120 (0.5 µg/ml) was subsequently captured for 60 minutes at room temperature. Soluble DC-SIGN-Fc supernatant (1.5 µg/ml) diluted 1:2 in TSM (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$) was added, and adhesion was performed for 90 min at 37°C. Bound DC-SIGN-Fc was detected with HRP labelled goat-anti human IgG antibody. Specificity was determined in the presence of 80 µg/ml AZN-D1, 5 µg/ml mannan or 5 mM EGTA.

To assess the ability of gp120 antibodies to compete with binding of DC-SIGN-Fc to gp120, gp120-coated wells were incubated with the indicated gp120 antibodies (50 µg/ml, 1:100 dilutions for antibody supernatants) for 90 minutes at room temperature to obtain maximal binding, followed by soluble DC-SIGN-Fc supernatant diluted in TSM incubation for another hour at room temperature. DC-SIGN-Fc

bound to gp120 was detected using the DC28 antibody (1 µg/ml), followed by HRP labelled goat-anti-mouse IgG antibody.

ELISA.
Flat bottom microtiter-plate wells (Maxisorb; Nunc, Copenhagen, Denmark) were coated overnight at 4℃ with gp120 (0.5 µg/ml) as indicated or gp120 antibody D7324 directed to the C5 region (Aalto Bioreagents) at a concentration of 10 µg/ml (500 ng/well) diluted in 0.2 M $NaHCO_3$ buffer pH 9.2, followed by blocking with 1% BSA for 30 min at 37°C. If D7324 was used, culture supernatants containing proteins of interested diluted 1:2 in TSM were subsequently captured for 60 minutes at room temperature. Next, antibodies of interest were added for 60 minutes at room temperature. Bound antibodies were detected with a suitable HRP labelled secondary antibody.

CD4-DC-SIGN-Fc binding assay.
For the CD4-DC-SIGN-Fc binding assay, gp120 was captured with D7324, and DC-SIGN-Fc (0.75 µg/ml) was added and incubated for 1 hour at room temperature. After washing, the wells were incubated with sCD4 (0.5 µg/ml) for 1 hour at room temperature. CD4 binding was detected using CD4v4 antibody (1 µg/ml), followed by HRP labelled goat-anti-mouse IgG antibody depleted of cross-reactivity to human IgG1 (Jackson Immunoresearch, West Grove, PA, USA). To assess the modulation of CD4 upon binding of DC-SIGN-Fc to gp120, no wash was performed after addition of DC-SIGN-Fc. Specificity was determined by pre-incubation of DC-SIGN for 15 minutes with mannan (5 µg/ml).

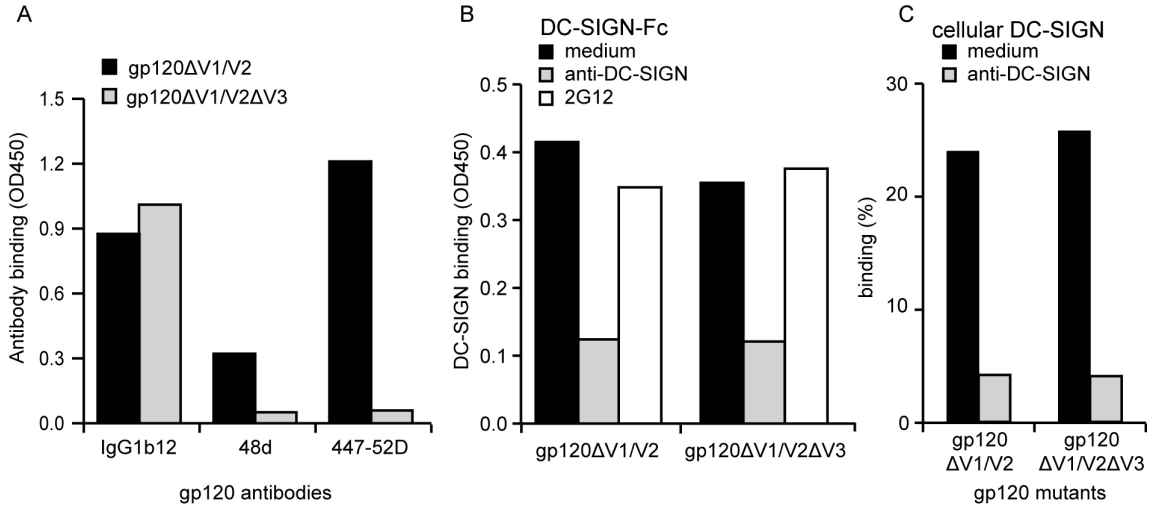
HIV-1 capture by immature DCs.
Cells (100,000) were incubated with HIV-1 JR-CSF (100 ng of $p24^{gag}$) for 60 minutes at 37°C. Specificity was determined in the presence of 20 µg/ml anti-DC-SIGN antibody AZN-D1, polyclonal antiserum against non-glycosylated gp120 (1:50), or EGTA (5 mM). The cells were washed twice and subsequently lysed and virus binding was determined by measuring the p24 concentration using the $p24^{gag}$ detection kit as described by the manufacturers (Beckman-Coulter, Mijdrecht, The Netherlands).

Results

Effect of gp120-specific antibodies on the DC-SIGN-gp120 interaction.
The interaction of DC-SIGN with gp120 provides an attractive target for intervention with HIV-1 transmission. A specific anti-DC-SIGN antibody can inhibit DC-SIGN function, as a anti-DC-SIGN antibodies efficiently inhibit gp120 binding in low or high avidity interactions assays, the ELISA-based DC-SIGN-Fc and the fluorescent beads adhesion assays respectively [3;16]. However, blocking the role of DC-SIGN in HIV-1 transmission will inhibit the immunological function of DC-SIGN [3;6]. Therefore we investigated the ability of antibodies against gp120 to block the interaction with DC-SIGN. A panel of monoclonal antibodies with defined epitopes on gp120 was used to compete with DC-SIGN for binding to gp120. The ability of antibodies in saturating concentrations to block the DC-SIGN-gp120 interaction was measured in a low avidity DC-SIGN-Fc adhesion assay. As summarized in table 5.1, although some gp120 antibodies were able to reduce the binding of DC-SIGN to gp120, we did not observe a complete block of the DC-SIGN-gp120 interaction.

The gp120 V3 loop antibodies.
The gp120 V3 loop antibodies, 257-D IV, 268-D IV and 447-52D, interfered most strongly with DC-SIGN binding to gp120 BaL. Similar results were obtained when using gp120 derived from HIV-1 IIIB (data not shown). Combinations of the V3 antibodies did not result in better inhibition compared to the individual antibodies (data not shown), which may be due to the overlapping

Figure 5.1



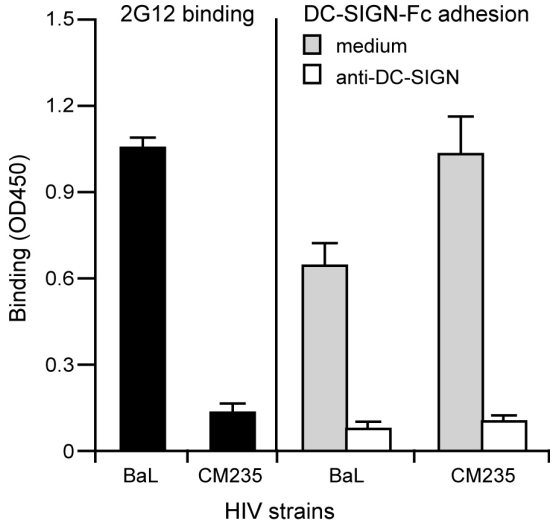
DC-SIGN binding to the HIV-1 gp120ΔV1/V2 ΔV3 mutant.

The ability of DC-SIGN to bind the gp120ΔV1/V2 and gp120ΔV1/V2/ΔV3 mutants was assessed in a DC-SIGN-Fc adhesion assay. (A) The captured gp120 glycoproteins, gp120ΔV1/V2 (black bars), gp120ΔV1/V2/ΔV3 (gray bars) were incubated with the indicated gp120 antibodies (1 µg/ml), and detected as described with HRP-labelled goat-anti human IgG antibody. (B) DC-SIGN-Fc (0.75 µg/ml) binding to captured gp120 glycoproteins was measured (black bars) and inhibition was investigated using the anti-DC-SIGN antibody AZN-D1 (gray bars) and the anti-gp120 antibody 2G12 (white bars). One representative experiment out of three is shown. (C) Cellular DC-SIGN binds the gp120 mutants ΔV1/V2 and ΔV1/V2/ΔV3. DC-SIGN transfectants were incubated with gp120-coated beads and the binding was measured by flow cytometry. Specificity was determined using antibodies against DC-SIGN (AZN-D1, gray bars). One representative experiment out of two is shown.

epitopes of the antibodies. The epitopes of these antibodies are located between residues 305 to 315 of gp120, at the tip of the V3 loop. The gp120 V3 loop, together with a highly conserved element in the C4 domain, has been implicated in chemokine receptor binding [38-40]. Inhibition was not observed for the V3 loop antibodies F425 B4a1 and F425 B4e8, which are directed to epitopes at the base of the V3 loop [31]. A decrease in DC-SIGN binding to gp120 was observed with the 697-D antibody directed to a conformational epitope in the V2 loop. A combination of the blocking V3 257-D IV and V2 697-D antibodies did not result in a stronger inhibition (data not shown). The 670-D antibody directed to the C5 region also exerted a minor inhibition, although this was not observed using gp120 derived from other HIV-1 strains (data not shown). In summary, only the V3 loop antibody 447-52D could significantly inhibit binding of DC-SIGN. This suggests that interactions of DC-SIGN with gp120 may take place in close proximity of the V3 loop.

To further analyze the role of the gp120 V3 loop in the DC-SIGN-gp120-interaction, we tested binding of DC-SIGN to a V3 loop deletion mutant of gp120. This deletion was introduced in the gp120ΔV1/V2 protein of the HIV_{YU2} strain that lacks the V1/V2 variable loops of the gp120 glycoprotein [38]. Both the gp120ΔV1/V2 and gp120ΔV1/V2ΔV3 mutant proteins were transiently produced by transfection of 293T cells. Integrity of the V3 loop deletion mutant was confirmed by binding of CD4 binding site (CD4bs) antibody IgG1b12 to the gp120ΔV1/V2ΔV3 mutant protein and absence of the V3 loop antibody 447-52D epitope (Fig. 5.1A). The CD4 induced (CD4i) antibody 48d also did not bind, as previously reported [25] (Fig. 5.1A). Both recombinant DC-SIGN and cellular DC-SIGN were able to bind the gp120 mutants ΔV1/V2 and ΔV1/V2ΔV3 (Fig. 5.1B and 1C). Binding could specifically be inhibited with the anti-DC-SIGN antibody AZN-D1, but not with the antibody 2G12 (Fig. 5.1B and 1C). Notably, although the V2 loop antibody 697-D interfered with DC-SIGN binding to gp120 (Table 5.1), DC-SIGN was able to bind the gp120ΔV1/V2 protein, lacking the V1/V2 loops.

Figure 5.2



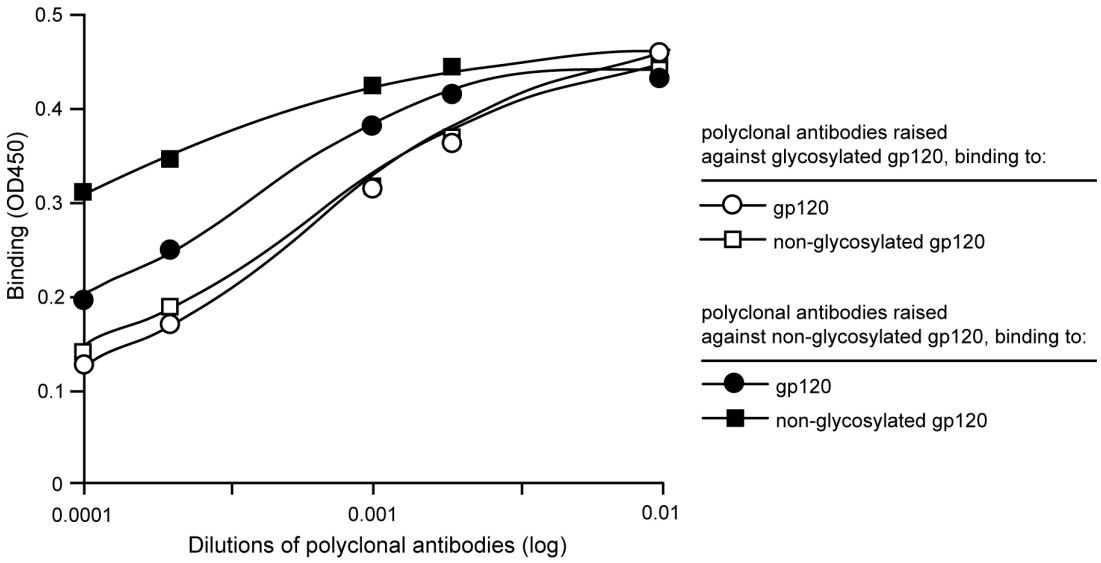
Binding of DC-SIGN and 2G12 to gp120 glycoproteins of different HIV-1 strains.
Binding of the 2G12 antibody (1 µg/ml; black bars) to gp120 glycoproteins of HIV-1 strains BaL and CM235 (1 µg/ml gp120) was assessed by ELISA. Using the soluble DC-SIGN-Fc adhesion assay, binding of soluble DC-SIGN-Fc was also evaluated (gray bars). Binding could be blocked by pre-incubation of DC-SIGN-Fc with anti-DC-SIGN antibody AZN-D1 (white bars).

These data indicated that both the V3 loop antibodies and the V2 loop antibody presumably sterically hinder the DC-SIGN-gp120 interaction, as both the V2 loop and the V3 loop are not essential for DC-SIGN binding to gp120.

The CD4bs and CD4i antibodies.
The CD4bs antibodies F105 and IgG1b12 that compete with CD4 for binding gp120 [41], did not block the binding of DC-SIGN. The 654-D antibody caused a decrease in DC-SIGN binding to gp120. Carbohydrates are implicated in the 654-D epitope, which could explain the interference of the 654-D antibody with the DC-SIGN binding to gp120 [27].
CD4 binding to gp120 exposes epitopes that are recognized by the CD4i antibody F425 A1g8. This antibody can also bind in the absence of CD4 with a lower affinity and inhibits binding of gp120 to the chemokine receptor [25]. We observed no significant interference with the DC-SIGN-gp120 interaction using the CD4i antibodies (Table 5.1). These data demonstrate that CD4bs and CD4i antibodies do not efficiently block the DC-SIGN-gp120 interaction, indicating that the CD4 binding site and the CD4i epitope are not directly involved in the interaction with DC-SIGN.

The carbohydrate-dependent antibody 2G12.
The human monoclonal antibody 2G12 has previously been demonstrated to bind to a carbohydrate-dependent epitope on gp120 [42]. 2G12 has a multivalent interaction with a conserved cluster of Man(α)1-2Man-linked high-mannose type carbohydrates on gp120 [43]. A similar structure is present in the mannose-cap of ManLAM, a mycobacterial cell wall component that is a known ligand for DC-SIGN [44].
However, 2G12 was not able to block the binding of DC-SIGN to gp120 (Table 1). Next, we investigated binding of both 2G12 and DC-SIGN to gp120 glycoproteins of another HIV-1 strain, since it has been demonstrated that 2G12 does not recognize clade E HIV-1 strains [42]. Indeed, we did not observe binding of 2G12 to the clade E $gp120_{CM235}$ (Fig. 5.2), whereas DC-SIGN efficiently interacted with the clade E $gp120_{CM235}$ glycoprotein, indicating that the 2G12 epitope is not recognized by DC-SIGN.

Figure 5.3



Binding characteristics of the polyclonal antibodies raised against wild type and non-glycosylated gp120. Binding of polyclonal antibodies raised against wt gp120 (open) and non-glycosylated gp120 (filled) to gp120 (circles) and non-glycosylated gp120 (squares) by ELISA. Different dilutions of the polyclonal antibodies were tested. Binding was detected with an HRP-labelled mouse-anti goat IgG antibody.

Polyclonal antibodies raised against non-glycosylated gp120 can block the DC-SIGN-gp120 interaction.

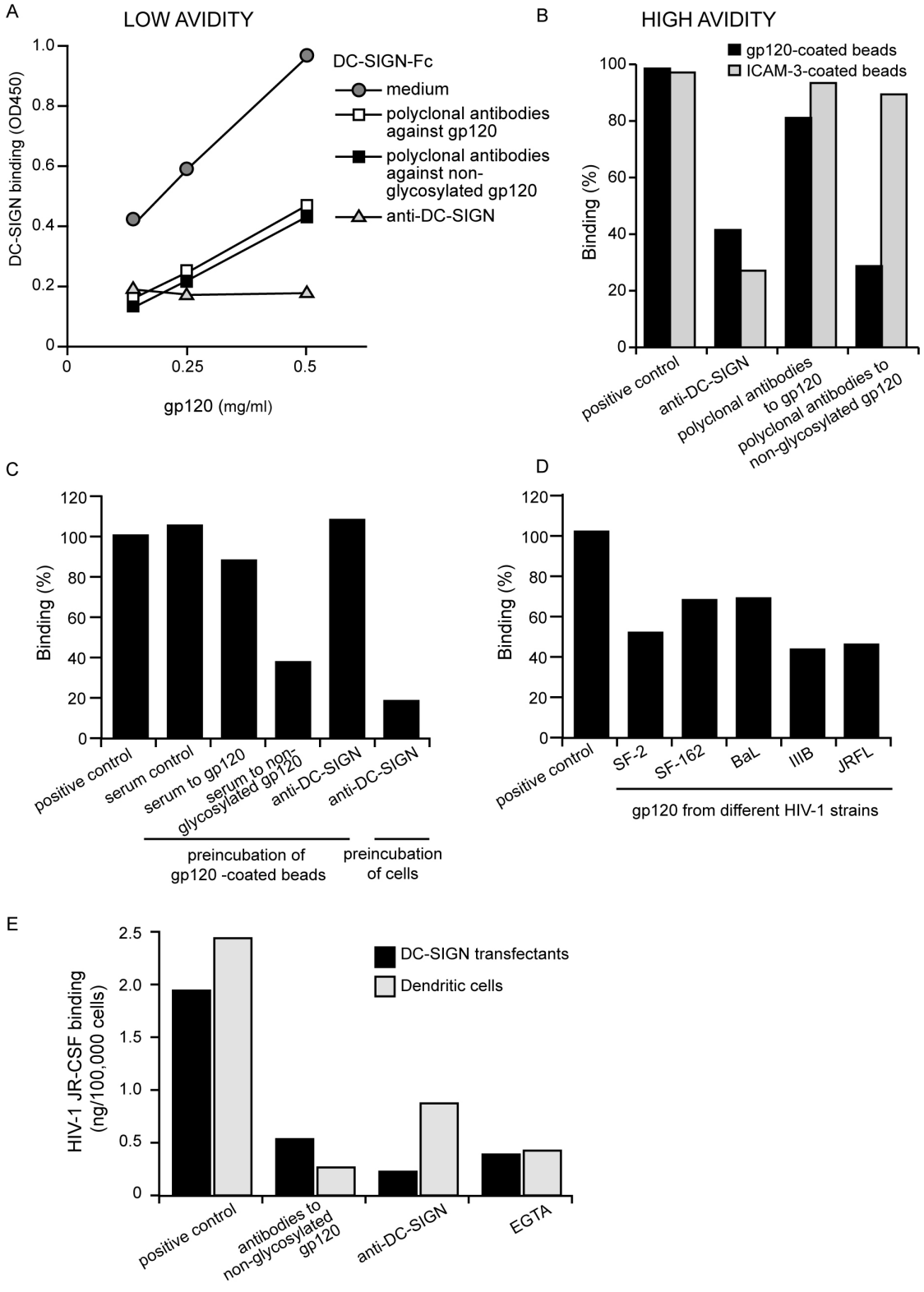
Monoclonal gp120 antibodies only gave partial inhibition of the DC-SIGN-gp120 interaction. Moreover, the antibodies able to interfere with DC-SIGN-Fc binding to gp120 were not able to interfere with gp120 binding to cellular DC-SIGN due to the high avidity of cellular tetrameric DC-SIGN for gp120. We hypothesized that the glycan shield of gp120 may prevent the generation of blocking antibodies. Therefore, we compared the potency of polyclonal antibodies raised against either glycosylated gp120 or non-glycosylated gp120 to block the interaction of DC-SIGN with gp120.

The binding characteristics of polyclonal antibody mixtures raised against gp120 and non-glycosylated gp120 were assessed by means of ELISA (Fig. 5.3). The polyclonal antibodies raised against gp120 recognized both glycosylated and non-glycosylated gp120. Binding to both forms of gp120 was also observed for the polyclonal antibodies raised against non-glycosylated gp120, demonstrating that epitopes recognized by the polyclonal antibodies raised against non-glycosylated gp120 are exposed in glycosylated gp120. Furthermore, the antibodies raised against both non-glycosylated and glycosylated gp120 bound to a similar extent to glycosylated gp120.

Next, we investigated the potency of these polyclonal antibodies to block the DC-SIGN-gp120 interaction. Polyclonal antibodies raised against both glycosylated and non-glycosylated gp120 were equally efficient in inhibiting the DC-SIGN-gp120 interaction in the low avidity DC-SIGN-Fc adhesion assay (Fig. 5.4A).

Since both polyclonal antibodies mixtures were as efficient in blocking the recombinant DC-SIGN binding, we tested inhibition of cellular DC-SIGN binding to polyvalent gp120 by the polyclonal antibody mixtures in the high avidity fluorescent beads adhesion assay (Fig. 5.4B). Strikingly, gp120 binding to immature DCs was inhibited in the presence of polyclonal antibodies raised against non-glycosylated gp120 to a similar extent as an antibody against

Figure 5.4

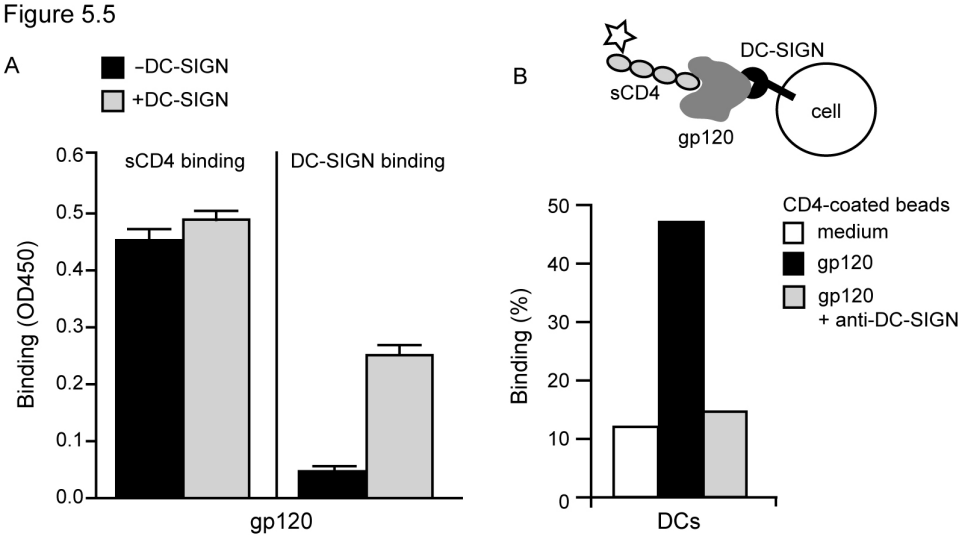

A
LOW AVIDITY
DC-SIGN binding (OD450)
1.0
0.8
0.6
0.4
0.2
0
0
0.25
0.5
gp120 (mg/ml)
DC-SIGN-Fc
medium
polyclonal antibodies against gp120
polyclonal antibodies against non-glycosylated gp120
anti-DC-SIGN
B
HIGH AVIDITY
gp120-coated beads
ICAM-3-coated beads
Binding (%)
100
80
60
40
20
0
positive control
anti-DC-SIGN
polyclonal antibodies to gp120
polyclonal antibodies to non-glycosylated gp120
C
Binding (%)
120
100
80
60
40
20
0
positive control
serum control
serum to gp120
serum to non-glycosylated gp120
anti-DC-SIGN
anti-DC-SIGN
preincubation of gp120 -coated beads
preincubation of cells
D
Binding (%)
120
100
80
60
40
20
0
positive control
SF-2
SF-162
BaL
IIIB
JRFL
gp120 from different HIV-1 strains
E
HIV-1 JR-CSF binding (ng/100,000 cells)
2.5
2.0
1.5
1.0
0.5
0
DC-SIGN transfectants
Dendritic cells
positive control
antibodies to non-glycosylated gp120
anti-DC-SIGN
EGTA

Polyclonal antibodies raised against non-glycosylated gp120 inhibit HIV-1 binding to DC-SIGN.
(A) For the DC-SIGN-Fc adhesion assay, gp120-coated wells were incubated with the polyclonal antibodies (1:100 dilution) or AZN-D1 (80 µg/ml), followed by DC-SIGN-Fc, without washing. (B) For the fluorescent beads adhesion assay, immature DCs were incubated with the polyclonal antibodies (1:10 dilution). Next, binding of the gp120-coated beads (black bars) and ICAM-3-coated beads (gray bars) in the presence of the polyclonal antibodies to cellular DC-SIGN was assessed. (C) The polyclonal antibodies against non-glycosylated gp120 specifically block the gp120-DC-SIGN interaction. Fluorescent gp120-coated beads were preincubated with the antisera for 20 minutes at 37°C. The beads were washed and added to DC-SIGN transfectants (THP-DC-SIGN). Adhesion is measured after 45 minutes at 37°C. (D) The polyclonal antibodies against non-glycosylated gp120 inhibit binding of DC-SIGN to different strains of HIV-1 gp120. The binding assay was performed as described in figure 5.4B. (E) The polyclonal antibodies against non-glycosylated gp120 inhibit HIV-1 binding to cellular DC-SIGN. Both immature DCs and THP-DC-SIGN transfectants are incubated with HIV-1 JR-CSF (p24 100 ng/100,000 cells) for 60 minutes at 37°C in presence of different antibodies. Binding is measured after washing and lysing the cells using a p24 ELISA.

DC-SIGN. This inhibition was specific since the polyclonal antibodies did not block binding of DC-SIGN to the cellular ligand ICAM-3. In contrast, the addition of the polyclonal antibodies raised against glycosylated gp120 caused only a minor inhibition of the binding of gp120 to immature DCs. In order to investigate the specificity of the polyclonal antibodies for gp120 and exclude any other components that may interfere with DC-SIGN binding, DC-SIGN transfectants were incubated with gp120-coated beads, which had been pre-incubated with the different antibodies and extensively washed to remove unbound antibodies (Fig. 5.4C). DC-SIGN-gp120 binding was blocked by treating the gp120-coated beads with polyclonal antibodies against non-glycosylated gp120, whereas both polyclonal antibodies against gp120 and pooled normal goat serum did not inhibit the interaction (Fig. 5.4C). As expected, antibodies against DC-SIGN were only able to inhibit the interaction when pre-incubated with cells but not with gp120 beads (Fig. 5.4C). These results demonstrate that polyclonal antibodies against non-glycosylated gp120 block the gp120 interaction with DC-SIGN by specifically binding to gp120. Moreover, the polyclonal antibodies raised against non-glycosylated gp120 SF-2 demonstrated a broad specificity, since the antibodies inhibited DC-SIGN binding to gp120 derived from different HIV-1 strains (Fig. 5.4D).
In order to asses their ability to prevent DC-SIGN binding to trimeric gp120 as present on viruses, we investigated the ability of the antibodies to inhibit HIV-1 capture by both DC-SIGN transfectants and immature dendritic cells. DC-SIGN transfectants captured whole HIV-1 virions as measured by a $p24^{gag}$ ELISA (Fig. 5.4E). The capture was blocked by the polyclonal antibodies against non-glycosylated gp120 to a similar extent as observed with both EGTA and antibodies against DC-SIGN (Fig. 5.4E). Moreover, the polyclonal antibodies against non-glycosylated gp120 also completely inhibited virus capture by immature DCs, similarly as antibodies against DC-SIGN (Fig. 5.4E).
In summary, the polyclonal antibodies raised against non-glycosylated gp120 could specifically block the DC-SIGN-gp120 interaction, and prevent HIV-1 capture by DCs. These promising results may lead to the use of non-glycosylated gp120 as an immunogen to elicit blocking antibodies to prevent HIV-1 capture by DC-SIGN.

Distinct binding sites on gp120 for DC-SIGN and CD4.
The receptor binding site for CD4 on gp120 is a highly conserved region and has been characterized extensively [8]. As the CD4bs antibodies were not able block the DC-SIGN-gp120 interaction (Table 5.1), we analyzed binding of soluble human CD4 (sCD4) to gp120 in the presence of DC-SIGN using an ELISA-based binding assay. Binding of sCD4 to gp120 was examined using a CD4 antibody that does not interfere with the gp120 binding site on CD4. As seen in figure 5.5A, binding of sCD4 to gp120 was not inhibited by pre-incubation of

Figure 5.5

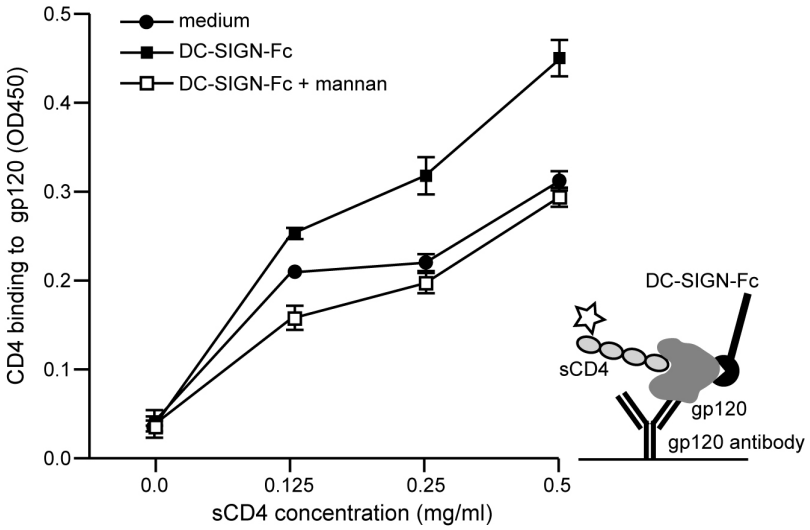


Simultaneous binding of CD4 and DC-SIGN to gp120.
(A) Binding of sCD4 (0.5 µg/ml) to gp120-coated wells in the presence of DC-SIGN-Fc, was determined using the CD4v4 antibody that does not bind to the gp120 binding site on CD4. Standard deviation < 0.02. (B) Simultaneous binding of DC-SIGN and CD4 to gp120 was also determined using the fluorescent beads adhesion assay. Monomeric gp120 (5 µg/ml) was added to K652-DC-SIGN stably transfected with DC-SIGN, the non-transfected cell line K652 and immature DCs. Binding of the CD4-coated fluorescent beads to gp120 was analyzed. CD4 binding in the absence of gp120 (white bars); CD4 binding in the presence of gp120 (black bars); binding of CD4 was absent when cells were pre-incubated with anti-DC-SIGN antibody AZN-D1 (20 µg/ml) (gray bars). One representative experiment of three is shown.

gp120 with DC-SIGN, demonstrating that DC-SIGN binding to gp120 did not interfere with the interaction of CD4 with gp120. Next, we examined whether CD4 could bind to gp120 that has been captured by cellular DC-SIGN (Fig. 5.5B). We observed binding of CD4 to immature DCs when gp120 was present, but not when gp120 was absent. This corroborates our observation with the ELISA-based binding assay, demonstrating that simultaneous binding of DC-SIGN and CD4 to gp120 can take place. Binding was inhibited when cells were pre-incubated with an anti-DC-SIGN antibody that blocks the binding of gp120 to DC-SIGN, demonstrating that binding was specific for DC-SIGN. Thus, we have demonstrated that CD4 and DC-SIGN have distinct binding sites on gp120.

Enhanced CD4 binding to gp120 induced by DC-SIGN.
Next, we examined whether DC-SIGN binding to gp120 could modulate binding of CD4 to gp120 in an ELISA-based binding assay. The molecular mechanism by which DC-SIGN enhances the infection of T cells is not fully understood. Therefore, besides concentrating virus particles on the surface of DCs, DC-SIGN may induce conformational changes in gp120 that enable a more efficient interaction of gp120 with CD4 by influencing conformational masking. The DC-SIGN-dependent enhancement of HIV-1 infection led us to investigate whether DC-SIGN binding to gp120 induces an increase of CD4 binding to gp120 [3].
As seen in figure 5.6, binding of CD4 to gp120 was increased upon DC-SIGN interaction with gp120. We demonstrate that in the presence of DC-SIGN, less CD4 was required for a similar extent of binding to gp120 compared to CD4 alone, suggesting that DC-SIGN binding to gp120 increased binding of CD4 to gp120. The increase is not due to cross-reactivity of the secondary anti-mouse antibody to the human IgG1-Fc region of DC-SIGN-Fc, since no binding is observed in the absence of sCD4. The increased binding is specific for DC-SIGN binding to

Figure 5.6



Enhancement of CD4 binding to gp120 by the DC-SIGN-gp120 interaction.
CD4 binding to gp120 captured onto microtiter wells using the D7324 antibody was assessed in the presence of DC-SIGN (0.75 µg/ml), with or without the DC-SIGN inhibiting polysaccharide mannan using different concentrations of sCD4. One representative experiment of three is shown.

gp120 since it was not observed when DC-SIGN binding to gp120 was inhibited with the polysaccharide mannan (Fig. 5.6). In the presence of DC-SIGN, an increase in CD4 binding to gp120 of 145 to 200% was repeatedly observed. The gp120 glycoprotein is very flexible and it seems likely that conformational changes in gp120 are induced upon binding of DC-SIGN thereby enhancing CD4 binding. This appears analogous to CD4 that triggers conformational reorganization in gp120 allowing high-affinity binding to the chemokine receptor [45;46].

Discussion

The important role of DC-SIGN in HIV-1 transmission makes it an attractive target to intervene with HIV-1 dissemination. Blocking antibodies against DC-SIGN inhibit HIV-1 capture by DCs [3], but also interfere with the immunological role of DC-SIGN [3;18]. Therefore, inhibitors targeting HIV-1 gp120 to prevent DC-SIGN capture seem warranted to interfere with HIV-1 dissemination. More knowledge on the DC-SIGN-gp120 interaction is essential for development of these inhibitors. Here, we have investigated the potency of monoclonal and polyclonal antibodies against gp120 to inhibit this interaction.

We have evaluated specific competition of DC-SIGN with antibodies against gp120. Our results demonstrate that defined monoclonal gp120 antibodies could not completely inhibit the interaction of DC-SIGN with gp120. V3 loop antibodies that recognize the tip of the loop [47] reduced binding of DC-SIGN up to 50%, in contrast to the V3 loop antibodies directed to the base of the loop that had no effect on DC-SIGN binding to gp120. However, the V3 loop itself is not involved in the interaction with DC-SIGN, as DC-SIGN binding was not affected by deletion of the V3 loop from gp120. Thus, V3 loop antibodies might modulate DC-SIGN binding by sterically hindering DC-SIGN from accessing its binding site. N-linked glycosylation high mannose structures, but not complex oligosaccharide structures, are specifically recognized by DC-SIGN [7], and studies on the gp120 carbohydrate composition indicate that high mannose structures are observed on the gp120 core closely clustered to the V3 loop [10]. Our inhibition data indicate that DC-SIGN interacts with these carbohydrates in relative close proximity to the V3 loop. The 2G12 gp120 antibody binds to specific conserved cluster of high-mannose type

carbohydrates on gp120 in proximity of the V3 loop, which could be involved in DC-SIGN binding. However, we did not observe inhibition of the DC-SIGN-gp120 interaction by 2G12. This data was supported by results showing that DC-SIGN binds to clade E $gp120_{CM235}$ that lacks the 2G12 epitope. This is in agreement with the data of Hong *et al.* [14], demonstrating that DC-SIGN and 2G12 have distinct binding sites on gp120. Future studies could include the use of antigen-binding fragments of the antibody instead of the entire antibody to minimize the effect of steric hindrance caused by the size of the antibody.

Our data demonstrate that monoclonal gp120 antibodies do not block the DC-SIGN-gp120 interaction efficiently. We hypothesized that the presence of a glycan shield may prevent the generation of blocking antibodies. Therefore, we have investigated the potency of polyclonal antibodies raised against both glycosylated and non-glycosylated gp120 to inhibit binding of DC-SIGN to gp120. Although both polyclonal antibodies raised against either glycosylated and non-glycosylated gp120 reacted similarly to glycosylated gp120 (Fig. 5.3), we observed differences in the blocking of the DC-SIGN-gp120 interaction (Fig. 5.4).

Polyclonal antibodies against glycosylated gp120 inhibited the DC-SIGN-gp120 interaction in the low avidity interactions ELISA-based DC-SIGN-Fc adhesion assay, but not in the high avidity fluorescent beads adhesion assay requiring binding of polyvalent gp120 to cellular DC-SIGN. In contrast, polyclonal antibodies raised against non-glycosylated gp120 inhibited binding of gp120 to DC-SIGN in both assays. Thus polyclonal antibodies raised against non-glycosylated gp120 have a high affinity for gp120 or may be directed to gp120 structures that become exposed when carbohydrates are absent. This suggests that the glycan shield formed by the carbohydrate cluster on gp120 [48], in addition to preventing binding of neutralizing antibodies, might also prevent the generation of gp120 antibodies against the DC-SIGN binding site.

DC-SIGN plays an important cellular function by mediating adhesion of DCs to T cells through ICAM-3 binding. Antibodies raised against non-glycosylated gp120 inhibit the gp120-DC-SIGN interaction without altering the immunological function of DC-SIGN (Fig. 5.4). These polyclonal antibodies evidently bind gp120 in a way that inhibits DC-SIGN binding. Furthermore, the polyclonal antibodies against non-glycosylated gp120 were able to inhibit capture of HIV-1 by both DC-SIGN transfectants and immature DCs, to a similar level as observed with antibodies against DC-SIGN (Fig. 5.4). Thus, the polyclonal antibodies efficiently block the interaction of DC-SIGN to both monomeric and trimeric gp120 as present on viruses. Even though the precise nature of the blocking capability of the polyclonal antibodies against the non-glycosylated gp120 remains yet to be identified, these data hold promise for the future. In vaccination strategies, it may be feasible to target a defined structure on gp120 to block DC-SIGN binding to elicit blocking antibodies, while the binding of cellular ligands to DC-SIGN remains unaffected.

It has recently been suggested that CD4 and DC-SIGN compete for gp120 binding [49]. In contrast, we have demonstrated that the primary HIV-1 receptor CD4 and DC-SIGN can bind simultaneously to gp120, establishing that the DC-SIGN binding site is distinct from the CD4 binding site on gp120 (Fig. 5.5). Similar results were obtained with a K562 transfectant expressing DC-SIGN (data not shown). This was supported by data demonstrating that CD4bs antibodies did not block the DC-SIGN-gp120 interaction (Table 5.1).

Binding of DC-SIGN to gp120 on HIV-1 is implicated to increase the local concentrations of virus particles on the DC surface in the proximity of entry receptors [5;50-52]. Here we have demonstrated that DC-SIGN binding to gp120 increases CD4 binding to gp120, suggesting that DC-SIGN induces conformational changes that favour CD4 binding. Hence, DC-SIGN may

not merely act to concentrate virus at the cell surface but may also increase CD4 binding to gp120.

Furthermore, besides a glycan shield to mask the potential vulnerable CD4 receptor binding site on gp120 to escape neutralization, conformational masking has been suggested to protect the receptor binding site [53]. This conformational masking influences the efficiency with which CD4 binding site antibodies are generated. It is tempting to speculate that this strategy to evade recognition by the immune system, might also affect the binding of the CD4 receptor itself. Therefore, DC-SIGN may play an essential role by enhancing CD4 binding to gp120. Taken together, the observed increased binding of gp120 to CD4 provides an additional explanation for DC-SIGN in enhancement of HIV-1 transmission. Future studies are required to investigate this intriguing possibility.

In summary, this study contributes to further understanding of the DC-SIGN-gp120 interaction. More importantly, these data will be valuable in the development of novel inhibitory agents to prevent capture of HIV-1 by DC-SIGN in future vaccination strategies.

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Lewis^{x} component in human milk binds DC-SIGN and inhibits HIV-1 transfer to CD4^{+} T lymphocytes

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Abstract

DC-specific ICAM3-grabbing nonintegrin (DC-SIGN), which is expressed on DCs, can interact with a variety of pathogens such as HIV-1, hepatitis C, Ebola, cytomegalovirus, Dengue virus, *Mycobacterium*, *Leishmania*, and *Candida albicans*. We demonstrate that human milk can inhibit the DC-SIGN-mediated transfer of HIV-1 to $CD4^+$ T lymphocytes as well as viral transfer by both immature and mature DCs. The inhibitory factor directly interacted with DC-SIGN and prevented the HIV-1 gp120 envelope protein from binding to the receptor. The human milk proteins lactoferrin, α-lactalbumin, lysozyme, ß-casein, and secretory leukocyte protease inhibitor did not bind DC-SIGN or demonstrate inhibition of viral transfer. The inhibitory effect could be fully alleviated with an Ab recognizing the $Lewis^x$ (Le^x) sugar epitope, commonly found in human milk. Le^x in polymeric form or conjugated to protein could mimic the inhibitory activity, whereas free Le^x sugar epitopes could not. We reveal that a Le^x motif present in human milk can bind to DC-SIGN and thereby prevent the capture and subsequent transfer of HIV-1 to $CD4^+$ T lymphocytes. The presence of such a DC-SIGN-binding molecule in human milk may both influence antigenic presentation and interfere with pathogen transfer in breastfed infants.

Introduction

DCs can capture an array of infectious agents and present their antigens to T lymphocytes. DCs express, among other receptors, the DC-specific ICAM3-grabbing nonintegrin (DC-SIGN) receptor, a C-type lectin, which contains an external calcium-dependent mannose-binding lectin domain [1;2]. DC-SIGN interacts with the envelope glycoprotein gp120 of HIV-1, HIV-2, and SIV [3-8], as well as other pathogens such as hepatitis C [9;10], Ebola [11], cytomegalovirus [12], Dengue virus [13], *Mycobacterium* [14-16], *Leishmania* [17;18], *Candida albicans* [19], and *Helicobacter pylori* [20;21]. DC-SIGN has been implicated as playing an important role in HIV-1 transmission and the establishment of infection [4;6;22-25]. The interaction of HIV-1 with DC-SIGN can lead to infection of the DCs, or alternatively the virus can be internalized into a trypsin-resistant compartment prior to undergoing transfer to its main target cells, and this mechanism has been shown to greatly enhance infection of T cells in vitro [26;27].

Mother-to-child transmission (MTCT) of HIV-1 accounts for the majority of HIV-1 infections among children [28]. Transmission can occur either in utero, intrapartum, or through breastfeeding [29]. It has been recognized that HIV-1 transmission via breastfeeding accounts for 40% of all MTCTs of HIV-1 [30;31]. Relatively little is known with regard to HIV-1 present within human milk and how the virus is transmitted from mother to child. Children exposed to HIV-1 through breastfeeding will encounter both free viral particles as well as cell-associated virus [32;33]. Macrophages in human milk are found at varying concentrations during lactation [34] and are preferentially infected with viruses that utilize the CCR5 coreceptor for viral entry (R5) and that are typically associated with HIV-1 transmission [35;36]. Mammary epithelial cells are present in human milk, and these cells are predominantly infected with viruses utilizing the CXCR4 coreceptor (X4), virus strains that seldom undergo transmission [37;38]. $CD4^+$ T lymphocytes are also present in human milk, and these cells can be infected with both R5 and X4 strains, depending on the cellular phenotype and cell activation status [39;40]. MTCT of HIV-1 via breastfeeding is likely to require the transfer of virus across a mucosal barrier or via breaches in the mucosal surface. The interaction of the HIV-1 gp120 protein with DCs, shown to be present at high concentrations in the tonsils, the upper rim of the oesophagus [41;42], and the intestinal tract [43], may therefore heighten virus capture and transmission. Although DC-SIGN expression has not been analyzed on DCs from all the above-mentioned anatomical sites, high levels of expression have been reported in tonsil material [6].

Several of the proteins present in human milk have been shown to possess antimicrobial activity. Lactoferrin [44-46], lysozyme [47], and secretory leukocyte protease inhibitor (SLPI) [48;49] have all been shown to exhibit anti-HIV-1 properties in vitro. On the contrary, ß-casein has been shown to enhance HIV-1 infection of $CD4^+$ T lymphocytes [44]. Oligosaccharides present in human milk include lactodifucotetraose; lacto-*N*-fucopentaose (LNFP) I, II and III; and monofucosyllacto-*N*-hexaose III; among others [50-52]. A number of sugar epitopes to be found in human milk are composed of the Lewis group of antigens, including Lewis^x (Le^x) and Lewis^y (Le^y) [53], which can be part of a larger oligosaccharide, regardless of whether the sugar is protein associated or not. The Le^x epitope (Galß1-4[Fucα1-3]GlcNAc-R) is also included within the LNFP III sugar moiety. This same sugar epitope has been identified in bodily fluids, including saliva, blood, and human milk and has also been shown to be present in a number of pathogens [21] and pathogen extracts [54]. Some of the human milk

oligosaccharides have been shown to protect against toxins and pathogens involved in childhood diseases [55].

Material and Methods

Cells.
The Raji control cell line and the cell lines expressing either DC-SIGN (Raji-DC-SIGN) or L-SIGN (Raji-L-SIGN) were cultured as previously described [4]. PBMCs were isolated from buffy coats by standard Ficoll-Hypaque density centrifugation, activated with phytohemagglutinin (3 µg/ml), and cultured in RPMI medium containing 10% FCS, penicillin (100 units/ml), and streptomycin (100 units/ml). On day 3 the cells underwent $CD4^+$ enrichment by incubation with CD8 immunomagnetic beads (Dynal Biotech) and were negatively selected according to the manufacturer's instructions and cultured with IL-2 (100 U/ml). DCs for the single-cycle transmission assay were generated from fresh PBMCs with cells layered on a standard Percoll gradient (Amersham Pharmacia). The light fraction with predominantly monocytes was collected, washed, and seeded in 24 well or 6 well culture plates at a density of 500,000 or 250,000 cells per well, respectively. After 60 minutes at 37℃, the adherent cells were cultured to obtain iDCs in Iscove's modified Dulbecco's medium (IMDM) with gentamicin (86 µg/ml) and 10% foetal clone serum (HyClone) supplemented with GM-CSF (500 U/ml) and IL-4 (250 U/ml). Culture medium was refreshed on day 3, with cell maturation induced at day 6 by culturing with poly(I:C) (20 µg/ml; Sigma-Aldrich). After 2 days, mature $CD14^-CD1b^+CD83^+$ DCs were obtained, washed, and utilized. The culture conditions for LuSIV cells with an integrated long terminal repeat-luciferase reporter construct have been described previously [56].

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 [57]. Subtype B molecular cloned viruses JR-CSF (R5), LAI (X4), and SF-162 (R5) and subtype B primary isolates NSI-18 (R5) and SI-19 (X4) were used in the experiments.

Human milk fractions and commercial milk products.
Internal review board approval was not required because samplings were taken from discarded material from a single time point. Human milk samples were sequentially centrifuged at 400g and 530g for 10 minutes, with pipette removal of the lipid layers. Samples were sterilized by filtration through both 0.45 µm and 0.2 µm syringe filters (Schleicher & Schuell BioScience Inc.) and stored at -80℃. Human lactoferrin (Sigma-Aldrich), bovine lactoferrin (Sigma-Aldrich), human α-lactalbumin (Sigma-Aldrich), bovine ß-casein (NIZO Food Research), human lysozyme (Sigma-Aldrich), LNFP III (Calbiochem), SLPI (Sigma-Aldrich), Le^x trisaccharide (Calbiochem), Le^x-BSA, 14-atom spacer (Calbiochem), PAA-Le^x (Syntesome), anti-human Le^x (mouse) IgM (Calbiochem), and anti-human ARA-LAM (mouse) IgM were used. All compounds were utilized at physiologically relevant concentrations by dilution in PBS containing 10% FCS.

Direct HIV-1 infection assay.
Enriched $CD4^+$ lymphocytes were plated in 96 well plates at 500,000 cells/well in IL-2-containing culture medium. Cells were incubated for 2 hours with human milk diluted in PBS containing 10% FCS and spiked with 3.7 log $TCID_{50}$/ml of HIV-1. After 2 hours the cells were washed and fresh medium added. Alternatively, after a 2-hour incubation of human milk with HIV-1, the spiked milk was diluted with PBS containing 10% FCS before addition to $CD4^+$ T lymphocytes. On day 7 of culture, viral capsid (CA-p24) levels were determined by ELISA.

DC-SIGN-mediated HIV-1 transfer assay.
The Raji and Raji-DC-SIGN cells were plated at 20,000 cells/well in a 96-well format. Dilutions of human milk or human milk compounds were made in PBS containing 10% FCS and spiked with 3.7 log

$TCID_{50}$/ml of the appropriate virus before addition to the Raji-DC-SIGN cells. As a control, PBS containing 10% FCS was spiked with the same $TCID_{50}$/ml of the corresponding virus before addition to Raji or Raji-DC-SIGN cells. After incubation the culture was washed with PBS before addition of $CD4^+$-enriched T lymphocytes at a concentration of 100,000 cells/well, with CA-p24 determined on day 7.

DC-SIGN-specific Ab binding after exposure to human milk.
Human milk was incubated with 50,000 Raji-DC-SIGN or iDCs for 15 minutes at 37℃, after which the cells were washed with TSM (20 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$) and the cells were incubated at 4℃ for 45 minutes with 5 µg/ml of the specific DC-SIGN Ab, AZN-D1 [6], AZN-D2 [12], or anti-stalk 4. Subsequently, the cells were washed and incubated with goat anti-mouse FITC for 45 minutes at 4℃. The cells were washed and resuspended in 100 µl TSM containing 0.5% BSA (Fraction V, Fatty Acid-Free; Calbiochem), and the adhesion was measured by flow cytometry (BD Biosciences).

DC-SIGN binding assay.
For preincubation of HIV-1 with human milk, a 1:4 dilution of human milk or PBS containing 10% FCS was incubated with a high virus titer (5.6 log $TCID_{50}$/ml) of SF-162 for 1 hour at 37℃ before diluting the mixture to 1:667 by addition of 270,000 Raji-DC-SIGN cells in a 24-well format. The cells were incubated for 2 hours at 37℃ before washing with PBS and addition of $CD4^+$ T lymphocytes at a concentration of 1 x 10^6 cells/ml followed by measuring CA-p24 production on day 15. For preincubation of Raji-DC-SIGN cells with human milk, a dilution of the human milk (1:4) or PBS containing 10% FCS was incubated with 270,000 Raji-DC-SIGN cells in a 24-well format for 1 hour at 37℃ before washing with PBS and addition of SF-162 (2.8 log $TCID_{50}$/ml). The cells were incubated for 2 hours before washing and adding 1 x 10^6 cells/well of $CD4^+$-enriched lymphocytes, with CA-p24 production measured on day 15.

gp120 bead adhesion assay.
Beads were prepared as previously described [6]. In short, streptavidin was covalently coupled to carboxylate-modified TransFluoSpheres (488/645 nm excitation/emission, 1.0 µm; Invitrogen Corp.). The streptavidin beads were incubated with biotinylated F(ab')2 fragment goat-anti-human IgG (6 µg/ml; Jackson ImmunoResearch Laboratories Inc.) and subsequently incubated overnight with gp120-Fc chimera. 50,000 Raji-DC-SIGN cells, iDCs, or Raji-L-SIGN cells were preincubated with human milk or milk compounds, AZN-D1 [6], AZN-D2 [12], EGTA, or mannan for 30 minutes at room temperature. The ligand-coated beads (20 beads/cell) were added to the preincubated cells and incubated for 30 minutes at 37℃, 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).

DC-SIGN-Fc binding ELISA.
This assay utilized the DC-SIGN-Fc chimera, which contained the extracellular portion of DC-SIGN (amino acids 64-404) fused at the C terminus to a human IgG1 Fc fragment as previously described [58]. Human milk or human milk compounds were diluted in 0.2 M $NaHCO_3$, coated on ELISA plates (Maxisorp plate; Nunc), and incubated overnight at 4℃ or 2 hours at 37℃. This was followed by blocking with TSM containing 1% BSA for 30 minutes at 37℃ before addition of soluble DC-SIGN-Fc (5 µg/ml) for 2 hours at room temperature; the binding was determined by incubation of a peroxidase-labelled anti-IgG1 Ab for 30 minutes at room temperature. DC-SIGN-Fc binding specificity was determined by preincubation of the DC-SIGN-Fc with either 50 µg/ml DC-SIGN-specific mouse Ab AZN-D1 [6] or 10 mM EGTA.

Single-cycle-replication transmission assay.
The assay was performed as previously described [59]. In short, mDCs and iDCs were incubated in a 96 well plate (35,000 to 50,000 DCs/well) with human milk for 30 minutes at 37℃ before addition of virus (5 ng CA-p24/well), which was incubated for 2 hours at 37℃. The DCs were washed twice with PBS before addition of 50,000 LuSIV cells. After 24 hours, LuSIV cells were harvested and resuspended in 50 µl lysis buffer (25 mM Tris-Cl 7.8, 2 mM DTT, 2 mM CDTA, 10% glycerol, 1% Triton X-100). The cells were then incubated for 45 minutes at room temperature while shaking, followed by 10 minutes centrifugation at 3,200g. The supernatant was transferred to a white-solid 96 well plate (Costar; Corning), and 150 µl of buffer (100 µg/ml BSA, 6.6 mM ATP, 15 mM $MgSO_4$, 25 mM glycylglycine) was added. One hundred

microliters of DE(-)Luciferin (Roche Diagnostics GmbH) was injected per well (0.28 mg/ml lucibuffer excluding ATP). LuSIV cells (50,000) grown without DC or HIV-1 were used to obtain the background luciferase value.

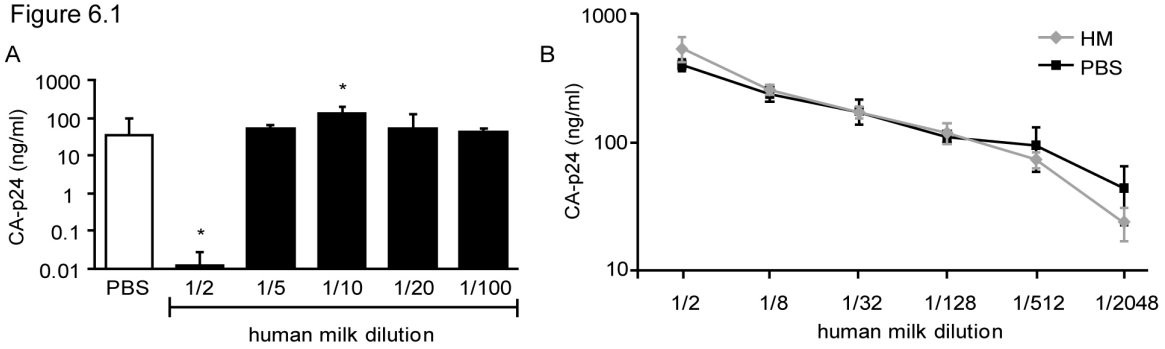
Capture assay.
To measure the capture of HIV-1 by iDCs, the cells were incubated in a 96 well plate (50,000 DCs/well) with human milk for 30 minutes at 37℃ before addition of the virus (5 ng CA-p24/well), which was incubated for 2 hours at 37℃. The iDCs were washed twice with PBS before the CA-p24 concentration was determined by standard ELISA.

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

Results

The effect of human milk on direct HIV-1 infection of CD4$^+$ T lymphocytes and DC-SIGN-mediated HIV-1 transfer.
We investigated the effect of human milk on direct infection of CD4$^+$ T lymphocytes by incubating HIV-1 in the presence of several dilutions of human milk from an HIV-1-negative donor taken 6 months into lactation. The 2-fold dilution of human milk demonstrated a significant degree of viral inhibition (>90%; $P<0.05$) in comparison with the PBS control (Fig. 6.1A), which corresponds with previous reports [44-49]. We also identify an enhancement to infection at the 1:10 dilution similar to findings from a previous study [38]. Preincubation of the same human milk sample (1:2) with HIV-1 before addition to CD4$^+$ T lymphocytes (Fig. 6.1B) showed no inhibitory effect in comparison to the PBS control, indicating that the inhibition shown in figure 6.1A is not due to a direct virucidal effect of the milk and that the effect is likely conferred on the CD4$^+$ lymphocyte. The experiments were repeated with human milk from 2 other mothers, with equivalent results obtained (data not shown).
DC-SIGN-expressing cells can enhance infection of CD4$^+$ T lymphocytes [4;8]. To study the effect of human milk on DC-SIGN-mediated transfer of HIV-1, we utilized the Raji cell line expressing the DC-SIGN receptor (Raji-DC-SIGN) [8]. The same human milk sample as used in the direct infection assay or PBS spiked with HIV-1 primary isolates was incubated with the Raji-DC-SIGN cells for 30 minutes or 2 hours, after which the cells were washed and incubated with activated CD4$^+$ T lymphocytes and the culture monitored for viral replication. Raji-DC-SIGN cells preincubated with PBS spiked with HIV-1 showed efficient transfer to CD4$^+$ T lymphocytes (Fig. 6.2A, black line). The Raji cell line not expressing DC-SIGN showed no viral transfer, demonstrating that the effect we observed is DC-SIGN dependent (data not shown). Surprisingly, preincubation of Raji-DC-SIGN cells with HIV-1-spiked human milk significantly reduced or blocked transfer of HIV-1 depending on the incubation time (Fig. 6.2A, gray line). Through testing cell viabilities, we demonstrated that the observed inhibition was not due to induced cell death of Raji-DC-SIGN by human milk (data not shown). The transfer experiment revealed that human milk blocks the transfer of HIV-1 by Raji-DC-SIGN cells irrespectively of viral coreceptor phenotype. We next performed limiting dilutions of the same human milk sample and found that R5 and X4 HIV-1 variants were completely inhibited at a 1:128 but not at a 1:512 dilution (Fig. 6.2B). Similar results were observed with the same assays using human milk from 2 other HIV-1-negative donors (data not shown).

Figure 6.1



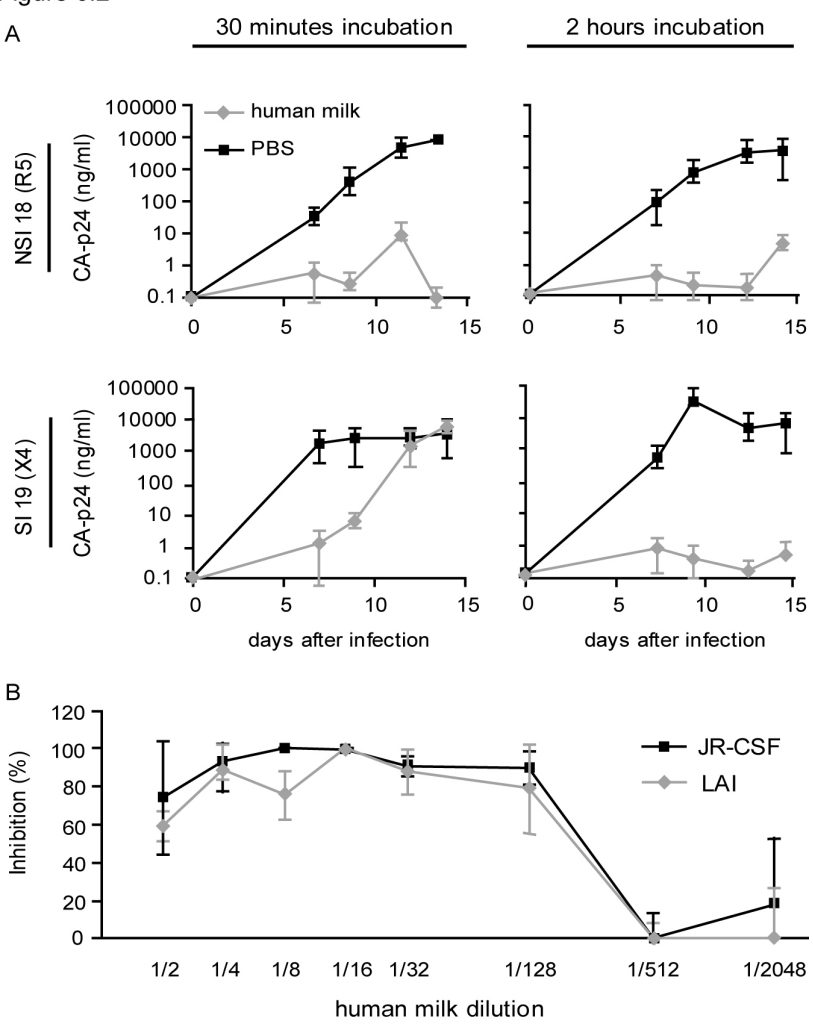
Direct infection of CD4$^+$ T lymphocytes in the presence of human milk.
(A) PBS or several dilutions of human milk from an uninfected mother were spiked with LAI (X4) and added to CD4-enriched T lymphocytes. After a 2-hour incubation, the CD4$^+$ T lymphocytes were washed, and fresh medium was added. (B) LAI (X4) was incubated with a 1:2 dilution of human milk or PBS for 2 hours, after which several dilutions were made and added to CD4$^+$ T lymphocytes. For both experiments the CA-p24 concentration was determined on day 7. * *P*<0.05 compared with the PBS control.

Human milk compound(s) bind to DC-SIGN, thereby preventing transfer of HIV-1 to CD4$^+$ T lymphocytes.

To determine whether the inhibitory effect of human milk on Raji-DC-SIGN-mediated viral transfer was caused by interaction of human milk with HIV-1 or DC-SIGN, we conducted preincubation experiments of either Raji-DC-SIGN cells or HIV-1 with human milk. To test for binding of the inhibitory factor to Raji-DC-SIGN cells, we preincubated the cells with either a known inhibitory concentration of human milk (1:4) or PBS before washing and then adding virus and subsequently CD4$^+$ T lymphocytes. Alternatively, to test binding of components in human milk to the virus, we incubated a high-titer virus stock with either PBS or human milk, after which the Raji-DC-SIGN cells were added, thereby diluting the milk to a noninhibitory concentration (1:667); CD4$^+$ T lymphocytes were then added. Preincubation of virus with human milk demonstrated a slight reduction in viral transfer compared with the PBS control (Fig. 6.3A), likely reflecting residual inhibitory effects of the human milk. In contrast, preincubation of the Raji-DC-SIGN cells with human milk provided a highly significant reduction in viral transfer compared with the PBS control (*P*<0.01; Fig. 6.3B). To test whether the observed inhibition was due to downmodulation of DC-SIGN expression, we investigated the surface expression of DC-SIGN in the presence of human milk. We demonstrate that with 2 DC-SIGN-specific mAbs, AZN-D2 and anti-stalk 4, cell surface expression was not altered, whereas the binding of AZN-D1 was reduced when DC-SIGN cells were preincubated with human milk (Fig. 6.3C). These results suggest that the inhibitory effect is mediated via the binding of factor(s) in human milk to the DC-SIGN molecule and prevention of its interaction with HIV-1 as opposed to the downmodulation of the DC-SIGN molecule at the cell surface.

To show direct binding of human milk compound(s) to DC-SIGN, we introduced 2 previously described assays [6;58], the gp120 bead adhesion assay and the DC-SIGN-Fc binding ELISA. In the gp120 bead adhesion assay, the effect of human milk on the binding of gp120-coated fluorescent beads to cellular DC-SIGN was studied. The binding of the gp120 beads to both Raji-DC-SIGN and immature DCs (iDCs) was inhibited by human milk in comparison with the control (*P*<0.01; Fig. 6.4A and B, respectively). To demonstrate DC-SIGN-specific binding, the cells were preincubated with a DC-SIGN-specific Ab (AZN-D1), the DC-SIGN-binding sugar mannan, and the Ca^{2+} chelator EGTA. These agents were found to block binding of gp120 beads to the DC-SIGN-expressing cells to the same extent as human milk (Fig. 6.4A, B). To

Figure 6.2

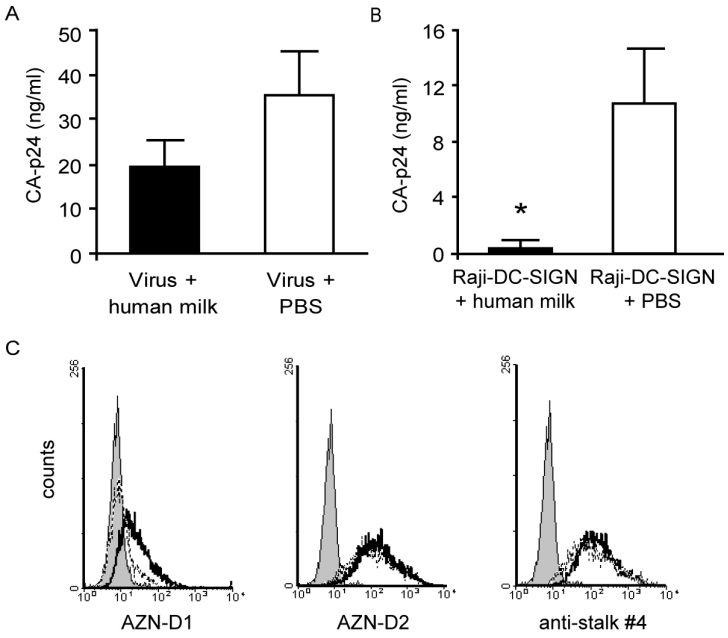


DC-SIGN–dependent transfer of HIV-1 to CD4$^+$ T lymphocytes is inhibited in the presence of human milk. (A) A 1:2 dilution of human milk of an uninfected mother or PBS was spiked with primary isolates NSI-18 (R5) or SI-19 (X4) before addition to Raji-DC-SIGN cells. After an incubation of 30 minutes or 2 hours, the cells were washed, and activated CD4$^+$ T lymphocytes were added. Viral replication was measured on days 7, 9, 12, and 14 after infection by determining CA-p24 values using a standard ELISA. The bars represent maximum and minimum CA-p24 values. (B) PBS or serial dilutions of human milk were spiked with JR-CSF (R5) or LAI (X4) before addition of Raji-DC-SIGN cells; after an incubation of 2 hours, the cells were washed with PBS, and stimulated CD4$^+$ T lymphocytes were added. At day 7, CA-p24 concentrations were determined by standard ELISA. Percent inhibition was determined in reference to the CA-p24 concentration of the corresponding spiked PBS control.

confirm the direct interaction of DC-SIGN with human milk, we performed a DC-SIGN-Fc binding ELISA where we demonstrated that DC-SIGN-Fc binding to human milk was specific, since preincubation of DC-SIGN-Fc with AZN-D1 or EGTA completely abrogated binding (P<0.01; Fig. 6.4C).

The liver and lymph node-specific homolog of DC-SIGN (liver and lymph node-specific ICAM3-grabing nonintegrin (L-SIGN)) is also capable of interacting with HIV-1 and enhancing viral infectivity [60]. To investigate the effect of human milk on the interaction of L-SIGN and gp120, we incubated Raji cells expressing the L-SIGN molecule (Raji-L-SIGN) with human milk in the gp120 bead adhesion assay, and as a control, the cells were preincubated with AZN-D1, AZN-D2 (an L-SIGN-specific Ab), mannan, and EGTA. The results demonstrated that human milk did not inhibit the interaction of gp120 with L-SIGN (Fig. 6.4D), indicating a specificity of the human milk compound for DC-SIGN.

Figure 6.3



The human milk compound(s) interact with the DC-SIGN receptor, which does not lead to DC-SIGN downmodulation.
(A) Human milk (1:4) or PBS was preincubated with a high-titer stock of LAI before adding to Raji-DC-SIGN cells at a dilution known not to inhibit viral replication. After incubation, the cells were washed, and $CD4^+$ T lymphocytes were added, with CA-p24 values measured on day 15 by standard ELISA (P>0.01). (B) Human milk (1:4) or PBS were incubated with Raji-DC-SIGN, after which the cells were washed to remove unbound human milk components before addition of LAI. After incubation, the cells were washed again, and $CD4^+$ T lymphocytes were added, with the CA-p24 values measured on day 15 by standard ELISA. * P<0.01. (C) Raji-DC-SIGN cells were incubated with TSM or human milk (1:2) before the binding of AZN-D1, AZN-D2, and anti-stalk 4 DC-SIGN-specific Abs were determined. The filled histograms represent the isotype control; the black lines represent the Ab binding without human milk preincubation; and the dotted lines represent the Ab binding after the cells were incubated with human milk.

Human milk inhibits both iDC- and mature DC–dependent transfer of HIV-1.
To study the biological relevance of the inhibitory properties of human milk, we utilized apreviously described single-cycle HIV-1 transmission assay [59] using both iDCs and mature DCs (mDCs). Either iDCs or mDCs (matured by poly(I:C)) were incubated with several dilutions of human milk before addition of HIV-1 LAI (X4) and target cells. After 24 hours, luciferase activity was measured, representing transmission of HIV-1 to the LuSIV cells. Human milk was found to inhibit HIV-1-mediated transfer by both iDCs and mDCs (Fig. 6.5A) in a dose-dependent manner, with the 2- and 5-fold dilutions showing significant inhibition for both cell types (P<0.01). Washing after the preincubation of mDCs with human milk (1:2) before addition of HIV-1 also showed a significant reduction (P<0.01) in HIV-1 transfer in comparison to the PBS control (data not shown), indicating that the observed inhibition shown in figure 6.5A was not due to a direct virucidal effect of the human milk. We also demonstrated that a 1:2 and 1:5 dilution of human milk significantly reduces HIV-1 capture by iDCs (P<0.01; Fig. 6.5B). We also tested for DC-SIGN downmodulation through cell-surface staining with the different Abs directed against DC-SIGN (AZN-D1, AZN-D2, and anti-stalk 4) in the presence or absence of human milk. Both AZN-D2 and anti-stalk 4 showed no significant difference in binding before and after human milk exposure, indicating that the DC-SIGN receptor is not downmodulated by the interaction with human milk (Fig. 6.5C), while AZN-D1 does show a reduced binding after human milk exposure. We expect that similar results would be obtained with an R5 virus and a CCR5-expressing cell, since we demonstrated that R5 and X4 viruses are equally inhibited with human milk (Fig. 6.2).

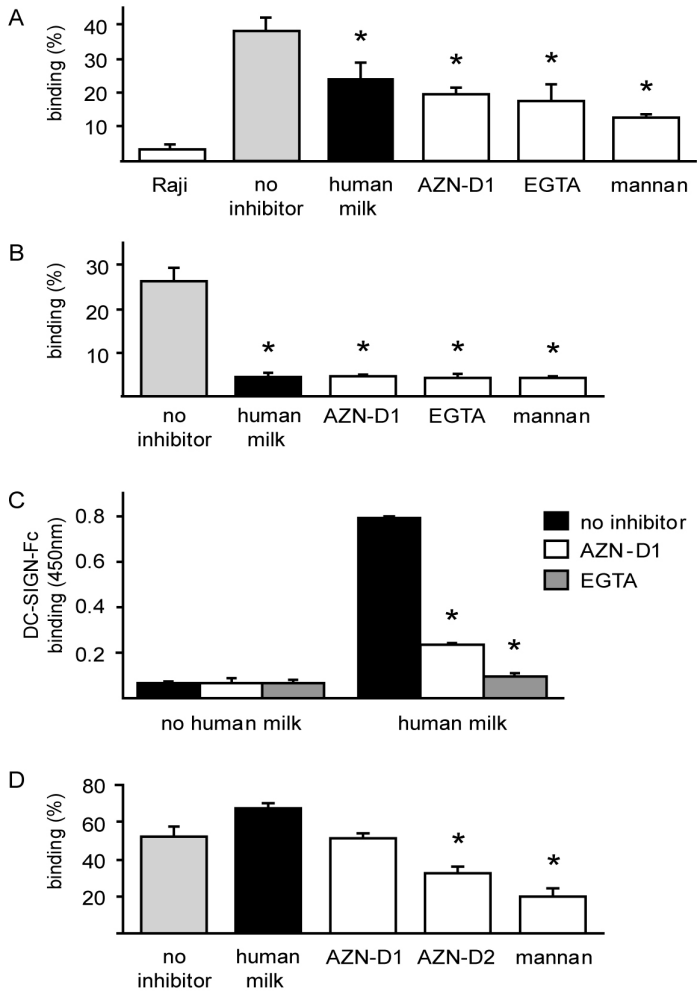
Major human milk proteins do not bind DC-SIGN nor inhibit HIV-1 transfer to CD4$^+$ T lymphocytes.

Since inhibition by human milk is present at relatively high dilutions (Fig. 6.2B), we hypothesized that one of the major proteins present in human milk may be responsible for the activity. We therefore tested human lactoferrin, bovine ß-casein, human lysozyme, human α-lactalbumin, and human SLPI, which have been shown to posses modulatory effects on HIV-1 replication in vitro [44-49]. All these compounds were tested in the gp120 bead adhesion assay and the DC-SIGN-Fc binding ELISA (Fig. 6.6). None of the tested human milk compounds could inhibit gp120 binding to Raji-DC-SIGN or iDCs (Fig. 6.6A and B, respectively). Furthermore, DC-SIGN-Fc showed no binding to the selected milk compounds (Fig. 6.6C). As a control, bovine lactoferrin was used, which has previously been shown to bind DC-SIGN and prevent viral transfer [59].

Preincubation of human milk with an anti-Le^x Ab lifted the inhibition of HIV-1 transfer.

Since DC-SIGN can bind sugars, we hypothesized that one of the abundant sugar motifs in human milk may provide the inhibitory activity [50-52]. The fact that we demonstrated that gp120 binding to L-SIGN could not be inhibited with human milk (Fig. 6.4D) and it is known that L-SIGN does not bind Le^x motifs, we predicted that Le^x could be contributing to the observed inhibition. To test our hypothesis, we preincubated human milk with an anti-Le^x IgM Ab or an

Figure 6.4

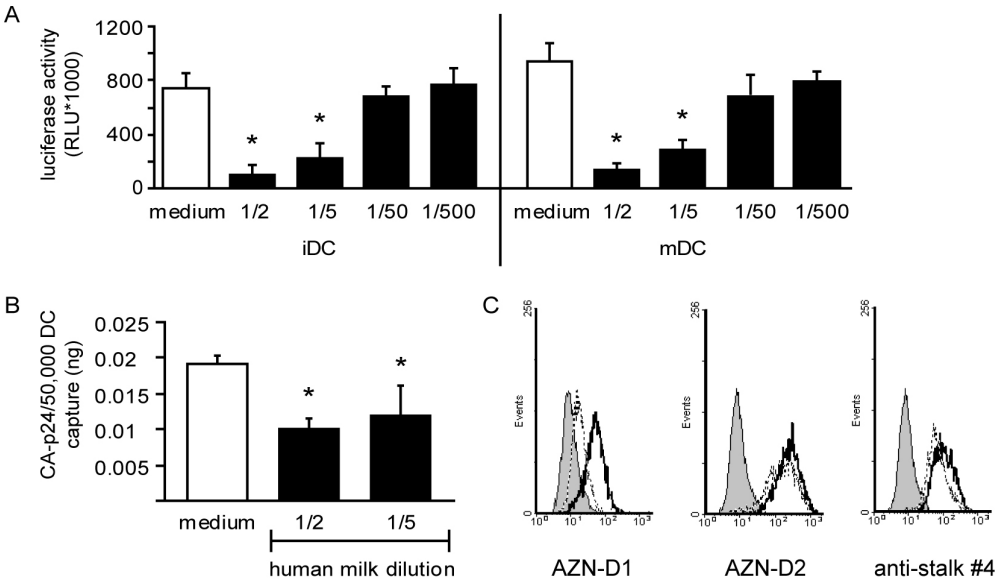


DC-SIGN-Fc binding ELISA and the gp120 bead adhesion assay demonstrate the interaction of the human milk compound(s) with DC-SIGN.
(A and B) Raji-DC-SIGN cells or iDCs, respectively, were incubated with human milk (1:20) before addition of fluorescent gp120-coated beads. DC-SIGN-positive cells and mock Raji cells were incubated with buffer as controls. To determine the specificity of the observed binding, the cells were incubated with AZN-D1, EGTA, and mannan before addition of the gp120 beads. * *P*<0.05 compared with the PBS control. (C) Human milk (1:20) was coated before addition of DC-SIGN-Fc. The specificity of the observed binding was determined by the preincubation of DC-SIGN-Fc with AZN-D1 and EGTA. ** *P*<0.01 compared with the noninhibitory control. (D) Raji cells expressing the L-SIGN receptor were incubated with buffer, human milk (1:20), AZN-D1, AZN-D2, or mannan before addition of the gp120 fluorescent beads. # *P*<0.01 compared with the binding without an inhibitor.

IgM isotype control before use in the culture transfer assay. The results demonstrated a dose-dependent lifting of the inhibitory effects of human milk on viral transfer after a preincubation with the Le^x-specific Ab but not with the control Ab (Fig. 6.7A). In contrast, the gp120 bead adhesion assay did not show reduced binding of DC-SIGN with Le^x Ab (data not shown). The most likely explanation for this difference is that the low-affinity Le^x-specific Ab was unable to block the high-avidity interaction of cellular multimeric DC-SIGN to the unknown human milk component. The DC-SIGN-Fc binding ELISA (Fig. 6.7B) demonstrated a reduction in DC-SIGN-Fc binding after preincubation of the coated human milk with the Le^x-specific Ab but again not with the control Ab. These results indicate that Le^x is critical for the human milk compound that binds to the DC-SIGN molecule.

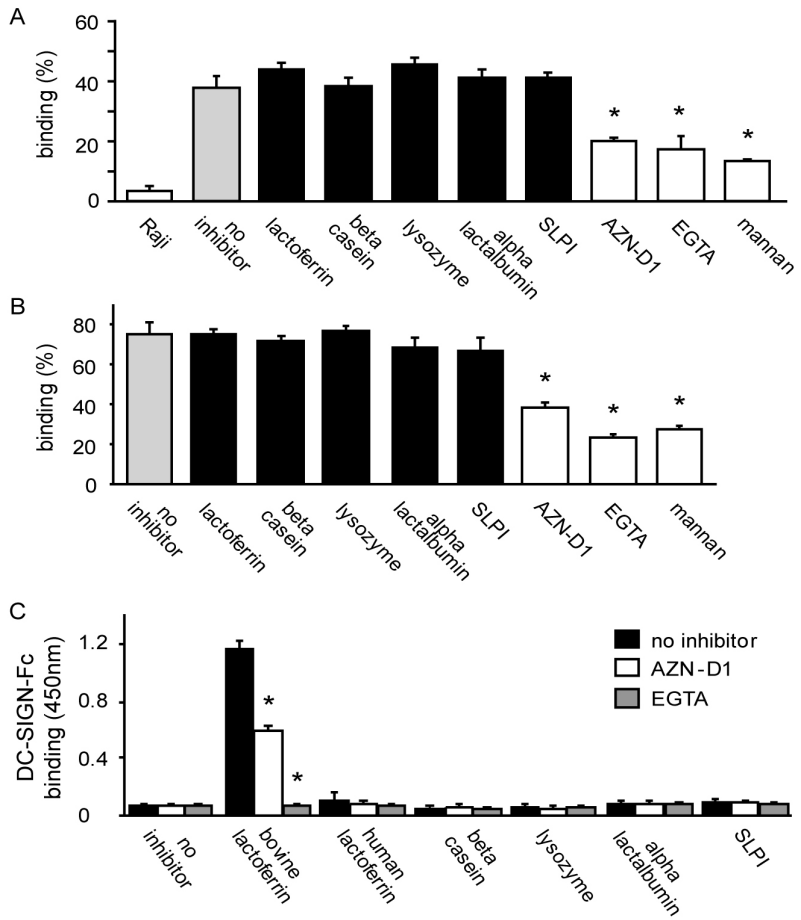
Not all Le^x-containing complexes can inhibit HIV-1 interacting with the DC-SIGN receptor.
We tested several compounds containing one or more Le^x epitopes for inhibitory and DC-SIGN binding activity. In the transfer culture assay, Le^x coupled to biotinylated polyacrylamide (PAA-Le^x) and Le^x-BSA were both able to inhibit DC-SIGN-mediated HIV-1 transfer (Fig. 6.8A). On the contrary, both LNFP III, a Le^x-containing oligosaccharide present in human milk, and the Le^x trisaccharide itself were unable to prevent HIV-1 transfer (Fig. 6.8A), even though LNFP III has been shown previously to bind DC-SIGN [61]. The compounds were also tested in the gp120 bead adhesion assay, but none of the tested compounds were able to block the interaction between DC-SIGN and gp120 (data not shown). Most likely, PAA-Le^x and Le^x-BSA are able to block the trimeric gp120 interaction, while binding of DC-SIGN to monomeric gp120

Figure 6.5



Human milk inhibits the transfer of HIV-1 by iDCs and mDCs.
(A) Both iDCs and mDCs from the same donor were incubated with several dilutions of human milk for 30 minutes before addition of LAI (X4). After 2 hours the cells were washed, and LuSIV cells were added; after 24 hours the LuSIV cells were washed, and the luciferase activity was determined as described in Methods. The asterisks represent statistical differences in infections (P<0.05). (B) After an incubation of iDCs with human milk or PBS, the cells were washed and LAI was added. After an incubation of 2 hours, the cells were washed again, and captured CA-p24 levels were monitored via ELISA. ** P<0.05 compared with the corresponding control value for both experiments. (C) iDCs were incubated with TSM or human milk (1:2) before the binding of AZN-D1, AZN-D2, and anti-stalk 4 DC-SIGN-specific Abs were determined. The filled histograms represent the isotype control; the black lines represent the Ab binding without human milk preincubation; and the dotted lines represent the Ab binding after the cells were incubated with human milk.

Figure 6.6



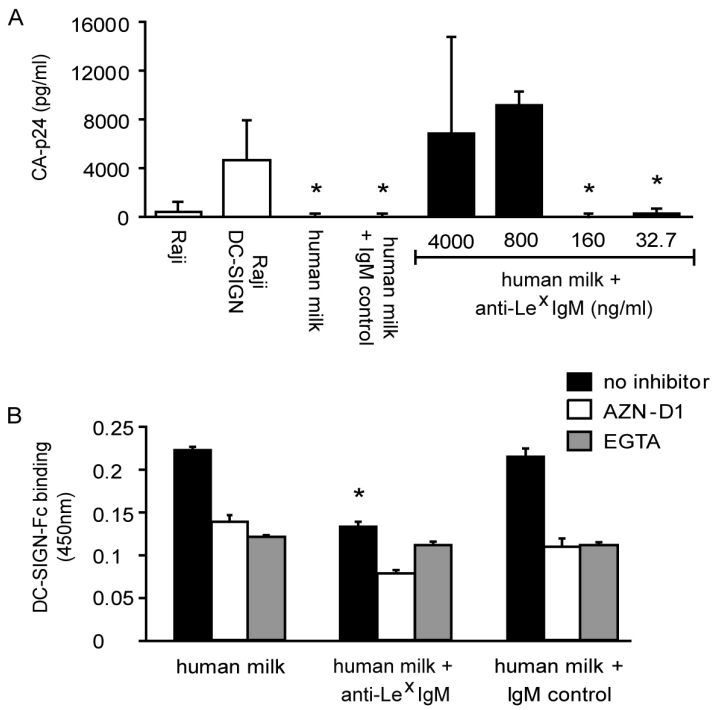
The major milk proteins are not responsible for the inhibitory effect of human milk.
(A and B) Raji-DC-SIGN cells or iDCs were incubated with the major milk proteins before addition of fluorescent gp120-coated beads; control cells were incubated with buffer. To determine the specificity of the observed binding, the cells were incubated with AZN-D1, EGTA, and mannan before addition of the gp120 beads. The asterisks represent $P<0.01$ compared with noninhibitory control. (C) The major milk proteins were coated on ELISA plates, and DC-SIGN-Fc binding was measured. To determine the specificity of the observed binding, the DC-SIGN-Fc was preincubated with AZN-D1 and EGTA. * $P<0.01$ compared with both the AZN-D1 and EGTA control. In all experiments the major proteins were diluted to a 1:20 dilution of their physiological concentration in human milk.

expressed on the fluorescent beads is still possible. Due to the inability to coat saccharides onto plates in the DC-SIGN-Fc binding ELISA, we only tested Le^x-BSA and the BSA control. Le^x-BSA indeed demonstrates binding to DC-SIGN-Fc, whereas BSA showed no binding, indicating that the DC-SIGN-Fc binding is Le^x specific (Fig. 6.8B).

Discussion

C-type lectins, such as the DC-SIGN molecule, expressed on DCs have been postulated to play an important role in HIV-1 transmission and the establishment of initial HIV-1 infection [1;2;4;6;22-25]. The interaction of DC-SIGN with the HIV-1 gp120 surface antigen can result in the efficient presentation of the virus to its target cells and can greatly heighten infection and virus replication [4;6;22-25]. This mechanism is likely to play a role in MTCT of HIV-1 through breastfeeding, since virus transfer is required across a mucosal barrier. Virus present in human milk will likely encounter DCs situated at high concentrations within the tonsils, the oesophagus [41;42], and the intestines [43] that will thereby aid virus capture and subsequent transfer to $CD4^+$ lymphocytes at the site of infection or in localized lymph nodes. In this study we demonstrate that human milk has a strong inhibitory effect on the DC-SIGN-mediated transfer

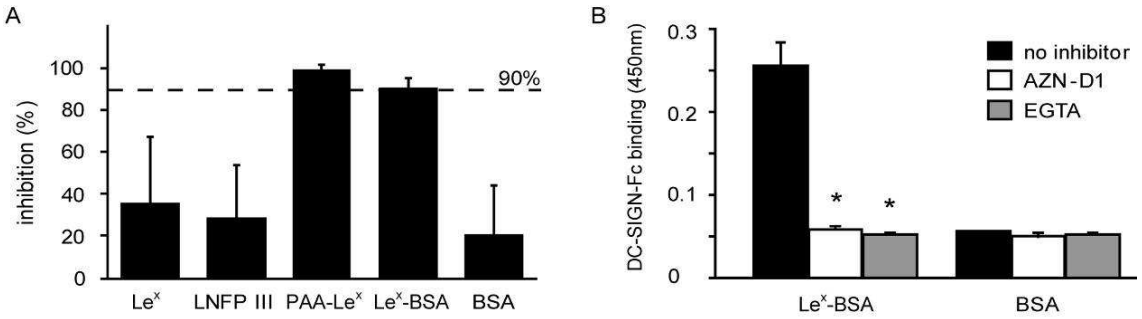
Figure 6.7



Incubation of human milk (1:20) with Le^x IgM Ab relieves the inhibitory properties of human milk on DC-SIGN-mediated transfer of HIV-1 to CD4$^+$ T lymphocytes.
(A) A 1:200 dilution of human milk was incubated alone, with IgM control Ab (4,000 ng/ml), or with serial dilutions of Le^x IgM Ab (4,000 to 32.7 ng/ml) before addition of Raji-DC-SIGN cells. LAI was added, and following a short incubation, the cells were washed, and activated CD4$^+$ T lymphocytes were added, with CA-p24 values determined at day 7. * *P*<0.05 compared with the Raji-DC-SIGN control. (B) Human milk (1:200) was coated and preincubated with anti-Le^x IgM Ab (4,000 ng/ml) or an IgM control Ab (4,000 ng/ml) before addition of DC-SIGN-Fc to determine binding. DC-SIGN-Fc was preincubated with AZN-D1 and EGTA to determine the specificity of the observed binding. ** *P*<0.01 compared with the human milk binding without Ab present.

virus replication [4;6;22-25]. This mechanism is likely to play a role in MTCT of HIV-1 through breastfeeding, since virus transfer is required across a mucosal barrier. Virus present in human milk will likely encounter DCs situated at high concentrations within the tonsils, the oesophagus [41;42], and the intestines [43] that will thereby aid virus capture and subsequent transfer to CD4$^+$ lymphocytes at the site of infection or in localized lymph nodes. In this study we demonstrate that human milk has a strong inhibitory effect on the DC-SIGN-mediated transfer of HIV-1 to CD4$^+$ T lymphocytes, with R5 and X4 viruses being inhibited to the same extent. A component in human milk is interacting with the DC-SIGN molecule that prevents HIV-1 from binding to the receptor. We showed that lactoferrin, α-lactalbumin, lysozyme, ß-casein, and SLPI were not responsible for the observed inhibition of HIV-1 interaction with DC-SIGN, even though these proteins can alter the direct infection of CD4$^+$ T lymphocytes in vitro [44-49]. Our results demonstrating that multimeric and protein-associated Le^x motifs can mimic the inhibitory activity of human milk and that the activity could be alleviated with a mAb recognizing the Le^x epitope suggest that a Le^x component in human milk is providing for the inhibition.
In the majority of our experiments, we utilized the DC-SIGN-expressing cell line (Raji-DC-SIGN) and the control Raji cell lacking DC-SIGN in order to demonstrate the DC-SIGN specificity of our results [8]. We have also shown that human milk inhibits transfer of HIV-1 by both iDCs and mDCs, illustrating the biological relevance of the phenomenon, again with the inhibition being due to prevention of binding rather than DC-SIGN downmodulation. The expression levels of DC-SIGN have previously been shown to be higher on iDCs than mDCs [62]; however, the human milk inhibited viral transfer by both cell types. This result indicates that the observed inhibition of mDC-dependent transfer may reflect the ability of human milk to block transfer of HIV-1 by other C-type lectins, such as the mannose receptor or the unidentified trypsin-resistant receptor [63]. Whether the same Le^x-containing compound(s) or whether other sugar motifs in human milk can bind to other C-type lectin receptors remains to

Figure 6.8



Multimeric and protein-associated Le^x inhibits DC-SIGN-mediated viral transfer.
(A) Several Le^x-containing compounds showed a difference in their ability to block DC-SIGN-dependent transfer of HIV-1 to CD4$^+$ T lymphocytes. The Le^x trisaccharide, LNFP III, PAA-Le^x, Le^x-BSA, and control BSA were tested in the Raji-DC-SIGN culture transfer assay at concentrations of 10 µg/ml. The inhibition is depicted as a percentage of the Raji-DC-SIGN incubated with PBS. (B) Le^x-BSA and BSA as a control were coated before addition of DC-SIGN-Fc to determine the binding. DC-SIGN-Fc was preincubated with AZN-D1and EGTA to determine the specificity of the observed binding. * *P*<0.01 compared with both the AZN-D1 and EGTA control.

be determined. We did demonstrate, however, that the binding of HIV-1 to the DC-SIGN homologue L-SIGN is not inhibited by human milk. Our result corresponds with the previous observation that Le^x present in *Schistosoma mansoni* egg antigens [54] is able to interact with DC-SIGN but not with L-SIGN [64], reconfirming Le^x as a candidate for the observed inhibition of DC-SIGN-mediated transfer of HIV-1.

PAA-Le^x and Le^x-BSA successfully inhibited the DC-SIGN-mediated transfer of HIV-1. Conversely, both monomeric Le^x trisaccharide and LNFP III, a human milk sugar, do not inhibit viral transfer. Even though LNFP III has been shown previously to bind to DC-SIGN [61], this interaction does not lead to sufficient blocking of the HIV-1 interaction with DC-SIGN nor prevent viral transfer to CD4$^+$ T lymphocytes. Multimeric Le^x was not able to inhibit gp120-coated beads binding to cellular DC-SIGN, possibly due to the high avidity of this interaction or the difference in interaction of the monomeric expression of gp120 on the fluorescent beads in comparison to trimeric expression of gp120 on the HIV-1 particle. The observed difference in the ability of Le^x Ab to block the inhibitory effect of human milk in the Raji-DC-SIGN transfer assay and the ELISA assay is possibly the result of differences in DC-SIGN configuration. On the cell surface, DC-SIGN is present as a tetramer, whereas in the DC-SIGN-Fc ELISA, the receptor is most likely a monomer or dimer, possibly leading to a weaker interaction. These differences in the configurations of the DC-SIGN molecules tested in the different assays suggest that caution should be applied when interpreting the results. It has been reported in previous studies that the Le^x and the gp120 binding sites are distinct but overlapping [54;58;65]. An explanation for our observed inhibition by human milk could be that the larger compounds block the interaction of gp120 and DC-SIGN through steric hindrance by the larger tail. DC-SIGN forms a tetrameric structure on the cell surface [1] which could also cause occlusion of the gp120 binding site by interaction of several Le^x residues with different monomeric DC-SIGN receptors. Most likely the interaction of several Le^x motifs with the tetrameric DC-SIGN leads to a much stronger interaction of the compound and again prevents sufficient binding of gp120 and subsequent viral transfer.

It has been previously demonstrated that DC-SIGN can interact with an array of other pathogens, including hepatitis C [9;10], Ebola [11], cytomegalovirus [12], and Dengue virus [13], as well as *Mycobacterium* [14-16], *Leishmania* [17;18], *Candida albicans* [19], and *Helicobacter pylori* [20;21]. Interaction of Le^x-containing human milk compounds with DC-SIGN

may influence the immune responses mounted in the child to incoming pathogens by preventing their interaction with the DCs and thereby prevent the presentation of pathogen-specific antigens and the subsequent activation of $CD4^+$ T lymphocytes. This may be a mechanism whereby levels of immune activation are buffered in a newborn to prevent overstimulation of the immune system or to skew specific immune responses in a certain direction. Indeed, it has previously been reported that mice immunized intranasally with LNFP III-HSA demonstrated a stronger induction of Th2-type immune responses versus Th1 [66]. This result corresponds with the fact that Le^x-positive *Helicobacter pylori* interactions with DC-SIGN can block Th1-induced cell responses, which results in a relative enhancement of the Th2 cell response [21], suggesting that Le^x compounds can influence the immune responses generated and that Le^x compounds in human milk can function as immunomodulatory factors. It will therefore be interesting to investigate whether breastfed versus bottle-fed infants have differences in their immune responses mounted against orally transmitted pathogens. It has also been reported that the baseline activation of lymphocytes is higher in bottle-fed than in breastfed infants [67]. A number of explanations have been hypothesized, including differences in exposure to foreign proteins or a higher variety in intestinal microflora. Another possibility is that human milk compounds containing the Le^x motif buffers the immune response by binding DC-SIGN and that these compounds are absent in formula or cow milk.

Future work will have to determine the biological significance of the interaction of the Le^x-containing compounds with DC-SIGN with regard to the stimulation of the immune system and the relevance for MTCT of HIV-1 and other pathogens. Further identification and characterization of the specific Le^x-containing compound(s) interacting with DC-SIGN may have major implications for the development of antimicrobial agents aiming at preventing HIV-1 transmission not only through breastfeeding but also through sexual activity.

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7

Interactions of DC-SIGN with Mac-1 and CEACAM1 regulate contact between dendritic cells and neutrophils

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Abstract

Early during infection neutrophils are the most important immune cells that are involved in killing of pathogenic bacteria and regulation of innate immune responses at the site of infection. It has become clear that neutrophils also modulate adaptive immunity through interactions with dendritic cells (DCs) that are pivotal in the induction of T cell responses. Upon activation, neutrophils release TNF-α and induce maturation of DCs that enables these antigen-presenting cells to stimulate T cell proliferation and to induce T helper 1 polarization. DC maturation by neutrophils also requires cellular interactions that are mediated by binding of the DC-specific receptor DC-SIGN to Mac-1 on the neutrophil. Here, we demonstrate that also CEACAM1 is an important ligand for DC-SIGN on neutrophils. Binding of DC-SIGN to both CEACAM1 and Mac-1 is required to establish cellular interactions with neutrophils. DC-SIGN is a C-type lectin that has specificity for Lewis^x, and we show that DC-SIGN mediates binding to CEACAM1 through Lewis^x moieties that are specifically expressed on CEACAM1 derived from neutrophils. This indicates that glycosylation-driven binding of both Mac-1 and CEACAM1 to DC-SIGN is essential for interactions of neutrophils with DCs and enables neutrophils to modulate T cell responses through interactions with DCs.

Introduction

Neutrophils are important in early immunity to bacterial infection. The early arrival of neutrophils at the site of infection depends on their ability to rapidly respond to chemotactic stimuli released by bacteria and inflamed epithelium. At the infection site the main function of neutrophils involves phagocytosis of bacteria and killing of phagocytized and extracellular bacteria by anti-bacterial enzymes and reactive oxygen species [1]. Besides this role in innate immune defence, neutrophils have also been implicated in the modulation of adaptive immune responses. Through the release of chemokines such as MIP-1α, MIP-3α and MIP-3β and chemotactic peptides such as α-defensin neutrophils are able to selectively attract other immune cells like T cells, monocytes, macrophages and dendritic cells (DCs) [2-4]. Moreover, in mice, effective T-cell responses against *Candida albicans*, *Legionella pneumophila*, and *Helicobacter pylori* depend on the presence of neutrophils [5-7]. Depletion of neutrophils abolishes polarization of T cells towards T helper 1 (Th1) cells and results in development of ineffective Th2 responses [5-7]. Recently, we have shown in humans that neutrophils may modulate T-cell responses through interactions with DCs [8]. DCs are antigen-presenting cells that play a pivotal role in the induction of T-cell responses, as they capture pathogens in the periphery, carry these to the lymph nodes, and present peptides derived from these pathogens to T cells [9]. DCs require maturation signals to effectively stimulate T cells. We and others have shown that activated neutrophils are able to induce maturation of DCs [8;10]. Furthermore, the cross-talk with neutrophils induces DCs to strongly activate T-cell proliferation and to instruct Th1 polarization [8]. This indicates that neutrophils may regulate adaptive immune responses indirectly through interactions with DCs.

We have reported previously that the interaction between DCs and neutrophils is mediated by DC-SIGN and Mac-1 (CD11b/CD18) [8]. Here, we describe that DC-SIGN not only binds Mac-1, but also CEACAM1 on neutrophils. Similar to Mac-1, CEACAM1 is a strong ligand of DC-SIGN, and binding of DC-SIGN to both Mac-1 and CEACAM1 is required to establish cellular interactions with neutrophils. DC-SIGN is a C-type lectin, and has specificity for high-mannose and Lewis^x moieties [11-13]. DC-SIGN mediates interactions of DCs with ICAM-2 on endothelial cells and with ICAM-3 on T cells through high-mannose moieties that are expressed on these counterstructures [13;14]. In contrast, CEACAM1 has recently been described to contain Lewis^x moieties on neutrophils [15], and interactions between DC-SIGN and neutrophil CEACAM1 are completely mediated by these Lewis^x moieties. This indicates that interactions of DC-SIGN with Mac-1 and CEACAM1 define a novel pathway of cellular adhesion between DCs and neutrophils that may play a crucial role in bridging the innate and the adaptive immune response.

Materials and methods

Antibodies and reagents.

The following monoclonal antibodies were used: 12A2 (mouse IgG1 isotype control) [16], F3 (mouse IgM isotype control, Calbiochem, La Jolla, CA), SPV-L7 (LFA-1) [17], KIM225 (Mac-1, kind gift of M. Robinson, Celltech Inc., Slough, UK), CLB-gran/10 (CEACAM1, CEACAM3 and CEACAM6, Sanquin, Amsterdam, Netherlands), col-1 (CEACAM3, Beckton Dickinson, San Jose, CA), 9A6 (CEACAM6,

Genovac GmbH, Freiburg, Germany), 6H3 (Lewis^x, kind gift of B.J. Appelmelk, VU University Medical Centre, Amsterdam, Netherlands), AZN-D1 (DC-SIGN) [13] and AZN-D2 (DC-SIGN and L-SIGN) [13]. DC-SIGN-Fc consists of the extracellular portion of DC-SIGN (aa residues 64-404) fused at the C terminus to the Fc domain of human IgG1 [18]. DC-SIGN-Fc was produced in Chinese hamster ovary K1 cells after transfection with the DC-SIGN-Sig-pIgG1-Fc vector (20 µg). We used ICAM-3-Fc (kind gift of D.L. Simmons, Celltech Inc., Slough, UK) that contains an Fc-domain of the same isotype as a negative control (control-Fc).

Cells.
Stable transfectants of K562 expressing wildtype or mutant DC-SIGN were obtained by electroporation of pRC-CMV-DC-SIGN (400 V, 960 µF) as described [18;19]. EBV-DC-SIGN and EBV-L-SIGN were generated by lentiviral transduction of EBV cells by LV-DC-SIGN and LV-L-SIGN, respectively [20]. Lipofectamin (Invitrogen, Carlsbad, CA) transfection of CHO cells with pRC-CMV-DC-SIGN was used to create CHO-DC-SIGN. Neutrophils were isolated from fresh blood of healthy volunteers. After Ficoll gradient centrifugation neutrophils were cleared from contaminating erythrocytes using erylysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA). Isolated neutrophils were over 95% pure (data not shown). Immature DCs were cultured from monocytes as described [21;22]. Briefly, monocytes were isolated from buffy coats of healthy donors by Ficoll centrifugation and MACS sorting for the monocyte marker CD14 (Miltenyi Biotec, Bergisch Gladbach, Germany). Isolated monocytes were cultured for seven days on RPMI 1640 10% FCS in the presence of IL-4 (600 U/ml) and GM-CSF (800 U/ml) to obtain 95% pure cultures of immature DCs. Immature DCs expressed high levels of DC-SIGN, low levels of CD80, CD86 and MHC class II and did not express the DC maturation marker CD83 (data not shown).

Immunoblotting.
Neutrophils were lyzed in lysis buffer (1% Triton-X-100, 10 mM TEA pH 8.2, 150 mM NaCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$) containing a cocktail of EDTA-free protease inhibitors (Roche Diagnostics, Penzberg, Germany) for 1h at 4℃. CEACAM1 and Mac-1 were immu noprecipitated from neutrophil lysates with anti-CEACAM1 and anti-Mac-1 antibodies coated onto protA sepharose beads (CL-4B, Pharmacia, Uppsala, Sweden). Immunoprecipitated CEACAM1 was run on SDS-PAGE (7% gel, reduced conditions), transferred onto blot, and stained with DC-SIGN-Fc and secondary peroxidase-conjugated goat anti-human antibodies (Jackson ImmunoResearch Lab. Inc., West Grove, PA) to determine DC-SIGN-Fc adhesion to CEACAM1 and Mac-1. Similarly, DC-SIGN ligands were immunoprecipitated with DC-SIGN-Fc coated onto protA sepharose beads (CL-4B, Pharmacia), and immunoblotted with anti-CEACAM1 and anti-Mac-1 antibodies and secondary peroxidase-conjugated goat anti-mouse antibodies (Jackson ImmunoResearch Lab. Inc.).

ELISA.
The ELISA to analyze DC-SIGN-Fc binding to native ligands from neutrophils was performed as described [8]. Briefly, goat anti-mouse antibodies (4 µg/ml, Jackson Immunoresearch Lab. Inc.) were coated onto ELISA plates (NUNC maxisorp, Nalge Nunc Int., Rochester, NY) for 1h at 37℃ followed by incubation with TSM (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$) 1% bovine serum albumin (BSA) for 30min at 37℃. Next, the EL ISA plate was incubated for 1h at 37℃ with mouse antibodies (1 µg/ml) directed against the indicated antigens, washed, and incubated for 18h at 4℃ with neutrophil lysate (20×10^6cells/ml) to allow capture of neutrophil antigens. DC-SIGN-Fc (1 µg/ml) was added and allowed to bind for 2h at room temperature. Unbound DC-SIGN-Fc was washed away and adhesion was determined using a peroxidase-conjugated goat anti-human Fc antibody (Jackson Immunoresearch Lab. Inc.) that was incubated for 30min at room temperature. Similarly, the expression of Lewis^x on neutrophil-derived antigens was determined using anti-Lewis^x antibodies and secondary goat anti-mouse IgM antibodies (Nordic Immunologic Laboratories, Tilburg, Netherlands).

Fluorescent bead adhesion.
Neutrophil-derived DC-SIGN ligands were coated onto carboxylate-modified TransFluoSpheres (488/645 nm, 1.0 µm, Molecular Probes, Eugene, OR) as described previously [8;23]. Briefly, streptavidin was covalently coupled onto TransFluoSpheres as described by manufacturer. To enable coupling of DC-SIGN ligands from neutrophils, streptavidin-coated beads were allowed to bind to

biotinylated goat anti-mouse Fc Fab_2 fragments (10 µg/ml, Jackson ImmunoResearch Lab. Inc.) for 2h at 37℃ in PBS 0.5% BSA. Subsequently, beads were washed, incubated for 18h at 4℃ with mouse antibodies directed against CEACAM1 or Mac-1, washed again, and incubated for 48h at 4℃ with neutrophil lysate (20×10^6 cells/ml) to capture CEACAM1 or Mac-1 onto the beads. After preincubation with blocking anti-DC-SIGN and anti-L-SIGN antibodies (20 µg/ml), mannan (50 µg/ml) or EGTA (10 mM) for 10min at room temperature in TSM 0.5% BSA, cells were incubated with ligand-coated beads for 45 min at 37℃. After washing, bead adhesion to the cells was analyzed by flow cytometry (FACS Calibur, Beckton Dickinson, San Jose, CA).

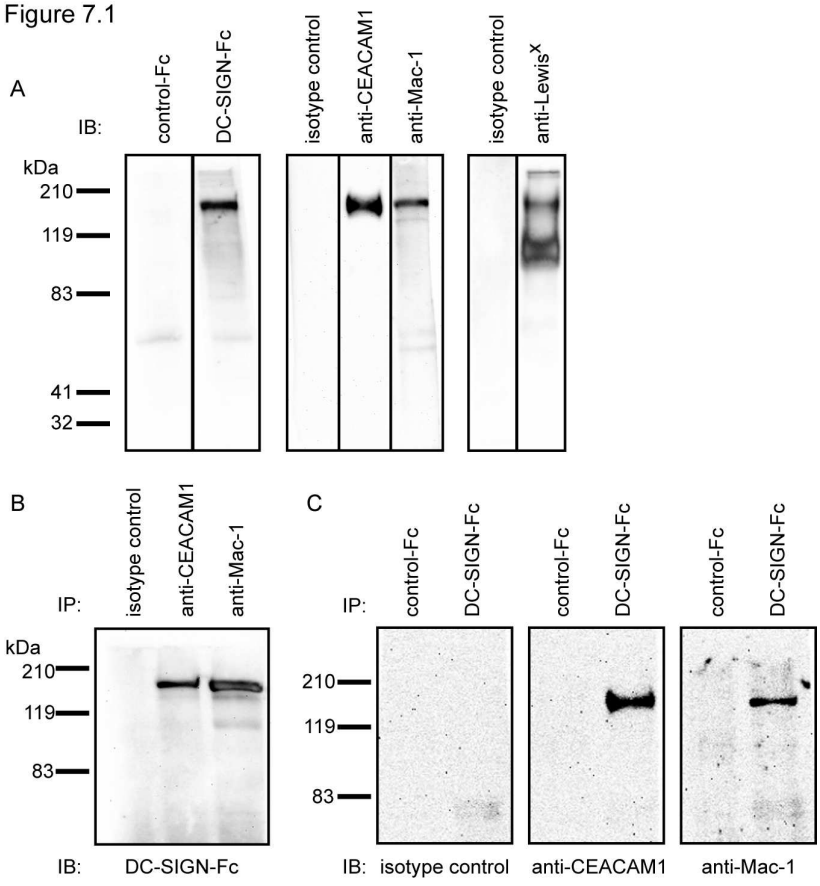
Cell-cell adhesion.

To examine cellular interactions of DC-SIGN with neutrophils CHO and CHO-DC-SIGN were grown overnight to confluent cultures in flat-bottom 96 well plates. Neutrophils were labelled with the green fluorescent dye Calcein-AM (1 µM, Molecular Probes) for 15min at 37℃ and incubated with mock and DC-SIGN transfected CHO cells in TSM 0.5% BSA for 2h at 37℃. To determine specific adhesion the CHO transfectants were preincubated with anti-DC-SIGN, anti-Mac-1 or anti-CEACAM1 antibodies (20 µg/ml) for 30 min at 37℃. Unbound neutrophils were washed away, and remaining cells were lyzed in lysis buffer (50 mM Tris-HCl, pH 8.0, and 0.1% SDS). Cell-cell adhesion was measured by fluorimetry of cell lysates at 488 nm (Fluostar Galaxy, BMG Labtechnologies Inc., Durham, NC). Cell-cell adhesion was calculated as the percentage of total added neutrophils that was considered as 100% adhesion.

Co-culture experiments.

Neutrophils were pulsed for 1h with LPS (100 ng/ml), rigorously washed, and then incubated with allogenic immature DCs at a ratio of 3:1 for 18h at 37℃ in RPMI 1640 10% FCS. Maturation of DCs was assessed by flow cytometry analysis of the expression of the DC maturation marker CD83, and analysis of the production of IL-12p40 in the supernatant using sandwich ELISA according to the manufacturer's protocol (Biosource, Nivelles, Belgium).

Figure 7.1



CEACAM1 is an alternative DC-SIGN ligand on neutrophils.
(A) Neutrophil lysate was immunoblotted with control-Fc, DC-SIGN-Fc, mouse IgG1 isotype control antibodies, anti-CEACAM1, -3 and -6 antibodies (on immunoblot the anti-CEACAM1, -3 and -6 specific antibody CLB-gran/10 only recognizes CEACAM1 [53]), anti-Mac-1 antibodies, mouse IgM isotype control antibodies and anti-Lewis^x antibodies. (B) Neutrophil lysate was immunoprecipitated using mouse IgG1 isotype control antibodies, anti-CEACAM1, -3 and -6 antibodies and anti-Mac-1 antibodies, and immunoblotted with DC-SIGN-Fc. (C) Immunoblots with mouse IgG1 isotype control antibodies, anti-CEACAM1, -3 and -6 antibodies and anti-Mac-1 antibodies were performed on neutrophil lysate that had been immunoprecipitated using control-Fc and DC-SIGN-Fc.

Results

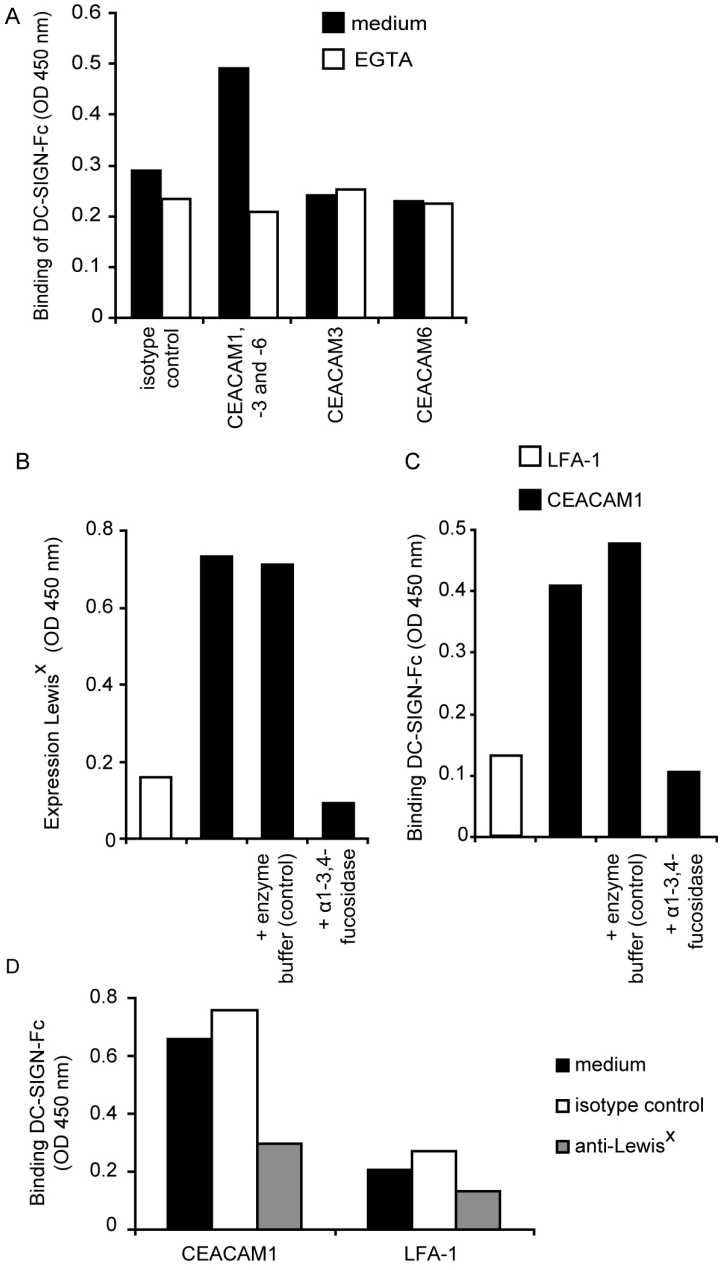
DC-SIGN binds Mac-1 and CEACAM1 on neutrophils.

Recently, we have demonstrated that DCs interact with neutrophils through binding of DC-SIGN to Mac-1, and that the DC-neutrophil interaction results in maturation of DCs and predisposes DCs to DC1 that induce Th1 responses [8]. DC-SIGN has specificity for Lewis^x moieties that are expressed at high levels on neutrophils [11;24]. These Lewis^x structures are present on few proteins on neutrophils, in particular on proteins of 100 and 160 kD (Fig. 7.1A). Mac-1 is a carrier molecule of Lewis^x moieties on neutrophils and contains Lewis^x on both the α-chain (CD11b) of 160 kD and the β-chain (CD18) of 100kD [25;26]. We have shown before that binding of DC-SIGN to neutrophil Mac-1 is mediated in part through its Lewis^x moieties [8]. Neutrophils express Lewis^x not only on Mac-1 but also on CEACAM1 [15;26]. Therefore, we investigated whether CEACAM1 is also an important ligand of DC-SIGN on neutrophils. We have shown that DC-SIGN binds a 160 kD ligand on neutrophils [8], of which the molecular weight not only corresponds to that of the previously identified DC-SIGN-ligand Mac-1, but also to that of the similarly-sized glycoprotein CEACAM1 (Fig. 7.1A). Although both the α- and β-chain of Mac-1 express Lewis^x, DC-SIGN specifically binds the α-chain of Mac-1, suggesting that expression of Lewis^x does not fully predict binding of DC-SIGN [8]. To investigate whether DC-SIGN binds both CEACAM1 and Mac-1, we immunoprecipitated these proteins from neutrophils using anti-CEACAM1 and anti-Mac-1 antibodies and analyzed binding to DC-SIGN-Fc on blot (Fig. 7.1B). DC-SIGN bound to CEACAM1 as well as to the α-chain, but not to the β-chain, of Mac-1 (Fig. 7.1B). The anti-CEACAM1 antibody also recognizes the homologous molecules CEACAM3 and -6 of 30 and 90 kD respectively, but they are not bound by DC-SIGN (Fig. 7.1B). Thus, both CEACAM1 and Mac-1 are ligands of DC-SIGN on neutrophils, and they may compete for binding to DC-SIGN. To examine whether DC-SIGN binds both ligands simultaneously, neutrophil lysate was immunoprecipitated with DC-SIGN-Fc, and analyzed on immunoblot for the presence of CEACAM1 and Mac-1. We were able to detect CEACAM1 as well as Mac-1 on immunoblot (Fig. 7.1C), demonstrating that under these settings both DC-SIGN ligands do not outcompete each other. This indicates that interactions between neutrophils and DCs require binding of DC-SIGN to both CEACAM1 and Mac-1.

CEACAM1 interacts with DC-SIGN through Lewis^x moieties.

We have recently developed an ELISA-based bindings assay that enables rapid screening of DC-SIGN-Fc binding to multiple potential ligands isolated from a native source [8]. Neutrophils express CEACAM1, -3, -6 and -8 of the immunoglobulin family of CEA-related proteins [27]. We were able to detect CEACAM1, -3 and -6 in neutrophil lysate using sandwich ELISA (data not shown). Capture of CEACAM1, -3 and -6 from neutrophil lysate and detection with DC-SIGN-Fc showed that DC-SIGN binds CEACAM1 and possibly to the homologous molecules CEACAM3 and -6 (Fig. 7.2A). Binding of DC-SIGN-Fc was not observed to either CEACAM3 or -6 that was captured from neutrophil lysate using specific antibodies (Fig. 7.2A), demonstrating that only CEACAM1 is a ligand of DC-SIGN. The carbohydrate recognition domain of DC-SIGN has specificity for high-mannose moieties and Lewis antigens [11;12]. As has been reported, CEACAM1 from neutrophils contains Lewis^x moieties [15;26]. To examine the contribution of Lewis^x moieties on CEACAM1 in binding to DC-SIGN, CEACAM1 was

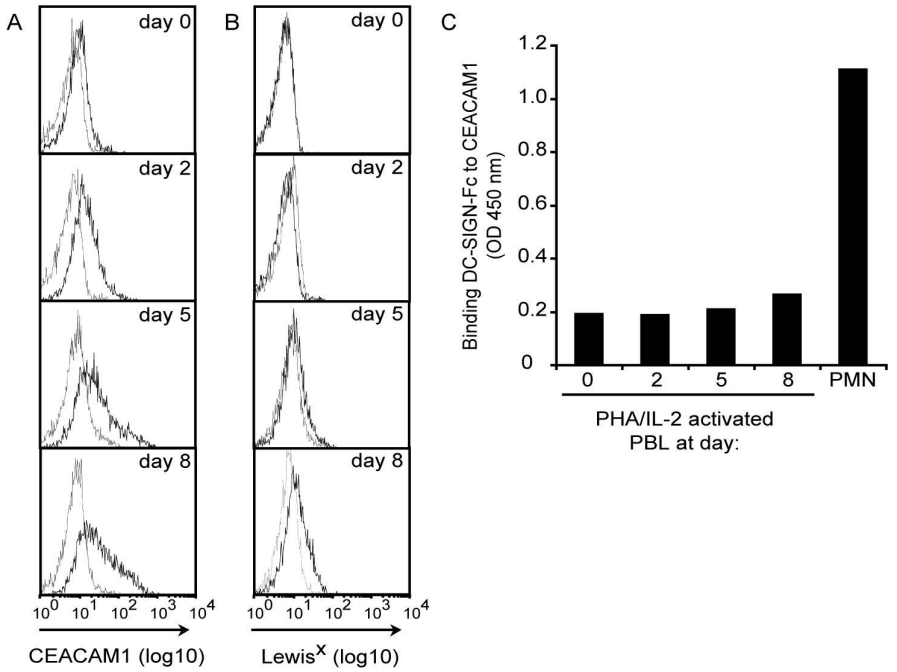
Figure 7.2



DC-SIGN binds Lewis^x on neutrophil CEACAM1.
(A) Binding of DC-SIGN-Fc to neutrophil-derived CEACAM1, -3 and -6 was analyzed using ELISA. Results are representative of three independent experiments. (B) Expression of Lewis^x was measured using ELISA on neutrophil-derived LFA-1 (negative control) and CEACAM1, -3 and -6 that was left untreated, or that had been treated after capture onto ELISA plates for three days with enzyme buffer (50 mM sodium phosphate buffer pH 5.0) in the presence or absence of α1-3,4-fucosidase (from *Xanthomonas*, Calbiochem, La Jolla, CA) at 37℃. Similar results were obtained in three independent experiments. (C) CEACAM1, -3 and -6 was incubated for three days in ELISA buffer, or in enzyme buffer with and without α1-3,4-fucosidase at 37℃, and analyzed for binding to DC-SIGN-Fc using ELISA. Experiment was performed three times with similar results. (D) LFA-1 and CEACAM1, -3 and -6 were captured from neutrophil lysate, incubated with IgM isotype control antibodies or blocking anti-Lewis^x antibodies, and binding to DC-SIGN-Fc was examined using ELISA. Three independent experiments with similar results were performed.

treated with α1-3,4-fucosidase that specifically removes the fucose moiety from Lewis^x that is essential for DC-SIGN binding [28]. Sandwich ELISA for CEACAM1 demonstrated that treatment with α1-3,4-fucosidase did not disrupt integrity of CEACAM1 (data not shown). The enzymatic treatment removed the Lewis^x epitope from CEACAM1 (Fig. 7.2B), and completely abrogated binding of DC-SIGN-Fc to neutrophil-derived CEACAM1, demonstrating that Lewis^x moieties mediate binding of DC-SIGN to CEACAM1 on neutrophils (Fig. 7.2C). In addition, blocking anti-Lewis^x antibodies inhibited binding of DC-SIGN-Fc to CEACAM1 from neutrophils, confirming that Lewis^x moieties on CEACAM1 are essential for DC-SIGN binding (Fig. 7.2D). In contrast to DC-SIGN binding to neutrophil CEACAM1, binding of DC-SIGN to Mac-1 on neutrophils was only partially dependent on Lewis^x [8].

Figure 7.3



T cell-derived CEACAM1 does not bind DC-SIGN. (A and B) Peripheral blood lymphocytes (PBLs) were activated with IL-2 (400 U/ml) and PHA (0.2 µg/ml) and expression of isotype controls (thin lines) and (A) CEACAM-1 (thick lines) and (B) Lewis^x (thick lines) was determined at day 0, 2, 5 and 8 using FACS analysis. (C) Binding of DC-SIGN-Fc to CEACAM-1, -3 and/or -6 derived from neutrophils and CEACAM1 from PBLs activated for 0, 2, 5 and 8 days with IL-2 and PHA was measured using ELISA. Experiment was performed three times with similar results.

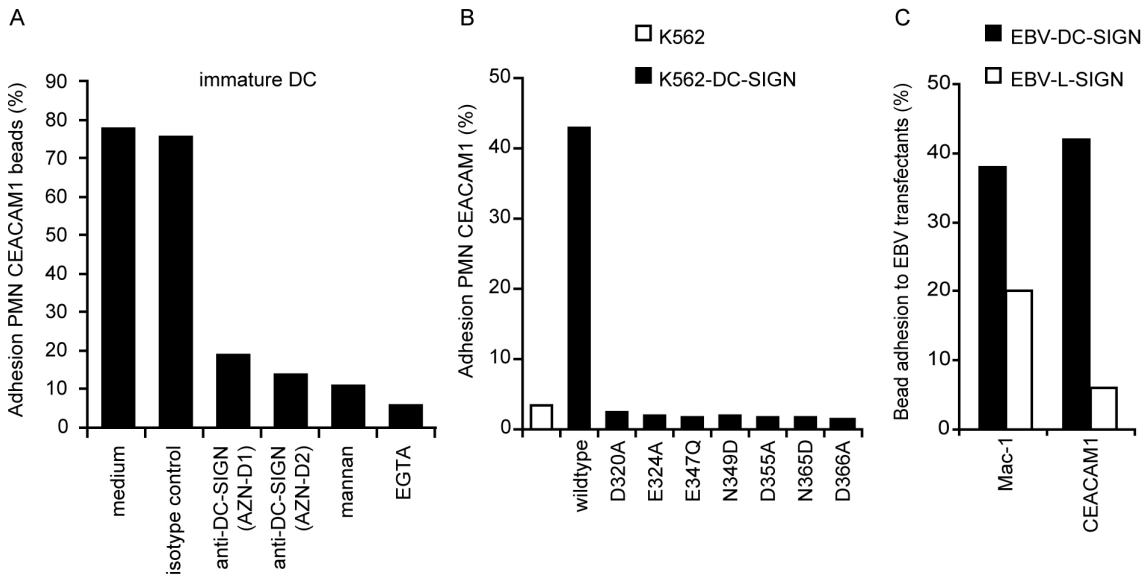
Cell-specific glycosylation of CEACAM1 regulates binding of DC-SIGN.

In contrast to CEACAM3 and -6, CEACAM1 is not only expressed on neutrophils, but also on activated T cells [29;30]. However, CEACAM1 on activated T cells is differently glycosylated and does not contain Lewis^x [30]. Indeed, activation of T cells with PHA and IL-2 induced expression of CEACAM1 on T cells at day 5 and 8 after stimulation (Fig. 7.3A), but did not upregulate Lewis^x expression on these T cells (Fig. 7.3B). We were able to capture and detect CEACAM1 from neutrophils and T cells using sandwich ELISA (data not shown). In contrast to neutrophil-derived CEACAM1, CEACAM1 from activated T cells did not bind to DC-SIGN, which correlates with the expression levels of Lewis^x on CEACAM1 (Fig. 7.3C). Thus, CEACAM1 is differently glycosylated on neutrophils and T cells, and the glycosylation pattern of CEACAM1 on these cell types regulates binding of DC-SIGN.

Cellular DC-SIGN but not L-SIGN binds neutrophil-derived CEACAM1.

DC-SIGN is present at high levels on monocyte-derived immature DCs [13]. On these cells, DC-SIGN is the dominant receptor that binds glycoconjugates of Lewis^x, and Lewis^x-expressing pathogens such as *Schistosoma mansoni* and *Helicobacter pylori* [11;31]. Since Lewis^x on CEACAM1 mediates binding to DC-SIGN, we investigated the adhesion of immature DCs to neutrophil CEACAM1. Therefore, we captured CEACAM1 from neutrophils onto fluorescent beads and analyzed binding of these beads to immature DCs. The antibody used to capture CEACAM1 onto the beads also recognizes CEACAM3 and -6, but these molecules do not interact with DC-SIGN (Fig. 7.2A). Monocyte-derived immature DCs bound neutrophil CEACAM1, and anti-DC-SIGN antibodies, the polysaccharide mannan, and the Ca^{2+} chelator EGTA blocked adhesion, demonstrating that these cells use DC-SIGN as an adhesion receptor for neutrophil CEACAM1 (Fig. 7.4A). The C-type lectin domain of DC-SIGN is involved in binding of neutrophil CEACAM1, since mutation of essential amino acids within this domain that mediate ligand binding and positioning of the Ca^{2+} ions abrogate adhesion of neutrophil CEACAM1 to DC-SIGN (Fig. 7.4B) [12;18]. L-SIGN (DC-SIGNR) is a close

Figure 7.4



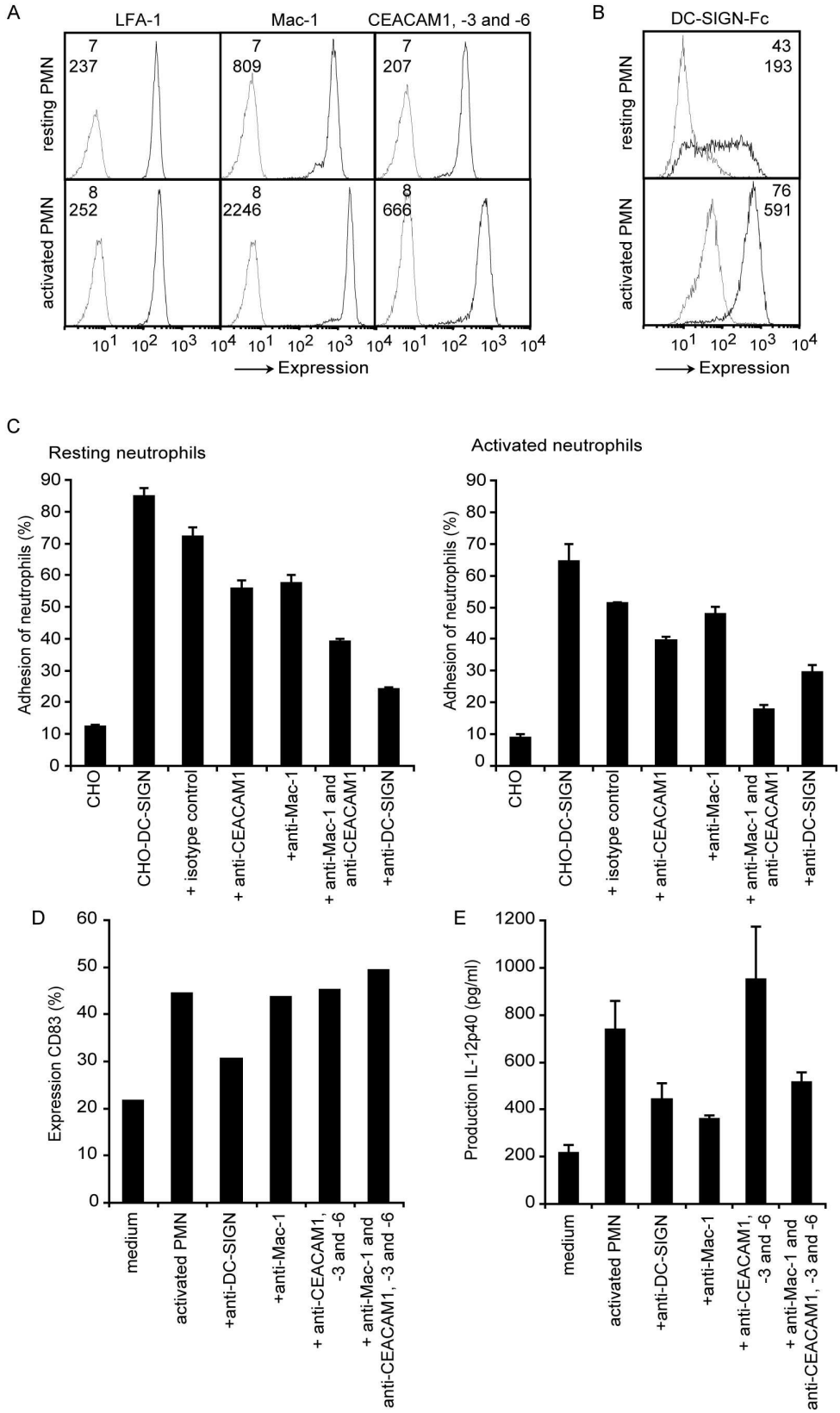
CEACAM1 is recognized by cellular DC-SIGN, but not by cellular L-SIGN.
(A) Neutrophil-derived CEACAM1, -3 and -6 was captured onto fluorescent beads, and the percentage of immature DCs that bound one or multiple beads was examined using FACS analysis. Specificity of adhesion was determined using isotype control antibodies, anti-DC-SIGN antibodies, mannan and EGTA. Similar results were obtained in three independent experiments. (B) Fluorescent beads coated with neutrophil CEACAM-1, -3 and -6 were incubated with mock-transfected K562 cells and with K562 cells transfected with wildtype or mutant DC-SIGN containing mutations in the C-type lectin domain, and adhesion was measured using FACS analysis. Results are representative of three independent experiments. (C) Adhesion of beads coated with Mac-1 or CEACAM-1, -3 and -6 from neutrophils to EBV cells transduced with DC-SIGN or L-SIGN was analyzed using FACS. Experiment was performed three times with similar results.

homologue of DC-SIGN that is not expressed on DCs, but on endothelial cells in liver and lymph node [16;32]. Although the binding specificities of DC-SIGN and L-SIGN are highly similar, Lewis^x moieties are bound by DC-SIGN, but not by L-SIGN [33;34]. Since binding of neutrophil-derived Mac-1 and CEACAM1 to DC-SIGN is mediated by Lewis^x, we analyzed adhesion of these proteins to L-SIGN. Therefore, we coated Mac-1 and CEACAM1 from neutrophils onto fluorescent beads, and measured adhesion of these beads to EBV-DC-SIGN and EBV-L-SIGN by FACS analysis. Neutrophil Mac-1 bound to DC-SIGN as well as L-SIGN, although adhesion of Mac-1 to L-SIGN was reduced compared to DC-SIGN, whereas neutrophil CEACAM1 bound to DC-SIGN but not to L-SIGN (Fig. 7.4C). This corresponds with the partial Lewis^x-dependency of DC-SIGN binding to Mac-1 and the complete Lewis^x-dependency of DC-SIGN binding to CEACAM1 [8], and indicates that Mac-1 contains glycans other than Lewis^x that are involved in binding to L-SIGN and DC-SIGN.

DC-SIGN mediates cellular interactions with resting and activated neutrophils through Mac-1 and CEACAM-1.

Neutrophil activation induces degranulation, and this results in the release of anti-microbial components and the upregulation of membrane molecules. The expression levels of CEACAM1, -3 and/or -6 and Mac-1, but not of LFA-1 are increased on neutrophils that are activated with both LPS and FMLP (Fig. 7.5A). This suggests that activated neutrophils bind stronger to DC-SIGN than resting neutrophils. Indeed, binding of DC-SIGN-Fc is increased to LPS- and FMLP-activated neutrophils compared to resting neutrophils (Fig. 7.5B). Previously, we have shown that DC-SIGN mediates cellular interactions between immature DCs and

Figure 7.5


A
LFA-1
Mac-1
CEACAM1, -3 and -6
resting PMN
activated PMN
7
237
7
809
7
207
8
252
8
2246
8
666
Expression
B
DC-SIGN-Fc
43
193
76
591
C
Resting neutrophils
Activated neutrophils
Adhesion of neutrophils (%)
CHO
CHO-DC-SIGN
+ isotype control
+ anti-CEACAM1
+anti-Mac-1
+ anti-Mac-1 and anti-CEACAM1
+anti-DC-SIGN
D
Expression CD83 (%)
medium
activated PMN
+anti-DC-SIGN
+anti-Mac-1
+ anti-CEACAM1, -3 and -6
+ anti-Mac-1 and anti-CEACAM1, -3 and -6
E
Production IL-12p40 (pg/ml)

CEACAM1 and Mac-1 on resting and activated neutrophils are involved in cellular interactions with DC-SIGN.
(A) Resting neutrophils and activated neutrophils that were incubated for 1h with LPS and in the final 15min with FMLP at 37℃ were analyzed on FACS for expression o f isotype control (thin line), LFA-1, Mac-1, or CEACAM-1, -3 and -6 (thick line). Insets represent mean fluorescence intensity. (B) Binding of DC-SIGN-Fc to resting and activated neutrophils was examined using FACS analysis (thick line). Specificity of binding was determined using anti-DC-SIGN antibodies (thin line). Insets represent mean fluorescence intensity. (C) CHO and CHO-DC-SIGN cells were incubated with Calcein-AM labelled resting or activated neutrophils, and cellular adhesion was examined after washing and cell lysis using fluorimetry. Specificity of adhesion was determined using isotype control antibodies and blocking antibodies against DC-SIGN, CEACAM1, -3 and -6, or Mac-1. The experiment was performed in triplicate three times with similar results. Error bars represent standard error of the mean of triplicates. (D and E) DCs were co-cultured with activated neutrophils during 18h to investigate neutrophil-induced DC maturation. (D) Expression of the DC maturation marker CD83 was examined using FACS analysis and (E) production of the cytokine IL-12p40 was analyzed within the supernatants using sandwich ELISA. Specificity of the interaction between DCs and neutrophils was determined with blocking antibodies against DC-SIGN, CEACAM-1, -3 and -6, or Mac-1. Similar results were obtained in three independent experiments. Error bars represent standard error of the mean of triplicates.

neutrophils [8]. To examine whether binding of DC-SIGN to both Mac-1 and CEACAM1 was required to support cellular interactions with neutrophils, we incubated neutrophils with CHO transfectants expressing DC-SIGN, and analyzed adhesion in the absence and presence of blocking antibodies. In contrast to the parental CHO cells, DC-SIGN-expressing CHO transfectants strongly interacted with both resting and activated neutrophils (Fig. 7.5C). Although DC-SIGN-Fc bound stronger to activated than to resting neutrophils, activation of neutrophils did not further enhance binding of neutrophils to CHO-DC-SIGN (Fig. 7.5C). Adhesion was inhibited in the presence of blocking anti-DC-SIGN antibodies, demonstrating that DC-SIGN mediated the cellular interaction with neutrophils (Fig. 7.5C). Preincubation with both anti-Mac-1 and anti-CEACAM1, -3 and -6 antibodies strongly blocked neutrophil adhesion to CHO-DC-SIGN, in contrast to preincubation with either anti-Mac-1 or anti-CEACAM1, -3 and -6 antibodies (Fig. 7.5C). Thus, binding to both Mac-1 and CEACAM1 is required for DC-SIGN to mediate cellular interactions with neutrophils. Since we have observed simultaneous binding of the antibodies and DC-SIGN to Mac-1 and CEACAM1 in ELISA (Fig. 7.2A), it is unlikely that they recognize the same epitope. This indicates that the inhibition of the anti-Mac-1 and anti-CEACAM1, -3 and -6 antibodies in cellular adhesion is through steric hindrance. Activated but not resting neutrophils induce upregulation of the DC maturation marker CD83 and production of IL-12p40 by DCs through DC-SIGN-dependent cellular interactions (Fig. 7.5D and E) [8]. We investigated whether neutrophil-induced DC maturation required binding of DC-SIGN to both Mac-1 and CEACAM1 using blocking antibodies against these DC-SIGN ligands. Surprisingly, neutrophil-induced upregulation of CD83 on DCs was not inhibited with anti-Mac-1 and anti-CEACAM1, -3 and -6 antibodies (Fig. 7.5D). In contrast, neutrophil-induced IL-12p40 release by DCs was sensitive to anti-Mac-1, but not anti-CEACAM1, -3 and -6 antibodies (Fig. 7.5E). This may indicate that binding of DC-SIGN to Mac-1 and CEACAM1 does not play a role in the induction of DC maturation by neutrophils, and that DC-SIGN-Mac-1 interactions are more important for neutrophils than DC-SIGN-CEACAM1 interactions to induce IL-12p40 release by DCs.

Discussion

Previously, we have shown that DCs interact with neutrophils through binding of DC-SIGN to Mac-1. The interaction results in maturation of DCs and primes these DCs to induce strong T-cell responses of the T helper 1 type. Here, we have identified CEACAM1 as an alternative

ligand of DC-SIGN on neutrophils. Both CEACAM1 and Mac-1 are essential ligands on neutrophils for DC-SIGN to establish cellular interactions. DC-SIGN binds neutrophil-derived CEACAM1 through Lewis^x moieties, but not high-mannose moieties. In contrast to DC-SIGN, the homologous molecule L-SIGN does not bind to CEACAM1 and poorly to Mac-1.

Neutrophils have a unique glycosylation pattern, and in contrast to other leukocytes, express non-sialylated Lewis^x carbohydrates on the cell surface [24]. Little is known on the regulation of this, but the differential expression of fucosyltransferases likely plays a role. These enzymes incorporate a fucose through α3-linkage on the *N*-acetyl-glucosamine moiety of a non-sialylated lactosamine unit to form a Lewis^x glycan. Neutrophils express high levels of Lewis^x, but only few proteins on neutrophils are carrier molecules of Lewis^x, and these include Mac-1 and CEACAM1 [15;25;26]. Currently, it is unknown what determines that particularly CEACAM1 and Mac-1 on neutrophils express Lewis^x epitopes. Neutrophils express the fucosyltransferases 4 and 9 that may be involved in Lewis^x synthesis on neutrophil proteins [35], but future research is required to determine whether they are involved in the formation of Lewis^x on CEACAM1 and Mac-1. Previously, we have described that the neutrophil-specific glycosylation of Mac-1 enables DC-SIGN to bind Mac-1 on neutrophils, but not on other cells [8]. Similar to Mac-1, CEACAM1 has a broad tissue expression and has been described on other leukocytes besides neutrophils such as B cells, a subset of NK cells and IL-2 activated T cells [30]. In contrast to CEACAM1 on neutrophils, DC-SIGN did not bind to CEACAM1 derived from PHA- and IL-2-activated T cells. This supports that glycosylation of CEACAM1 is cell-specifically regulated, and that expression of Lewis^x on neutrophils but not on activated T cells enables binding of DC-SIGN. Indeed, inhibition with fucosidase treatment and anti-Lewis^x antibodies showed that binding of DC-SIGN to neutrophil CEACAM1 critically depended on Lewis^x moieties. Thus, binding of DC-SIGN to neutrophil-specific glycosylation of CEACAM1 may contribute together with DC-SIGN-Mac-1 interactions to cellular adhesion of immature DCs and neutrophils to enable cross-talk between the cells.

CEACAM1 belongs to a small subfamily of immunoglobulins that in humans consists of CEACAM1 to -8 and 11 pregnancy specific glycoproteins. Neutrophils express the CEACAM1 homologues CEACAM3, -6 and -8 besides CEACAM1 [27]. Consistent with the absence of the DC-SIGN adhesion determinant Lewis^x on these molecules [36], DC-SIGN did not bind to CEACAM1 homologues. Carcinoembryonic antigen (CEA) is a homologue of CEACAM1 that is not expressed on neutrophils, but on normal colon epithelium and has retained or enhanced expression on colorectal cancer cells [37]. Glycosylation of CEA is distinct on normal and tumour colon epithelium, and the expression of Lewis^x and Lewis^y on colorectal cancer is increased compared to normal colon epithelium [38-41]. Consistent with its carbohydrate specificity, DC-SIGN binds to CEA from normal but not tumour colon epithelium [42]. Thus, glycosylation modifications that result in acquisition of Lewis^x and Lewis^y antigens regulate binding of DC-SIGN to CEACAM1 and homologues of CEACAM1.

Neutrophils induce DC maturation through DC-SIGN-dependent interactions with immature DCs and the release of TNF-α. DC-SIGN may establish a cellular contact zone or synapse between neutrophils and DCs through interactions with Mac-1 and CEACAM1 that enable efficient targeting of the DC maturation-inducing factor TNF-α to DCs. [8]. We observed that binding to Mac-1 and CEACAM1 is required for DC-SIGN to mediate cellular interactions with neutrophils, indicating that these DC-SIGN ligands are involved in the induction of DC maturation by neutrophils. However, anti-Mac-1 and anti-CEACAM1 antibodies did not block DC maturation, and anti-Mac-1 antibodies, but not anti-CEACAM1 antibodies, inhibited IL-12p40 release by DCs. Conclusions from these functional experiments should be drawn with caution, since anti-CEACAM1 and anti-Mac-1 antibodies may induce signalling and activate

neutrophils to release DC maturation factors such as TNF-α. Indeed, CEACAM1 has been implicated in neutrophil activation. Although CEACAM1 contains a complete inhibitory ITIM motif, and associates with the phosphatases SHP-1 and SHP-2 in epithelial tumour cells [43], it associates with the protein tyrosine kinases lyn and hck in neutrophils [44]. Antibodies that target CEACAM1 potentiate the oxidative burst of neutrophils in response to FMLP [45], induce degranulation of secondary granules containing lactoferrin [46], increase LFA-1-mediated adhesion of neutrophils to endothelium, and trigger upregulation of Mac-1 and shedding of L-selectin [47]. Similarly, galectin-3 has been shown to bind CEACAM1 and to enhance the oxidative burst in FMLP-activated neutrophils [48]. Galectin-3 is a C-type lectin that is released by macrophages under inflammatory conditions, and in contrast to DC-SIGN has high affinity for β-galactoside in lactosamine glycans. This may indicate that cross-talk between neutrophils and DCs is bi-directional, and that DC-SIGN is involved in the induction of neutrophil activation. However, we did not observe that DC-SIGN triggered stimulation of neutrophils that results in production of oxygen radicals or degranulation (data not shown). This may be explained by binding of DC-SIGN to Lewis^x on CEACAM1 that is a different epitope than those recognized by anti-CEACAM1 antibodies or galectin-3. During infection, neutrophils are likely present prior to the arrival of DCs, indicating that activation of neutrophils by inflammatory mediators or pathogenic stimuli is more important than activation by DCs. DC-SIGN not only establishes cellular interactions with resting but also with activated neutrophils. Mac-1 and CEACAM1 are upregulated upon activation of neutrophils, and this results in stronger binding of DC-SIGN to activated than to resting neutrophils. Thus, DC-SIGN may not be involved in activation of neutrophils, but perhaps it plays a role in regulation of later events such as neutrophil apoptosis, cytokine and chemokine production by neutrophils, and differentiation of neutrophils into DCs.

Neutrophils are very efficient in degrading internalized antigens in contrast to DCs that keep antigens intact for prolonged periods in endocytic vesicles [49]. The DC-SIGN-CEACAM1 mediated interaction may enable DCs to transfer captured bacteria to neutrophils, which would facilitate efficient internalization and destruction of the bacteria. Indications that neutrophils phagocytize bacteria tethered to other cells have been found in rat liver, in which it was shown that intravenously administered *Listeria monocytogenes* were initially bound to Kupffer cells, and thereafter accumulated within neutrophils [50]. Phagocytosis and processing of pathogens by neutrophils may provide a rich source of pathogen-derived peptides that enables DCs to specifically activate pathogen-specific T cells. Indeed, some evidence indicates that antigens that are phagocytized and processed by neutrophils are transferred to DCs for presentation to T cells [51;52]. Here, we have established CEACAM1 as a novel ligand of DC-SIGN that besides Mac-1 may be involved in interactions between neutrophils and DCs to enable cross-talk between the cells. It will be interesting to examine whether interactions of CEACAM1 and Mac-1 with DC-SIGN play a role in antigen transfer from DCs to neutrophils and vice versa.

Acknowledgements

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7

L-SIGN mediates adhesion and rolling of neutrophils

ADDENDUM

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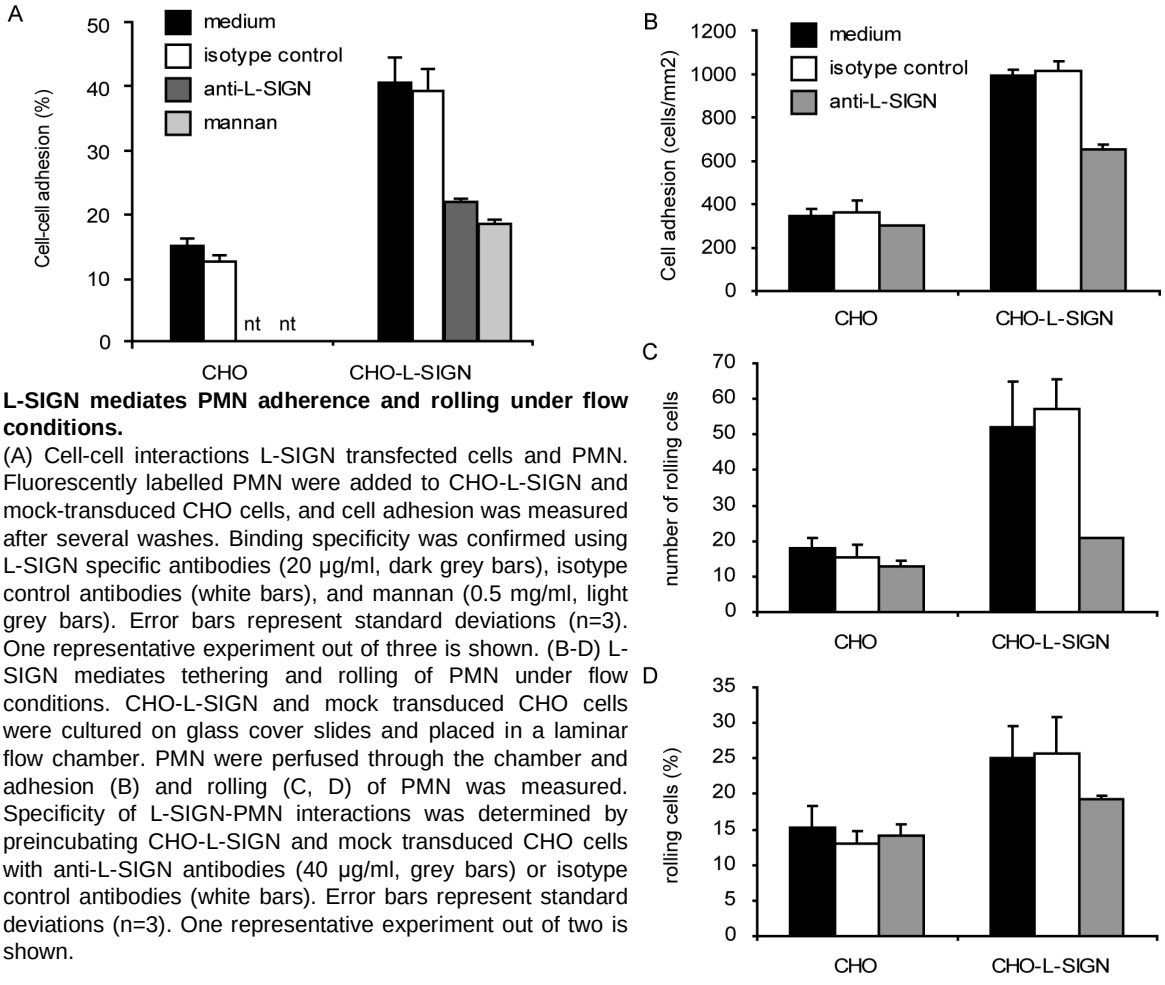
DC-SIGN mediates dendritic cell (DC)-PMN interactions by binding Mac-1 and CEACAM1 on PMN [1-3]. PMN adherence to DC-SIGN-positive cells could be blocked with anti-Mac-1 and with anti-CEACAM1 antibodies (Fig. 7.5C), indicating that both Mac-1 and CEACAM1 interactions with DC-SIGN participate in the adherence of PMN to DC-SIGN. DC-SIGN recognizes Lewis^x (Le^x) and Le^y moieties on Mac-1 and Le^x on CEACAM1 (Fig. 7.2C, [1;3]). The DC-SIGN homologue L-SIGN also binds Mac-1 isolated from PMN, but does not interact with PMN derived CEACAM-1 (Fig. 7.4C). This discrepancy is due to the lack of Le^x recognition by L-SIGN [4].

Since L-SIGN only binds PMN Mac-1 and not CEACAM1, we questioned whether PMN could adhere to L-SIGN-positive cells. Therefore we performed a cell-cell based adhesion assay in which PMN were allowed to adhere to a monolayer of L-SIGN-positive cells. PMN adhered strongly to L-SIGN-positive cells. This interaction was L-SIGN specific since the interaction was specifically blocked by incubating the L-SIGN-positive cells with anti-L-SIGN antibodies or mannan (Fig. 7.6A). This indicates that Mac-1 on PMN is sufficient for adherence of PMN to L-SIGN-positive cells under static conditions.

In vivo, PMN travel in the blood stream and can encounter L-SIGN-positive sinusoidal

Figure 7.6



L-SIGN mediates PMN adherence and rolling under flow conditions.

(A) Cell-cell interactions L-SIGN transfected cells and PMN. Fluorescently labelled PMN were added to CHO-L-SIGN and mock-transduced CHO cells, and cell adhesion was measured after several washes. Binding specificity was confirmed using L-SIGN specific antibodies (20 µg/ml, dark grey bars), isotype control antibodies (white bars), and mannan (0.5 mg/ml, light grey bars). Error bars represent standard deviations (n=3). One representative experiment out of three is shown. (B-D) L-SIGN mediates tethering and rolling of PMN under flow conditions. CHO-L-SIGN and mock transduced CHO cells were cultured on glass cover slides and placed in a laminar flow chamber. PMN were perfused through the chamber and adhesion (B) and rolling (C, D) of PMN was measured. Specificity of L-SIGN-PMN interactions was determined by preincubating CHO-L-SIGN and mock transduced CHO cells with anti-L-SIGN antibodies (40 µg/ml, grey bars) or isotype control antibodies (white bars). Error bars represent standard deviations (n=3). One representative experiment out of two is shown.

endothelial cells in the liver [5]. Under these conditions L-SIGN-PMN interactions have to resist shear stress induced by the blood stream, although the blood flow in the sinusoids is low. It has been demonstrated previously that DC-SIGN on DCs interacts with ICAM-2 and this interaction is strong enough to mediate adherence and rolling under shear stress [6]. This DC-SIGN-ICAM-2 interaction is involved in trans-endothelial cell migration of DCs [6]. However, DC-SIGN-ICAM3 interactions are not strong enough to induce adherence under shear stress [6]. To investigate PMN-L-SIGN interactions under shear stress, we used a flow chamber in which a coverslip with a confluent monolayer of CHO-L-SIGN was placed. PMN were perfused into the chamber and adhesion to the cells was measured. Under flow conditions PMN adhered significantly more to L-SIGN-positive cells than to L-SIGN-negative cells (Figure 7.6B). The adherence could specifically be blocked with anti-L-SIGN antibodies but not with isotype control antibodies (Figure 7.6B). Furthermore we investigated rolling of PMN under these conditions. A significant greater number of PMN were rolling over L-SIGN-positive cells than over L-SIGN-negative CHO cells (Figure 7.6C). This rolling was L-SIGN dependent since antibodies against L-SIGN reduced the number of rolling cells to back ground levels (Figure 7.6C). The higher number of rolling PMN was not just a reflection of the higher number of PMN adhering to L-SIGN-positive cells, since also the percentage of rolling PMN was increased compared to the percentage of PMN rolling over mock-transduced CHO cells (Figure 7.6D).
These data indicate that L-SIGN strongly interacts with PMN, and PMN could adhere to L-SIGN-positive LSECs in the liver. L-SIGN has also been implicated in the binding of hepatocytes [Chapter2] and Kupffer cells [Chapter 8], and therefore might act as a key player in cellular interactions in the liver.
During inflammation PMN rapidly sequester in the liver, even when the infection is outside the liver [7]. Binding of PMN to L-SIGN-positive cells in the liver could therefore result in the ending of the inflammation by the clearance of PMN. During bacterial infections, PMN are the main inducers of hepatic injury [8]. In this process the binding of PMN to L-SIGN on LSECs could facilitate PMN transmigration across the endothelial cell layer, and induce subsequently hepatic damage. Since PMN have increased expression of Mac-1 upon activation, especially activated PMN might be trapped inside the sinusoids.
Altogether these data suggest that PMN are retained in the liver by binding L-SIGN either resulting in clearance or in transendothelial migration.

Materials and methods

Antibodies and cells.
The following monoclonal antibodies were used: AZN-D1 (mouse IgG1 isotype control) [9], and AZN-D2 (DC-SIGN and L-SIGN) [9].
CHO-L-SIGN cells were generated by lentiviral transduction of CHO cells with LV-L-SIGN [10]. Neutrophils were isolated from fresh blood of healthy volunteers. After Ficoll gradient centrifugation, neutrophils were cleared from contaminating erythrocytes using erylysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA). Isolated neutrophils were over 95% pure (data not shown).

Static cell-cell interaction.
To examine cellular interactions of L-SIGN with neutrophils CHO and CHO-L-SIGN were grown overnight to confluent cultures in flat-bottom 96 well plates. Neutrophils were labeled with the green fluorescent dye Calcein-AM (1 µM, Molecular Probes) for 15 min at 37℃ and incubated with mock and DC-SIGN transfected CHO cells in TSM 0.5% BSA for 2 h at 37℃. To determine specific adhesion the CHO transfectants were preincubated with anti-L-SIGN, or isotype control antibodies (20 µg/ml) for 30

min at 37℃. Unbound neutrophils were washed away, and remaining cells were lyzed in lysis buffer (50 mM Tris-HCl, pH 8.0, and 0.1% SDS). Cell-cell adhesion was measured by fluorimetry of cell lysates at 488 nm (Fluostar Galaxy, BMG Labtechnologies Inc., Durham, NC). Cell-cell adhesion was calculated as the percentage of total added neutrophils that was considered as 100% adhesion.

Cell-cell interaction under flow conditions.
CHO and CHO-L-SIGN were coated on glass coverslips, and grown confluent overnight. PMN (2 x 10^6 cells/ml) were perfused over CHO(-L-SIGN) for 10 minutes at a flow rate of 100 µl/min. Wall shear stress was calculated to be 0.8 dyne/cm^2. During perfusion, the flow chamber [11;12] was mounted on a microscope stage (Axiovert 25; Zeiss, Oberkochen, Germany), which was equipped with a black-and-white charge-coupled device video camera (Sanyo, Osaka, Japan) and was coupled to a VHS video recorder. Video images were evaluated for the number of adherent cells, with dedicated routines made in the image analysis software OPTIMAS 6.1 (Media Cybernetics Systems, Silverspring, Md). PMN that were in contact with the surface appeared as bright white-centred cells after proper adjustment of the microscope during recording. L-SIGN specific interaction with PMN was determined by preincubation of CHO cells with anti-L-SIGN antibodies (AZN-D2, 40 µg/ml) or isotype control antibodies (AZN-D1, 40 µg/ml).

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C-type lectins are involved in monocyte/Kupffer cell migration in the liver

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Abstract

Kupffer cells (KCs), the resident liver macrophages, originate from blood monocytes and reside in the liver sinusoids. KCs function in clearance of harmful material from the portal circulation in combination with liver sinusoidal endothelial cells (LSECs). Therefore they express several receptors. LSEC express amongst others the C-type lectins mannose receptor, L-SIGN in human and its homologue mSIGNR1 in mouse. These C-type lectins recognize carbohydrate moieties expressed on pathogens. However, most C-type lectins recognize both non-self and self-ligands. Here we demonstrate that cellular L-SIGN is recognizing a self-ligand on monocytes, the KC precursors, and these monocytes strongly adhere to L-SIGN-positive cells. Using an *in vivo* model we furthermore demonstrate that the murine L-SIGN homologue mSIGNR1 expressed on LSECs influences KC numbers in the liver, because mSIGNR1 knock out mice have reduced numbers of KC. The effect of mSIGNR1 on KC recruitment appears after the initial phase of KC repopulation. Altogether, L-SIGN could be involved in the recruitment of KC precursors in the human liver.

Introduction

Kupffer cells (KCs) are the resident macrophages of the liver, and form the largest population of resident tissue macrophages [1]. They reside in the liver sinusoids where they access the hepatocytes through the endothelial cell layer and the space of Disse [2]. KCs participate in the clearance of particulate and foreign material from the portal circulation [3] and phagocytosis of apoptotic cells [4;5]. By the release of several cytokines they modulate immune responses [6]. KCs are also involved in defence against tumour metastasis in the liver sinusoid [7]. The KC population remains constant by recruitment of monocytes from the blood that differentiate locally into macrophages. How the recruitment of monocytes is regulated is largely unknown. The life span of KC is estimated to be almost 4 days in mice. However in monocytopenic mice lacking monocytes, KCs are maintained in the liver for more than 6 weeks [8;9], and in human, donor KCs can persist in transplanted liver even for up to 1 year [10], indicating that KC have a long life span and might proliferate locally.

The liver sinus is lined with liver sinusoidal endothelial cells (LSECs) which form, in contrast to conventional endothelium, a discontinuous, fenestrated endothelial cell layer lacking a basement membrane [11]. LSECs participate, in combination with KCs, in the clearing function of the liver. Therefore, LSECs express several scavenger receptors and C-type lectin receptors, such as mannose receptor (MR) and liver/lymph node specific ICAM-3 grabbing non-integrin (L-SIGN) [12-14]. MR binds besides pathogens, such as bacterial antigens [15], also self-antigens, such as serum glycoproteins [16] and thereby participates in the clearance of harmful exogenous and endogenous material [17]. L-SIGN is also a pathogen receptor and binds several pathogens, such as HCV [18-21] and HIV-1 [13;14] and can transmit these viruses to target cells [13;14;22]. Moreover, we have demonstrated that L-SIGN is also involved in antigen presentation of viral antigens to T cells [Ludwig, in preparation]. Although the recognition of pathogens is extensively described, the function during homeostasis of L-SIGN on LSEC remains elusive. L-SIGN is mediating LSEC-neutrophil interactions via Mac1 [23] and thereby it could function in clearance of PMN or in attenuating PMN responses. Here we demonstrate that L-SIGN interacts with monocytes, suggesting that L-SIGN might have a role in monocyte-LSEC interactions. Since KCs originate from monocytes and are located in close proximity of L-SIGN^{+} LSECs, we investigated the KC population in mouse liver from wild type and mSIGNR1 deficient mice. Strikingly, mSIGNR1$^{-/-}$ mice have a reduced KC population. Therefore, mSIGNR1, and possibly L-SIGN in human liver, is participating in KC homeostasis.

Materials and methods.

Mice.

mSIGNR1$^{-/-}$ mice were a generous gift from A. McKenzie (Cambridge, UK). C57BL/6x129 wild type and mSIGNR1$^{-/-}$ mice were bred in the animal facility of the VU University Medical Centre under specific pathogen-free conditions. The Animal Care and Use Committee of the VU University approved all experiments.

Antibodies.
The following antibodies were used: goat-anti-mSIGNR1 (R&D systems, Oxon, UK), F4/80 (eBioScience, San Diego, CA, USA), MOMA-2 [24], anti-DC-SIGN mouse IgG1 AZN-D1 and anti-DC-SIGN/L-SIGN mouse IgG1 AZN-D2 [25;26], mouse anti-L-SIGN/DC-SIGN-stalk, mouse anti-L-SIGN (R&D Systems, Minneapolis, MN, USA), anti-mouse CD31 (eBioScience, San Diego, CA, USA), mouse anti-human CD3-PE (Becton Dickinson), mouse anti-human CD4-PE (Becton Dickinson), mouse anti-human CD14 (Becton Dickinson).

Cells.
Human PBMCs were isolated from buffy coats, obtained from blood bank donors, by centrifugation on a Ficoll gradient as previously described [27].
Stable transfectants of K562 expressing L-SIGN and mSIGNR1 were obtained by electroporation of pRC-CMV-L-SIGN and pCR-CMV-mSIGNR1 (400 V, 960 µF) as previously described [28]. CHO-L-SIGN cells were generated by lentiviral transduction with LV-DC-SIGN or LV-L-SIGN (kind gift of Vincent Piquet). Transfection and transduction efficiency was tested by staining the cells with anti-L-SIGN AZN-D2 antibodies.
Murine LSECs were isolated from mouse liver by perfusing the liver via the portal vein with successively HEPES buffer (8.3 mg/ml NaCl, 0.5 mg/ml KCl, 2.4 mg/ml HEPES) containing 0.19 mg/ml EGTA, HEPES buffer without EGTA and collagenase solution (3.9 mg/ml NaCl, 0.5 mg/ml KCl, 0.7 mg/ml $CaCl_2.2H_2O$, 2.4 mg/ml HEPES, 0.4 mg/ml collagenase). The livers were dissociated and single cell suspensions were made by passing the cells through a nylon mesh. Cells were washed in PBS and centrifuged 2 times 2 minutes at 50 g. The pellet containing hepatocytes was removed after each centrifugation step. The non-parenchymal cells were washed twice in PBS (350 g, 7 minutes) and layered on a double percoll gradient (2 ml 75%, 1.5 ml 35% percoll), and subsequently centrifuged at 900 g for 20 minutes without brakes. The lower ring containing KCs and LSECs was collected, washed and seeded in serum free IMDM in 6 well plates. After 30 minutes incubation at 37°C, non-adherent LSECs were collected by vigorous washing and seeded on fibronectin coated culture plates. After 6 to 10 days of cultured, purity of LSEC cultures was determined by staining for CD31 and was >90%.

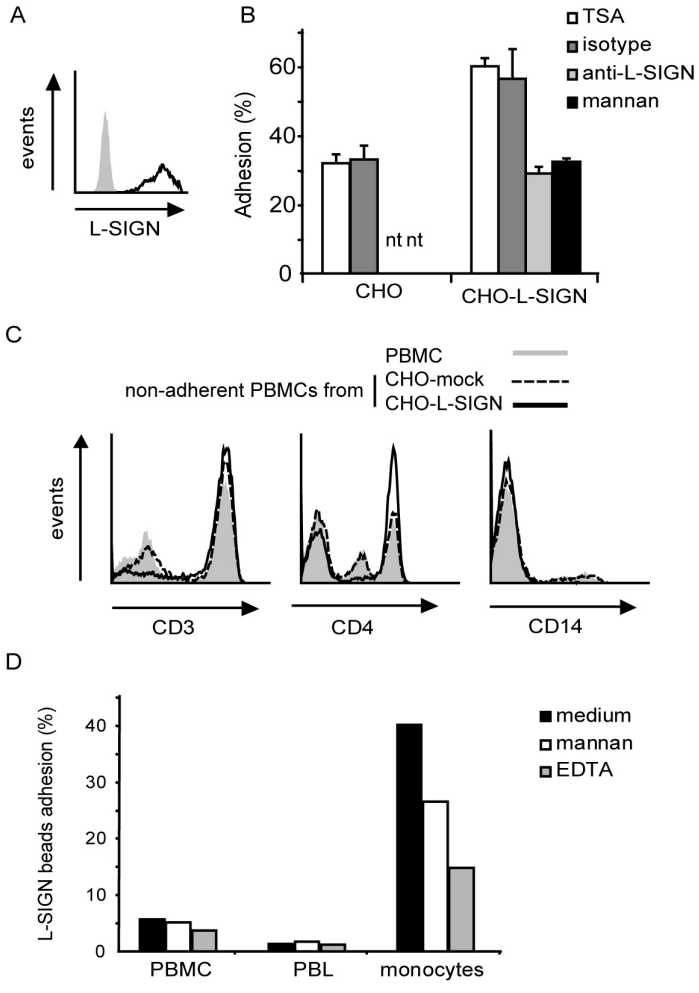
Cell-cell adhesion.
To examine cellular interactions of L-SIGN with PBMC, CHO and CHO-L-SIGN were grown overnight to confluent cultures in flat-bottom 96 well plates. PBMC were labeled with the green fluorescent dye Calcein-AM (1 µM, Molecular Probes) for 15 min at 37°C and incubated on mock or L-SIGN transduced CHO cells in TSM 0.5% BSA for 1 h at 37°C. To determine specific adhesion the CHO cells were preincubated with mannan (1 mg/ml), anti-L-SIGN antibody (20 µg/ml), or an isotype control (20 µg/ml) for 30 min at 37°C. Unbound PBMC were washed away, and remaining cells were lyzed in lysis buffer (50 mM Tris-HCl, pH 8.0, and 0.1% SDS). Cell-cell adhesion was measured by fluorimetry of cell lysates at 488 nm (Fluostar Galaxy, BMG Labtechnologies Inc., Durham, NC). Cell-cell adhesion was calculated as the percentage of total added PBMC that was considered as 100% adhesion. To investigate the cell population adhering to the CHO cells, non-adherent cells were collected by vigorous washing and stained with anti-CD3, CD4, and CD14 antibodies. Expression of these markers was measured by flow cytometry and compared with the initial PBMC population.

Beads adhesion.
Binding of ligand-coated beads to cells was performed as described by Geijtenbeek et al. [29]. In short, streptavidin was covalently coupled to the carboxylate-modified TransFluorSpheres (488/645 nm, 1.0 µm; Molecular Probes, Eugene, OR, USA). The streptavidin-coated beads were incubated with biotinylated $F(ab')_2$ fragment goat anti-mouse IgG (6 µg/ml; Jackson Immunoresearch), followed by an overnight incubation with mouse-anti-L-SIGN antibodies (anti-stalk) at 4°C. The beads were washed and incubated with K562-L-SIGN lysate overnight at 4°C. gp120 coated beads were made by incubating streptavidin-coated beads with biotinylated $F(ab')_2$ fragment goat anti-mouse IgG (6 µg/ml; Jackson Immunoresearch), followed by an overnight incubation with supernatant containing gp120-Fc chimera.
The fluorescent beads adhesion assay was performed as described before [30]. In short, 50,000 cells were incubated with beads for 45 minutes at 37°C. Mannan (0.5 mg/ml), EGTA (10 mM) and blocking antibodies against L-SIGN (20 µg/ml) were used to determine specificity of adhesion. Binding was measured by flow cytometry.

Liver section staining and cell counting.
Mouse liver tissue was obtained from wild type and mSIGNR1 knock out mice. 6 µm cryosections were placed on gelatine-coated slides and stored at -80°C. Sections were fixed with acetone and stained with goat-anti-mSIGNR1 and donkey-anti-goat-Alexa Fluor 488, or with rat-anti-mouse-CD31, F4/80 or MOMA-2 antibodies and counterstained with goat-anti-rat-Alexa Fluor 594. Sections were analyzed with a Nikon Eclipse E800 fluorescence microscope and pictures were captured with a digital NIKON DXM1200 camera at 20X objective. KC content was determined by counting F4/80 or MOMA-2 positive cells in 20 visual fields in every section for two or three sections per liver.

Clodronate depletion of Kupffer cells.
Clodronate (Roche Diagnostics, Mannheim, Germany) was encapsulated in liposomes as previously described. After removal of free clodronate, liposomes were resuspended in NaCl (0.9%). Depletion of KCs was achieved by injection of multi-lamellar clodronate liposomes (iv) [31]. Efficiency of KC depletion was determined by staining liver sections with F4/80.

Figure 8.1



L-SIGN is interacting with monocytes. (A) CHO cells were transduced with L-SIGN and expression levels were tested by staining the cells with the anti-L-SIGN antibody AZN-D2 (black line). Grey histogram represents isotype staining. (B) Interaction of PBMCs with L-SIGN was investigated by incubating calcein-AM labelled PBMCs on monolayers of CHO and CHO-L-SIGN cells. Cell-cell adhesion was determined after washing and cell lysis using fluorimetry. L-SIGN specificity was determined by incubating by pre-incubation of CHO-L-SIGN cells with mannan (0.5 mg/ml, black bar), blocking anti-L-SIGN antibodies (20 µg/ml, light grey bar) or isotype control antibodies (dark grey bar). Error bars represent standard deviations of triplicates. One representative experiment out of three is shown. (C) The L-SIGN binding subset in the PBMC population was investigated by incubating CHO and CHO-L-SIGN cells with PBMC. Non-adherent cells were collected during washing and stained for CD3, CD4, and CD14. Expression was measured using flow cytometry. Expression levels of the indicated markers were compared for cells not adhering to CHO (dashed lines) or CHO-L-SIGN (black lines) and PBMC (filled histograms). One representative experiment out of three is shown. (D) Fluorescent beads coated with cell derived L-SIGN were incubated with PBMCs. Adhesion was measured by flow cytometry. T cells and monocytes were gated based on forward sideward scatter. Specificity of L-SIGN binding was confirmed by blocking with mannan and EGTA. One representative experiment out of three is shown.

Results

L-SIGN interacts with human monocytes.

L-SIGN is a pathogen receptor involved in the transmission of viruses, such as HCV and HIV-1 [13;14;22], and also in antigen presentation of these viruses [Ludwig, in preparation]. However, little is known about cellular interactions mediated by L-SIGN. In order to investigate the interaction of L-SIGN with peripheral blood mononuclear cells (PBMC), a cell-cell adhesion assay with L-SIGN-positive cells (Fig. 8.1A) and PBMC was performed. PBMCs were allowed to adhere to a monolayer of L-SIGN-positive or mock-transduced cells and about 30% of total PBMC adhered to L-SIGN-positive cells (Fig. 8.1B). This cellular interaction was L-SIGN specific since blocking with anti-L-SIGN antibodies or mannan reduced adhesion to background levels (Fig. 8.1B).

Since PBMCs are a mixed cell population, consisting of B cells, T cells and monocytes, we addressed the question which subset was interacting with L-SIGN. The non-adherent cells were collected and stained for CD4, CD3, and CD14. Cell distribution of non-adherent cells from parental CHO cells resembled that of the starting population of PBMCs. Strikingly, in the non-adherent cell population from CHO-L-SIGN cells, the $CD14^{+}$, $CD3^{-}$, $CD4^{intermediate}$ population was completely absent (Fig. 8.1C, Table 1) strongly indicating that the adherent population consists primarily of monocytes.

To confirm specific interaction of L-SIGN with monocytes, a beads adhesion assay was performed. Fluorescent beads were coated with native L-SIGN, derived from L-SIGN-positive cells, and these beads were incubated with PBMCs. Binding was analyzed using flow cytometry. L-SIGN-coated beads did not interact with PBLs, but as expected, they strongly interacted with monocytes (Fig. 8.1D). Moreover, the interaction of L-SIGN beads with monocytes was specific since it was blocked by mannan and EGTA (Fig. 8.1D). Thus this adhesion experiment using L-SIGN-coated beads supports the cell-cell adhesion assay demonstrating that L-SIGN interacts with monocytes.

mSIGNR1 is expressed on LSEC and functionally resembles L-SIGN.

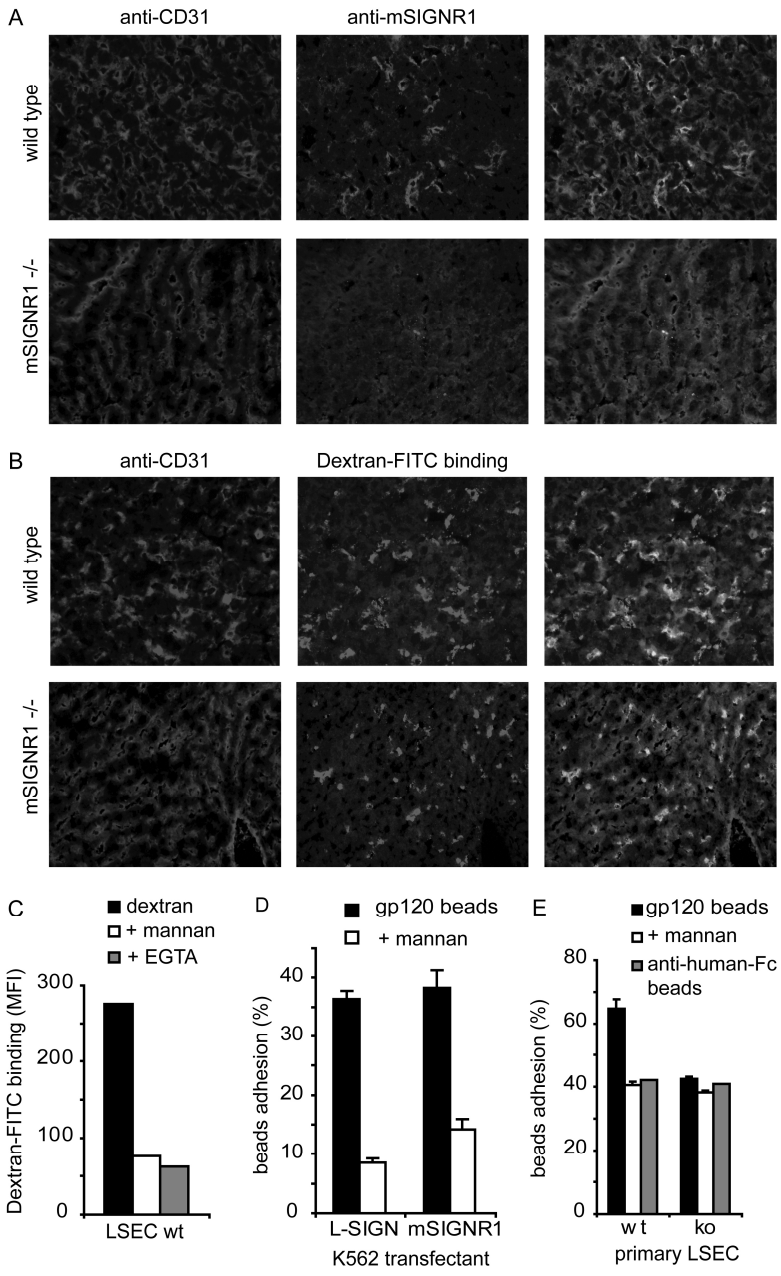
L-SIGN is expressed on liver sinusoidal endothelial cells in human liver [13]. Because of the difficulty of isolating sufficient LSECs from human tissue and the availability of an mSIGNR1 knock out mouse, we used a mouse model in order to investigate the role of L-SIGN-monocyte

Table 8.1.
Identification of PBMC subset binding to L-SIGN

		non-adherent cells from[a]	
	PBMC	CHO-mock	CHO-L-SIGN
CD3	54	60[b]	76
CD4 high	29	32	56
CD4 int	20	18	6
CD14	14	11	1

[a] PBMC were incubated on monolayers of CHO-mock or CHO-L-SIGN. Non-adherent cells were collected and stained for CD3, CD4 and CD14 expression.
[b] percentage positive cells

Figure 8.2



mSIGNR1 is the murine homologue of L-SIGN on LSECs. (A) Wild type and mSIGNR1$^{-/-}$ mouse liver sections were stained with anti-mSIGNR1 (green) and anti-CD31 (red) antibodies and counterstained with anti-goat Alexa Fluor 488 and anti-rat Alexa Fluor 595. Sections were analyzed with fluorescent microscopy. (B) Wild type and mSIGNR1$^{-/-}$ mouse liver sections were incubated with dextran-FITC and subsequently stained for CD31 (red). Dextran-FITC binding and antibody staining were analyzed with fluorescent microscopy. (C) LSECs were isolated from wild type liver by perfusion with collagenase and percoll gradient. At day 6 after isolation cells were incubated with dextran FITC and binding was analyzed by flow cytometry. Specificity of mSIGNR1 binding was determined by blocking with EGTA and mannan. One representative experiment out of three is shown. (D) K562 cells transfected with L-SIGN or mSIGNR1 were incubated with gp120-coated beads. Specificity was determined by blocking with mannan (0.5 mg/ml) Adhesion was measured using flow cytometry. Error bars represent standard deviations of triplicates. One representative experiment out of three is shown. (E) Primary LSECs from wild type or mSIGNR1$^{-/-}$ liver were, after 6 days of culture, incubated with gp120 coated fluorescent beads. Adhesion was measured by flow cytometry. Specificity of mSIGNR1 binding was determined by blocking with EGTA and mannan. Error bars represent standard deviations of triplicates. One representative experiment out of two is shown.

interactions in vivo. In mice the DC-SIGN/L-SIGN homologue mSIGNR1 is also expressed on CD31 positive LSEC (Fig. 8.2A and [32]). Thus we used mSIGNR1 knock out mice to further investigate the role of mSIGNR1 in liver. mSIGNR1$^{-/-}$ liver sections did not stain for mSIGNR1, confirming that these mice were lacking any mSIGNR1 (Fig. 8.2A).

Next we investigated whether mSIGNR1 on LSECs is functional and whether it has a similar binding specificity as L-SIGN. To investigate the functionality of mSIGNR1 on LSECs we studied binding of dextran-FITC, a known mSIGNR1 ligand, to murine liver sections. In wild type liver sections, a CD31 positive and a CD31 negative cell population bound dextran-FITC (Fig. 8.2B). In mSIGNR1$^{-/-}$ mice liver tissue dextran-FITC is only bound by the CD31 negative cell population (Fig. 8.2B). The CD31 negative cells reside in the sinusoids and probably are KC. The finding that in wild type mice, in contrast to mSIGNR1$^{-/-}$ mice, also CD31 positive cells bind dextran-FITC indicates that mSIGNR1 on LSEC is functional in wild type mice.

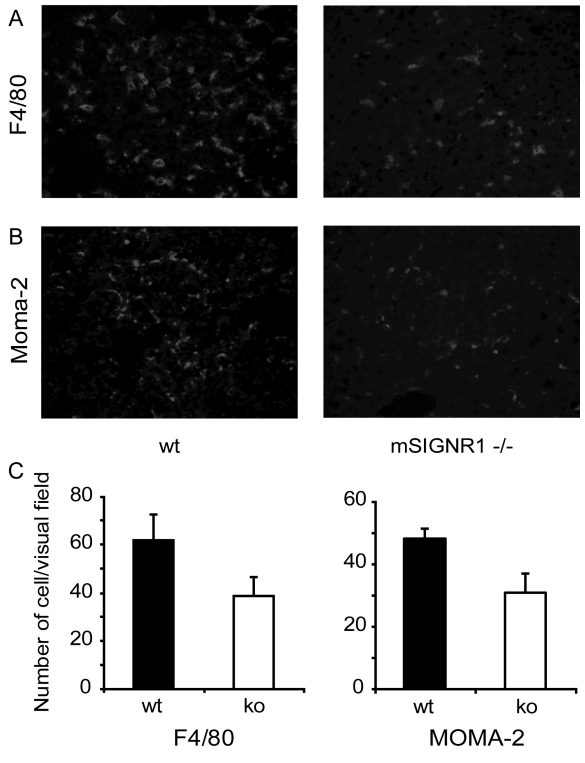
Next, we isolated primary LSEC from liver by perfusion with collagenase buffer and subsequent centrifugation on a percoll density gradient. Primary LSEC were positive for CD31 and able to bind dextran (data not shown and Fig. 8.2C). Dextran-FITC binding was specific since mannan and EGTA blocked the interaction (Fig. 8.2C). Additionally, we compared mSIGNR1 function with L-SIGN by investigating the binding of HIV-1 envelope glycoprotein gp120. Recombinant gp120-Fc was coupled to fluorescent beads and adhesion to K562 cells transfected with L-SIGN or mSIGNR1 was investigated. Both K562-L-SIGN and K562-mSIGNR1 cells efficiently bound gp120 beads as was previously described (Fig. 8.2D and LSECs (Fig. 8.2E). Furthermore, wild type LSECs displayed binding significantly above background levels, whereas mSIGNR1$^{-/-}$ cells did not, and this binding could be specifically blocked by mannan (Fig. 8.2E). These data indicate that mSIGNR1 resembles L-SIGN both in expression pattern and function on LSEC.

mSIGNR1 is regulating Kupffer cell homeostasis.

Next we investigated the role of mSIGNR1 in KC homeostasis, because we had observed that L-SIGN interacts with monocytes, which are precursors of KCs. KCs are in close proximity of mSIGNR1-positive LSECs in the liver sinusoids. Therefore we compared KC population of wild type mice and mSIGNR1 knock out mice by staining liver cryosections with the KC markers F4/80 and MOMA-2. Sections were analyzed and quantified by fluorescent microscopy (Fig. 8.3A). Strikingly, mSIGNR1$^{-/-}$ mice had less KC in liver compared to wild type mice (Fig. 8.3B). The amount of KCs was decreased with 40% in the absence of mSIGNR1. Thus mSIGNR1 in mouse liver sinusoids is participating in the maintenance of KC population.

KCs originate from monocytes that enter the liver sinusoid through the bloodstream and locally differentiate to macrophages. The KC population furthermore is maintained constant by local

Figure 8.3

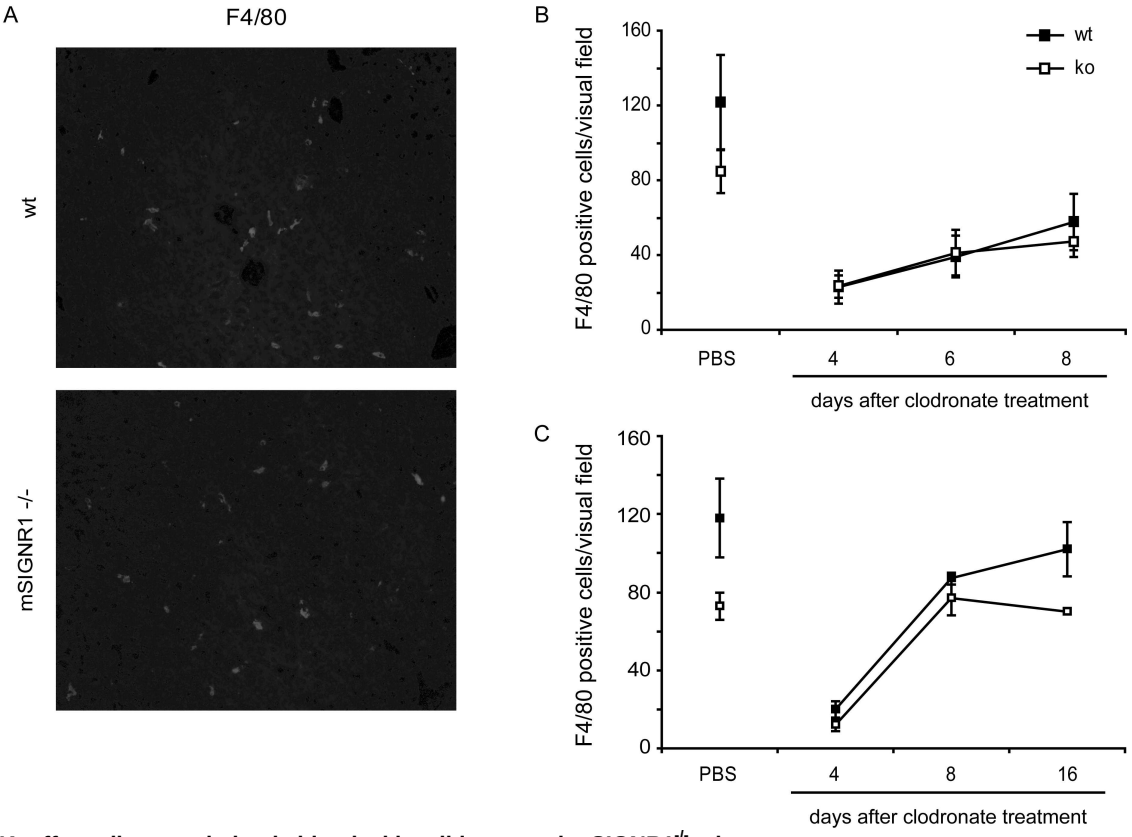


KC population in liver is decreased in mSIGNR1$^{-/-}$ mice.

(A and B) Liver sections of wild type (left panels) and mSIGNR1$^{-/-}$ (right panels) mice were stained with F4/80 (A) or MOMA-2 (B) antibodies and counterstained with goat-anti-rat Alexa Fluor 594 and analyzed with fluorescence microscopy. (C) F4/80 or MOMA-2 positive cells were counted in 10 visual fields of three sections per liver. Error bars represent standard deviations.

proliferation of cells that are already present in the liver sinusoid. As mSIGNR1 seems necessary for KC homeostasis (Fig. 8.3), we hypothesized that mSIGNR1 is either involved in the recruitment of monocytes or in the proliferation of KCs. In order to address the question whether mSIGNR1 is involved in the recruitment of KCs we depleted all KCs from the liver by i.v. administration of liposome-encapsulated clodronate. With the administration of liposome-encapsulated clodronate, macrophages are specifically removed, as these cells phagocytose the liposomes and after lysosomal degradation cytotoxic clodronate is released [34]. Mice were sacrificed at different days after clodronate administration, livers were removed, and the amount of KCs was quantified. Four days after clodronate injection, almost no KCs were present in the sections (Fig. 8.4A). Onwards, the KC population gradually increased over time and reached normal numbers after 8 to 16 days after KC depletion. Until day 8, KC repopulation kinetics were similar in both wild type and mSIGNR1$^{-/-}$ mice (Fig. 8.4B and C), suggesting that mSIGNR1 is not involved in the initial repopulation of KCs in the liver. After this initial phase, when KC number in mSIGNR1$^{-/-}$ liver has reached its maximum, the KC population in wild type mice increased further (Fig. 8.4C). These data suggest that KC repopulation in a KC deprived liver is a biphasic process of which the first phase is mSIGNR1 independent and the second phase is mSIGNR1 dependent resulting in more KC in the liver in the presence of mSIGNR1. This could be due to the recruitment of more monocytes to the liver or by influencing turnover of residing KC.

Figure 8.4



Kupffer cell repopulation is identical in wild type and mSIGNR1$^{-/-}$ mice.
Wild type and mSIGNR1$^{-/-}$ mice were given liposome-encapsulated clodronate or PBS i.v.. In two separate experiments livers were removed at day 4, 6 and 8, or at day 4, 8 and 16, and liver sections were stained with F4/80 to stain KCs. F4/80 positive KCs were counted in 10 randomly chosen visual field at 20 times magnification of two sections per liver. One mouse out of two per group is shown. Error bars represent standard deviations.

Discussion

C-type lectin receptors, like MR and DC-SIGN, are acting as pathogen recognition receptors on DCs to take up antigens for presentation. However, C type lectins also interact with self-ligands. MR binds to bacterial products [15], but also to endogenous ligands [16]. DC-SIGN is recognizing several pathogens like HIV-1, HCV, and mycobacterium tuberculosis and self ligands on endothelial cells, T cells and neutrophils [35-37]. The DC-SIGN homologue L-SIGN has a comparable binding pattern to DC-SIGN, although due to the lack of Le^x recognition L-SIGN does not bind Le^x containing ligands [38]. Little is known about the cellular function of L-SIGN. Here we demonstrate that L-SIGN, present on LSEC in the liver, interacts with monocytes and the murine L-SIGN homologue mSIGNR1 affects the KC population, which differentiate from monocytes, in the liver.

The origin of KCs, the resident liver macrophages, remains elusive. It has been hypothesized that KCs are derived from recruited monocytes that differentiate locally into KCs in response to chemokines. This is in line with the theory of the mononuclear phagocyte system [39]. According to this theory, all macrophages derive from non-proliferating monocytes which are differentiated via promonocytes from monoblasts. However, although the estimated life span of KC in mice is about 4 days, in monocytopenic mice lacking monocytes, and therefore have no monocyte recruitment to the liver, KC reside in the liver for up to 6 weeks [8;9]. This argues for a proliferative capacity of KCs that contributes to keeping the KC population constant.

We demonstrate here that the lack of mSIGNR1 in the liver results in a reduced amount of KCs in the liver (Fig. 8.3). mSIGNR1 and L-SIGN are both expressed on LSECs in the liver in mice and human respectively (Fig. 8.2, [13;32]) and have comparable binding patterns (Fig. 8.2, [33]). Therefore, it is possible that the decrease of KCs in the absence of mSIGNR1 can be accounted to the importance of mSIGNR1-monocyte interactions, promoting monocyte arrest in the liver sinusoid. The interaction of murine monocytes with mSIGNR1 is not investigated yet, but the reduced numbers in KCs are indicative for this interaction. It is demonstrated that L-SIGN-positive cells promote neutrophil rolling and adhesion under shear stress [Ludwig, chapter 7 addendum], a mechanism that could also function in monocyte-L-SIGN interactions.

Upon depletion of all macrophages from the liver with clodronate liposomes, the initial KC repopulation is similar in both wild type and mSIGNR1$^{-/-}$ mice, suggesting that mSIGNR1 is not involved in the initial recruitment of monocytes to the liver. Furthermore, because of the small diameter of the liver sinusoids, incoming monocytes might not even need initial attachment to stay in the sinusoids. Indeed, passing leukocytes can roll over sinusoidal endothelium independent of selectin-adressin interactions normally needed due to the small size and low and intermittent blood flow [40], before adherence to the endothelial cell layer. Therefore recruitment of monocytes after depletion of all KC, might occur independent of cellular interactions and chemokines. Our data implicate that the return of KC in a KC depleted liver is a biphasic process and the difference in KC numbers between wt and mSIGNR1$^{-/-}$ mice is due to differences in the second phase of KC repopulation. In this second phase, mSIGNR1 might influence KC homeostasis in several ways. First, mSIGNR1 might create more binding sites for monocytes entering the sinusoid, besides mSIGNR1 independent binding sites. After the initial ‘filling’ phase, when all other sites are occupied, monocytes will pass the liver without retention in the absence of mSIGNR1. However in the presence of mSIGNR1, more monocytes could be retained in the sinus. Second, mSIGNR1 could influence the cell turn over of the KC

present in the sinusoids, by reducing apoptosis or promoting proliferation. However, this hypothesis only holds when the mSIGNR1/L-SIGN ligand expressed by monocytes is still expressed after differentiation into KCs. Although the precise mechanism is not known, these data indicate for a role of C-type lectins in monocyte binding and KC homeostasis.

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9

General discussion

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General discussion

DC-SIGN is a C-type lectin expressed by DCs that recognizes self, altered-self and pathogen-derived ligands, such as cellular ICAM-2, tumour associated CEA, HIV-1 and HCV [1-4]. DC-SIGN functions as an adhesion molecule but upon binding, particulate ligands can also be internalized, resulting in antigen processing and presentation to $CD4^+$ T cells [5;6]. DC-SIGN also mediates cross presentation of antigens to $CD8^+$ T cells [7]. The cytoplasmic domain of DC-SIGN contains three putative internalization motifs; a tyrosine-based YxxL motif, a di-leucine based motif and a triacidic cluster [8]. These motifs are associated with internalisation of receptor-ligand complexes and targeting to lysosomes. However, mutagenesis of these motifs demonstrated that only the di-Leucine motif is necessary for internalization [9;10].
The DC-SIGN homologue L-SIGN contains two internalization motifs, the di-leucine motif and a triacidic cluster, but it lacks the tyrosine based motif. L-SIGN is expressed on specialized endothelial cells in the liver. These liver sinusoidal endothelial cells (LSECs) can act as APC. The cells have a great internalisation capacity and contain large amounts of lysosomal enzymes [11] [12]. In mice, LSECs have shown to be able to present antigens to T cells, both $CD4^+$ and $CD8^+$, without the need of prior activation [13;14]. In contrast to conventional endothelial cells, LSECs constitutively express MHC class II [15;16], and mouse LSECs also express the costimulatory molecules CD40, CD80 and CD86 [17;18]. Strikingly, human LSECs express CD40 but lack expression of CD80 and CD86 [19]. Previously, it was suggested that L-SIGN lacks the capacity to internalise antigens for antigen presentation, and that L-SIGN merely functions as an adhesion receptor [20]. However, we have demonstrated that L-SIGN is able to internalise ligands [chapter3 [3]] resulting in presentation to $CD4^+$ T cells [chapter 4]. Thus L-SIGN functions also as an antigen uptake receptor. The presentation of antigens by LSECs might result in tolerance, as these cells lack the expression of some co-stimulatory molecules. Concomitantly, in mice, LSEC promote the induction of tolerogenic $CD4^+$ and $CD8^+$ T cells [13;14]. Therefore, it has been hypothesized that pathogens target L-SIGN for internalization by LSECs and thereby inducing tolerance.

Carbohydrate recognition profile of DC-SIGN and L-SIGN

The amino acid similarity of the carbohydrate recognition domains of DC-SIGN and L-SIGN is over 80%, and both lectins contain the EPN motif associated with the recognition of mannose and fucose structures [8;21]. DC-SIGN recognizes high mannose and fucose-containing carbohydrates such as $Lewis^a$ (Le^a), $Lewis^b$ (Le^b), $Lewis^x$ (Le^x), and $Lewis^y$ (Le^y) [20;22-24]. Sulfation of Le^a and Le^x does not abrogate DC-SIGN recognition, whereas sialylation of these Lewis antigens results in loss of recognition [22;24]. This specificity has been confirmed with different assays using both soluble recombinant DC-SIGN [20;22] and cellular DC-SIGN [22;24]. In spite of the high homology between the CRDs of DC-SIGN and L-SIGN there are differences in carbohydrate recognition. Soluble recombinant L-SIGN was shown to bind only high mannose containing glycoconjugates [20]. In contrast, L-SIGN expressed on cells was shown to recognize also fucose-containing glycoconjugates, Le^a, Le^b and Le^y [24]. The discrepancy is most likely due to incorrect clustering of the soluble L-SIGN. In contrast to DC-

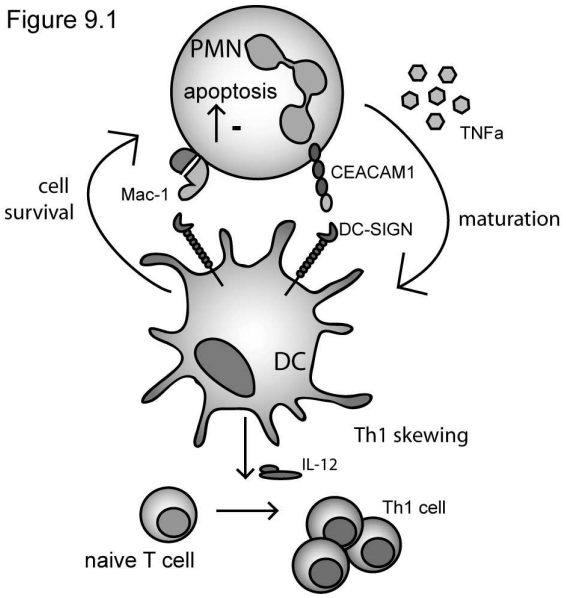
SIGN, L-SIGN is not able to interact with Le^x. The disparity in Le^x binding between DC-SIGN and L-SIGN is based on one amino acid difference in the CRD [25]. Whereas DC-SIGN has a valine at position 351 (Val351), L-SIGN has at this same position a serine (Ser363) [20;25]. Le^x binding capacity is restored by replacing Ser363 in L-SIGN with a Valine residue, thereby creating a hydrophobic pocket [25].
Besides this dissimilarity in Le^x binding, there is yet another difference in carbohydrate binding profile. L-SIGN recognizes particular sialic acid containing glycoconjugates [chapter2], in contrast to DC-SIGN carbohydrate recognition, which is abrogated upon sialylation of carbohydrates [22;24]. Recognition of these sialylate carbohydrates is dependent on the specific protein backbone, since sialylated glycoconjugates on a polyacrylamide carrier were neither recognized by soluble L-SIGN [20] nor L-SIGN expressed on cells [24].
In conclusion, high mannose and certain fucose-containing structures are recognized by both DC-SIGN and L-SIGN. DC-SIGN and L-SIGN specificity is distinct in their recognition of Le^x and sialylated carbohydrates. Thus, although the binding specificity of Dc-SIGN and L-SIGN is very similar, both DC-SIGN and L-SIGN can interact with specific pathogens and self-structures based on their exclusive recognition of Le^x and sialic acids respectively.

Cellular interactions of DC-SIGN and L-SIGN

DC-SIGN and L-SIGN interactions with neutrophils.
During homeostasis polymorphonuclear neutrophils **(**PMN) and DCs are located in different compartments, in the bloodstream and in peripheral tissue respectively. However, during infection they both are located at the site of inflammation. Upon microbial infection, PMN produce chemokines that attract immature DCs [26]. Recently it has been shown that neutrophils and DCs form clusters when cocultured [27;28]. This suggests that PMN and DC scan each other's surface continuously when they are near each other. The interaction with PMN induces maturation of DC as shown by the upregulation of costimulatory molecules HLA-DR, CD86 and CD40. Besides the maturation of DCs, PMN also induce IL-12 production by DCs [27]. This results in a greater capacity of these 'primed' DCs to activate T cells [28]. The maturation of DCs is mediated by TNF-α production by neutrophils. However, when PMN and DCs are in direct contact the effect is greatly enhanced [28;29]. This implicates that cell-cell interactions have an additional effect on the effect of TNF-α.

Direct cell-cell contact between PMN and DCs is dependent on CD18 [28]. CD18 is expressed in heterodimers with CD11b, forming Mac-1, and with CD11a forming LFA1. Recently it was shown that the C-type lectin DC-SIGN on DCs can interact with the CD11b/CD18 heterodimer Mac-1 [27], and CEACAM1 [chapter7][30;31]. Indeed, DC maturation by PMN was shown to be dependent on DC-SIGN on the DC side [27]. There is some controversy about the activation state of the PMN that can induce DC maturation. Whereas van Gisbergen [27] shows that only activated PMN, which have increased Mac-1 expression, can induce DC maturation, others [28] show that also non-activated PMN are able to induce DC maturation. This can be due to differences in isolation methods resulting in an already activated state of the PMN, resulting in an upregulation of Mac-1, CEACAM1 and TNF-α expression. Whereas probably low expression of Mac-1 and CEACAM1 is sufficient for clustering, a stronger interactions and TNF-α is needed to induce DC maturation.

Figure 9.1



DC interaction with PMN.
DCs and PMN influence each other. DC-SIGN binds Mac-1 and CEACAM on PMN and induces cell survival. Via this interaction and TNF-α production by PMN, DCs are instructed to regulated T-cell differentiation towards Th1.

Mac-1 (CD11b/CD18) on PMN is involved in pathogen recognition and binding and is redirecting them to phagosomes in PMN. Mac-1 can bind directly to several pathogens [32] but is also a receptor for complement and thus serves as a receptor for opsonized pathogens [32]. Mac-1 also functions in the regulation of apoptosis with a twofold effect: signalling of Mac-1 alone promotes cell survival [33], whereas in combination with an apoptosis signal, Mac-1 triggering is resulting in higher apoptosis induction [34]. The other DC-SIGN ligand on PMN, CEACAM1 (CD66a), mediates cell-cell adhesion via homophilic binding. This interaction regulates cell proliferation and it delays apoptosis of PMN [35]. CEACAM1 is expressed on more cells from myeloid origin, however, it differs in its glycosylation pattern between these different cell types [36]. CEACAM1 on PMN is expressing high amounts of Le^x [37], and indeed DC-SIGN binding to CEACAM1 is entirely based on the expression of Le^x structures on PMN CEACAM1 [chapter7][30]. Mac-1-DC-SIGN interactions are based on Le^x and Le^y expressed on Mac-1 [27].

PMN induce DC maturation and indeed these DCs efficiently induce T-cell proliferation [27]. DCs matured by LPS induce a mixed type differentiation of T cells, IFN-γ producing Th1 and IL-4 producing Th2 cells are induced to the same extent. However, when DCs are simultaneously interacting with PMN, the balance is shifting from a mixed type to a Th1 based response [27] (Fig. 9.1). This is reflected *in vivo* during Crohn's disease, a disease characterized by inflammation of the colon mucosa and Th1 responses. In the mucosa of Crohn's disease patients DCs and PMN can be found in close vicinity [27] and both the DC-$SIGN^+$ and the DC-$SIGN^-$ DC population are increased [38]. However only the DC-$SIGN^+$ DCs are interacting with PMN and further more these are responsible for the production of Th1 cytokines [27;38]. This implies the importance of the interaction of PMN with DC-$SIGN^+$ DCs in the development of the vigorous Th1 response in Crohn's disease.

Based on the chemokine expression pattern of DCs it was hypothesized that DCs play a role in the recruitment of PMN to inflamed tissues [39]. Indeed, DC conditioned medium attracts PMN. Both immature and mature DC can induce PNM migration, however mature DCs are more

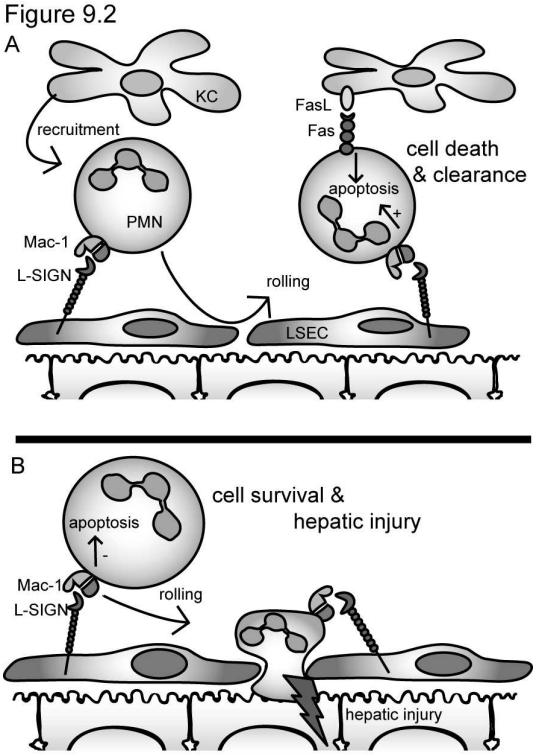
potent because of higher IL-8 production [40]. Furthermore the cytokines produced by DCs enhance the phagocytic capacity of PMN [41].
DCs interact through DC-SIGN with both Mac-1 and CEACAM1 on PMN. Since both CEACAM1 and Mac-1 are involved in the regulation of PMN apoptosis, it is likely that binding of DCs to these molecules has an effect on PMN apoptosis. Indeed it was shown that interaction of DCs with PMN results in a lack of down regulation of several markers such as CD13, CD15, CD16, and CD11b/CD18 (Mac-1) on the PMN on a subset of the PMN [28]. The down regulation of these markers is associated with induction of apoptosis [42;43]. This suggests that the interaction of DCs with PMN prolongs the lifespan of neutrophils (Fig. 9.1). Upon DC maturation, DC-SIGN expression is decreased, and although also mature DCs are binding PMN, this is less than the binding of immature DCs [27]. This implies that the binding of an individual mature DC to a PMN is less strong than that of immature DC. It is possible that the decrease in binding results in a change from an anti-apoptotic signal in an apoptotic signal by which the inflammation is ended.

L-SIGN positive cells are localized at a strategic site in the liver, and L-SIGN can mediate PMN adherence and rolling over liver sinusoidal endothelial cells [Chapter 7 addendum]. PMN rapidly sequester in the liver upon inflammation, even when the inflammation is at a remote site. PMN are recruited to the liver by Kupffer cell produced factors [44]. The liver functions in the clearance of old and redundant PMN, and the recruitment of PMN during inflammation could result in the ending of the inflammation by this process. Upon interaction with bacterial products Kupffer cells upregulate Fas ligand (FasL) expression [45]. FasL-Fas engagement on PMN will induce apoptosis [46]. Strikingly, PMN also induce severe hepatic injury upon bacterial infections. They are even the main players in this process as depletion of PMN protects against septic liver injury [47]. This argues that it is of utmost importance that PMN are cleared from the liver before they generate severe liver damage.

The effect of the L-SIGN-PMN interaction *in vivo* remains to be elucidated; however it is tempting to suggest a role in apoptosis induction. L-SIGN is binding PMN, however because L-SIGN is not able to bind to Le^x, this interaction is not via CEACAM1 but only via Mac-1 [30][chapter 7]. When PMN apoptosis is already triggered, L-SIGN association with Mac-1 could enhance apoptosis induction. During inflammation this is likely as the phagocytosis of opsonized particles itself is an apoptotic signal for PMN [48;49]. Further more, Kupffer cells can provide an apoptotic signal as they up regulate FasL after contact with bacterial products [45]. However, when PMN are not programmed yet to undergo apoptosis, the interaction of Mac-1 with L-SIGN could have a reverse effect by inhibiting spontaneous apoptosis and even mediate transmigration of activated PMN across liver sinusoidal endothelium into the parenchyma during bacterial infections (Fig. 9.2).

Liver cells interacting with L-SIGN
Besides the interaction with neutrophils, L-SIGN interacts with a yet unknown ligand on monocytes [chapter 8]. Monocytes are adhering to both L-SIGN-positive cells and recombinant L-SIGN [chapter 8]. Therefore, L-SIGN could function in rolling and arrest of monocytes in the liver sinusoid, similar as for neutrophils [chapter 7 addendum][50]. Although in our assays no DC-SIGN binding to monocytes was demonstrated, others show that DC-SIGN interacts weakly with monocytes [51]. This binding is increased when Le^x structures are exposed after

Figure 9.2

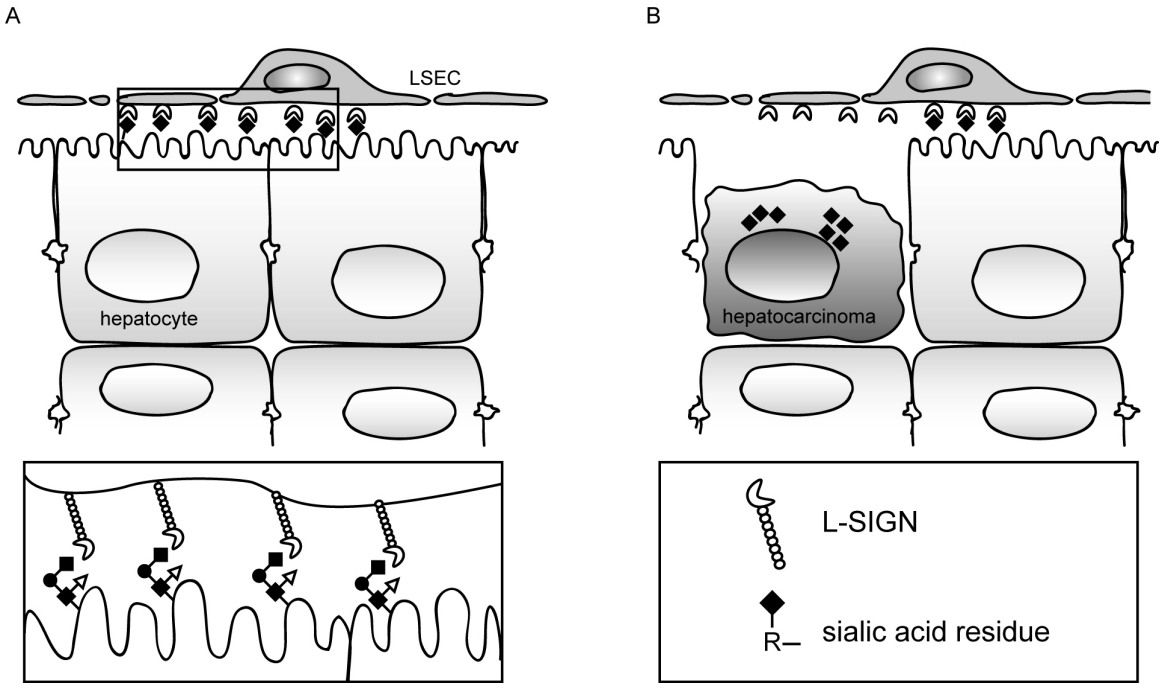


PMN in liver sinusoid.
PMN are recruited to the liver by Kupffer cells were they role over and adhere to sinusoidal endothelial cells. (A) When Kupffer cells via FasL induce PMN apoptosis this is enhanced by L-SIGN-Mac-1 interactions. (B) Alternatively, when an apoptotic signal is absent, activated PMN can role over the L-SIGN^{+} endothelial cells and induce severe hepatic injury.

acids. Notably, we observed that mice deficient in the murine DC-SIGN/L-SIGN homologue neuraminidase treatment [51], indicating that most of the Le^{x} structures are masked by sialic acids. Notably, we observed that mice deficient in the murine DC-SIGN/L-SIGN homologue mSIGNR1 had lower numbers of KCs in the liver sinusoids [chapter 8]. mSIGNR1 is expressed by LSEC similar as L-SIGN, and combined with the finding that KCs originate from monocytes it can be hypothesized that monocytes interact with L-SIGN and mSIGNR1 in order to settle in the liver sinusoids. These receptors might also provide a signal to the monocytes, that induces monocyte differentiation into KCs.

We also observed that L-SIGN interacts with the well-differentiated hepatoma cell line HUH7 through sialic acid-containing glycoconjugates [chapter 2]. Healthy hepatocytes also express large amount of α-2,6-sialic acid on their surface mainly confined to the sinusoidal side of hepatocytes [52], suggesting that L-SIGN might interact with the hepatocytes. The interaction between LSEC and hepatocytes, which are not connected with extracellular matrix [53], could contribute to maintenance of hepatic sinus architecture.
Oncogenic transformation often results in a perturbation of the glycosylation machinery [54], which has severe effects on the metastatic properties of the tumour. Sialic acid expression on the cell membrane is lost upon malignant transformation of hepatocytes into hepatocarcinoma cells and sialic acids are solely expressed around the nucleus in the cytoplasm [52]. The loss of sialylated ligands would loosen the L-SIGN-hepatocellular carcinoma cell interaction and the hepatocellular carcinoma cells would more easily transmigrate into the bloodstream and form metastases at distant sites (Fig. 9.3). Indeed this loss of sialic acid expression is indicative for a poor patient survival [55].

Figure 9.3



L-SIGN binds sialylated carbohydrates expressed by hepatocytes.
(A) L-SIGN on LSEC binds to sialic acid containing glycan structures expressed by hepatocytes. (B) The surface expression of sialylated carbohydrates is abrogated in hepatocellular carcinoma cells, and sialylated carbohydrates are confined intracellular. The lack of cell surface expression of the L-SIGN ligand results in loss of interaction between the carcinoma cell and the endothelial cell layer, and the carcinoma cell could subsequently extravasate.

Role of L-SIGN in metastasis of colon carcinomas

In contrast to the loss of sialic acid glycoconjugates during malignant transformation of hepatocellular carcinoma cells, colon cells display an increased [56-58] or *de novo* [59] expression of sialylated carbohydrates upon malignant transformation. The increased expression of ST6Gal1, the enzyme catalyzing the generation of α-2,6 sialylated glycans, in colorectal carcinoma cells correlated to the metastasizing capacity of colon carcinomas in the liver [60] and negatively correlated with survival [61]. Expression of sialylated glycoconjugates on the cell surface metastasizing colon carcinoma cells could facilitate adherence to L-SIGN on LSEC, and thereby enhancing the formation of liver metastasis. Indeed, removal of sialic acid residues by neuraminidase treatment reduces metastatic capacity [62] and desialylation with the sialyltransferase inhibitor KI-8110 completely abolished liver metastasis of certain tumours [63].

DC-SIGN can mediate DC-tumour cell interaction by recognition of CEA and CEACAM1 on colon carcinoma cells [4]. Besides the upregulation of sialylation, malignant transformation of colon epithelial cells results also in an increased expression of Le^x and Le^y epitopes [64;65]. As a consequence, DC-SIGN can discriminate between normal and colon carcinoma tissue derived CEA and CEACAM1. Notably we also demonstrated an increased binding of tumour CEA over normal colon derived CEA to L-SIGN [chapter 2], suggesting that this interaction might be involved in metastation of the tumour. Metastasizing colon carcinoma cells reach the hepatic sinus via the blood stream and the interaction between L-SIGN and the α-2,6-sialic acid structures and Le^y epitopes expressed by the tumour cells. This would in addition to

getting constricted by the narrow vasculature of the sinus, explain the frequent metastasis formation of colon carcinomas in the liver.

In conclusion, L-SIGN mediates cellular interactions with PMN, monocytes and hepatocytes, important for homeostasis in the liver.

DC-SIGN and L-SIGN in virus interactions

DC-SIGN interacts with several pathogens such as (myco)bacteria and viruses. (Myco)bacteria tend to modulate DC-mediated immune responses by binding DC-SIGN. For example, *Mycobacteria tuberculosis* is preventing DC maturation [66], and both *Lactobaccilus reuteri* and *Lactobacillus casei* direct DCs to induce the development of IL-10 producing regulatory T cells [67]. Viruses, on the other hand, target DC-SIGN to infect DCs and for viral transmission to target cells [1;68-73].
DC-SIGN and L-SIGN interact with several viruses such as HIV-1, HCV, Ebola virus, and cytomegalovirus virus [chapter3][1;3;70;71;74-76]. All these viruses express highly glycosylated envelope proteins, and these carbohydrates structures exposed on the surface of the virus mediate interactions between the virus and the C-type lectins. The HCV envelope is formed by two heavily N-glycosylated type I transmembrane envelope glycoproteins E1 (31 kD) and E2 (70 kD) expressed as heterodimers [77-79]. Most of the glycosylation sites in the viral envelope proteins are well conserved, since these sites are important for proper folding and heterodimerisation of the proteins [80], and for viral entry [81]. Indeed, besides DC-SIGN and L-SIGN several other receptors have been described recognizing the envelope proteins; CD81 [82], ASGPR [83], and SR-B1 [84]. By binding these receptors the virus not only mediates attachment and entry but might also interfere with immune responses. HCV E2 interaction with CD81 on NK cells, for instance, leads to inhibition of proliferation and activation [85;86].

Dendritic cells in HCV infections.
Although hepatocytes are the main target of the HCV virus, other cells are also affected during HCV infections. The effect of HCV on DC function is under debate and there are indications for impairment of DC function but also for normal DC function. In chronic HCV infected patients, myeloid and plasmacytoid DC counts are lower than in healthy individuals [87-90]. Maturation and immune stimulatory functions of these DCs are impaired, especially in inducing Th1 responses [87;88;91-93]. On the contrary, in resolved HCV infections DC function and numbers are not impaired [92]. Also monocyte-derived DCs of chronic HCV infected individuals are impaired in stimulating T cells [92-94]. In contrast, other studies demonstrated that monocyte-derived DCs [95;96], myeloid, and plasmacytoid DCs [89;97] from patients suffering from chronic HCV infection are functionally normal. Thus, more research is necessary to understand the role of DCs in the establishment of HCV infections.
DCs express the HCV binding receptors CD81, which recognizes E2 [82;98], and DC-SIGN, which binds both E1 and E2 [3;74-76]. DCs strongly interact with HCV envelope proteins, which is mediated by DC-SIGN since blocking DC-SIGN almost completely abrogates DC-HCV interactions [chapter3][3;76]. Upon interaction with HCV envelope proteins, the particles are internalized by DCs and, in contrast to other carbohydrate ligands, targeted to non-

lysosomal compartments [chapter3][3]. The normal routing after internalisation is directed through early endosomes to lysosomes. Indeed, antibodies [5] and carbohydrate ligands of DC-SIGN [chapter3][3] are targeted to lysosomes for degradation and presentation to T cells. Remarkably this specific internalization pathway of HCV is cell-type specific and only occurs in antigen presenting cells, both primary DCs and B-cell lines expressing DC-SIGN, but not in non-APC cell lines [chapter3][3]. A similar internalization pathway and cell-dependency has been described for the HIV-1 virus [99]. HIV-1 is also retained in non-lysosomal vesicles after uptake via DC-SIGN, and similar to HCV targeting this differential routing is cell type specific [99]. The mechanism by which this differential targeting is regulated remains elusive. Clustering of DC-SIGN binding sites on and the size of the internalized particle could be involved, by mediating the formation of DC-SIGN aggregates on the cell surface, whereas less multimeric ligands with less binding sites for DC-SIGN are targeted to lysosomes. A recent study demonstrated that the interaction of DC-SIGN with the leukocyte-specific protein-1 (LSP1) directs DC-SIGN-captured ligands to the proteasome [100], suggesting that HCV interaction might modulate this interaction and prevent degradation. The retention of HCV virus in non-lysosomal compartments protects the virus from degradation and can lead to transmission to target cells [68].

However, besides protection and transmission, DCs also mediate HCV antigen presentation [101]. HCV presentation is dependent on the interaction with DC-SIGN [chapter 4]. Although HCV is partially protected by DC-SIGN, a part is diverted to the lysosomes, processed and derived antigens are presented to $CD4^+$ T cells [chapter 4]. The degradation of HCV-VLPs is enhanced in the presence of HCV specific antibodies [chapter 4], indicating that Fc-receptors may participate in the internalization of HCV, and diverting HCV from the non-lysosomal route into the lysosomal route. This study implies that DCs process and present HCV antigens more efficiently through Fc interactions when HCV is bound by HCV specific antibodies.

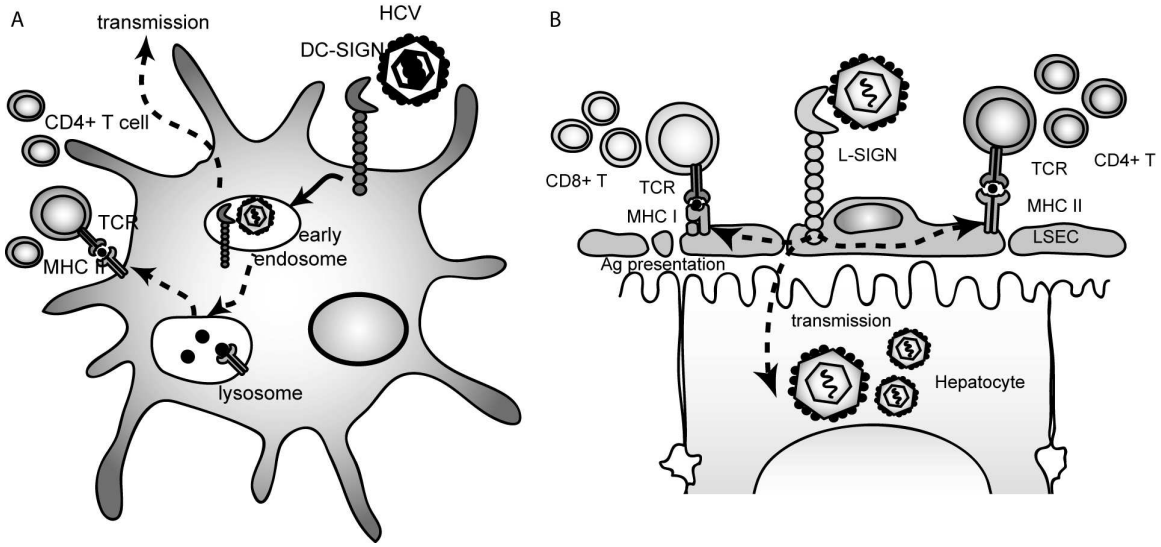
LSEC in HCV infections

L-SIGN recognizes, similar to DC-SIGN both E1 and E2 viral envelope glycoproteins [3;74-76]. Furthermore, cell lines expressing L-SIGN rapidly internalize HCV through L-SIGN, HCV-VLPs are retained in early endosomes [chapter3][3], suggesting that this pathway might be involved in transmission of HCV. Indeed, L-SIGN mediates transmission to hepatocyte cell lines [68]. This is actively mediated by L-SIGN since the number of neck repeats determines the efficiency of virus binding and transmission [102].

L-SIGN is expressed by LSECs and these cells form a barrier between the blood and the hepatocyte layer. Thus, the HCV in blood can be captured by L-SIGN and transmitted to hepatocytes. The transmission of a virus from LSECs to hepatocytes was also demonstrated for Duck hepatitis B virus [103].

Since LSECs function as APC, uptake of HCV in LSECs can also result in antigen presentation. However, LSECs lack costimulatory molecules and therefore presentation of HCV antigens to T cells might lead to tolerance. In the liver, Th2 responses are preferentially induced, whereas Th1 cells are suppressed [104;105]. Indeed, LSEC presentation on MHC II leads to differentiation to Th2 cells [13]. Furthermore, presentation on MHC I results in tolerogenic, non-cytotoxic $CD8^+$ T cells, and there are indications for liver specific selective retention and clearance of activated $CD8^+$ T cells [106;107]. This tolerance induction can be overcome when simultaneously with antigen presentation by LSEC viral E2 binds to CD81 on the T cell [108].

Figure 9.4



HCV escapes immune responses by targeting CLRs on DCs and LSECs.
(A) DC-SIGN on DCs binds HCV envelope proteins, and HCV is internalized. Upon internalization HCV is partially degraded in lysosomes and presented to $CD4^+$ T cells. However, HCV may also be retained in early endosomes and transmitted to target cells. (B) L-SIGN on LSECs binds HCV envelope proteins and internalizes the virus. Upon internalization HCV can be transmitted to its main target cells, the hepatocytes. Alternatively, HCV might be degraded and presented to $CD4^+$ or $CD8^+$ T cells.

The E2-CD81 ligation provides the co-stimulatory signals for T-cell activation. But because of the low viral load, the chance of simultaneous interaction of HCV and LSEC with T cells is low.

DC-SIGN and L-SIGN in HIV-1 infection

T cells are infected by HIV-1 through interaction of the envelope protein gp120 with CD4 and the chemokine co-receptors CCR5 or CXCR4 [109]. During sexual transmission, DCs are among the first cells encountered by HIV-1 in the mucosal tissues [110]. T cell infection is greatly enhanced by the presence of DCs, although DCs themselves are poorly infected [111;112]. The discovery of DC-SIGN shed light on the molecular mechanism of HIV-1 transmission by DCs. DC-SIGN expressed by DCs, captures and internalizes HIV-1 through the HIV-1 envelope glycoprotein gp120 [1]. Similar to HCV, HIV-1 is retained in non-lysosomal vesicles [1;113]. This compartment is $CD81^+$ and studies suggest that the virus stays infectious for several days [1;113]. DCs migrate subsequently to the lymph nodes, where the virus is transmitted to T cells. The DC-T cell interaction causes translocation of the virus to the DC-T cell interface, the so called 'infectious synaps', which leads to concentration of HIV-1 in close proximity to CD4, thereby facilitating infection of the target cells [114-117]. Recent data demonstrate that the transmission consists of two phases: initially virus is captured by DCs and retained intracellular for transmission, whereas the second phase is due to *de novo* synthesis of HIV-1, which is subsequently transmitted. DC-SIGN seems to be involved in both phases. DC-SIGN captures HIV-1 for direct transmission [118], whereas infection of DCs themselves is enhanced by DC-SIGN expression [119]. However, upon internalization HIV-1 is also partially degraded and presented to $CD4^+$ and $CD8^+$ T cells [6;7]. A similar role for L-SIGN has been suggested; L-SIGN interacts with gp120 and is able to transmit captured HIV-1 to T cells [120-122].

DC-SIGN recognizes gp120 through carbohydrate structures expressed on this glycoprotein. Gp120 is heavily glycosylated, and carbohydrate structures compose 50% of its mass

[123;124]. Since carbohydrates are clustered together on the outer domain of the gp120 molecule, this region is weakly immunogenic and known as the 'silent' face [125;126]. Furthermore, it has been implicated that the carbohydrate cluster forms a 'glycan shield' preventing neutralizing antibodies from binding through steric inhibition [127]. Differential glycosylation of the envelope glycoproteins affects DC-SIGN binding and enhancement [Chapter 5][128;129]).
It has been suggested that DC-SIGN and CD4 compete for gp120 binding [9], however antibodies against the CD4 binding site do not block DC-SIGN binding and moreover there is enhancement of CD4 binding upon DC-SIGN interaction with gp120 [chapter5][129]. Thus DC-SIGN binding to gp120 induces conformational changes in gp120 and is thereby enhancing CD4 binding to T cells [chapter5][129], and CD4 binding in turn is a prerequisite for CXCR4/CCR5-gp120 interactions [130].
Thus, DC-SIGN is enhancing transmission of HIV-1 and therefore an attractive target for preventing HIV-1 transmission.

Intervention of DC-SIGN mediated HIV transmission.
Blocking the interaction between DC-SIGN and HIV-1 is a good strategy to reduce T cell infection and spreading the virus from initial infection site to the lymph node. Carbohydrate based ligands for DC-SIGN are good possible candidates for this purpose. The bovine glycoprotein lactoferrin from cow milk for instance, is efficient in blocking DC-SIGN mediated HIV-1 transmission [131]. Although human lactoferrin has anti-viral activity [132], human lactoferrin is not interfering with the DC-SIGN-HIV-1 interactions [chapter 6][133]. However human milk does inhibit at high concentrations direct infection of T cells, and at lower concentrations it efficiently abrogates DC-SIGN mediated transmission to T cells [chapter 6][133]. Bile salt stimulated lipase (BSSL) was identified as the glycoprotein responsible for this inhibition [chapter 6][133;134]. BSSL has been demonstrated to have antiviral characteristics [135], and indeed BSSL blocks HIV-1 infectivity. However, unlike other reports only describing antiviral activity on the virus itself, we demonstrate that BSSL furthermore prevents HIV-1 transmission by competing with HIV-1 gp120 for the binding site of DC-SIGN [chapter 6][133]. BSSL is highly glycosylated and contains many Le^x structures. These Le^x structures are responsible for the blocking capacity of BSSL [chapter 6][133;134]. Thus human milk is a promising source for anti-HIV-1 therapy. However, milk is also a source of HIV-1. In human breast milk HIV-1 is present as free virus particles or within cells, and can be transmitted via the gastrointestinal tract. Infection during mother to child transmission (MTCT) occurs in the tonsils and mucosa [136-138], and children fed with breast milk from HIV-1 infected mothers have an increased risk in becoming infected with HIV-1 compared to infants fed with formula milk [139]. However infants fed with a combination of both breast milk and formula milk had an even more increased risk to become infected [140], implicating that breast milk also has beneficial effects.
The C-type lectin Langerin, which is expressed on Langerhans cells [141], is also interacting with HIV-1 gp120 [142]. In contrast to the HIV-1 interaction with DC-SIGN, which leads to viral dissemination, the interaction of HIV-1 with Langerin results in degradation of the virus. Even more, blocking Langerin result in susceptibility of Langerhans cells for HIV-1 [142]. Therefore, therapeutic use of BSSL for reducing HIV-1 spreading is advantageous because it impedes DC-SIGN mediated transmission but does not affect Langerin mediated viral degradation.

Preventing HIV-1 transmission by direct blocking of DC-SIGN is a promising approach in combating HIV-1 infections, however these interventions also interfere with the immunological

role of DC-SIGN [1;2;143;144]. Therefore, inhibitors targeting HIV-1 gp120 to prevent DC-SIGN capture seem warranted to interfere with HIV-1 dissemination. Although HIV-1 efficiently escapes the immune response, antibodies against HIV-1 are present in infected patients.
Although some antibodies promote HIV-1 survival, others can block DC-SIGN-gp120 interactions and thereby inhibit in cis and in trans infection [chapter 5][129;145;146]. Anti-V3 loop antibodies partially block DC-SIGN-gp120 interactions although DC-SIGN is not directly binding to the V3 loop itself [chapter 5][129]. Especially the blocking function of the antiV3 loop antibody 447-52D is of interest. This antibody demonstrated the highest block of DC-SIGN-gp120 interactions [chapter 5][129]. Although the epitope recognized by the human antibody 447-52D is situated in a variable loop of gp120, the crown of the V3 loop is rather conserved [147]. Furthermore polyclonal goat serum inhibited DC-SIGN-HIV-1 interactions. HIV-1 gp120 is highly glycosylated and this glycan shield forms a relative non-immunogenic site on gp120. Therefore serum from goats immunized with glycosylated or non-glycosylated, lacking the glycan shield, were compared for inhibition of DC-SIGN gp120 binding. Both polyclonal sera bound efficiently to glycosylated gp120, however polyclonal serum raised against non-glycosylated gp120 was more efficient in blocking DC-SIGN gp120 interactions [chapter 5][129].
Therefore, vaccinations could be more successful when non-glycosylated gp120 is used as a vaccine rendering potent neutralizing antibodies compared to vaccinations in which glycosylated gp120 is used.

General conclusions

DC-SIGN mediates several interactions with self-ligands, tumours and pathogens. The function of DC-SIGN on primary cells has been extensively studied and it has become clear that there is a delicate balance in the outcome of the DC-SIGN-mediated interactions. Therefore one should be careful with interfering in DC-SIGN-pathogen interactions at the side of DC-SIGN.
We have described here that L-SIGN also mediates important interactions with cells, such as PMN, monocytes and tumour cells, and with pathogens. However research into the function of L-SIGN on primary cells has been hampered by the difficulty to isolate sufficient amounts of L-SIGN-positive LSECs from human liver. Therefore, most studies have used cell lines expressing L-SIGN as a model. Here we demonstrate that L-SIGN function *in vivo* can be investigated using a mouse model. mSIGNR1 is expressed on homologues cells in the murine liver and has a comparable carbohydrate recognition profile [24]. Therefore, the mSIGNR1 deficient mice will be important in further exploring the function of L-SIGN.

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Summary

Summary

The C-type lectin receptors (CLRs) DC-SIGN and L-SIGN are both expressed on antigen presenting cells; dendritic cells (DCs) and liver sinusoidal endothelial cells (LSECs) respectively. These cells capture and process ligands for antigen presentation to T cells.
DC-SIGN and L-SIGN recognize pathogens and self-ligands through recognition of specific carbohydrate moieties on glycoconjugates. Due to a high degree of homology between the carbohydrate recognition domains of DC-SIGN and L-SIGN, both CLRs have affinity for high mannose structures and the fucose containing Lewis^{a} (Le^{a}), Le^{b}, and Le^{y} antigens, with the exception of Le^{x}, which is only bound by DC-SIGN.
In **chapter 2** a novel L-SIGN specific carbohydrate ligand is described, which is not recognized by DC-SIGN. This ligand is neuraminidase sensitive, suggesting that L-SIGN recognizes a sialicacid containing glycan structure that is not recognized by DC-SIGN. This makes L-SIGN comparable to its murine homologue mSIGNR1 that similar to L-SIGN is expressed on LSECs in the liver and also is able to bind sialylated carbohydrates.

Cellular ligands for DC-SIGN and L-SIGN
DC-SIGN recognizes several cellular ligands on different cells, for example ICAM-2 on endothelial cells and ICAM-3 on T cells. Furthermore, DC-SIGN interacts with neutrophils via Mac-1 binding. Early during infection neutrophils are involved in killing of pathogenic bacteria and regulation of innate immune responses at the site of infection. It has become clear that neutrophils also modulate adaptive immunity through interactions with DCs. Upon activation, neutrophils release TNF-α and combined with Mac1-DC-SIGN interactions, induce maturation of DCs that enables these APCs to stimulate T cell proliferation and to induce T helper 1 polarization. In **chapter 7**, we demonstrate that also CEACAM1 is an important ligand for DC-SIGN on neutrophils. Binding of DC-SIGN to both CEACAM1 and Mac-1 is required to establish cellular interactions with neutrophils. L-SIGN on the other hand, only recognizes Mac-1 on neutrophils. Neutrophils are able to adhere to and roll over L-SIGN positive cells, indicating a role for L-SIGN in neutrophil homing to the liver sinusoid.
Besides the interaction with neutrophils, L-SIGN also interact with a yet unknown ligand on monocytes (**chapter 8**). These cells are amongst others precursors of resident tissue macrophages like the Kupffer cells in the liver. Using an *in vivo* model we demonstrate that the murine L-SIGN homologue mSIGNR1 expressed on LSECs influences Kupffer cell (KC) numbers in the liver, because mSIGNR1 deficient mice have reduced numbers of KCs. The effect of mSIGNR1 on KC recruitment appears after the initial phase of KC repopulation, indicating that L-SIGN could be involved in the recruitment of KC precursors in the human liver.
We furthermore demonstrate in **chapter 2** that L-SIGN recognizes a carbohydrate ligand on hepatoma cells. Hepatocytes express high amounts of sialylated glycans, which are abrogated in metastasizing hepatocellular carcinoma cells. The interaction between LSEC and hepatocytes, which are not connected with extracellular matrix, could contribute to maintenance of hepatic sinus architecture. The loss of sialylated ligands would loosen the L-SIGN-hepatocellular carcinoma cell interaction and the hepatocellular carcinoma cells would more easily transmigrate into the bloodstream and form metastases at distant sites. Indeed this loss of sialic acid expression is indicative for a poor patient survival.

DC-SIGN mediates interactions with colon carcinoma cells, which express high levels of Le^y- and Le^x-decorated CEA and CEACAM1. In **chapter 2** we show that, similar to DC-SIGN, L-SIGN recognizes tumour derived CEA, probably because of the presence of Le^y on CEA, and therefore could participate in colon cancer liver metastasis. Furthermore, in contrast to hepatocellular carcinoma cells, colon cells display an increased or *de novo* expression of sialylated carbohydrates upon malignant transformation. The binding of these tumour-associated glycoconjugates to L-SIGN could, in addition to getting constricted by the narrow vasculature of the sinus, explain the frequent metastasis formation of colon carcinomas in the liver.

DC-SIGN/L-SIGN-pathogen interactions

Besides self ligands, DC-SIGN and L-SIGN also recognize a range of pathogens. DC-SIGN was originally identified as a human immunodeficiency virus 1 (HIV-1) gp120 binding receptor. DC-SIGN plays a key-role in the dissemination of HIV-1 by DCs through HIV-1 gp120 binding. Upon binding the virus is internalized and with the DC transported to the lymph node where it infects T cells efficiently.

Since the capture of HIV-1 by DC-SIGN enhances T cell infection, the interaction of DC-SIGN with gp120 provides an attractive target for intervention of HIV-1 transmission. In **chapter 5** and **6** we describe two ways to inhibit the DC-SIGN-gp120 interactions. In **chapter 6** we describe that human milk contains a component that inhibits the DC-SIGN-mediated transfer of HIV-1 to T cells. This Le^x containing inhibitory factor directly interacted with DC-SIGN, and prevented the HIV-1 gp120 envelope protein from binding to DC-SIGN thereby prevent the capture and subsequent transfer of HIV-1 to T cells. The presence of such a DC-SIGN-binding molecule in human milk may both influence antigenic presentation and interfere with pathogen transfer in breastfed infants. Interactions of L-SIGN with HIV-1 gp120 are not affected by this milk component since L-SIGN can not bind Le^x carbohydrates.

In **chapter 5**, we have investigated the potency of gp120 antibodies to inhibit the DC-SIGN-gp120 interaction. We demonstrate that antibodies against the V3 loop partially inhibit DC-SIGN binding, suggesting that these antibodies sterically hinder DC-SIGN binding to gp120. Polyclonal antibodies raised against non-glycosylated gp120 inhibited DC-SIGN-gp120 interactions in contrast to polyclonal antibodies raised against glycosylated gp120. Thus, glycans present on gp120 may prevent the generation of antibodies that block the DC-SIGN-gp120 interactions. Moreover, the polyclonal antibodies against gp120 efficiently inhibited HIV-1 capture by both DC-SIGN transfectants and immature dendritic cells. Therefore, non-glycosylated gp120 may be an attractive immunogen to elicit gp120 antibodies that block the binding to DC-SIGN. Furthermore, we demonstrate that DC-SIGN binding to gp120 enhanced CD4 binding, suggesting that DC-SIGN induces conformational changes in gp120, which may provide new targets for neutralizing antibodies.

In **chapter 3** and **4** the interaction of DC-SIGN and L-SIGN with the hepatitis C virus (HCV) glycoproteins E1 and E2 is described. HCV targets hepatocytes in the liver. However, the mechanism of hepatocyte infection is largely unknown. DC-SIGN and L-SIGN are important receptors for HCV E1 and E2. DC-SIGN is the main HCV receptor on DCs and L-SIGN is the main HCV receptor in liver since interaction can be inhibited by specific anti-DC-SIGN and L-SIGN antibodies. Mutagenesis analyses demonstrates that both HCV E1 and E2 bind the same binding site on DC-SIGN as the pathogens HIV-1 and mycobacteria, which is distinct from the cellular ligand ICAM-3.

HCV virus-like particles (VLPs) are efficiently captured and internalized by DCs through binding of DC-SIGN. Interestingly, internalized HCV-VLPs are partially targeted to non-lysosomal compartments within DCs, where they are protected from degradation similarly as was demonstrated for HIV-1. This suggests that HCV may target DC-SIGN to 'hide' within DCs and to facilitate viral dissemination. L-SIGN expressed by APC cell-lines internalizes HCV particles into similar non-lysosomal compartments, suggesting that L-SIGN on LSECs may capture HCV from blood and transmit it to hepatocytes, the primary target for HCV. However, in **chapter 4** we describe that HCV-VLPs are also susceptible for degradation by DCs, and degradation is more efficient in the presence of HCV-specific antibodies. We demonstrate that degradation of the HCV-antibody complexes leads to MHC class II presentation by DCs. Moreover, induction of DC maturation by HCV-VLPs on an aluminium carrier strongly enhances this antigen presentation by DCs. Thus, antibody targeting and maturation-induction directs DC-SIGN-captured ligands into lysosomal compartments and efficient antigen presentation. Moreover, we demonstrate that L-SIGN captures HCV-antibody complexes leading to antigen presentation to $CD4^+$ T cells, demonstrating that L-SIGN is an antigen receptor on LSECs. Human LSECs express both L-SIGN and MHC class II molecules and our data suggest that LSECs are important as antigen presentation cells of viral antigens in the liver. However this presentation may lead to erroneous T cell activation since LSEC are known to induce T cell tolerance.

Final conclusion

DC-SIGN mediates several interactions with self-ligands, tumours and pathogens. The function of DC-SIGN on primary cells has been extensively studied and it has become clear that there is a delicate balance in the outcome of the DC-SIGN-mediated interactions. Therefore one should be careful with interfering in DC-SIGN-pathogen interactions at the side of DC-SIGN.
We have described here that L-SIGN also mediates important interactions with cells, such as neutrophiles, monocytes and tumour cells, and with pathogens. However research into the function of L-SIGN on primary cells has been hampered by the difficulty to isolate sufficient amounts of L-SIGN-positive LSECs from human liver. Therefore, most studies have used cell lines expressing L-SIGN as a model. Here we demonstrate that L-SIGN function *in vivo* can be investigated using a mouse model. mSIGNR1 is expressed on homologues cells in the murine liver and has a comparable carbohydrate recognition profile. Therefore, the mSIGNR1 deficient mice will be important in further exploring the function of L-SIGN.

Nederlandse samenvatting voor niet ingewijden

Nederlandse samenvatting voor niet ingewijden

Het menselijke lichaam kan zich op vele manieren goed beschermen tegen binnendringende ziekteverwekkers. Hierbij speelt het immuunsysteem een zeer belangrijke rol. Het immuunsysteem in de mens bestaat uit twee nauw met elkaar samenwerkende onderdelen: het aangeboren (innate) en het aangeleerde (adaptieve) immuunsysteem. Het aangeboren afweersysteem bestaat onder andere uit macrofagen en neutrofiele cellen. Deze cellen herkennen algemeen voorkomende patronen op ziekteverwekkers en zullen vervolgens de ziekteverwekker direct bestrijden. Het adaptieve immuunsysteem bestaat uit B en T cellen die zeer specifieke eiwitstructuren herkennen en een 'geheugen' functie hebben. Dendritische cellen (DCs) vormen een belangrijke brug tussen deze twee onderdelen van het immuunsysteem. Deze cellen zijn in staat vrij algemeen voorkomende structuren op ziekteverwekkers te herkennen, waarna ze de ziekteverwekker opnemen. Na opname in de DC wordt de ziekteverwekker afgebroken tot kleine eiwitten, zogenaamde antigenen, welke vervolgens op het oppervlakte worden gepresenteerd aan T cellen. Op het moment dat de DC deze antigenen aan de T cel presenteert, wordt de T cel niet alleen geactiveerd maar de DC bepaald ook hoe de T cel zijn werk zal doen. Op deze manier regiseren de DCs de zeer specifieke immuunrespons uitgevoerd door T cellen. De DC is onder andere gepositioneerd op strategische plekken in het lichaam waar veel contact is met de buitenwereld. Hier vervullen ze een poortwachterfunctie door continu de omgeving af te tasten.

De DCs herkennen ziekteverwekkers met behulp van verschillende moleculen op hun celoppervlak: zogenaamde Toll like receptoren en C-type lectine receptoren. De verschillende Toll like receptoren herkennen vaste patronen op ziekteverwekkers zoals lipopolysaccharide van Gram-negatieve bacteriën. Als een TLR een ligand heeft gebonden leidt dit tot activatie van de DC en zal deze naar een lymfe klier verplaatsen. De C-type lectine receptoren echter herkennen specifieke suikerstructuren op ziekteverwekkers. DCs hebben vele verschillende C-type lectines op hun oppervlak met elk hun eigen suikerherkenningspatroon. De C-type lectines zorgen voor de opname van ziekteverwekkers in de cel, waarna deze worden afgebroken en gepresenteerd aan T cellen. Naast ziekteverwekkers brengen ook lichaamseigen cellen deze suikermoleculen tot expressie. Hiermee zorgen de C-type lectines ervoor dat DCs kunnen communiceren met andere cellen.
DC-SIGN is een van deze C-type lectine receptoren die voorkomen op DCs. DC-SIGN is ontdekt als receptor voor een eiwit dat aan de buitenkant van het AIDS veroorzakende HIV-1 virus zit, het gp120. Door binding van DC-SIGN aan de suikermoleculen op dit HIV-1 eiwit, wordt het virus in de cel opgenomen. Deels zal het virus vervolgens worden afgebroken en gepresenteerd aan T cellen. Maar het virus heeft ook een methode ontwikkeld waardoor het zich in de DC kan verschuilen. Het virus wordt vervolgens door de DC naar de T cel rijke lymfe knopen getransporteerd. Hier wordt het virus door de DC overgegeven aan de T cel, de ideale gastheer cel voor het HIV-1 virus. Op deze manier verhoogt het HIV-1 virus via de binding aan DC-SIGN de infectie van de T cellen.

In de lever komt een receptor voor die heel erg lijkt op DC-SIGN, L-SIGN genaamd. De lever is een belangrijk orgaan, dat een rol speelt bij de zuivering van het bloed dat komt vanaf het maagdarmstelsel. In de lever bevinden zich verschillende soorten gespecialiseerde cellen die

er voor zorgen dat dit zo goed mogelijk gebeurt. De hepatocyten zijn voornamelijk verantwoordelijk voor het omzetten van stoffen. Van veel van de niet hepatocyten wordt gedacht dat ze een immunologische rol spelen. Veel lichaamsvreemde materialen passeren de lever als ze het lichaam via de maag zijn binnen gekomen. Deze lichaamsvreemde materialen zijn over het algemeen niet gevaarlijk en de lever zorgt ervoor dat het lichaam deze stoffen niet aanvalt. In de lever bevinden zich verschillende soorten cellen die hier aan mee werken. Een van deze celtypen is de lever sinusoid endotheel cel (LSEC). Deze LSEC vormen de begrenzing tussen de hepatocyten en het passerende bloed. In tegenstelling tot andere endotheel cellen, zitten deze specifieke endotheel cellen niet vast aan een onderliggende matrix en hebben ze vele 'vensters' die een soort zeefplaten vormen. Hierdoor zijn ze in staat om actief te bepalen welke materialen uit de bloedbaan de hepatocyten bereiken. De LSECs nemen stoffen op die met het bloed vanuit de darmen naar de lever zijn gevoerd. Nadat deze stoffen zijn opgenomen in de cellen worden ze afgebroken en aan T cellen gepresenteerd, net als gebeurt in DCs. Normaal gesproken als een cel een stukje materiaal, een antigen, aan deze T cellen presenteert, worden de T cellen geactiveerd en aangezet om een immuunreactie uit te voeren. Maar in het geval van deze LSECs blijft deze activatie achterwege en resulteert het presenteren van de antigenen in tolerantie voor deze stoffen. Dit is in vele gevallen gunstig omdat een heftige immuunreactie tegen antigenen afkomstig van voedsel leidt tot voedselallergie. Maar in het geval dat er een ziekteverwekker met het voedsel het lichaam is binnengedrongen is een adequate immuunrespons gewenst.
Op deze LSECs bevindt zich L-SIGN. Deze receptor is familie van DC-SIGN, en herkent, net als DC-SIGN, suikerstructuren die zich bevinden op lichaamseigen en lichaamsvreemde componenten. L-SIGN en DC-SIGN herkennen veel dezelfde suikers, maar er zijn ook verschillen in herkenning. DC-SIGN en L-SIGN kunnen beide hoog mannose bevattende suikers, en bepaalde fucose suikers binden. Er is een bepaalde fucose bevattende suiker die alleen door DC-SIGN, en niet door L-SIGN wordt herkend; Lewis^x (Le^x). Dit patroon van suikerherkenning bepaalt welke lichaamseigen en -vreemde structuren worden herkend door DC-SIGN en L-SIGN.

In dit proefschrift is de functie binnen het immuunsysteem van zowel DC-SIGN als L-SIGN verder uitgediept. Hiervoor zijn verschillende aspecten onderzocht: de suikerherkenning, interacties met lichaamseigen materialen en interacties met virussen.

Allereerst is de suikerherkenning van L-SIGN is verder in kaart gebracht. Ondanks de grote gelijkenis van de beide receptoren zijn er verschillen in de suikerherkenning. Naast het al eerder beschreven verschil, het gebrek aan herkenning van het zogenaamde Le^x door L-SIGN, wordt in **hoofdstuk 2** een ander verschil beschreven. Wij hebben aangetoond dat L-SIGN in staat is een siaalzuur bevattende suiker te herkennen, welke niet herkend wordt door DC-SIGN. Deze siaalzuur bevattende suikers komen veel tot expressie op hepatocyten. Deze hepatocyten vormen het grootste deel van de lever en grenzen aan de LSECs die L-SIGN op hun oppervlak hebben. L-SIGN is inderdaad in staat om aan deze cellen te binden. De specifieke suiker evenals het eiwit waarop deze suiker zit is nog niet gevonden en dit zal verder onderzocht gaan worden. Mogelijkerwijze heeft deze binding een rol in het behouden van de structuur van de lever. Het L-SIGN bevattende endotheel zit namelijk niet zoals conventioneel endotheel vast aan de onderlaag met behulp van een soort matrix maar zit vrij los. Door de binding van L-SIGN aan de hepatocyten zouden de endotheel cellen aan de

hepatocyten verankerd kunnen zijn en daarmee de structuur van de lever in stand kunnen houden. Het is inderdaad bewezen dat het verlies van deze siaalzuur bevattende suikers van het oppervlak van de hepatocyten tijdens het proces van tumorvorming een verhoogde kans geeft op het vormen van uitzaaiingen.

Naast de interactie met hepatocyten, gaat L-SIGN ook binding aan met monocyten (**hoofdstuk 8**). Monocyten zijn voorlopercellen van macrofagen, een soort alleseters. Monocyten zitten in het bloed en als ze naar weefsels verhuizen veranderen zij in macrofagen. L-SIGN is in staat met deze voorlopercellen een interactie aan te gaan. Met behulp van een muismodel, is deze interactie verder onderzocht. De muis heeft een homologe vorm van L-SIGN in de lever: mSIGNR1. Deze receptor lijkt zowel met betrekking op suikerherkenning alsmede de plekken waar het tot expressie komt in de lever veel op L-SIGN. Dit maakt de muis een belangrijk model om de rol van L-SIGN *in vivo*, dus in een geheel organisme, te kunnen onderzoeken. Met behulp van deze muis en een muis waar mSIGNR1 specifiek is uitgeschakeld is bepaald dat de aanwezigheid van mSIGNR1 in de lever de hoeveelheid macrofagen in de lever verhoogt. Omdat deze macrofagen zijn ontstaan uit monocyten, is het aannemelijk dat mSIGNR1, en ook L-SIGN, een rol speelt bij het rekruteren van monocyten uit het bloed en de verandering van deze cellen in lever macrofagen.

In **hoofdstuk 7** staat de interactie van DC-SIGN en L-SIGN met neutrofielen beschreven. Neutrofielen zijn belangrijke cellen binnen het aangeboren immuunsysteem. Neutrofielen verzamelen zich snel op de plek van een infectie. Doormiddel van twee eiwitten, Mac-1 en CEACAM1, op hun oppervlak sturen zij DCs aan. Na binding van deze eiwitten aan DC-SIGN op de DC, zal de DC zodanig veranderen dat deze een specifieke soort T cel reactie op gang zal brengen. L-SIGN is in staat één van deze twee eiwitten te binden, namelijk Mac-1. CEACAM1 wordt niet herkend door L-SIGN. Tijdens een ontsteking verzamelen neutrofielen zich massaal in de lever, zelfs als de ontsteking zich niet in de lever bevindt. Omdat L-SIGN zich op een strategische plaats in de lever bevindt, is het waarschijnlijk dat L-SIGN een rol speelt bij het vasthouden in de lever van passerende neutrofielen. Neutrofielen kunnen namelijk zo sterk aan L-SIGN binden dat ze niet door de stroming van het bloed mee gevoerd kunnen worden. Vervolgens kunnen de neutrofielen opgeruimd worden door de LSEC of de lever macrofagen. Ook is het mogelijk dat ze het leverweefsel binnendringen nadat ze zijn vastgehouden door L-SIGN op het endotheel.

Het was al bekend dat DC-SIGN in staat is verschil te herkennen tussen eiwitten uit gezond weefsel en tumorweefsel. Wij hebben in **hoofdstuk 2** aangetoond dat L-SIGN ook in staat is dit verschil te herkennen. Een bepaald eiwit op darmcellen, CEA, verandert dusdanig tijdens het proces van tumorvorming in darmweefsel dat het andere suikers tot expressie gaat brengen. Dit leidt tot een betere herkenning door DC-SIGN en dus ook L-SIGN. In het geval van L-SIGN kan deze verbeterde herkenning leiden tot het vasthouden van de tumorcellen op het moment dat ze uitzaaiingen gaan vormen. Darmtumoren vormen vaak uitzaaiingen in de lever. Dit komt doordat de bloedvatstructuur in de lever erg nauw is waardoor de verplaatsende tumorcellen daar gemakkelijk vast komen te zitten. Maar ook de binding van de tumorcellen aan L-SIGN op het endotheel in de lever sinusoiden kan leiden tot het blijven hangen van de tumorcellen die vervolgens een secundaire tumor kunnen gaan vormen.

Naast interacties met lichaamseigen eiwitten zijn L-SIGN en DC-SIGN ook belangrijke receptoren voor ziekteverwekkers, zoals virussen, bacteriën en parasieten. In de **hoofdstukken 3** tot en met **6** is de interactie van de twee receptoren met virussen onderzocht. In **hoofdstuk 3** en **4** is de interactie van L-SIGN en DC-SIGN met het hepatitis C virus (HCV) onderzocht. Dit virus wordt omgeven door een envelop bestaande uit eiwitten waarop veel hoog-mannose structuren zitten. DC-SIGN en L-SIGN binden aan deze structuren en nemen het virus mee de cel in. Eenmaal in de cel kan het virus worden afgebroken en worden gepresenteerd aan T cellen. Het virus heeft echter ook een methode ontwikkeld waardoor het uit het zicht blijft van het immuunsysteem. Na opname in de cel verstopt het zich in bepaalde compartimenten in de cel waarna het, als de cel een geschikte andere cel tegenkomt, naar deze andere cel overgegeven kan worden. Hierin lijkt het HCV virus veel op het HIV-1 virus.
Wij hebben aangetoond dat in de lever L-SIGN het belangrijkste molecuul is dat aan HCV envelop eiwitten bindt. Het blokkeren van L-SIGN in leverweefsel heeft tot gevolg dat de HCV envelop eiwitten nauwelijks meer aan het leverweefsel binden. Tevens hebben we aangetoond dat L-SIGN op cellijnen HCV envelop eiwitten gecombineerd met antilichamen kan opnemen en presenteren aan T cellen. Op het moment dat L-SIGN in de lever HCV eiwitten presenteert aan T cellen zou dit kunnen leiden tot tolerantie voor het virus, aangezien deze cellen verantwoordelijk zijn voor tolerantie inductie. Inderdaad is het aangetoond dat veel T cellen die specifiek HCV eiwitten herkennen het virus niet effectief aanvallen. Dit betekent dat het HCV virus twee manieren heeft ontwikkeld om via L-SIGN een effectieve immuunrespons te omzeilen: ten eerste door zich te verstoppen in de LSEC en ten tweede door via de LSEC tolerantie op te wekken.
Op DCs is DC-SIGN het belangrijkste molecuul dat de HCV eiwitten herkent. Na opname in de DC blijft een deel van het virus een lange tijd intact. Maar ook in de DC wordt een deel van het virus afgebroken en gepresenteerd aan T cellen. Als de HCV eiwitten op een aluminium drager zitten, een methode die veel in vaccinaties gebruikt wordt, worden de eiwitten effectiever aan T cellen gepresenteerd door DCs. Dit suggereert dat dit een veel belovende methode is om een goed werkend vaccin tegen HCV te ontwikkelen.

In **hoofdstuk 5** en **6** is onderzocht hoe de interactie van het HIV-1 virus met DC-SIGN op DCs kan worden geblokkeerd. Door middel van antilichamen die specifieke delen van het gp120 envelop eiwit van HIV-1 binden te gebruiken en te bepalen in welke mate dit een effect heeft op de binding van DC-SIGN aan het eiwit hebben wij aangetoond dat bepaalde eiwitten beter zijn in het blokkeren van deze interactie dan andere antilichamen. Een verrassend resultaat is dat antilichamen die zijn opgewekt tegen suikerloos gp120 goed zijn in het blokkeren van de interactie van suiker bevattend gp120 met DC-SIGN. Dit betekent dat de suikers een belemmering vormen voor de antilichaam binding die DC-SIGN binding verhindert. Practisch gezien houdt dit in dat eventuele vaccinatie met gp120 eiwitten beter kan plaatsvinden met suikervrije gp120 dan met suiker bevattend gp120. Tevens hebben we aangetoond dat na binding van DC-SIGN aan gp120, gp120 makkelijker aan CD4 bindt. Het HIV-1 virus gebruikt de interactie tussen gp120 en CD4 op de T cel om de T cel te infecteren. Dit betekent dat HIV-1 door het binden aan DC-SIGN niet alleen door de DC naar T cel rijke gebieden kan worden getransporteerd maar dat het eenmaal daar aangekomen ook makkelijker de T cel binnendringt.
Een andere methode om DC-SIGN gp120 interacties te voorkomen is het blokkeren van DC-SIGN. In **hoofdstuk 6** staat beschreven dat in moedermelk een eiwit zit dat de overdracht van HIV-1 door DCs kan verhinderen. Dit eiwit bevat veel Le^x suiker structuren waaraan DC-SIGN bindt. Op het moment dat dit eiwit aan DC-SIGN gebonden heeft kan het HIV-1 virus niet meer

binden en kan het zich dus niet meer verschuilen in de DC. Dit melk eiwit wordt niet herkend door L-SIGN dus de remmende werking van moeder melk heeft geen effect op HIV-1 dat de lever heeft kunnen bereiken.

In conclusie: DC-SIGN op DCs en L-SIGN op LSECs zijn belangrijk voor de communicatie met andere cellen zoals neutrofielen, monocyten en hepatocyten. De virussen HCV en HIV-1 gebruiken DC-SIGN en L-SIGN in hun eigen voordeel. Maar als de binding van deze virussen verhinderd wordt door de functie van DC-SIGN en L-SIGN te remmen zal dit als nadelig effect hebben dat ook de interactie met de lichaamseigen componenten geremd worden. Daarom is verder onderzoek nodig waar het muismodel een goed hulpmiddel zal zijn.

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Kampong dames: mijn voetbal carrière begon ongeveer tegelijk met mijn AIO carrière. Helaas schoot daardoor vaak het voetballen te kort. Maar het was heerlijk om af en toe de frustraties er uit te kunnen trappen met jullie. Ook alle gezelligheid buiten het voetbal om heeft vaak geholpen om de zinnen te verzetten. Hopelijk heb ik vanaf nu meer tijd voor de 1ste, 2de of de 3de helft. VSC, here we come! Jan, bedankt voor de eindeloze hoeveelheden koffie als het weer eens nodig was. Winkelen met jou is een feest en ik hoop dat het ons gelukt is met de jurk. Joris, bedankt voor je relativerende woorden aan het eind van dit traject.

Annemarie, jammer dat je nu niet letterlijk achter me kunt staan op het podium, maar ik weet dat je er figuurlijk wel bent. Bedankt voor al je wetenschappelijke en vooral ook voor je niet wetenschappelijke steun, alle leuke en minder leuke momenten die we hebben gedeeld. Veel geluk met de kleine.
Camiel, ik ben heel blij dat je (nog maar een keer) naast me staat als pinguïn. Ik heb het gemist. Esther, een betere reserve had ik me niet kunnen wensen. Bedankt ook voor de Rob&Rick kwartiertjes, de adviezen en de mogelijkheid om m'n gal te spuwen.
Lieve papa, mama, Hilde en Nora en ook Kasper. Voor jullie was het vaak abracadabra. Ik hoop dat ik het op de laatste bladzijden een beetje heb kunnen uitleggen. Bedankt voor jullie steun.

En verder iedereen die ik vergeten ben hier te noemen, maar het wel verdient: **Bedankt!**

Irene

Curriculum Vitae

Irene Stephanie Ludwig werd geboren op 13 juni 1977 te Grootegast. In 1995 behaalde zij haar VWO diploma aan het Nienoordcollege te Leek. In dat zelfde jaar ging zij biologie studeren aan de Universiteit Utrecht. Tijdens de doctoraalfase heeft ze een wetenschappelijke stage gedaan bij de vakgroep Veterinaire Farmacie Farmacologie en Toxicologie aan de faculteit Diergeneeskunde onder begeleiding van Kasper Hoebe en prof. dr. Johanna Fink-Gremmels. Tijdens deze stage heeft ze onderzoek verricht aan het effect van endotoxinen op de leverfunctie. Vervolgens heeft ze een wetenschappelijke stage verricht bij de afdeling immunologie aan het Nederlands Kanker Instituut te Amsterdam onder begeleiding van dr. Yinka Zevering en prof. dr. Ada Kruisbeek. Hierin werd het effect van endogeen myelin basic protein expressie in antigeen presenterende cellen op het T cel repertoire en op potentiële verschillen in antigeen processing door verschillende klassen antigeen presenterende cellen. Haar doctoraal scriptie werd geschreven onder begeleiding van professor dr. Hans Clevers van de afdeling Immunologie aan het Universitair Medisch Centrum te Utrecht. Hierin behandelde ze verschillende mechanismen waarop apoptotische cellen worden herkend door fagocyterende cellen.
In 2002 is ze gaan werken als promovendus bij de afdeling Moleculaire Celbiologie en Immunologie van het VU medisch centrum te Amsterdam. Onder begeleiding van prof. dr. Yvette van Kooyk en dr. Teunis Geijtenbeek verrichte zij door de Maag Lever Darm Stichting en Aids Fonds gefinancierd onderzoek naar de C-type lectines DC-SIGN en L-SIGN waarvan de resultaten staan beschreven in dit proefschrift.
Momenteel is werkt zij als postdoc bij de vakgroep immunotoxicologie van het Institute for Risk Assesment Sciences van de Universiteit Utrecht aan de onderliggende mechanismen van het ontstaan van medicijn allergieën.

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Ludwig, IS, EA Koppel, GJ van der Bij, M van Egmond, Y van Kooyk, and TBH Geijtenbeek. mSIGNr1 regulates Kupffer cell population maintenance in the liver sinusoid. *In preparation*.

Colour figures

Figure 3.3

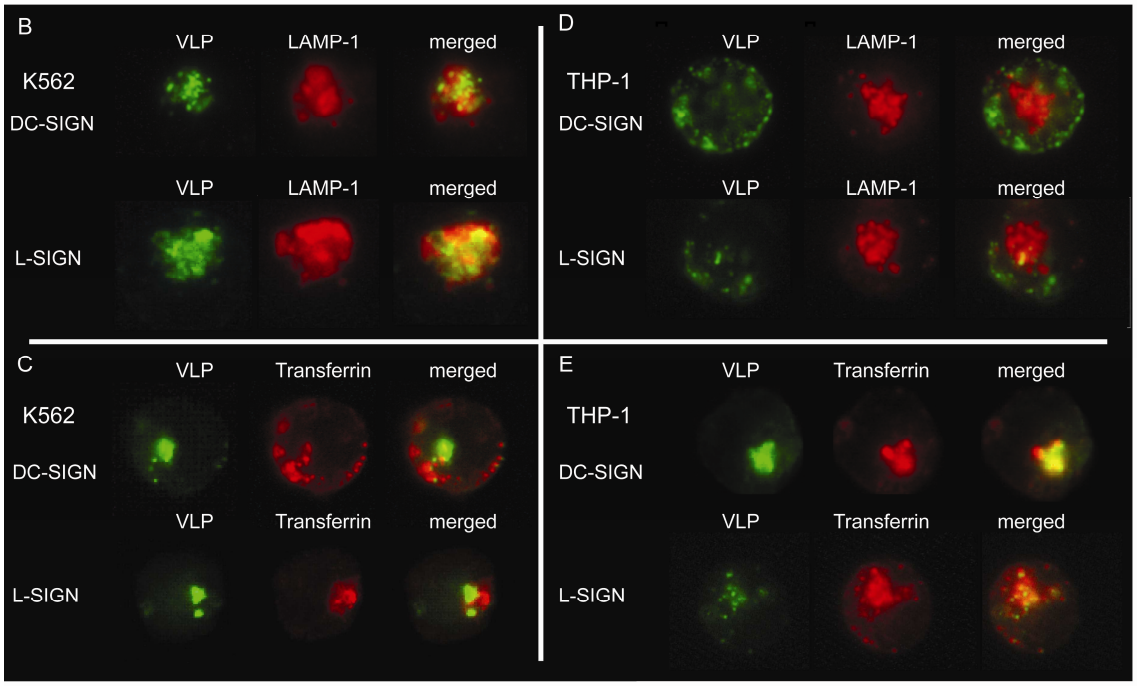
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Figure 3.5

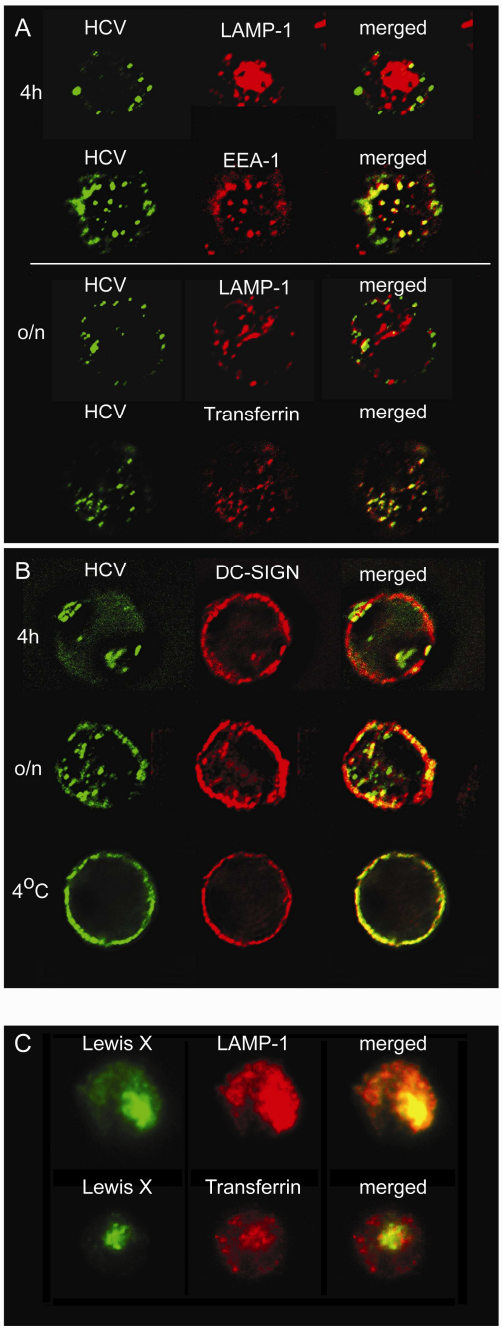

Figure 3.6

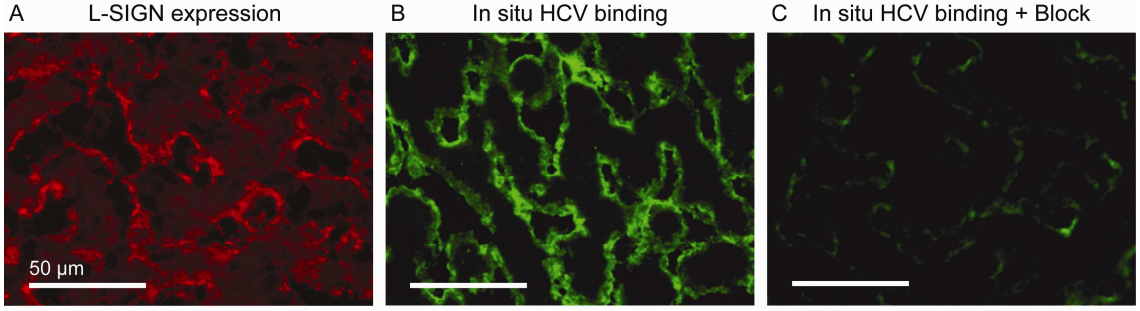

Figure 4.1

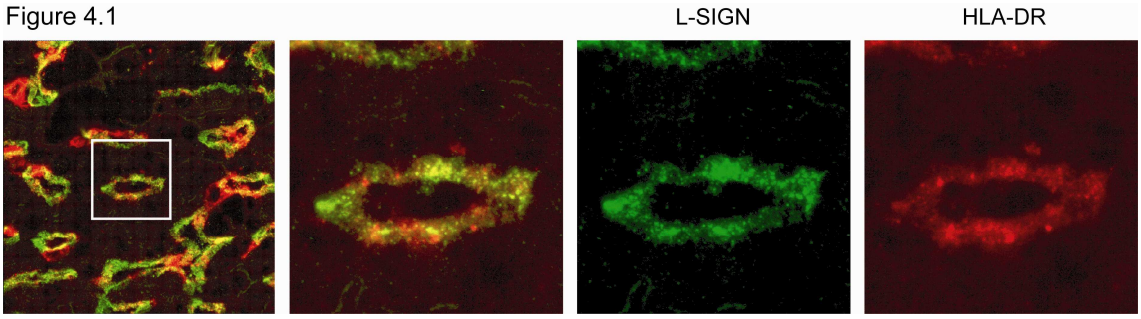

Figure 8.2

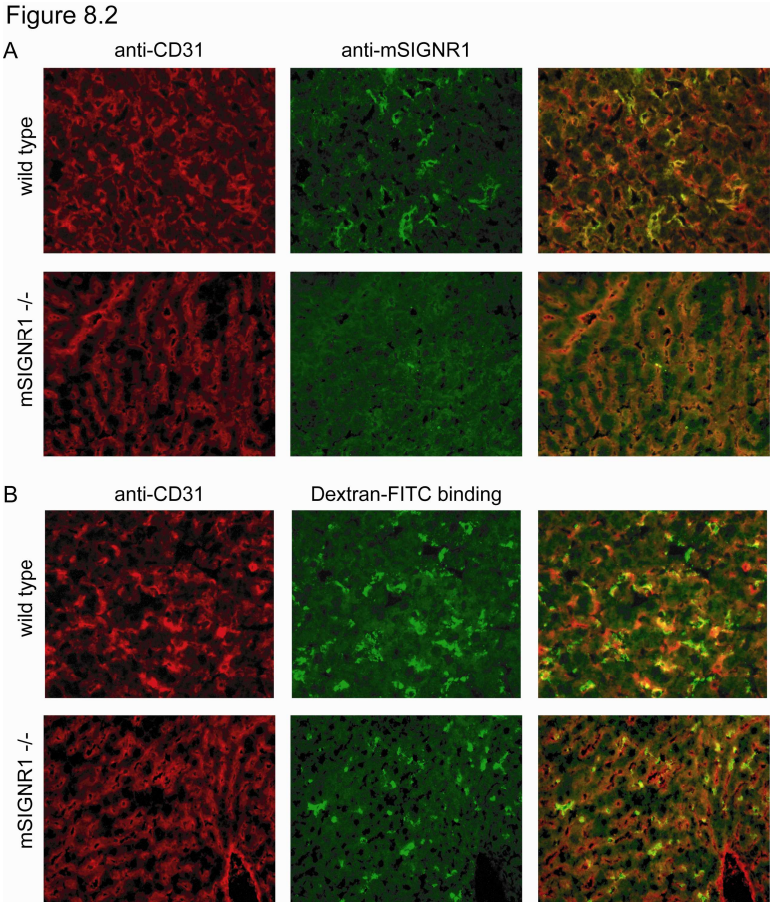

Figure 8.3

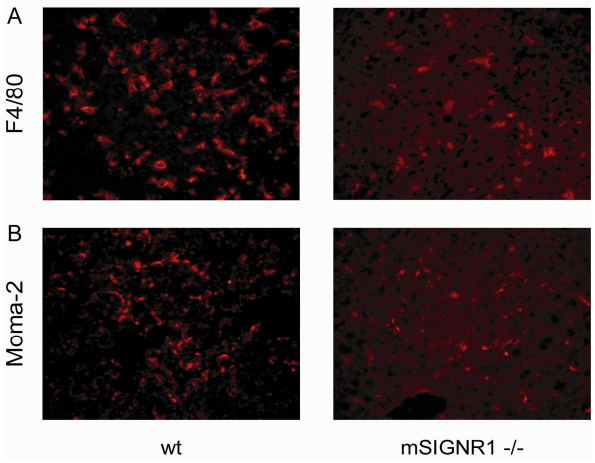

Figure 8.4