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Interactions of LSECTin and DC-SIGN/DC-SIGNR with viral ligands: Differential pH dependence, internalization and virion binding

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Abstract

The calcium-dependent lectins DC-SIGN and DC-SIGNR (collectively termed DC-SIGN/R) bind to high-mannose carbohydrates on a variety of viruses. In contrast, the related lectin LSECTin does not recognize mannose-rich glycans and interacts with a more restricted spectrum of viruses. Here, we analyzed whether these lectins differ in their mode of ligand engagement. LSECTin and DC-SIGNR, which we found to be co-expressed by liver, lymph node and bone marrow sinusoidal endothelial cells, bound to soluble Ebola virus glycoprotein (EBOV-GP) with comparable affinities. Similarly, LSECTin, DC-SIGN and the Langerhans cell-specific lectin Langerin readily bound to soluble human immunodeficiency virus type-1 (HIV-1) GP. However, only DC-SIGN captured HIV-1 particles, indicating that binding to soluble GP is not necessarily predictive of binding to virion-associated GP. Capture of EBOV-GP by LSECTin triggered ligand internalization, suggesting that LSECTin like DC-SIGN might function as an antigen uptake receptor. However, the intracellular fate of lectin–ligand complexes might differ. Thus, exposure to low-pH medium, which mimics the acidic luminal environment in endosomes/lysosomes, released ligand bound to DC-SIGN/R but had no effect on LSECTin interactions with ligand. Our results reveal important differences between pathogen capture by DC-SIGN/R and LSECTin and hint towards different biological functions of these lectins.

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Introduction

The lectin DC-SIGN (CD209) and the related lectin DC-SIGNR (L-SIGN, CD209L) bind to a virtually identical spectrum of viral and non-viral pathogens in a carbohydrate-dependent

manner (Cambi et al., 2005; Koppel et al., 2005). Both lectins recognize high-mannose carbohydrates. In addition, DC-SIGN binds to Lewis X sugars (Appelmek et al., 2003; Feinberg et al., 2001; Guo et al., 2004; Lin et al., 2003; van Die et al., 2003; Van Liempt et al., 2004). DC-SIGN is expressed by dendritic cells (DCs) (Geijtenbeek et al., 2000), platelets (Boukour et al., 2006; Chaipan et al., 2006), activated primary B-cells (He et al., 2006; Rappocciolo et al., 2006) and certain macrophages (Soilleux et al., 2001, 2002), whereas DC-SIGNR is found on vascular

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endothelium in the placenta and on liver and lymph node sinusoidal endothelial cells (Bashirova et al., 2001; Pöhlmann et al., 2001c). In liver (Lai et al., 2006), lymph node and placenta (Soilleux et al., 2001, 2002; Pöhlmann et al., 2001c), both lectins are co-expressed. DC-SIGN and DC-SIGNR (collectively referred to as DC-SIGN/R) can concentrate virions on the cell surface, thereby facilitating engagement of the cognate cellular receptors and subsequent infectious viral entry into target cells.

DC-SIGN-mediated capture of human immunodeficiency virus type-1 (HIV-1) by DCs in the anogenital mucosa was proposed to facilitate dissemination of sexually transmitted HIV-1 (Geijtenbeek et al., 2000). However, several reports indicate that the contribution of DC-SIGN to augmentation of HIV-1 infectivity by DCs is minor (Boggiano et al., 2007; Gummuluru et al., 2003). Nevertheless, DC-SIGN on platelets or activated B-cells might promote HIV-1 spread once the virus has reached the blood stream (Boukour et al., 2006; Chaipan et al., 2006; Rappocciolo et al., 2006). DC-SIGNR in lymph nodes and liver might also impact spread of HIV-1 and hepatotropic viruses, like hepatitis C virus (HCV). Indeed, polymorphisms in the DC-SIGNR neck region modulate susceptibility to HIV-1 infection (Liu et al., 2006), and maybe more impressively, a correlation between the DC-SIGNR genotype and the viral load in HCV infected patients has been documented (Nattermann et al., 2006). While the former finding is controversial (Lichterfeld et al., 2003), the observations that liver sinusoidal endothelial cells (LSEC) are permissive for HIV-1 infection (Steffan et al., 1992) and capture HCV envelope protein E2 in a DC-SIGNR-dependent manner (Lai et al., 2006; Ludwig et al., 2004; Pöhlmann et al., 2003) indicate that DC-SIGNR and possibly other lectins on LSECs might impact spread of several clinically relevant human viruses.

The DC-SIGN/R and CD23 genes are located in a cluster of lectin encoding genes on chromosome 19p13.3 (Soilleux et al., 2000). Liu and colleagues (2004) reported that the gene for LSECtin, another calcium-dependent (C-type) lectin, is also localized in this cluster. They found that LSECtin is co-expressed with DC-SIGNR in liver and lymph node and, like DC-SIGN/R, binds to mannose (Liu et al., 2004). We previously discovered that LSECtin, like DC-SIGN/R, augments Ebola virus (EBOV) and SARS coronavirus infection (Gramberg et al., 2005). However, in contrast to DC-SIGN/R, LSECtin failed to augment HCV and HIV-1 infection and virus interactions with LSECtin were not inhibited by the mannose polymer mannan (Gramberg et al., 2005). Thus, LSECtin might have an expression pattern similar to DC-SIGNR but it seems to interact with a more restricted spectrum of viruses and seemingly in a mannose-independent manner. Interestingly, a recent study confirmed interactions between LSECtin and EBOV and detected LSECtin on dendritic cells and macrophages under certain conditions (Dominguez-Soto et al., 2007), suggesting some similarities between the expression pattern of LSECtin and DC-SIGN. However, both the natural function and the role of LSECtin in virus infection are incompletely understood.

Here, we sought to elucidate similarities and differences between DC-SIGN/R and LSECtin interactions with ligands in order to better understand the biological functions of these lectins. We confirmed co-expression of DC-SIGNR and LSECtin on LSECs

and found that both lectins bind to soluble EBOV glycoprotein (GP) with similar affinities. LSECtin also bound to HIV-1-GP but failed to capture HIV-1 particles, demonstrating that capture of soluble GP is not necessarily predictive of virion capture. Finally, LSECtin like DC-SIGN was able to internalize ligand. However, ligand bound to LSECtin could not be dissociated by endosomal low pH, indicating that the intracellular fate of internalized LSECtin and DC-SIGN might be different.

Results

Generation of LSECtin-specific monoclonal antibodies

In order to investigate LSECtin expression and function, we generated LSECtin-specific monoclonal antibodies by immunization of mice with bacterially expressed LSECtin, followed by generation of clonal B-cell hybridomas as described previously (Baribaud et al., 2001). Screening of hybridoma supernatants by ELISA identified four supernatants that specifically reacted with the antigen used for immunization. Antibodies from the respective supernatants were purified. All antibodies were reactive against recombinant LSECtin as assessed by ELISA and allowed detection of both recombinant and cellular LSECtin by Western blot (Table 1; Fig. 1A). However, only the D18 antibody was reactive against native, cell surface expressed LSECtin as judged by FACS analysis (Fig. 1A) and was therefore chosen for further analysis. LSECtin is a type II transmembrane protein, which, based on sequence analysis, exhibits the following domain structure: N-terminal cytoplasmic domain (CD), transmembrane domain, neck domain and C-terminal carbohydrate recognition domain (CRD). In order to identify the LSECtin domain recognized by D18, various portions of LSECtin were bacterially produced (Fig. 1B) and D18 reactivity against the recombinant proteins assessed by ELISA. D18 specifically reacted with the purified neck domain and all proteins containing this domain (Fig. 1C), indicating that the D18 epitope is located in the LSECtin neck region.

DC-SIGNR and LSECtin are co-expressed in liver, lymph node and bone marrow, but only DC-SIGNR is found in placental tissue

We next employed D18 to investigate tissue expression of LSECtin. Immunohistochemical staining of parental 293 cells and cells engineered to express LSECtin confirmed the specificity of the antibody (Fig. 2A, B). Staining of tissue sections revealed no evidence for LSECtin expression in any major organ/tissue, except lymph node (Fig. 2C, D), liver (Fig. 2G) and bone marrow (Fig. 2J), where LSECtin was detected with a

Table 1
Reactivity of LSECtin MAbs

	ELISA	WB	FACS	IHC	Subclasses
D18	+	+	+	+	IgG 2a
C 12	+	+	–	n.d.	n.d.
D 9	+	+	–	n.d.	n.d.
C17	+	+	–	n.d.	n.d.

mAb, monoclonal antibody; WB, Western blot; IHC, immunohistochemistry.

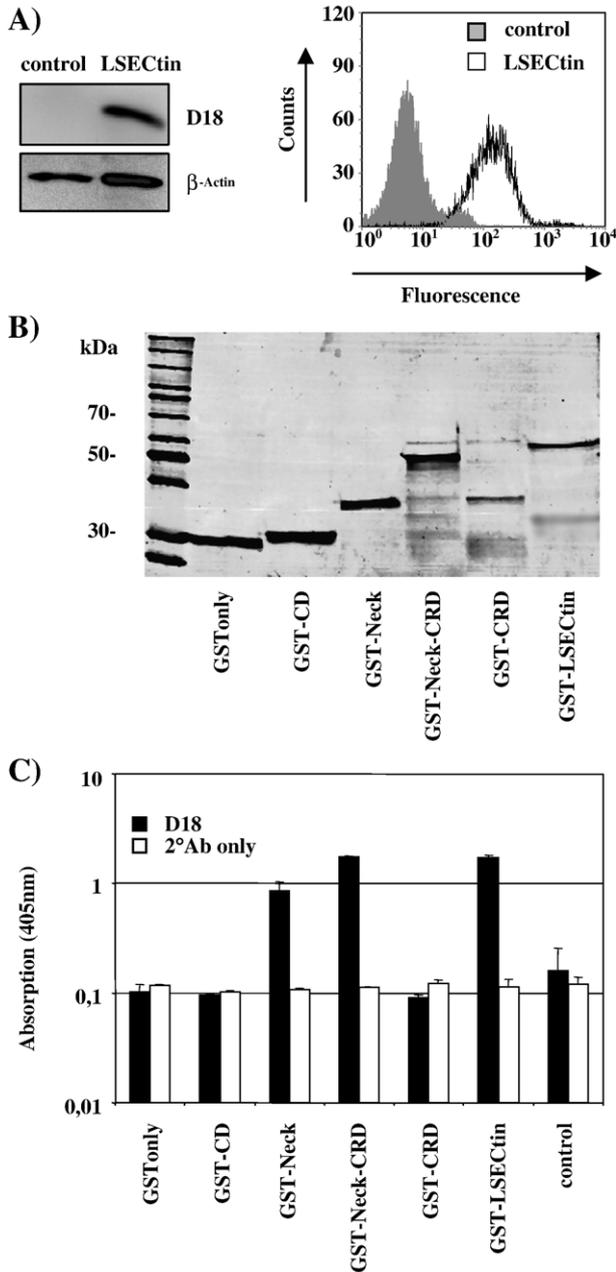


Fig. 1. Characterization of the LSECtin-specific monoclonal antibody D18. (A) D18 allows detection of LSECtin by Western blot and FACS analysis. LSECtin was transiently expressed in 293T cells and expression detected by Western blot (left panel) or FACS (right panel). Cells transfected with empty vector were analyzed as negative control. Staining for β -actin served as loading control for the Western blot analysis. (B) Analysis of recombinant LSECtin variants. Wild-type LSECtin or the indicated LSECtin domains fused to GST were purified from bacteria, separated by SDS-gel electrophoresis and visualized by Coomassie staining. (C) The D18 epitope is located in the LSECtin neck region. The indicated recombinant LSECtin proteins were coated onto 96-well plates and D18 reactivity against the proteins was analyzed by ELISA. D18 staining of uncoated control wells and staining with secondary antibody alone served as negative controls. The results of a representative experiment are shown and were confirmed in two separate experiments.

continuous staining pattern along the sinusoids, in keeping with expression by sinusoidal endothelial cells rather than Kupffer cells, which would give a discontinuous staining pattern. Very similar staining was observed with the DC-SIGN/R-specific antibody DC28 (Baribaud et al., 2001) (Fig. 2E, H, K) and was

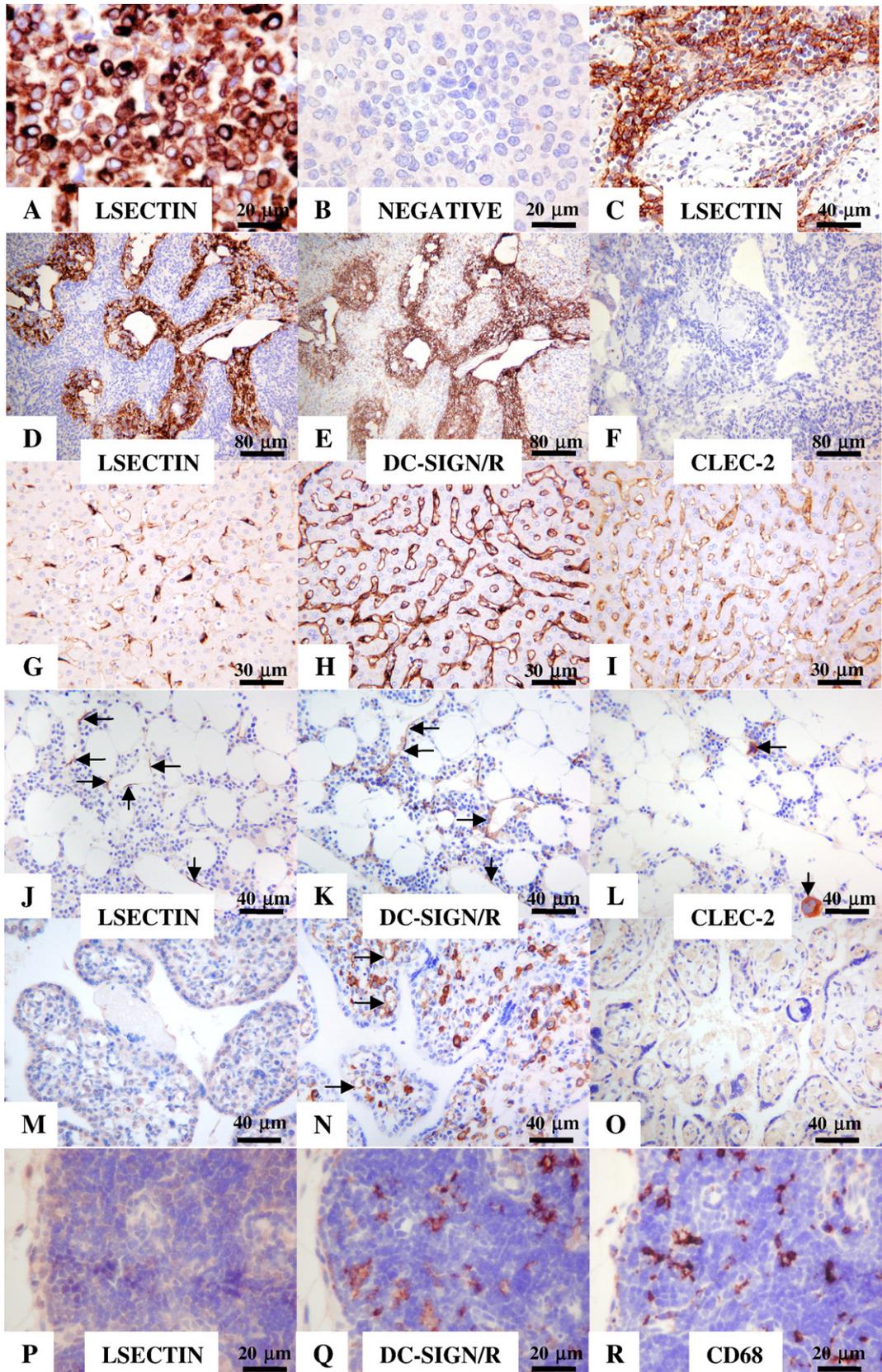
mainly due to DC-SIGNR expression (Bashirova et al., 2001; Lai et al., 2006; Pöhlmann et al., 2001c). Sinusoidal endothelial cells in liver (Fig. 2I), but not lymph node (Fig. 2F), also expressed CLEC-2, a novel HIV-1 attachment factor (Chaipan et al., 2006). In the liver, anti-LSECtin immunostaining was more similar to anti-CLEC-2 immunostaining, in keeping with LSECtin expression by sinus endothelial cells, rather than sinus endothelial cells and Kupffer cells, which have been reported to express both DC-SIGN and DC-SIGNR, and DC-SIGN alone, respectively (Lai et al., 2006). Finally, DC-SIGNR (Fig. 2N), but not LSECtin (Fig. 2M) or CLEC-2 (Fig. 2O), was expressed by vascular cells in the placenta. Thus, in agreement with previous results (Liu et al., 2004), LSECtin shows a restricted tissue expression and is only found in liver, lymph node and bone marrow sinusoids, where it is co-expressed with DC-SIGNR. In light of recent published data suggesting expression of LSECtin by human peripheral blood and thymic dendritic cells isolated *ex vivo* (Dominguez-Soto et al., 2007), normal thymic tissue was immunostained for LSECtin (Fig. 2P). No positive immunostaining was identified, although DC-SIGN/R (Fig. 2Q) and CD68 immunostaining (Fig. 2R) demonstrated the presence of abundant cells of macrophage/dendritic cell lineage.

LSECtin and the Langerhans cell-specific lectin Langerin bind to soluble HIV-1 envelope protein but do not capture and transmit HIV-1

Our previous analyses failed to detect LSECtin interactions with HIV-1 (Gramberg et al., 2005). It was unclear, however, whether LSECtin simply failed to bind to the surface unit Gp120 of the HIV-1 envelope protein or whether the failure to capture HIV-1 was due to more complex reasons. We therefore analyzed binding of a soluble HIV-1-Gp120-Fc fusion protein to CD4-negative HeLa cells transiently expressing DC-SIGN, LSECtin, Langerin and CD23. Langerin, which is exclusively expressed by Langerhans cells (Valladeau et al., 1999), was included as a positive control since we previously showed robust HIV-1-Gp120 binding to this lectin (Turville et al., 2002). CD23 served as a negative control. Cell surface expression of all lectins was readily detectable (Fig. 3A, upper panel). DC-SIGN and Langerin but not CD23 readily bound to HIV-1-Gp120 (Fig. 3A, lower panel), as expected (Geijtenbeek et al., 2000; Turville et al., 2002). To our surprise, LSECtin also captured HIV-1-Gp120 with high efficiency (Fig. 3A, lower panel). The mannose polymer mannan blocked binding of HIV-1-Gp120 and, with slightly lower efficiency, binding of soluble EBOV-GP-Fc to DC-SIGN and Langerin (Fig. 3B). In contrast, mannan did not inhibit ligand binding to LSECtin (Fig. 3B), providing further evidence that LSECtin does not recognize mannose residues on the surface of ligands. Given the similar binding of soluble Env by DC-SIGN, LSECtin and Langerin, we next compared the ability of these lectins to support HIV-1 transfer to target cells (trans-infection). While DC-SIGN-expressing cells captured and transmitted HIV-1 to target cells with high efficiency, LSECtin and Langerin-positive cells failed to bind and transmit HIV-1 (Fig. 4). Langerin expression also

failed to enhance EBOV-GP-driven infection, while DC-SIGN and LSEctin augmented infection efficiency, as expected (Gramberg et al., 2005). Finally, all cell lines exhibited com-

parable susceptibility to infection with a control virus bearing the G-protein of vesicular stomatitis virus (VSV-G). Thus, despite efficient binding to HIV-1-Gp120, LSEctin and



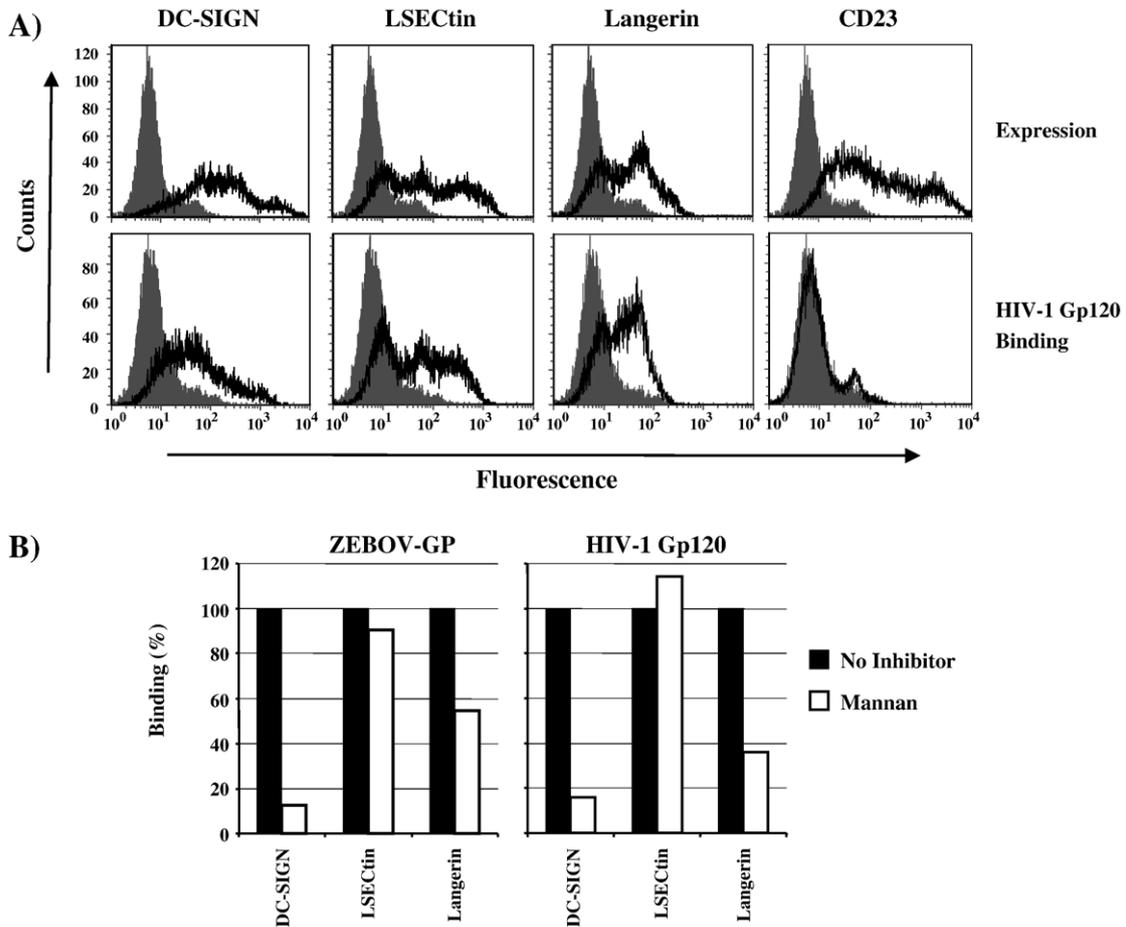


Fig. 3. LSECtin binds to soluble HIV-1-Gp120 in a mannose independent fashion. (A) HeLa cells were transiently transfected with plasmids encoding the indicated lectins and lectin expression and HIV-1-Gp120 binding analyzed in parallel. Lectin expression was determined by staining with an antibody directed against a C-terminal AU1 antigenic tag (DC-SIGN, Langerin, CD23) or with the LSECtin-specific monoclonal antibody D18 (top panel). Lectin binding to an HIV-1-Gp120-Fc fusion protein was assessed by incubating lectin expressing cells with concentrated supernatant containing HIV-1-Gp120 followed by detection of bound protein with an Fe-specific antibody (bottom panel). Similar results were obtained in two separate experiments. (B) Inhibition of HIV-1-Gp120 binding by mannane. Binding of HIV-1-Gp120 to lectin expressing cells was analyzed as described for panel (A); however, cells were pre-incubated with PBS or mannane before addition of soluble envelope protein. Binding to lectin expressing cells in the absence of inhibitor was set as 100%. The results of a representative experiment are shown and were confirmed in two independent experiments.

Langerin do not interact with HIV-1, at least under the conditions tested and thus may not support HIV-1 spread in infected patients.

LSECtin and DC-SIGNR bind to soluble EBOV-GP with similar affinities

Liver and lymph nodes are important early targets of EBOV infection (Geisbert et al., 2003a). In these tissues, LSECtin and DC-SIGNR are co-expressed on sinusoidal endothelial cells and both lectins are capable of augmenting infectious cellular

entry of EBOV (Alvarez et al., 2002; Bashirova et al., 2001; Pöhlmann et al., 2001c; Simmons et al., 2003). Because DC-SIGN/R and LSECtin differ in their carbohydrate specificities (Gramberg et al., 2005), we asked if these differences translate into different affinities for EBOV-GP, which might have important implications for EBOV attachment to liver and lymph node sinusoidal endothelial cells. In order to assess affinities of LSECtin and DC-SIGNR for EBOV-GP, 293 cells expressing roughly comparable amounts of these lectins (Fig. 5A) were incubated with purified soluble EBOV-GP-Fc fusion protein and binding was assessed by flow cytometry (Fig. 5B). Quantification

Fig. 2. LSECtin is co-expressed with DC-SIGNR on liver, lymph node and bone marrow sinusoidal endothelial cells. Expression of LSECtin on stably transfected 293T-REx cells was induced by doxycycline (A) or PBS (B) and the cells were subsequently formalin fixed, paraffin embedded and immunostained (brown) for LSECtin. Nuclei were stained with hematoxylin (blue). Lymph node was immunostained (brown) for LSECtin (C and D), DC-SIGNR (E) and CLEC-2 (F). Liver sections immunostained for LSECtin (G), DC-SIGNR (H) and CLEC-2 (I) demonstrated a continuous staining pattern around hepatic sinusoids for all the lectin molecules, indicative of expression of all three molecules by sinusoidal endothelial cells. Serial sections of bone marrow demonstrated expression of LSECtin (arrows on J) and DC-SIGNR (arrows on K), but not CLEC-2 (L) on sinusoidal endothelial cells. DC-SIGN was also expressed by some megakaryocytes (K) and possibly other bone marrow cell types, whereas CLEC-2 was expressed by megakaryocytes only (arrows on L). Sections of placenta were negative for LSECtin (M) and CLEC-2 (O) but demonstrated DC-SIGNR expression by vascular endothelial cells (arrows on N) and DC-SIGN expression by Hofbauer cells (N). Sections of thymus showed no expression of LSECtin (P), although abundant macrophages/dendritic cells were demonstrated by immunostaining for DC-SIGNR (Q) and CD68 (R).

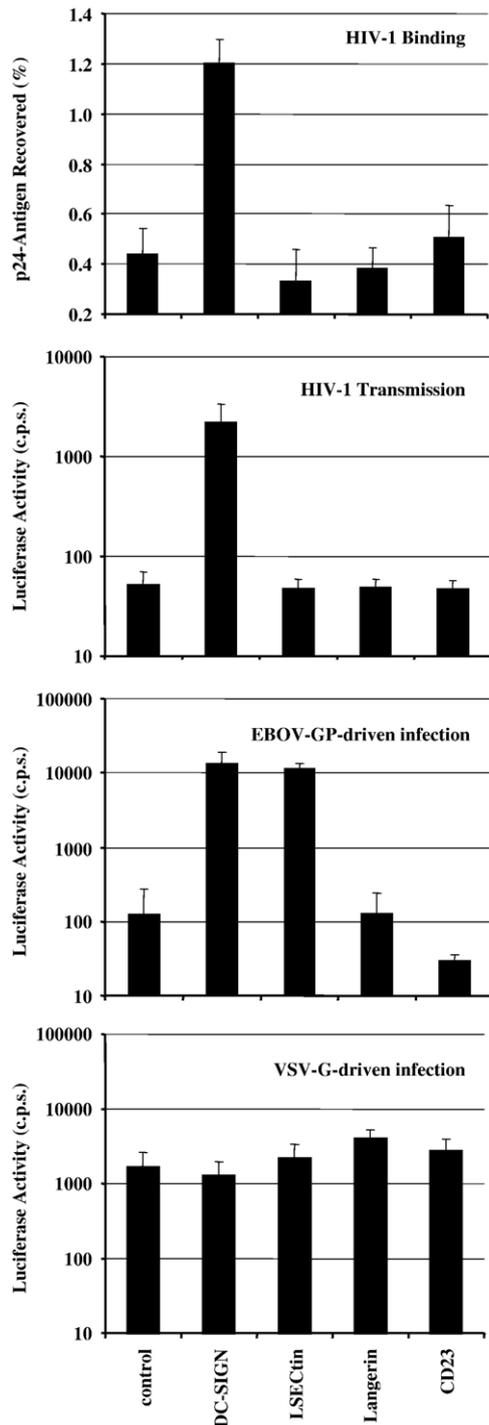


Fig. 4. LSECtin does not capture and transmit HIV-1 to target cells. HeLa cells transiently expressing the indicated lectins or control transfected cells were pulsed with HIV-1 or HIV-1 pseudotypes bearing EBOV-GP or VSV-G. All viruses encoded the luciferase gene in place of nef. In order to determine HIV-1 binding, the cells were washed and the amount of p24-antigen in cell lysates determined (top panel). Results are shown as percent of recovered input antigen. HIV-1 transmission was assessed by coculture of virus-exposed cells with target cells followed by measurement of luciferase activities in culture lysates (second panel). The efficiency of EBOV-GP and VSV-G-driven infection was determined by quantifying luciferase activities in the lysates of infected HeLa cells (third and fourth panel). The results of representative experiments carried out in triplicate are presented. Error bars indicate standard deviation (SD). Similar results were obtained in two independent experiments.

of binding efficiency and assessment of K_D values employing a previously described method (Lozach et al., 2003) revealed comparable high affinity binding for LSECtin ($K_D=21$ nM) and DC-SIGNR ($K_D=26$ nM) (Fig. 5C). Thus, LSECtin and DC-SIGNR on liver and lymph node sinusoidal endothelial cells might be equally adept at capturing soluble EBOV-GP and possibly EBOV particles.

LSECtin internalizes soluble EBOV-GP and acidic pH does not dissolve the lectin–ligand complex

DC-SIGN internalizes ligand for intracellular processing and subsequent MHC presentation (Engering et al., 2002). We investigated whether LSECtin was also capable of ligand internalization. B-THP cells (Wu et al., 2004) expressing exogenous DC-SIGN or LSECtin, but not control B-THP cells, readily captured soluble Alexa-labeled EBOV-GP. Binding was lectin dependent because a cell population double positive for lectin and EBOV-GP was readily detectable by flow cytometry. The population of double positive B-THP LSECtin cells was relatively heterogeneous and less prominent than that observed with B-THP DC-SIGN cells due to reduced expression of LSECtin relative to DC-SIGN. When cells were maintained at 4 °C, EBOV-GP binding could be abrogated by trypsin digestion, which cleaved both DC-SIGN and LSECtin (Fig. 6A and data not shown, the remaining LSECtin signal was most likely due to unspecific reactivity of B-THP LSECtin cells with antibodies; T.G. and S.P., unpublished observations), suggesting that EBOV-GP was localized to the cell surface under these conditions. In contrast, shifting cells to 37 °C for 5 or 15 min before trypsin digestion abrogated lectin expression but preserved the EBOV-GP signal, indicating that the soluble protein had been internalized. After successful cellular uptake, the lectin–ligand complexes might be dissociated by low pH in endosomal compartments. Alternatively, the complexes might remain stable at low pH and might traffic to lysosomes. In order to distinguish between these possibilities, we assessed whether exposure to low pH released EBOV-GP from DC-SIGN/R and LSECtin expressing T-Rex cells. At neutral pH, all lectin expressing cells bound to EBOV-GP with comparable efficiency (data not shown). Upon treatment with medium of pH 6.0 or lower, EBOV-GP was released from DC-SIGN/R, with the association of DC-SIGNR with ligand being slightly more resistant to low pH than DC-SIGN–ligand complexes (Fig. 6B). In contrast, even exposure to pH 5.5 did not dissolve LSECtin–EBOV-GP complexes (Fig. 6B), suggesting that intact LSECtin–ligand complexes might traffic to lysosomes.

Discussion

We generated and employed an LSECtin-specific monoclonal antibody to analyze LSECtin expression and function. We show that LSECtin is co-expressed with DC-SIGNR on liver, lymph node and bone marrow sinusoidal endothelial cells but, unlike DC-SIGNR, is absent from vascular endothelial cells in the placenta. LSECtin bound to soluble dimeric EBOV-GP and HIV-1-GP and enhanced EBOV-GP-driven entry. However, LSECtin failed to capture and transmit HIV-1 to target cells,

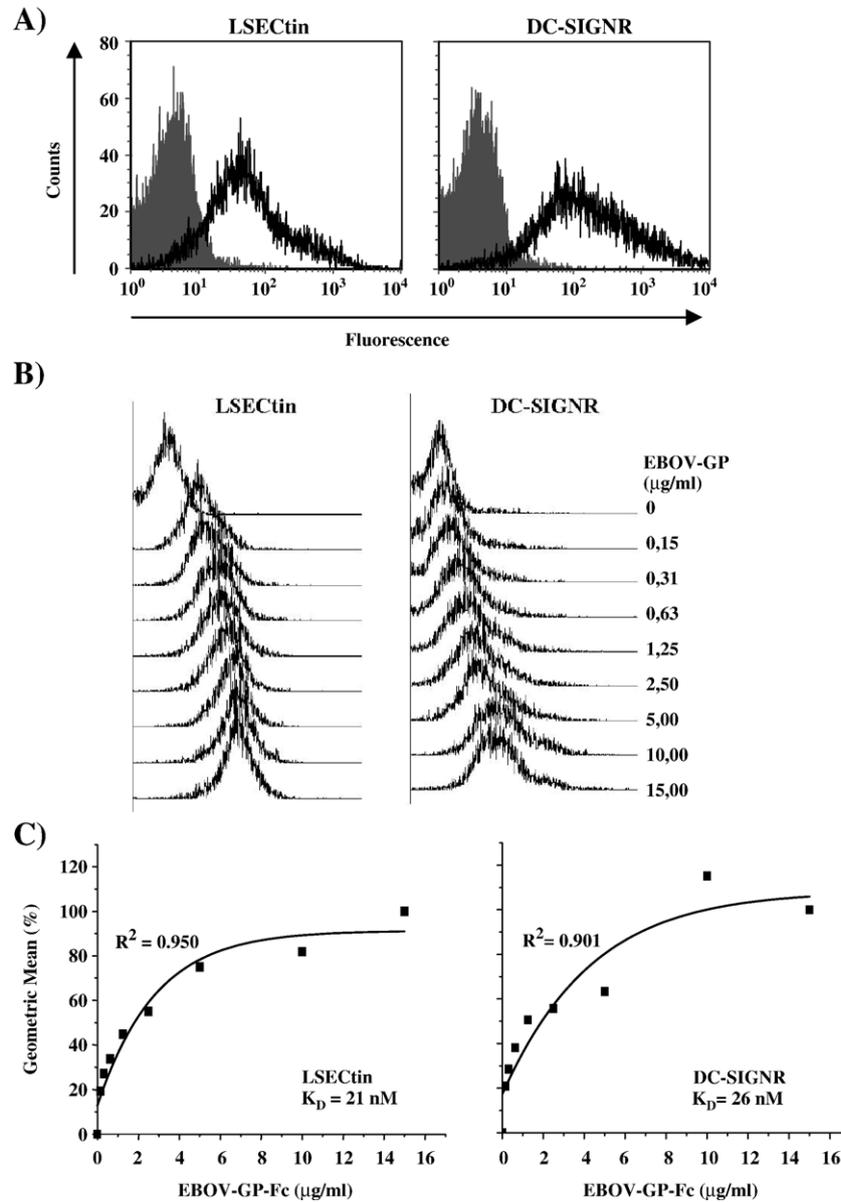


Fig. 5. LSECTin and DC-SIGNR exhibit similar affinities for EBOV-GP. 293T-REx cells (Pöhlmann et al., 2001a,b; Simmons et al., 2003) were induced to express comparable amounts of LSECTin or DC-SIGNR, incubated with the indicated amounts of purified soluble EBOV-GP-Fc fusion protein and binding determined by FACS. Results of a representative experiment are shown in (A, lectin expression) and (B, GP binding). The average of four independent experiments, for which the relative geometric mean channel fluorescence values were determined, is shown in panel C. Geometric mean values observed under saturating conditions were set as 100%. Error bars indicate standard deviation. The results were used to calculate K_D values employing a previously described method (Lozach et al., 2003).

suggesting that LSECTin does not interact with trimeric virion-associated HIV-1-GP. Binding of EBOV-GP to LSECTin triggered internalization and lectin–ligand complexes remained stable at low pH, indicating that intact LSECTin–ligand complexes might be transported into lysosomes. Thus, LSECTin like DC-SIGN might function as an antigen uptake receptor, but the intracellular fate of their ligands might be different. Notably, LSECTin has different specificities for virus interactions compared to DC-SIGN and whether it might promote cis- or trans-infection of other viruses remains to be determined. The LSECTin-specific antibody we have developed will be an important tool in these analyses.

This antibody recognized cell surface expressed LSECTin through an epitope present in the LSECTin neck region (Fig. 1)

but did not appreciably diminish EBOV-GP interactions with LSECTin (data not shown), which occur via the CRD. Staining of tissue sections revealed that LSECTin and DC-SIGNR are co-expressed by liver, lymph node and bone marrow sinusoidal endothelial cells (Fig. 2), which is in agreement with a previous report (Liu et al., 2004). LSECs are exposed to blood from the systemic circulation and from the gut and exhibit powerful scavenger capabilities (Lalor et al., 2006). Lectins like mannose receptor (MR), DC-SIGNR and LSECTin might contribute to the scavenger function of LSECs (Lalor et al., 2006). Whether the interaction of these lectins with viruses present in the blood stream preferentially leads to viral uptake, processing and MHC presentation or augmentation of viral infectivity remains to be determined. Notably, LSECs are permissive for HIV-1 infection

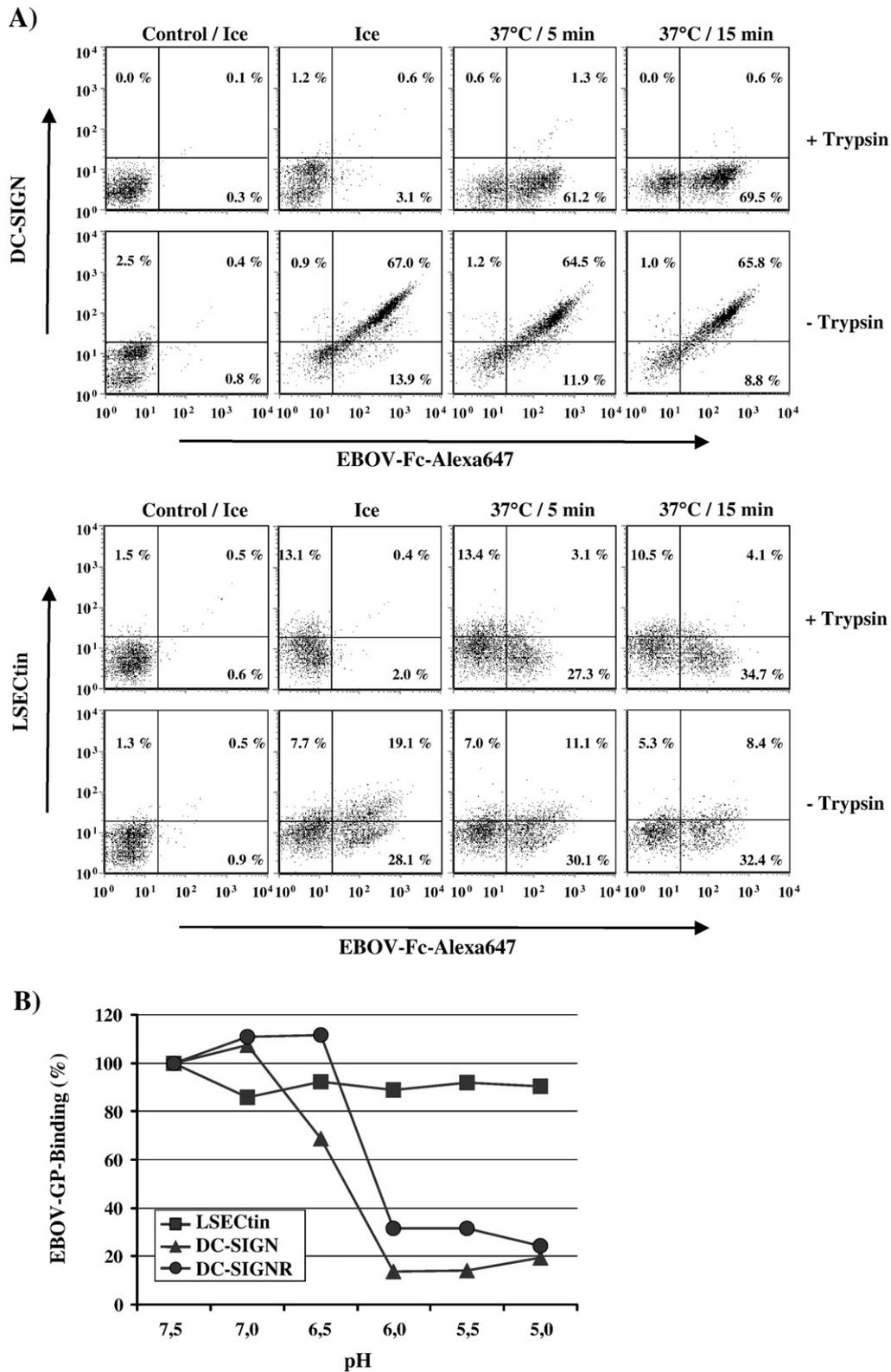


Fig. 6. LSECtin internalizes EBOV-GP and lectin–ligand complexes are not dissolved by low pH. (A) LSECtin internalizes ligand. B-THP cells engineered to express DC-SIGN or LSECtin or control B-THP cells (Wu et al., 2004) were incubated with Alexa647-labeled soluble EBOV-GP-Fc fusion protein on ice; subsequently, cells were kept on ice or shifted to 37 °C for 5 min or 15 min to allow internalization of bound antigen. After trypsin or control treatment lectin expression and EBOV-GP binding were analyzed. The results of a representative experiment are shown and were confirmed in two separate experiments. (B) Lysosomal pH does not dissociate LSECtin–ligand complexes. 293T-REX cells induced to express comparable amounts of the indicated lectins were incubated with soluble EBOV-GP-Fc fusion protein, unbound protein was washed away and the cells were treated for 20 min with media adjusted to the indicated pH values. Bound protein was detected by FACS analysis. Binding at pH 7.5 was set as 100%. The results of a representative experiment are presented and were confirmed in three independent experiments.

(Steffan et al., 1992) and polymorphisms in the DC-SIGNR neck region might impact susceptibility to HIV infection (Liu et al., 2006). Moreover, LSECs capture HCV envelope protein via DC-SIGNR (Lai et al., 2006; Ludwig et al., 2004) and certain variations in the DC-SIGNR neck region are associated with a reduced viral load in HCV patients (Nattermann et al., 2006). Thus, DC-SIGNR on LSECs and lymph node and bone marrow sinusoidal endothelium might promote HIV-1 and particularly HCV infection. By contrast, LSECTin does not interact with these viruses but our cell culture data indicate that LSECTin could facilitate filovirus interactions with sinusoidal endothelium in liver, lymph node and bone marrow. Potentially increased expression of LSECTin due to elevated cytokine production in the context of EBOV infection might facilitate these interactions. Liver and particularly lymph nodes are prominent early targets of EBOV in experimentally infected macaques, whereas infection of bone marrow is observed at later stages (Geisbert et al., 2003a, b). Because infection of the vascular endothelium in these organs is only detected during the later stages of EBOV replication (Geisbert et al., 2003b), one might postulate that LSECTin and DC-SIGNR mainly promote viral spread by virus capture and transmission to adjacent target cells, like hepatocytes or Kupffer cells. In fact, LSECs can bind and transmit duck hepatitis B virus to adjacent hepatocytes but do not become infected themselves (Breiner et al., 2001), suggesting that these cells, like DCs, might promote viral infection in trans.

LSECTin did not bind or transmit HIV-1 (Fig. 4), which is in agreement with our previous results (Gramberg et al., 2005). However, LSECTin captured a soluble HIV-1 Gp120-Fc fusion protein with high efficiency (Fig. 3), suggesting that the binding interface present in soluble Gp120 is obscured or absent in Env trimers on HIV-1 particles. Notably, similar results were obtained with Langerin (Figs. 3 and 4), a C-type lectin specifically expressed on Langerhans cells (Valladeau et al., 1999), probably the first cell type to come into contact with sexually transmitted HIV-1. The discrepancy between Gp120 and virion binding by LSECTin indicates that soluble and virion-associated Gp120 might differ in terms of content and/or surface exposure of glycans. Alternatively, the multimerization status of Gp120 (soluble Fc fusion protein is most likely dimeric while virion-associated protein is trimeric) or the lectin expression level might play a role. For Langerin, the situation might be different because it has recently been proposed that Langerin efficiently captures HIV-1 and targets bound virions for degradation in intracellular compartments (de Witte et al., 2007). In light of these results, our finding that Langerin expressing HeLa cells did not capture HIV-1 can be explained in three ways. Exogenous Langerin on HeLa cells, unlike endogenous Langerin on Langerhans cells, might not be capable of binding HIV-1, maybe because a cofactor present on Langerhans cells is missing. Alternatively, as for LSECTin, lectin expression levels might play a role. Thus, higher copy numbers of surface expressed Langerin might be required for binding to HIV-1 as compared to binding to soluble gp120. In this context, it needs to be pointed out that Langerhans cells most likely express substantially higher levels of Langerin relative to the transfected cells used in the present study. Finally, Langerin-bound HIV-1 might be rapidly endocytosed and degraded in HeLa cells. However, the absence of any specific HIV-1 binding

to Langerin expressing HeLa and 293T cells even early after exposure (Fig. 4 and data not shown) suggests that HIV-1 degradation might not account for the effects observed. In any case, it is notable that binding to soluble Gp120 is not necessarily predictive for binding to virus particles (present manuscript) and capture of virions does not necessarily result in transmission (Baribaud et al., 2001; Pöhlmann et al., 2001b). Detailed analysis of the determinants involved might yield important insights into the molecular mechanisms behind lectin-mediated augmentation of viral infectivity.

Ligand captured by DC-SIGN, but not DC-SIGNR (Guo et al., 2004), is endocytosed and transported into late endosomes/lysosomes (Engering et al., 2002). The acidic milieu in this compartment dissociates the lectin–ligand complex and DC-SIGN might be recycled to the cell surface (Engering et al., 2002). Soluble EBOV glycoprotein bound to LSECTin expressing cells at 37 °C but not at 4 °C was protected against protease digestion (Fig. 6A), suggesting that LSECTin–ligand complexes were also endocytosed. A recent study by Dominguez-Soto and colleagues (2007) confirms and extends this observation by showing that tyrosine- and diglutamic-containing motifs in the cytoplasmic domain mediate rapid LSECTin internalization. LSECTin like DC-SIGN might therefore function as an antigen uptake receptor. However, the fate of the endocytosed lectins might be different. Low pH dissociated DC-SIGN/R–ligand complexes, whereas LSECTin–ligand complexes remained stable, even in an acidic milieu similar to that observed in lysosomes (Fig. 6B). Thus, both LSECTin and ligand might be degraded in lysosomes whereas DC-SIGN might be recycled to the cell surface. Obviously, EBOV escapes degradation upon LSECTin-mediated uptake and uses this lectin for augmentation of infectivity (Fig. 4; Gramberg et al., 2005). These results are not unexpected because endosomal/lysosomal enzymes were shown to dissociate GP1 (which is bound by LSECTin) from GP2 (which drives membrane fusion) (Chandran et al., 2005; Schornberg et al., 2006), thereby most likely releasing EBOV from LSECTin and allowing the virus to fuse with the vesicle membrane.

In summary, LSECTin shows important similarities to DC-SIGN/R, including expression by liver, lymph node and bone marrow sinusoidal endothelial cells, interaction with viral glycoproteins and internalization of bound ligands. However, important differences are also apparent. For one, LSECTin promotes infection by a more restricted spectrum of viruses and might have no impact on HIV-1 and HCV spread in infected individuals. Secondly, complexes between ligand and DC-SIGN/R but not LSECTin–ligand complexes are dissociated by a low pH milieu, suggesting differential intracellular trafficking of the endocytosed lectins.

Materials and methods

Cell culture

293T cells were propagated in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), penicillin and streptomycin. 293T-REx cells expressing DC-SIGN,

DC-SIGNR or LSECTin were described previously (Gramberg et al., 2005; Pöhlmann et al., 2001a,b; Simmons et al., 2003) and maintained in medium containing DMEM, 10% FBS, 50 µg/ml zeocin (Invitrogen, CA, USA), 2.5 µg/ml of blasticidin (Invitrogen, CA, USA), penicillin and streptomycin. Expression was induced by culturing the cells in medium containing 0.1 µg/ml of doxycycline (Sigma-Aldrich, Germany). 293T-REx parental cells were maintained in the same medium as lectin expressing cell lines; however, no zeocin was added. CEMx174 5.25 (Hsu et al., 2003) and HeLa cells were maintained in RPMI 1640 containing 10% FBS, penicillin and streptomycin. Parental B-THP cells, which are derived from Raji-B-cells (Wu et al., 2004), and B-THP cells expressing DC-SIGN and DC-SIGNR were maintained in RPMI 1640 containing 10% FBS, penicillin and streptomycin. B-THP cells expressing LSECTin were generated via electroporation and FACS sorting and were maintained in RPMI 1640 supplemented with 10% FBS, penicillin, streptomycin and 500 µg/ml G418. All cells were grown at 37 °C and 5% CO₂.

Plasmid construction and in vitro mutagenesis

Expression plasmids encoding the indicated lectins were described previously (Pöhlmann et al., 2001a,b; Simmons et al., 2003). The glycoprotein (GP) expression plasmids employed for the generation of pseudotyped viruses were also described previously (Simmons et al., 2003). For regulated lectin expression, the open reading frames (ORFs) were inserted into pcDNA4TO (Invitrogen, CA, USA) as described previously (Gramberg et al., 2005; Marzi et al., 2004). For expression of soluble EBOV-GP and soluble HIV-1 Gp120, the extracellular portion of the respective GP-ORF was fused to the Fc part of human IgG₁ via PCR and cloned in frame with the amino-terminal murine IgG kappa signal peptide in the eukaryotic expression vector pAB61, as described (Gramberg et al., 2005; Marzi et al., 2004). For prokaryotic expression of LSECTin, PCR fragments spanning the entire LSECTin ORF or sequences encoding the indicated protein domains were inserted into pGEX6P1 (Amersham Biosciences, UK) via *Bam*HI and *Xho*I. All PCR-amplified sequences were confirmed by automated sequence analysis.

Antigens and antisera

A mouse monoclonal antibody (MAb) directed against the AU1 antigenic tag was purchased from Covance Research Products, CA, USA. The MAbs 507 (DC-SIGN specific), 526, DC28, DCN46 (DC-SIGN/R specific) and 604 (directed against DC-SIGNR) were obtained via the NIH AIDS Research and Reference Reagent Programme. FITC-conjugated anti-mouse and Cy5-conjugated anti human secondary antibodies were purchased from Jackson ImmunoResearch, PA, USA, and Diaova, Germany, respectively. The anti-LSECTin antibodies D18, C12, D9 and C17 were obtained by immunizing NMRI mice with 20 µg of purified GST–LSECTin fusion protein, as described elsewhere (Gramberg et al., 2005). Fusion proteins were obtained by overexpression in *Escherichia coli* DH10B fol-

lowed by purification of the proteins employing Glutathione Sepharose 4B Beads (Amersham Biosciences, UK). After a final boost, splenocytes were removed from immunized mice and fused with SP 2.0 myeloma cells. Clonal hybridoma cells were cultured in HAT selection medium and screened for anti LSECTin reactivity via enzyme-linked immunosorbent assay (ELISA), Western blot analysis and flow cytometry. Subsequently, antibodies were purified from supernatants reactive in Western blot (C12, D9, C17) or flow cytometry (D18) via HighTrap™ ProteinG-Columns (Amersham Biosciences) according to the manufacturers instructions.

Binding of soluble glycoproteins to lectin expressing cells

Soluble EBOV-GP-Fc and HIV-1-Gp120-Fc fusion proteins or control Fc were obtained by harvesting the supernatant of 293T cells 48 h after transient transfection with the respective expression vectors. The supernatants containing the respective proteins were concentrated using Centricon Plus-20 centrifugal filters (Millipore, USA). To measure lectin binding, we incubated comparable amounts of soluble protein, as judged by Western blot, with transiently transfected HeLa cells for 45 min on ice. After washing with FACS buffer, cells were stained with Cy5-conjugated anti-human IgG (Jackson ImmunoResearch, USA) at a final concentration of 15 µg/ml for 45 min on ice. Cells were then washed and diluted in FACS buffer and binding was measured via flow cytometry using a FACS-Calibur flow cytometer (Becton Dickinson). To determine the pH dependence of GP binding, we inoculated lectin expressing 293 cells with the indicated soluble GPs and subsequently incubated for 20 min in ice-cold PBS (pH 5.0–7.5). Staining with secondary antibody and detection via flow cytometry was performed as described above.

Internalization assay

Soluble EBOV-GP-Fc was directly labeled with the Alexa647-fluorochrome using the AlexaFluor647-labeling Kit (Invitrogen, CA, USA). To assess internalization, we incubated lectin expressing B-THP cells with labeled protein on ice, washed and incubated at 37 °C or on ice, respectively. After fixation of the cells with 2% PFA (10 min/ice), surface molecules were cleaved off by treatment with 1mg/ml trypsin TPCK (Invitrogen). Subsequently, cells were stained for lectin expression on ice (lectin-specific antibody, Cy3-labeled secondary antibody) and were analyzed via flow cytometry, as mentioned above.

Production of reporter viruses and infection assays

HIV-1-derived pseudotypes were generated by co-transfection of 293T cells with GP expression plasmids coding for the indicated glycoproteins and pNL4-3 E⁻R⁻Luc plasmid, as described previously (Connor et al., 1995; Simmons et al., 2003). The production of replication competent HIV-1 NL4-3 reporter virus bearing the luciferase gene in place of nef has been described previously (Pöhlmann et al., 2001a). The culture supernatants were harvested 48 h after transfection, passed through 0.4 µm pore size filters, aliquoted and stored at –80 °C.

To assess lectin-mediated enhancement of infection, we seeded 293 cells or HeLa cells expressing the indicated lectins onto 96-well plates at a density of 1.0×10^4 cells per well and incubated with viral supernatants normalized for comparable luciferase activity upon infection of control cells. Generally, the medium was replaced 12 h after infection and luciferase activities in culture lysates were determined 72 h after infection with a commercially available kit, following the recommendations of the manufacturer (Promega, WI, USA).

HIV-1 binding and transmission assays

HeLa cells transiently expressing the indicated lectins were seeded onto 96-well plates and incubated with HIV-1 NL4-3 reporter virus normalized for capsid protein (p24) content by ELISA (Murex, Abbott Diagnostics, IL, USA). After 3 h incubation at 37 °C, the cells were washed three times with PBS and either lysed in 1% Triton X-100 followed by quantification of the amount of bound virus by p24-ELISA or cocultured with CEMx174 5.25 cells. Luciferase activities in cell lysates were determined 72 h after cocultivation as described above.

Assessment of K_D values

To compare the affinities of LSEctin and DC-SIGNR for ligand, we determined the dissociation constant K_D of both proteins for binding to soluble EBOV-GP-Fc. To this end, we incubated 3×10^5 293T-Rex cells (Invitrogen, CA, USA) induced to express comparable amounts of DC-SIGNR and LSEctin with indicated amounts of soluble EBOV-GP-Fc (0–15 µg/ml) or control-Fc (0–15 µg/ml) for 45 min on ice. Pilot experiments showed saturation binding upon incubation of cells with 15 µg/ml soluble EBOV-GP-Fc (data not shown). After washing with FACS buffer, cells were stained with Cy5-conjugated secondary antibody for 45 min on ice. Cells were then washed and diluted in FACS buffer and binding was measured via flow cytometry. After subtracting unspecific binding, specific binding curves were fit in by non-linear regression. K_D values were calculated by the use of the Origin 6.0 software (Microcal Software, USA), following a previously described protocol (Lozach et al., 2003).

ELISA reactivity of anti LSEctin MAbs

To determine the reactivity and domain specificity of anti-LSEctin antibodies, we coated 1.5 µg/ml of the indicated GST-fusion proteins or GST-control protein onto 96-well ELISA plates. After blocking free binding sites with blocking buffer (TBS buffer+2% BSA), coated fusion proteins were incubated with 100 ng/µl of the indicated anti LSEctin antibodies or a control antibody for 2 h at room temperature. After extensive washing, specific interactions of the LSEctin–MAbs with fusion proteins were assessed by measuring the reactivity of alkaline phosphatase bound to secondary antibody (Vector-Laboratories, USA) with 5 mM *p*-nitrophenylphosphate (Vector Labs, USA) in an ELISA plate reader (SpectraMAX 190, Molecular Devices, USA) at 405 nm.

Immunostaining of tissue sections

Expression of LSEctin on 293T-Rex cells was induced by doxycycline or PBS, the cells were pelleted, fixed in formalin, embedded in paraffin wax and immunostained as described for tissues. Tissue microarrays (a generous gift from Professor Kevin Gatter, University of Oxford) and anonymised sections of a range of normal tissues were obtained from the Department of Cellular Pathology, John Radcliffe Hospital, Oxford, United Kingdom, with local Research Ethics Committee approval. These samples were immunostained as described previously (Pöhlmann et al., 2001c) with mouse monoclonal anti-LSEctin or isotype-matched negative control, mouse MAb DC28, mouse MAb CD68 clone PGM1 (Dako) or goat polyclonal anti-CLEC-2 (R&D Systems) and detected using standard staining kits from Dako and Vector Laboratories. All sections were carefully reviewed by an experienced histopathologist (E.S.). Images were taken at room temperature with an Olympus BX40 microscope equipped with a 40/0.75 0.17 lens and a Nikon Coolpix 950 digital camera. Images were analyzed with Adobe Photoshop version 7 software.

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