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LSECTin interacts with filovirus glycoproteins and the spike protein of SARS coronavirus

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Abstract

Cellular attachment factors like the C-type lectins DC-SIGN and DC-SIGNR (collectively referred to as DC-SIGN/R) can augment viral infection and might promote viral dissemination in and between hosts. The lectin LSECTin is encoded in the same chromosomal locus as DC-SIGN/R and is coexpressed with DC-SIGNR on sinusoidal endothelial cells in liver and lymphnodes. Here, we show that LSECTin enhances infection driven by filovirus glycoproteins (GP) and the S protein of SARS coronavirus, but does not interact with human immunodeficiency virus type-1 and hepatitis C virus envelope proteins. Ligand binding to LSECTin was inhibited by EGTA but not by mannan, suggesting that LSECTin unlike DC-SIGN/R does not recognize high-mannose glycans on viral GPs. Finally, we demonstrate that LSECTin is N-linked glycosylated and that glycosylation is required for cell surface expression. In summary, we identified LSECTin as an attachment factor that in conjunction with DC-SIGNR might concentrate viral pathogens in liver and lymph nodes.

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Introduction

The membrane glycoproteins (GP) of enveloped viruses facilitate infection of target cells through the interaction with cellular receptors. However, binding of virions to target cells is not necessarily mediated by GP interactions with receptor, but can be facilitated by GP binding to attachment factors.

Attachment factor engagement is not sufficient to allow infectious entry, but can augment infection. Several cellular lectins can serve as attachment factors. Thus, dendritic cell specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN), a C-type (calcium dependent) lectin which is expressed at high levels on dendritic cells (DCs), binds human immunodeficiency virus (HIV) envelope protein (Env) and promotes viral transfer to susceptible cells (“infection in trans”) (Geijtenbeek et al., 2000). DC-SIGN-mediated HIV infection in trans could aid viral dissemination upon sexual transmission of HIV (Geijtenbeek et al., 2000). Besides HIV, DC-SIGN interacts with a

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range of other viral (Alvarez et al., 2002; Gardner et al., 2003; Halary et al., 2002; Klimstra et al., 2003; Lozach et al., 2003, 2004; Marzi et al., 2004; Navarro-Sanchez et al., 2003; Pöhlmann et al., 2003; Simmons et al., 2003; Tassaneeritthep et al., 2003; Yang et al., 2004) and non-viral pathogens (Van Kooyk and Geijtenbeek, 2003), which use DC-SIGN to target DCs. Notably, DC-SIGN-mediated enhancement of HIV infection is not limited to concentrating viral particles onto the cell surface (Pöhlmann et al., 2001b), but involves internalization and complex intracellular trafficking of virions (Kwon et al., 2002; McDonald et al., 2003).

The lectin DC-SIGNR (also known as L-SIGN) shares 77% amino acid identity with DC-SIGN and also interacts with pathogens (Bashirova et al., 2001; Mummidi et al., 2001; Pöhlmann et al., 2001c). DC-SIGNR is expressed on sinusoidal endothelial cells in lymph nodes and liver (Bashirova et al., 2001; Mummidi et al., 2001; Pöhlmann et al., 2001c), and the latter cell type shares several features with DCs (Limmer et al., 2000). Liver sinusoidal endothelial cells (LSECs) have been implicated in transmission of duck hepatitis B virus (DHBV) to adjacent hepatocytes, a process that involves endocytosis and intracellular transport of viral particles (Breiner et al., 2001). Enhancement of viral infection by LSECs and DCs might involve similar pathways, and hepatotropic viruses might specifically target LSECs to promote their spread in liver tissue (Breiner et al., 2001).

DC-SIGN and DC-SIGNR (collectively referred to as DC-SIGN/R) recognize high-mannose carbohydrates on the surface of ligands (Appelmelk et al., 2003; Feinberg et al., 2001; Lin et al., 2003), and albeit a role for protein–protein contacts in the interaction of DC-SIGN with HIV has been reported (Geijtenbeek et al., 2002), a large body of evidence suggests that carbohydrate recognition is the sole determinant of DC-SIGN/R ligand specificity (Appelmelk et al., 2003; Feinberg et al., 2001; Guo et al., 2004; Lin et al., 2003). In fact, while DC-SIGNR is strictly high-mannose carbohydrate specific, DC-SIGN can interact with other carbohydrates. These differences in carbohydrate specificity account for differences in the ligand spectrum of these lectins (Guo et al., 2004; Van Liempt et al., 2004).

Recently, LSEctin (liver and lymph node sinusoidal endothelial cell C-type lectin), a lectin which is coexpressed with DC-SIGNR on sinusoidal endothelial cells in liver and lymph node has been identified (Liu et al., 2004). The LSEctin gene is located on chromosome 19p13.3 in the vicinity of the genes encoding DC-SIGN/R and CD23 (Liu et al., 2004). Sequence analysis revealed that LSEctin harbors all invariant and most of the highly conserved amino acid residues characteristic for C-type lectins (Liu et al., 2004). The carbohydrate recognition domain (CRD) of LSEctin exhibits 39% and 38% amino acid sequence identity with the respective domains of DC-SIGN/R and was shown to interact with various carbohydrates, including mannose, *N*-acetylglucosamine (GlcNAc), and fucose (Liu et al., 2004). However, the ligand(s) of this lectin are unknown.

Here, we demonstrate that LSEctin interacts with the GPs of filoviruses and the spike (S) protein of the severe acute respiratory syndrome (SARS) coronavirus (CoV) and enhances viral infection. Augmentation of filovirus infection was observed with lentiviral pseudotypes bearing filovirus GPs and with replication-competent Ebolavirus of the Zaire strain (ZEBOV), indicating that LSEctin might promote filovirus infection *in vivo*. In contrast, LSEctin did not functionally interact with HIV and hepatitis C virus (HCV) Env proteins. LSEctin interactions with viral GPs were dependent on calcium, but were not blocked by mannan, indicating that LSEctin and DC-SIGN/R might differ in their carbohydrate specificities. Finally, we present evidence that LSEctin is N-linked glycosylated and that glycosylation is important for cells surface expression.

Results

Regulated expression of LSEctin on a 293 cell line and endogenous expression on LSECs

The LSEctin ORF was amplified from liver RNA, cloned, and 293 cell lines expressing the lectin upon induction with doxycycline were generated as described (Pöhlmann et al., 2001a). Alternatively, LSEctin was transiently expressed in 293T cells. LSEctin expression was verified with an antiserum generated by immunization of mice with a GST-LSEctin fusion protein produced in bacteria. FACS analysis revealed that serum from immunized mice, but not serum obtained prior to immunization, reacted with cells induced to express LSEctin (Figs. 1B, E). In contrast, no reactivity was observed with uninduced cells (Fig. 1A) or cells expressing DC-SIGN or DC-SIGNR (Figs. 1C, D), indicating that reactivity was specific and that LSEctin was expressed at the cell surface to appreciable levels and in a regulated fashion. Similar levels of LSEctin expression were also obtained with transiently transfected 293T cells (data not shown). LSEctin expression in LSEC has previously been demonstrated by immunofluorescence analysis of tissue sections (Liu et al., 2004). We employed the LSEctin specific mouse serum to investigate if LSEctin is expressed on the surface of primary LSECs obtained from human liver tissue. Analysis of LSECs obtained from three different donors revealed readily detectable surface expression of LSEctin (Fig. 1F), suggesting that this lectin might contribute to the recognition of blood-borne antigens by LSECs.

LSEctin expression augments infection driven by filovirus GPs and the S protein of SARS-CoV

We first investigated if LSEctin expression enhances infection by lentiviral luciferase reporter viruses bearing the GPs of ZEBOV, Marburgvirus (MARV), Lassa virus, SARS-CoV, murine leukemia virus (MLV), and vesicular

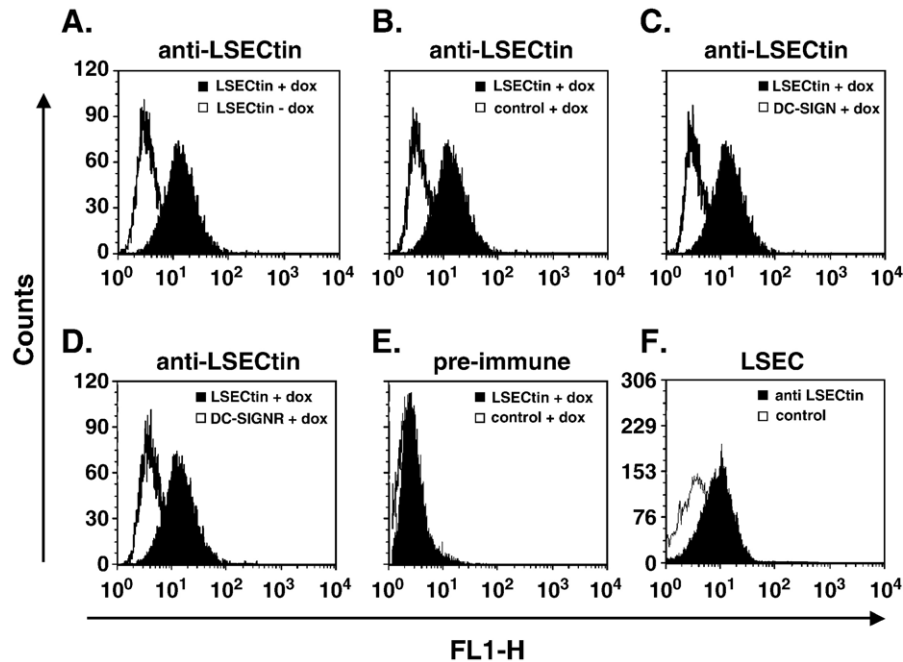


Fig. 1. Inducible expression of LSECTin on 293 T-REx cells and endogenous expression on LSEC. (A–E) Regulated LSECTin expression on cell lines. Cell lines were generated by stable transfection and lectin expression was analyzed with an antiserum raised against a GST-LSECTin fusion protein (A–D) or with pre-immune serum (E). Lectin expression on doxycycline (dox)-induced LSECTin T-REx cells (black) was compared to the following cells (white): (A) uninduced LSECTin T-REx cells, (B and E) induced T-REx control cells, (C) induced DC-SIGN T-REx cells, (D) induced DC-SIGNR T-REx cells. (F) Expression of LSECTin on LSECs. Primary human LSECs were obtained from human liver tissue and analyzed for LSECTin expression as described above. Similar results were obtained with cells from three different donors.

stomatitis virus (VSV). Enhancement of infection was analyzed with 293T cells as targets, since these cells are permissive to infectious cellular entry driven by the above listed GPs. Virus stocks were generated as described (Simmons et al., 2003), normalized for comparable luciferase production upon infection of untransfected 293T control cells, and used for infection of 293T cells transiently expressing DC-SIGN/R, LSECTin, or empty vector (Fig. 2A). Infection is shown relative to infectious entry into control cells, which was set as 100%. In agreement with previous reports (Alvarez et al., 2002; Jeffers et al., 2004; Marzi et al., 2004; Simmons et al., 2003; Yang et al., 2004), expression of DC-SIGN/R strongly augmented infection driven by filovirus GPs and the SARS-CoV-S protein, but had no appreciable effect on infectious entry mediated by VSV-, MLV-, and Lassa-GP (Fig. 2A). Expression of LSECTin enhanced filovirus GP driven infection with high efficiency and moderately augmented entry by SARS-CoV-S bearing reporter viruses (Fig. 2A). LSECTin expression on non-permissive HeLa cells did not render these cells susceptible to SARS-CoV-S bearing pseudotypes. In contrast, expression of the SARS-CoV receptor ACE2 (Li et al., 2003) allowed for efficient infection and infectious entry was enhanced upon coexpression of LSECTin (Fig. 2B), indicating that LSECTin functions as an attachment factor, but not as a viral receptor. Comparable results were obtained for DC-SIGN (data not shown), and these observations are in agreement with our previously published results (Marzi

et al., 2004). Antiserum raised against LSECTin abrogated LSECTin-, but not DC-SIGN-mediated enhancement of ZEBOV-GP driven infection (Fig. 2C), and augmentation of infection was dependent on induction of lectin expression (data not shown), confirming that enhanced infection was indeed due to LSECTin expression. Thus, LSECTin joins DC-SIGN/R as a potent attachment factor which augments infection driven by certain viral GPs.

LSECTin does not interact with HIV-1 and pseudotypes bearing hepatitis C virus envelope proteins

LSECs have been shown to bind HCV E2 protein (Ludwig et al., 2004) and to be permissive to HIV infection (Steffan et al., 1992). We therefore investigated if these viruses engage LSECTin. In order to assess if LSECTin interacts with HIV-1, T-REx cell lines were induced to express the indicated lectins, pulsed with replication-competent HIV-1 NL4-3 harboring the luciferase gene in place of *nef* (Pöhlmann et al., 2001a), washed three times, and either lysed or cocultivated with CEMx174 5.25 target cells. The content of bound virions in cell lysates was measured by p24 antigen capture ELISA, while transmission to target cells was determined 3 days after cocultivation by assessing luciferase activity in cell lysates. Cells expressing DC-SIGN bound and transmitted HIV-1 about 10-fold more efficiently than control cells and the interaction was inhibited by mannan (Fig. 3A). In contrast,

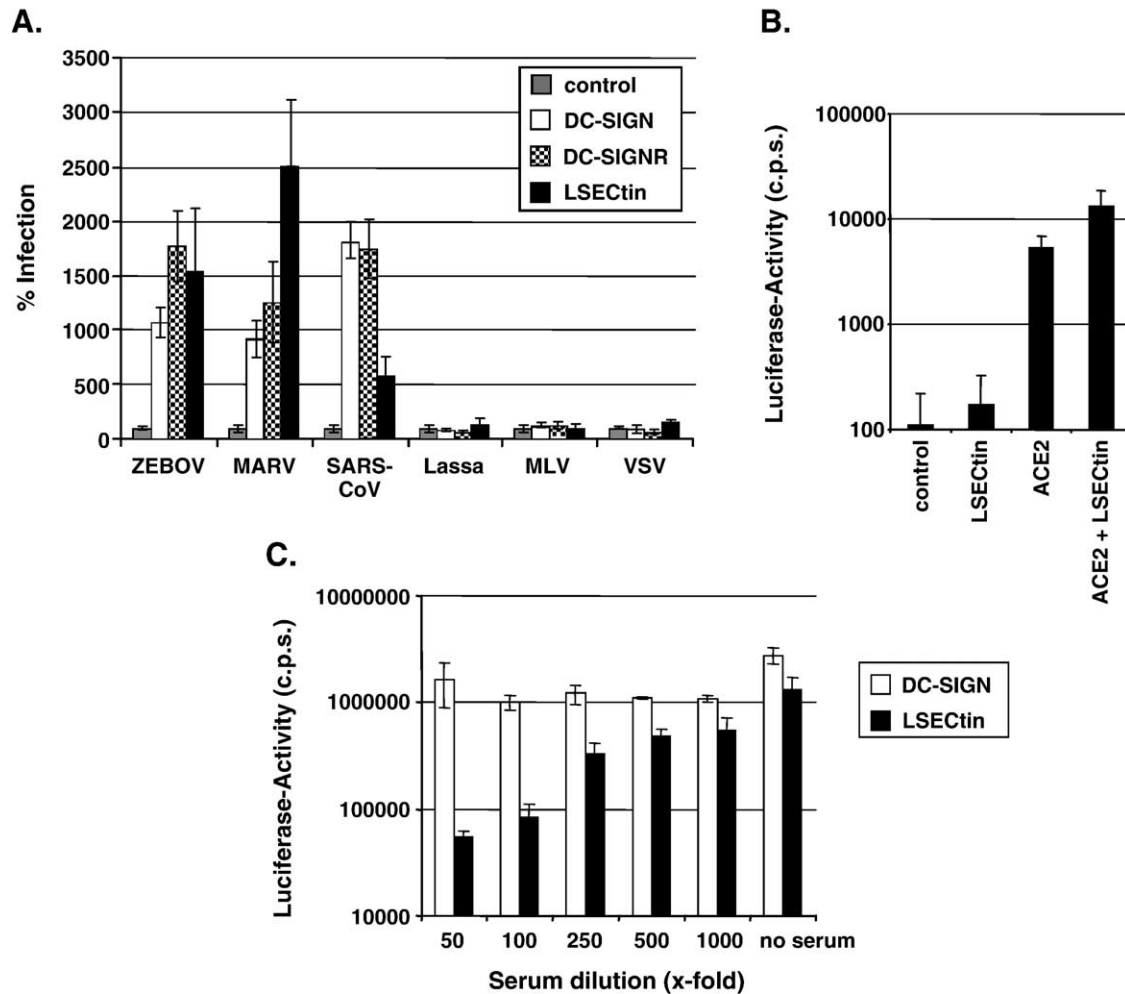


Fig. 2. Impact of LSEctin expression on viral infection. (A) LSEctin and DC-SIGN/R augment infection by lentiviral pseudotypes bearing filovirus GPs or SARS-CoV-S. Lentiviral pseudotypes harboring the GPs of the indicated viruses were normalized for comparable infection of 293T control cells and used to infect 293T cells transiently expressing DC-SIGN/R, LSEctin, or empty vector. The reporter viruses employed contain the luciferase gene, which is expressed only upon integration of the proviral DNA into the cellular genome. Three days after infection, the cells were lysed and luciferase activity was determined. Infection is shown relative to infectious entry into control cells, which was set as 100%. Upon infection of control cells with ZEBOV-GP, SARS-CoV-S, or VSV-G bearing pseudotypes, luciferase activities of 9049 ± 1546 , $19,810 \pm 6809$, and 6341 ± 676 counts per second (c.p.s.) were measured. The result of a representative experiment is shown, similar results were obtained in three independent experiments. Error bars indicate standard deviation (SD). (B) LSEctin expression does not allow SARS-CoV-S driven infection of non-permissive cells. HeLa cells, which do not express the SARS-CoV receptor ACE2 and are refractory to SARS-CoV-S driven infection, were transiently transfected with LSEctin or ACE2 or cotransfected with both plasmids. The transfected cells were subsequently infected with SARS-CoV-S bearing pseudotypes and luciferase activity was determined as described under panel A. The results of a representative experiment performed in quadruplicates are shown, error bars indicate SD. Similar results were obtained in two independent experiments. (C) Inhibition of infection by LSEctin-specific antiserum. T-REX cell lines were induced to express DC-SIGN or LSEctin, incubated with LSEctin-specific antiserum and infected with ZEBOV-GP harboring pseudotypes, which were normalized for equal luciferase activity upon infection of control cells. A representative experiment is shown, error bars indicate SD. Similar results were obtained in an independent experiment.

binding and transmission by LSEctin expressing cells was similar to that observed with control cells (Fig. 3A), indicating that LSEctin does not interact with HIV-1. LSEctin interactions with infectious lentiviral particles bearing the HCV E1 and E2 proteins were investigated in a binding experiment as described for Fig. 3A. Pseudoparticles containing the HCV envelope proteins bound to DC-SIGN expressing cells about 3-fold more efficiently than to control cells (Fig. 3B). In contrast, binding of pseudovirions to LSEctin expressing cells was comparable to binding to

control cells (Fig. 3B), suggesting that the HCV E1 and E2 proteins are not recognized by LSEctin. Thus, LSEctin exhibits a different ligand specificity compared to DC-SIGN/R.

LSEctin binding to pathogens cannot be blocked by mannan and depends on the presence of calcium

We next determined if differential ligand binding by DC-SIGN/R and LSEctin is reflected by differences in

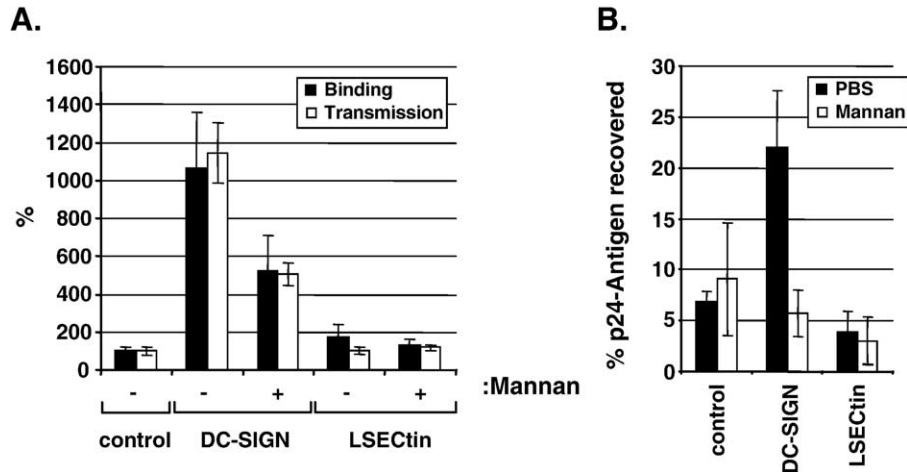


Fig. 3. LSECtin does not interact with HIV-1 and infectious pseudovirions bearing the HCV E1 and E2 proteins. (A) Binding and transmission of HIV-1 by lectin expressing T-REX cells. T-REX cells were induced to express the indicated lectins, preincubated in the presence or absence of mannan and incubated for 3 h with replication-competent NL4-3 reporter virus harboring the luciferase gene in place of *nef*. Thereafter, the cells were washed and either processed to analyze viral binding or transmission to target cells. Binding was assessed by lysing the cells in 1% Triton X-100 and quantification of bound antigen by p24-antigen capture ELISA. Transmission was determined by cocultivation of virus pulsed cells with CEMx174 5.25 target cells and analysis of luciferase production in the cultures 3 days after the start of the cocultivation. Results are shown as percent (%) HIV-1 binding (black bars) or transmission (white bars) by control cells, which was set as 100%. Error bars indicate SD, comparable results were obtained in an independent experiment. (B) Binding of pseudovirions harboring the HCV GP proteins to lectin expressing T-REX cells. The binding assay was carried out as described in the legend of panel A, except that HIV-1 pseudovirions harboring HCV E1 and E2 proteins in their envelope were used as input. The amount of bound virus is shown relative to the amount of input virus. A representative experiment is shown, the results were confirmed in an independent experiment. Error bars indicate SD.

carbohydrate specificities. Target cells were incubated with the indicated carbohydrates at a final concentration of 100 μ g/ml and infected with ZEBOV-GP bearing pseudotypes. Preincubation with D(+)-glucose, L(-)-fucose, *N*-acetylglucosamine (Glc-NAc), *N*-acetylgalactosamine (Gal-Nac), and methyl α -D-galacto-pyranoside (MetGal) had no appreciable effect on DC-SIGN/R- and LSECtin-mediated enhancement of infection (Fig. 4A). In agreement with published data (Geijtenbeek et al., 2000), preincubation of target cells with mannan or mannose strongly reduced DC-SIGN/R-mediated enhancement of ZEBOV-GP driven infection (Fig. 4A and data not shown). In contrast, these carbohydrates had no appreciable effect on LSECtin-dependent augmentation of infectious cellular entry, indicating that LSECtin interactions with pathogens might not be mediated through recognition of high-mannose glycans. Complexation of calcium by the DC-SIGN/R and LSECtin lectin domains has been shown to be important for carbohydrate binding (Geijtenbeek et al., 2000; Liu et al., 2004). Indeed, preincubation of LSECtin and DC-SIGN/R expressing cells with 5 mM EGTA strongly diminished ZEBOV-GP driven infection (Fig. 4B). In contrast, infectious entry driven by VSV-G, which is not augmented by the lectins tested (Fig. 2A), was only moderately reduced, probably because of toxic effects of EGTA (Fig. 4B). Similarly, the G_1 subunit of ZEBOV-GP fused to human immunoglobulin (Ig) bound efficiently to LSECtin expressing cells but not to control cells and binding could be abrogated by preincubation with EGTA (Fig. 4C). Thus, LSECtin requires calcium ions for function as an attachment factor.

N-linked glycosylation is required for surface expression of LSECtin on transfected cells

LSECtin contains two consensus signals for *N*-linked glycosylation, both located in the neck domain of the protein. We assessed if these signals are utilized and if *N*-linked glycosylation is required for expression of LSECtin at the cell surface. DC-SIGN, LSECtin, and LSECtin variants with mutated glycosylation signals were transiently expressed in the presence or absence of tunicamycin, an inhibitor of *N*-linked glycosylation, and expression was analyzed by Western blot. Tunicamycin treatment augmented the gel mobility of DC-SIGN, which is in agreement with published results (Pöhlmann et al., 2001a), and of LSECtin, indicating that these proteins are modified by *N*-linked glycosylation (Fig. 5A). Inactivation of the glycosylation signal at amino acids 73–75 resulted in faster gel migration compared to wt LSECtin, and gel mobility was further increased upon addition of tunicamycin to the culture medium, suggesting that both glycosylation signals are utilized (Fig. 5A). In fact, inactivation of the second glycosylation signal located at amino acids 159–161 also augmented gel mobility, albeit a band with the size of wt LSECtin was still detected. Upon mutation of both glycosylation sites, a prominent band of the size of tunicamycin-treated LSECtin was observed, further substantiating that both asparagine 73 and 159 are modified by glycans. Analysis of surface expression and attachment factor function of the LSECtin variants with altered glycosylation signals revealed that mutation of asparagine 73 alone strongly reduced surface expression and consequently

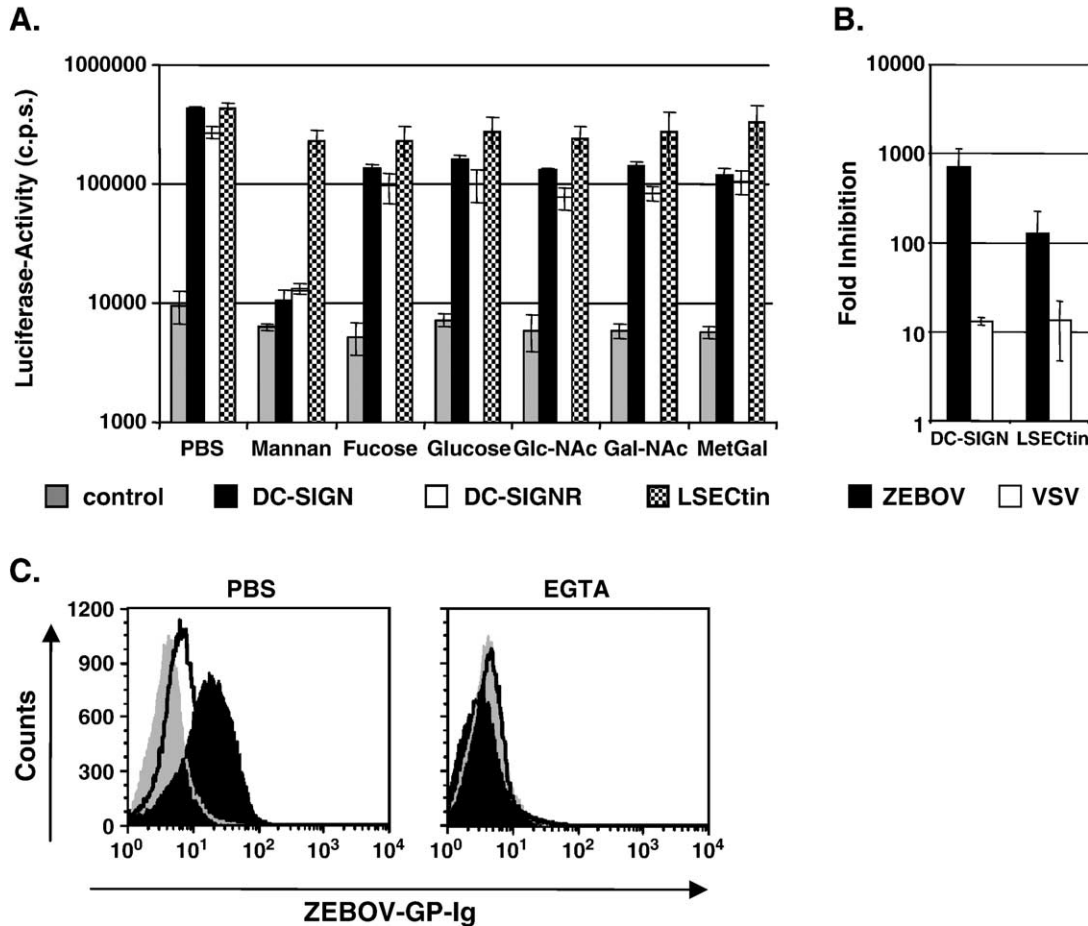


Fig. 4. Inhibition of lectin-mediated enhancement of ZEBOV-GP driven infection by carbohydrates and EGTA. (A) Carbohydrate inhibition of ZEBOV-GP mediated infection of lectin expressing cells. Lectin expression was induced on T-REX cells; the cells were incubated with the indicated carbohydrates (100 $\mu\text{g/ml}$ final concentration) and infected with ZEBOV-GP harboring pseudotypes. A representative experiment performed in quadruplicates is shown, error bars indicate SD. The results were confirmed in two independent experiments. (B) EGTA inhibition of ZEBOV-GP mediated infection of lectin expressing cells. DC-SIGN and LSEctin expression was induced on T-REX cells, the cells treated with EGTA (5 mM final concentration) and infected with pseudovirions harboring either ZEBOV-GP or VSV-G. Input viruses were normalized for comparable luciferase production upon infection of control cells. The results are presented as fold inhibition compared to infection of target cells treated with PBS. A representative experiment is shown, error bars indicate SD. Comparable results were obtained in an independent experiment. (C) EGTA inhibition of ZEBOV-GP binding to LSEctin. Induced control cells (black line) or induced LSEctin T-REX cells (black area) were preincubated with PBS or EGTA, incubated with ZEBOV-GP-Ig fusion protein concentrated from cellular supernatants, and ZEBOV-GP-Ig binding was detected with an antibody specific for the Fc portion of the fusion protein. Reactivity is shown relative to untreated control cells incubated with secondary antibody (gray area). A representative experiment is shown, similar results were obtained in an independent experiment.

augmentation of ZEBOV-GP driven infection (Fig. 5B). These results suggest that glycosylation of LSEctin, at least in the context of 293T cells, is essential for efficient expression on the cell surface.

The GPs of all EBOV subspecies functionally interact with LSEctin, and LSEctin augments infection with replication-competent ZEBOV

Engagement of cellular attachment factors might profoundly impact EBOV tropism, especially at the early stages of infection (Baribaud et al., 2002). However, the GPs of the four EBOV subspecies bind to attachment factors with differential efficiencies (Lin et al., 2003; Simmons et al., 2003; Takada et al., 2004). Therefore, we asked if LSEctin

augments infectious entry driven by the GPs of all EBOV subspecies. T-REX cells expressing DC-SIGN or LSEctin or T-REX control cells were infected with infectivity normalized pseudotypes bearing the GPs of the EBOV subspecies Zaire, Sudan (SEBOV), Ivory Coast (ICEBOV), and Reston (REBOV) (Fig. 6A). As observed previously (Alvarez et al., 2002; Lin et al., 2003; Simmons et al., 2003), DC-SIGN expression particularly enhanced infection driven by ZEBOV- and REBOV-GP, whereas augmentation of entry mediated by SEBOV- and ICEBOV-GP was somewhat less pronounced. Similar results were obtained for LSEctin-mediated enhancement of infection; however, LSEctin was repeatedly more adept in enhancing ICEBOV-GP-dependent infection when compared to DC-SIGN. Thus, LSEctin can interact with the GPs of all EBOV subspecies with

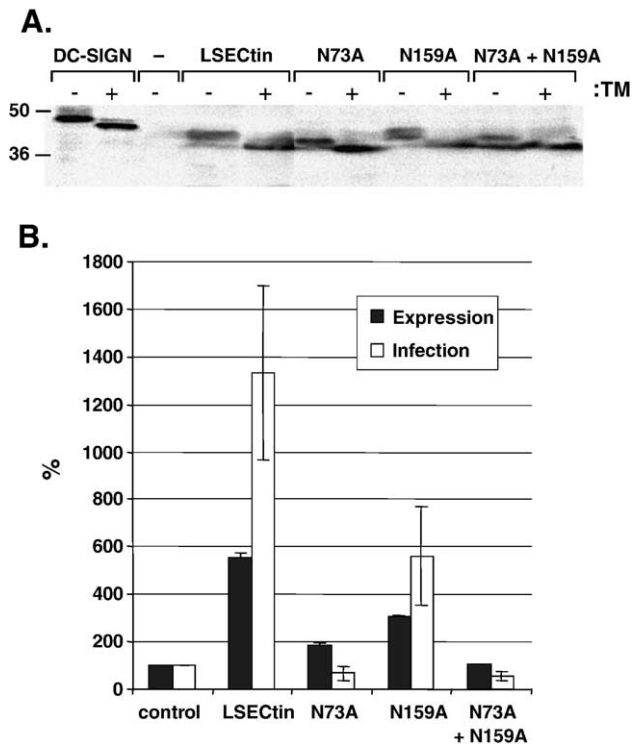


Fig. 5. Glycosylation of LSEctin in transfected cells. (A) Inhibition of N-linked glycosylation by tunicamycin. DC-SIGN, LSEctin, and LSEctin variants with alterations in consensus signals for N-linked glycosylation were transiently transfected into 293T cells, the cells cultivated in the presence or absence of tunicamycin (1 μ g/ml) and lectin expression was analyzed by Western blot 48 h after transfection. The results were confirmed in three independent experiments. TM, Tunicamycin. (B) Enhancement of ZEBOV-GP driven infection by LSEctin variants with defective N-linked glycosylation signals. LSEctin and the indicated LSEctin variants with defects in glycosylation signals were transiently expressed in 293T cells and lectin expression and enhancement of ZEBOV-GP driven infection analyzed in parallel. Expression was determined by staining with a LSEctin-specific antiserum and FACS analysis, while augmentation of ZEBOV-GP driven infection was assessed as described in the legend of Fig. 2A. Surface expression and augmentation of infection are shown relative to pcDNA3 transfected control cells, which was set as 100%. The average of three independent experiments performed in duplicates (surface expression) or quadruplicates (infection) is shown, error bars indicate standard error of the mean.

appreciable efficiency and might augment infection by these viruses. Since lentiviral and filoviral particles differ in size and shape and GP interactions with attachment factors might depend on the nature of the viral particle on which GPs are presented, we finally assessed if LSEctin expression also enhances infection by replication-competent ZEBOV. T-Rex cell lines were induced to express DC-SIGN, LSEctin, or empty vector, inoculated with ZEBOV and infected cells were detected by immunostaining (Fig. 6B). DC-SIGN and LSEctin expression enhanced ZEBOV infection compared to control cells, indicating that both lectins functionally interact with replication-competent filovirus particles.

Discussion

We demonstrated that LSEctin augments infectious entry driven by filovirus GPs and SARS-CoV-S protein and enhances infection by replication-competent ZEBOV. In contrast to the attachment factors DC-SIGN/R, LSEctin does not interact with HIV-1 and HCV GPs and GP binding to LSEctin was not blocked by mannan, suggesting that LSEctin has a different carbohydrate and ligand specificity than DC-SIGN/R. Thus, LSEctin is a potent attachment factor, which in concert with DC-SIGN/R might concentrate certain viral pathogens in liver and lymph nodes.

Engagement of LSEctin and related attachment factors might be of particular relevance for filovirus infection. Filoviruses exhibit an extremely broad cell tropism *in vitro* (Wool-Lewis and Bates, 1998), and viral proteins can be detected in many cell types and organs during the late stage of infection (Geisbert et al., 2003a). In sharp contrast, the viral tropism during the early stages of infection is quite narrow. Thus, macrophages and DCs are early and sustained target cells, and spleen and liver are among the first organs infected (Geisbert et al., 2003a). The discrepancy between the broad tropism late in infection and the limited range of targets early in infection is unlikely to be due to differential receptor expression, but might be explained by EBOV engagement of attachment factors expressed on specific target cells (Simmons et al., 2003). Thus, LSEctin and DC-SIGN/R on LSECs might capture and transmit virus to adjacent hepatocytes and Kupffer cells, the latter known to be early targets of filovirus infection (Geisbert et al., 2003a). Capture of DHBV particles and transmission to adjacent hepatocytes has been observed in infected animals (Breiner et al., 2001), providing evidence that LSECs are capable of promoting viral infection *in trans*. Endothelial cells are targeted by ZEBOV, and in the context of lymph node and liver sinusoids, LSEctin and DC-SIGN/R might augment direct infectious entry into this cell type (“infection *in cis*”). In fact, endothelial cells of hepatic cortical sinusoids are the first type of endothelial cells found to be infected in cynomolgus monkeys experimentally inoculated with ZEBOV (Geisbert et al., 2003b). Of note, the lectins DC-SIGN, human macrophage C-type lectin specific for galactose and Glc-Nac (hMGL), and asialoglycoprotein receptor (ASGP-R) also augment filovirus infectious entry *in vitro* (Alvarez et al., 2002; Becker et al., 1995; Lin et al., 2003; Simmons et al., 2003; Takada et al., 2004) and might promote infection of macrophages, DCs (DC-SIGN, hMGL), and hepatocytes (ASGP-R) *in vivo*, providing further evidence for a potential role of lectins in filovirus tropism. Development of specific inhibitors and the analysis of their impact on filovirus infection of primary cells are required to assess the contribution of each lectin to viral spread.

SARS is a respiratory disease (Peiris et al., 2003a), and a role for LSEctin in SARS-CoV spread in patients is less obvious. However, increasing evidence suggests that SARS-

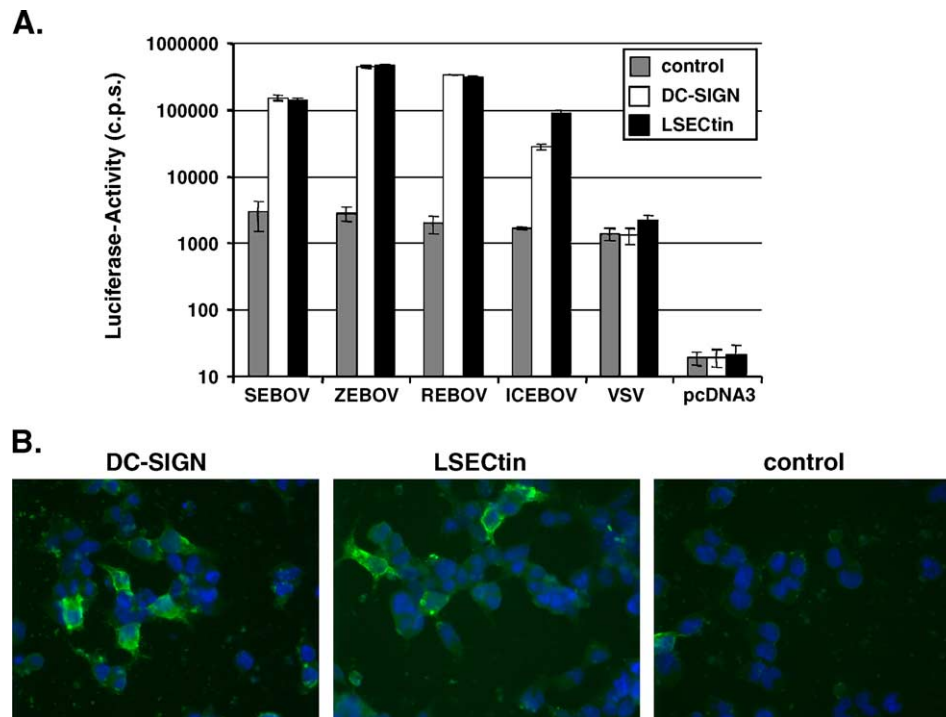


Fig. 6. Interaction of LSECTin with the GPs of the EBOV subspecies and with replication-competent ZEBOV. (A) LSECTin enhances infection driven by the GPs of the four EBOV subspecies. T-REx cells were induced to express the indicated lectins or control vector, inoculated with infectivity normalized pseudotypes bearing the EBOV-GPs, VSV-G, or no GP (pcDNA3), and luciferase activity in cell lysates was determined as described in the legend to Fig. 2A. (B) LSECTin expression enhances infection by replication-competent ZEBOV. T-REx cell lines were seeded in chamber slides, induced to express the indicated lectins and infected with replication-competent ZEBOV. Cells producing ZEBOV antigen were detected by immunofluorescence at 24 h after infection.

CoV infection is not limited to lung tissue (Hofmann and Pohlmann, 2004), but can be detected in several different tissues and cell types (Ding et al., 2004; To et al., 2004) including hepatocytes (Ding et al., 2004). Some hepatoma cell lines were found to be susceptible to SARS-CoV-S driven infection (Hofmann et al., 2004; Simmons et al., 2004; Yang et al., 2004) and viral replication (Hofmann et al., 2004), further underlining that SARS-CoV might productively infect hepatocytes. In the light of these results, it cannot be excluded that direct infection of liver tissue might contribute to the altered levels of liver-specific enzymes commonly observed in SARS-CoV-infected patients (Ding et al., 2003; Lang et al., 2003; Peiris et al., 2003b). Thus, similar as for filoviruses, it can be speculated that LSECTin in conjunction with DC-SIGNR might promote infection of LSECs and/or might target SARS-CoV to adjacent hepatocytes, while SARS-CoV engagement of DC-SIGN on DCs might facilitate viral dissemination (Marzi et al., 2004; Yang et al., 2004). In contrast to the efficient interaction of LSECTin with SARS-CoV and filovirus GPs, no binding of HIV-1 and HCV GPs to LSECTin expressed on 293T cells was detected. Thus, LSECTin might not be involved in the interactions of lymph node and liver sinusoidal endothelial cells with these pathogens.

One possibility to evaluate the relative contributions of LSECTin and DC-SIGNR to, e.g., LSEC interactions with

ZEBOV and SARS-CoV is to exploit differences in the carbohydrate specificities of these lectins. Thus, DC-SIGNR binds exclusively to high-mannose carbohydrates (Feinberg et al., 2001; Guo et al., 2004), and lectin engagement is blocked by preincubation with mannan (Bashirova et al., 2001; Pöhlmann et al., 2001c), a mannose polymer obtained from the cell wall of yeast. In contrast, mannan did not block ligand binding to LSECTin, indicating that LSECTin might not recognize mannose residues on the GPs tested. Therefore, mannan and related sugars are suitable to inhibit DC-SIGNR but not LSECTin. Notably, apart from mannan, fucose and Glc-Nac also failed to prevent binding of ZEBOV-GP to LSECTin, albeit these carbohydrates have previously been shown to block binding of a mannosylated ligand to a soluble LSECTin-Fc fusion protein (Liu et al., 2004). This discrepancy might be due to differences in the experimental systems used. Thus, carbohydrate inhibition of binding of a mannosylated ligand to a soluble LSECTin-Fc fusion protein was assessed in the previous study (Liu et al., 2004), while we investigated the interaction of cellular LSECTin, which was reported to form tetramers (Liu et al., 2004), with multimeric GPs presented on the surface of virions. Nevertheless, LSECTin interactions with ligand were dependent on the presence of calcium, confirming the previously reported classification of LSECTin as a C-type lectin (Liu et al., 2004). Further analysis will be

required to define which carbohydrates on viral GPs are bound by LSECTin and to investigate whether this lectin recognizes other pathogens, which display the appropriate glycosylation pattern.

LSECTin contains two consensus sites for N-linked glycosylation located in the neck domain. Analysis of LSECTin variants with altered glycosylation signals and treatment of lectin expressing cells with tunicamycin, an inhibitor of N-linked glycosylation, provided evidence that both glycosylation sites are utilized and that glycosylation is required for efficient cell surface expression and thus for enhancement of ZEBOV-GP driven infection. In contrast, tunicamycin treatment had relatively little effect on DC-SIGN expression and attachment factor function (data not shown), confirming a previous study, which demonstrated that mutation of a unique, utilized N-linked glycosylation signal in DC-SIGN does not affect interactions with HIV (Pöhlmann et al., 2001a). LSECTin and DC-SIGN/R might also differ in regards to internalization and intracellular trafficking upon ligand binding, a function known to be important for DC-SIGN-mediated HIV transmission by DCs (McDonald et al., 2003). Thus, the DC-SIGN/R cytoplasmic tails harbor LL motifs which, at least in case of DC-SIGN, might be required for efficient receptor internalization and HIV transmission (Kwon et al., 2002). No such motif is present in the cytoplasmic tail of LSECTin; however, sequence analysis identified a YSKW sequence which resembles a YSEI motif in the CD23 cytoplasmic tail known to be involved in internalization of this lectin (Liu et al., 2004). The role of internalization in DC-SIGN- and LSECTin-mediated augmentation of viral infection in cis remains to be determined.

In summary, LSECTin joins DC-SIGN/R, ASGP-R, and hMGL as an attachment factor for ZEBOV, which might modulate viral tropism in infected individuals. Ultimately, the relevance of these factors for viral spread in vivo can only be determined in animal models. A mouse model for ZEBOV replication and pathogenesis has been established (Bray et al., 1998). The characterization of mouse homologues of LSECTin and DC-SIGN/R in vitro and the analysis of their impact on viral tropism in infected animals are thus of particular interest.

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. 293 T-REx cells expressing DC-SIGN/R were described previously (Pöhlmann et al., 2001a, 2001b) and maintained in DMEM containing, 10% FBS, 50 µg/ml zeocin (Invitrogen, CA, USA), 2.5 µg/ml blasticidin (Invitrogen, CA, USA), penicillin, and streptomycin. A T-REx cell line expressing

LSECTin was generated as described for DC-SIGN/R T-REx cells (Pöhlmann et al., 2001a). DC-SIGN/R and LSECTin expression was induced by culturing the cells in medium containing 0.1 µg/ml doxycycline (SIGMA-Aldrich, Germany). 293 T-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) cells were maintained in RPMI 1640 containing 10% FBS, penicillin, and streptomycin. All cells were grown at 37 °C and 5% CO₂.

Plasmid construction and in vitro mutagenesis

For construction of LSECTin expression vectors, a fragment encoding LSECTin was amplified from reverse-transcribed liver RNA (Clontech) using the Superscript One Step RT-PCR kit (Invitrogen, CA, USA) and cloned in the pcDNA3.1Zeo vector (Invitrogen, California, USA) via *Hind*III and *Xba*I. Expression plasmids encoding DC-SIGN and DC-SIGNR were described previously (Pöhlmann et al., 2001a, 2001c). For regulated LSECTin expression, the open reading frame was inserted in pcDNA4TO (Invitrogen, CA, USA) using the restriction enzymes *Hind*III and *Xba*I. For prokaryotic expression of LSECTin, a PCR-fragment spanning the entire LSECTin open reading frame was inserted into pGEX6P1 via *Bam*HI and *Xho*I (Amersham Biosciences, UK) to yield the plasmid pGEX6P1-LSEC. LSECTin variants with defects in two consensus signals for N-linked glycosylation were generated by PCR mutagenesis employing overlapping oligonucleotides. Oligonucleotides 5' Lsec-mut 73AAS75 (GGCCACGACCTGCTGAGGACAGCCGCTCGAAGCAGACGGCGGCG) and 3' Lsec-mut 73AAS75 (CGCCGCCGTCTGCTTCGAGGCGGC-TGTCCTCAGCAGGTCGTGGCC) were employed to alter the glycosylation signal NAS (amino acids 73 to 75), while oligonucleotides 5' Lsec-mut 159ANS161 (GGAGGCCGTGAGGCTCCAGGCCAACTCCTGCGAGCCGTGCC) and 3' Lsec-mut 159ANS161 (GGGGCACGGCTCGCAGGAGTTGGCCTGGAGCCTCACGGCCTCC) were used to change the glycosylation signal 159NNS161 to 159ANS161. PCR-fragments encoding LSECTin variants with altered glycosylation signals were cloned via *Hind*III and *Xba*I into pcDNA3.1Zeo. An expression vector for ZEBOV-GP protein fused to the Fc portion of human immunoglobulin was obtained by inserting the sequence of ZEBOV GP₁ in the construct pAB61 (Birkmann et al., 2001). All PCR-amplified sequences were confirmed by automated sequence analysis. The GP expression plasmids employed for the generation of pseudotyped viruses have been described previously (Hofmann et al., 2004; Kolesnikova et al., 2004; Simmons et al., 2003; Volchkov et al., 1998).

Antigens and antisera

A mouse monoclonal antibody (MAb) directed against the AU1 antigenic tag was purchased from Covance

Research Products, CA, USA. FITC-conjugated anti-mouse IgG-Fc γ and Cy5-conjugated anti-human secondary antibodies were purchased from Jackson ImmunoResearch, PA, USA and Dianova, Germany, respectively. Expression of a GST-LSEctin fusion protein was induced in the *E. coli* strain DH10B carrying the plasmid pGEX6P1-LSEC by adding IPTG (1 mM) followed by purification of the fusion protein employing Glutathione Sepharose 4B Beads (Amersham Biosciences, UK). Anti LSEctin sera were obtained by immunizing NMRI mice with the purified GST-LSEctin fusion protein.

Expression analysis by flow cytometry

To assess cell surface expression of lectins, fluorescence-activated cell sorting analysis (FACS) was performed. Transiently transfected 293T cells or doxycycline-induced T-REx cell lines were harvested, washed, and resuspended in ice-cold FACS buffer (PBS with 3% FBS; 0.01% NaN₃). Approximately 2×10^5 cells were incubated with MAb AU1 at 10 μ g/ml or sera from LSEctin-immunized mice were diluted 1:100 in a total volume of 100 μ l FACS buffer for 45 min on ice. Cells were washed and incubated with phycoerythrin-conjugated anti-mouse IgG at a final concentration of 5 μ g/ml for 45 min on ice. Thereafter, the cells were washed, reconstituted in FACS buffer, and analyzed by FACS employing a FACSCalibur flow cytometer (Becton Dickinson). Analysis of LSEctin expression on LSEC was carried out in an analogous fashion; however, cells were detached from culture plates using non-enzymatic cell dissociation solution (Sigma, UK).

Production of reporter viruses and infection assays

Simian immunodeficiency virus (SIV) pseudotypes were generated by cotransfection of 293T cells with the indicated GP expression plasmids in combination with the SIV_{mac} 239 Δ env Δ nef Luc expression plasmid as described previously (Kirchhoff et al., 1997). HIV-derived pseudotypes were generated by cotransfection of 293T cells with GP expression plasmids and pNL4-3 E⁻R⁻Luc plasmid as described previously (Connor et al., 1995). 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- μ m-pore-size filters, aliquotted, and stored at -80 °C. To assess lectin-mediated enhancement of infection, 293T cells or T-REx cells induced to express the indicated lectins were seeded onto 96-well plates at a density of 1.0×10^4 and incubated with viral supernatants normalized for comparable luciferase activity upon infection of 293T cells. For neutralization assays, cells were preincubated with serial dilutions of immune sera 3 h before infection. For carbohydrate or EGTA inhibition assays, cells were preincubated with 100 μ g/ml of the

indicated carbohydrate (SIGMA-Aldrich, Germany) or 5 mM EGTA (SIGMA-Aldrich, Germany) 30 min before infection. Generally, the medium was replaced 12 h after infection and luciferase activity was determined 72 h after infection with a commercially available kit as recommended by the manufacturer (Promega, WI, USA).

Analysis of lectin-mediated binding and transmission of reporter viruses

T-REx cells were seeded onto 96-well plates, induced to express the indicated lectins and incubated with HIV reporter viruses normalized for capsid protein (p24) content by enzyme-linked immunosorbent assay (ELISA) (Murex; Abbott Diagnostics, IL, USA). After a 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 (Hsu et al., 2003), and luciferase activity in cell lysates was determined 72 h after cocultivation.

ZEBOV infection and immunofluorescence analysis

ZEBOV was grown and passaged under biosafety level 4 conditions as described elsewhere (Modrof et al., 2002). Infection of doxycycline-induced T-REx cell lines (6×10^4 per well) with ZEBOV was performed in chamber slides (Nunc, Germany). Cells were inoculated with viral supernatant at a multiplicity of infection (MOI) of 1. After 1 h, viral supernatant was removed and cells were maintained in DMEM, 2% FBS, and antibiotics for 24 h. For immunofluorescence analysis, cells were fixed for 24 h in 4% paraformaldehyde at 4 °C and permeabilized with an ice-cold acetone–methanol mix. Cells were incubated with an anti-ZEBOV goat immune serum diluted 1:100, washed and incubated with FITC-conjugated F(ab')₂Fragment anti-goat IgG (Dianova, Germany) diluted 1:200 and DAPI (SIGMA-Adlrich, Germany) 1:10,000. Images were recorded using a fluorescence microscope (Axiophot, Zeiss) and processed using Spot software (Diagnostic Instruments, MI, USA).

Isolation and culture of intrahepatic endothelial cells

Primary intrahepatic endothelial cultures were isolated from human liver tissue surplus to surgical requirements as described previously (Lalor et al., 2002). Approximately 30 g of tissue was enzymatically digested using 2 mg/ml collagenase Type 1A (Sigma,UK), filtered and further purified by density gradient centrifugation using 25% (w/v) metrizamide (Nycomed, Norway). Human sinusoidal endothelial cells were extracted using negative magnetic selection with antibody HEA125 (Progen Biotechnik, Germany) to deplete biliary epithelial cells followed by positive selection of endothelial cells with CD31 antibody

(Dako, UK) and Dynabeads conjugated with sheep anti-mouse immunoglobulin (Dyna, UK). Endothelial cultures (greater than 95% purity) were maintained in collagen-coated flasks in complete medium (10% [v/v] human AB serum; HD Supplies, UK, 10 ng/ml vascular endothelial growth factor and 10 ng/ml hepatocyte growth factor [R&D Systems, UK] both in human endothelial basal medium supplemented with penicillin and streptomycin) at 37 °C.

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