Langerhans cells and dendritic cells in innate defense against pathogens

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Chapter 9

Branched oligosaccharide structures on HBV prevent interaction with both DC-SIGN and L-SIGN

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Abstract

Hepatitis B virus (HBV) is a DNA virus that infects the liver as primary target. Currently, a high affinity receptor for HBV is still unknown. The dendritic cell specific C-type lectin DC-SIGN is involved in pathogen recognition thrsough mannose- and fucose containing carbohydrates leading to the induction of an anti-viral immune response. Many glycosylated viruses subvert this immune surveillance function and exploit DC-SIGN as a port of entry and for *trans*-infection of target cells. The glycosylation pattern on HBV surface antigens (HBsAg) together with the tissue distribution of HBV would allow interaction between HBV and DC-SIGN and its liver-expressed homologue L-SIGN. Therefore, a detailed study to investigate the binding of HBV to DC-SIGN and L-SIGN was performed. For HCV, both DC-SIGN and L-SIGN are known to bind envelope glycoproteins E1 and E2. Soluble DC-SIGN and L-SIGN specifically bound HCV virus like particles, but no interaction with either HBsAg or HepG2.2.15 derived HBV was detected. Also, neither DC-SIGN nor L-SIGN transfected Raji cells bound HBsAg. In contrast, highly mannosylated HBV, obtained by treating HBV producing HepG2.2.15 cells with the α-mannosidase I inhibitor kifunensine, is recognized by DC-SIGN. The α-mannosidase I trimming of N-linked oligosaccharide structures thus prevents recognition by DC-SIGN. On the basis of these findings, it is tempting to speculate that HBV exploits mannose trimming as a way to escape recognition by DC-SIGN and thereby subvert a possible immune activation response.

Introduction

Hepatitis B virus (HBV) infects the liver as primary target, resulting in the majority of cases in self-limiting acute hepatitis. Nevertheless, more than 350 million people are chrsonically infected with HBV worldwide (1). Despite the high incidence of infection, a cellular receptor for HBV entry is still unknown. Several putative binding factors have been described for the HBV surface antigens, such as human serum albumin (2), asialoglycoprotein receptor (3), heparin (4) and mannose binding lectin (5), but their exact role in HBV attachment and uptake remains unclear (6).

HBV is a DNA virus, consisting of a core particle enveloped by small (S), middle (M) and large (L) surface antigens, generally referred to as HBsAg. All thrsee surface antigens contain a common S domain, both M and L proteins contain a preS2 domain and L exclusively contains a preS1 domain (7). The liver and peripheral blood of HBV infected individuals can reach levels of 10^9-10^{10} infectious particles per ml. In addition, HBsAg is secreted from infected hepatocytes as spherical subviral particles and filaments, which can accumulate up to 100 µg/ml in peripheral blood (7;8). S is the main component of both HBV virions and HBsAg subviral particles, while M and L are highly enriched on HBV virions. Post-translational modifications of the surface antigens are crucial for HBV life cycle; myristoylation of the preS1 domain is essential for infectivity (6;9), while inhibition of N-glycosylation of the preS2 domain prevents secretion of viral particles (10;11). Recently, post-translational N-glycosylation of the preS1 domain has been reported as well and although it seems dispensable for HBV morphogenesis it might be involved in viral attachment (12).

The first step in clearance of a viral infection involves recognition of the virus by the innate immune system, mediated thrsough host pattern recognition receptors (PRR) such as Toll-like receptors and C-type lectins (13;14). C-type lectins recognize highly conserved pathogen-derived carbohydrate structures, leading to internalization, antigen processing and presentation to T cells (14). A prototypical member of the C-type lectin family is the dendritic cell-specific lectin DC-SIGN (15), which recognizes a broad range of glycosylated pathogens thrsough mannose- or fucose-containing carbohydrates, including HIV-1, hepatitis C virus, Ebola virus, Dengue virus, measles virus, human herpesvirus 8, SARS coronavirus, cytomegalovirus, *Mycobacterium tubercolosis, Helicobacter pylori, Streptococcus pneumonia* and *Neisseria meningitidis* (16-18). The outcome of this interaction is pathogen-dependent. Many of these DC-SIGN-binding pathogens have evolved to subvert the immune surveillance function of DC-SIGN for their own benefit and re-direct the internalization route to non-lysosomal compartments for protection and transmission to target cells (16). Some pathogens can also modulate adaptive immune responses thrsough interaction with DC-SIGN; e.g. *Mycobacterium tuberculosis* exploits DC-SIGN to escape immune surveillance by inhibition of the immunostimulatory function of dendritic cells (16;19).

It is well established that envelope glycoproteins E1 and E2 of Hepatitis C virus (HCV) interact with both DC-SIGN (15) and its homologue L-SIGN (20) expressed on sinusoidal endothelial cells in liver and lymph nodes. For HCV virus-like particles, DC-SIGN interaction leads to efficient capture, internalization and transport to non-lysosomal compartments within immature dendritic cells, thereby protecting the virus from degradation (21;22). Similarly, HCV virus-like particles interact with L-SIGN expressed on liver sinusoidal endothelial cells (LSEC) *in situ*, and are targeted to non-lysosomal early endosomes in L-SIGN transfected Raji cells (22). DC-SIGN and L-SIGN are also thought to play an important role in HCV viral dissemination by transferring HCV from the circulation to hepatocytes, the main HCV target cells (23).

Several reports have shown the presence of HBV on or within dendritic cells (24-27), suggesting the involvement of a dendritic cell-specific receptor such as DC-SIGN. Patient-derived HBsAg is preferentially internalised by human LSEC in a mixed culture with human hepatocytes (K. Esser and U. Protzer, unpublished data) - as it has also been reported for duck HBV (27) - implying a potential interaction between HBV and L-SIGN. The HBV glycosylation pattern together with the cellular localization prompted us to investigate the possible role of both DC-SIGN and L-SIGN in binding of HBV.

Results

Characterisation of purified HBV particles

Secreted HBsAg subviral particles outnumber the HBV virions at least 100-fold in both patient serum and culture supernatant of HepG2.2.15 cells (7). To enrich for HBV virions, culture supernantant of HepG2.2.15 cells was fractionated over a heparin column and different elution fractions were assessed by HBV L, M and S-specific ELISA. The input fraction contained mainly the S protein, representing the

relative high level of secreted spherical subviral particles (Figure1A). Elution fraction 1 and 2 however, were highly enriched for both M and L proteins confirming the increased level of HBV virions in these fractions. Quantification of HBV-DNA of the input fraction and elution fraction 1-3 confirmed an almost 50-fold enrichment of HBV virions in fraction 1 (0,026 to 1,27x10^9 HBV particles per ml, Figure 1B).

HBsAg and HBV do not interact with recombinant DC-SIGN-Fc

The interaction between DC-SIGN and recombinant HBsAg or HepG2.2.15-derived HBV was determined in a binding ELISA where coated HBsAg, HBV, HCV VLP or HepG2 medium controls were incubated with soluble recombinant DC-SIGN-Fc. The detected signal of the HepG2 medium control was never above background. Strikingly, DC-SIGN did not interact with HBsAg nor with whole virus particles, whereas DC-SIGN did interact with HCV virus like particles, consisting of purified yeast recombinant E1 and E2 HCV envelope proteins (Figure 2A,(22)). Specificity of binding was determined by blocking with either mannan, a yeast cell derived polycarbohydrate that competitively binds the carbohydrate binding site of mannose-specific lectins, or the calcium chelator EGTA, and both agents indeed reduced DC-SIGN binding to HCV to background level. Coating controls demonstrated that approximately equal amounts of HBsAg, HBV and HCV VLP were coated.

Based on their oligosaccharide specificity, several plant lectins were used in a glycan analysis of HBsAg and heparin purified HBV (Figure 2B). Strong binding to Con A, GNA, WGA, PNA, RCAII and LTA demonstrated the presence of high and/or complex mannose, N-acetylglucosamine, galactose and fucose containing carbohydrate structures on both HBsAg and HBV. For DC-SIGN-glycoprotein interaction the high mannose and fucose containing oligosaccharide structures are most important (28). Of note, carbohydrate structures on both HCV VLP and mannan mainly consist of mannose, as indicated by the exclusive binding to Con A and GNA.

HBsAg and HBV do not interact with soluble DC-SIGN from cell-lysates

To exclude the possibility that the lack of interaction between HBV and recombinant DC-SIGN was because of improper multimerization of DC-SIGN-Fc, binding of HBsAg and HBV to native DC-SIGN was studied with DC-SIGN derived from transfected Raji cells (29;30). As described before, flow cytometric analysis of Raji transfectants showed high expression levels of DC-SIGN (Figure 3A, (22)). In a bindings ELISA, coated HBsAg or HCV VLP were incubated with lysates of mock or DC-SIGN transfected Raji cells and bound DC-SIGN was detected with an anti-DC-SIGN antibody. DC-SIGN derived from Raji transfectants did not bind HBsAg nor whole HBV particles, whereas HCV VLP showed mannan-sensitive binding to DC-SIGN (Figure 3B). Control incubation with anti-HCV and anti-HBsAg specific antibodies indicated equal amounts were coated. No background binding was observed for either HBsAg or HCV VLP using the lysate of untransfected Raji cells (data not shown).

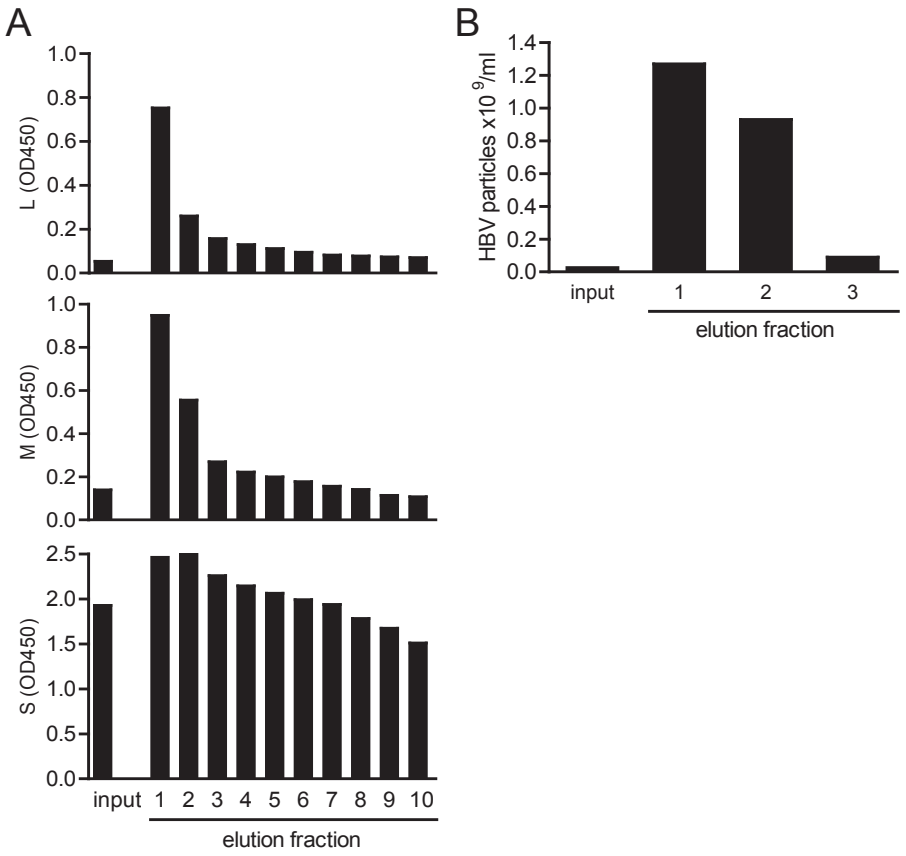

Figure 1. Characterisation of purified HBV particles. (A) HBV particles were purified from HepG2.2.15 culture supernatant by heparin column and both input and different elution fractions were assessed by L, M and S-specific capture ELISA. (B) Quantification of HBV particles in both input and elution fraction 1, 2 and 3 by COBAS® TaqMan HBV Test.

HBsAg does not interact with cellular DC-SIGN

In addition to ELISA, HBsAg interaction with cellular DC-SIGN was also determined. DC-SIGN is highly expressed on moDC (Figure 3A). Therefore, both Raji-DC-SIGN and moDC were incubated with HBsAg and binding was measured by flow cytometry. Neither DC-SIGN-positive Raji cells nor moDC interacted with HBsAg after 2 and 18 hrs incubation (Figure 3C and data not shown). In contrast, both DC-SIGN-transfected Raji cells and moDC efficiently internalised the DC-SIGN ligand dextran-FITC in a mannan-sensitive manner (Figure 3C). Mock transfected Raji cells remained dextran-FITC negative (data not shown). Figure 3D summarizes the results of two independent binding assays.

HBsAg and HBV do not interact with soluble L-SIGN

In a similar manner, the interaction between cellular L-SIGN and both HBsAg and HBV was determined using L-SIGN-transfected Raji cells. Flow cytometric analysis showed L-SIGN expression levels equal to DC-SIGN expression (Figure 4A, (22)). Soluble L-SIGN interacted neither with HBV nor with HBsAg in ELISA, whereas L-SIGN did interact with HCV VLP (Figure 4B). Medium controls were always at background level. Moreover, cellular L-SIGN expressed by Raji transfectants did not bind to HBsAg, while it bound dextran-FITC in a mannan-sensitive manner (Figure 4C,D).

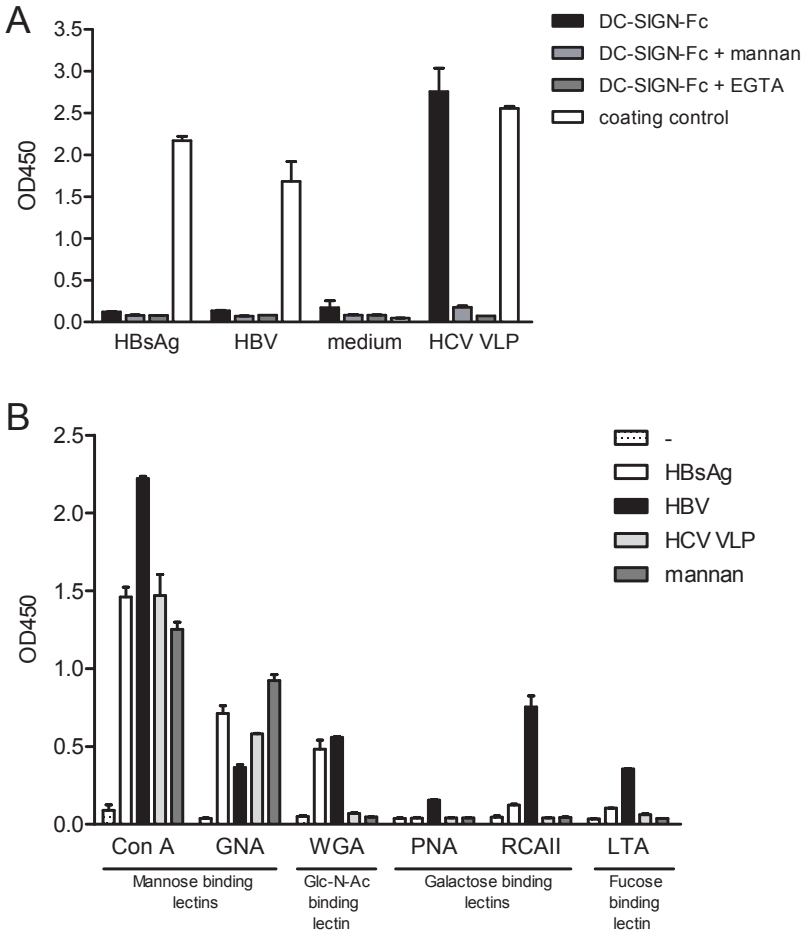

Figure 2. HBV and its surface antigen HBsAg do not interact with recombinant DC-SIGN. (A) DC-SIGN interaction with CHO-derived recombinant HBV surface antigen HBsAg and HepG2.2.15-derived HBV was determined in an Fc-based ELISA, as described in Methods. Supernatant of the non-virus producing cell line HepG2 was used as negative control and HCV VLP were used as a positive control. Specificity of binding was determined in the presence of mannan or the calcium chelator EGTA. To assess the coating efficiency, HBsAg, HBV and HCV VLP were detected with specific antibodies. Data are shown as mean±sd of duplicate measurements; one representative experiment out of four is shown. (B) Glycan analysis of HBsAg and HBV by plant lectin ELISA. For abbreviations and detailed oligosaccharide specificity of the indicated lectins see Methods.

DC-SIGN binds highly mannosylated HBV

Thus, neither HBsAg nor HBV interacts with DC-SIGN or L-SIGN, whereas both C-type lectins interact with HCV and other viruses such as HIV-1. These data suggest that the N-linked glycosylation of HBV is distinct from that of other viruses. To evaluate whether indeed native HBV glycosylation does not meet the requirements for DC-SIGN interaction, glycan modified HBV was generated by treating HBV-producing HepG2.2.15 cells with the α-mannosidase I inhibitor kifunensine. Kifunensine causes the accumulation of $Man_{7\text{-}9}GlcNAc_2$ oligosaccharides on glycoproteins by inhibiting mannose trimming in the endoplasmic reticulum (31). Carbohydrate analysis of glycan modified HBV indeed demonstrated an increased amount of mannose structures compared to native HBV, shown by a 2-fold increase in binding to the mannose-specific plant lectins Con A and GNA while binding to

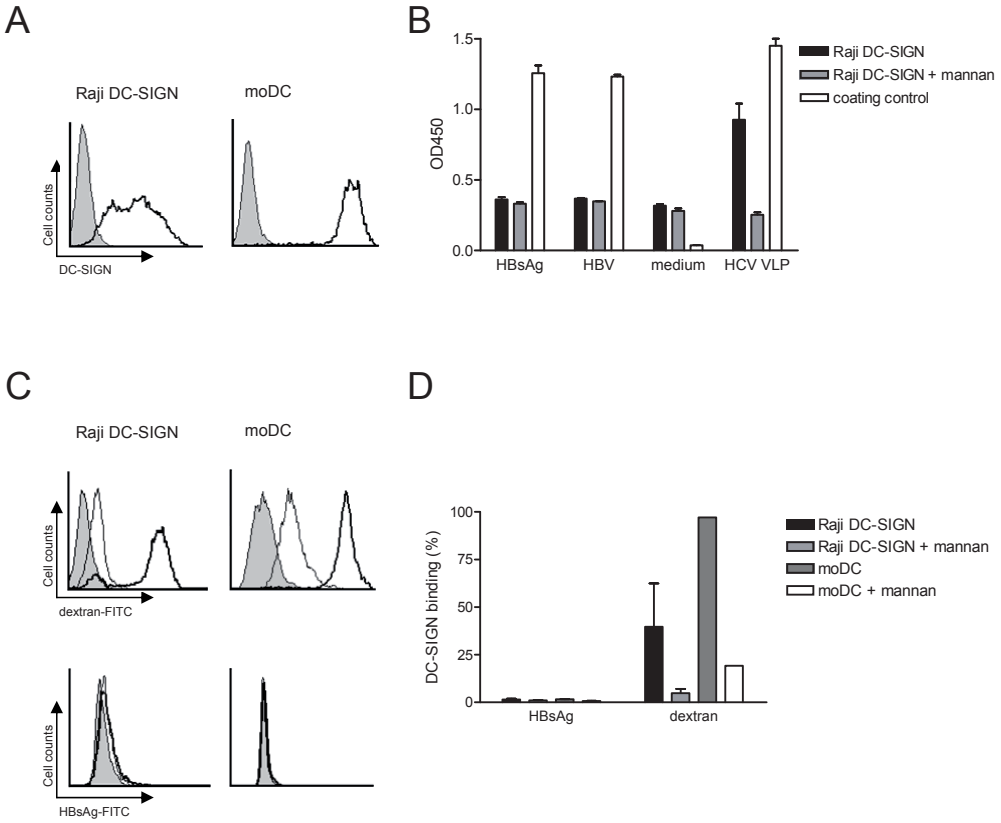

Figure 3. HBsAg and HBV do not interact with cellular DC-SIGN. (A) Expression levels of DC-SIGN on DC-SIGN transfected Raji cells and monocyte-derived dendritic cells (moDC) were determined by flow cytometry with AZN-D2 (dark line). Filled graphs represent isotype controls. (B) HBsAg and HBV interaction with cellular DC-SIGN was determined by the soluble DC-SIGN lysate ELISA, as described in Methods. Interaction with HCV VLP was determined as a positive control. Specificity of binding was determined in the presence of mannan. To assess the coating efficiency, HBsAg, HBV and HCV VLP were detected with specific antibodies. Data are shown as mean±sd of duplicate measurements; one representative experiment out of thrsee is shown. (C, D) Interaction of HBsAg with DC-SIGN transfected Raji cells (n=2) or DC-SIGN expressing moDC (n=1) in culture was determined by flow cytometry, after 2 hrs HBsAg incubation at 37°C. Specificity of binding was determined by 30 min pre-incubation with mannan. Lectin-mediated binding of dextran-FITC to DC-SIGN was determined as a positive control. (C) Histogram plots of dextran-FITC (upper panel) and HBsAg (lower panel) binding to Raji DC-SIGN and moDC. Dotted lines represent background without substrate, bold black lines represent substrate binding at 37°C and grey lines show the specific inhibition with mannan. (D) Summary of DC-SIGN binding experiments, data are shown as mean±sd.

the N-acetylglucosamine-specific lectin WGA was unchanged (Figure 5B). Control incubations with an anti-HBsAg specific antibody showed that similar amounts of native and highly glycosylated HBV were coated (Figure 5A). The interaction between highly mannosylated HBV and DC-SIGN was studied by DC-SIGN-Fc-based ELISA (Figure 5C). Strikingly, HBV binding to DC-SIGN was observed after kifunensine treatment of the virus producing cells, and the binding could be inhibited by both mannan and EGTA. The protein structure of HBV is therefore compatible with DC-SIGN binding, but the native glycosylation, that exclusively determines DC-SIGN interaction, is not. This observation also excludes the possibility that lack of DC-SIGN-HBV interaction is observed due to too low sensitivity of the DC-SIGN assays used in this study. Supernatant of untreated HepG2.2.15 and HepG2 cells showed

no binding to DC-SIGN. Minor background binding was observed with supernatant of kifunensine-treated HepG2 cells, due to the accumulation of large amounts of highly mannosylated proteins in the medium during treatment. These data indicate that mannose trimming of the oligosaccharide structures present on HBV prevents interaction with DC-SIGN.

Discussion

One of the key questions in HBV biology focuses on the attachment and entry mechanisms used by HBV to infect hepatocytes, the main target cells. Several putative binding receptors are proposed, but none of them are confirmed as high affinity receptors for HBV. Related to the issue of HBV entry is the way HBV reaches the hepatocyte: Is there direct contact between HBV in the circulation and hepatocytes, possibly thrsough fenestrations in the LSEC (32)? Or are other cell types involved in HBV dissemination, similar to HIV transport by dendritic cells for trans-infection of T cells (29) and HCV capture by LSEC promoting infection of hepatocytes (33)?

Since HBV surface antigens are glycoproteins, the involvement of a C-type lectin receptor in viral recognition seems plausible. The best-characterised C-type lectins are the dendritic cell-expressed DC-SIGN and its LSEC-expressed homologue L-SIGN, both capable of recognizing a broad range of glycosylated pathogens, including viruses, bacteria and protozoa (16). The fact that HBV has been found attached to both dendritic cells (24-27), and LSEC (34) leads to DC-SIGN and L-SIGN as likely candidate receptors.

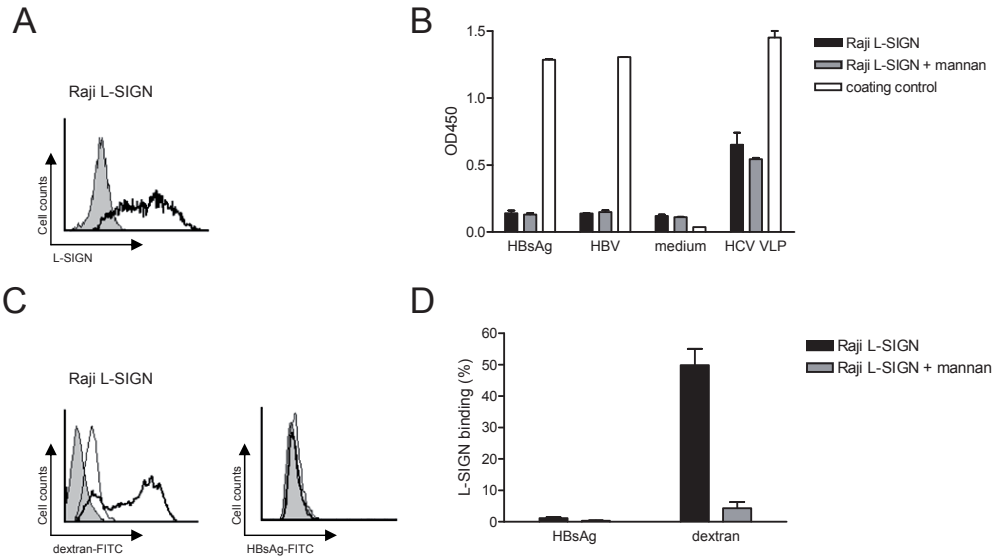

Figure 4. HBsAg and HBV do not interact with cellular L-SIGN. (A) Expression level of L-SIGN on L-SIGN transfected Raji cells was determined by flow cytometry with AZN-D2 (dark line). Filled graph represents isotype control. (B) HBsAg and HBV interaction with cellular L-SIGN was determined by soluble L-SIGN lysate ELISA. Data are shown as mean±sd of duplicate measurements; one representative experiment out of thrsee is shown. (C, D) Interaction of HBsAg with L-SIGN transfected Raji cells in culture was determined by flow cytometry, as described in Figure 3. L-SIGN binding of dextran-FITC was determined as a positive control. (C) Histogram plots of dextran-FITC (upper panel) and HBsAg (lower panel) binding to Raji L-SIGN. Dotted lines represent background without substrate, bold black lines represent substrate binding at 37°C and grey lines show the specific inhibition with mannan. (D) Results of two independent L-SIGN binding experiments, data are shown as mean±sd.

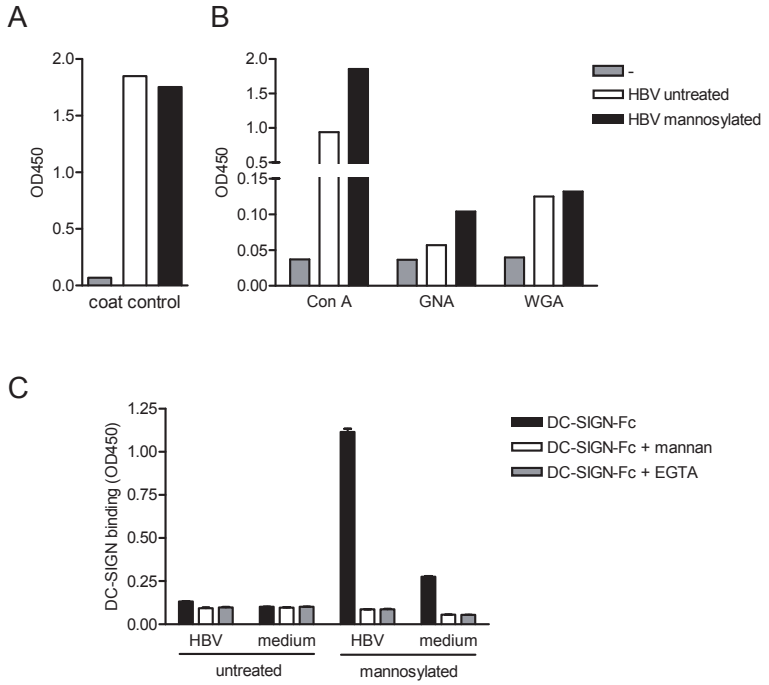

Figure 5. DC-SIGN specifically binds highly mannosylated HBV. Highly mannosylated HBV was generated by treating HBV producing HepG2.2.15 cells with the α-mannosidase I inhibitor kifunensine (20 µg/ml) for 5 days. Untreated HepG2.2.15 cells and kifunensine treated HepG2 cells served as negative controls. (A, B) Glycan analysis of untreated and mannosylated HBV by the mannose-specific lectins Con A and GNA and the N-acetylglucosamine-specific lectin WGA. In the coating control, the captured amount of untreated and mannosylated HBV is detected with an anti-HBS specific antibody. (C) The interaction of highly mannosylated HBV with DC-SIGN was studied by DC-SIGN-Fc ELISA, as described in Figure 2. Specificity of binding was determined in the presence of mannan or EGTA. Supernatant of untransfected HepG2 cells is shown as medium control. Data are shown as mean±sd of duplicate measurements; one representative experiment out of thrsee is shown.

The present study conclusively shows the lack of interaction between DC-SIGN and purified HepG2.2.15-derived HBV with its native glycosylation. Moreover, recombinant HBV surface antigens were recognized by neither DC-SIGN nor L-SIGN. Glycan analysis of both HBV and HBsAg demonstrated that lack of DC-SIGN binding was not due to aberrant glycosylation of virions and glycoproteins used in this study, since the observed glycosylation pattern was consistent with previously reported complex-type carbohydrate structures on HBV (35). Notably, while mannose and N-acetylglucosamine seem to be present in similar amounts on both HBV and HBsAg, glycan structures on virions are enriched for galactose, N-acetylgalactosamine and fucose. This difference in observed glycosylation pattern might be the result of O-glycans present on the preS1 domain (35).

DC-SIGN has been shown to interact with high mannose-containing glycans on various viruses, such as HCV, HIV and Ebola (19). Even though data demonstrate that these viruses can exploit DC-SIGN for their own benefit, the majority of the captured virus is degraded and routed into the antigen presentation pathway (36-38), thereby allowing the induction of an anti-viral immune response. Although HBV glycoproteins are involved in viral recognition, as they are known to bind mannose binding lectin and the asialoglycoprotein receptor, here it is shown that α-mannosidase I trimming of N-linked oligosaccharide structures prevents recognition by DC-SIGN. HBV is

thus recognized by DC-SIGN as soon as one of the enzymes in the formation of complex glycans, α-mannosidase I, is inhibited and highly mannosylated virions are generated. Previous reports have shown the importance of HBV glycosylation for viral secretion (11;39) and indeed we observe a decrease in secretion of highly mannosylated versus native HBV, under similar culture conditions.

We cannot rule out that patient sera contain a subpopulation of these highly glycosylated viremia that would allow interaction with DC-SIGN. Preliminary data on HBV derived from several patient sera with a high viral load did not show binding to DC-SIGN-Fc (data not shown), thereby indicating that DC-SIGN may not be involved in recognition of HBV *in vivo* either. On the basis of our findings, it is tempting to speculate that HBV exploits mannose trimming as a way to escape recognition by DC-SIGN and thereby subvert a possible immune activation response. Further studies are needed to address this issue.

Materials and methods

Antibodies and viral glycoproteins. The following antibodies were used: DC-SIGN and L-SIGN specific antibody AZN-D2 (15;22); mouse anti-DC-SIGN conjugated with fluorescein isothiocyanate (FITC) (clone DCN46; BD Biosciences, San Jose, CA); mouse anti-HCV E2 (4H6B2; Innogenetics, Ghent, Belgium); sheep anti-HBsAg preS1, mouse anti-HBsAg preS2 and biotinylated human anti-HBsAg (F-9H9-E, all kind gifts of R. Heijtink, Erasmus MC, Rotterdam, the Netherlands); rabbit anti-HBsAg-FITC (Acris Antibodies GmbH, Hiddenhausen, Germany); peroxidase-conjugated goat anti-human immunoglobulin G1 (Jackson Immunoresearch, West Grove, PA); peroxidase-conjugated rabbit anti-FITC (Dako, Glostrup, Denmark); peroxidase-conjugated goat anti-mouse (Caltag; Carlsbad, CA); peroxidase-conjugated streptavidin (Dako).

Recombinant HBV surface antigen HBsAg (containing both S and preS2 domains) purified from transfected Chinese Hamster Ovary (CHO) cells was kindly provided by M. van Roosmalen (bioMérieux, The Netherlands). Yeast cell-derived HCV glycoproteins E1 and E2 reconstituted as virus like particles (VLP) were kindly provided by S. Depraetere (Innogenetics, Belgium).

HepG2.2.15-derived HBV. HepG2.2.15 cells (40) were grown until confluence in Williams' E medium (Gibco, Paisley, UK) supplemented with 5% FCS. Secreted HBV and subviral particles were concentrated from the medium as described before (27), to a final concentration of $0.96x10^9$ HBV particles/ml. To enrich for HBV over subviral particles, HepG2.2.15 culture supernatants were loaded on a PBS equilibrated 1 ml bed volume Hi Trap™ Heparin HP column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). After extensive washing, HBV particles were eluted in an elution buffer (350 mM NaCl, 20 mM Tris/HCl, pH 7.4) and collected in elution 2 ml fractions. Elution fractions were dialyzed against PBS. As a negative control, the same procedure was followed with supernatant from untransfected HepG2 cells. The elution fractions were assessed for the presence of HBV particles with L, M and S-specific ELISA, by capturing HBV with anti-preS1, anti-preS2 and anti-S specific antibodies. Bound HBV was detected with a biotinylated human anti-HBsAg (F-9H9-E). Quantification of HBV particles was done by COBAS® TaqMan HBV Test (Roche Diagnostics GmbH, Mannheim, Germany) after viral DNA isolation using the High Pure System Viral Nucleic Acid Kit (Roche Diagnostics). To generate highly mannosylated HBV, HepG2.2.15 cells were treated with the α-mannosidase I inhibitor kifunensine (5 days, 20 µg/ml; Calbiochem, Darmstadt, Germany). Untreated HepG2.2.15 cells and kifunsine treated HepG2 cells served as negative controls. Final concentrations of highly mannosylated and native HBV were $1.4x10^8$ and $2.2x10^8$ HBV particles/ml.

Cells. Stable Raji transfectants expressing wild-type DC-SIGN or L-SIGN were generated as previously described (29;30). Monocyte-derived dendritic cells (moDC) were cultured from monocytes 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 moDC was confirmed by flow cytometric analysis (22). Expression levels of DC-SIGN and L-SIGN on transfected Raji cells and moDC were determined by flow cytometry with the DC-SIGN and L-SIGN specific AZN-D2 antibody.

Plant lectin ELISA. Heparin purified, glycan modified or native HepG2.2.15-derived HBV were captured on enzyme-linked immunosorbent assay (ELISA) plates (Maxisorb, Nunc, Roskilde, Denmark) with mouse anti-preS2. Recombinant HBsAg (0.5 µg/well), HCV VLP (0.25 µg/well) and mannan (0.25 µg/well; Sigma-Aldrich, St. Louis, MO) were coated directly onto the plate for 18 hrs at 4°C. After blocking with 1% bovine serum albumin for 1 hr at 37°C, the following biotinylated plant lectins were added for 2 hrs at room temperature at a concentration of 5 µg/ml: Con A (*Concavalin A*; recognizes α-glucose and α-mannose), GNA (*Galanthus nivalis* agglutinin; α-mannose), WGA (Wheat germ agglutinin; N-acetylglucosamine), PNA (*Arachis hypogaea* agglutinin; β-galactose, N-acetylgalactosamine), RCAII (*Ricinus communis* agglutinin; β-galactose, N-acetylgalactosamine) or LTA (*Lotus tetragonolobus* agglutinin; fucose; all from Sigma Aldrich (41)). Lectin binding was detected using peroxidase-conjugated streptavidin and absorbance was measured at 450 nm.

Recombinant DC-SIGN-Fc binding ELISA. The DC-SIGN-Fc binding assay was performed as previously described (15). In short, recombinant HBsAg (0.5 µg/well), HepG2.2.15-derived HBV and HepG2 controls were captured on Maxisorb ELISA plates (Nunc) with mouse anti-preS2. HCV VLP (0.25 µg/well) were coated directly on the plates. After blocking with 1% bovine serum albumin for 30 min at 37°C, soluble DC-SIGN-Fc was added and bound DC-SIGN was detected after incubation with peroxidase-labelled anti-human immunoglobulin G1 antibody. Specificity of DC-SIGN-Fc binding was determined by blocking with either mannan (100 µg/ml; Sigma-Aldrich) or EGTA (10 mM; Sigma-Aldrich). To assess the coating efficiency, HBsAg and HBV were detected with biotinylated human anti-HBsAg (F-9H9-E) and HCV VLP were detected with mouse anti-HCV E2 (4H6B2). HBV concentrated by centrifugation or purified by heparin column gave similar results in DC-SIGN-Fc binding assays.

Soluble DC-SIGN/L-SIGN lysate ELISA. Raji DC-SIGN/L-SIGN transfectants were lysed for 4 hrs at 4°C in lysis buffer (1% NP40, 150 mM NaCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$ in PBS) supplemented with EDTA-free protease inhibitors (Roche Diagnostics, Penzberg, Germany). HBsAg, HBV and medium controls were captured on ELISA plates with a mouse anti-preS2 antibody. HCV VLP were coated directly on the plates. After blocking with 5% bovine serum albumin for 30 min at 37°C, Raji DC-SIGN/L-SIGN lysates were added for 2 hrs at RT. Bound DC-SIGN/L-SIGN was detected with a FITC-conjugated DC-SIGN/L-SIGN specific antibody (clone DCN46) followed by a peroxidase-conjugated rabbit anti-FITC antibody. Specificity of binding was determined in the presence of mannan (100 µg/ml; Sigma-Aldrich). To assess the coating efficiency, HBsAg, HBV and HCV VLP were detected with specific antibodies. HBV concentrated by centrifugation or purified by heparin column gave similar results in soluble DC-SIGN/L-SIGN lysate ELISA.

Cellular DC-SIGN/L-SIGN bindings assay. Untransfected Raji cells, DC-SIGN or L-SIGN-transfected Raji cells and moDC were incubated with or without HBsAg (5 µg/ml) for 2 or 18 hrs at 37°C and binding was measured by flow cytometry after intracellular staining with an anti-HBsAg-FITC antibody (Acris Antibodies GmbH). Uptake was compared to lectin-mediated binding of dextran-FITC (100 µg/ml, 40.000 MW; Molecular Probes, Invitrogen, Carlsbad, CA). Specificity of binding was determined by 30 min pre-incubation with mannan (100 µg/ml).

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References

1. 2004. WHO. Hepatitis B vaccines. 255-263.
2. Machida,A., Kishimoto,S., Ohnuma,H., Miyamoto,H., Baba,K., Oda,K., Nakamura,T., Miyakawa,Y., and Mayumi,M. 1983. A hepatitis B surface antigen polypeptide (P31) with the receptor for polymerized human as well as chimpanzee albumins. *Gastroenterology* **85**:268-274.
3. Treichel,U., Meyer zum Buschenfelde,K.H., Stockert,R.J., Poralla,T., and Gerken,G. 1994. The asialoglycoprotein receptor mediates hepatic binding and uptake of natural hepatitis B virus particles derived from viraemic carriers. *J Gen. Virol.* **75 (Pt 11)**:3021-3029.
4. Zahn,A., and Allain,J.P. 2005. Hepatitis C virus and hepatitis B virus bind to heparin: purification of largely IgG-free virions from infected plasma by heparin chrsomatography. *J Gen. Virol.* **86**:677-685.
5. Chong,W.P., To,Y.F., Ip,W.K., Yuen,M.F., Poon,T.P., Wong,W.H., Lai,C.L., and Lau,Y.L. 2005. Mannose-binding lectin in chrsonic hepatitis B virus infection. *Hepatology* **42**:1037-1045.
6. Glebe,D., and Urban,S. 2007. Viral and cellular determinants involved in hepadnaviral entry. *World J Gastroenterol.* **13**:22-38.
7. Seeger,C., and Mason,W.S. 2000. Hepatitis B virus biology. *Microbiol. Mol. Biol. Rev.* **64**:51-68.
8. Bozdayi,A.M., Uzunalimoglu,O., Turkyilmaz,A.R., Aslan,N., Sezgin,O., Sahin,T., Bozdayi,G., Cinar,K., Pai,S.B., Pai,R. et al 2003. YSDD: a novel mutation in HBV DNA polymerase confers clinical resistance to lamivudine. *J Viral Hepat.* **10**:256-265.
9. Gripon,P., Le,S.J., Rumin,S., and Guguen-Guillouzo,C. 1995. Myristylation of the hepatitis B virus large surface protein is essential for viral infectivity. *Virology* **213**:292-299.
10. Lu,X., Mehta,A., Dwek,R., Butters,T., and Block,T. 1995. Evidence that N-linked glycosylation is necessary for hepatitis B virus secretion. *Virology* **213**:660-665.
11. Mehta,A., Lu,X., Block,T.M., Blumberg,B.S., and Dwek,R.A. 1997. Hepatitis B virus (HBV) envelope glycoproteins vary drastically in their sensitivity to glycan processing: evidence that alteration of a single N-linked glycosylation site can regulate HBV secretion. *Proc. Natl. Acad. Sci. U. S. A* **94**:1822-1827.
12. Lambert,C., and Prange,R. 2007. Posttranslational N-glycosylation of the hepatitis B virus large envelope protein. *Virol. J* **4**:45.
13. Thoma-Uszynski,S., Stenger,S., Takeuchi,O., Ochoa,M.T., Engele,M., Sieling,P.A., Barnes,P.F., Rollinghoff,M., Bolcskei,P.L., Wagner,M. et al 2001. Induction of direct antimicrobial activity thrsough mammalian toll-like receptors. *Science* **291**:1544-7.
14. Figdor,C.G., van Kooyk,Y., and Adema,G.J. 2002. C-type lectin receptors on dendritic cells and Langerhans cells. *Nature Rev Immunol* **2**:77-84.
15. Geijtenbeek,T.B., Torensma,R., van Vliet,S.J., van Duijnhoven,G.C., Adema,G.J., van Kooyk,Y., and Figdor,C.G. 2000. Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell* **100**:575-585.
16. Koppel,E.A., van Gisbergen,K.P., Geijtenbeek,T.B., and van Kooyk,Y. 2005. Distinct functions of DC-SIGN and its homologues L-SIGN (DC-SIGNR) and mSIGNR1 in pathogen recognition and immune regulation. *Cell Microbiol.* **7**:157-165.
17. van Vliet,S.J., den Dunnen,J., Gringhuis,S.I., Geijtenbeek,T.B., and van Kooyk,Y. 2007. Innate signaling and regulation of Dendritic cell immunity. *Curr. Opin. Immunol.* **19**:435-440.
18. Zhou,T., Chen,Y., Hao,L., and Zhang,Y. 2006. DC-SIGN and immunoregulation. *Cell Mol. Immunol.* **3**:279-283.
19. van Kooyk,Y., and Geijtenbeek,T.B. 2003. DC-SIGN: escape mechanism for pathogens. *Nat. Rev. Immunol.* **3**:697-709.
20. Bashirova,A.A., Geijtenbeek,T.B., van Duijnhoven,G.C., van Vliet,S.J., Eilering,J.B., Martin,M.P., Wu,L., Martin,T.D., Viebig,N., Knolle,P.A. et al 2001. A dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN)-related protein is highly expressed on human liver sinusoidal endothelial cells and promotes HIV-1 infection. *J. Exp. Med.* **193**:671-678.

21. Pohlmann,S., Zhang,J., Baribaud,F., Chen,Z., Leslie,G.J., Lin,G., Granelli-Piperno,A., Doms,R.W., Rice,C.M., and McKeating,J.A. 2003. Hepatitis C virus glycoproteins interact with DC-SIGN and DC-SIGNR. *J. Virol.* **77**:4070-4080.
22. Ludwig,I.S., Lekkerkerker,A.N., Depla,E., Bosman,F., Musters,R.J., Depraetere,S., van Kooyk,Y., and Geijtenbeek,T.B. 2004. Hepatitis C virus targets DC-SIGN and L-SIGN to escape lysosomal degradation. *J. Virol.* **78**:8322-8332.
23. Cormier,E.G., Durso,R.J., Tsamis,F., Boussemart,L., Manix,C., Olson,W.C., Gardner,J.P., and Dragic,T. 2004. L-SIGN (CD209L) and DC-SIGN (CD209) mediate transinfection of liver cells by hepatitis C virus. *Proc. Natl. Acad. Sci. U. S. A* **101**:14067-14072.
24. Beckebaum,S., Cicinnati,V.R., Dworacki,G., Muller-Berghaus,J., Stolz,D., Harnaha,J., Whiteside,T.L., Thomson,A.W., Lu,L., Fung,J.J. et al 2002. Reduction in the circulating pDC1/ pDC2 ratio and impaired function of ex vivo-generated DC1 in chrsonic hepatitis B infection. *Clin Immunol.* **104**:138-150.
25. Beckebaum,S., Cicinnati,V.R., Zhang,X., Ferencik,S., Frilling,A., Grosse-Wilde,H., Broelsch,C.E., and Gerken,G. 2003. Hepatitis B virus-induced defect of monocyte-derived dendritic cells leads to impaired T helper type 1 response in vitro: mechanisms for viral immune escape. *Immunology* **109**:487-495.
26. Tavakoli,S., Schwerin,W., Rohwer,A., Hoffmann,S., Weyer,S., Weth,R., Meisel,H., Diepolder,H., Geissler,M., Galle,P.R. et al 2004. Phenotype and function of monocyte derived dendritic cells in chrsonic hepatitis B virus infection. *J Gen. Virol.* **85**:2829-2836.
27. Untergasser,A., Zedler,U., Langenkamp,A., Hosel,M., Quasdorff,M., Esser,K., Dienes,H.P., Tappertzhofen,B., Kolanus,W., and Protzer,U. 2006. Dendritic cells take up viral antigens but do not support the early steps of hepatitis B virus infection. *Hepatology* **43**:539-547.
28. Appelmelk,B.J., van Die,I., van Vliet,S.J., Vandenbroucke-Grauls,C.M., Geijtenbeek,T.B., and van Kooyk,Y. 2003. Cutting edge: carbohydrate profiling identifies new pathogens that interact with dendritic cell-specific ICAM-3-grabbing nonintegrin on dendritic cells. *J. Immunol.* **170**:1635-1639.
29. Geijtenbeek,T.B., Kwon,D.S., Torensma,R., van Vliet,S.J., van Duijnhoven,G.C., Middel,J., Cornelissen,I.L., Nottet,H.S., KewalRamani,V.N., Littman,D.R. et al 2000. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell* **100**:587-597.
30. Wu,L., Martin,T.D., Carrington,M., and KewalRamani,V.N. 2004. Raji B cells, misidentified as THP-1 cells, stimulate DC-SIGN-mediated HIV transmission. *Virology* **318**:17-23.
31. Vallee,F., Karaveg,K., Herscovics,A., Moremen,K.W., and Howell,P.L. 2000. Structural basis for catalysis and inhibition of N-glycan processing class I alpha 1,2-mannosidases. *J Biol. Chem.* **275**:41287-41298.
32. Warren,A., Le Couteur,D.G., Fraser,R., Bowen,D.G., McCaughan,G.W., and Bertolino,P. 2006. T lymphocytes interact with hepatocytes thrsough fenestrations in murine liver sinusoidal endothelial cells. *Hepatology* **44**:1182-1190.
33. Gardner,J.P., Durso,R.J., Arrigale,R.R., Donovan,G.P., Maddon,P.J., Dragic,T., and Olson,W.C. 2003. L-SIGN (CD 209L) is a liver-specific capture receptor for hepatitis C virus. *Proc. Natl. Acad. Sci. U. S. A* **100**:4498-4503.
34. Breiner,K.M., Schaller,H., and Knolle,P.A. 2001. Endothelial cell-mediated uptake of a hepatitis B virus: A new concept of liver targeting of hepatotropic microorganisms. *Hepatology* **34**:803-8.
35. Schmitt,S., Glebe,D., Tolle,T.K., Lochnit,G., Linder,D., Geyer,R., and Gerlich,W.H. 2004. Structure of pre-S2 N- and O-linked glycans in surface proteins from different genotypes of hepatitis B virus. *J Gen. Virol.* **85**:2045-2053.
36. Moris,A., Nobile,C., Buseyne,F., Porrot,F., Abastado,J.P., and Schwartz,O. 2004. DC-SIGN promotes exogenous MHC-I-restricted HIV-1 antigen presentation. *Blood* **103**:2648-2654.
37. Moris,A., Pajot,A., Blanchet,F., Guivel-Benhassine,F., Salcedo,M., and Schwartz,O. 2006. Dendritic cells and HIV-specific CD4+ T cells: HIV antigen presentation, T cell activation, viral transfer. *Blood* **108**:1643-51.
38. Turville,S.G., Santos,J.J., Frank,I., Cameron,P.U., Wilkinson,J., Miranda-Saksena,M., Dable,J., Stossel,H., Romani,N., Piatak,M., Jr. et al 2004. Immunodeficiency virus uptake, turnover, and 2-phase transfer in human dendritic cells. *Blood* **103**:2170-2179.
39. Lazar,C., Durantel,D., Macovei,A., Zitzmann,N., Zoulim,F., Dwek,R.A., and Branza-Nichita,N. 2007. Treatment of hepatitis B virus-infected cells with alpha-glucosidase inhibitors results in production of virions with altered molecular composition and infectivity. *Antiviral Res.* **76**:30-37.
40. Sells,M.A., Chen,M.L., and Acs,G. 1987. Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. *Proc. Natl. Acad. Sci. U. S. A* **84**:1005-1009.
41. Lotan,R., Beattie,G., Hubbell,W., and Nicolson,G.L. 1977. Activities of lectins and their immobilized derivatives in detergent solutions. Implications on the use of lectin affinity chrsomatography for the purification of membrane glycoproteins. *Biochemistry* **16**:1787-1794.