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Capture and transmission of HIV-1 by the C-type lectin L-SIGN (DC-SIGNR) is inhibited by carbohydrate-binding agents and polyanions

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ABSTRACT

It was recently shown that capture of HIV-1 by DC-SIGN-expressing cells and the subsequent transmission of HIV to $CD4^+$ T-lymphocytes can be prevented by carbohydrate-binding agents (CBAs), whereas polyanions were unable to block virus capture by DC-SIGN. In this study, we could show that a short pre-exposure of HIV-1 to both mannose- and *N*-acetylglucosamine (GlcNAc)-specific CBAs or polyanions dose-dependently prevented virus capture by L-SIGN-expressing 293T-REx/L-SIGN cells and subsequent syncytia formation in co-cultures of the drug-exposed HIV-1-captured 293T-REx/L-SIGN cells and uninfected C8166 $CD4^+$ T-lymphocytes. Additionally, the inhibitory potential of the compounds against L-SIGN-mediated HIV-1 capture and transmission was more pronounced than observed for DC-SIGN-expressing 293T-REx/DC-SIGN cells. The excess value of CBAs and polyanions to prevent HIV-1 capture and transmission by DC-SIGN and L-SIGN-expressing cells to susceptible T-lymphocytes could be of interest for the development of new drug leads targeting HIV entry/fusion.

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1. Introduction

The human immunodeficiency virus (HIV) is one of the major human infectious pathogens with more than 33 million infected individuals. Since the first onset of the epidemic, some 25 million people have died of AIDS-related illnesses worldwide (UNAIDS, 2008). HIV establishes a persistent infection that is characterized by variable viremia and by an escape from the immune system through several strategies, e.g. continuous antigenic variation, downregulation of host MHC molecules and the destruction of infected and uninfected cells through apoptosis (Gougeon, 2003). Currently there is no curative treatment or vaccine available for HIV infection (Letvin, 2006).

The most common route for HIV-1 infection is through sexual transmission across the genital mucosa. Langerhans cells (LCs) that specifically express the C-type lectin langerin and dendritic cells (DCs) expressing the specific intercellular adhesion molecule-3 (ICAM-3)-grabbing nonintegrin (DC-SIGN or CD209) are part of the innate immune system and are present in mucosal tissues (de Witte et al., 2007). LCs reside in the epidermis of the skin and in most mucosal epithelia, whereas DC-SIGN-expressing DCs exist in the subepithelium layer (Geijtenbeek et al., 2000a; de Witte et al., 2007; Patterson et al., 2002). DCs in the mucosal tissues are thought to transmit HIV-1 to T-cells through capture of the virus particles by DC-SIGN (Geijtenbeek et al., 2000b; Turville et al., 2001, 2002).

DC-SIGN is a 44 kDa type II integral membrane protein with a short amino-terminal cytoplasmic domain and a carboxyl-terminal C-type (i.e. calcium-dependent) lectin domain (Geijtenbeek et al., 2000a). When the mucosal tissue is exposed to HIV-1, the viral surface glycoprotein gp120 will specifically bind to the DC-SIGN-expressing DCs that subsequently transport HIV to the draining lymph node, thereby facilitating infection of susceptible target cells *in trans* (Geijtenbeek et al., 2000b).

A significant proportion of the heavily glycosylated surface glycoprotein gp120 is highly (terminally) mannosylated (Geyer et al., 1988; Leonard et al., 1990). It is assumed that the variability of glycosylation of the HIV-1 gp120 envelope surface modulates the immunogenicity of gp120 as this glycoprotein is the main target for neutralising antibodies during HIV-1 infection. The continuous change in the glycan shield enables the virus to persist the presence of the evolving antibody repertoire (Wei et al., 2003).

Liver-specific ICAM-3-grabbing nonintegrin (L-SIGN), DC-SIGN-related (DC-SIGNR) or CD209L has functional similarity in its interactions with ICAM-3 and HIV-1 (Bashirova et al., 2001; Geijtenbeek et al., 2002; Pöhlmann et al., 2001b). The sequences of DC-SIGN and DC-SIGNR are 88% identical, with 93% identity in the neck domains, 79% identity in the carbohydrate-recognition domains (CRDs), and 63% identity in the cytoplasmic tail and

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transmembrane domains. It is interesting that apparently minor sequence differences in each of these regions have important functional consequences, leading to differences in sugar-binding specificity and intracellular trafficking (Yu et al., 2009). Endothelial cells, but not DCs, of the liver and lymph nodes express high levels of L-SIGN. Similar to DC-SIGN, L-SIGN can bind to mannose residues of viral glycoproteins, for example HIV-1 gp120 and Hepatitis C virus (HCV) E2, through a C-terminal carbohydrate-binding domain (Feinberg et al., 2001; Gardner et al., 2003; Lozach et al., 2004, 2003; Pöhlmann et al., 2003). The oligomerisation of the extracellular domain of both the L-SIGN and DC-SIGN receptors was shown to be important for high affinity binding of these viral glycoproteins (Feinberg et al., 2001; Guo et al., 2004; Mitchell et al., 2001). Thus, L-SIGN and DC-SIGN function as capture receptors for HIV and HCV and may fulfil critical roles in viral pathogenesis and cell tropism. However, the real physiological functions of L-SIGN still remain unclear.

Carbohydrate-binding agents (CBAs) that directly interact with the intact viral envelope glycans compromise the efficient entry of the virus into its susceptible target cells. In addition CBAs could force the virus to delete parts of its glycan shield to escape CBA drug pressure, which might trigger an immune response against previously hidden uncovered immunogenic epitopes (Balzarini, 2005; Balzarini, 2007). Recently it was shown that the capture of HIV-1 by DC-SIGN and subsequent transmission to $CD4^+$ T-lymphocytes and macrophages was also efficiently prevented by CBAs (Balzarini et al., 2007a; Turville et al., 2005). Also, CBAs could block capture of HIV-1 particles by the macrophage mannose receptor (MMR) present on primary monocyte-derived macrophage cell cultures (Pollicita et al., 2007). In addition, CBAs were proven to efficiently block the entry of HCV into its target cells (Helle et al., 2006; Bertaux et al., 2007).

Given the different location and possibly also the different physiological function of L-SIGN *versus* DC-SIGN, we extended our studies on the inhibitory activity of CBAs against DC-SIGN-directed HIV-1 capture and transmission to L-SIGN. We showed that different classes of CBAs but also polyanions could prevent the capture and transmission of HIV-1 to T-lymphocytes by L-SIGN-expressing cells (293T-REx/L-SIGN). In addition, we confirmed that CBAs efficiently inhibit the capture and transmission of HIV-1 to T-lymphocytes by DC-SIGN-expressing cells (293T-REx/DC-SIGN). The significant inhibition of L-SIGN-mediated HIV-1 capture and transmission by polyanions show that its mode of capture and/or transmission must differ from the DC-SIGN-modulated transmission because polyanions did not show a prominent activity in DC-SIGN-directed virus capture and transmission. The potential of the CBAs to impair the L-SIGN receptor in its capacity to capture and to transmit HIV to T-lymphocytes could be an important property in the continuing search for new CBA lead compounds as members of a novel functional class of antivirals.

2. Materials and methods

2.1. Test compounds

The mannose-specific plant lectins from *Galanthus nivalis* (GNA), *Hippeastrum* hybrid (HHA), *Narcissus pseudonarcissus* (NPA) and *Cymbidium* hybrid (CHA) and the *N*-acetylglucosamine (GlcNAc)-specific plant lectin from *Urtica dioica* (UDA) were derived and purified from these plants as described previously (Van Damme et al., 1988a,b). Dextran sulfate (M_r 5000) and suramin were purchased from Sigma (St. Louis, MO). Cyanovirin (CV-N) was provided by Dr. J.B. Mc-Mahon (National Institutes of Health (NIH), Bethesda, MD) and Dr. C. Bewley (NIH, Bethesda, MD). The sulfonated polyvinyl alcohol (PVAS) was synthesized by Dr. S. Görög (Budapest, Hungary). Pradimicin A (PRM-A) was obtained from Prof. T. Oki and Prof. Y. Igarashi (Toyama, Japan).

2.2. Cells

Human T-lymphocyte C8166 cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA) and were cultivated in RPMI-1640 medium (Invitrogen, Merelbeke, Belgium) supplemented with 10% fetal calf serum (FCS) (Cambrex Bio Science Verviers, Verviers, Belgium), 1% penicillin/streptavidin, 2 mM L-glutamine and 75 mM $NaHCO_3$. The human embryonic kidney cells 293T were purchased from the ATCC and cultivated in DMEM supplemented with 10% FCS, 1% penicillin/streptavidin and 75 mM $NaHCO_3$. U87/$CD4^+$/$CXCR4^+$/$CCR5^+$ cells were cultivated in DMEM containing 10% FCS supplemented with 0.4% geneticin and 1% puromycin for selection of CD4, CCR5 and CXCR4, respectively (Princen et al., 2004). 293T-REx/DC-SIGN and 293T-REx/L-SIGN cells were a kind gift from Dr. A. Marzi (Erlangen, Germany). The T-REx cell lines had been constructed by a stable transfection of 293T cells with DC-SIGN or L-SIGN Tet-On plasmids, making the respective nonintegrin expressions inducible with doxycycline (Sigma) (Simmons et al., 2003). The cells were maintained in 293T medium, as described above, supplemented with zeocine (50 μg/ml) and blasticidine (2.5 μg/ml).

2.3. Viruses

The pNL4.3-EGFPΔenv construct that was used for production of wild-type NL4.3 virus through homologous recombination with the *env* gene, was provided by M. Quiñones-Mateu of The Cleveland Clinic Foundation (Cleveland, OH) (Weber et al., 2006). All rights, title, and interest in these materials are owned by The Cleveland Clinic Foundation. After a standard calcium dichloride based transfection of pNL4.3-EGFPΔenv and the wild-type *env* gene into 293T cells, the produced HIV-NL4.3-EGFP virus was transferred to U87/$CD4^+$/$CXCR4^+$/$CCR5^+$ cells for higher virus production. The produced virus in the cell culture supernatants was harvested after 3 days post-infection and 1 ml aliquots were frozen at −80 °C until use. HIV-1_{IIIB} and HIV-1_{BaL} were provided by R.C. Gallo and M. Popovic (at that time at the National Cancer Institute (NCI), National Institutes of Health, Bethesda, MD). HIV-1_{HE} is a clinical isolate derived from a Belgian AIDS patient in 1987 and later propagated in MT-4 cells.

Fluorescent virions were produced by transient co-transfection of pNL4-3 and pGFP-Vpr, a plasmid expressing the GFP-Vpr fusion protein (a kind gift from Dr G.N. Pavlakis, NCI, Frederick, MD). Supernatants holding GFP-Vpr containing HIV-1 were collected 60 h after transfection. Cell debris were removed from the supernatant by centrifugation (450 × *g* for 10 min) and the supernatant was used for infection or stored at −80 °C.

2.4. Flow cytometric analysis

293T, 293T-REx/DC-SIGN and 293T-REx/L-SIGN cells were stained with the monoclonal anti-human DC-SIGN-phycoerythrin antibody and the L-SIGN specific monoclonal anti-human DC-SIGN2-fluorescein antibody (R&D System, UDA) and were processed by flow cytometry. In a 48-well plate 40,000 293T cells and 50,000 293T-REx/DC-SIGN and 293T-REx/L-SIGN cells were seeded on day 0. On day 1, the cells were stimulated with doxycycline or mock-treated. On day 2, the supernatant was removed and replaced by 200 μl of fresh medium supplemented with one of the monoclonal antibodies. After incubation for 30 min at 37 °C, the cells were washed three times with phosphate buffered saline (PBS). Finally, the cell pellet was resuspended in 500 μl 2% paraformaldehyde

and analysed with the FACSCalibur using CellQuest software (BD Biosciences).

293T, 293T-REx/DC-SIGN and 293T-REx/L-SIGN cells were also stained with PE-conjugated mouse anti-human CD4 antibody, PE-conjugated mouse anti-human CD195 (CCR5) and PE-conjugated mouse anti-human CD184 (CXCR4) antibody (Becton Dickinson Pharmingen, USA). The same amount of cells were seeded and stimulated as stated previously, but on day 2, the supernatant was removed and replaced by 200 μl PBS with 2% FCS. After addition of the antibody, the plates were incubated for 30 min at 4 °C, washed three times with PBS, suspended in 500 μl paraformaldehyde and analysed with the FACSCalibur using CellQuest software (BD Biosciences).

2.5. Capture of different amounts of HIV-$1_{NL4.3}$ by 293T-REx/L-SIGN or 293T-REx/DC-SIGN in function of time

An amount of 140,000 293T-REx/L-SIGN or 293T-REx/DC-SIGN cells were seeded in 24-well plates. After stimulation with doxycycline for 24 h to induce L-SIGN or DC-SIGN expression the cells were exposed to different amounts (a total of 5000–10,000–25,000 and 50,000 pg p24) of HIV-NL4.3-EGFP for different incubation times (1, 2 or 3 h). After incubation, unadsorbed virus was thoroughly removed by several successive washing steps. The remaining total amount of virus present in the supernatant of the last washing step was below the detection limit of the assay, i.e. 10 pg HIV-1 p24.

2.6. Capture of different isolates of HIV-1 by 293T-REx/L-SIGN cells

293T-REx/L-SIGN cells were seeded and treated as described above. On day 2 the induced cells were exposed to a total amount of 25,000 pg p24 of HIV-$1_{NL4.3}$ (HIV-NL4.3-EGFP), HIV-1_{IIIB}, HIV-1_{HE} and HIV-1_{BaL} for 2 h. After incubation, unadsorbed virus was thoroughly removed by several successive washing steps as described below. Unstimulated cells that were incubated with the different HIV-1 isolates were used as p24 background.

2.7. Inhibition of capture of HIV-$1_{NL4.3}$ and HIV-1_{BaL} by 293T-REx/L-SIGN and $HIV_{NL4.3}$ by 293T-REx/DC-SIGN cells

For the assay measuring the inhibition of capture by CBAs and polyanions, the cells were exposed on day 2 for 2 h to mixtures of 200 μl virus and 200 μl compound at 37 °C. These mixtures consisted of a total of 25,000 pg HIV-1_{BaL} or HIV-1_{NL3} that was pre-incubated for 30 min with several dilutions of different classes of CBAs and polyanions. Subsequently, the cells were thoroughly washed to remove unadsorbed virus as described below. After removal from the wells, the cells were resuspended in 15 ml tubes with 6 ml DMEM and centrifuged at 1500 rpm for 5 min after which 5.9 ml supernatant was carefully removed. The virus-exposed cells were resuspended in 5 ml DMEM and centrifuged a second time. After removal of 4.9 ml supernatant the cells were resuspended in 10 ml DMEM and centrifuged for a third time. After removal of 9.9 ml of supernatant, the cell pellet was transferred into a microcentrifuge tube and resuspended in 1.5 ml DMEM. At the end, the pellet of the virus-exposed cells was resuspended in 200 μl of 10% Triton X100 for p24 Ag determination by an HIV-1 p24 Ag enzyme-linked immunosorbent assay (Perkin Elmer, Zaventem, Belgium) to quantify the captured virus on the DC-SIGN or L-SIGN receptor of the 293T-REx cells. The p24 content from the supernatant of the last washing step was below the detection limit. Therefore, the p24 values measured in the pellets solely represent the virus particles that were captured by the 293T-REx/L-SIGN or 293T-REx/DC-SIGN cells.

For fluorescence microscopy, the cells were pelleted after the washing steps, resuspended in a 3% paraformaldehyde solution and transferred to an eight-well glass-bottom Labtek chamber before imaging.

2.8. Effect of mannan on to the capture of HIV-1 by 293T-REx/L-SIGN cells in the presence of CBAs or polyanions

In this protocol, the capture assay was performed as described above. However, the dilutions of CBAs or polyanions were pre-mixed with 2 mg/ml mannan during 30 min at 37 °C before the addition of wild-type HIV-NL4.3-EGFP.

2.9. Exposure of 293T-REx/DC-SIGN and 293T-REx/L-SIGN cells to HIV-1 (virus capture) and subsequent co-cultivation with C8166 cells (transmission)

In a 48-well plate 50,000 293T-REx/DC-SIGN and 239T-REx/L-SIGN cells were seeded on day 0. On day 1 the exponentially growing cells were stimulated as described above. As a control, non-stimulated cells were used. On day 2, i.e. after 24 h induction with doxycycline, the cells were exposed to a 200 μl mixture of 100 μl of wild-type HIV-NL4.3-EGFP (a total of 25,000 pg) that was pre-exposed to 100 μl of several test compounds for 30 min. The cells were exposed for 2 h to the virus-test compound mixture at 37 °C. After virus exposure, the cells were removed from the wells and washed as described above to remove carefully unadsorbed virus particles and test compounds before addition of uninfected C8166 T-lymphocytes. After the last centrifugation, the supernatant was discarded and the cell pellet was resuspended in 500 μl of RPMI and transferred to a 48-well plate. This procedure allowed to dilute unadsorbed virus and test compound by >2 million-fold from the cell pellets. Then, 60,000 uninfected C8166 lymphocytes were added to each well. These co-cultures were incubated at 37 °C in a CO_2-controlled humidified incubator. After 72 h of virus exposure/co-cultivation, the cells were fixed in 2% paraformaldehyde and the expression of EGFP as % of control was measured by a FACSCalibur and analysed by CellQuest software (BD Biosciences, San Jose, CA). The 50% effective concentration (EC_{50}) corresponds to the compound concentration required to prevent EGFP expression (transmission) by 50% in the virus-infected C8166 cell cultures

2.10. Confocal microscopy

Images of the cells were acquired with a laser scanning SP5 confocal microscope (Leica Microsystems, Mannheim, Germany) equipped with an AF 6000 microscope and an AOBS, using a HCX PL APO 63× (NA 1.2) water immersion objective magnification. GFP was monitored with the Ar laser using the 488 nm line for excitation, and emission was detected between 492 and 558 nm.

3. Results

3.1. Receptor expression on 293T, 293T-REx/L-SIGN and 293T-REx/DC-SIGN cells

The expression of the different cellular receptors that are important in HIV-1 binding and/or transmission was investigated on 293T, 293T-REx/L-SIGN and 293T-REx/DC-SIGN cells (Fig. 1). The flow cytometric analysis showed that 63.4% of the doxycycline-stimulated 293T-REx/L-SIGN cells expressed L-SIGN on their membrane while only 0.1% of the wild-type 293T cells exposed to doxycycline expressed L-SIGN. 293T-REx/DC-SIGN cells stimulated with doxycycline showed 51.1% DC-SIGN positive cells while only 0.1% of the stimulated 293T cells were DC-SIGN positive (data not shown). Non-stimulated 293T-REx/L-SIGN and 293T-REx/DC-SIGN cells showed ~3% L-SIGN- and DC-SIGN-positive cells. Expression of

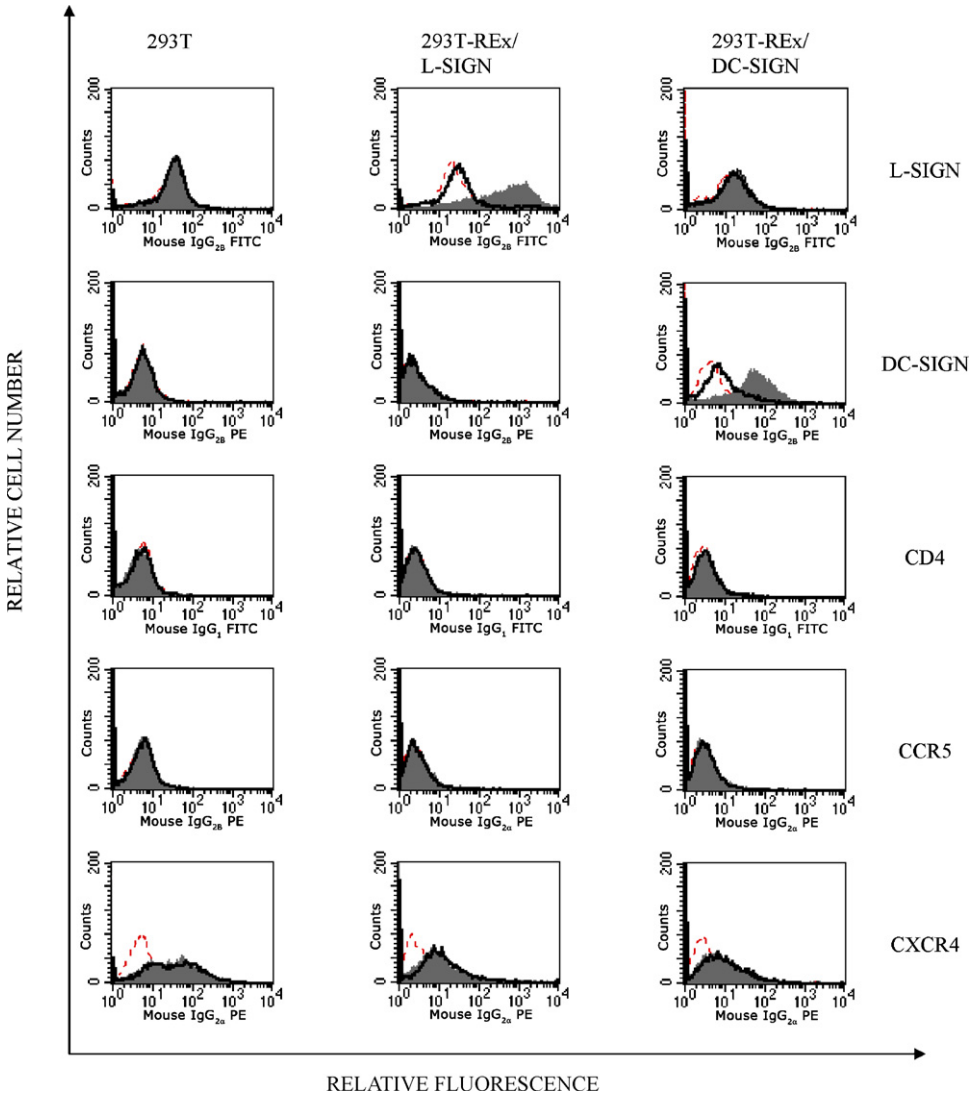

Fig. 1. Cell surface expression of the receptors L-SIGN, DC-SIGN, CD4, CCR5 and CXCR4 on 293T (left), 293T-REx/L-SIGN (middle) and 293T-REx/DC-SIGN (right) cells. The expression of the receptors after doxycycline stimulation is shown as a grey histogram and before stimulation as a black histogram. An isotypic mAb was used as negative control (dotted red line).

CD4 and/or CCR5 was not detectable in all these cell lines, whereas CXCR4 was continuously expressed on all of them (Fig. 1).

3.2. Exposure of 293T-REx/L-SIGN cells to HIV-$1_{NL4.3}$, HIV-1_{IIIB}, HIV-1_{HE} and HIV-1_{Bal}

The capture of HIV-1 by 140,000 stimulated 293T or unstimulated 293T-REx/L-SIGN cells only resulted in a low background amount of p24 (approximately 75 pg). The p24 levels associated with the doxycycline-stimulated 293T-REx/L-SIGN cells could be reliably measured when the total initial virus input was 25,000 pg p24. We found that the capture of the HIV-1_{HE} isolate by 293T-REx/L-SIGN cells was more efficient than for the HIV-$1_{NL4.3}$ and HIV-1_{IIIB} isolates. However, no clear difference in capture by 293T-REx/L-SIGN between the X4-tropic isolates (HIV-$1_{NL4.3}$, HIV-1_{IIIB} and HIV-1_{HE}) and the R5-tropic HIV-1_{Bal} was observed (Fig. 2).

3.3. Correlation of different amounts of virus captured in function of time

A clear linear correlation was observed between $HIV_{NL4.3}$ input and the total amount of captured virus by 140,000 stimulated 293T-REx/L-SIGN cells (Fig. 3) and similar results were obtained with DC-SIGN-mediated capture (data not shown). Incubation times longer than 1–2 h (i.e. 3 h) did only result in a moderate increase of the number of captured HIV-NL4.3-EGFP particles. An incubation time of 2 h with a total initial amount of 25,000 pg virus was chosen

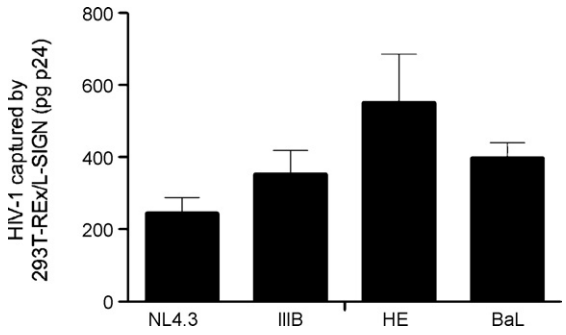

Fig. 2. Capture of different HIV-1 strains by stimulated 293T-REx/L-SIGN cells. A total amount of 25,000 pg p24 was used as virus input and the virus was exposed for 2 h to 140,000 cells. After removal of unbound virus by several washing steps, the cell-associated virus was quantified by p24 ELISA.

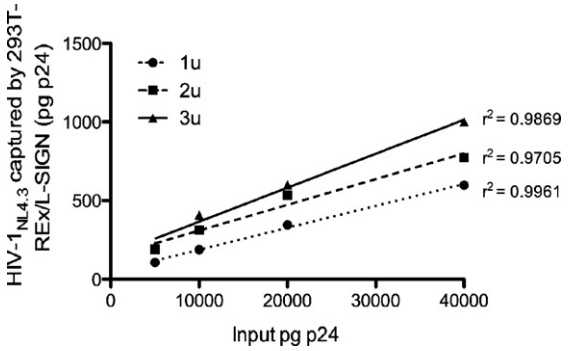

Fig. 3. Correlation between total input of HIV-$1_{NL4.3}$ and the total capture of virus after 1, 2 and 3 h of incubation with 140,000 stimulated 293T-REx/L-SIGN cells.

to be the optimal condition for the virus capture assays. Under these conditions the virus capture is still in the linear phase where no saturation of the receptor is present. Therefore, the minor differences in expression levels between 293T-REx/L-SIGN and 293T-REx/DC-SIGN cells could be accounted for the observed differences in sensitivity to polyanions.

3.4. Inhibitory effect of CBAs and polyanions on the capture of HIV-NL4.3-EGFP or HIV-1_{BaL} by 293T-REx/L-SIGN and 293T-REx/DC-SIGN

The data resulting from the inhibition by different CBAs and polyanions of the HIV-1 capture are represented as the percentage of the mean captured p24 by the 293T-REx/L-SIGN or 293T-REx/DC-SIGN cells incubated with HIV-NL4.3-EGFP or HIV-1_{BaL} in the absence of CBAs (control cultures) (Fig. 4).

The results show that the mannose-specific HHA, GNA and NPA and the GlcNAc-specific UDA CBAs dose-dependently inhibit the capture of HIV-NL4.3-EGFP by 293T-REx/L-SIGN cells (Fig. 4A). The most active CBA was the mannose-specific HHA with an IC_{50} for HIV-1 capture by L-SIGN-expressing cells around 0.04 μM. UDA had a ~10-fold higher IC_{50}. The capture of HIV-NL4.3-EGFP by 293T-REx/DC-SIGN cells was also inhibited by CBAs but at markedly higher concentrations compared with the HIV capture inhibition by

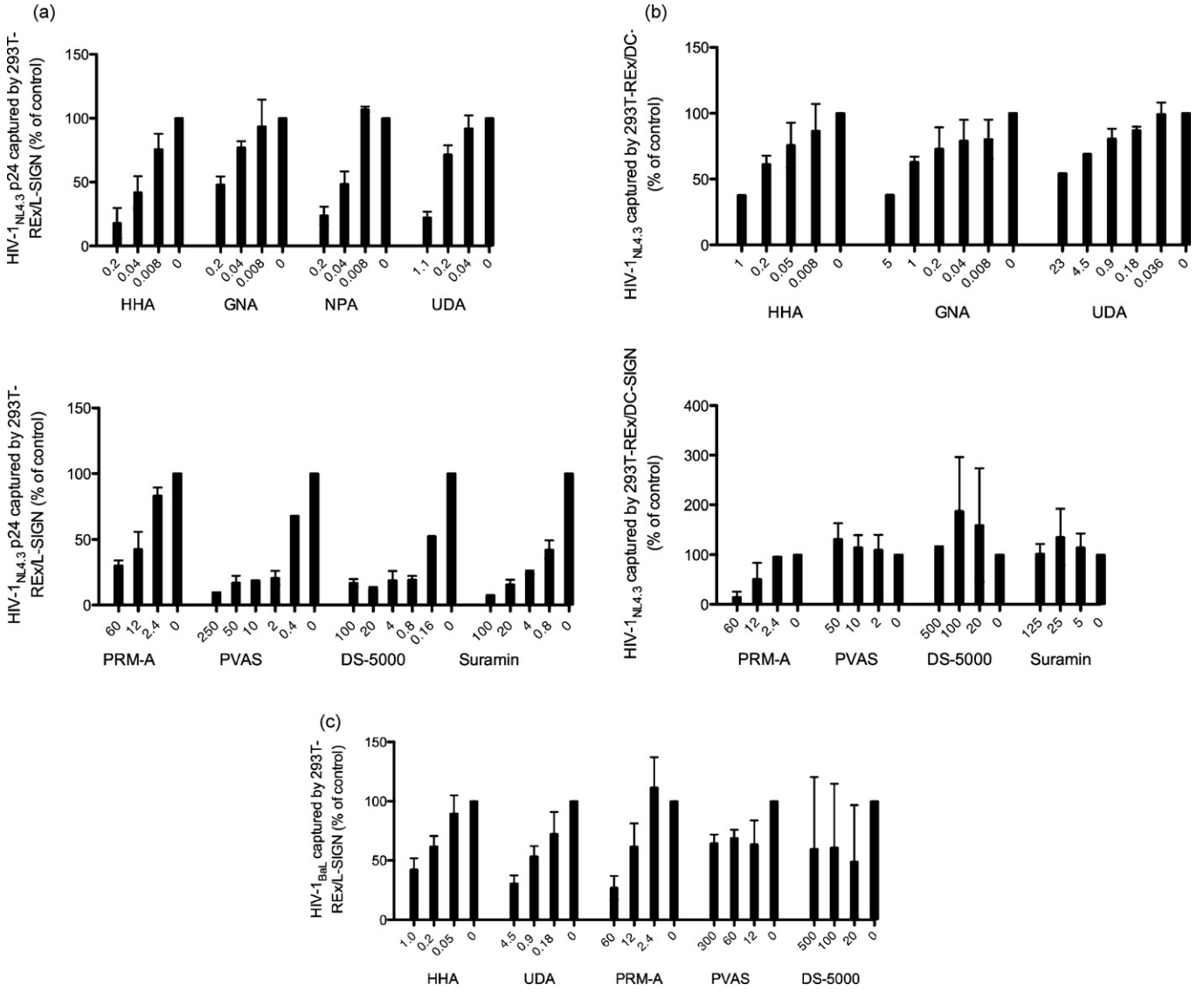

Fig. 4. Capture of HIV-NL4.3-EGFP (panel A and B) or HIV-1_{BaL} (panel C) by 293T-REx/L-SIGN (panels A and C) or 293T-REx/DC-SIGN cells (panel B) in presence or absence of CBAs or polyanions. HIV-NL4.3-EGFP or HIV-1_{BaL} were exposed to different dilutions of CBAs or polyanions for 30 min prior to administration to 293T-REx/L-SIGN (panels A and C) or 293T-REx/DC-SIGN cells (panel B) for 2 h. After removal of unbound virus by several washing steps, the cell-associated virus was quantified by p24 ELISA. CBA, and suramin (MW 1500) concentrations are in μM; PVAS and DS-5000 in μg/ml.

L-SIGN (Fig. 4B). Interestingly, the low-size, non-peptidic mannose-specific pradimicin A showed similar inhibitory values in both assay systems ($IC_{50} \sim 10\ \mu M$) (Fig. 4A and B). The polyanions PVAS and DS-5000 and also suramin were inhibitory against the capture of HIV-NL4.3-EGFP by 293T-REx/L-SIGN in contrast to the capture by 293T-REx/DC-SIGN where these compounds show no activity, even at high concentrations (Fig. 4A and B). In addition, HHA, UDA and PRM-A were able to prevent the capture of the R5-tropic HIV-1_{BaL} by 293T-REx/L-SIGN dose-dependently, while the polyanions PVAS and DS-5000 have no significant inhibitory effect against the capture by L-SIGN (Fig. 4C).

3.5. Blockage of the inhibitory effect of CBAs on the capture of HIV-NL4.3-EGFP by 293T-REx/L-SIGN cells by the addition of mannan

About 2 mg/ml mannan was administered to serial dilutions of different CBAs and polyanions for 30 min at 37 °C. Subsequently HIV-NL4.3-EGFP particles were exposed to these compound mixtures. The further treatment and washing of the 293T-REx/L-SIGN cells was similar as described above. Our results show that the dose-dependent inhibition of the capture of HIV-NL4.3-EGFP by 293T-REx/L-SIGN by HHA, GNA, UDA and PRM-A was virtually fully blocked by the addition of 2 mg/ml mannan (Fig. 5). This pronounced blocking effect of mannan was not observed for the inhibition of HIV-NL4.3-EGFP capture by 293T-REx/L-SIGN cells in the presence of suramin and the polyanions PVAS and DS-5000 (Fig. 5).

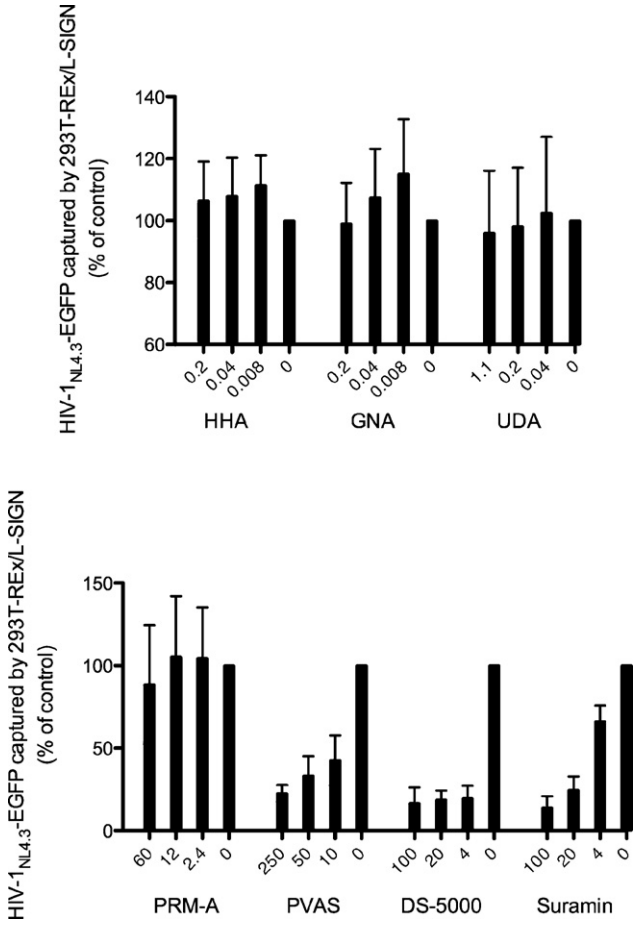

Fig. 5. Capture of HIV-NL4.3-EGFP by 293T-REx/L-SIGN in the presence or absence of CBAs or polyanions. The dilutions of CBAs or polyanions were first incubated with 2 mg/ml mannan for 30 min prior to their administration to the virus. CBA, and suramin (MW 1500) concentrations are in μM; PVAS and DS-5000 in μg/ml.

Table 1
Inhibitory activity of CBAs and polyanions against transmission of HIV-NL4.3-EGFP from virus-captured 293T-REx/DC-SIGN or 293T-REx/L-SIGN cells to uninfected C8166 cells.

Compound	EC_{50} (μM)	
	DC-SIGN	L-SIGN
HHA	0.022 ± 0.008	0.014 ± 0.005
GNA	0.098 ± 0.014	0.070 ± 0.032
UDA	0.38 ± 0.13	0.10 ± 0.03
CA	0.23 ± 0.10	0.042 ± 0.014
PRM-A	35 ± 6.2	11 ± 6.2
CV-N	0.001 ± 0.0006	0.001 ± 0.0002
DS-5000 (μg/ml)	>250	37 ± 26
PVAS (μg/ml)	>250	0.21 ± 0.18
Suramin	>50	0.34 ± 0.15

3.6. Co-cultivation of C8166 T-lymphocytes and HIV-NL4.3-EGFP-exposed 293T-REx/L-SIGN and 293T-REx/DC-SIGN cells

To mimic the *in vivo* transmission of HIV-1 to T-lymphocytes by DC-SIGN-expressing DCs or L-SIGN-expressing endothelial cells, an *in vitro* assay studying the transmission of virus from HIV-captured 293T-REx/L-SIGN or 293T-REx/DC-SIGN cells to human C8166 T-lymphocytes was developed. When non-exposed 293T-REx/L-SIGN (and 293T-REx/DC-SIGN) cells were co-cultured with uninfected C8166 cells no EGFP expression was observed (Fig. 6D). Virus-exposed 293T-REx/L-SIGN (and 293T-REx/DC-SIGN) cells could not be infected by HIV-1 (Fig. 6B) and have a similar morphology as non-exposed 293T-REx/L-SIGN (and 293T-REx/DC-SIGN) cells (Fig. 6A). Virus capture by 293T-REx/L-SIGN cells exposed to GFP-Vpr labelled NL4.3 virions was shown in Fig. 6C (HIV-capturing cell in the middle of panel C). We observed that in co-cultures of uninfected C8166 cells with HIV-NL4.3-EGFP-exposed 293T-REx/L-SIGN or 293T-REx/DC-SIGN cells, C8166 cell clusters appear with abundant EGFP expression and visible giant cells (Fig. 6E).

3.7. Inhibitory activity of CBAs and polyanions against HIV-NL4.3-EGFP transmission from virus-exposed 293T-REx/L-SIGN and 293T-REx/DC-SIGN cells to C8166 T-lymphocytes

Most CBAs showed a clear dose-dependent inhibitory activity in this assay both for inhibiting L-SIGN- or DC-SIGN-mediated virus transmission to T-lymphocytes (Fig. 7A and B, respectively). The EC_{50} values range between 1 nM (for CV-N) and 35 μM (for PRM-A) as shown in Table 1. It is noteworthy that most CBAs generally show a higher potency against L-SIGN-mediated transmission of HIV-1 than DC-SIGN-mediated transmission (up to sixfold). The polyanions PVAS and DS-5000 and also suramin show a clear inhibitory activity in L-SIGN-mediated transmission while no inhibitory activity against DC-SIGN-directed virus transmission was observed ($EC_{50} > 250\ \mu g/ml$).

4. Discussion

It has previously been described that L-SIGN functions similarly as DC-SIGN with regard to virus attachment and transmission (reviewed in Koppel et al., 2005; Khoo et al., 2008). Like DC-SIGN, L-SIGN appears to function as an attachment factor for primate lentiviruses, affording binding and transmission of HIV-1, HIV-2 and SIV strains (Pöhlmann et al., 2001a). L-SIGN is present on endothelial cells in human placenta, lymph node sinuses and hepatic sinoids (Bashirova et al., 2001; Pöhlmann et al., 2001b; Soilleux et al., 2002). In particular, the abundant expression of L-SIGN in lymph nodes is

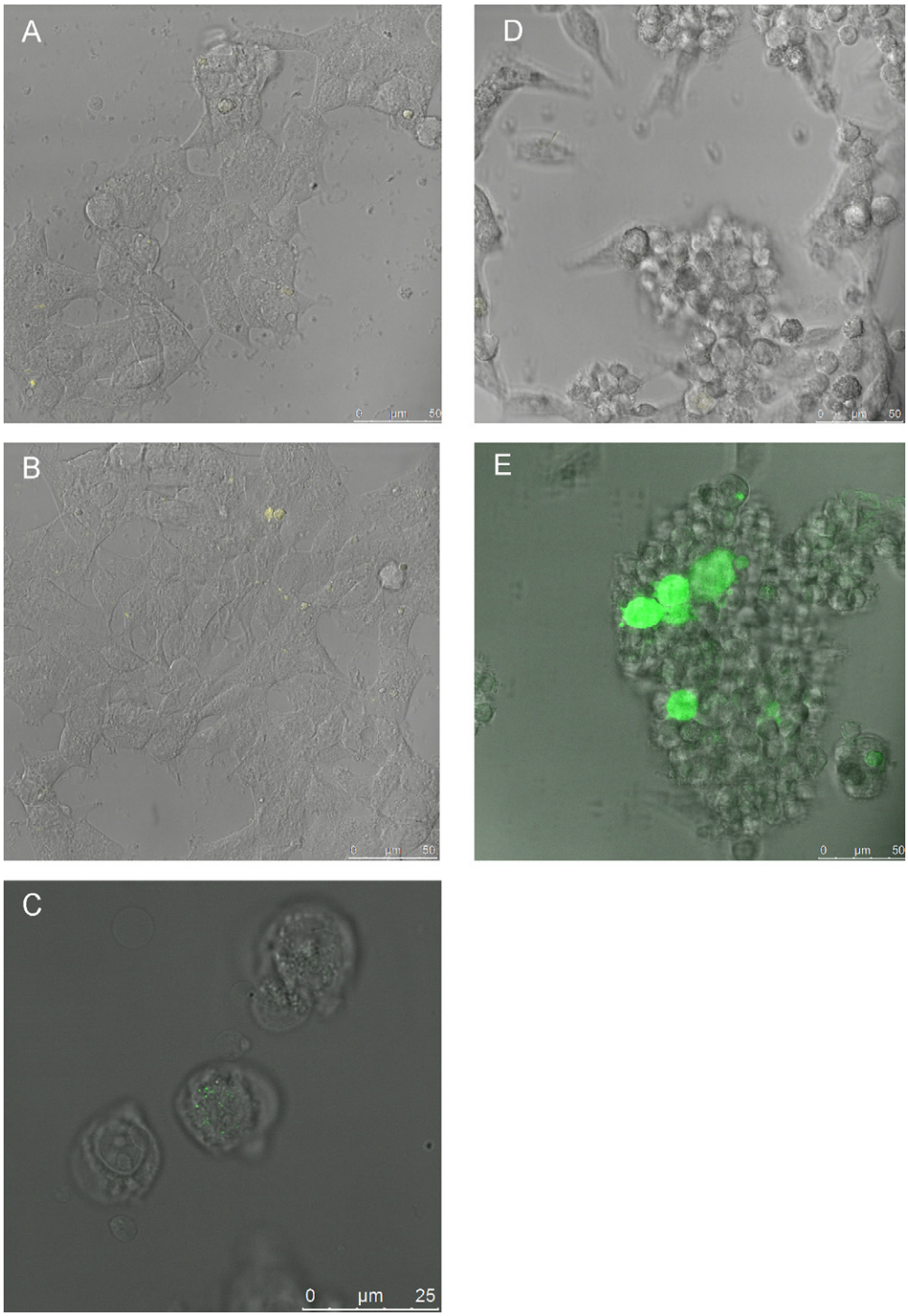

Fig. 6. Microscopic view of the following cell cultures: (A) 293T-REx/L-SIGN cells. (B) HIV-NL4.3-EGFP exposed 293T-REx/L-SIGN cells. (C) 293T-REx/L-SIGN cells exposed to GFP-Vpr labelled NL4.3 virions. (D) Co-cultures (2 days after initiation) of C8166 cells with non-exposed 293T-REx/L-SIGN cells. (E) Co-cultures (2 days after initiation) of C8166 cells with pre-exposed (HIV-NL4.3-EGFP)-293T-REx/L-SIGN cells. Before co-cultivation the non-adsorbed virus to the 293T-REx/L-SIGN cells was carefully removed after a 30 min virus-exposure time. All 293T-REx/L-SIGN cells were stimulated with doxycycline 1 day before initiation of the experiments. Pictures shown are the overlays of the differential interference contrast (DIC) image with the fluorescence image. Scale bar: 25 µm.

of importance as lymph node sinuses represent a major site and reservoir for HIV replication. The presence of this efficient virus attachment factor on multiple endothelial cell types indicates that L-SIGN could play a role in the vertical transmission of primate lentiviruses, in enabling HIV to traverse the capillary endothelium in some organs, and in the presentation of virus to CD4-positive cells in multiple locations of the body including lymph nodes (Pöhlmann et al., 2001a). The discovery of these C-type lectins of the innate immune system, which share similar attachment mechanisms, had an important impact on the understanding of the mechanism of virus attachment and spread in infected individuals. Moreover, DC-SIGN but also L-SIGN could represent an important therapeutic target to prevent HIV capture and subsequent transmission to T-lymphocytes.

In this study we used 293T-REx/L-SIGN cells that can capture and transmit HIV-1 particles to T-lymphocytes as a reliable and relevant

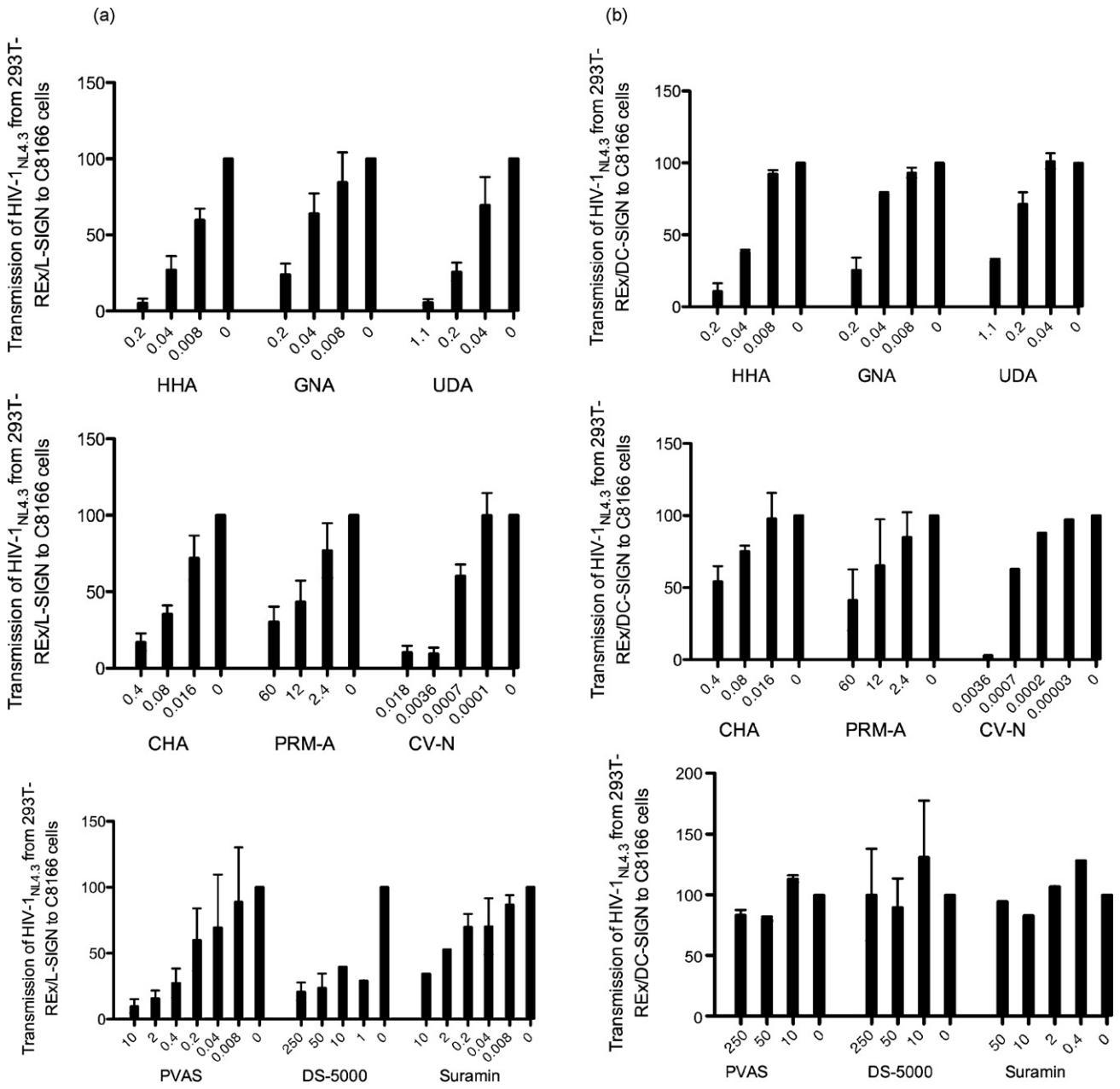

Fig. 7. Effect of CBAs and polyanions on the virus transmission of HIV-NL4.3-EGFP-captured 293T-REx/L-SIGN and 293T-REx/DC-SIGN cells to uninfected C8166 cells. HIV-NL4.3-EGFP was exposed for 30 min to various concentrations of CBAs or polyanions prior to administration to 293T-REx/L-SIGN (panel A) or 293T-REx/DC-SIGN (panel B) cells. After removal of unbound virus by several washing steps, the L-SIGN- and DC-SIGN-transduced 293T-REx cells were co-cultivated with C8166 cells. The virus production was quantified by flow cytometric detection of EGFP expression. CBA and suramin (MW 1500) concentrations are in μM; PVAS and DS-5000 in μg/ml.

model system to mimic capture and transmission of HIV particles to T-lymphocytes by L-SIGN-expressing endothelial cells (Simmons et al., 2003). In addition, we compared 293T-REx/L-SIGN cells with the corresponding 293T-REx/DC-SIGN cells for their performance in HIV-1 capture and transmission. Under our experimental conditions both cell types could not be infected with HIV-1, but they were able to specifically capture HIV-NL4.3-EGFP particles and to transmit them to uninfected C8166 T-lymphocytes. In addition, we have shown that stimulated 293T-REx/L-SIGN cells were able to capture both X4 (HIV-$1_{NL4.3}$, HIV-1_{IIIB} and HIV-1_{HE}) and R5 viruses (HIV-1_{BaL}).

A variety of HIV CBA entry inhibitors with relatively high molecular weight, such as the plant lectins GNA, HHA, UDA, NPA and CHA, and the cyanobacterium-derived CV-N, but also the low-size non-peptidic PRM-A antibiotic (MW: 838) (Balzarini et al., 2007b) were included in our study. In addition, three non-CBA entry inhibitors like the polyanions PVAS and DS-5000 and the negatively charged, lower-size suramin (MW: 1500) were also included in our study. All of the CBAs evaluated in our study invariably showed a pronounced inhibitory activity against the transmission of HIV-NL4.3-EGFP particles from virus-captured 293T-REx/L-SIGN and 293T-REx/DC-SIGN cells to uninfected C8166 cells. This observation was very similar to the results found for inhibition by CBAs against the transmission of HIV-1 from virus-captured Raji/DC-SIGN cells to uninfected C8166 T-lymphocytes (Balzarini et al., 2007a). Overall we observed that the CBAs inhibit L-SIGN-mediated virus transmission at a ~5 times better extent than DC-SIGN-mediated virus capture and transmission. This is surprising because there is a very high similarity between both L-SIGN and DC-SIGN molecules. It was even reported that the highest degree of homology between

DC-SIGN and L-SIGN is located in the carbohydrate recognition domain indicating that DC-SIGN and L-SIGN have very similar ligands (Guo et al., 2004). Like DC-SIGN, L-SIGN recognizes and binds high-mannose-containing ligands but, in contrast to DC-SIGN, L-SIGN does not bind to the fucose-containing Lewis^x antigens. The slight difference in carbohydrate recognition between L-SIGN and DC-SIGN is thought to be due to the presence of Val351 in DC-SIGN and Ser363 in L-SIGN resulting in the elimination of the van der Waals interaction with the –2OH group of fucose (Guo et al., 2004). It is unclear, however, whether these differences in carbohydrate recognition between L-SIGN and DC-SIGN might account for the higher activity of CBAs against L-SIGN-mediated capture and transmission because the CBAs are reported to predominantly have a GlcNAc (UDA) or mannose oligomer (the other CBAs) specificity. Probably, subtle differences in the structure and/or affinity of L-SIGN and DC-SIGN may account for the superior effects of the CBAs on virus capture by L-SIGN.

The polyanions PVAS and DS-5000 and also suramin showed a clear inhibition of HIV-NL4.3-EGFP-capture and transmission from the virus-captured adherent 293T-REx/L-SIGN cells to uninfected C8166 T-lymphocytes. These findings were surprising since polyanions were not active in capture and transmission assays using Raji/DC-SIGN cells. In contrast, they even stimulated the capture of HIV-1 by Raji/DC-SIGN cells (Balzarini et al., 2007a), which was confirmed in our current study using 293T-REx/DC-SIGN cells. For the R5-tropic HIV-1_{BaL} no inhibitory effect of the polyanions for virus capture by L-SIGN was observed, likely pointing to differences in the envelope between the R5 and X4 virus strains to be recognized by L-SIGN.

The addition of mannan was able to decrease the inhibitory activity of the CBAs against the capture of virus particles by 293T-REx/L-SIGN cells but had no marked influence on the inhibitory activity of the polyanions and suramin. These results indicate that the CBAs preferentially bind to the mannose oligomers of the virus envelope and that this binding is interfering with the capture of virus particles by L-SIGN. However, the inhibition of the L-SIGN-directed capture of virus particles by polyanions and suramin is not mediated by binding to carbohydrate residues as their mode of binding to gp120 is different from CBAs. In this respect it was previously found that polyanions (DS-5000 and PVAS) and suramin also inhibited CD4-binding of HIV-1 (Schols et al., 1989; Baba et al., 1990) and CXCR4-binding (Moulard et al., 2000), while CBAs (HHA and GNA) had no effect on the CD4-binding of HIV-1 (Balzarini et al., 1991). The pronounced inhibition of L-SIGN-mediated capture and transmission of HIV-NL4.3-EGFP by polyanions and suramin might point to a different binding mechanism of HIV-1 particles to L-SIGN than to DC-SIGN. It has to be mentioned that L-SIGN has a total amino acid charge of +3 while DC-SIGN is neutral. However, it seems unlikely that this difference can explain their different behaviour towards polyanions. The differences in inhibition of L-SIGN-mediated capture by polyanions between X4 and R5 viruses might be explained by the fact that the V3 loop previously was demonstrated to be the major determinant for polyanion interaction with HIV-1 gp120 (Moulard et al., 2000) and that the V3 loop overall changes may differ between X4 and R5 viruses. In this study it was also shown that – unlike for X4 virus – the polyanions showed only weak if any binding to gp120 of an R5-tropic virus.

In conclusion, we have now established an *in vitro* assay that enables the determination of the inhibitory activity of different (entry) compounds against the selective capture of HIV-1 particles by the L-SIGN receptor expressed on monolayer 293T-REx/L-SIGN cells. This assay model may mimic the behaviour of endothelial L-SIGN-expressing cells present *in vivo*. In addition, the inhibition of capture and subsequent transmission of HIV-NL4.3-EGFP by L-SIGN-expressing cells to uninfected C8166 T-lymphocytes by different compounds can easily be detected and quantified by flow cytometric analysis. We observed that CBAs but also polyanions were able to prevent capture and transmission of HIV-NL4.3-EGFP particles by L-SIGN more efficiently than by DC-SIGN. The potential of CBAs to inhibit L-SIGN- and also DC-SIGN-mediated transmission of HIV-1 to uninfected lymphocytes in latent reservoirs like lymph nodes could be a very important property and of interest for the development of new drug leads targeting HIV entry/fusion.

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