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DC-SIGN and DC-SIGNR Bind Ebola Glycoproteins and Enhance Infection of Macrophages and Endothelial Cells

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Ebola virus exhibits a broad cellular tropism *in vitro*. In humans and animal models, virus is found in most tissues and organs during the latter stages of infection. In contrast, a more restricted cell and tissue tropism is exhibited early in infection where macrophages, liver, lymph node, and spleen are major initial targets. This indicates that cellular factors other than the broadly expressed virus receptor(s) modulate Ebola virus tropism. Here we demonstrate that the C-type lectins DC-SIGN and DC-SIGNR avidly bind Ebola glycoproteins and greatly enhance transduction of primary cells by Ebola virus pseudotypes and infection by replication-competent Ebola virus. DC-SIGN and DC-SIGNR are expressed in several early targets for Ebola virus infection, including dendritic cells, alveolar macrophages, and sinusoidal endothelial cells in the liver and lymph node. While DC-SIGN and DC-SIGNR do not directly mediate Ebola virus entry, their pattern of expression *in vivo* and their ability to efficiently capture virus and to enhance infection indicate that these attachment factors can play an important role in Ebola transmission, tissue tropism, and pathogenesis.

INTRODUCTION

Ebola (EBO) virus has caused several major outbreaks of severe hemorrhagic fever in Central Africa over the last 25 years with documented fatality rates approaching 90% for subspecies Zaire (Who, International Study Team, 1978). EBO and Marburg virus constitute the only two members of the Filovirus family of negative-stranded RNA viruses. EBO encodes a single heavily glycosylated (over 50% of total mass) viral surface protein, GP. GP is a major determinant of cellular and tissue tropism, presumably by mediating receptor binding and virus entry. Recently, folate receptor-α has been implicated in playing a role in EBO entry into some, but not all, cell types (Chan *et al.,* 2001). Folate receptor-α and other receptors for EBO appear to be widely expressed as many cell lines from multiple avian and mammalian species are susceptible to infection by pseudotype viruses bearing EBO-GP (Wool-Lewis and Bates, 1998). In addition, EBO can be detected in virtually all organs and many cell types, including tissue macrophages, endothelial cells, hepatocytes, and fibroblasts in the terminal stages of disease (Baskerville *et al.,* 1985; Fisher-Hoch and McCormick, 1999; Ryabchikova *et al.,* 1999a,b). In experimental primate studies, however, cells of the monocyte/macrophage lineage constitute a predominant early cell target for infection and are thought to be responsible for disseminating the virus to multiple target organs (Ryabchikova *et al.,* 1999a,b; Schnittler and Feldmann, 1999). Detection of EBO replication in organs also appears to follow a distinct order, with liver, spleen, and lymph nodes being early targets. Thus, specific receptors or attachment factors may promote infection of certain cell types. Identification of such factors will aid in the understanding and potential prevention of EBO transmission and pathogenesis.

The C-type lectin DC-SIGN is expressed on a variable proportion of alveolar macrophages, placental macrophages, and some types of dendritic cells, while its closely related homolog DC-SIGNR is expressed on endothelial cells in the liver and lymph node sinuses (Bashirova *et al.,* 2001; Geijtenbeek *et al.,* 2000; Jameson *et al.,* 2002; Pöhlmann *et al.,* 2001c; Soilleux *et al.,* 2000, 2002). Both proteins efficiently bind and transmit human immunodeficiency virus (HIV) to receptor positive cells in a carbohydrate-dependent manner and may promote HIV dissemination in and between hosts (Bashirova *et al.,* 2001; Geijtenbeek *et al.,* 2000; Pöhlmann *et al.,* 2001c). Similar to HIV gp120, virion-associated filovirus GP con-

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tains hybrid and high mannose carbohydrates (Geyer *et al.,* 1992) and it has recently been demonstrated that DC-SIGN/DC-SIGNR selectively interact with high mannose sugars (Feinberg *et al.,* 2001). Thus, the ability of these lectins to interact with EBO-GPs and to influence virus infection was investigated.

RESULTS

DC-SIGN and DC-SIGNR enhance binding and transduction of EBO-GP pseudotypes

EBO-GP from the highly pathogenic Zaire subspecies (EBOZ-GP) as well as the envelope proteins (Env) of amphotropic murine leukemia virus (MLV) and vesicular stomatitis virus (VSV) were pseudotyped onto HIV virus cores. Binding of virus pseudotypes to DC-SIGN or DC-SIGNR expressing 293T cells was analyzed at 4°C, since under these conditions virus transduction of target cells is negligible (Fig. 1A and data not shown). The presence of DC-SIGN or DC-SIGNR had little or no effect on binding of virions bearing the VSV or MLV Env glycoproteins (Fig. 1A). In contrast, EBOZ-GP pseudotype binding to DC-SIGN/DC-SIGNR-expressing cells was strongly enhanced compared to control cells. EBOZ-GP pseudotypes bound poorly to cells transfected with empty vector, despite the fact that such pseudotypes transduce 293T cells (Wool-Lewis and Bates, 1998). EBOZ-GP binding to DC-SIGN/DC-SIGNR was not limited to HIV-based pseudotypes as MLV core particles pseudotyped with EBOZ-GP also bound to DC-SIGN- and DC-SIGNR-expressing cells but only poorly to 293T cells expressing vector alone (data not shown).

To determine whether enhanced binding of EBO-GP pseudotypes to cells expressing DC-SIGN or DC-SIGNR led to enhanced transduction, we challenged transiently transfected 293T cells with HIV luciferase reporter viruses bearing EBOZ-GP, the GP from the less pathogenic EBO Reston (EBOR-GP) subspecies, or the VSV-G protein (Fig. 1B). 293T cells are susceptible to EBO pseudotype transduction (Wool-Lewis and Bates, 1998), yet entry of both types of EBO-GP pseudotypes into 293T cells was enhanced over 8-fold by DC-SIGN and 14-fold by DC-SIGNR expression (Fig. 1B). In contrast, DC-SIGN or DC-SIGNR expression did not enhance transduction of VSV-G-pseudotyped particles (Fig. 1B). Enhanced transduction of EBOZ-GP particles due to DC-SIGNR expression was blocked by DC-SIGNR-specific monoclonal antibody 604, while enhanced transduction due to DC-SIGN expression was blocked by monoclonal antibody 526 that recognizes both DC-SIGN and DC-SIGNR (Fig. 1C) (Baribaud *et al.*, 2002). Enhanced transduction was also prevented by mannan, a carbohydrate that blocks interactions between DC-SIGN and its ligands (Geijtenbeek *et al.,* 2000), indicating that carbohydrate recognition was important for EBO-GP interactions with DC-SIGN/DC-SIGNR (Fig. 1C). These results are in agreement with a recent study by Alvarez *et al.,* who also found that expression of DC-SIGN or DC-SIGNR on cell lines enhanced virus pseudotype binding and transduction (Alvarez *et al.,* 2002).

To prove that enhancement of EBO-GP pseudotype transduction was not limited to conditions of high DC-SIGN surface expression, quantitative fluorescence-activated cell sorting (Q-FACS) analysis was used to determine the cell-surface copy numbers of DC-SIGN. Q-FACS relies on a series of precalibrated beads that bind fixed numbers of IgG molecules, allowing calculation of the absolute number of antibody-binding sites on a cell (Lee *et al.,* 1999). As few as 40,000 copies of cell surface expressed DC-SIGN or DC-SIGNR molecules were sufficient to significantly enhance EBOZ-GP-mediated transduction using an inducible expression system to vary levels of DC-SIGN or DC-SIGNR (Fig. 1D). Importantly, immature dendritic cells typically express more than 250,000 copies of DC-SIGN (Fig. 1E), indicating that the effects we observed due to DC-SIGN/DC-SIGNR expression *in vitro* occurred at surface expression levels that are physiologically relevant. To address the specificity of interactions between DC-SIGN and Ebola virus pseudotypes, we also examined the ability of replication-competent herpes simplex virus type 1 (HSV-1) to engage DC-SIGN (Fig. 1F), as this virus harbors several glycosylated Env proteins, as well as HIV pseudotypes bearing the glycoproteins from Lassa fever virus, MLV, or VSV (Fig. 1G). Lassa fever virus and MLV-GP are both heavily glycosylated, with Lassa-GP of the Josiah strain containing 11 potential N-linked glycosylation sites (Auperin and McCormick, 1989) and MLV-GPs harboring up to seven N- and seven O-linked glycosylation sites (Linder *et al.,* 1994). In contrast, VSV-G contains only two utilized glycosylation sites (Hunt and Summers, 1977). We found that HSV-1 infected target cells expressing high and low levels of DC-SIGN/DC-SIGNR or the C-type lectin CD23 with similar efficiency and that the presence of DC-SIGN did not enhance transduction of Lassa or VSV-G virus pseudotypes (Fig. 1G). Infection by virus pseudotypes bearing the MLV Env protein was enhanced slightly by high levels of DC-SIGN expression (average of 295 cps at high DC-SIGN levels compared to 65 cps at low levels). In contrast, expression of DC-SIGN/DC-SIGNR but not CD23 strongly enhanced transduction by EBOZ-GP-bearing pseudotypes (Fig. 1G). Thus, the mere presence of multiple carbohydrates in a viral Env protein is not sufficient for functional DC-SIGN/DC-SIGNR engagement, and enhancement of EBOZ-GP-mediated infection was not observed with a heterologous C-type lectin, indicating that the DC-SIGN/DC-SIGNR interaction with EBO-GP is specific.

DC-SIGN and DC-SIGNR do not directly mediate EBO-GP pseudotype transduction

The ability of DC-SIGN and DC-SIGNR to enhance EBO-GP-mediated transduction prompted us to investi-

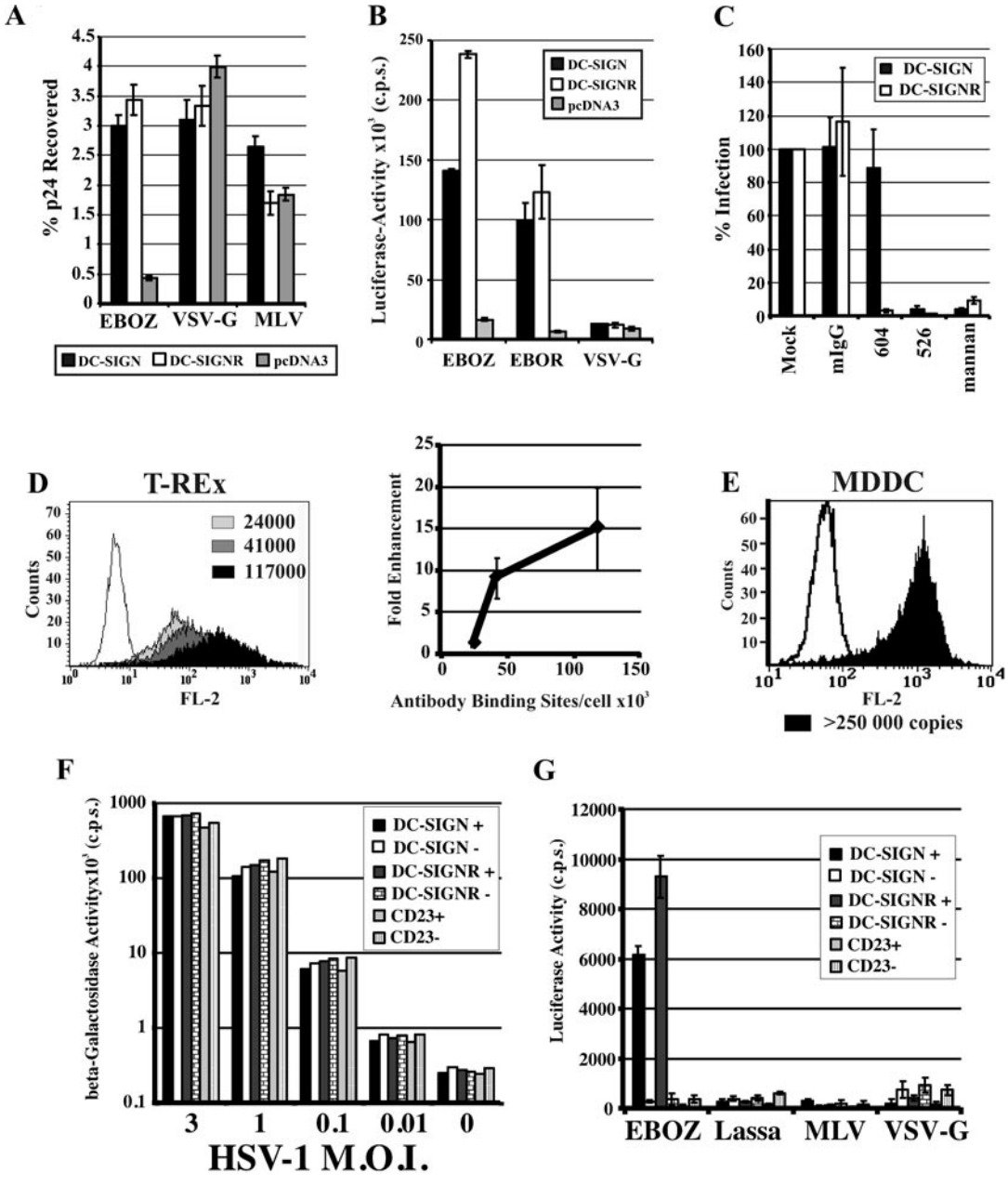


FIG. 1. Enhancement of EBOZ-GP pseudotype binding and transduction by DC-SIGN and DC-SIGNR. (A) Binding of HIV pseudotypes bearing EBOZ-GP, VSV-G, or MLV Env to DC-SIGN, DC-SIGNR, or a control plasmid expressing 293T cells. The data are presented as % of recovered p24-antigen and represent the average ± standard error of the mean (SEM) of three independent experiments. (B) DC-SIGN/DC-SIGNR-mediated enhancement of transduction with EBO-GP-bearing HIV particles. Transduction of 293T cells transiently expressing DC-SIGN, DC-SIGNR, or a control plasmid with HIV-luciferase reporter viruses pseudotyped with the EBOZ-GP, EBOR-GP, or VSV-G envelopes. The average ± standard deviation (SD) of a single experiment carried out in triplicate is shown. Similar results were obtained in two additional independent experiments. (C) Inhibition of EBOZ-GP bearing HIV pseudotype transduction by DC-SIGN- or DC-SIGNR-specific antibodies. DC-SIGN/DC-SIGNR T-REx cells were induced with doxycycline and preincubated with 20 μg/ml of the indicated antibodies or mannan. MAb 604 is specific for DC-SIGNR, while MAb 526 recognizes both DC-SIGN and DC-SIGNR. mIgG indicates control murine IgG. Results are expressed as a percentage of virus transduction in the absence of inhibitors, which resulted in approximately 15,000 and 45,000 counts per second (cps) for DC-SIGN and DC-SIGNR, respectively. The results of a single experiment ± SD carried out in triplicate are shown; comparable results were obtained in an independent experiment. (D) Assessment of DC-SIGN copy numbers required for enhancement of EBOZ-GP-mediated transduction. T-REx DC-SIGN cells were seeded in duplicate, induced with doxycycline, and DC-SIGN expression and transduction efficiency analyzed in parallel. DC-SIGN expression was assessed by Q-FACS, with the number of antibody-binding sites obtained after each induction is indicated (left panel). The induced cells were transduced as described above and luciferase activity determined 3 days after transduction (right panel). The results of a single experiment ± SD are shown. The results were confirmed in an independent experiment. (E) Quantification of DC-SIGN copy numbers on monocyte-derived dendritic cells as determined by Q-FACS. The value obtained exceeded the linear range of the assay, indicating that these cells express at least 250,000 copies of DC-SIGN. Similar results were obtained in independent experiments with MDDC from different donors (Baribaud *et al.,* 2002). (F and G) Infection of DC-SIGN-, DC-SIGNR-, and CD23-expressing T-REx cells with herpes simplex virus 1 (HSV-1) or transduction by HIV pseudotypes bearing the EBOZ, Lassa fever virus, MLV, or VSV glycoproteins. T-REx cells expressing the indicated lectins were infected with a herpes virus simplex type-1 reporter virus (F) or with the indicated pseudotypes (G). Infection of fully induced cells was compared to infection of uninduced cells, expressing relatively low levels of the indicated lectins as determined by FACS (data not shown). The results of a single experiment ± SD are shown. The results were confirmed in an independent experiment.

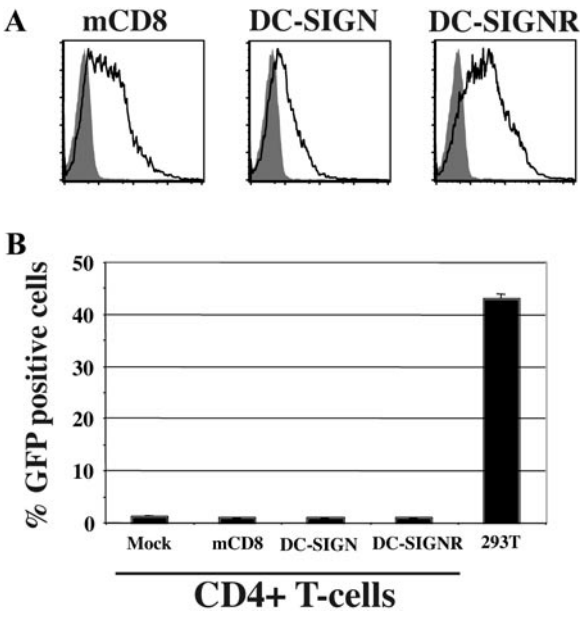


FIG. 2. EBOZ-GP-mediated transduction of DC-SIGN/DC-SIGNR-expressing primary T cells. (A) Purified CD4+ T cells were prepared from whole blood, transduced with lentiviruses encoding DC-SIGN, DC-SIGNR, or murine CD8, and expression verified by FACS. (B) The transduced T cells as well as control 293T cells were transduced with EBOZ-GP bearing HIV-GFP pseudotypes and the percentage of GFP-positive CD4-expressing T cells or 293T control cells analyzed by FACS. The results of a single experiment carried out in triplicate ± SD are shown. The results were confirmed in an independent experiment.

gate if these lectins function as cellular receptors for EBO. Lentiviral vectors encoding DC-SIGN, DC-SIGNR, or murine CD8 used as a negative control led to high levels of expression upon transduction into primary $CD4^+$ T cells which are not targets for EBO transduction (Fig. 2A). Expression of DC-SIGN or DC-SIGNR did not result in T cell transduction with EBOZ-GP pseudotypes (Fig. 2B), although control 293T cells were efficiently transduced. Moreover, VSV-G pseudotypes transduced the T cells efficiently, confirming that the block to transduction was at the level of virus entry (data not shown). In addition, stable expression of DC-SIGN in the promonocytic cell-line THP, which cannot be efficiently transduced by EBOZ-GP pseudotypes, did not increase susceptibility to transduction (data not shown). In contrast MLV pseudotypes transduced these cells efficiently (data not shown). Thus, these proteins function as highly efficient attachment factors for EBO, but do not function as primary entry receptors in the cell types examined.

DC-SIGN and DC-SIGNR significantly enhance transduction of primary cell types

In primates and humans, the liver is a primary target organ of EBO infection (Ryabchikova *et al.,* 1999a). DC-SIGNR is expressed on liver sinusoidal endothelial cells (Bashirova *et al.,* 2001; Pöhlmann *et al.,* 2001c) where it may help target virus to this organ. Indeed, in both human postmortem material and animal models extensive virus replication is seen in liver sinusoidal endothelial cells, as judged by inclusion bodies formed by viral core protein accumulation (Zaki *et al.,* 1999). To explore this possibility, we used lentivirus vectors to transduce DC-SIGNR, DC-SIGN, or CCR5 as a control into primary human umbilical vein endothelial cells (HUVECs). Expression of DC-SIGN or DC-SIGNR enhanced the transduction of HUVECs with EBOZ-GP HIV pseudotypes (Fig. 3A). In contrast, DC-SIGN/DC-SIGNR expression did not enhance VSV-G-mediated entry. When GFP-expressing virus pseudotypes were used, FACS confirmed that the DC-SIGNR-expressing endothelial cells were preferentially transduced (Fig. 3B). Blocking experiments with MAb 526, which binds to both DC-SIGN and DC-SIGNR,

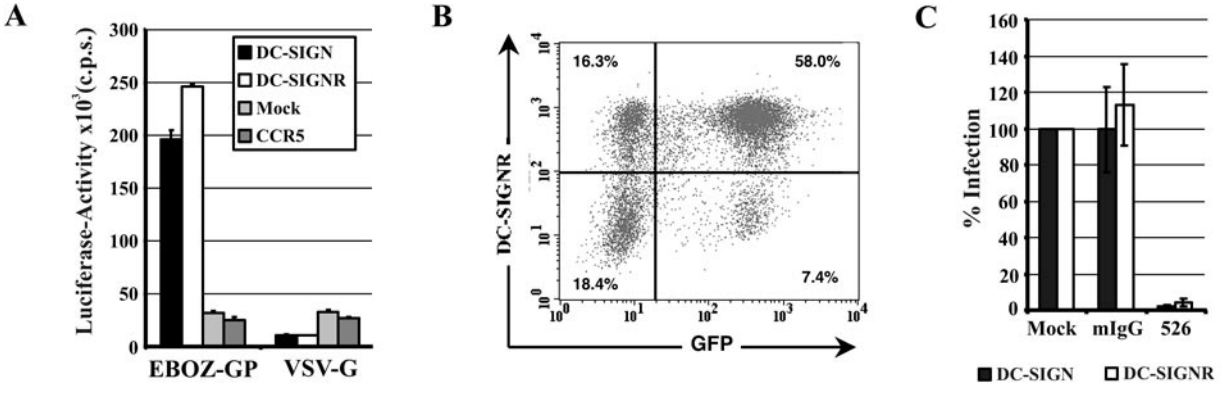


FIG. 3. EBOZ-GP-mediated transduction of DC-SIGN/DC-SIGNR expressing endothelial cells. (A) HUVECs transduced with DC-SIGN, DC-SIGNR, or CCR5 encoding lentiviruses were transduced with EBOZ-GP or VSV-G bearing HIV pseudotypes. One representative experiment of three is shown. Error bars indicate SD. (B) EBOZ-GP-bearing pseudotypes preferentially transduce DC-SIGN/DC-SIGNR-expressing cells. HUVECs were transduced with a lentiviral vector encoding DC-SIGNR and challenged with EBOZ-GP bearing HIV GFP reporter virus. The amount of DC-SIGNR/GFP-expressing cells was analyzed by FACS. (C) Inhibition of lentivirus-transduced HUVEC infection by DC-SIGN/DC-SIGNR-specific antibodies. Results are expressed as a percentage of virus transduction in the absence of inhibitors. A representative experiment performed in triplicate is shown; similar results were obtained in an independent experiment.

confirmed that EBO-GP engagement of DC-SIGN/DC-SIGNR was responsible for the enhanced entry into HUVECs (Fig. 3C).

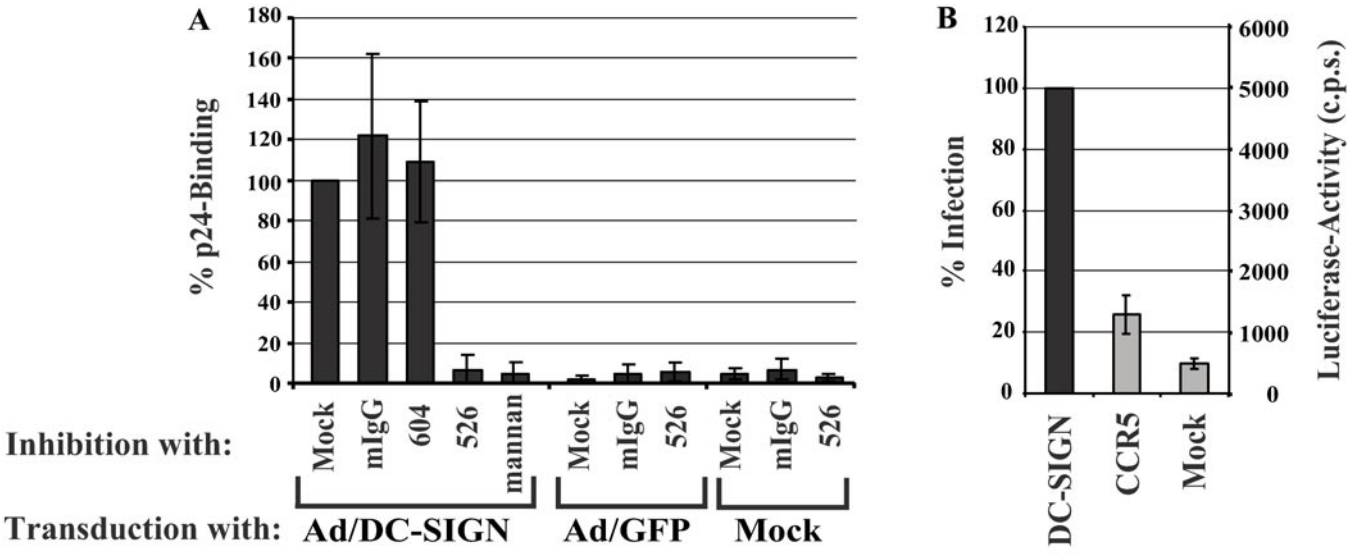
Cells of the monocyte/macrophage lineage represent the predominant cell type infected early in infection and are believed to be critical for the dissemination of virus to target organs (Ryabchikova *et al.,* 1999a,b; Schnittler and Feldmann, 1999). DC-SIGN is expressed on a variable fraction of human alveolar macrophages as well as on placental macrophages. High levels of DC-SIGN expression on blood monocyte-derived macrophages (MDM) can be induced by inflammatory cytokines such as IL-13 *in vitro* (Soilleux *et al.,* 2002), indicating that other macrophage types may express DC-SIGN during immune stimulation *in vivo*. Indeed, systemic levels of inflammatory cytokines are elevated during EBO infection (Hutchinson *et al.,* 2001; Leroy *et al.,* 2001). We investigated if DC-SIGN expression on MDM enhanced interactions with EBO-GP. MDM were transduced with a DC-SIGN encoding adenovirus (Ad/DC-SIGN) and incubated with EBOZ-GP pseudotyped HIV particles, and the amount of bound virus quantified (Fig. 4A). Virus particles bound to DC-SIGN expressing MDM far more efficiently than to control cells and binding was inhibited by preincubation of MDM with MAb526 (Fig. 4A). DC-SIGN-transduced MDM were about 5 to 12 times more susceptible to EBO-GP pseudotypes than control macrophages (Fig. 4B). Q-FACS demonstrated that the transduced MDM expressed similar levels of DC-SIGN as both cytokine-induced macrophages and dendritic cells (data not shown), indicating that the expression levels which promoted EBO-GP pseudotype infection were similar to DC-SIGN levels on primary cells. To determine whether these results reflect infection with replication-competent Ebola virus, a similar experiment was performed with live Ebola virus Zaire (Figs. 4C–4F). MDM were transduced with lentiviral vectors and Ebola virus infection was detected by immunostaining for Ebola proteins. Despite a transduction efficiency of only about 15% as judged by FACS using MAb 526, the expression of either DC-SIGN (Fig. 4E) or DC-SIGNR (Fig. 4F) rendered the MDMs sixfold more susceptible to Ebola viral infection than mock-transduced cells or cells transduced with control vector (Figs. 4C and 4D).

DISCUSSION

The filoviruses exhibit a very broad cell tropism in culture, animal models, and infected humans. The widely expressed folate receptor-α has been reported to mediate entry of both Ebola and Marburg viruses in some cell types (Chan *et al.,* 2001). Nonetheless, tropism in various animal models is much more limited early after infection. Consequently, cellular factors other than the primary receptor must contribute to EBO tropism and pathogenesis. Our results indicate that attachment factors such as DC-SIGN and DC-SIGNR might be responsible for the characteristic pattern of cell types and organs that are predominantly infected during discrete stages of the disease by efficiently capturing virus and enhancing virus infection. DC-SIGN is expressed on dendritic cells (DCs), which often encounter invading pathogens at mucosal surfaces. It has been proposed that HIV adsorbed to the surface of DCs via DC-SIGN interactions may be transported to secondary lymphoid organs and ultimately transmitted to receptor-positive cells (Geijtenbeek *et al.,* 2000). Our finding that EBO attaches to DC-SIGN suggests that if the virus encounters DCs *in vivo*, which is likely, it will bind to these cells efficiently and either infect them or be transmitted to other susceptible cell types. Indeed, a recent study by Alvarez *et al.* has shown that virus pseudotypes bearing the EBO-GP can bind to DC-SIGN on monocyte-derived dendritic cells and can be transmitted to other cells once bound (Alvarez *et al.,* 2002). They also found that the expression of DC-SIGN or DC-SIGNR on cell lines markedly enhanced infection by virus pseudotypes. DC-SIGN is also expressed on alveolar macrophages that are early targets for infection following aerosol transmission (Johnson *et al.,* 1995), and the DC-SIGN homolog DC-SIGNR is expressed on sinusoidal endothelial cells in the liver, an organ that is an important early target for filovirus infection.

Binding of EBO to DC-SIGN and DC-SIGNR was inhibited by addition of excess amounts of the carbohydrate mannan, indicating that attachment of EBO to these lectins is due to interactions between their carbohydrate recognition domains and one or more N-linked carbohydrate structures on the EBO-GP. The recently solved structure of these lectin domains coupled with carbohydrate-binding studies indicate that both DC-SIGN and DC-SIGNR preferentially bind to high mannose carbohydrates, which may in part account for the specificity exhibited by these lectins (Feinberg *et al.,* 2001; Mitchell *et al.,* 2001). EBO-GP, similar to HIV-1 Env, is heavily glycosylated with a variable number of its N-linked carbohydrate structures being fully processed upon transport through the Golgi. The presence of immature, high-mannose carbohydrate structures may be required for efficient binding to DC-SIGN. However, since most lectins bind to their ligands with relatively low affinity, the presence of several high mannose chains that could interact with several subunits in the DC-SIGN tetramer might be needed for efficient binding (Mitchell *et al.,* 2001). If so, this could explain the efficiency with which both HIV Env and EBO-GP bind to DC-SIGN and why other viruses that bear one or more glycoproteins do not.

Most of our experiments, as well as all of the experiments by Alvarez *et al.* (Alvarez *et al.,* 2002), used retrovirus pseudotypes bearing EBO-GPs. While useful experimental models, retrovirus pseudotypes differ from replication-competent filoviruses, which are long and filamentous. Thus, it was important that we confirmed



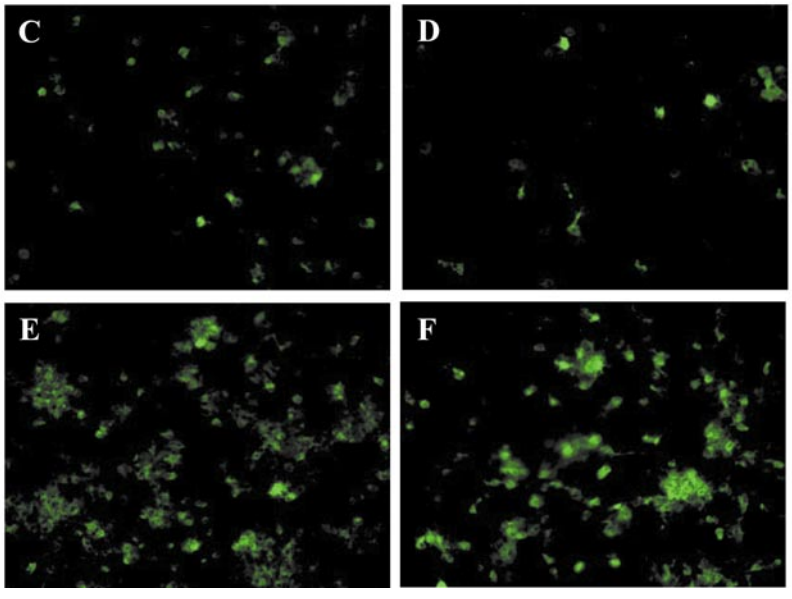


FIG. 4. EBOZ-GP-mediated binding and transduction of DC-SIGN-expressing macrophages. (A) Binding of EBOZ-GP-bearing HIV pseudotypes to Ad/DC-SIGN or Ad/GFP transduced monocyte-derived macrophages. Macrophages were preincubated with DC-SIGN/DC-SIGNR-specific antibodies or mannan. Binding is shown relative to levels of p24 observed with untreated, Ad/DC-SIGN-infected macrophages. Similar results were obtained in an independent experiment. (B) Macrophages were transduced with lentiviruses expressing DC-SIGN or CCR5 as described above and 48 h later transduced with HIV particles bearing EBOZ-GP. The cells were lysed 3 days later and luciferase activity was determined. The results represent the average ± SEM of four independent experiments. (C–F) Infection of macrophages with replication-competent EBO virus. Monocyte-derived macrophages were transduced with the indicated lentiviruses and infected with the Zaire strain of Ebola virus using an m.o.i. of 0.1. Infected cells were detected by immunostaining and the number of positive cells quantified. Representative fields are shown in (C–F). Of the mock-transduced cells about 2% stained positive for EBO (C), while approximately 1% of the CD8-transduced cells were virus positive (D). In contrast, virus infection was detected in about 7% of the DC-SIGN (E) and about 8% of the DC-SIGNR-transduced cells (F). Similar levels of enhancement were obtained upon infection with various m.o.i.s.

that DC-SIGN expression on macrophages enhanced infection by wild-type Ebola virus. Whether the effects of DC-SIGN on infection by wild-type virus is less than, similar to, or greater than the effects observed with viral pseudotypes will require additional experiments. In addition, Ebola virus infected cells secrete a soluble version of the viral glycoprotein. We have found that this soluble protein also binds to DC-SIGN and DC-SIGNR

(data not shown). Whether the presence of soluble glycoprotein will affect virus interactions with DC-SIGN and DC-SIGNR *in vivo* is not known, though the presence of multiple glycoproteins on virus particles will likely result in an avid interaction with the surface of cells expressing high levels of DC-SIGN.

Once bound to DC-SIGN, infection of either cell lines or primary cell types by EBO pseudotypes or Ebola virus was markedly enhanced. The mechanism for this enhancement is not entirely clear at present. However, virus infection can be diffusion limited and the presence of an efficient, high-affinity virus attachment factor may enhance virus infection by capturing virus at the cell surface where interactions with the receptors needed for infection occur (O'Doherty *et al.,* 2000; Pöhlmann *et al.,* 2001d). DC-SIGN might also enhance EBO infection by modifying GP conformation in a manner that enhances its membrane fusion activity. In addition, DC-SIGN contains endocytosis motifs in its cytoplasmic domain, and once bound to DC-SIGN EBO, may be efficiently endocytosed to acidic compartments where membrane fusion is thought to take place (Wool-Lewis and Bates, 1998). Additional work will be needed to fully characterize the mechanism by which EBO binds to DC-SIGN and to characterize how binding results in enhanced virus infection.

The potential importance of C-type lectins in filovirus infection is also underlined by the observation that Marburg virus, which is closely related to EBO, uses the C-type lectin asialoglycoprotein receptor (ASGP-R) for efficient infection of hepatocytes (Becker *et al.,* 1995). The significance of DC-SIGN, DC-SIGNR, and the folate receptor for filovirus infection *in vivo* will have to await the development of specific antibodies or antagonists that efficiently prevent interactions with the viral GP. However, the expression of DC-SIGN and DC-SIGNR *in vivo* coupled with the known tissue tropism of the filoviruses early after infection and our demonstration that these proteins serve as efficient attachment factors for EBO virus suggest that these proteins can play important roles in the early stages of rapidly progressive filovirus infections. More generally, our results suggest that other pathogens may take advantage of cell-surface proteins to mediate efficient attachment and, as a result, significantly enhance infection efficiency and dissemination within the host.

MATERIALS AND METHODS

Plasmids

cDNAs encoding the Zaire and Reston subtypes of EBO-GP were subcloned into the pCB6 expression plasmid as described previously (Simmons *et al.,* 2002). The coding sequence of CD23 was amplified from tissue cDNA and cloned into pcDNA4 (Invitrogen). To detect surface expression, an AU-1 tag was added using PCR-based mutagenesis. The Lassa virus (strain Josiah) glycoprotein was PCR amplified and cloned into pCMV (Clontech). pHIT60 (MLV gag/pol), pHIT111 (*lac*Z), and DC-SIGN/DC-SIGNR expression plasmids have been previously described (Pöhlmann *et al.,* 2001a,c; Soneoka *et al.,* 1995).

HIV pseudotype production and binding assay

HIV pseudotypes were generated by cotransfection of 293T cells with the indicated Env-encoding plasmids together with the pNL4-3-Luc-R^-E^- plasmid as described (Connor *et al.,* 1995). The viral supernatants were p24-normalized and the binding assay performed as previously described (Pöhlmann *et al.,* 2001a,c), with the exception that pseudotypes and cells were incubated at 4°C to prevent infection.

Quantification of DC-SIGN copy numbers

Endogenous expression of cell-surface DC-SIGN was analyzed on monocyte-derived dendritic cells (MDDCs). Immature MDDCs were generated by purifying monocytes from PBMC by discontinuous Percoll gradient centrifugation and plastic adhesion and by culturing the cells in RPMI 1640 supplemented with GM-CSF (50 ng/ml) and IL-4 (100 ng/ml) for 7 days. The immature MDDC had the following phenotype: HLA-DR^{high}, $CD11c^{high}$, $CD14^-$, $CD80^+$, $CD83^-$, as judged by flow cytometry (data not shown). DC-SIGN expression on these cells was assessed using a quantitative FACS technique (Q-FACS) as described (Lee *et al.,* 1999; Pöhlmann *et al.,* 2001a). In brief, commercially available microbeads (Sigma, St. Louis, MO) harboring defined numbers of antibody-binding sites were included in the FACS analysis and processed similar to regular samples. The binding capacity of the microbeads was used as a standard to extrapolate the antibody-binding sites on the cells analyzed.

Transduction of 293T and DC-SIGN/DC-SIGNR-expressing T-REx cells

293T cells were transiently transfected with pcDNA3, DC-SIGN, or DC-SIGNR plasmids and seeded into 24-well or 96-well plates. Forty-eight hours posttransfection, cells were challenged overnight with 200 μl (for 24-well plate) of medium containing 800 $TCID_{50}$ (as determined on 293T cells) of HIV-luciferase pseudotypes bearing EBOZ-GP, EBOR-GP, or VSV-G envelopes (Figs. 1B and 1G). Alternatively, virus stocks normalized for p24 antigen content by ELISA (Coulter Beckman) were used for infection (Fig. 1C). Fresh medium was added 12 h thereafter and luciferase expression was quantified 48 or 72 h after transduction using a commercially available kit (Promega, Madison, WI). T-REx cells (Invitrogen) that inducibly express DC-SIGN/DC-SIGNR have been described previously (Pöhlmann *et al.,* 2001a,b). Cells with inducible CD23 expression were similarly generated us-

ing the T-REx system. Gene expression was induced with doxycycline overnight and cells were transduced with pseudotypes bearing the EBOZ, Lassa fever virus, VSV, or MLV glycoproteins as described for the transiently transfected 293T cells. Infection by HSV-1 was assessed using KOS/tk12, a replication-competent HSV-1 reporter virus which carries a β-galactosidase expression cassette in the thymidine kinase locus of the viral genome (Warner *et al.,* 1998). β-Galactosidase enzyme activity was assessed 7 h after infection using a commercially available kit (Tropix). To correlate DC-SIGN copy numbers and enhancement of infection, T-REx DC-SIGN cells were seeded in duplicate wells and induced overnight with increasing concentrations of doxycycline and one panel was used for Q-FACS analysis, whereas the second panel was used for infectivity studies as described above.

Transduction and infection of PBMCs, HUVECs, and macrophages with HIV pseudotypes bearing EBO-GP

Recombinant lentivirus pseudotypes expressing DC-SIGN, DC-SIGNR, or CCR5 were generated by cotransfection of 293T cells with plasmids encoding VSV-G envelope (pMD.G), a packaging construct (pMDLg/pRRE), a Rev expression construct (RSV-Rev), and a modified transfer vector construct (pRRL.SIN-18), in which the HIV-1 cPPT element was added as previously described (Dull *et al.,* 1998). Recombinant adenoviruses expressing DC-SIGN and GFP (Ad/DC-SIGN), or GFP alone (Ad/GFP), were generated from pAdTrack-CMV/DC-SIGN or pAdTrack-CMV/5RCC adenovirus shuttle vectors and pAdEasy-1 adenoviral backbone using homologous recombination in bacteria as previously described (He *et al.,* 1998). PBMC were Ficoll isolated from whole blood of healthy donors, CD4+ T cells were purified by negative magnetic bead selection and PHA and IL-2 stimulated for 3 days, and seeded in 24-well dishes. Human umbilical vein endothelial cells (Clonetics) were set-up in a 48-well plate at 2×10^4 cells per well in EGM medium (Clonetics). Monocytes were purified by elutriation of an apheresis product from a healthy donor. Monocyte-derived macrophages were differentiated in RPMI containing 10% human serum for 6 days and seeded in 48-well dishes. The next day cells were challenged overnight with 150 μl of the lentiviral vectors at a 1:2 dilution or adenovirus vectors at an m.o.i. of 75. After addition of 400 μl of medium, the cells were incubated for 2 days and then challenged overnight with 150 μl of medium containing 400 $TCID_{50}$ (as determined on 293T cells) of HIV-luciferase pseudotypes bearing EBOZ-GP or VSV-G envelopes, or 2000 $TCID_{50}$ of EBO-Z HIV-GFP pseudotypes (Figs. 2B and 3A). Alternatively, cells were infected with p24-normalized virus stocks (Figs. 3C and 4B). Fresh medium was added the following day and luciferase expression quantified 48 h after transduction or percentage of GFP-positive cells determined by FACS analysis 72 h after transduction. Binding assays using Ad/DC-SIGN-transduced macrophages were performed as for 293T cells.

Infection of macrophages with EBO virus

Day 5 monocyte-derived macrophages were seeded overnight at 2×10^5 in eight-well chamber slides (Becton-Dickinson, NJ). Cells were transduced with lentiviral vectors as described above and 2 days posttransduction were challenged with an m.o.i. of 0.1 of live EBO Zaire, Mayinga strain. After a further 2 days the cells were fixed and immunostained using polyclonal guinea pig sera raised against EBO followed by an anti-guinea pig–FITC conjugate. Slides were then observed by fluorescence microscopy and numbers of EBO antigen-positive cells per well were quantified.

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