

# HIV-1 gp120 Glycoprotein Interacting with Dendritic Cell-specific Intercellular Adhesion Molecule 3-grabbing Non-integrin (DC-SIGN) Down-Regulates Tight Junction Proteins to Disrupt the Blood Retinal Barrier and Increase Its Permeability\*

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Approximately 70% of HIV-1 infected patients acquire ocular opportunistic infections and manifest eye disorders during the course of their illness. The mechanisms by which pathogens invade the ocular site, however, are unclear. Under normal circumstances, vascular endothelium and retinal pigment epithelium (RPE), which possess a well developed tight junction complex, form the blood-retinal barrier (BRB) to prevent pathogen invasion. We hypothesize that disruption of the BRB allows pathogen entry into ocular sites. The hypothesis was tested using *in vitro* models. We discovered that human RPE cells could bind to either HIV-1 gp120 glycoproteins or HIV-1 viral particles. Furthermore, the binding was mediated by dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) expressed on RPE cells. Upon gp120 binding to DC-SIGN, cellular NF- $\kappa$ B signaling was triggered, leading to the induction of matrix metalloproteinases, which subsequently degraded tight junction proteins and disrupted the BRB integrity. DC-SIGN knockdown or prior blocking with a specific antibody abolished gp120-induced matrix metalloproteinase expression and reduced the degradation of tight junction proteins. This study elucidates a novel mechanism by which HIV, type 1 invades ocular tissues and provides additional insights into the translocation or invasion process of ocular complication-associated pathogens.

Highly active antiretroviral therapy (HAART)<sup>4</sup> effectively reduces the HIV type 1 (HIV-1) viral load to below the limit of detection in most infected individuals. Nevertheless, an HIV-1 reservoir persists in a small number of infected cells. It is now known that the gastrointestinal mucosa, lymph nodes, genital tract, and central nervous system represent the major anatomical reservoirs (1–6). Occasionally, HIV-1 RNA or antigens can also be detected in intraocular tissues such as the conjunctiva and retinal vascular endothelium and sometimes even in contact lenses (7–10). Our previous study has demonstrated the detection of HIV-1 RNAs in the tears of HIV-1 patients, including those whose viral loads were completely suppressed by HAART therapy. Moreover, HIV viral loads in intraocular tissues were higher than in plasma in some individuals (11), suggesting that intraocular tissues might also be a reservoir for HIV-1.

A proportion of HIV-1 patients have ocular complications (12–15), mainly attributable to various ocular opportunistic infections. For instance, cytomegalovirus, varicella-zoster virus and herpes simplex virus have been described as the most common causes for retinitis, iridocyclitis, keratitis, and other ocular diseases in HIV-1 patients (13, 16, 17). In a cross-sectional study of 787 cases of HAART-treated HIV-1 patients in eastern China, we have found the prevalence of ocular complications to be 26.3%; among them, cytomegalovirus retinitis had the highest prevalence (10.6%), followed by ocular microangiopathy (9.4%) (15). Other than HIV-1 and AIDS-associated opportunistic pathogens, Ebola virus had been detected in ocular tissues among survivors during convalescence (18). One patient manifested severe unilateral uveitis during convalescence from

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<sup>4</sup> The abbreviations used are: HAART, highly active antiretroviral therapy; HIV-1, HIV, type 1; RPE, retinal pigment epithelium; BRB, blood-retinal barrier; JAM, junctional adhesion molecule; ZO, zonula occludens; BBB, blood-brain barrier; DC-SIGN, dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin; MMP, matrix metalloproteinase; MFI, mean fluorescence intensity; PDTTC, pyrrolidine dithiocarbamate; TEER, trans-epithelial electrical resistance; VLP, virus-like particle; PE, phycoerythrin; RPE, retinal pigment epithelium; IKK, inhibitor of nuclear factor  $\kappa$ -B kinase; CRISPR/Cas9, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) 9.

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Ebola; viable amounts of Ebola virus were detected in aqueous humor at the onset of disease as well as 9 weeks after the clearance of viremia (19). How pathogens invade the ocular tissues and cause diseases is poorly understood.

The human retina is a highly specialized neural tissue located at the posterior of the eye between the vitreous body and the choroid (20, 21). It is comprised of vascular cells, pigment epithelium, neurons, and microglia or resident macrophages that are organized into distinct layers. Both the vascular endothelium and RPE possess a well developed tight junction complex to form the BRB, which confers a stringent control of solute and fluid permeability and maintains the appropriate environment for a functional retina (21).

The tight junction complex includes the tight junction and adherens junction. Tight junctions form an apical impermeable barrier to fluid (22, 23). Although many proteins contribute to the formation of the tight junction, the major types are transmembrane proteins, including claudins, occludins, and junctional adhesion molecules (JAMs), and the intracellular scaffolding protein zonula occludens (ZO) family (22, 24). The transmembrane proteins are linked to the cytoskeleton via an interaction with the ZO family of scaffolding proteins. The adherens junctions, such as endothelial cadherin-based or nectin-based adherens junctions, are essential for development of the barrier function and influence formation of the tight junction (22, 23).

HIV-1 envelope glycoprotein gp120 mediates viral entry by binding to receptors on host cells. Notably, gp120 can induce the degradation of tight junction proteins in human brain microvascular endothelial cells and has been associated with increased permeability of the blood brain barrier (BBB) during progressive HIV-1-associated dementia (25–29). The enhanced BBB permeability induced by HIV-1 gp120 has also been observed in transgenic mice (26, 30). Treatment with HIV-1 gp120 down-regulated the expression of tight junction proteins in human RPE cells and led to increased monolayer permeability and consequent translocation of HIV-1 and bacteria across the epithelium (31). The molecular mechanism by which gp120 down-regulates tight junction proteins remains elusive.

In this study, we found that HIV-1 gp120 or viral particles could be captured by human RPE cells through binding to DC-SIGN expressed on their surface. The binding then triggered cellular NF- $\kappa$ B signaling for the induction of matrix metalloproteinase (MMP), which degraded tight junction proteins and thus caused disruption of BRB integrity. These data elucidate a novel mechanism by which HIV-1 invades intraocular tissues and suggests a common pathway for ocular invasion by pathogens.

## Results

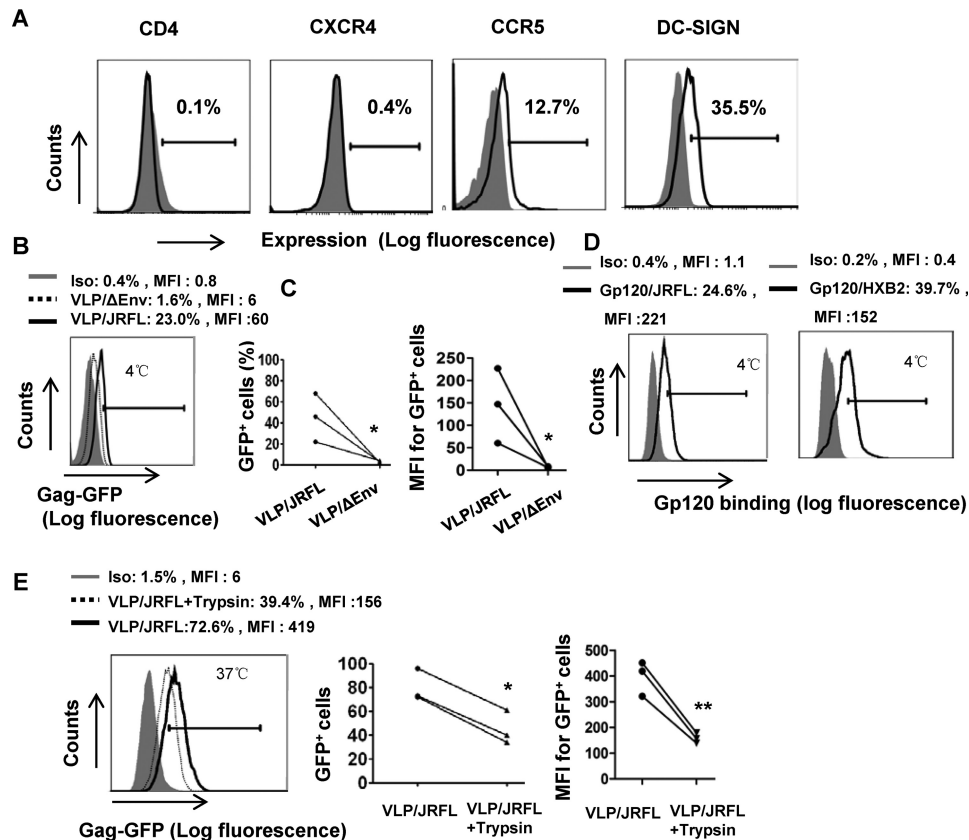
*ARPE-19 Cells Mediate the Uptake of HIV-1 Particles*—ARPE-19 is a human retinal pigment epithelial cell line. To evaluate whether ARPE-19 cells have the molecular basis for interacting with HIV-1, we first examined their expression of the HIV-1 receptor CD4 and co-receptors CCR5 and CXCR4 as well as the DC-SIGN molecule, which has been demonstrated previously to mediate HIV-1 binding (32). The results from

flow cytometry showed that APRE-19 cells expressed DC-SIGN and CCR5 but not CD4 and CXCR4 (Fig. 1A).

Next we investigated whether HIV-1 can bind to ARPE-19 cells. VLPs pseudotyped with envelope proteins from HIV-1 JRFL was used as a representative of the HIV virion, and the VLP/ $\Delta$ Env that do not incorporate HIV-1 envelope proteins were used as a control for monitoring nonspecific binding. At 4 °C, 23% of cells displayed Gag-GFP<sup>+</sup>, whereas nonspecific control VLP/ $\Delta$ Env showed less than 2% binding (Fig. 1B). The results from three independent repeats are summarized in Fig. 1C. To confirm the envelope-dependent binding, we examined the binding of recombinant HIV-1 gp120 glycoprotein to ARPE-19 cells and found that both HIV-1 JRFL and HXB2-derived gp120 glycoprotein bound to these cells (Fig. 1D). When the temperature was increased to 37 °C to enable endocytosis, there was increased VLP/JRFL uptake, and more than 72.6% of cells showed Gag-GFP<sup>+</sup> (Fig. 1E). Trypsin treatment could significantly but not completely remove the capture of VLPs, suggesting that both surface binding and internalization of viruses occurred (Fig. 1E). Collectively, these data demonstrate that HIV-1 VLPs can bind to ARPE-19 cells.

*DC-SIGN Expressed on ARPE-19 Cells Mediates the Binding of HIV-1 Particles through the Interaction with Viral gp120 Glycoprotein*—The DC-SIGN molecule has been demonstrated previously to mediate HIV-1 binding (32). We next evaluated the role of the DC-SIGN molecule in the capture of VLP. Cells were pretreated with an anti-DC-SIGN-specific antibody and then pulsed with VLP. The results showed that blocking of DC-SIGN diminished VLP capture at 4 °C, resulting in a decrease in the percentage and MFI value for the Gag-GFP<sup>+</sup> cell population (Fig. 2, A and B). When the DC-SIGN gene was knocked out by the CRISPR/Cas9 targeting system (Fig. 2, C and D), the binding of gp120 glycoprotein was dramatically decreased to the basal level (Fig. 2E); the same was true for the binding of VLP/JRFL or VLP/HXB2 to cells (Fig. 2F). These data demonstrate the crucial role of DC-SIGN molecules in mediating the capture of VLPs. Collectively, these data demonstrate that DC-SIGN molecules expressed on the surface of these cells mediate VLP binding through the interaction with viral envelope glycoprotein.

*Binding of HIV-1 gp120 Glycoprotein to ARPE-19 Cells Down-regulates Tight Junction Proteins*—The disruption of PRE barrier integrity was associated with the down-regulation of several tight junction proteins (21, 31). Gp120 glycoprotein can induce the down-regulation of tight junction proteins in human RPE cells (31). It would be logical to see whether the same is true in ARPE-19 cells. To do this, we cultured ARPE-19 cells in the presence of gp120 glycoprotein for 48 h and then monitored the expression of tight junction proteins, including ZO-1, Claudin-5, Occludin, and JAM-2. Both gp120 glycoproteins derived from either HIV-1 JRFL or HXB2 down-regulated the expression of ZO-1, Claudin-5, Occludin, and JAM-2 (Fig. 3, A and B). Intriguingly, blocking with anti-DC-SIGN antibody before the addition of gp120 prevented the down-regulation of these tight junction proteins (Fig. 3, B and C), suggesting that gp120 binding to DC-SIGN triggered downstream signaling involved in the modulation of tight junction protein expression. Gp120 glycoproteins-induced disruption of the tight junction



**FIGURE 1. ARPE-19 cells capture HIV-1 particles.** *A*, expression of DC-SIGN and HIV-1 receptors on ARPE-19 cells. Cells were immunostained with specific antibodies and analyzed using flow cytometry. The percentage of positive cells is shown. *B* and *C*, HIV-1 VLP binding to ARPE-19 cells. Cells were pulsed with HIV-Gag-GFP-VLP/JRFL or VLP/ΔEnv for 1 h at 4 °C and analyzed using flow cytometry. Results from three independent repeats were summarized and are presented (*C*). *D*, binding of recombinant gp120 to ARPE-19 cells. Cells were pulsed with recombinant gp120 glycoprotein for 1 h at 4 °C and then immunostained with mouse anti-gp120 antibody, followed by immunostaining with secondary anti-mouse IgG-FITC. Cells were analyzed using flow cytometry, and the percentage of positive cells and the MFI are shown. *E*, ARPE-19 cell capture of HIV-1 VLP. ARPE-19 cells were incubated with HIV-Gag-GFP-VLP/JRFL for 1 h at 37 °C, treated with trypsin for 5 min or left untreated, and analyzed using flow cytometry. Gag-GFP<sup>+</sup> cell percentage and MFI are presented. One representative result from four repeats is shown. \*\*,  $p < 0.01$ ; \*,  $p < 0.05$ . Iso, isotype.

proteins ZO-1 and Occludin was also observed under confocal microscopy (Fig. 3D). Notably, knockdown of DC-SIGN abolished the gp120-induced disruption of these tight junction proteins (Fig. 3D). Together, these data demonstrate that gp120 glycoprotein induces down-regulation of tight junction proteins on RPE cells through the interaction with DC-SIGN.

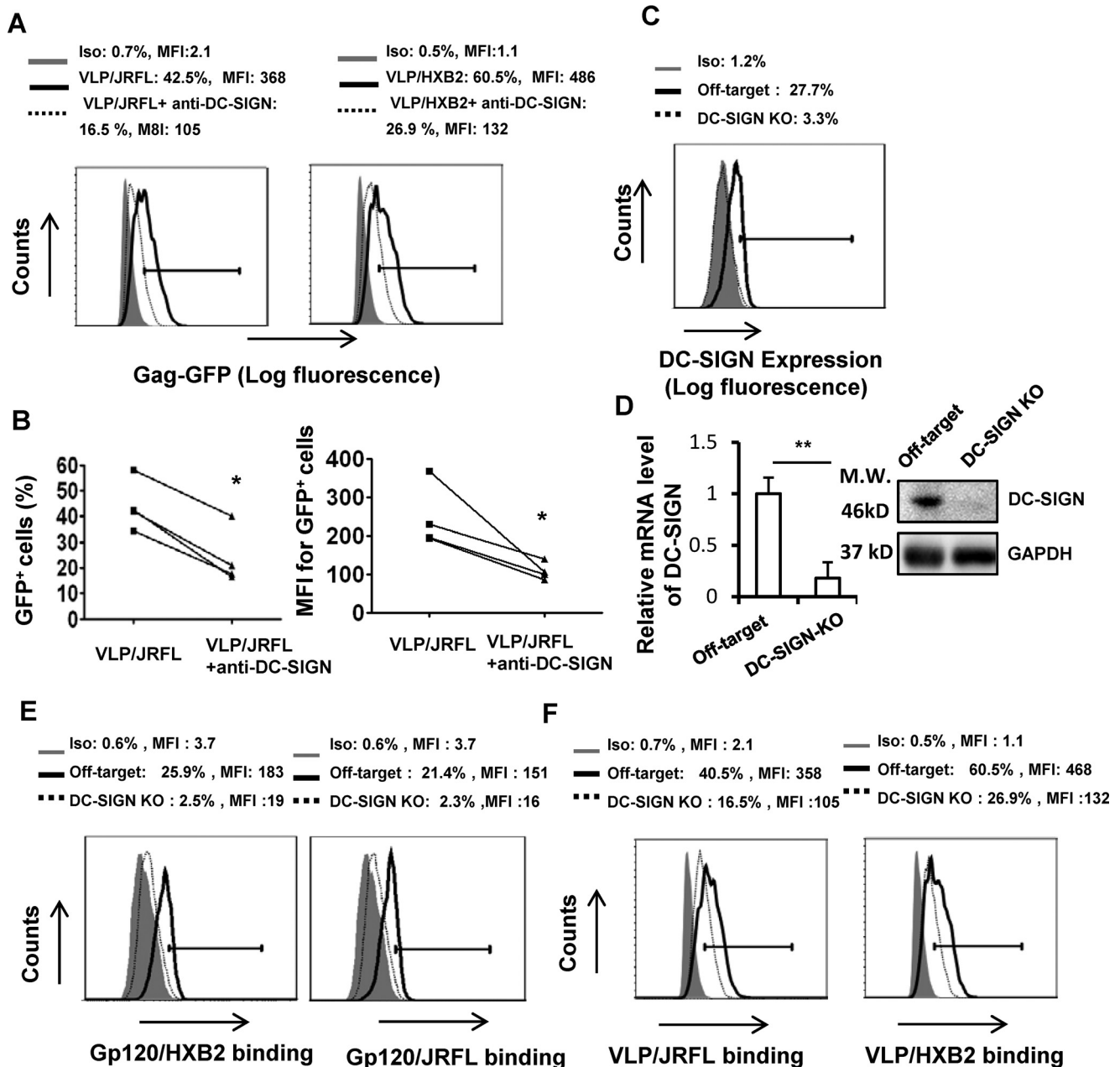
**MMPs Induced by gp120 Binding to DC-SIGN Are Responsible for the Down-regulation of Tight Junction Proteins**—MMPs are zinc-dependent endopeptidases that require calcium to function. They are responsible for degradation of extracellular matrix proteins and tight junction proteins. Increased expression of MMPs is well documented in various neurological disorders (33–35). HIV-1 gp120 can induce the expression of MMPs, resulting in disruption of BBB tight junctions (26–28). To investigate whether gp120 could also induce MMPs in ARPE-19 cells, we treated them with recombinant gp120 glycoprotein derived from HIV-1 JRFL. The expression of MMPs was measured at both transcription and translation levels. Gp120 glycoprotein treatment induced a 10- to 15-fold increase of MMP2 and MMP9, mRNA levels (Fig. 4, *A* and *B*) and markedly increased their protein expression (Fig. 4C). Coincidentally, treatment with gp120 also significantly induced expression of the inflammation factors IL-8, CCL-2, and TNF- $\alpha$  (Fig. 4A). To prove the role of the DC-SIGN-gp120 interaction in the induc-

tion of MMPs, an anti-DC-SIGN-specific antibody was used to treat ARPE-19 cells before the addition of gp120 protein. The treatment abolished the induction of MMP2 and MMP9 (Fig. 4, *B* and *C*). Moreover, when the DC-SIGN gene was knocked out by the CRISPR/Cas9 targeting system, treatment with gp120 glycoprotein was no longer able to induce MMP2 and MMP9 expression (Fig. 4D). These data demonstrate that the binding of gp120 to DC-SIGN induces the expression of MMPs.

To verify the role of induced MMPs in the down-regulation of tight junction proteins of RPE cells, a broad-spectrum inhibitor of MMPs, GM 6001, was used during gp120 treatment. The results show that the addition of GM6001 prevented gp120-induced down-regulation of the tight junction proteins ZO-1, Claudin-5, Occludin, and JAM-2 (Fig. 4E), confirming the role of MMPs in gp120-induced down-regulation of tight junction proteins. Taken together, these data demonstrate that the binding of gp120 to DC-SIGN induces MMP expression and that MMPs are responsible for the down-regulation of tight junction proteins.

**Gp120 Triggers Activation of the Cellular NF- $\kappa$ B Pathway Upstream of MMP Induction**—The binding of HIV-1 gp120 to DC-SIGN could induce varied cellular signaling, including that of the NF- $\kappa$ B pathway (36–38). To examine the involvement of this signaling pathway, we added gp120 to RPE cells and

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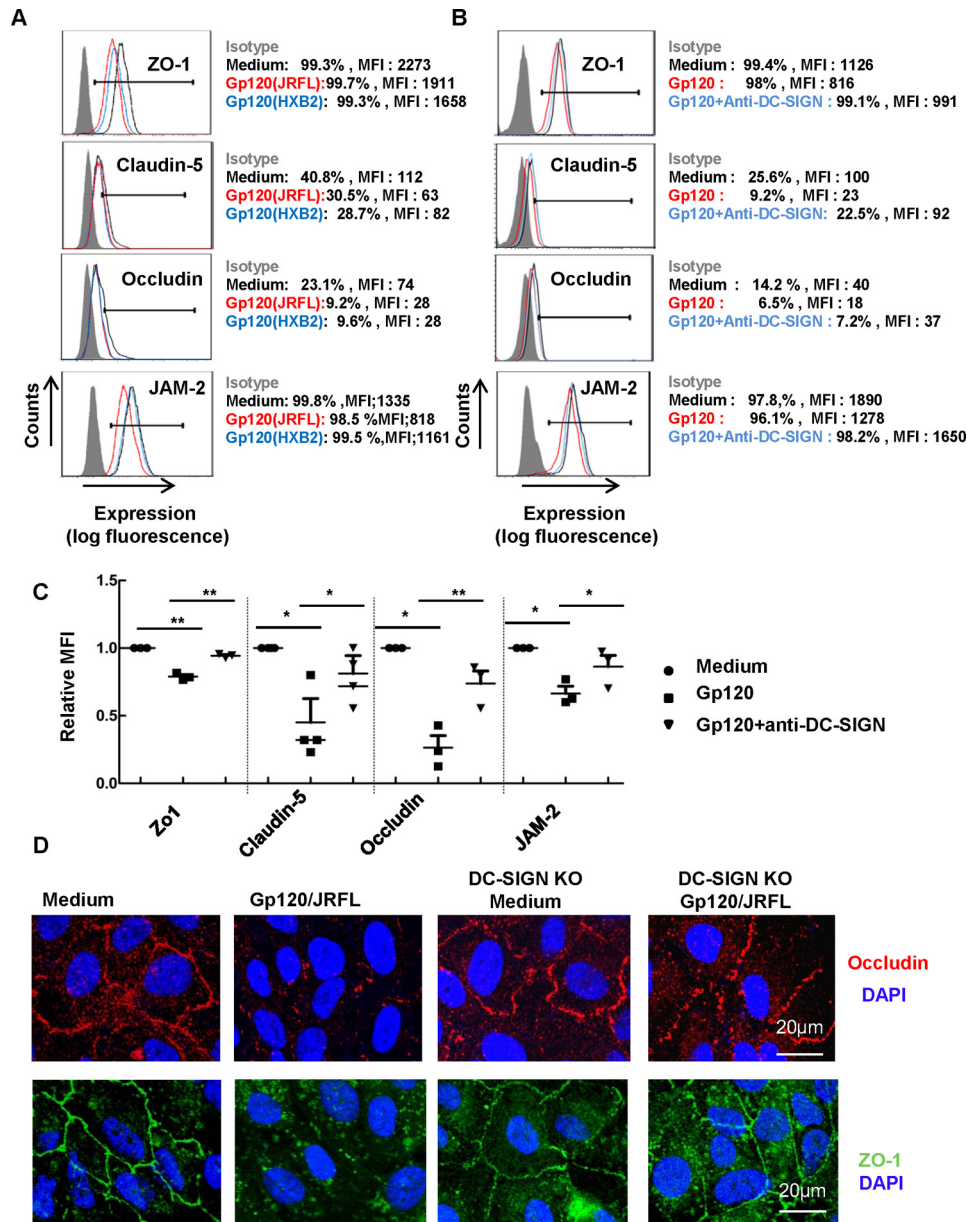


**FIGURE 2. DC-SIGN mediates HIV-1 binding to ARPE-19 cells.** *A* and *B*, preblocking with anti-DC-SIGN antibody diminishes HIV-1 VLP binding. ARPE-19 cells were treated with anti-DC-SIGN antibody before VLP loading at 4 °C performed as above. Results from four independent repeats were summarized and are presented (*B*). \*,  $p < 0.05$ . *C–E*, DC-SIGN knockout abolished HIV-1 gp120 binding. ARPE-19 cells with or without DC-SIGN knockout were pulsed with HIV-1 gp120 glycoproteins at 4 °C, and gp120 binding was detected as above. One representative result from three repeats is shown. Data are mean  $\pm$  S.D. (*D*). *F*, DC-SIGN knockout decreased the binding of VLP/JRFL or VLP/HXB2. ARPE-19 cells with or without DC-SIGN knockout were pulsed with VLP/JRFL or VLP/HXB2 for 1 h at 4 °C, and VLP binding was detected with flow cytometry. *M.W.*, molecular weight; *Iso*, isotype.

observed the induction of NF- $\kappa$ B activation, as demonstrated by the elevated phosphorylation of IKK- $\alpha/\beta$  and degradation of the inhibitory I $\kappa$ B subunit (Fig. 5A). To validate the role of the NF- $\kappa$ B pathway in gp120-induced expression of MMPs, an NF- $\kappa$ B inhibitor, pyrrolidine dithiocarbamate (PDTC), was used during the gp120 treatment. It has been reported that PDTC prevents the dissociation of inhibitory subunit I $\kappa$ B from NF- $\kappa$ B in the cytoplasm and thus suppresses the translocation of active NF- $\kappa$ B to the cell nucleus as a consequence of the inhibition of NF- $\kappa$ B activation and the reduced production of inflammatory cytokines (39). In our experimental system,

PDTC treatment suppressed dissociation of the I $\kappa$ B subunit for degradation (Fig. 5A), and consequently the gp120-induced expression of MMP2 and MMP9 and the production of inflammatory cytokines were abolished (Fig. 5, A–C). When the DC-SIGN gene was knocked out by the CRISPR/Cas9 targeting system, treatment with gp120 glycoprotein failed to activate the NF- $\kappa$ B pathway and the subsequent induction of MMP2 and MMP9 expression (Fig. 5A).

*HIV-1 gp120 Induces Breakdown of the RPE Barrier and Increases Endothelial Cell Permeability*—Having shown that gp120 could down-regulate the expression of tight junction

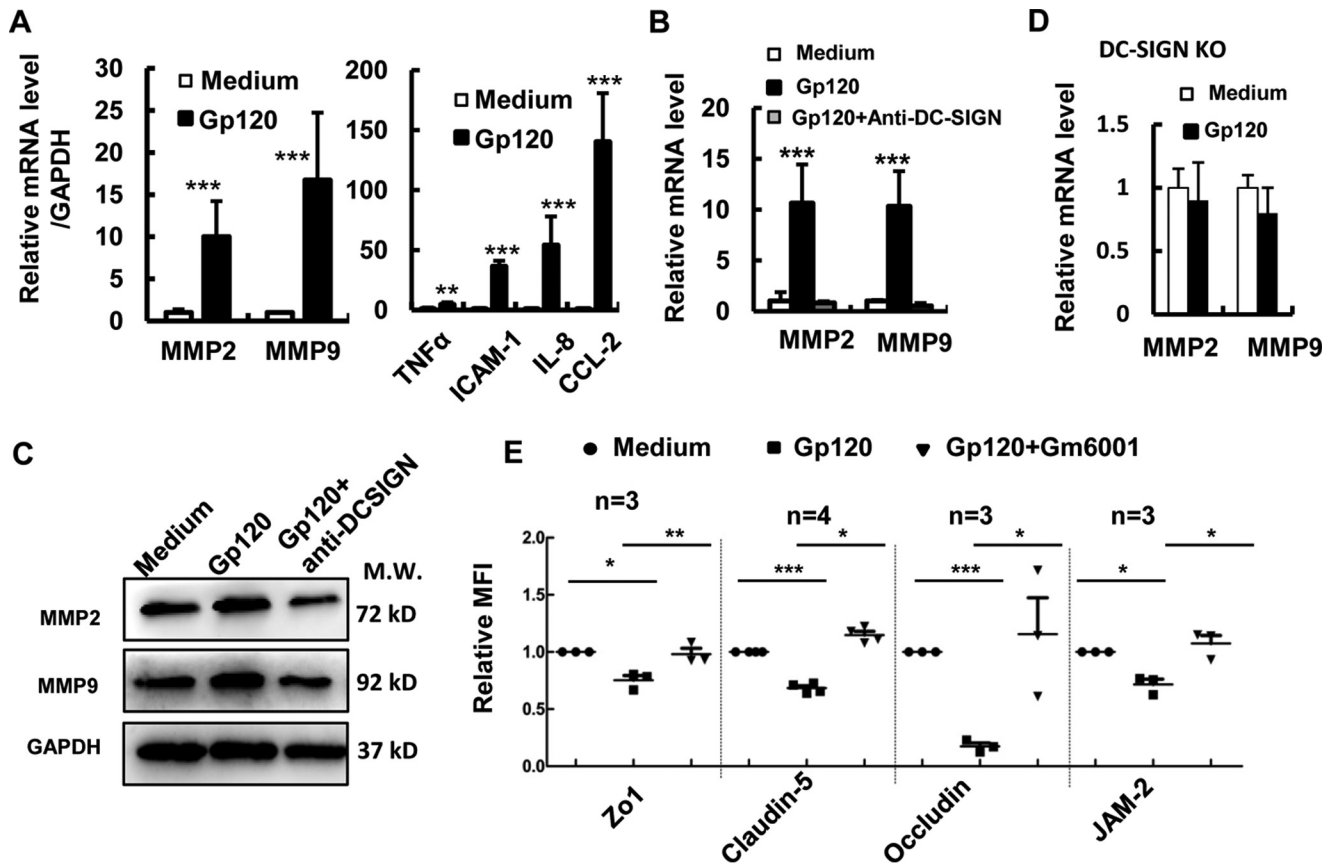


**FIGURE 3. Gp120 binding to ARPE-19 cells down-regulates tight junction proteins.** *A*, Gp120 induced down-regulation of tight junction proteins. ARPE-19 cells were treated with recombinant gp120 glycoproteins for 48 h and then immunostained with specific antibodies against ZO1, Claudin-5, Occludin, or JAM-2, followed by immunostaining with anti-rabbit or anti-mouse IgG-FITC secondary antibodies. Cells were analyzed using flow cytometry. The percentage of positive cells and MFI are presented. *B* and *C*, prior blocking with anti-DC-SIGN antibody diminishes gp120-induced down-regulation of tight junction proteins. Anti-DC-SIGN antibody was used to treat cells before adding gp120<sub>JRFL</sub>, and the expression of ZO1, Claudin-5, Occludin, and JAM-2 was detected as above. The data from three repeats were summarized and are presented, and the lines indicate the mean value (*C*). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ . *D*, Gp120 induced-disruption of tight junction proteins observed under confocal microscopy. ARPE-19 cells with or without DC-SIGN knockout were treated with HIV-1 gp120 glycoproteins or medium for 48 h at 37 °C. The expression of ZO-1 and Occludin was observed under confocal microscopy after immunostaining with specific antibodies. Nuclei were stained with DAPI.

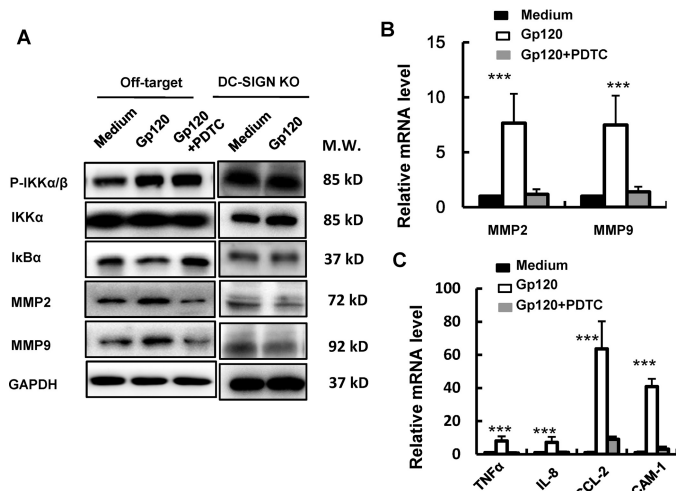
proteins, we next examined whether it could disrupt the PRE barrier. We seeded ARPE-19 cells into a transwell to form a monolayer that mimics the RPE barrier, as described previously (31, 40), and then monitored the trans-epithelial electrical resistance (TEER) values (31) and the FITC-dextran flux to evaluate the permeability of the monolayer barrier. Results showed that the TEER value reached a steady level of around 88 ohm when the ARPE-19 cells form a monolayer barrier (Fig. 6A). The addition of gp120 for 2 days in cell culture significantly decreased the TEER values (Fig. 6A) and increased the dextran flux (Fig. 6B). Moreover, prior blocking with an anti-DC-SIGN

antibody or treatment with an inhibitor of MMPs, Gm6001, prevented these changes (Fig. 6, A and B). These data prove that HIV-1 gp120 induces breakdown of the RPE barrier and increases its permeability through the induction of MMPs after binding of gp120 to DC-SIGN.

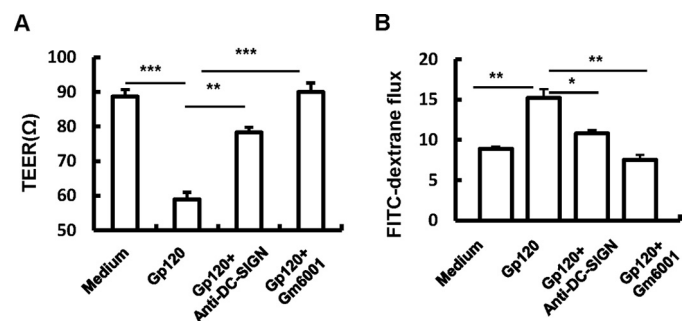
*Binding of HIV-1 gp120 to DC-SIGN Induces the Expression of MMPs in Primary Human RPE Cells*—After establishing the facts in cell lines, we then used primary human RPE cells, HRPEpiC, to confirm the induction of MMPs triggered by gp120 binding to DC-SIGN. Similar to ARPE-19 cells, HRPEpiC cells express DC-SIGN and CCR5 but not CD4 and



**FIGURE 4. Binding of gp120 to DC-SIGN induced MMP expression for down-regulation of tight junction proteins.** A, induction of MMPs and cytokines/chemokines. ARPE-19 cells were treated with recombinant gp120 glycoproteins for 6 h, and the expression of MMP2, MMP9, TNF- $\alpha$ , ICAM-1, IL-8, and CCL-2 was detected at the mRNA level by semiquantification RT-PCR and normalized with GAPDH. B-D, prior blocking with anti-DC-SIGN antibody or DC-SIGN knockout abolished the induction of MMPs. ARPE-19 cells or DC-SIGN knockout cells were pretreated or not with anti-DC-SIGN antibody for 1 h before incubation with gp120. The expression of MMP2 and MMP9 was assessed by semiquantification RT-PCR and Western blotting. E, treatment with MMP inhibitor abolished the down-regulation of tight junction proteins. ARPE-19 cells were pretreated with or without MMP inhibitor GM6001 for 1 h before further incubation with gp120 for 48 h, and the expression of ZO-1, Claudin-5, Occludin, and JAM-2 was analyzed using flow cytometry. The data from three or four repeats were summarized and are presented, and the lines indicate the mean value. Data are mean  $\pm$  S.D. (A, B, and D). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . M.W., molecular weight.

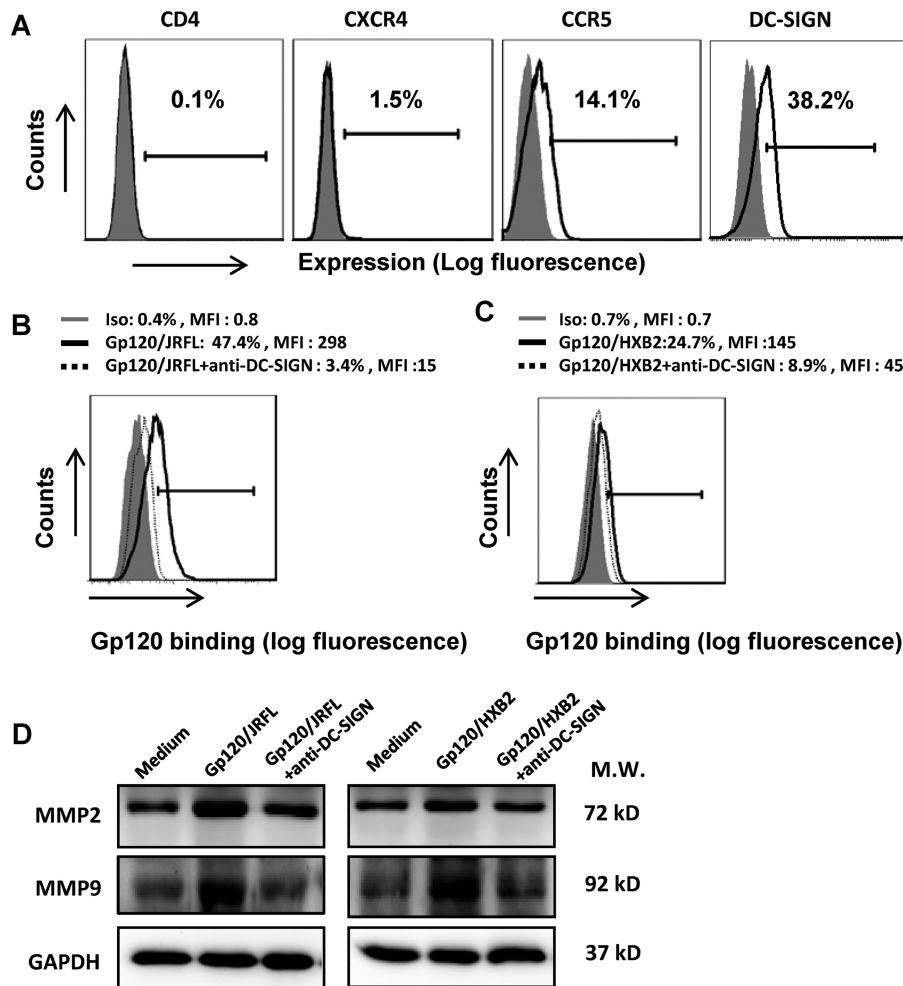


**FIGURE 5. Gp120 triggers activation of the cellular NF- $\kappa$ B pathway for MMPs induction.** A, ARPE-19 DC-SIGN knockout cells were pretreated with or without the NF- $\kappa$ B inhibitor PDTC for 1 h before further incubation with gp120 for an additional 4 h, and the expression of IKK $\alpha$ , p-IKK $\alpha$ / $\beta$ , I $\kappa$ B, MMP2, and MMP9 was monitored with Western blotting. B and C, the expression of MMP2, MMP9, and cytokines or chemokines was quantified at the mRNA level by semiquantification RT-PCR and normalized with GAPDH. Data are mean  $\pm$  S.D. M.W., molecular weight. \*\*\*,  $p < 0.001$ .



**FIGURE 6. HIV-1 gp120 induces breakdown of the RPE barrier and increases permeability.** ARPE-19 cells were seeded on Millicell inserts for growth to form the monolayer. Monolayers were treated with or without recombinant HIV-1 gp120 glycoprotein during the last 2 days of cell culture. Some samples were pretreated with anti-DC-SIGN antibodies or GM6001 before incubation with gp120. A, the TEER value was measured. B, the paracellular permeability for FITC-dextran flux was assessed. Data represent mean  $\pm$  S.D. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .  $\Omega$ , ohm.

CXCR4 (Fig. 7A) and are capable of capturing recombinant gp120 glycoproteins derived from either HIV-1 JRFL or HXB2 (Fig. 7, B and C). Also, prior blocking with an anti-DC-SIGN antibody in HRPEpiC cells diminished gp120 capture (Fig. 7, B and C), demonstrating the role of DC-SIGN in mediating gp120



**FIGURE 7. Binding of HIV-1 gp120 to DC-SIGN induces expression of MMPs in human primary RPE cells.** *A*, expression of DC-SIGN and HIV-1 receptors on HRPEpiC cells. Cells were immunostained with specific antibodies and analyzed using flow cytometry. The percentage of positive cells is shown. *B* and *C*, prior blocking with anti-DC-SIGN antibody diminishes gp120 binding. HRPEpiC cells were treated with anti-DC-SIGN antibody for 1 h before gp120 loading for an additional 1 h at 4 °C, and then cells were immunostained with mouse anti-gp120 antibody, followed by immunostaining with secondary anti-mouse IgG-FITC. Cells were analyzed using flow cytometry. The percentage of positive cells and the MFI are indicated. *D*, prior blocking with anti-DC-SIGN antibody abolished the induction of MMPs. HRPEpiC cells were pretreated with or without anti-DC-SIGN antibody for 1 h before incubation with gp120. The expression of MMP2 and MMP9 was detected by Western blotting. *Iso*, isotype; *M.W.*, molecular weight.

uptake in these primary cells. Consistent with ARPE-19 cells, recombinant gp120 glycoproteins induced expression of MMP2 and MMP9 in HRPEpiC cells (Fig. 7D), and blocking with an anti-DC-SIGN antibody abolished the induction of MMPs triggered by gp120 binding (Fig. 7D), thus confirming the crucial role of DC-SIGN in the induction of MMPs. Taken together, these data confirm that the binding of HIV-1 gp120 to DC-SIGN induces the expression of MMPs in human primary RPE cells.

## Discussion

Cumulative data have demonstrated that ocular tissues can be breached by a number of pathogens. However, the molecular mechanisms by which these pathogens enter ocular tissues remain elusive. Here we used HIV-1 as a model to investigate the ocular invasion process. We found that HIV-1 gp120 glycoprotein could bind to DC-SIGN molecules expressed on the surface of human RPE cells. The binding then triggers cellular NF- $\kappa$ B signaling for the induction of MMPs. The MMPs can

then mediate the degradation of tight junction proteins, resulting in the disruption of BRB integrity (Fig. 8).

DC-SIGN is a promiscuous viral attachment factor that binds to a wide range of pathogens, including those invading ocular tissues. Cellular binding by envelope glycoproteins of human cytomegalovirus, Ebola virus, and Kaposi's sarcoma-associated herpesvirus has been shown to promote viral transmission (41, 42). In the case of Ebola virus, it appears that DC-SIGN can mediate infection both in *cis* and in *trans* (42, 43). The finding that DC-SIGN-mediated intracellular signaling induced by HIV-1 glycoproteins in human RPE cells might provide a clue for the understanding of ocular invasion by these pathogens.

HIV-1 gp120 could induce varied cellular signaling in a DC-SIGN-dependent or -independent manner. Binding of gp120 to DC-SIGN on the dendritic cell (DC) surface promotes apoptosis signal regulating kinase 1-dependent apoptosis of cells induced by CD40 ligation or by exposure to lipopolysaccharide or the pro-inflammatory cytokines TNF- $\alpha$  or IL-1 $\beta$ .

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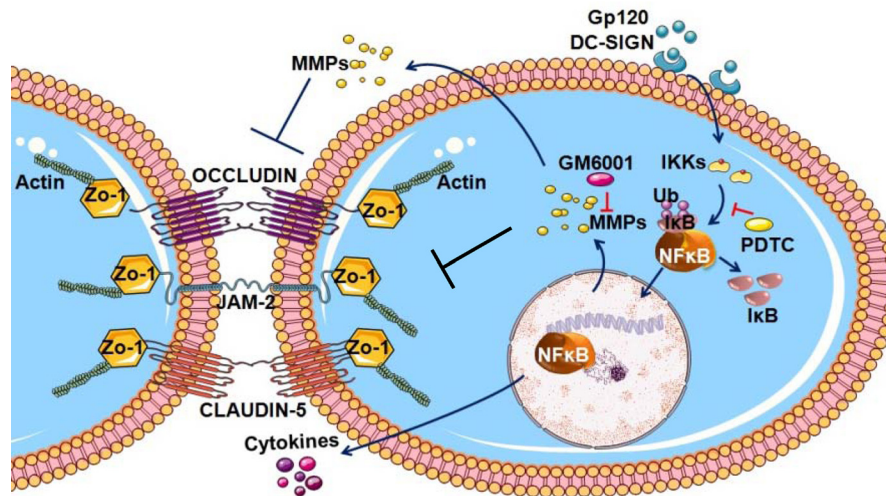


FIGURE 8. **Schematic of the gp120-DC-SIGN interaction, which down-regulates tight junction proteins and induces disruption of the blood-retinal barrier.** HIV-1 gp120 glycoprotein binds to the DC-SIGN molecules expressed on human RPE cells. The binding triggers cellular NF- $\kappa$ B signaling for the induction of MMPs, which then mediate the degradation of tight junction proteins and consequent disruption of BRB integrity.

This finding partially explains the DC depletion in chronically infected HIV-1 patients (36). On the other hand, HIV-1 replication in DCs requires DC-SIGN signaling triggered by gp120 and binding of gp120 to DC-SIGN-induced kinase Raf1-dependent phosphorylation of the NF- $\kappa$ B subunit p65, which could recruit the transcription elongation factor pTEF-b, demonstrating that DC-SIGN signaling triggered by gp120 is essential for HIV-1 transcription elongation (37).

Here we showed that binding of gp120 to DC-SIGN induced NF- $\kappa$ B-dependent expression of MMPs in RPE cells. MMPs are calcium-requiring, zinc-containing endopeptidases capable of degrading the extracellular matrix of the basal membrane and tight junction proteins (34, 35). Human RPE cells express several types of MMPs and are an important source of MMP production. Overexpression of MMP-2 and 9 seems to be of special importance for the progression of choroidal neovascularization in patients with age-related macular degeneration (45–47).

The BRB is comprised of both tight and adherens junction complexes, and the tight junctions form an apical impermeable barrier to fluid (22, 23, 48). Down-regulation of tight junction proteins is strongly associated with the disruption of PRE barrier tightness. The tight junction is mainly formed by transmembrane proteins, including claudins, occludins, and JAMs, and intracellular ZO scaffolding proteins. In the RPE, the expression of claudins-1, 2, and 5 has been detected in the embryogenesis of chick retinal pigment epithelium (49, 50). It has been reported that treatment with HIV-1 gp120 down-regulated the expression of the tight junction proteins ZO-1, occludin, and claudin 1–5, leading to increased permeability of the monolayer formed by human RPE cells, and thus allowed translocation of HIV-1 and bacteria across the epithelium (31). Here we further demonstrate a crucial role of DC-SIGN on PRE cells in mediating gp120-induced cellular signaling for the induction of MMPs and down-regulation of tight junction proteins.

HIV gp120 glycoprotein can disrupt the integrity of the BBB and cause HIV-associated neurocognitive disorders (51–53). The blood-retinal barrier has a similar nature to the BBB and is

derived from the same embryonic primordium. Exposure of neurons to HIV gp120 glycoprotein can increase oxidative stress and promote production of inflammatory cytokines, and increased expression of pro-inflammatory cytokines, including IL-6, IL-8, and CCL5, was observed in astrocytes upon exposure to gp120 glycoprotein (52–54). Exposure to gp120 results in increased oxidative stress in astrocytes, including decreased GSH/GSSG ratios and reduced levels of glutathione peroxidase and glutathione reductase (51). Our data also demonstrate that gp120 could increase the production of IL-8, CCL-2, and TNF- $\alpha$ , and these pro-inflammatory cytokines may also play a role in the breakdown of the BRB.

### Experimental Procedures

**Cells**—Human retinal pigment epithelial cells (ARPE-19) were cultured in DMEM/F12 medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). Human primary RPEiC cells, which were isolated from human retina, were purchased from ScienCell Research Laboratories and cultured in 6540 medium (EpiCM consisting of 500 ml of basal medium, 10 ml of fetal bovine serum, 5 ml of epithelial cell growth supplement, and 5 ml of penicillin/streptomycin). The first four passages of HPREiC cells were used. All cells were incubated at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>.

**Generation of DC-SIGN Knockout Cell Lines**—ARPE-19 cells were transduced with LentiCRISPR (Addgene plasmid 49535) containing DC-SIGN exon 3-targeting guide RNA (guide RNA 205). Cells were selected by puromycin (1  $\mu$ g/ml, Sigma) 24 h after transduction, and the single cell clone was expanded to generate the stable cell lines. The following target sequences were used: DC-SIGN exon 3 (guide RNA 205), 5'-ACT CCT CTC CTT CAC GCT CT-3'; GFP (control), 5'-GTG AAC CGC ATC GAG CTG AA-3'. To verify the disruption of the DC-SIGN open reading frame in the clonal population of ARPE-19 cells, genomic DNA was extracted for PCR. A 135-nt fragment encompassing exon 3 was amplified by using primers 5'-CAC TGG CAG GCT GAC GCA TGT-3' and 5'-GGA CCC CAG



ACC CTC AGA ACC T-3'. Knockout was confirmed by sequencing (Biosune Corp., Shanghai, China). The expression of DC-SIGN was also assessed with flow cytometry, SYBR Green-based semiquantitative RT-PCR, and Western blotting. The following primers were used: DC-SIGN-F, 5'-AATGGCT-GGAACGACGACAAA-3'; DC-SIGN-R, 5'-CAGGAGGCTG-CGGACTTTTT-3'. The purified mouse anti-human DC-SIGN antibody (clone 120507, Abcam) was used for Western blotting.

**HIV-1 Viral Stocks**—HIV virus-like particles (VLPs) were generated by co-transfecting HEK293T cells with or without a plasmid containing Gag-GFP and plasmids expressing JRFL or HXB2 HIV envelope proteins, respectively (44, 55). Cell-free supernatant was harvested, filtered, and titrated using a p24<sup>gag</sup> capture ELISA.

**Viral Binding and Internalization**—ARPE-19 or RPEiC cells ( $2 \times 10^5$ ) were incubated with HIV-1 VLP or VLP/ $\Delta$ Env (1 ng of p24<sup>gag</sup>) for 1 h at 4 °C and then washed. The amount of cell-associated Gag-GFP was quantified by flow cytometry, and the GFP<sup>+</sup> cell percentage and the mean fluorescence intensity were calculated. Some VLP-pulsed cells were treated with 0.25% trypsin (without EDTA) (Invitrogen) for 5 min at room temperature to remove VLPs bound to the cell surface. To perform the HIV-1 gp120 binding assay, the cells were incubated with 5  $\mu$ g/ml recombinant gp120 (JRFL or HXB2) (eEnzyme) in adherent buffer (1 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, and 5% BSA (pH 7.4)) for 1 h at 4 °C. The cells were then fixed with 4% paraformaldehyde (Sigma-Aldrich) for 10 min and stained with goat anti-gp120 antibodies (Sigma-Aldrich, SAB3500463). Subsequently, the cells were stained with FITC-conjugated secondary anti-goat antibodies (sc-2356, Santa Cruz Biotechnology), and gp120 binding was quantified by flow cytometry. Some cells were pretreated with a purified antibody against DC-SIGN (120507, Abcam) before VLP binding. To allow internalization, VLP binding was performed at 37 °C for 2 h. The cells were treated with 0.25% trypsin, and the intracellular VLPs were quantified by flow cytometry.

**Real-time (RT-) PCR**—Total RNAs from differently treated RPE cells were extracted by using TRIzol reagent (Invitrogen) and then reverse-transcribed into cDNA with a synthesis kit (Takara) according to the instructions of the manufacturer. Real-time PCR was carried out by using SYBR quantitative PCR mix (Toyobo) with the following thermal cycling conditions: initial denaturation at 95 °C for 10 min, amplification with 40 cycles of denaturation at 95 °C for 15 s, primer annealing at 60 °C for 15 s, and extension at 72 °C for 30s, followed by final extension at 72 °C for 6 min. The data were analyzed by green-based SYBR, semiquantified, and normalized with GAPDH. Real-time PCR was performed on the ABI 7900HT real-time PCR system. The primers used were as follows: MMP2-F, 5'-GCCAAGTGGTCCGTGTGAAGTA-3'; MMP2-R, 5'-GCCGTACTTGCCATCTCTCA-3'; MMP9-F, 5'-CCTTCTACGGC CACTACTGTGC-3'; MMP9-R, 5'-GCCAGTACTTCCCATCCTTGAAC-3'; TNF $\alpha$ -F, 5'-CCCAGGCAGTCAG ATCATCTTC-3'; TNF $\alpha$ -R, 5'-GTGAGGAGCACATGGGTGGAG-3'; CCL-2-F, 5'-CGCTCAGCCAGATGCAATCAA-3'; CCL-2-R, 5'-GTGGTCCATGGA ATCCTGAACC-3'; ICAM-1-F, 5'-AGCCA ACCAATGTGCTATTCAAAC-3';

ICAM-1-R, 5'-CACCTGGCAGCGTAGG GTAA-3'; IL-8-F, 5'-CTGATTTCTGCAG CTCTGTGTGA-3'; IL-8-R, 5'-GGTCCAG ACAGAGCTCTTCCA-3'; GAPDH-F, 5'-ATCCCATCACCATCTTCCAGG-3'; and GAPDH-R, 5'-CCTTCTCCATGGTGGTG AAGAC-3'.

**Detection of Tight Junction Proteins, HIV-1 Receptors, and DC-SIGN with Flow Cytometry**—The expression of CD4, CCR5, CXCR4, DC-SIGN, or the tight junction proteins ZO-1, Claudin-5, Occludin, and JAM-2 was assessed with flow cytometry. Some cells were stimulated with recombinant gp120 glycoproteins. In some experiments, an anti-DC-SIGN antibody (10  $\mu$ g/ml, 120507, Abcam) or GM6001 (ilomastat or Galardin, 10 nM, Selleck) was used to treat cells before stimulation with gp120 glycoprotein. Cells were stained with specific mAbs or isotype-matched IgG controls, detected with an LSRFortessa flow cytometer (BD Biosciences), and analyzed with FlowJo 7.6.1 software (Tree Star Inc.). Monoclonal antibodies against the following markers were used (clone numbers and resources are given in parentheses): phycoerythrin (PE)-CD4 (L3T4, eBioscience), allophycocyanin-Cy7-CCR5 (2D7, BD Pharmingen), allophycocyanin-CXCR4 (12G5, BD Pharmingen), and PE-DC-SIGN (eB-h209, eBioscience). Some purified primary antibodies were used, followed by a secondary anti-mouse IgG-FITC or anti-rabbit IgG-FITC antibodies: mouse anti-Occludin mAb (OC-3F10, Invitrogen), rabbit anti-Claudin 5 mAb (EPR7583, Abcam), rabbit anti-ZO-1 polyclonal antibody (Invitrogen), and rabbit anti-JAM-2 (EPR2489.2, Abcam). A permeabilizing agent (0.2% saponin, Abcam) was used for ZO-1 intracellular staining.

**Western Blotting**—ARPE-19 or RPEiC cells ( $2 \times 10^5$ ) were treated with recombinant HIV-1 gp120 glycoproteins (0.2  $\mu$ g/ml) for 4 h at 37 °C, and, in some experiments, cells were pretreated with anti-DC-SIGN antibody (10  $\mu$ g/ml, 120507, Abcam) or PDTC (50 nM, Sigma-Aldrich). Harvested cells were lysed with Nonidet P-40 lysis buffer containing protease inhibitor mixture (Sigma-Aldrich). Cell lysates were resolved by electrophoresis with 10% SDS-polyacrylamide gel and transferred onto PVDF membranes. The components from the NF- $\kappa$ B pathway sampler kit (Cell Signaling Technology) were used for Western blotting, including phospho-IKK $\alpha$ / $\beta$  (Ser-176/180, 16A6) rabbit mAb, IKK $\alpha$  (3G12) mouse mAb, and I $\kappa$ B $\alpha$  (L35A5) mouse mAb (amino-terminal antigen). The anti-MMP2 polyclonal antibody (SAB), anti-MMP9 mAb (EP1254, Abcam), and anti-GAPDH antibody (ab82633, Abcam) and the peroxidase-conjugated secondary antibody were also used in Western blotting. The blots were visualized using an enhanced chemiluminescence kit.

**Confocal Microscopy**—ARPE-19 cells were seeded onto poly-L-lysine-coated microscope slides (PolyScience) and then treated with recombinant HIV-1 gp120 glycoproteins (0.2  $\mu$ g/ml) for 48 h at 37 °C. Cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) and permeabilized with 1% Triton X-100 in PBS. Cells were blocked with PBS containing 5% BSA at room temperature for 1 h and then incubated with primary antibodies in blocking solution for 2 h at room temperature. Primary antibodies against ZO-1 (2.5  $\mu$ g/ml, 40-2200, Invitrogen) and Occludin (2.5  $\mu$ g/ml, OC-3F10, Invitrogen) were used. Next, cells were washed with PBS three times and then incu-

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bated with FITC-labeled goat anti-rabbit or PE-labeled goat anti-mouse IgG (2 µg/ml, Invitrogen) for 1 h at room temperature. Nuclei were counterstained with DAPI. Slides were mounted with fluorescent mounting medium (Dako) and observed using a laser-scanning confocal microscope (Olympus, FV1200).

**Measurement of TEER**—ARPE-19 cells ( $1 \times 10^5$ ) were seeded on Millicell inserts with a pore size of 0.4 µm (Millipore Corp.) for growth for 7 days to form the monolayer, and the medium was changed every other day. Monolayers were treated with or without recombinant HIV-1 gp120 glycoprotein during the last 2 days of cell culture. Some samples were pretreated with anti-DC-SIGN antibodies or GM6001 before incubation with gp120. The TEER values were detected to monitor monolayer confluency with a Millicell-electrical resistance system (ERS) volt-ohm meter using “chopstick” electrodes (EVOM-2 STX2, World Precision Instruments), according to the instructions of the manufacturer. Net TEER values were calculated by subtraction of the resistance value of the filters alone.

**Measurement of RPE Monolayer Permeability**—ARPE-19 cells ( $1 \times 10^5$ ) were seeded on Millicell inserts with a pore size of 0.4 µm (Millipore Corp.) for growth for 7 days to form the monolayer, and monolayers were treated with recombinant HIV-1 gp120 glycoprotein during the last 2 days of cell culture or left untreated. Some samples were pretreated with anti-DC-SIGN antibodies or GM6001 before incubation with gp120. The movement of 0.02 µg/ml FITC-dextran (4 kDa, Sigma-Aldrich) from the apical to basolateral compartment of the insert, over 1 h of incubation, was measured to determine the paracellular permeability. FITC-dextran was measured with Varioskan Flash (Thermo) with excitation at 488 nm and emission at 520 nm.

**Statistics**—Statistical analysis was performed using an unpaired *t* test with SigmaStat 2.0 (Systat Software, San Jose, CA).

**Author Contributions**—J. H. W. conceived the project, designed the study, and wrote the manuscript. Y. W. Q., C. L., and A. P. J. conducted the experiments. J. W., Y. W. Q., C. L., and Z. L. W. analyzed the results. X. J., T. S. L., S. G., P. G., and X. F. advised the study. All authors reviewed the results and approved the final version of the manuscript.

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