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HIV-1 impairs human retinal pigment epithelial barrier function: possible association with the pathogenesis of HIV-associated retinopathy

Suiyi Tan[1], Heng Duan[1,2], Tianrong Xun[1], Wei Ci[1], Jiayin Qiu[1], Fei Yu[1,3], Xuyan Zhao[1], Linxuan Wu[1], Lin Li[1], Lu Lu[4], Shibo Jiang[1,3,4] and Shuwen Liu[1]

The breakdown of human retinal pigment epithelial (HRPE) barrier is considered as the etiology of retinopathy, which affects the quality of life of HIV/AIDS patients. Here we demonstrate that HIV-1 could directly impair HRPE barrier function, which leads to the translocation of HIV-1 and bacteria. HRPE cells (D407) were grown to form polarized, confluent monolayers and treated with different HIV-1 infectious clones. A significant increase of monolayer permeability, as measured by trans-epithelial electrical resistance (TEER) and apical–basolateral movements of sodium fluorescein, was observed. Disrupted tightness of HRPE barrier was associated with the downregulation of several tight junction proteins in D407 cells, including ZO-1, Occludin, Claudin-1, Claudin-2, Claudin-3, Claudin-4, and Claudin-5, after exposure to HIV-1, without affecting the viability of cells. HIV-1 gp120 was shown to participate in the alteration of barrier properties, as evidenced by decreased TEER and weakened expression of tight junction proteins in D407 monolayers after exposure to pseudotyped HIV-1, UV-inactivated HIV-1, and free gp120, but not to an envelope (Env)-defective mutant of HIV. Furthermore, exposure to HIV-1 particles could induce the release of pro-inflammatory cytokines in D407, including IL-6 and MCP-1, both of which downregulated the expression of ZO-1 in the HRPE barrier. Disrupted HRPE monolayer allowed translocation of HIV-1 and bacteria across the epithelium. Overall, these findings suggest that HIV-1 may exploit its Env glycoprotein to induce an inflammatory state in HRPE cells, which could result in impairment of HRPE monolayer integrity, allowing virus and bacteria existing in ocular fluids to cross the epithelium and penetrate the HRPE barrier. Our study highlights the role of HIV-1 in the pathogenesis of HIV/AIDS-related retinopathy and suggests potential therapeutic targets for this ocular complication.

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The application of highly active antiretroviral therapy (HAART) has ensured a prolonged lifespan of HIV-1-positive patients. However, various complications threatening to the quality of life of HIV/AIDS patients have emerged. In particular, the incidence of retinopathy, the most common HIV-associated ocular disease, could be as high as 75%, even in the era of HAART.[1,2] Clinical presentation of HIV-associated retinopathy varies from retinal microvasculopathy to vision defect, and even blindness. Various opportunistic infections, including cytomegalovirus (CMV) retinitis, varicella zoster virus (VZV), or herpes simplex virus (HSV) associated with progressive outer retinal necrosis (PORN),[3,4] toxoplasma chorioretinitis, fungal retinitis, syphilitic retinitis, and retinal lymphoma,[5] have all been widely documented in HIV/AIDS-related retinal complications.

Limited studies have revealed the etiology and mechanism involved in the process of HIV-associated retinal pathology. The retina is guarded by human retinal pigment epithelial (HRPE) cells that constitute the outer blood–retina barrier (oBRB). The oBRB has well-developed tight junctions that contribute mostly to the barrier function of epithelial cells and strictly regulate the movement of nutrients and fluid into

[1]School of Pharmaceutical Sciences, Southern Medical University, Guangzhou, China; [2]Department of Pharmacy, Guangdong Provincial Stomatological Hospital, Guangzhou, China; [3]Lindsley F. Kimball Research Institute, New York Blood Center, New York, NY, USA and [4]Key Laboratory of Medical Molecular Virology of Ministries of Education and Health, Shanghai Medical College and Institute of Medical Microbiology, Fudan University, Shanghai, China
Correspondence: Dr SW Liu, MD, PhD, School of Pharmaceutical Sciences, Southern Medical University, 1838 Guangzhou Avenue North, Guangzhou 510515, China. E-mail: liusw@smu.edu.cn

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the outer retina. However, in the case of HIV-associated retinopathy, considerable clinical reports have demonstrated the presence of HIV-1 in the retina.[6,7] HIV-1 antigens were documented in all layers of the retina.[8] Therefore, the breakdown of the HRPE barrier and subsequent leakage of HIV-1 across the epithelium has been proposed as part of the pathogenesis for HIV-associated retinopathy.

Pathways by which HIV-1 enters various tissues across the epithelium have been documented, including direct infection, transcytosis, sequestration, and diffusive percolation.[9–16] Other studies revealed that direct contact of HIV-1 with the endothelium, which share tight junction structures in common with the epithelium,[17–19] could lead to the disruption of endothelial integrity and subsequent increased viral leakage across the endothelium. HIV-1 gp120 and Tat protein have been associated with the disruption of tight junctions in the endothelium.[17,18,20] Viruses, such as rotavirus and astrovirus, as well as bacteria, such as enteropathogenic *E. coli* and *C. difficile*, are known to increase intestinal permeability by disrupting tight junctions to more easily invade the host.[21–23]

Although HIV-1 has been found in various ocular fluids, including tears, aqueous humor, subretinal fluids, or vitreous body,[24–27] it is not clear how HIV-1 in ocular fluids crosses the HRPE and penetrates the inner retina. *In vitro* cell culture models are valuable tools for studying the physiology and pathophysiology of human naive tissue, particularly under circumstances where access to fresh sample is limited. Here we exploit an HRPE cell line, D407, which can retain epithelial characteristics, even after prolonged culture, to set up an *in vitro* oBRB in a transwell system to investigate the interaction between HIV-1 and oBRB in anticipation that the results will illuminate the role of HIV-1 in the pathogenesis of HIV-associated retinopathy.

MATERIALS AND METHODS

Reagents

Rabbit anti-ZO-1 (Mid) antibody, rabbit anti-Occludin antibody, mouse anti-Claudin-4 antibody, TRLzol reagent, HRP-labeled goat anti-mouse-IgG, and goat anti-rabbit-IgG were purchased from Invitrogen (San Francisco, CA, USA). Rabbit anti-Claudin-1 antibody was purchased from Thermo Scientific. The D407 cell line was obtained from the Center of Experimental Animals of Sun Yat-sen University. FITC-labeled goat anti-rabbit IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). DAPI (4',6-diamidino-2-phenylindole) was from Sigma-Aldrich (St Louis, MO, USA). Human recombinant (hr) IL-6, hr MCP-1, and various cytokine ELISA kits were purchased from Dakewe Bioengineering (Shenzhen, China). Plasmids of CXCR4-tropic NL4-3 and CCR5-tropic 92TH014.12 infectious clones were kindly provided by Jan Münch of Ulm University, Ulm, Baden-Württemberg, Germany. Antibodies against CD4, CXCR4, and CCR5 were obtained from the NIH AIDS Research and Reference Reagent Program. The soluble gp120 of the HIV-1_{JRFL} was from Haiyuan Protein Biotech (Jiangsu, China).

Preparation of Viral Particles and Viral Infection

HIV-1 infectious clones, pseudotyped HIV-1, and Env-defective mutant (HIV_{Env}^{-}) were produced by polyethylenimine-mediated transfection of 293T cells with DNA proviral expression plasmids according to the manufacturer's protocol. Viral supernatants were subjected to concentration with a PEG-it virus precipitation solution (System Biosciences, Mountain View, CA, USA). Viral particles were suspended in fresh DMEM and stored in aliquots at $-80\,^{\circ}C$. HIV-1 infection assay was performed in TZM-bl cells.[28] UV-inactivated HIV-1 was prepared by exposing HIV-1 R5-tropic strain to UV light in the hood with an emission wavelength of 254 nm for 30 min. UV inactivation of the virus was confirmed by titration in TZM-bl cells.

Quantitative Real-time Reverse Transcriptase PCR of Tight Junction mRNA

Quantization of tight junction gene expression in D407 was done by real-time quantitative reverse transcriptase PCR (RT-PCR) with SYBR Green. D407 monolayers exposed to HIV-1, or unexposed to HIV-1, were lysed by TRIzol. Real-time quantitative RT-PCR was performed with PrimeScript RT reagent Kit with SYBR Primxi Ex Taq (TaKaRa) on an ABI7500 sequence detection PCR system. The sequence of primers for each gene was either taken from published papers or designed with Primer Premier 5.0 (Table 1).

Measurement of TEER

Confluent D407 monolayers were formed onto 0.4-μm pore-size polyester membrane tissue culture inserts within 4–7 days. Next, serum concentration of the culture medium was reduced to 1% in order to form tight junction structure. The confluence of D407 monolayers was monitored microscopically and by measurement of trans-epithelial electrical resistance (TEER), using a Millicell ERS-2 Volt-Ohm Meter (Millipore), according to the manufacturer's instructions. TEER was reported as $\Omega \times cm^2$.

Permeability Assay

D407 cells were seeded on 12-well tissue culture inserts and grown to confluence. After exposure of HIV-1 to the apical surface of confluent D407 monolayers for 24 h, fresh medium was replaced in the lower and upper wells. Sodium fluorescein at 25 μg/ml was added in the upper well of each insert. At various time points, basolateral medium was sampled and replaced by equal volume of fresh medium. Samples were transported to 96-well black culture plates to measure fluorescence.[20]

Western Blot Analysis

D407 cells were collected in radioimmunoprecipitation buffer containing protease and phosphatase inhibitors,

Table 1 Primers used for real-time RT-PCR

Gene	Accession number	Primer names	Primer sequence 5′—3′
ZO-1	NM_003257	ZO-1 F	TGTGAGTCCTTCAGCTGTGGAA
		ZO-1 R	GGAACTCAACACACCATTG
Occludin	NM_002538	Occludin F	CATTGCCATCTTTGCCTGTG
		Occludin R	AGCCATAACCATAGCCATAGC
Claudin 1	NM_021101	Claudin 1 F	CCCAGTCAATGCCAGGTACG
		Claudin 1 R	GGGCCTTGGTGTTGGGTAAG
Claudin 2	NM_020384	Claudin 2 F	CCCAAACCCACTAATCACATC
		Claudin 2 R	GCCACTGCTTCTCCTTCC
Claudin 3	NM_001306	Claudin 3 F	CAGGCTACGACCGCAAGGAC
		Claudin 3 R	GGTGGTGGTGGTGGTGTTGG
Claudin 4	NM_001305	Claudin 4 F	GGCGTGGTGTTCCTGTTG
		Claudin 4 R	AGCGGATTGTAGAAGTCTTGG
Claudin 5	NM_003277	Claudin 5 F	GCAGCCCCTGTGAAGATTGA
		Claudin 5 R	GTCTCTGGCAAAAAGCGGTG
Claudin 6	NM_021195	Claudin 6 F	GCTCTGCTGTTTCTCACCCTTGGA
		Claudin 6 R	GCAAAGCCAGCACAGCAAGCA
Claudin 7	NM_001185022	Claudin 7 F	AATGTACGACTCGGTGCTCG
		Claudin 7 R	AATCTGATGGCCATACCAGG
GAPDH	NM_002046	GAPDH F	TCTCTGCTCCTCCTGTTC
		GAPDH R	CTCCGACCTTCACCTTCC

and subjected to western blotting, as previously described.[29]

Immunofluorescent Staining for Tight Junction Proteins

D407 cells, post-exposure to HIV-1 for 48 h, or unexposed to HIV-1, were fixed with 4% paraformaldehyde for 30 min at room temperature in the dark. The fixed cells were washed three times with PBS for 5 min each and then blocked with PBS containing 5% BSA at room temperature for 1 h. Cells were next incubated with primary antibodies (at 3 μg/ml) in blocking solution for 2 h at room temperature. Normal rabbit serum was used as a negative control. Next, cells were washed with PBS three times and incubated with FITC-labeled goat anti-rabbit IgG (1:200) for 1 h at room temperature in the dark. Nuclei were counterstained with DAPI (1:5000). After extensive washing, target proteins in D407 were visualized using fluorescence microscopy (Nikon).

Cell Viability Assay

Cell viability was determined by an MTT assay as published elsewhere.[29]

Cytokine Analysis

Production of multiple cytokines, including MCP-1, IL-6, IL-10, and TNF-α, in the supernatant in the upper compartment, post-exposure to HIV-1, was measured by ELISA assay. All immunoassays were performed according to the manufacturer's instructions.

Bacterial and HIV-1 Translocation

For HIV-1 translocation experiments, HIV-1 was added to the apical surface of confluent D407 monolayers. At different time points, basolateral supernatants were collected and mixed with equal volume of 5% Triton X-100 for determination of p24 by ELISA assay. Meanwhile, the infectivity of translocated viruses was determined by a coculture system. D407 cells were grown to confluence on the upper chamber, while the lower compartment was loaded with HIV-1 target cells. HIV-1 was added to the upper chamber. At different time intervals, the upper chamber and the medium were removed. Cells in the lower chamber continued to grow for another 72 h, and luciferase activity was determined.

For bacterial translocation experiments, D407 cells were grown to confluence on 3.0-μm-pore-size filters (BD Falcon, Canada). HIV-1 was exposed to the monolayers in the upper chamber for 24 h. Next, nonpathogenic *E. coli* strain HB101 (10^8 CFU/ml) was added. At 3, 6, and 12 h, 50 μl of basolateral supernatants were collected, diluted, plated on LB agar, and incubated for 24 h, followed

by bacterial colony counts. Wells treated with bacteria alone in the upper chamber were controls.

Statistical Analysis

Results were expressed as mean ± s.e.m. Statistical analysis was done by SPSS 13.0. Student's two-tailed *t*-test was used to compare data between two groups. One-way ANOVA, followed by Dunnett's or Tukey–Kramer's test, was applied to multiple comparisons. *P*-values < 0.05 were considered statistically significant. All experiments were repeated at least once to ensure reproducibility.

RESULTS

HIV-1 Exposure Impairs the Integrity of the HRPE Barrier

An HRPE cell line, D407, was evaluated for the effect of HIV-1 on HRPE barrier function by monitoring TEER, as a marker of epithelial integrity. When culturing on microporous filters, D407 cells could form a tight monolayer. The TEER of the D407 monolayer increased rapidly during the initial 7 days, reaching a plateau around day 8, and this level was maintained during the next 5 days (data not shown). Experiments began when D407 monolayers reached a steady state around day 8 by exposing replication-competent HIV-1 clones (CXCR4-tropic NL4-3 and CCR5-tropic 92TH014.12, respectively) apically to the monolayers. Changes in TEER were monitored at the indicated time points. The TEER of control HRPE monolayer remained stable throughout the experiments. It was found that both CCR5-tropic and CXCR4-tropic HIV-1 clones could significantly decrease TEER 4 h post exposure and that continuous culture of HRPE cells with HIV-1 for longer periods resulted in further reduction of TEER ($P < 0.001$) (Figure 1a). Exposure to HIV-1 clones led to a drop in TEER by ~60% after exposure for 48 h.

Monolayer permeability was further evaluated by measuring the transepithelial diffusion rate of sodium fluorescein through the D407 monolayer. After treatment of D407 monolayer with HIV-1 NL4-3 or HIV-1 92TH014.12 for 24 h, the percentage of sodium fluorescein leakage was recorded. Results showed that values of leaked sodium fluorescein, as measured at 30, 60, 90, and 120 min, were all significantly higher than those of cells in the standard medium, respectively, and followed a time-dependent manner (Figure 1b). This result was consistent with that of TEER measurement,

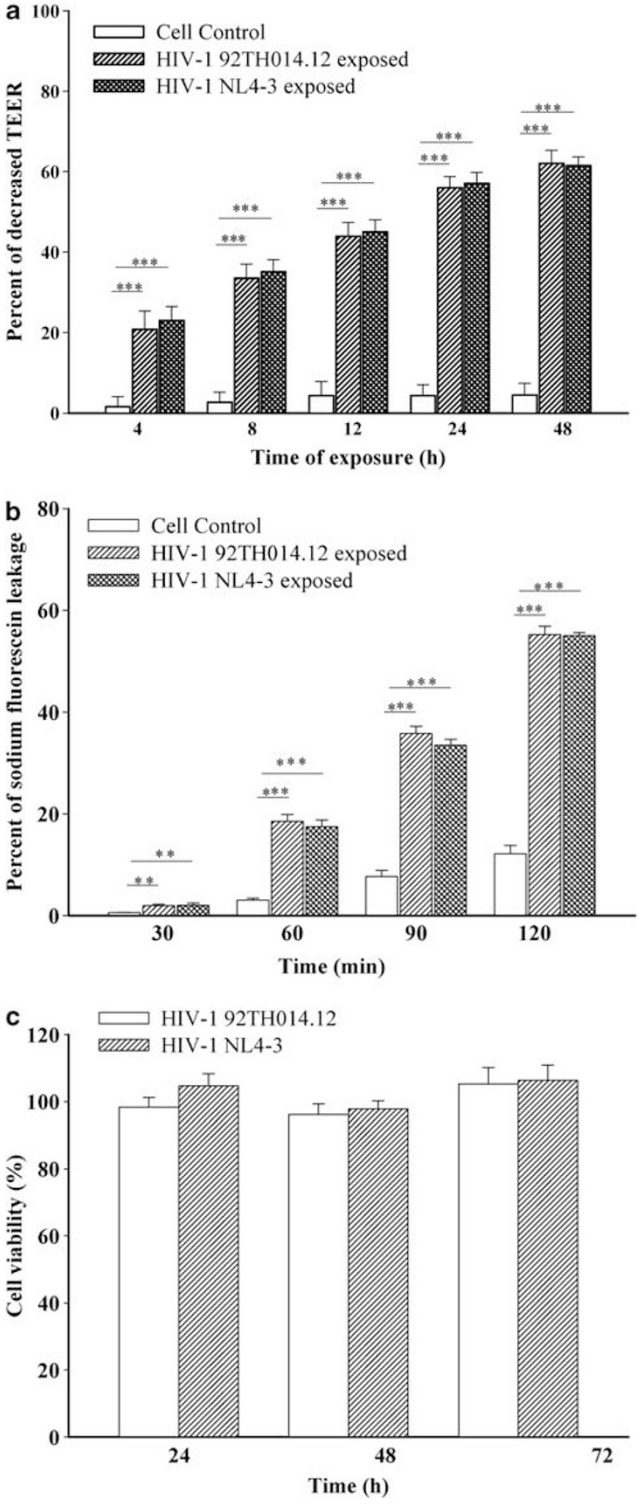

Figure 1 HIV-1 infectious clones impair the integrity of HRPE cells. D407 monolayers were exposed to 10^6 infectious viral units/ml of HIV-1 clones, including NL4-3 (CXCR4-tropic) and 92TH014.12 (CCR5-tropic). Corresponding p24 values were as follows: NL4-3 (1200 ng/ml) and 92TH014.12 (1000 ng/ml). TEER was measured before and post exposure to HIV-1 (**a**). Data are representative of the average of sextuplicate values s.d.). Experiments were repeated once, and similar results were found. ***$P < 0.001$, compared with cell control. After treatment of HIV-1 to D407, the leakage of sodium fluorescein across monolayer was determined (**b**). Values are presented as means ± s.d. ($n = 3$). Experiments were repeated once. ***$P < 0.001$, **$P < 0.01$, compared with cell control. Viability of D407 cells was assessed by MTT assay after exposure to HIV-1 92TH014.12 (1500 ng/ml) and NL4-3 (2000 ng/ml) 24, 48, and 72 h later, respectively (**c**). Data are representative of two separate experiments (± s.d.).

indicating that exposure to HIV-1 triggered an increased permeability of D407 monolayers.

The reduced TEER and increased monolayer permeability after HIV-1 exposure to D407 monolayers may be ascribed to the cell death, which can cause cellular morphological changes and formation of holes in the D407 monolayers. Therefore, we used MTT assay to determine the cell viability. No cytotoxicity was observed when cells were exposed to HIV-1 at a concentration higher than that used in the permeability experiments (Figure 1c). The results suggested that the significant breakdown of HRPE integrity was not due to cell death.

Notably, previous study showed that viral propagation medium containing cytokines could affect epithelial barrier function.[30] To avoid this problem, we used HIV-1 particles pelleted from culture supernatants by a PEG-based virus precipitation solution[31] in all the experiments.

HIV-1 Exposure Downregulates Expression of Tight Junction Genes and Proteins in HRPE Barrier

Tight junction proteins are critical components to epithelial barrier integrity. We next determined whether D407 monolayer abnormality was correlated with an abnormal expression of several tight junction proteins. Results of real-time quantitative RT-PCR demonstrated that several genes encoding tight junction proteins were downregulated in D407 monolayer 48 h post exposure to HIV-1. A significant reduction in expression of genes encoding tight junction proteins included ZO-1, Occludin, Claudin-1, Claudin-2, Claudin-3, Claudin-4, and Claudin-5 ($P<0.05$), while expression of the genes encoding Claudin-6 and Claudin-7 proteins was not influenced (Figure 2a). After treatment with HIV-1, subsequent western blotting confirmed that the expressed proteins, including ZO-1, Occludin, Claudin-1, and Claudin-4, in the D407 monolayer were reduced (Figure 2b).

Results of immunohistochemistry showed that control D407 cells displayed a continuous and intact membrane-associated staining pattern for both ZO-1 and Occludin. However, exposure of the monolayer to HIV-1 led to a profound reduction in distribution of ZO-1 and Occludin (Figures 3a and b). After HIV-1 treatment, D407 cells showed a loss of continuous membrane staining of either ZO-1 or Occludin, whereas DAPI staining for nuclei showed the uniform distribution of D407 cells over the monolayer.

The above results showed that decreased barrier tightness after exposure of the monolayer to HIV-1 was associated with the decreased expression of several tight junction proteins in D407 cells.

HIV-1 gp120 Mediates the Disruption of HRPE Barrier Integrity

HIV-1 gp120 is a viral surface envelope glycoprotein (Env) and free gp120 exists *in vivo*. Oh *et al*[32,33] reported that the estimated concentration of gp120 in the sera of HIV-infected

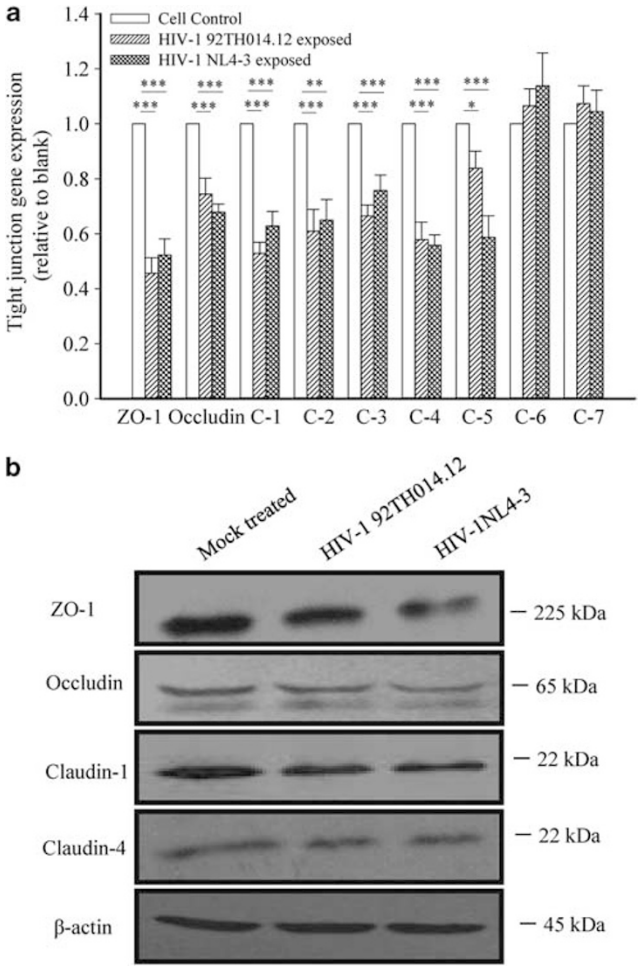

Figure 2 Exposure to HIV-1 downregulates tight junction expression in HRPE monolayers. Confluent monolayers of D407 were treated with HIV-1 (NL4-3, 1200 ng/ml and 92TH014.12, 1000 ng/ml). Total RNA was extracted and cDNA was synthesized. Real-time quantitative RT-PCR was performed for various tight junction gene expressions. Gene GAPDH was used as an internal control (**a**). Results are means ± s.d. from two independent experiments in which a duplicate test was performed. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, compared with cell control. C, Claudin. After exposure of D407 monolayers to HIV-1, tight junction protein expressions were monitored by western blotting (**b**). β-Actin was used to evaluate protein loading. These results are one representative of two independent experiments.

patients was about 120 ~ 960 ng/ml, while others recorded lower gp120 concentrations in the patients' sera[34,35] In the present study, we examined whether gp120 mediated the impairment of HRPE barrier function using a soluble gp120 of the HIV-1_{JRFL} at a reasonable level (100 ng/ml) in the *in vitro* experiments. Pseudotyped HIV-1 (CCR5-tropic HIV-1_{JRFL} and CXCR4-tropic HIV-1_{HXB2}) and the UV-inactivated HIV-1 were included as controls. In addition, an Env-defective mutant of HIV_{Env-} lacking both gp120 and gp41 was treated as negative control. TEER was significantly decreased following exposure of pseudotyped HIV-1 particles and HIV-1 gp120 to D407 monolayers, respectively

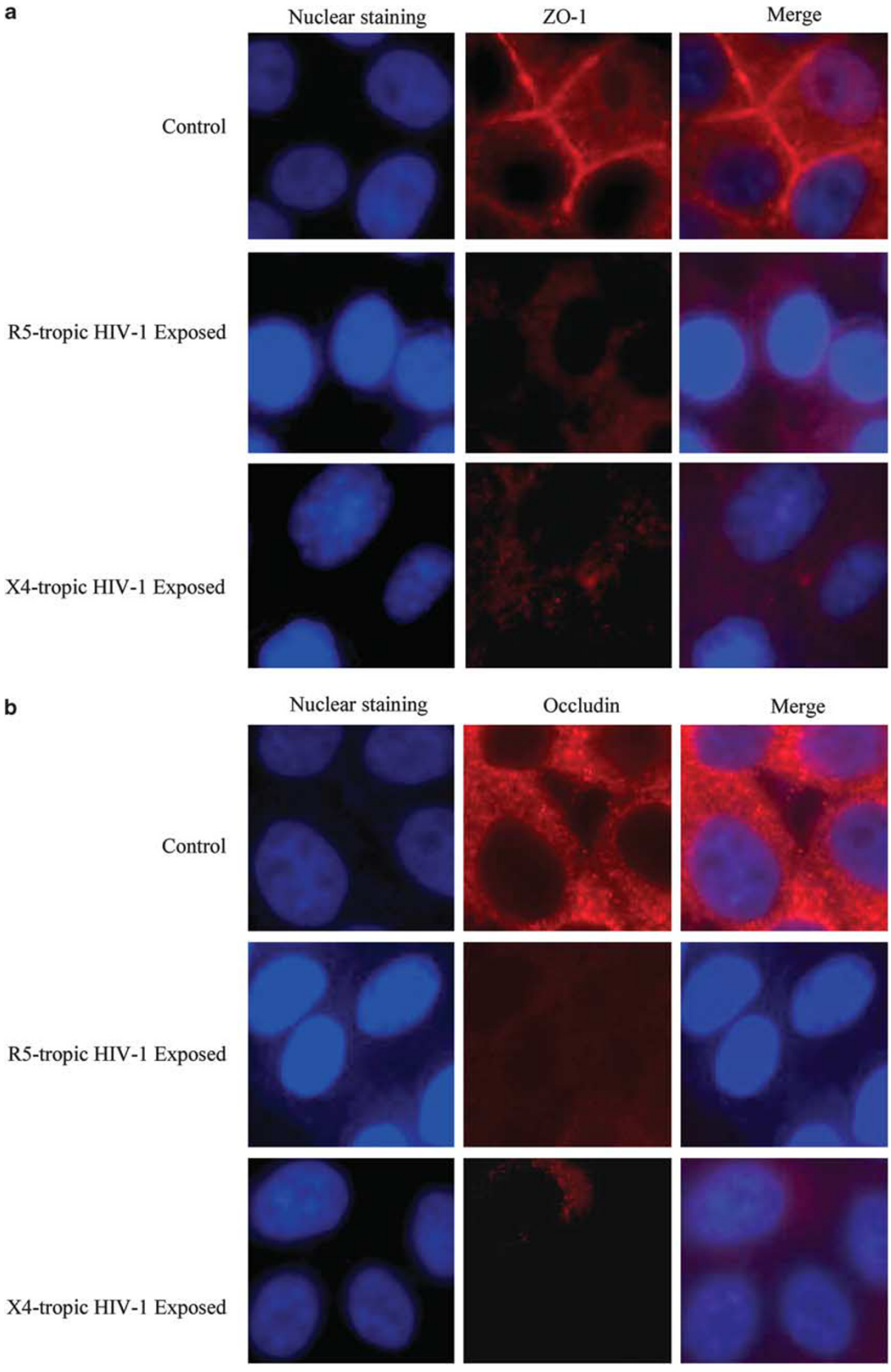

Figure 3 Exposure of HIV-1 to D407 monolayers disrupts tight junction proteins by immunofluorescent staining. Representative staining is shown for ZO-1 (**a**) and Occludin (**b**) at 48 h post exposure. Magnification: $\times 600$. Data shown are one representative of two independent experiments.

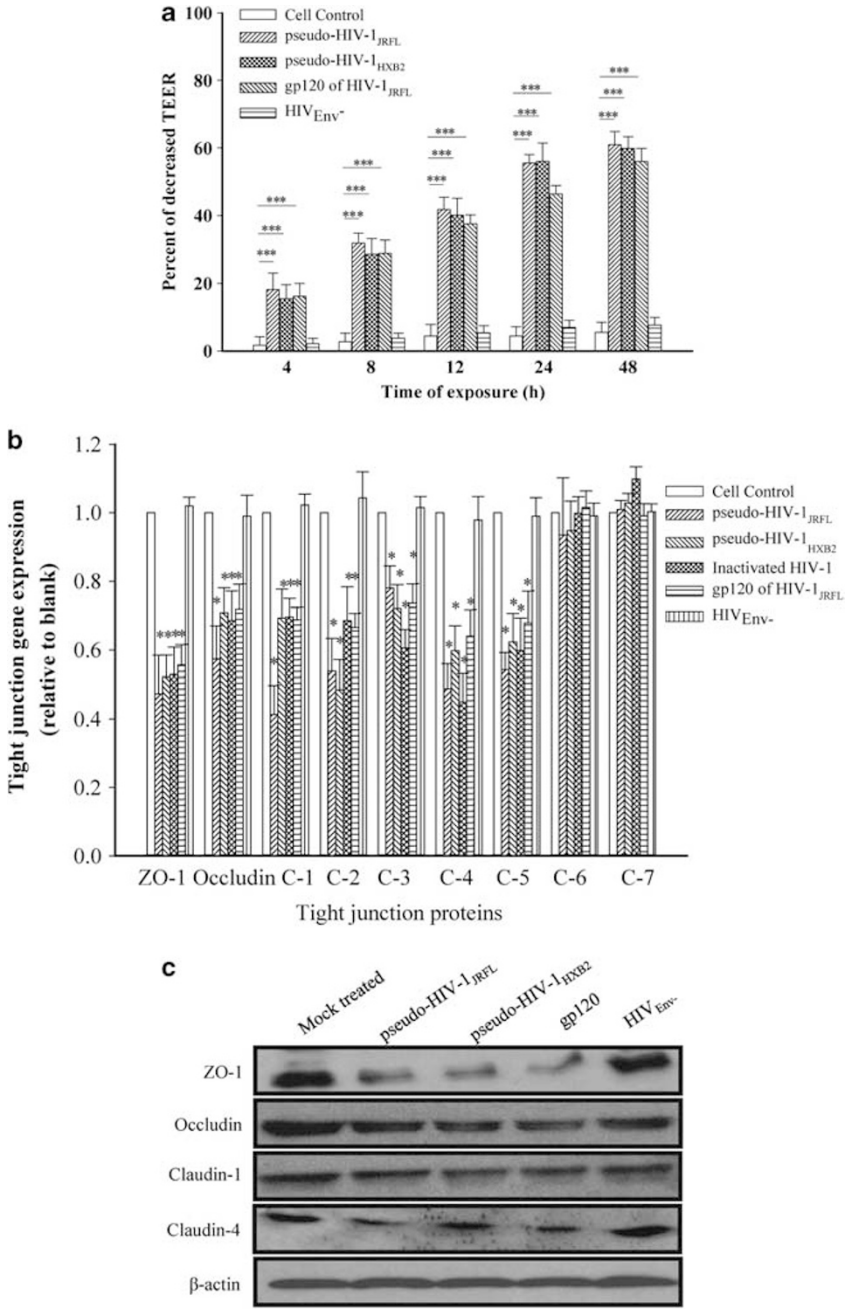

Figure 4 HIV-1 gp120 mediates impairment of barrier function of HRPE cells. After treatment of D407 monolayers with pseudotyped HIV-1_{JRFL} (p24 at 1200 ng/ml), HIV-1_{HXB2} (p24 at 2000 ng/ml), gp120 alone (at 100 ng/ml), and HIV-envelope mutant HIV_{Env}^{-} (p24 at 1500 ng/ml), TEER was measured before and post exposure to evaluate barrier functions (**a**). ***$P<0.001$, compared with cell control, $n=6$. In addition, D407 cells were collected and subjected to real-time quantitative RT-PCR to determine the effect of various HIV-1 pseudo-particles, UV-inactivated HIV-1 and gp120 on tight junction gene expression (**b**). *$P<0.001$, compared with cell control, $n=4$. C, Claudin. After treatment of D407 monolayers with HIV-1 pseudo-particles or gp120, the expression of tight junctions was analyzed by western blotting (**c**). All data shown in this figure are average values (± s.d.) from one of two independent experiments that yielded similar results.

(Figure 4a). In contrast, the HIV-1 Env mutant did not affect the permeability of D407 monolayer throughout the experimental period. As shown in Figures 4b and c, the greater permeability of the HRPE monolayer exposed to pseudo-HIV-1, UV-inactivated HIV-1, and HIV-1 gp120 was associated with the decreased expression of tight junction genes and proteins, including ZO-1, Occludin, Claudin-1, Claudin-2, Claudin-3, Claudin-4, and Claudin-5 ($P<0.01$).

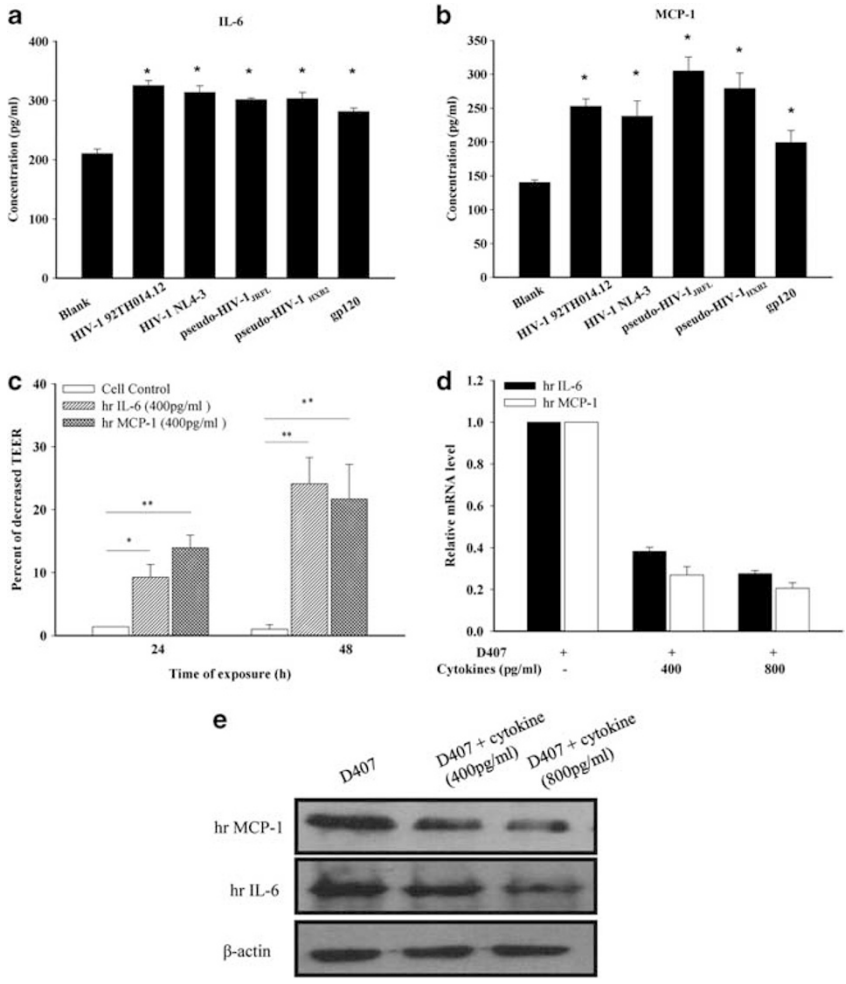

Figure 5 HIV-1 exposure to D407 cells promotes cytokine production that leads to the impairment of barrier function. HRPE monolayers were exposed to HIV-1 (NL4-3 at 1200 ng/ml, 92TH014.12 at 1000 ng/ml, pseudo-HIV_{JRFL} at 1200 ng/ml, and pseudo-HIV_{HXB2} at 2000 ng/ml), and the supernatants in the apical chamber were collected 48 h post exposure and assayed for the following cytokines by ELISA assay: (**a**) IL-6, (**b**) MCP-1. $^{*}P<0.05$, compared with cell control ($n=4$). To determine the effect of cytokines on the integrity of HRPE monolayers, D407 monolayers were treated with hr IL-6 and hr MCP-1, and TEER was measured at different time points (**c**). $^{*}P<0.05$, $^{**}P<0.01$, compared with cell control, $n=6$. In addition, ZO-1 gene expression in D407 cells by real-time quantitative RT-PCR (d), $n=3$, and ZO-1 protein expression by western blotting (**e**) in D407 cells were determined. All data shown in this figure were average values (± s.d.) from one of two independent experiments that yielded similar results.

Production of Proinflammatory Cytokines in HRPE Cells after Exposure to HIV-1 is Partially Responsible for the Impairment of Barrier Function

Various proinflammatory cytokines, including TNF-α, IL-6, IL-10, and MCP-1, were detected in the supernatants of D407 cells post exposure to HIV-1. The results showed that treatment of HIV-1 and gp120 significantly enhanced the production of IL-6 ($P<0.05$) and MCP-1 ($P<0.05$) in D407 monolayers (Figures 5a and b). However, we could not detect the release of TNF-α or IL-10 in all samples (data not shown). Failure to detect TNF-α and IL-10 could be explained by the low limit of sensitivity used in the experimental method or the fact that D407 cells do not secrete these two cytokines. The detection limit for TNF-α is 8 pg/ml, while that for IL-10 is 5 pg/ml. Next, we found that D407 monolayers exhibited a significant decrease in paracellular permeability in the presence of IL-6 and MCP-1, as determined by TEER ($P<0.05$) (Figure 5c). Disruption of barrier permeability by IL-6 or MCP-1 was associated with decreased expression of tight junction protein ZO-1, as revealed by real-time RT-PCR and western blotting (Figures 5d and e). The disruption of barrier function by IL-6 or MCP-1 appeared to be dose dependent (Figures 5d and e). However, addition of IL-6 or MCP-1 to the D407 monolayer did not influence the expression of other tight junction proteins, including Occludin, Claudin-1, Claudin-2, Claudin-3, Claudin-4, Claudin-5, Claudin-6, or Claudin-7 (data not shown). Thus, exposure to HIV-1 triggers the production of IL-6 and MCP-1 in the HRPE monolayer, allowing further inflammatory impairment of HRPE integrity.

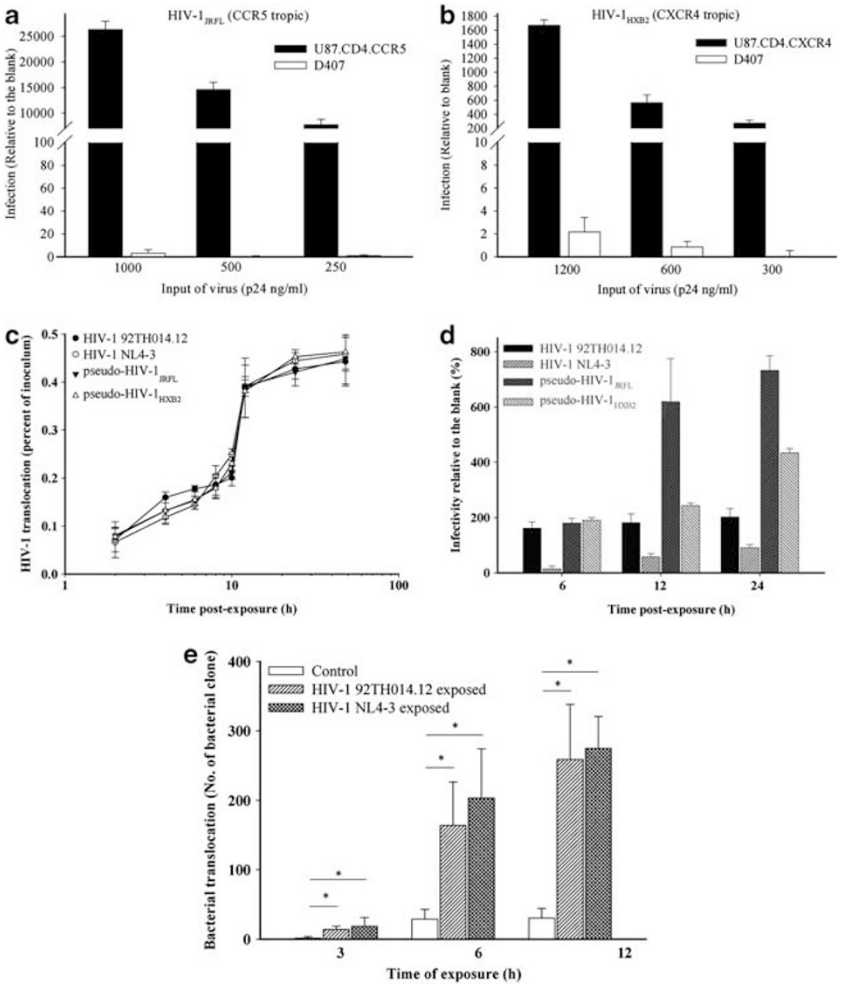

Figure 6 HIV-1 and bacteria translocate across HRPE monolayers following HIV-1 exposure. Infection of pseudotyped HIV-1_{JRFL} (**a**) and HIV-1_{HXB2} (**b**) in viral target cells and D407 cells. (**c**) Viral translocation was examined in D407 monolayers exposed to different HIV-1 on the apical side (NL4-3 at 1200 ng/ml, 92TH014.12 at 1000 ng/ml, pseudo-HIV_{JRFL} at 1200 ng/ml, and pseudo-HIV_{HXB2} at 2000 ng/ml). Basolateral supernatants were collected after different time intervals. Viral antigen p24 was determined to calculate the viruses that translocated across the barrier. (**d**) In a coculture system where D407 cells formed monolayers in the upper well and HIV-1 target cells were cultured in the lower well, HIV-1 was added to the upper chamber. At different time points, viruses and the upper chamber were removed, and the cells in the lower chamber were allowed to continue in culture for another 72 h. Infection was determined by luciferase activity using a luciferase assay kit. (**e**) Disruption of HRPE barrier function by HIV-1 led to increased bacterial transmigration across the barrier. *E. coli*, accompanied by HIV-1 or not, was added in the upper compartment of transwell. Basolateral medium was collected and bacterial counts were done. Data shown are representative of two separate experiments. Each experiment represents triplicate measurements.

Impairment of HRPE Function Correlates with Translocation of Virus and Bacteria across the Epithelial Monolayer

D407 cells do not express HIV-1's primary receptor CD4 and its coreceptor, CXCR4 or CCR5 (Supplementary Material, Supplementary Figure S1). Therefore, neither CCR5-tropic HIV-1_{JRFL} nor CXCR4-tropic HIV-1_{HXB2} could infect D407 cells, while JRFL or HXB2 strain could infect target cells U87.CD4.CCR5 or U87.CD4.CXCR4, respectively (Figures 6a and b). Disruption of HRPE barrier by exposure to HIV-1 did not result from HIV-1 infection in D407 cells. Weakened tightness of D407 barrier allowed HIV-1 transmigration through D407 monolayer as early as at 2 h (Figure 6c). Using a coculture system, we found that the transmigrating HIV-1 could still infect the target cells in the lower chamber (Figure 6d). Furthermore, we found that barrier dysfunction caused by exposure to HIV-1 could allow easier translocation of other pathogens. Specifically, when D407 monolayers were treated with *E. coli* alone, only a small number of *E. coli* could transmigrate across the monolayer. However, when accompanied with HIV-1, *E. coli* could significantly transmigrate across the D407 monolayer ($P<0.05$, Figure 6e).

DISCUSSION

In this study, we demonstrate that exposure of HRPE monolayer to HIV-1 impairs barrier function, as characterized by decreased TEER and greater permeability of the monolayer without affecting the cellular viability (Figure 1). Barrier

dysfunction is associated with reduction in the expression of tight junction proteins, including ZO-1, Occludin, Claudin-1, Claudin-2, Claudin-3, Claudin-4, and Claudin-5, whereas the expression of Claudin-6 and Claudin-7 are unaltered (Figures 2 and 3). Tight junction proteins are the apical most junctional complex in the epithelium and contribute to the barrier function of epithelial cells.[36] They strictly regulate the passage of various molecules through paracellular spaces.[37] As such, any abnormality of these tight junctions can have various pathological consequences.[37] Previously, HIV-1 has been proposed to disrupt the integrity of brain endothelial cells, which possess tight junction structures as barrier components similar to those of HRPE cells.[17,38–41] Other viruses, such as rotavirus and astrovirus, as well as some bacteria, are known to increase intestinal permeability by disrupting tight junctions as part of their pathogenesis.[21–23] Here we show that HIV-1 could also induce disruption of tight junction structures in HRPE cells. Notably, we used HIV-1 in the *in vitro* experiments at a high level resembling the high viral load in the HIV-1-infected patients in the acute phase of infection or in AIDS stage,[42] reflecting the facts that viruses might cause this ocular pathological abnormality at either early phase of infection or the late stage of infection.

Disruption of barrier function of HRPE monolayer is mediated by HIV-1 gp120 (Figure 4). Env-expressing HIV-1 pseudovirus and free gp120 can decrease the TEER of D407 monolayer. Pseudoviruses, UV-inactivated HIV-1, and gp120 could downregulate the expression of several tight junction proteins, while an HIV mutant lacking the Env has no disruptive effect on the HRPE barrier. HIV-1 gp120 is the viral surface glycoprotein, and free gp120 exists *in vivo*. Although previous reports showed that HIV-1 Tat could disrupt HRPE barrier function by altering the expression of tight junction proteins, it is not clear whether Tat exists in the extracellular space.[43] Therefore, our study only focused on the effects of HIV-1 gp120 on the properties of HRPE barrier. Our data are consistent with other results showing that HIV-1 gp120 downregulates the expression of tight junction proteins in the blood–brain barrier in a manner similar to the effect of HIV-1 gp120 on HRPE cells possessing well-developed tight junctions.[38,44]

Viral infection is often associated with inflammation. Therefore, we next pointed out that the D407 monolayer is induced to release IL-6 and MCP-1 on exposure to HIV-1 (Figures 5a and b). In the presence of hr IL-6 and hr MCP-1, the expression of ZO-1 proteins was downregulated in D407 cells (Figures 5d and e), thereby impairing HRPE monolayer integrity (Figure 5c). TNF-α is often associated with viral infection and has been reported to impair the intestinal epithelial tight junction barrier;[45] however, in D407 cells we could not detect the release of TNF-α, possibly because different epithelial cells show distinct properties in response to viral infection. In addition, IL-6 and MCP-1 exerted no effect on the expressions of Occludin, Claudin-1, Claudin-2, Claudin-3, Claudin-4, or Claudin-5. An elevated level of IL-6 and MCP-1 induced by HIV-1 could partially compromise the barrier function of D407 monolayer. Other as yet unknown mechanisms implicated in the impairment of barrier function of HRPE cells still need to be elucidated.

Our results show that the breakdown of the HRPE barrier leads to the translocation of HIV-1 across the epithelium (Figure 6c) and that the transmigrating HIV-1 can still infect CD4 + cells (Figure 6d). A similar finding demonstrated that the loss of tight junctions in squamous epithelium correlated to easier penetration of HIV-1 across the monolayer.[11] In the case of retinopathy, the entry of HIV-1 into the retina is often associated with the breakdown of retinal vascular endothelium. However, our results suggest that the impairment of the HRPE barrier by HIV-1, which allows the opening of the paracellular route in the HRPE monolayer, might be an alternative pathway for viral access into the retina. In the retinal inner structures, where microglia cells are supportive for HIV-1 infection,[46,47] transmigrating HIV-1 possessing infectivity might establish latency in the neuroretina. Moreover, leaking HIV-1 could further damage the inner retinal neuronal system,[48,49] as by neuronal apoptosis or neurotoxicity.[50,51] This is consistent with the clinical observation that a significant thinning of the retinal nerve fiber layer is found in HIV-1-positive patients without CMV retinitis.[48] Such damage to the retinal nerve system leads to subtle vision abnormalities, including reduced contrast sensitivity, altered color vision, and loss of visual field in HIV-positive people.[48,52] It should also be noted that disruption of the HRPE barrier results in easier leakage of bacteria, implying a greater chance that a secondary infection might occur in the retina of HIV/AIDS patients. Clinically, the manifestation of retinopathy involves many opportunistic infections, such as CMV retinitis,[4] which is the leading opportunistic ocular infection and the major reason for blindness in AIDS patients, VZV- or HSV-associated PORN,[3] toxoplasma chorioretinitis, fungal retinitis, or syphilitic retinitis.[5]

Overall, we underscore an important role of HIV-1 in the pathogenesis of HIV-associated retinopathy *via* the disruption of junctional tightness in the HRPE barrier. Based on our results, HIV-1 gp120 or several cytokines might be potential therapeutic targets for this ocular complication.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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