Exosomes Transport Anti-Human Immunodeficiency Virus Factors from Human Cervical Epithelial Cells to Macrophages

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Keywords

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Abstract

The female reproductive tract (FRT) is a major site of HIV sexual transmission. As the outermost layer of cells in the FRT, the human cervical epithelial cells (HCEs) have direct contact with HIV or infected cells. Our early work showed that supernatant (SN) from TLR3-activated HCEs contain the antiviral factors that could potentially inhibit HIV replication in macrophages. However, it remains to be determined how HCEs transport the anti-HIV factors to macrophages. This follow-up study examined the role of exosomes in HCE-mediated anti-HIV activity. We found that TLR3 activation of HCEs resulted in the release of exosomes that contained multiple IFN-stimulated genes (ISGs: *ISG56*, *OAS1*, *MxA*, and *Mx2*) and the HIV restriction microRNAs (miR-28, miR-29 family members, miR-125b, miR-150, miR-382, miR-223, miR-20a, and miR-198). The depletion of exosomes from SN of TLR3-activated HCEs diminished HCE-mediated anti-HIV activity in macrophages, indicating that HCE-derived exosomes are responsible for transporting the antiviral molecules to macrophages. These in vitro findings suggest a novel antiviral

mechanism by which HCEs participate in the FRT innate immunity against HIV infection. Further in vivo studies are necessary in order to develop an exosome-based delivery system for prevention and treatment of HIV infection through sexual transmission.

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Introduction

Human immunodeficiency virus (HIV) transmission to women through the female reproductive tract (FRT) accounts for about 40% of all HIV transmission events [1]. During sexual intercourse, HIV in male semen can pass the female genital epithelium [2, 3] and establish infection in the target cells of the underlying lamina propria, including CD4⁺ T cells, dendritic cells, and macrophages [4, 5]. As the first layer cells in the FRT, the human cervical epithelial cells (HCEs) are in the direct contact with HIV or infected cells. Therefore, to understand an important role of HCEs in the FRT innate immunity against viral infection is of significance and interest [6]. Several independent studies [7–9] have shown that HCEs have the immune protection function to identify pathogen-associated molecular patterns on pathogens through pattern recognition receptors. We have documented that the HCEs

possess a functional toll-like receptor 3/interferon (TLR3/IFN) signaling system [10], which could be activated by a TLR3 ligand, inducing the production of the antiviral factors that suppressed HIV replication in primary human macrophages [11]. These earlier studies, however, did not address the question of how HCE-released anti-HIV factors, particularly the IFN-stimulated genes and the HIV restriction miRNAs, are transported to macrophages.

There has been a rapid growth in investigating the role of the extracellular vehicles (EVs), including exosomes, ectosomes, oncosomes, microvesicles, apoptotic bodies, microparticles, and other EV subsets in cell-cell communications [12]. Among them, exosomes (EVs of endosomal origin) play a crucial role in the intercellular transportation [13–15]. Exosomes are formed through the fusion of multivesicular bodies (MVBs) with the host cell plasma membrane and then release of intraluminal vesicles (ILVs) as exosomes [16]. Specifically, exosomes originate in endosomal compartments called MVBs, which are late endosomes containing multiple ILVs formed by the invagination of the endosomal membrane [17]. When MVBs fuse with the plasma membrane, ILVs are released as exosomes. Differential ultracentrifugation is the most commonly used exosome separation. Isolated exosomes have a typical cup-shaped structure, with a diameter of 30–100 nm and are rich in endosome-associated proteins [18]. The most common exosome-carried proteins can act as specific markers, including *CD63*, *CD81*, Alix (apoptosis-linked gene 2-interacting protein x), LAMP2 (lysosome-associated membrane protein 2), TSG101 (tumor susceptibility gene 101 protein), and PECAM-1 (platelet and endothelial cell adhesion molecule-1) [19, 20]. In addition, the identity and purity of exosome can be determined by lacking of the cellular markers such as EEA1 (early endosomal antigen 1, an endosomal marker), cytochrome C (a mitochondrial marker), and GAPDH (cytoskeleton marker) [21]. In addition to exosome-associated proteins, exosomes carry various molecules in their lumen, including lipids, proteins, mRNAs, microRNAs (miRNAs), and long noncoding RNAs (lncRNAs) [22]. After their release into the extracellular space, exosomes can act locally or circulate through various bodily fluids, including blood and lymph, resulting in a systemic response [23]. The recent studies have shown that exosome-mediated intercellular communication also plays a crucial role in the antiviral innate immunity [21, 24, 25]. We, thus, examined the role of exosomes produced by HCEs in transporting the antiviral factors to HIV-infected macrophages. We also investigated the mechanisms by which the exosomes participate in HCE-mediated innate immunity against HIV.

Materials and Methods

Reagents

Rabbit antibodies against exosome-linked markers, ISGs, and HIV p24 protein were purchased from Cell Signaling Technology (Danvers, MA, USA). PKH67 fluorescent cell linker kits and PKH26 fluorescent kit were purchased from Sigma-Aldrich Co. LLC. Exosome-depleted fetal bovine serum (FBS) was purchased from System Biosciences, Inc (Mountain View, CA, USA). All culture plasticwares were obtained from Corning (Corning, NY, USA). Unless otherwise specified, all other culture reagents were purchased from Invitrogen (San Diego, CA, USA).

Cells Culture

The HCE line (End1/E6E7 cells) has been extensively studied and well established as a HCE model [26, 27]. The cells were cultured in keratinocyte growth medium (Gibco, Grand Island, NY, USA) supplemented with the provided recombinant epidermal growth factor (0.1 ng/mL) and bovine pituitary extract (50 µg/mL). Monocytes were obtained from Human Immunology Core at the University of Pennsylvania (Philadelphia, PA). The core has the institutional review board approval for blood collection from healthy donors. Monocytes isolated from peripheral blood differentiated into macrophages as described [28, 29]. Briefly, freshly isolated monocytes were cultured in a 48-well plate (2.5×10^5 cells/well) in complete DMEM containing 10% FBS and 1% penicillin-streptomycin without any growth factors. Monocyte-derived macrophages refer to monocytes cultured in vitro for 7 days.

Exosome Isolation

HCEs (End1/E6E7 cells) were transfected with Poly I:C for 4 h and cultured in fresh-culturing medium containing 10% exosome-free FBS. At 48 h post-transfection, End1/E6E7 cell supernatant (SN) was collected and exosomes were isolated by the differential centrifugation as described [24]. Briefly, SN was centrifuged at 300 g for 10 min, 2,000 g for 10 min, 10,000 g for 30 min, and 100,000 g for 70 min. For further purification, the pellets were washed with PBS and centrifuged at 100,000 g for 70 min. The pellets were then resuspended in 100 µL PBS and freshly used.

Electron Microscopy of Isolated Exosomes

Isolated exosomes from HCE SN were resuspended in 10 µL PBS and spotted onto Formvar-coated grids (200 mesh). Adsorbed exosomes were then fixed in 2% (vol/vol) paraformaldehyde at room temperature for 5 min. After fixation, the exosomes were negatively stained with uranyl acetate. The grids were observed under the electron microscope (CM100; Philips, Amsterdam, the Netherlands).

Labeling Exosomes with Fluorescent

Macrophages were cultured at a density of 2.5×10^5 cells/well in 48-well plates. Isolated exosomes from SN of HCE cultures were labeled with PKH67 fluorescent according to the manufacturer's protocol (Sigma-Aldrich). Purified PKH67 exosomes were incubated with macrophages and cultured at 37°C for 18 h in a CO₂ incubator. Macrophages were then stained with a PKH26 fluorescent for membrane and Hoechst 33342 for nuclei and then washed 3 times with 1 × PBS. The cells were photographed under a confocal microscope (Nikon A1R, Nikon, Japan).

Fig. 1. Characterization of exosomes of End1/E6E7 cells. **a** Electronic microscope observation of End1/E6E7 cell-derived exosomes. End1/E6E7 cells were transfected with or without Poly I:C (1 µg/mL) for 48 h, and cell culture SN was collected for exosome isolation. Isolated exosomes were then fixed in 2% (v/v) paraformaldehyde. The fixed exosomes were negatively stained with uranyl acetate and then observed by electron microscope. **b** Western blot assay of exosome markers and nonexosome markers. End1/E6E7 cells were cultured in exosome-free culture media for 48 h in a 48-well culture plate. Total proteins from End1/E6E7 cells and

the isolated exosomes were extracted for Western blot analysis. The expression of the specific exosomal markers (Alix, CD63, CD81, PECAM-1, and LAMP2) and nonexosomal markers (EEA1, cytochrome C, and GAPDH) are indicated. **c** The presence of exosome markers in End1/E6E7 cell-derived exosomes upon Poly I:C transfection. End1/E6E7 cells were transfected with or without Poly I:C (0.1, 1, 10 µg/mL) for 48 h. Cell culture SN was collected for exosome isolation and to detect the expression of the specific exosomal markers (Alix, CD63, CD81, PECAM-1, and LAMP2). SN, supernatant.

Real-Time PCR Quantification of mRNA and microRNA

Total RNA from cultured cells was extracted with Tri-Reagent (Molecular Research Center, OH, USA) as previously described [10]. Total RNA (1 µg) was subjected to reverse transcription using reagents from Promega (Promega, WI, USA). The resulting cDNA was then used as a template for qPCR quantification. The real-time PCR for the quantification of GAPDH, HIV gag, and ISG mRNA was performed with IQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) as described previously [30]. The levels of GAPDH mRNA were used as an endogenous reference to normalize the quantities of the tested mRNAs. The primers were synthesized by Invitrogen Inc., and the sequences of oligonucleotide primers are shown in Table 1. MicroRNA was extracted with the miRNeasy Mini Kit (Qiagen, Valencia, CA, USA) based on the manufacturer's instructions. RNA was reverse transcribed with the miScript Reverse Transcription Kit (Qiagen). Reverse transcription PCR for the quantification of a subset of miRNAs was carried out with

miScript Primer Assays and with the miScript SYBR Green PCR Kit from Qiagen as previously described [31]. Synthetic *Caenorhabditis elegans* miRNA-39 (cel-miR-39) was used as a spiked-in miRNA for normalization.

Western Blotting

Total cell lysates were prepared by the cell extraction buffer (Thermo Fisher Scientific, MA, USA) with 1% protease inhibitor cocktail (Sigma, MO, USA) according to the manufacturer's instructions. Equal amounts of protein lysates (30 µg) were separated on 4–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis precast gels and transferred to an Immobilon-P membrane (Millipore, Germany). The blots were incubated with primary antibodies in 5% nonfat milk in PBS with 0.05% Tween 20 overnight at 4°C (OAS1, 1:1,000; OAS2, 1:1,000; ISG15, 1:1,000; ISG56, 1:1,000; MxA, 1:1,000; Mx2, 1:1,000; GBP-5, 1:1,000; Alix, 1:1,000; LAMP2, 1:1,000; CD63, 1:1,000; CD81, 1:1,000; cytochrome C, 1:1,000; PECAM-1, 1:1,000; EEA1, 1:1,000; GAPDH,

Fig. 2. TLR3 signaling of End1/E6E7 cells released exosomes can be taken up by the macrophages. End1/E6E7 cells were cultured in exosome-free culture media for 48 h. Isolated exosomes were labeled with PKH67 fluorescent cell linker (green) and then added to Exosome Spin Columns. Purified PKH67-labeled exosomes

were incubated with macrophages for 48 h. Macrophages were then stained with Hoechst 33342 (blue) for nuclei and PKH26 fluorescent cell linker (red) and then observed under a fluorescence microscope (original magnification, $\times 100$).

Fig. 3. Effect of exosomes from Poly I:C-stimulated End1/E6E7 cell cultures on HIV infection of TZM-bl cells. TZM-bl cells were treated with or without exosomes (1, 2, 5, and 10 μ g) from End1/E6E7 cell cultures stimulated with Poly I:C for 24 h prior to (a), simultaneously (b), or after HIV infection (c). Luciferase activity

of TZM-bl cells was measured at 48 h post-HIV infection. The results are the mean $\pm$ SD of triplicate cultures, representative of 3 experiments ($*p < 0.05$ and $**p < 0.01$). HIV, human immunodeficiency virus.

Fig. 4. End1/E6E7 cell-derived exosomes inhibit HIV replication in macrophages. End1/E6E7 cells were transfected with or without Poly I:C (1 μ g/mL) for 48 h, and then, cell culture SN was collected for exosome isolation. **a–c** Morphologic observations of uninfected and HIV Bal-infected macrophages with or without exosomes pretreatment (arrows indicate syncytium, magnification $\times 100$). **d** Macrophages were pretreated for 24 h with or without End1/E6E7 cell-derived exosomes (1, 2, 5, and 10 μ g) and then infected with HIV Bal. Total RNA extracted from macrophages was sub-

jected to real-time PCR for HIV gag gene quantification 8 days post-HIV infection. **e** The intracellular p24 protein level was measured by Western blot. **f** The inhibition of HIV replication by End1/E6E7 cell SN with or without exosome depletion. The data were expressed as HIV gag levels relative (%) to control (without treatment, which is defined as 100%). The results are the mean $\pm$ SD of triplicate cultures, representative of 3 experiments (** $p < 0.01$). SN, supernatant; HIV, human immunodeficiency virus.

1:1,000; and HIV p24, 1:1,000). Horseradish peroxidase-conjugated appropriate second antibodies were diluted 1:2,000 to 1:8,000 in 5% nonfat milk PBS with 0.05% Tween 20. Blots were developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, MA, USA).

TZM-bl Assay

The anti-HIV activity was assessed with the TZM-bl, a commonly used cell line for evaluating the efficacy of neutralizing antibodies and small molecular inhibitors in HIV research [32]. TZM-bl cells contain the reporter gene (firefly luciferase) for the quantitative detection of HIV Tat-dependent luciferase activities after HIV infection. The cells were prepared and maintained as previously described.

End1/E6E7 Cell-Derived Exosomes Treatment and HIV Infection

TZM-bl cells were seeded in a 96-well microtiter plate (10^4 cells/well) and allowed to achieve confluence overnight at 37°C. The cells were then treated with or without End1/E6E7 cell-derived exosomes for 24 h prior to, simultaneously, or post-HIV (Bal strain) infection.

Luciferase activity of TZM-bl cells was measured at 48 h post-HIV infection. Macrophages were treated with or without End1/E6E7 cell-derived exosomes for 24 h and then infected with HIV Bal strain. At day 8 postinfection, the cells were collected for HIV gag gene expression. TZM-bl cells and primary human macrophages were infected with an equal amount (p24 30 ng/ 10^6 cells) of cell-free HIV Bal for 3 h at 37°C in the presence or absence of End1/E6E7 cell-derived exosomes. To deplete exosomes, the SN from Poly I:C-transfected HCEs was incubated with anti-CD63 antibody-conjugated with Dynabeads overnight at 4°C and then separated in a magnetic field.

Data Analysis

When appropriate, data were obtained from at least 3 independent experiments and expressed as mean $\pm$ SD. For comparison of the mean of 2 groups, the statistical significance was measured by Student's *t* test. To compare the difference between multiple groups, statistical significance was analyzed using ANOVA followed by Tukey's test. Calculations were performed with GraphPad Prism statistical software (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was defined as $p < 0.05$.

Fig. 5. Characterization of the antiviral factors in the exosomes of TLR3-stimulated End1/E6E7 cells. End1/E6E7 cells were transfected with or without Poly I:C (0.1, 1, and 10 µg/mL) and then cells and exosomes were collected to extract RNA and proteins. **a, c** Cellular RNAs (1 µg) and exosomal RNAs (1 µg) were subjected to real-time PCR for indicated genes (*OAS1*, *ISG56*, *MxA*,

and *Mx2*) and GAPDH RNA. Data are expressed as indicated gene RNA levels relative (fold) to untreated control, which is defined as 1. **b, d** Cellular proteins (20 µg) and exosomal proteins (20 µg) were subjected to Western blot assay using antibodies to *OAS1*, *ISG56*, *MxA*, and *Mx2*. The results are the mean ± SD of triplicate cultures, representative of 3 experiments (* $p < 0.05$ and ** $p < 0.01$).

Results

HCEs Produce Exosomes

To determine the role of exosomes in intercellular communications between HCEs and macrophages, we examined whether TLR3 signaling of End1/E6E7 cells can induce the production of exosomes. As shown in Figure 1a, electronic microscopy analysis of the purified pellets observed small membrane vesicles of 30–100 nm in diameter, which have a typical cup-shaped exosome structure. As shown in Figure 1b, Poly I:C-transfected cells produced exosomes that had the special exosomal markers (*Alix*, *CD63*, *CD81*, *LAMP2*, and *PECAM-1*) and lacked the cellular markers (*EEA1*, cytochrome C, and *GAPDH*). Because TLR3 localizes in the endosomal com-

partment of the cells, the transfection of Poly I:C is necessary for activating TLR3 in the cells. As shown in Figure 1c, Poly I:C transfection could increase the expression of the special exosomal markers (*Alix*, *CD63*, *CD81*, *LAMP2*, and *PECAM-1*) in End1/E6E7 cell-produced exosomes.

End1/E6E7 Cell-Derived Exosomes Can Be Taken Up by Macrophages

To visualize whether exosomes isolated from End1/E6E7 cell culture SN could be delivered into macrophages, macrophages were incubated with exosomes labeled with fluorescent dye PKH67 (green) for 18 h in a CO₂ incubator. Macrophages were then stained with a PKH26 (red) for membrane and Hoechst 33342 (blue) for nuclei. The cells were photographed under a confocal microscope

Fig. 6. Characterization of the microRNAs in the exosomes of TLR3-stimulated. End1/E6E7 cells cultured in exosome-free media were transfected with or without Poly I:C (0.1, 1, and 10 µg/mL) for 48 h. Cell culture SN was collected for exosome isolation. End1/E6E7 cells (**a**) and isolated exosomes (**b**) were subjected for RNA extraction. Total RNA (1 µg) was then subjected to real-time

RT-PCR to quantify the levels of miR-28, miR-29 family members, miR-125b, miR-150, miR-382, miR-223, miR-20a, and miR-198 in End1/E6E7 cells and exosomes. Synthetic *Caenorhabditis elegans* miRNA-39 (cel-miR-39) was used as a spiked-in miRNA for normalization. The levels of miRNAs were plotted as fold of control. SN, supernatant.

(Nikon A1R, Nikon, Japan). As shown in Figure 2, PKH67-labeled exosomes were observed within macrophages.

End1/E6E7 Cell-Derived Exosomes Inhibit HIV Infection in TZM-bl Cells

We first examined whether End1/E6E7 cell-derived exosomes can suppress HIV replication in TZM-bl cells. TZM-bl cells were treated with or without End1/E6E7 cell-derived exosomes for 24 h prior to, simultaneously, or post-HIV (Bal strain) infection. Luciferase activity of TZM-bl cells was measured at 48 h post-HIV infection. HIV infection in TZM-bl cells was positively correlated with the luciferase activity in the cells. As shown in Figure

3, exosomes from Poly I:C-transfected End1/E6E7 cells could inhibit HIV replication in TZM-bl cells. Comparing to the condition of the cells treated with exosome and infected with HIV simultaneously (Fig. 3c), the exosome pretreatment (Fig. 3b) was more effective in HIV inhibition (Fig. 3a). Therefore, we used the pretreatment condition to examine whether exosomes can inhibit HIV replication in primary human macrophages.

End1/E6E7 Cell-Derived Exosomes Inhibit HIV Replication in Macrophages

As demonstrated in Figure 4b, HIV infection could induce syncytia in macrophage cultures. In contrast,

Fig. 7. Effect of End1/E6E7 cell-derived exosomes on the expression of ISGs in macrophages. Primary human macrophages were treated with exosomes from Poly I:C-stimulated End1/E6E7 cells. The mRNA (**a**) and protein levels (**b, c**) of the ISGs were examined

by qRT-PCR and Western blot, respectively. Data were obtained from 3 independent experiments and expressed as the mean $\pm$ standard deviation (* $p < 0.05$ and ** $p < 0.01$).

treatment of HIV-infected macrophages with exosomes significantly reduced virus-induced giant syncytia (Fig. 4c). In addition, HIV replication was also significantly inhibited in macrophages pretreated with End1/E6E7 cell-derived exosomes at both genomic (Fig. 4d) and protein levels (Fig. 4e). To confirm the role of the exosomes in End1/E6E7 cell-mediated anti-HIV activity, we examined the antiviral potency of End1/E6E7 cell SN with or without exosome depletion. Figure 4f showed that while SN from Poly I:C-transfected End1/E6E7 cell cultures significantly suppressed HIV replication in macrophages, the depletion of exosomes from the culture SN diminished End1/E6E7 cell-mediated anti-HIV activity in macrophages.

Exosomes Contain anti-HIV ISGs and miRNAs

To determine the mechanism of the exosome-mediated anti-HIV activity, we next determined whether the exosomes carry the antiviral IFN-stimulated genes. As shown in Figure 5a, Poly I:C transfection of End1/E6E7

cells induced the mRNA expression of several key anti-HIV ISGs (ISG56, OAS1, MxA, and Mx2). The expression of these ISGs was also detected in the exosomes isolated from End1/E6E7 cell cultures (Fig. 5c). In addition, we also observed an increase of the ISGs in both End1/E6E7 cells (Fig. 5b) and End1/E6E7 cell-derived exosomes (Fig. 5d) at protein levels. It is known that miRNAs can be compartmentalized in cell-released exosomes and exert biologic functions on recipient cells [33, 34]. We, thus, examined whether the exosomes contain the anti-HIV miRNAs (miR-28, miR-29 family members, miR-125b, miR-150, miR-382, miR-223, miR-20a, and miR-198). As shown in Figure 6a and b, both End1/E6E7 cells and purified exosomes from Poly I:C-transfected End1/E6E7 cells induced the expression of the anti-HIV miRNAs.

End1/E6E7 Cell-Derived Exosomes Induce ISGs in Macrophages

We also examined the immune response in macrophages that were treated with End1/E6E7 cell-derived exo-

Fig. 8. Schematic diagram of mechanisms for exosome-mediated HIV inhibition in HCEs. Stimulation of HCEs with double-stranded RNA (Poly I:C) results in activation of TLR3 signaling pathway and production of the exosomes that contain the multiple cellular antiviral factors, including several key IFN-stimulated genes (ISGs: ISG56, OAS1, MxA, and Mx2) and the HIV restriction microRNAs

(miR-28, miR-29 family members, miR-125b, miR-150, miR-382, miR-223, miR-20a, and miR-198). These exosomes can be released by HCEs and taken up by HIV-infected macrophages where the released antiviral ISGs and the HIV restriction miRNAs can inhibit HIV at different steps of viral replication. HCEs, human cervical epithelial cells; HIV, human immunodeficiency virus.

somes. As shown in Figure 7, treatment of macrophages with the exosomes isolated from Poly I:C-transfected End1/E6E7 cells induced the expression of the anti-HIV ISGs at both mRNA (Fig. 7a) and protein levels (Fig. 7b, c).

Discussion

Our earlier work showed that SN from TLR3-activated HCEs contained the multiple antiviral factors that could effectively suppress HIV replication in macrophages [11]. However, the mechanisms by which HCEs confer immune protection to macrophages remain to be determined. The present study examined the role of exosomes in HCE-mediated anti-HIV activity. Exosomes are essential in the intercellular communication and play a role in host antiviral activities [21, 25]. Exosomes deliver their contents to recipient cells, through which they regulate host cell functions. This transporting function of exosomes is crucial in the bio-

logic processes, including immune response to viral infections [35, 36]. Therefore, it is of great interest to determine whether exosomes play a role in the intercellular communication between HCEs and macrophages. We showed that HCEs produced and released exosomes that could be taken up by macrophages (Fig. 1, 2). Importantly, exosomes isolated from HCEs, when added to HIV-infected macrophages, inhibited HIV replication (Fig. 4). This inhibitory effect on HIV is likely due to the antiviral ISGs and the HIV restriction miRNAs in the exosomes as the exosomes from Poly I:C-transfected HCEs were enriched with the antiviral ISGs at both mRNA and protein levels (Fig. 5). Studies have shown that cytosolic RNA is taken up into EVs undergoing inward budding from the cell membrane, and this budding-in process is designated as RNA loading into exosomes [37]. Importantly, RNA in exosomes is protected from RNase degradation and stable under various environment conditions [38].

Experimental evidence indicates that several ISGs, including *ISG15*, *ISG56*, *OAS1*, *MxA*, and *Mx2*, possess anti-HIV properties [39, 40]. For example, *ISG15* plays a crucial role in the late stages of HIV assembly and release [41, 42]; *OAS1* suppresses HIV by interfering with the viral RNA reverse transcription [43]; *Mx2* can reduce HIV replication as it has the ability to inhibit the capsid-dependent nuclear import of subviral complexes [44]. In addition to the ISGs, we also determined whether exosomes isolated from HCEs contain the HIV restriction miRNAs. Studies have shown that miRNAs can be packaged into and carried by exosomes at the immune synapse, regulating gene expression of the recipient cells [45, 46]. We demonstrated that both End1/E6E7 cells and End1/E6E7 cell-derived exosomes contained the HIV restriction miRNAs (miR-28, miR-29 family members, miR-125b, miR-150, miR-382, miR-223, miR-20a, and miR-198) (Fig. 6). It is known that miR-28, miR-125b, miR-150, miR-382, and miR-223 have the ability to bind to 3'UTR of HIV transcripts through sequence complementarity and inhibit viral translation [47]. The miR-29 family members can target a highly conserved site in various HIV subtypes and suppress viral translation/replication [48]. Studies by several groups reported that miR-20a can competitively bind to p300/CREB-binding protein-associated factor, an important cellular cofactor for the HIV Tat function, to inhibit HIV infection [48–50].

In summary, we demonstrated that HCEs could produce and release exosomes that contained the multiple antiviral factors. More importantly, the exosomes released by HCEs could be internalized by macrophages (Fig. 8). These findings suggest a novel anti-HIV mechanism through which HCEs as a bystander confer immune protection for HIV-infected macrophages. Because HIV has evolved several mechanisms to evade TLR3/IFN-me-

diated intracellular innate immunity in the host cells, such as macrophages [51, 52], the participation in host anti-HIV activities by non-HIV target and bystander cells in the FRT is necessary and important for control of HIV infection through sexual transmission. The observation that exosomes produced/released by HCEs have the ability to transport multiple antiviral factors to the macrophages suggests an alternative and feasible strategy for exosome-based immunotherapy for HIV disease.

Conflict of Interest Statement

The authors declare no conflict of interest.

Statement of Ethics

Monocytes were obtained from Human Immunology Core at the University of Pennsylvania (Philadelphia, PA, USA). The core has the institutional review board approval for blood collection from healthy donors.

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Author Contributions

X.-Q.X. and W.-Z.H. conceived and designed the experiments; X.-Q.X., Y.L., and L.G. performed the experiments; X.-Q.X. and B.Z. analyzed the data; F.-Z.M., W.-H.H., and X.W. contributed to reagents, materials, and analysis tools; X.-Q.X., W.-Z.H., and Adil Khan reviewed and revised the manuscript. All the authors have read, reviewed, and edited the manuscript and agreed for submission to this journal.

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