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Cell-cell contact viral transfer contributes to HIV infection and persistence in astrocytes

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Abstract

Astrocytes are the most abundant cells in the central nervous system and play important roles in HIV/neuroAIDS. Detection of HIV proviral DNA, RNA and early gene products but not late structural gene products in astrocytes *in vivo* and *in vitro* indicates that astrocytes are susceptible to HIV infection albeit in a restricted manner. We as well as others have shown that cell-free HIV is capable of entering CD4⁺ astrocytes through human mannose receptor-mediated endocytosis. In this study, we took advantage of several newly developed fluorescence protein-based HIV reporter viruses and further characterized HIV interaction with astrocytes. First, we found that HIV was successfully transferred to astrocytes from HIV⁺ infected CD4⁺ T cells in a cell-cell contact- and gp120-dependent manner. In addition, we demonstrated that compared to endocytosis-mediated cell-free HIV entry and subsequent degradation of endocytosed virions, the cell-cell contact between astrocytes and HIV-infected CD4⁺ T cells led to robust HIV infection of astrocytes but retained the restricted nature of viral gene expression. Furthermore, we showed that HIV latency was established in astrocytes. Lastly, we demonstrated that infectious progeny HIV was readily recovered from HIV latent astrocytes in a cell-cell contact-mediated manner. Taken together, our studies point to the importance of the cell-cell contact-mediated HIV interaction with astrocytes and provide direct evidence to support the notion that astrocytes are HIV latent reservoirs in the central nervous system.

Keywords

HIV; astrocytes; cell-cell contact; viral persistence; latency; gene expression

INTRODUCTION

HIV gains access to the central nervous system (CNS) soon after the systematic infection (1, 2) and causes a variety of neurological dysfunctions, collectively called HIV-associated neurocognitive disorder (HAND) (3, 4). Despite the success of combination antiretroviral therapy (cART) in suppressing HIV replication in the peripheral blood, improving immune

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Conflict of Interest

The authors declare no conflict of interest.

function and prolonging the lifespan of HIV-infected individuals (5, 6), HAND has remained prevalent (6–8). In light of the persistent effects of HIV on the CNS in the era of cART, a better understanding of HIV/neuroAIDS pathogenesis is undoubtedly warranted and urgently needed.

The biggest challenge in tackling HIV is the inability of cART to eradicate the virus. Two main reasons for this challenge are replication of the virus in immune-privileged sites with limited access to cART such as CNS and the ability of the virus to establish latent infection. Our knowledge about HIV latent reservoirs and their regulatory mechanisms is primarily derived from studies on two main peripheral HIV cellular reservoirs: macrophages (9, 10) and resting CD4⁺ T cells (11, 12). In comparison to the peripheral blood, the main HIV target cells in the CNS are macrophages/microglia, which can be actively, persistently, or latently infected with HIV (see review (13). Limited accessibility to cART and the ability of HIV to establish latent infection have made the CNS a unique HIV reservoir (14, 15). Astrocytes possess several characteristics that make them main players as HIV reservoirs in the CNS. These include susceptibility to HIV infection (see discussion below), the abundance, very low turnover (16, 17), and ability to produce infectious viruses to infect other cells when stimulated with pro-inflammatory cytokines TNF α or IL1- β , or when co-cultured with CD4⁺ T cells and monocytic cell lines (18–22). However, the exact roles of the astrocytes in serving as HIV reservoirs in the CNS and their contributions to HAND in the era of cART have not been defined.

HIV-1 infection of astrocytes has been documented both *in vivo* and *in vitro* (23–25), although the infection has primarily been characterized as one that is consistent with a restricted form, i.e., expression of early multiply spliced HIV-1 gene products such as Nef (26, 27), but no late structural gene products (18, 28). Restrictions in astrocytes are believed to take place at multiple levels, including entry (29, 30), transcription (31, 32), and post-transcription (22, 33–35). A recent study shows that up to 20% of perivascular astrocytes can be infected by HIV and that the percentage of HIV-infected astrocytes correlates with the severity of encephalitis and dementia (36), further confirming the important roles of HIV infection of astrocytes in HIV/neuroAIDS. The underlying mechanisms likely involve (1) HIV invasion into the CNS through astrocytes at the interface of blood-brain barriers (37–39); (2) Secretion of cytokines/chemokines by astrocytes to attract infiltration of monocytes/macrophages and CD4 T cells into the CNS and facilitate HIV spread among those cells and the CNS cells (18, 40–42); (3) Astrocyte activation (astrocytosis) and dysfunction (e.g., glutamate metabolism) and production of neurotoxins and cytokines/chemokines by astrocytes to cause neuronal injury (43–46). Importantly, latent HIV infection in the CNS has recently been linked to astrocyte activation, compromised neuronal integrity, and altered expression of epigenetic factors and cytokine/chemokines in the CNS (47). Nevertheless, it should be pointed out that all of the above-mentioned *in vitro* studies about HIV interaction with astrocytes are derived from use of cell-free HIV.

Cell-cell contact-mediated intercellular virus spread has recently been recognized as an important route of infection and transmission for a number of viruses including T cell leukemia virus type 1, human hepatitis C virus and HIV (48–50). Intercellular HIV transfer can occur among CD4 T lymphocytes, macrophages, dendritic cells, and renal epithelial

cells (51–54); it involves virological synapse formation (48, 55, 56) and viral factors such as Env and Gag and host factors such as CD4 and chemokine co-receptors CXCR4/CCR5 (56–58). This new route of HIV infection offers protection against anti-HIV neutralizing antibodies and exhibits decreased sensitivity to cART treatment (59, 60). Considering the compact nature of the cells in the CNS and the long perceived notion that HIV is introduced into the CNS by infiltrating HIV-infected macrophages/monocytes and CD4 T lymphocytes, we hypothesized that cell-cell contact plays important roles in HIV infection with astrocytes in the CNS and formation of HIV reservoirs in these cells. In the present study, we took advantage of several recently developed HIV reporter viruses and determined the possibility of cell-cell contact-mediated HIV infection of astrocytes. We found that compared to cell-free HIV infection, cell-cell contact between astrocytes and HIV-infected CD4 T lymphocytes led to robust HIV infection of astrocytes. Importantly, we demonstrated that HIV successfully maintains an extremely low level of ongoing HIV replication in astrocytes. Lastly, we showed that infectious progeny viruses were readily recovered from HIV latent astrocytes in a cell-cell contact manner.

MATERIALS AND METHODS

Cells

Human 293T, human T lymphoblastoid cell line Jurkat and human astrocytoma cell line U373.MG were obtained from American Tissue Culture Collection (Manassas, VA). Human T cell leukemia cell line MT4 were obtained from NIH AIDS Reagent Program (kindly donated by from Dr. Douglas Richman of University of California San Diego) (61). Jurkat stably expressing green fluorescent protein (GFP) (GFP-Jurkat) were established as previously described (62). Briefly, pEGFP was linearized with *Pvu*I and electroporated into Jurkat constitutively expressing the tTA using a gene pulser (Bio-Rad, Hercules, CA, USA). pTK-Hyg (Clontech) was included in the transfection to facilitate subsequent selection of stable cell clones. After electroporation, The clone expressing the highest level of GFP fusion protein was selected by using the limiting-dilution method in the presence of 800 µg/ml G418 (Invitrogen), 200 µg/ml hygromycin (Boehringer Mannheim), and 2 µg/ml Tet (Sigma, St. Louis, MO). GFP-Jurkat were maintained in the presence of 400 µg/ml G418 and 200 µg/ml hygromycin. Human primary astrocytes (HPA) were prepared from aborted human fetus brain tissues (15–20 weeks) (Advanced Bioscience Resources, Alameda, CA), as previously described (29). Briefly, fetus brain tissues were dissected, trypsinized, and passed through a 94 µm mesh for single cell suspension. Mixed brain cells were maintained in primary astrocytes culture medium for 3 passages to generate high purity astrocytes. Immunofluorescence staining was performed to assure a purity of 98% glial acidic fibrillary protein (GFAP)-positive cells, i.e., astrocytes. 293T and U373.MG cells were maintained in Dulbecco's modified Eagle's medium (DMEM); Jurkat, GFP-Jurkat, MT4 were maintained in Roswell Park Memorial Institute 1640 medium (RPMI-1640); HPA were maintained in Nutrient Mixture Kaighn's Modification F-12 medium. All cells were cultured at 37°C with 5% CO₂. All culture mediums were purchased from Lonza (Walkersville, MD) and supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 1% Penicillin-streptomycin-glutamine (Life Technologies, Grand Island, NY).

Antibodies and reagents

Rabbit anti-GFAP antibody (Z0334) was purchased from Dako (Carpinteria, CA). PE-conjugated mouse anti-p24 antibody (KC57) was purchased from Beckman Counter (Brea, CA). Sheep anti-HIV-1 gp120 serum was obtained from the NIH AIDS Reagent Program (kindly donated by Dr. M. Phelan of Division of AIDS of NIH/NIAID) (63). Rabbit anti-mCherry antibody (ab167453) was purchased from Abcam (Cambridge, MA). Goat anti-mouse Alexa-Fluor-555 and goat anti-rabbit Alexa-Fluor-488 antibodies were purchased from Molecular Probes (Eugene, Oregon, USA). PE-conjugated goat anti-rabbit antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Yeast mannan and dextran sulfate were purchased from Sigma-Aldrich (St. Louis, MO).

Plasmids

RGH plasmid were obtained from NIH AIDS Reagent Program (donated by Dr. I. Sadowski and Dr. S. Viviana of University of British Columbia, Canada) (64). HIV gagi and NLGi plasmids were generously provided by Dr. B. K. Chen of Mount Sinai School of Medicine (65, 66). pHCMV-G was a kind gift from Dr. J. Sodroski of Harvard School of Medicine, and it encodes the glycoprotein from vesicular stomatitis virus (VSV-G) under the control of the CMV promoter. pcDNA3, and pCMV- β gal were purchased from Clontech (Mountain View, CA). pLTR-Luc was obtained from NIH AIDS Reagent Program (kindly donated by Dr. R. Jeeninga and Dr. B. Berkhout of University of Amsterdam) (67, 68). pTat.myc was constructed as previously described (69).

Cell-free virus preparation, infection and spinoculation

NL4-3, gagi, NLGi and RGH/pHCMV-G (3:1) plasmids were transfected to 293T using a standard calcium phosphate precipitation method. Transfection medium was replaced with fresh medium 16 hr post transfection. Culture medium was collected 72 hr after the medium change, removed of cell debris, purified and concentrated by passing through a 20% sucrose cushion and spinning at 100,000 *g* for 2 hr, and then suspended in PBS, aliquoted and stored at liquid nitrogen. Viral titers were determined by the reverse transcriptase (RT) assay previously described (30) and expressed as counts per minute (cpm). For cell-free HIV infection, Jurkat or MT4 (1×10^6) were infected with HIV equivalent to 10,000 cpm RT in a final volume of 1 ml RPMI complete medium at 37°C, 5% CO₂ for 4 hr. At the end of the infection, unbound viruses were removed, the cells were rinsed with multiple PBS washes and then cultured in fresh medium at a density of $0.3\text{--}1.0 \times 10^6$ cells/ml. The percentage of infected cells was determined every other day by immunostaining for p24, followed by flow cytometry. For cell-free HIV infection of astrocytes, U373.MG or HPA were seeded to a 6-well plate at the 50–70% confluence, cultured for one day, and then infected with HIV (NL4-3, gagi, or NLGi) equivalent to 15,000 cpm RT at 37°C, 5% CO₂ for 4 – 16 hr as indicated. For spinoculation infection of astrocytes with VSV-G-pseudotyped RGH, HPA were seeded in a 6-well plate, VSV-G-pseudotyped RGH equivalent to 30,000 cpm RT were added in a final volume of 3 ml and spun at 600 *g*, room temperature for 2 hr. At the end of the spinoculation, unbound viruses were removed, the cells were rinsed with multiple PBS washes and then cultured in fresh medium.

Cell-cell contact-mediated HIV infection (co-culture) assay

For co-culture assay, Jurkat or GFP-Jurkat were infected with cell-free NL4-3 to achieve 50–70% infected cells (determined by p24 staining and flow cytometry) as above and used as the donor cells. HPA or U373.MG (5×10^4) were seeded and cultured in a 24-well plate and used as the target cells. Then, the donor cells (5×10^4) were added onto the target cells, i.e. at the ratio of 1:1 and cultured for 16 hr. At the end of the co-culture, the cells were fixed and subjected to immunostaining for p24. In parallel, as controls for co-culture infection, cell-free infection was set up as follows: the same number of HIV-infected donor cells (5×10^4) cells were cultured in fresh medium for 16 hr, the cell culture supernatants were collected, removed of cells and cell debris using 600 *g* centrifugation for 10 min plus 0.2 mm filtration, and used as cell-free viruses to infect HPA or U373.MG for 16 hr. In addition, the transwell infection was set up as follows: the same number of HIV-infected donor cells (5×10^4) were washed and seeded onto the insert of a transwell with HPA or U373.MG (5×10^4) cultured in the lower chamber and continued to culture for 16 hr. At the end of the cell-free and transwell infection, the cells were fixed and subjected to immunostaining for p24. HIV-infected cells, i.e., p24+ HPA or U373.MG were determined by flow cytometry or microscopic imaging.

Luciferase assay

The Firefly luciferase activity was measured using the luciferase assay system from Promega according to the manufacturer's directions. Briefly, HPA cells were transfected with the appropriate amount of pLTR-Luc with pCDNA3 or pTat.myc using a standard calcium phosphate precipitation method. pCMV-βgal were co-transfected to normalize transfection variations. MT4 cells were added to pLTR-Luc-transfected HPA 48 hr post the first transfection and cells were washed with PBS and collected in 1× firefly luciferase lysis buffer at 72 hr post transfection. Lysates were centrifuged briefly to obtain the clear cell lysates. The clear lysates were mixed with firefly luciferase substrate, and the luciferase activity was measured by an Opticomp Luminometer (MGM Instruments, Hamden, CT).

Flow cytometry

Adherent cells were collected using brief trypsin digestion, while suspension cells were collected by centrifugation at 600*g* for 10 min. Cells were either suspended in PBS and directly analyzed by flow cytometry, or the cells were fixed in 4% paraformaldehyde at RT for 15 min, then permeabilized in 0.1% triton in PBS at RT for 10 min, followed by staining with desired primary antibody at RT for 60 min, appropriate secondary fluorescence antibody at RT for 60 min. Cells were then washed with PBS between the staining steps above, suspend in PBS, and analyzed by flow cytometry.

Immunofluorescence microscopic imaging Expression of mCherry, GFP, and GFP fusing proteins was captured using a Zeiss Axiovert 200 microscope. For immunostaining/microscopic imaging, immunostaining was performed as described above except for that cells were blocked in PBS-BB (1% non-fat milk, 0.2% Bovine serum albumin, 0.3% Triton in PBS) before they were stained with appropriate primary and secondary antibodies. In addition, at the end of the staining, cells were mounted with Fluoromount G medium

(Southern Biotech, Birmingham, AL). All representative micrographs were taken using a Zeiss Axiovert 200 microscope.

Data analysis

Where appropriate, values were expressed as mean $\pm$ SD of triplicate experiments. All statistical analyses including one-way and two-way ANOVA followed by post hoc tests (bonferroni correction or Dunnett's test). A p value of < 0.05 was considered statistically significant (*), a p value < 0.01 highly significant (**). All data were representative of multiple repeated experiments.

RESULTS

Cell-free HIV-1 enters astrocytes through gp120- and human mannose receptor (hMR)-dependent endocytosis

We have shown that cell-free HIV infection of astrocytes is mediated by gp120- and human mannose receptor (hMR)-dependent endocytosis (29, 30). To ascertain the hMR-mediated endocytosis nature of cell-free HIV infection in astrocytes, human fetal astrocytes (HPA) were infected with cell-free HIV at two different temperatures, i.e., 37°C and 4°C, or in the presence of anti-HIV gp120 antibody, or hMR antagonist yeast mannan or dextran sulfate for 16 hr. Then the virus entry into astrocytes was determined by immunostaining for HIV p24 (red), while glial fibrillary acidic protein (GFAP, green) and 4',6-diamidino-2-phenylindole (DAPI, blue) staining was performed to identify the astrocytes and the nucleus, respectively. As expected (70, 71), p24 staining was detected only in HPA at 37°C, but not in HPA at 4°C (Fig. 1A). Treatment of anti-HIV gp120 antibody (Fig. 1B) and yeast mannan (Fig. 1C) or dextran sulfate (Fig. 1D) all led to significant decreases of p24 detection in HPA. These results confirm the important role of hMR-mediated endocytosis in cell-free HIV infection of astrocytes.

Degradation of endocytosed cell-free HIV-1 in astrocytes

Receptor-mediated endocytosis delivers ligands to early sorting endosomes, where ligands become dissociated from their receptors, then ligands traffic to late endosomes and lysosomes for degradation (72–74). Thus, it is highly conceivable that HIV uptake through hMR-mediated endocytosis into astrocytes would mainly lead to degradation and as a result, little infection. To determine this possibility, HPA were infected with cell-free HIV at 37°C for 16 hr to allow the virus endocytosis to take place. Then, the cells were removed of unbound viruses and continued to culture in fresh medium at 37°C for additional 24 hr and 48 hr. At the end of each time points, the cells were stained for p24, GFAP or DAPI as above. Compared to p24 staining in HPA at the beginning of the continued culturing (0 hr), there was a considerable decrease of p24 staining in the HPA at 24 hr and even more decrease of p24 staining in the HPA at 48 hr (Fig. 2A). To confirm the findings, a recently developed HIV reporter virus gag_i (48, 65) was used in the same experiments. The new reporter virus has the green fluorescence protein (GFP) coding sequence inserted at the cleavage site between matrix and capsid within the HIV *gag* gene (Fig. 2B), thus leading to production of GFP-incorporated (GFP-labeled) HIV, which will allow direct and accurate detection of HIV dynamics within the cells without immunostaining. The gag_i was used to

infect HPA as above, the dynamics of gag in HPA was monitored by flow cytometry. Consistent with the findings obtained by p24 staining, gag was significantly decreased at day 1, almost became undetectable at day 2 and beyond (Fig. 2C). Inability to detect HIV in astrocytes after 2 days of infection could be due to degradation of input viruses following endocytosis or lack of *de novo* Gag expression and infectious progeny viruses resulting from restricted HIV structural gene expression in astrocytes. In addition, to determine whether HIV uptake by hMR-mediated endocytosis would give rise to productive HIV infection in astrocytes, CD4 T lymphocytes MT4 were co-cultured with the HPA at day 5 post gag infection for additional 9 more days (Fig. 2C) and then analyzed by flow cytometry for gag+ cells. There were few gag+ MT4 cells detected in both mock- and gag-infected HPA (Fig. 2D), suggesting that despite being taken up by HPA, endocytosis of cell-free HIV might not likely lead to establishment of efficient HIV infection in HPA.

Cell-cell contact leads to successful entry of HIV-1 into astrocytes

Besides cell-free infection, HIV also infects its target cells through cell-cell contact (51–54). One early study also suggests the possibility of cell-cell contact HIV infection of astrocytes (75). Considering the notion that HIV gains access to the brain via infiltrating HIV-infected monocytes/macrophages and CD4 T lymphocytes and considering the highly compact nature of the cells in the brain (3, 76), cell-cell contact is likely to contribute to HIV infection in the brain cells such as astrocytes. To determine the possibility, we designed a unique experimental scheme (Fig. 3A) to compare the role of cell-free vs. cell-cell contact-mediated HIV infection in astrocytes, i.e., to culture HIV-infected CD4 T lymphocytes Jurkat in fresh medium for 16 hr, then the cell-free supernatant and the cells were separately collected. The supernatant was used to infect human astrocytoma cells U373.MG, while the same number of cells were cocultured with U373.MG or placed on the insert of a transwell in which U373.MG were cultured at the bottom. U373.MG from each infection were collected 16 hr post infection and stained for p24. p24 was only detected in the co-cultured U373.MG and only in the U373.MG that were in close contact with HIV-infected Jurkat, but not in cell-free and transwell infections (Fig. 3B). Similar results were obtained using HPA (data not shown). To accurately quantify the p24+ astrocytes and to validate the findings in HPA, stable GFP-expressing Jurkat (GFP-Jurkat) were used in place of regular Jurkat as donor cells and HPA as the target cells in the similar experiments. Use of GFP-Jurkat allows complete exclusion of input GFP-expressing Jurkat donor cells from the p24+ HPA in the samples by flow cytometry and accurate quantitation of the p24+ HPA in the samples by flow cytometry (Fig. 3C). Compared to the mock control, coculture had about 12% p24+GFP– HPA after 16 hr co-culture, but only negligible p24+GFP– HPA in cell-free infected or transwell-infected HPA (Fig. 3C & D). Infection of HPA with cell-free viruses produced from the equivalent number of the HIV-infected cells during the 16 hr culture and infection of HPA in the transwell setting did not give rise to any significant p24+GFP– HPA, which would also exclude the possibility that p24+GFP– HPA in the co-culture are derived from cell-free infection. These together results indicate that cell-cell contact between HIV-infected CD4 T lymphocytes and astrocytes results in more efficient HIV transfer into astrocytes than cell-free infection.

Cell-cell contact leads to successful establishment of HIV infection of astrocytes

In addition to being a higher infection efficiency, cell-cell contact-mediated viral infection has been shown to bypass cell-free infection-associated restrictions such as antibody neutralization and entry block (77, 78). HIV uptake into astrocytes by hMR-mediated endocytosis is extremely inefficient (29, 30). Thus, we next determined whether cell-cell contact-mediated HIV infection would lead to successful establishment of HIV infection in astrocytes. Considering the restricted nature of HIV structural gene expression in astrocytes, we took advantage of HIV reporter virus NLGi (48, 65). In NLGi, the *gfp* gene is inserted in-frame immediately preceding Nef (Fig. 4A). Since GFP protein is expressed alone without Nef, GFP is not incorporated into HIV virions; its expression can be used as a sensitive and direct indicator of HIV early gene expression, i.e., establishment of HIV infection in astrocytes. Thus, NLGi was used to infect MT4, and NLGi-infected MT4 were then co-cultured with HPA in a ratio of 1:1 as above. The co-culture was passaged every two day and continued to culture and monitored for GFP expression by fluorescence microscopic imaging. At day 1 after the first passage, GFP+ cells were more input MT4 cells, judged by the smaller cell size and the round cell morphology (Fig. 4B, upper panels). At day 8 after the first passage, GFP was detected in few MT4, instead in more HPA, which appeared to be larger and have cellular projections (Fig. 4B, lower panels). GFAP staining further confirmed that those GFP+ cells at day 8 were indeed GFAP+ astrocytes (Fig. 4C). No GFP signal was detected in HPA when co-cultured with uninfected MT4 or in cellfree virus-infected HPA (data not shown). controls such as co-culture of uninfected MT4 with These results provide the evidence that co-culture of astrocytes with HIV-infected CD4+ T cells leads to successful establishment of HIV infection in astrocytes.

Cell-cell contact-mediated HIV-1 infection of astrocytes is dependent on the number of input HIV-infected donor cells and HIV-1 envelope gp120

To further ascertain that the cell-cell contact is responsible for HIV infection from HIV-infected CD4 T lymphocytes to astrocytes, an increasing number of NLGi-infected MT4 was co-cultured with HPA at 37°C for 16 hr as above. At the end of the co-culturing, representative images of the co-cultures were taken under a fluorescence microscope. GFP+ cells were identified to be exclusively the input MT4 based on the small cell size and the round cell morphology (Fig. 5A). Then, the co-cultures were passaged every two days, followed by gentle shaking to gradually remove the input donor cells. Similar to our previous results (Fig. 4), GFP+ HPA began to show up at day 8 post co-culture. Manual counting of GFP+ HPA at day 12 showed that the number of GFP+ HPA gradually increased with the increasing number of input NLGi-infected MT4 (Fig. 5B). In parallel, the highest number of the input NLGi-infected MT4 cells (1×10^6) were cultured for 16 hr, the culture supernatants were collected and used as cell-free viruses to infect HPA. But, there were no GFP+ HPA identified in the cell-free infection (Fig. 5B). In addition, we pretreated the NLGi-infected MT4 with anti-HIV gp120 monoclonal antibody before the co-culture and performed the similar co-culture experiments in the presence of the same anti-gp120 antibody. Compared to the isotype-matched IgG control, anti-gp120 antibody treatment showed a significant decrease in the number of GFP+ HPA (Fig. 5C). Taken together, HIV transfer from HIV-infected CD4 T lymphocytes to astrocytes and ensuing infection in

astrocytes are dependent on the number of the infected donor cells and directly involve HIV-1 gp120.

Establishment of HIV latency in astrocytes

As discussed above, astrocytes possess several attributes to be HIV reservoirs. We next examined the possibility that astrocytes could serve as HIV reservoirs. To this end, we took advantage of a recently developed dual red-green fluorescent HIV reporter virus (RGH) (64). In RGH (Fig. 6A), the GFP coding sequence is inserted between matrix and capsid of the *gag* gene, exactly the same as that in *gag* (Fig. 2B), and a CMV-driven mCherry-expressing cassette is inserted in place of Nef gene. In addition, Env gene is disrupted by Kpn I digestion, blunted and re-ligation. Thus, expression of GFP, mCherry, or both in RGH-infected cells has been used to monitor infected but not integrated, latent infection, and integrated/active infection, respectively (Fig. 6B). As a standard protocol used in studies of HIV latency, VSV-Gpseudotyped RGH was used to infect HPA by spinoculation. HPA were then monitored for GFP+mCherry+ HPA (active infection) and GFP-mCherry+ HPA (latent infection) by flow cytometry. The number of GFP+mCherry+ HPA (active infection) showed an initial increase, representative of *de novo* infection and replication, followed by gradual decreases to a background level at day 25 (Fig. 6C). Conversely, the number of GFP-mCherry+ (latent infection) showed an initial increase, then a steep decrease (likely due to post-entry restrictions), followed by gradual increases until day 25 to 30. Micrographs taken at selected time points, i.e., day 1, 7 and 20 showed similar results (Fig. 6D). These results provide the definitive evidence of establishment of HIV latency in astrocytes and suggest that VSV-Gpseudotyped RGH-HPA could serve as an excellent platform to study the underlying molecular mechanisms of HIV latency in astrocytes.

Recovery of infectious progeny HIV from latent astrocytes in a cell-cell contact manner

One important criterion to define HIV latent cells/reservoirs is the potential to produce infectious progeny viruses upon the external stimuli (79). Thus, we determined whether HIV was truly latent in astrocytes after these cells acquired HIV, i.e., complete shutdown of HIV replication or maintain an extremely low level of HIV replication that is not be detectable by routine virology assays. To this end, first, HPA were transfected with *gag* (GFP as an indicator of HIV late structural genes) or NLGi (GFP as an indicator of HIV early genes) and monitored GFP expression. As expected, GFP in *gag*-transfected HPA was detected only at the beginning, i.e., day 3, but not at later time points day 7 and 19 (Fig. 7A). In contrast, GFP in NLGi-transfected HPA was detected throughout the experiments, i.e., day 3, 7 and 19 (Fig. 7B). However, the routine p24 ELISA, RT assay and qRT-PCR detected only HIV replication at day 3 but not at day 7 and 19 (data not shown). To determine there was no truly HIV replication in those cells, fresh MT4 cells were added to *gag*- and NLGi-transfected HPA at day 19, the co-cultures were continued for 3 more days. Direct microscopic imaging showed formation of clusters of GFP+ MT4 in both *gag*- and NLGi-transfected HPA/MT4 co-cultures (Fig. 7C & D), suggesting that those transfected astrocytes, despite of the restricted nature of HIV replication in astrocytes, are capable of producing an extremely lower level of progeny viruses to infect CD4 T lymphocytes. To further ensure the infectivity of the progeny viruses, the input MT4 were recovered from the co-cultures by gently shaking, continued to culture, and monitored for GFP+ cells by flow

cytometry. The supernatants of the transfected HPA at day 17 to day 19 were used to infect naive MT4 as cell-free infection controls, while GFP-transfected HPA and their supernatants were also included as mock controls in the experiments. The results showed that only MT4 derived from either gagi- (Fig. 7E) or NLGi-transfected HPA (Fig. 7F) exhibited continued HIV replication, but no HIV replication was detected in all other controls. Of note was that gagi consistently exhibited a delayed replication kinetics compared to NLGi. Then, we performed the similar HIV recovery experiment using the HPA that were pre-infected with cell free NL4-3, co-cultured with NL4-3-infected Jurkat and the transwell containing NL4-3-infected Jurkat as described in Fig. 3A and using naive Jurkat to recover the HIV, followed by p24 staining to monitor HIV replication. The results confirmed that only Jurkat from HPA that were co-cultured with NL4-3-infected Jurkat showed HIV replication, but none of others did (Fig. 8A). Taken together, all these results suggest that there is indeed a very low level of ongoing HIV replication in astrocytes about 2–3 weeks after they acquire HIV through either transfection or cell-cell contact-mediated infection and that cell-cell contact allows successful transfer to and infection of new target cells by the extremely low level of infectious progeny viruses. The cell-cell contact-mediated HIV recovery from HPA did not appear to result from cell-cell contact-induced activation of HIV LTR transcription, as MT4 co-culture with HPA did not lead to any increases of the LTR-driven luciferase reporter gene activity in HPA (Fig. 8B).

DISCUSSION

Here, we have determined that the degradation following the receptor mediated-endocytosis of cell-free HIV only leads to a transient p24 detection and failed to establish HIV infection and viral reservoir in astrocytes (Fig. 1 & 2). In addition, we have demonstrated that a novel route for HIV infection is mediated by direct cell-cell contact virus transfer from infected CD4 T lymphocytes to astrocytes and leads to successful infection as shown by HIV early gene expression (Fig. 3 & 4). This cell-cell contact-mediated HIV infection of astrocytes is cell density- and gp120-dependent (Fig. 5). Furthermore, we have shown that HIV infection of astrocytes leads to establishment of such a lower level of persistent replication in astrocytes that only cell-cell contact is able to recover the infectious progeny viruses as shown with use of HIV reporter viruses gagi, NLGi, RGH and NL4-3 (Fig. 6–8).

We have previously shown that cell-free virus entry into astrocytes is mediated through hMR-mediated endocytosis (29, 30). Other cell surface receptors including DC-SIGN, CCR5 and CD5 have also been reported to be involved in viral entry into astrocytes by interacting with viral envelope protein gp120 and initiated the receptor-dependent HIV endocytosis (29, 80–82). HIV virions are detected in clathrin-coated pits and cytoplasmic endocytotic vesicles by electron microscopy (29, 30, 82). GFP-labeled HIV viruses are found to co-localize with endosome and lysosome markers and become rapidly degraded in the lysosomes (80). Thus, endocytosis uptake of HIV is extremely inefficient and likely not an effective pathway leading to establishment of HIV infection in astrocytes (Fig. 1 & 2). Meanwhile, a recent study has reported up to 20% of HIV-infected astrocytes in the brain of HIV-infected subjects, which correlates with HIV-associated neurocognitive disorders (36), suggesting the existence of alternative route for HIV to gain access to astrocyte and its importance. In the current study, we have directly compared cell-free and cell-cell contact

HIV infection of astrocytes and unambiguously demonstrated that only cell-cell contact results in successful establishment of HIV infection in astrocytes (Fig. 3–5, & 8A). Our current study may offer the mechanistic support to substantial HIV infection of astrocytes *in vivo* and suggest that cell-cell contact could be the main route for HIV infection of astrocytes.

Cell-cell contact-mediated intercellular HIV transfer and infection have received a great deal of attention for the past several years (51–54). Intercellular HIV transfer between CD4+ T lymphocytes requires formation of virological synapse and host factors such as CD4, CXCR4/CCR5, cytoskeleton and viral factors such as gp120 and Gag involved (65, 83–85). Although astrocytes express two major HIV co-chemokine receptors CXCR4 and CCR5 (86, 87), they do not have a detectable cell-surface CD4 expression (18, 88) but have hMR (29) for HIV infection. Similar to cell-free HIV infection (29), we have shown that cell-cell contact-mediated HIV infection of astrocytes is dependent on gp120 (Fig. 5C). Whether hMR is also involved in this new pathway of HIV entry of astrocytes remains to be determined. In addition to virological synapses, there is a second means of direct cell-cell spread of HIV-1, known as long-range nanotubes or filopodial bridges (89, 90). It has been hypothesized that these cellular protrusions are remnants of virological synapses, left after cell separation, or that they are the initial step in virological synapse formation, establishing cell-cell connections that allow the infected and uninfected pair to come together more efficiently (91). Of particularly note is that nanotube formation between CD4+ T lymphocytes and astrocytes (Fig. 3B), suggesting that intercellular nanotube formation may be the HIV transfer mechanism between CD4 T lymphocytes and astrocytes. It is clear that the host and viral factors involved in the intercellular transfer between CD4 T lymphocytes and astrocytes and the roles of nanotube formation in the transfer process merit further investigation.

HIV infection of astrocytes has long been described to be non-productive, i.e., lack of late structural gene expression and as a result, no production of infectious progeny viruses due to multiple restrictions in astrocytes. HIV replication in astrocytes can only be briefly and partially restored by treatment with IL-1 β and TNF- α and removed of the Rev or nuclear export blocks (92–98). These findings suggest post-integration blocking mechanisms other than limited transcription factors in astrocytes. In this study, we have shown that HIV successfully establishes latency in astrocytes (Fig. 6). VSV-G-pseudotyped RGH, by way of spinoculation, appears to completely override not only the entry restriction, but also post-entry restrictions, as shown by GFP expression. Similar results have indeed been obtained in an early study (99). In contrast, HIV gene expression in HIV-transfected astrocytes does display gradual loss of HIV structural genes but not the early genes (Fig. 7A & B). This discrepancy is likely due to the different Rev level and its requirement for multimerization and the Rev biphasic function in HIV gene expression (100, 101). Most surprisingly, in the study, we have shown that there is a low level of production of infectious progeny viruses in HIV-transfected astrocytes (Fig. 7) or cell-cell contact-mediated HIV-infected astrocytes (Fig. 8A). Unlike cell-free infection, which could occur at a considerable distance from the infected donor cells, cell-cell contact-mediated HIV infection occurs when donor and target cells interact and are in close contact and thus is more readily achieved (102, 103). Consistent with this notion, the level of cell-free progeny viruses from astrocytes are so low

that do not enable infection of CD4 T lymphocytes, but the viruses can be successfully transmitted to CD4 T lymphocytes through cell-cell contact (Fig. 7B, C, E, F & 8A). Importantly, these findings suggest that HIV in astrocytes is indeed replication-competent albeit at an extremely low level. In addition, the VSV-G-RGH/astrocytes/spinoculation system (Fig. 6), the gag/astrocyte/MT4 co-culture system (Fig. 7A), or NLG1/astrocyte/MT4 co-culture system (Fig. 7B) shall offer very unique platforms to further elucidate the underlying molecular mechanisms of HIV latency in astrocytes as well as cell-cell contact-mediated HIV infection in the absence of the HIV primary receptor CD4.

Cell-cell contact-mediated HIV infection has been shown to have a dramatically decreased sensitivity to anti-retrovirals compared to cell-free infection (60, 102). Thus, it is reasonable to assume a similarly decreased ARV sensitivity in cell-cell contact-mediated HIV infection of astrocytes. If so, this certainly provides additional explanations for the HIV persistence in the CNS where the anti-retrovirals' access is already limited. Therefore, the new findings in this study shall encourage more studies to understand HIV infection of astrocytes and its roles in both HIV pathogenesis and HIV persistence in the CNS.

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Figure 1. Temperature-, gp120- and human mannose receptor-dependent HIV entry into astrocytes

A. Human primary fetal astrocytes (HPA) were infected with HIV NL4-3 equivalent to 15,000 cpm RT at 37°C or 4°C for 16 hr. Unbound viruses were removed by extensive washes with PBS, the cells were then fixed and stained for p24, GFAP and DAPI. **B–D.** HPA were incubated with HIV for 4 hr in the presence of anti-HIV gp120 antibody (**B**), yeast mannan (**C**), or dextran sulfate (**D**) before being processed for p24, GFAP and DAPI staining, followed by microscopic imaging (**B & D**) or manual counting (**C**). The micrographs (**A, B & D**) were representative of each treatment from four independent experiments; the cell counts were mean $\pm$ SD of triplicate and representative of three independent experiments.

Figure 2. Time-dependent degradation of endocytosed HIV in astrocytes

A. HPA were infected with HIV NL4-3 equivalent to 15,000 cpm RT at 37°C for 16 hr. Unbound viruses were removed by extensive washes with PBS, the cells continued to incubate for 0, 24 or 48 hr before being fixed and stained for p24, GFAP and DAPI. The micrographs were representative of each treatment from three independent experiments. **B.** Schematic of HIV reporter gag. Green fluorescence protein (GFP)-encoding gene was inserted in frame between HIV matrix gene (MA) and capsid gene (NC), so transfection of 293T with gag would allow production of GFP-labeled HIV. **C.** HPA were incubated with GFP-labeled HIV gag equivalent to 15,000 cpm RT at 37°C for 16 hr. Unbound viruses were removed by extensive washes with PBS, the cells continued to incubate for indicated lengths of time before collected for flow cytometry analysis for GFP mean fluorescence intensity (MFI). The relative MFI were mean $\pm$ SD of duplicate samples and representative of three independent experiments. Mock-infected HPA were included as a control (Ctrl). **D.** HPA from day 5 post-infection with gag were co-cultured with CD4-positive T lymphocytes MT4 for 9 more days. MT4 cells were recovered by gentle washing with PBS and analyzed by flow cytometry for GFP expression. Dot plots were representative of three independent experiments.

Figure 3. Cell-cell contact-mediated HIV infection of astrocytes

A. Experimental scheme to discern cell-cell contact-mediated HIV infection of astrocytes. An equal number of HPA/U373 MG (5×10^4) were plated and cultured in a 24-well plate or at the bottom of a 24-well transwell plate. HIV NL4-3-infected Jurkat (5×10^4), determined to be 50–70% infected by p24 staining and flow cytometry, were cultured in 500 μ l fresh culture medium for 16 hr. The culture supernatant was collected, removed of cell debris and saved as virus stock to infect HPA/U373 MG (Cell-free); NL4-3-infected Jurkat were collected, the infected cells (5×10^4) were used to co-culture with HPA/U373 MG (Co-culture) or plated onto the insert of the transwell in the bottom of which HPA/U373 MG

were cultured (Transwell). After incubation for 16 hr, the cells were fixed and stained for p24, GFAP and DAPI, followed by microscopic imaging. Representative micrographs were shown in **B. C.** Similar experiments as those in **A** were performed except for use of GFP-expressing Jurkat in place of regular Jurkat. HPA were removed of GFP-expressing NL4-3-infected Jurkat cells by extensive PBS washes, and then stained for p24, followed by flow cytometry analysis for p24+GFP⁻ HPA (top panel), or the cells were first gated for GFP⁻ cells, i.e., HPA (bottom left), and then analyzed for p24⁺ HPA (bottom right). Dot plots and histograms were representative of three independent experiments. **D.** p24+GFP⁻ HPA were expressed as mean $\pm$ SD of triplicate and representative of three independent experiments. **, $p < 0.01$.

Figure 4. Establishment of HIV infection of astrocytes through cell-cell contact

A. Schematic of NLGi HIV. A GFP-IRES-Nef cassette was inserted in frame in replace of the first 34 amino acid HIV Nef gene, it expressed GFP as an indicator of the early gene expression as well as Nef itself. **B.** MT4 were infected with NLGi HIV equivalent to 100,000 cpm/ml, the infected cells (0.2×10^6), determined to be 50–70% infected by flow cytometry for GFP expression, were co-cultured with HPA (0.2×10^6) for 1 or 8 days, followed by direct microscopic imaging. **C.** The cultures from day 8 were stained for GFAP and then

followed by direct microscopic imaging. The micrographs were representative of each treatment from three independent experiments.

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Figure 5. Cell density- and gp120-dependent cell-cell contact-mediated HIV-1 infection of astrocytes

A. HPA (0.2×10^6) were cultured in a 6-well plate. An indicated number of NLGi-infected MT4 cells were added onto HPA and co-culture for 16 hr. The unattached MT4 cells were removed, the images of the remaining cells were taken. **B.** The same co-cultures from A were cultured for 12 more days, the GFP+ HPA in each well were manually counted under a fluorescence microscope. The data were mean $\pm$ SD of triplicate samples and representative of two independent experiments. **C.** HPA were infected with cell-free NLGi equivalent to 30,000 cpm RT (Cell-Free) or co-cultured with NLGi-infected MT4 (1×10^6) in the presence of 5 μ g/ml anti-gp120 antibody (α -gp120) or an isotype-matched control (IgG) for 8 days. GFP+ HPA were manually counted under a fluorescence microscope as described above. The data were mean $\pm$ SD of triplicate samples and representative of two independent experiments. *, $p < 0.05$; **, $p < 0.01$; NS: not significant.

Figure 6. Establishment of HIV latency in astrocytes

A. Schematic of red green HIV (RGH). GFP was inserted in-frame between MA and NC gene, while CMV-mCherry cassette was inserted in place of Nef gene. Env gene was disrupted by Kpn I digestion, blunted and religated (filled-in). **B.** GFP expression in RGH is under the control of HIV LTR promoter, and mCherry in RGH is under the control of the CMV promoter and only expressed when RGH is integrated. Thus, RGH-infected cells can be gated into four distinct cell populations by flow cytometry analysis based on GFP and mCherry expression. **C.** HPA were inoculated with VSV-G-pseudotyped RGH equivalent to 30,000 cpm RT by centrifugation at 600 *g*, 4°C for 2 hr. Following the spin inoculation, unbound RGH were removed by extensive wash with PBS, the HPA were cultured for the indicated lengths of time before being collected for flow cytometry analysis for GFP or mCherry expression. The data were mean $\pm$ SD of triplicate samples and representative of two independent experiments. **D.** Representative micrographs of HPA infected with VSV-G-pseudotyped RGH were taken at day 1, 7 and 20 post spinoculation.

Figure 7. HIV recovery from latently transfected astrocytes in a cell-cell contact manner
A–F. HPA were transfected with gagi (**A & C**) or NLGi (**B & D**) and cultured for the indicated times. At day 19, MT4 cell were added into HPA and continued to co-culture for 11 more days (**C & D**). Representative micrographs were taken at indicated times (**A–D**). GFP was used as a control and not shown. The culture supernatants from day 17 to day 19 post transfection of HPA with gagi, NLGi, or GFP were collected and used to infect MT4 as cell-free infection controls. GFP+ MT4 in the co-cultures and in the cell-free infections were determined at every 3 days by flow cytometry (**E & F**). The data were mean $\pm$ SD of triplicate samples and representative of three independent experiments.

Figure 8. HIV recovery from latently infected astrocytes in a cell-cell contact manner and with no involvement of activation of HIV transcription

A. HPA in the Mock, Cell-free, Co-culture and Transwell experiments in Fig. 3A were extensively washed with PBS to remove any input NL4-3-infected Jurkat and continued to culture for 13 more days. Naive Jurkat (1×10^6) were then added into each HPA and co-cultured with HPA for 24 hr. Then the input Jurkat were recovered from the co-cultures, continued to culture for 0, 3 and 6 days, and stained for p24, followed by flow cytometry analysis for p24+ expression. **B.** HPA (1×10^5) were plated in a 24-well plate and transfected with LTR-Luc with or without Tat and cultured for 72 hr, or transfected with LTR-Luc and then co-cultured with MT4 (0.5×10^6) for 24 hr. HPA were then collected for the luciferase (Luc) reporter activity assay. cDNA3 was used to equalize the total amount of the plasmid DNA transfected, CMV- β Gal was used to normalize the transfection efficiency among all transfections. The data were mean $\pm$ SD of triplicate samples and representative of three independent experiments. **, $p < 0.01$; NS: Not significant.