# SUPPLEMENTAL INFORMATION

## METHODS

### Primary cells and cell lines

All culture media and supplements were obtained from Life Technologies (Grand Island, NY) unless indicated otherwise. All primary cells and cell lines were maintained at 37$_o$C in a humidified atmosphere with 5% CO2.

To obtain primary astrocytes, the meninges and blood vessels were removed and the tissue was dissociated using a syringe and a 20 gauge needle by a single stroke. The dissociated cells were centrifuged at 1000 rpm for 10 min, re-suspended in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (100x), incubated for 2 weeks and then passaged 1-2 times a week. After 6-8 passages, mature Progenitor-derived astrocytes were differentiated from human neural progenitor cells as described previously (Messam et al., 2003). Progenitor cells were grown in Minimum Essential Medium (MEM) with 10% FBS and 2 mM L-glutamine for astrocytic differentiation. This process lasted ~20 days.

LTR-Venus astrocytes were generated by transfecting human fetal astrocytes with pHIV LTRVenus using the 4D-Nucleofector™ system (Lonza Group Ltd, Muenchensteinerstrasse, Switzerland) and selected by G418 (Nagai et al., 2002).PBMCs were isolated via a Ficoll–Hypaque gradient centrifugation from blood of HIV-negative donors obtained from the New York Blood Center (de-identified), and stimulated with phytohemagglutinin P (PHA-P) (Sigma Chemical Corp., St. Louis, MO) and 20U/ml of recombinant interleukin-2 (IL-2) (Boehringer, Mannheim, Germany) in Roswell Park Memorial Institute Medium (RPMI)-1640 with 10% FBS and 1% antibiotic-antimycotic solution for 1-3 days.

Cell lines Jurkat-tat (JKT), MT4, U373-MAGI-CXCR4CEM and U373-MAGI-CCR5E were

obtained from the NIH AIDS Reagent Program. 293T cells originated from HEK 293 cells with SV40 Large T-antigen. T cell lines JKT and MT4 were cultured in RPMI 1640 with 10% FBS and 1% antibiotic-antimycotic solution and passaged twice a week. Other cell lines were cultured and passaged in DMEM medium as described above.

The cell line, hCMEC/D3, was selected from normal human brain endothelial cells transduced by lentiviral vector carrying human telomerase (Weksler et al., 2005). The cells were cultured in Endothelial Cell Growth Media (EGM)-2 media (Lonza, Walkersville, MD) substituting 2.5% human serum for FBS to allow for tighter junctions (Vu et al., 2009). The cells between passages 30-42 were used in the experiments.

**Preparation of HIV-1 viral stocks and infection**

Viral stocks (NLENG1, SF162R3) were prepared by transfecting 293T cells with HIV-1 infectious molecular clones using Lipofactamine 2000 (Life Technologies, Carlsbad, CA). HIV-1 IIIb stocks were prepared from H9/HTLV-IIIB NIH 1983 cell line (obtained from the NIH AIDS Reagent Program). Viral titers were determined in U373-MAGI-CXCR4$_{CEM}$ cells and U373-MAGI-CCR5E. HIV-1 p24 antigen was quantified using an ELISA kit (ZeptoMetrix Co., Franklin, MA). HIV-1 strains 92HT599 (X4) and 93US151 (R5X4) were obtained from the NIH AIDS Reagent Program and propagated in PBMCs.

**Time-lapse imaging**

Astrocytes were pre-seeded in a 35-mm petri dish, and then co-cultured with normal JKT cells or NLENG1-infected JKT cells. The cells were imaged with a Zeiss Axio Observer Z1 inverted fluorescence microscope equipped with a high-sensitivity CCD camera, environment control units and a Definitive-Focus module. Multiple-spot live cell images with 100x magnification were acquired under bright/phase-field or fluorescence-field every 30 min via Zeiss AxioVision software. The videos were edited by Adobe After Effects CS6$_{TM}$.

**Immunocytochemical staining and confocal microscopy**

The cells on coverslips were fixed in 4% paraformaldehyde (PFA) for 20-30 min, permeabilized

with 0.2% triton X-100 in PBS for 20 min. The cells were incubated with 1:500-1000 rabbit anti-GFAP antibody (Sigma-Aldrich) (in PBS) for 1hr at room temperature, washed 3 times with PBS; then incubated with 1:500 goat anti-rabbit IgG Alexa 594 (Life Technologies) for 30-60 min, washed with PBS; and stained with 4',6-diamidino-2-phenylindole (DAPI) to label the nuclei. The coverslips were mounted on slides with GEL-MOUNT (EMS, Hatfield, PA) and examined by a Zeiss 510-Meta confocal microscope. Photomicrographs were taken in the Z stack mode. The primary antibody was omitted in controls.

**Correlative electron microscopy and three-dimensional electron microscopy**

The samples were post-fixed in 1% (w/v) osmium, reduced with 0.8% (w/v) potassium ferrocyanide in 0.1M sodium cacodylate and en-bloc stained with 2% (w/v) uranyl acetate. The coverslips with locator grids in the center were embedded in epon and placed in polyethylene capsules (Size 00; Polysciences, Warrington, PA). Based on the locator grids and the images captured before the processing, the samples were trimmed to minimal size but maintained the pre-identified astrocytes as well as the surrounding JKT cells. Sections were cut and stained with uranyl acetate followed by lead citrate. The grids were viewed on a Hitachi 7600 transmission electron microscope and digital images were captured with an AMT 1 K x 1 K CCD camera.

One of the samples described above was processed for 3-dimensional electron microscopy (3D-EM) by Renovo Neural Inc. The region of interest in the block was identified based on the features from the light and fluorescent micrographs, and then the sample was trimmed to the region of interest and then precisely mounted on a sample pin. The block was imaged by SBFSEM in a Carl Zeiss Sigma VP scanning electron microscope fitted with a Gatan 3View in-chamber ultra-microtome and Gatan high sensitivity, low-kV BSE detector. A series of images were generated for creating 3-D stacks. A cut thickness of 75 nm was used for all image sequences. Block faces were imaged at 2.25 kV and high-resolution images were generated at a resolution of 5 nm per pixel. 3D images and videos were created using softwares ImageJ/Fiji,

Reconstruct and Amira.

Selected 3D-EM images were processed at Laboratory of Cell Biology, National Cancer Institute, NIH using focused ion beam scanning electron microscopy (FIB-SEM) (Felts et al., 2010). The images were segmented using Slicer3D software and alignment algorithms written by Bradley Lowekamp at the National Library of Medicine.

**Infection blocking assay**

Anti-CD4 (cell surface CD4 complex monoclonal B4), anti-CXCR4 (clone 44717), anti-dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-Sign; 9E9A8), anti-α4β7 integrin (Act-1), AMD3100 (bicyclam JM-2987) and T-20 (CD4 fusion Inhibitor) were obtained from the NIH AIDS Reagent Program. Anti-intercellular adhesion molecule-1(ICAM-1; IA29) and anti-lymphocyte function-associated antigen-1 (LFA-1; HI111) were purchased from BD Pharmingen (San Jose, CA). To investigate the mechanism of HIV-1 transmission from T lymphocytes to astrocytes, astrocytes were pre-incubated with the above antibodies (30 μg/ml for anti-α4β7 integrin, 20 μg/ml for others) for 1 hr at 37°C and then continuously maintained in the media of the co-cultures as described above. The cells were treated with 40-100 μM AMD3100 and 100-500 nM T20 in the same way.

**Migration of HIV-infected T lymphocytes through BBB model**

4 x$10^4$ astrocytes in 50 μl DMEM were seeded on each of the upside-down, collagen-coated 24-well Millipore transwells (8 μm pores)(EMD Millipore, Billerica, MA) that were placed in 12 well plates with 3.5 ml DMEM and cultured overnight. The inserts were flipped and placed in 24 well plates with 1 ml EGM-2 medium. 4 x $10^4$ HCMEC/D3 endothelial cells in 200 μl medium were seeded into each of the transwells and cultured for 5 days. The medium was changed at day 4. Transendothelial Electrical Resistance (TEER) was measured using an EVOM voltmeter with an EndOhm-6 chamber (World Precision Instruments, Sarasota, Florida) and the TEER values were calculated as Ω x 0.33 $cm^2$ with a subtraction of the TEER of a cell-free, collagen coated insert (Resistance=15Ω x $cm^2$). Only the inserts with a TEER value of >100 were used to ensure

tight junctions. 4 x $10^5$ NLENG1-infected JKT cells in 50 µl medium were added to the insert replacing the old media. 150 ng/ml of SDF-1 or RANTES was added in the bottom chamber. Migrated cells with EGFP were counted for the next 2 days. In an additional experiment, the membranes of the inserts were removed next day, fixated and mounted on slides for confocal microscopy.

## SUPPLEMENTARY REFERENCES


Felts, R.L., Narayan, K., Estes, J.D., Shi, D., Trubey, C.M., Fu, J., Hartnell, L.M., Ruthel, G.T., Schneider, D.K., Nagashima, K., *et al.* (2010). 3D visualization of HIV transfer at the virological synapse between dendritic cells and T cells. Proceedings of the National Academy of Sciences of the United States of America *107*, 13336-13341.

Messam, C.A., Hou, J., Gronostajski, R.M., and Major, E.O. (2003). Lineage pathway of human brain progenitor cells identified by JC virus susceptibility. Annals of neurology *53*, 636-646.

Nagai, T., Ibata, K., Park, E.S., Kubota, M., Mikoshiba, K., and Miyawaki, A. (2002). A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. Nature biotechnology *20*, 87-90.

Vu, K., Weksler, B., Romero, I., Couraud, P.O., and Gelli, A. (2009). Immortalized human brain endothelial cell line HCMEC/D3 as a model of the blood-brain barrier facilitates in vitro studies of central nervous system infection by Cryptococcus neoformans. Eukaryotic cell *8*, 1803-1807.

Weksler, B.B., Subileau, E.A., Perriere, N., Charneau, P., Holloway, K., Leveque, M., Tricoire-Leignel, H., Nicotra, A., Bourdoulous, S., Turowski, P., *et al.* (2005). Blood-brain barrier-specific properties of a human adult brain endothelial cell line. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *19*, 1872-1874.

## SUPPLEMENTARY FIGURES

**Figure S1. Infection of astrocytes with X4- or R5X4 viruses by cell-to-cell contact.** HIV infection in astrocytes was observed in the co-cultures with JKT cells pre-infected with NL4-3 (A), 92HT599 (X4) (B), and 93US151 (R5X4) (C) with a large syncytial cell. Magnification: 200x.

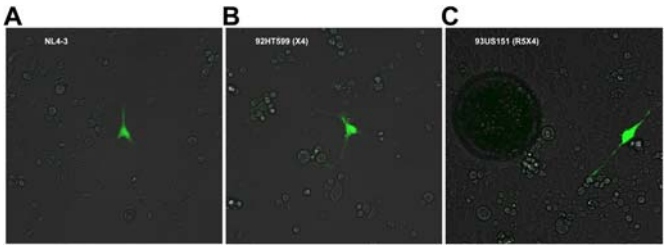


**Figure S2. Interactions of NLENG1-infected JKT cells with astrocytes.** (A) Serial images of confocal microscopy as illustrated in figure 3A showed that a NLENG1-infected JKT cell (arrow) was partially invaginated into an astrocyte which had been infected with the virus by cell-to-cell contact. (B) Serial images by confocal microscopy as shown in figure 3B demonstrated an interdigitated interface (arrow) between the two cells. Image magnification: 630x.

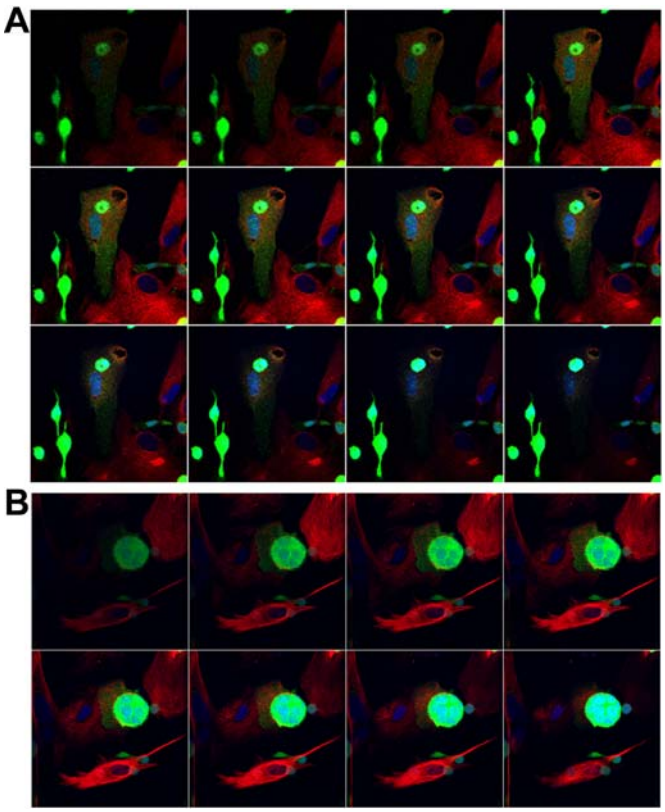


**Figure S3. Inhibition assay for infection of cell-free NLENG1 in astrocytes by antibodies.** Astrocytes were pre-treated with 20 µg/ml of each of the antibodies for 1 hour prior to infection with cell-free NLENG1 and maintained in antibody-containing media for 24 hours post-infection, following which the antibodies were removed by washing. Levels of HIV-1 p24 antigen were measured in supernatants of the cultures 8 days post-infection. No significant inhibition was seen with any of the antibodies. Data represents three independent experiments and was analyzed with one-way ANOVA.

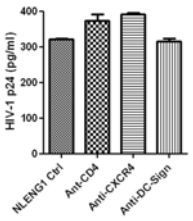
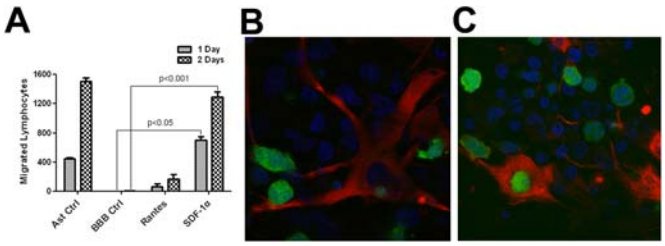


**Figure S4. NLENG1-infected JKT cells migrate through the BBB model along a gradient of SDF1α.** (A) Significant migration of NLENG1-infected JKT cells through the BBB was observed in the presence of SDF1α (150 ng/ml); however, no significant migration was seen with RANTES (150 ng/ml), but there was also no migration in the BBB control. In contrast, the infected JKT cells could pass through the membrane where only astrocytes were pre-seeded. The results were analyzed by Student's T-test. Data shown as mean ± SEM. (B) Cell-to-cell contacts between astrocytes and NLENG1-infected JKT cells were observed one day after SDF1α (150 ng/ml) was present in the bottom chamber. (C) The contacts were also observed when RANTES (150 ng/ml) was present in the bottom chamber, but were much fewer. Magnification: 630x in B and C.



**Movie S1. Time lapse imaging of interactions between astrocytes and JKT cells.** (A) Normal JKT cells dynamically interacted with human astrocytes. These cells might remain on, separate from, or undergo a process of attaching, detaching and re-attaching to astrocytes over time (16 hours). Magnification: 100x. (B) Astrocytes were co-cultured with NLENG1-infected JKT cells for 2 days and then analyzed by time-lapse imaging for another 24 hours. Arrow shows that a NLENG1-infected JKT cell as indicated by a red cycle was adherent to an astrocyte. This astrcoyte acquired green fluorescence suggesting that the infection occurred. Subsequently, the JKT cell detached from the infected astrocyte. Magnification: 100x.

**Movie S2. 3-dememsional reconstructions of SBF-SEM images of interactions between astrocytes and HIV-infected JKT cells.** (A) A low-resolution 3D movie shows the relationship between astrocytes and NLENG1-infected JKT cells as shown in Figure 3A-3C. (B) A highresolution

3D movie shows the interaction between A1 and T1 as shown in Figure 3B and 3D.

(C) A 3D movie shows the interaction between the processes from A1 and T2 as shown in Fig 3A-3C and 3E. (D) A 3D movie shows the interaction between A2 and T3 as shown in Figure 3A-3C and 3F.