STAT1 signaling modulates HIV-1-induced inflammatory responses and leukocyte transmigration across the blood-brain barrier

Running head: STAT1 modulates HIV-1-induced BBB dysfunction

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ABSTRACT

The relationship between neuroinflammation, blood-brain barrier (BBB) dysfunction, and progressive HIV-1 infection as they affect the onset and development of neuroAIDS is incompletely understood. One possible link is signal transducers and activators of transcription (STAT) pathways. These respond to pro-inflammatory and regulatory factors and could affect neuroinflammatory responses induced from infected cells and disease affected brain tissue. Our previous works demonstrated that HIV-1 activate pro-inflammatory and interferon-alpha-inducible genes in human brain microvascular endothelial cells (HBMEC) and that these genes are linked to the janus kinase (JAK)/STAT pathway. We now demonstrate that HIV-1 activates STAT1, induces IL-6 expression, and diminishes expression of claudin-5, ZO-1, and ZO-2 in HBMEC. The STAT1 inhibitor, fludarabine, blocked HIV-1-induced IL-6, diminished HIV-1-induced claudin-5 and ZO-1 downregulation, and blocked HIV-1- and IL-6-induced monocyte migration across a BBB model. Enhanced expression and activation of STAT1 and decreased claudin-5 was observed in microvessels from autopsy brains of patients with HIV-1-associated dementia. These data support the notion that STAT1 plays an integral role in HIV-1-induced BBB damage and is relevant to viral neuropathogenesis. Inhibition of STAT1 activation could provide a unique therapeutic strategy to attenuate HIV-1-induced BBB compromise and as such improve clinical outcomes.
INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) infection of the central nervous system (CNS) commonly results in behavioral, motor, and cognitive impairments. Although disease severity and progression has slowed, in part, as a result of anti-retroviral therapy, 76-83% of brain autopsies continue to show observable neuropathological abnormalities. Disease pathology ranges from mild brain atrophy and gliosis to robust viral replication, multinucleated giant cell formation, astro- and micro-gliosis, myelin pallor, and neuronal loss. These pathological findings are collectively termed HIV-1 encephalitis (HIVE). HIVE is a common correlate to the later stages of behavioral, motor, neuropsychiatric, and neurological consequences of disease termed HIV-1 associated dementia (HAD) [for recent reviews, see]. HIVE is fueled by viral infection and immune activation of brain mononuclear phagocytes (MP: blood-derived perivascular macrophages and microglia). Such MP- and virus-associated neuroinflammation promotes monocyte trafficking across the blood-brain barrier (BBB), MP infiltration into the CNS, and neurodegeneration. Thus, dysfunction of the BBB is one critical feature of HIV-1 neuropathogenesis.

Brain microvascular endothelial cells, a major component of BBB function and integrity, are connected by tight junctions (TJ) that limit paracellular flux and restrict permeability. Indeed, under normal physiologic conditions, the brain endothelium functions as an interface between the blood and the brain parenchyma, strictly regulating influx of ions, molecules, and leukocytes into the CNS. Nonetheless, in disease, a variety of environmental, toxic, degenerative, and microbial insults could cause BBB breakdown. Such a breakdown occurs during progressive HIV-1-infection and was documented in laboratory, animal models, human clinical observations, and autopsy studies. Underlying mechanisms of BBB dysfunction
and how it affects ongoing disease are incompletely understood. Dysfunction of the BBB enhances penetration of cell-free virus, ingress of activated HIV-1-infected monocytes across the BBB, accumulation of MP in the CNS, and spread of the virus to neighboring microglia and astrocytes. Thus, BBB breech is commonly associated with accelerated disease and the development of behavioral and cognitive deficits that are signatures of HAD. Based on these observations, the elucidation of the signaling pathways mediating BBB compromise can prove important for understanding disease mechanisms and development of new therapies.

Signal transducers and activators of transcription (STAT) proteins are latent cytoplasmic transcription factors that are phosphorylated by janus kinases (JAK) in response to pro-inflammatory and regulatory factors. STAT1 encodes a 91-kDa protein that is activated by type I (alpha and beta) and type II interferons (IFN), epidermal growth factor, platelet-derived growth factor, and interleukin-6 (IL-6). Moreover, STAT1 activation often correlates with cellular pro-inflammatory, anti-proliferative, and apoptotic activities. The JAK/STAT pathway plays a prominent role in cytokine-mediated inflammatory responses and as such, has been implicated in the pathogenesis of HIV infection. Indeed, relationships between neuroinflammatory activities and specific signaling pathways are well appreciated. Mechanistic studies reported herein arise from microarray analyses performed in our laboratories demonstrating that HIV-1 activates pro-inflammatory and IFN-inducible genes in human brain microvascular endothelial cells (HBMEC). We now demonstrate that HIV-1 and HIV-1-infected monocyte-derived macrophages (MDM) activate STAT1 and induce IL-6 expression in HBMEC through STAT1. A specific STAT1 inhibitor, fludarabine (FLUD), blocked HIV-induced STAT1 activation, decreased IL-6 expression, and secretion. Moreover, FLUD diminished HIV-induced downregulation of TJ, prevented HIV-1- and IL-6-induced monocyte migration across in vitro
BBB models. Most importantly, enhanced expression and activation of STAT1 was shown in microvessels acquired from autopsy brains of HIV-infected people, as well as in autopsy brains of patients who died with advanced HAD. These data strongly support the notion that STAT1 plays an integral role in HIV-induced BBB damage and is relevant to the pathogenesis of viral infection. Inhibition of STAT1 activation could provide a unique therapeutic strategy to prevent HIV-1-induced BBB compromise and as such improve clinical outcomes in infected people.

MATERIALS AND METHODS

**Endothelial cell culture**

Primary HBMEC were isolated from brain tissue obtained during surgical removal of epileptogenic cerebral cortex in adult patients\textsuperscript{34} and were provided by Drs. Marlys Witte and Michael Bernas (University of Arizona, Tucson, AZ). Routine evaluation for von Willebrand factor (vWF), \textit{Ulex europeus} lectin, and CD31 demonstrated that HBMEC were > 99\% pure. HBMEC were seeded in the upper chamber of collagen-I-coated 6- or 24-well tissue culture inserts (with 0.4-\textmu m pore size) or 6-well tissue culture plates and cultured to confluence in EGM\textsuperscript{TM}-2 BulletKit\textsuperscript{®} media (Cambrex, Walkersville, MD) supplemented with 5\% fetal bovine serum (FBS). Only cells at passage 1 to 4 were used in this study.

**Monocyte isolation, macrophage culture, and HIV-1 infection**

Monocytes were obtained from HIV-1, HIV-2, and hepatitis-B seronegative donor leukopaks, separated by countercurrent centrifugal elutriation and characterized as previously described\textsuperscript{20,35}. Freshly elutriated monocytes were differentiated into MDM by 7 days culture in Dulbecco’s Modified Eagles Media containing 2mM L-glutamine (Invitrogen, Carlsbad, CA), 1U/ml human
recombinant macrophage colony stimulating factor (a generous gift from Wyeth Inc., Cambridge, MA), 10% heat-inactivated human serum, 100µg/ml gentamicin and 10µg/ml ciprofloxacin (Sigma, St Louis, MO). MDM were infected with HIV-1\textsubscript{ADA} at a multiplicity of infection (MOI) of 0.01 then used for co-cultures with HBMEC at day 5 after viral inoculation. All reagents were prescreened for endotoxin (<10 pg/ml, Associates of Cape Cod, Woods Hole, MA) and mycoplasma contamination (Gen-probe II, Gen-probe, San Diego, CA). Levels of productive viral replication were measured in culture fluids by HIV-1 p24 ELISA (Beckman Coulter™, Miami, FL).

**Co-cultures of HIV-1 infected MDM and HBMEC**

In triplicate, HBMEC in transwell inserts (0.4-µm pore size) were co-cultured for 12h with HIV-1\textsubscript{ADA}-infected MDM in endothelial cell media (EGM\textsuperscript{TM}-2 BulletKit® media with 5% FBS). Triplicate samples of HBMEC co-cultured for 12h with non-infected MDM or untreated HBMEC served as controls. Following co-culture, media from the top (HBMEC side) and bottom (MDM side) wells of each insert were collected, centrifuged for 15 min at 3000 rpm. The recovered fluids were cryopreserved at -70°C. In parallel experiments, HBMEC in 6-well plates were exposed directly to purified HIV-1\textsubscript{ADA} (MOI 0.01) for 5 min to 24h; and the fluids prepared for analysis as described above.

**Monocyte migration through an *in vitro* BBB model**

For migration assays, 2x10\textsuperscript{4} HBMEC were seeded on collagen-coated FluoroBlok tinted tissue culture inserts (with 3µm pore size, BD Biosciences, Franklin Lakes, NJ). Because monolayers are not visible on these inserts, manual readings of transendothelial electric resistance were taken with a voltmeter (EVOM, World Precision Instruments, Sarasota, FL) to confirm monolayer
formation and cell confluence. Freshly elutriated, infected or non-infected monocytes were labeled with calcein-AM (Invitrogen) at 5µM/1x10^6 cells for 45 min and washed to eliminate residual label. Then, 2.5x10^5 labeled-monocytes were placed on HBMEC (upper chamber of the FluoroBlok insert) and allowed to migrate for 2h (37°C, 5% CO₂). Migrated monocytes were quantified with a fluorescence plate reader (absorbance 494nm; emission 517nm), with a standard curve derived from a serial dilution of known numbers of calcein-labeled cells. For migration experiments testing the effects of FLUD, HBMEC were exposed to FLUD (20µM) (Sigma) for 30 min before migration. We previously performed dose-dependent migration experiments with FLUD and determined that (20µM) was optimal in altering monocyte transendothelial migration.

Detection of IL-6

IL-6 levels in each sample were quantified using the Human IL-6 ELISA kit (eBioscience, San Diego, CA) according to the manufacturer’s instructions. IL-6 levels in media from infected and non-infected MDM and HBMEC prior to co-culture served as controls.

Microvessels (MV) isolation

Brain tissues (cortex) from HIV-1 seropositive patients without neuro-cognitive impairment, HAD patients, and aged-matched seronegative controls were obtained from the National NeuroAIDS Tissue Consortium (NNTC) and our Center for Neurovirology and Neurodegenerative Diseases (CNND) brain bank. The clinical histories of all brain MV donors are detailed in Table 1. The MV isolation procedure was as described by Brooks and collaborators. Briefly, brain tissues were homogenized in a buffer containing 103mM NaCl,
4.7mM KCl, 2.5mM CaCl₂, 1.2mM KH₂PO₄, 1.2mM MgSO₄, 14mM HEPES, 2.5mM NaHCO₃, 10mM D-glucose, 1mM sodium pyruvate, pH 7.4 and 1X protease inhibitor cocktail. Following homogenization each sample was passed sequentially through a 350, 112, and 40-µm nitex mesh filters (Sefar, Depew, NY). Following the last filtration, the 40-µm mesh were removed and rinsed to collect trapped MV. Isolated MV was pelleted by centrifugation at 5600-g for 10 min at 4°C. RNA and protein were extracted from each MV sample for real-time PCR and Western blot analyses. For immunofluorescence and confocal microscopy, 50µl of MV solution was spread onto poly-l-lysine- or fibronectin-coated glass coverslips, air-dried, fixed for 20 min with a 4% paraformaldehyde solution (pH 7.4), rinsed with phosphate buffered saline (PBS), air-dried, and used for immunostaining with antibodies to STAT1 and phospho-STAT1. Immunostaining with antibodies to the TJ protein claudin-5 and vWF was used to confirm the vascular identity of the isolated MV as previously described₃⁷,₃⁸.

**RNA isolation and Real-time PCR**

HBMEC and brain MV were lysed using the Trizol reagent (Invitrogen), total RNA was extracted from each sample using RNAeasy mini-kit (Qiagen, Valencia, CA) with DNase treatment according to the manufacturer’s instructions. RNA yield and quality were checked using a NanoDrop spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE) and for all samples, absorbance ratio of 260/280 was >2. The purity and quality of extracted RNA were further assessed using the RNA 6000 Nano LabChip® Kit and Agilent-2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) according to the manufacturer's instructions.

For each sample, cDNA was generated from 1µg RNA using random hexamers and Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen). Reverse transcription was
carried out for 1h at 42°C. The cDNA obtained was used for real-time quantitative PCR (qRT-PCR) using an ABI PRISM 7000 sequence detector (Applied Biosystems, Foster City, CA). A TaqMan gene detection system was employed and quantification performed using the standard curve method as described in user bulletin #2 supplied with ABI PRISM 7000 sequence detector. All PCR reagents and primers were obtained from Applied Biosystems and primer IDs were as follows: chemokine (C-X-C motif) ligand-1 (CXCL1): Hs00236937, IL-6: Hs00174131_m1, interferon-stimulated gene 15 (ISG15 or GIP2): Hs00192713_m1, tumor necrosis factor-alpha-induced protein-3 (TNFAIP3): Hs00234713_m1, TNF (ligand) superfamily 15 (TNFSF15): Hs00353710_s1, ubiquitin-D (UBD): Hs00197374_m1, RelB: Hs00232399_m1, STAT1: Hs00234829_m1, superoxide dismutase 2 (SOD2): Hs00167309_m1. For endogenous control each gene expression was normalized to GAPDH (Hs99999905_m1).

**Immunofluorescence microscopy**

Confluent HBMEC grown on glass coverslips were washed with PBS and fixed in methanol/acetone (1:1) for 20 min. Fixed MV and HBMEC were permeabilized with 0.1% triton X-100 and blocked for non-specific binding with 3% BSA in PBS (10 min at 4°C). Cells were incubated with primary antibodies (1:50 dilution) for 1h at room temperature, followed by staining (1h in the dark at room temperature) with secondary antibodies coupled with Alexa-488 or Alexa-594 (1:500 dilution, Invitrogen). Stained cells were mounted in Prolong Gold (Invitrogen) and examined using a fluorescent microscope (E800 Nikon, Melville, NY) connected to a color MagnaFire digital camera (Optronics, Goleta, CA).
Protein extraction and Western blot analyses

Protein extraction and quantification was performed as we previously described\textsuperscript{19,20}. Twenty-five \( \mu \text{g} \) of protein was fractionated in a 4-15\% gradient gel and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked for 1h with SuperBlock\textsuperscript{\textregistered} T-20 (Pierce Rockford, IL), blotted 2h or overnight with the appropriate primary antibodies, 1h with the secondary antibody, washed, and visualized using SuperSignal\textsuperscript{\textregistered} West Pico Substrate (Pierce). All STAT and phospho-STAT antibodies were from Cell Signaling Technology (Danvers, MA). Claudin-5, ZO-1, and ZO-2 antibodies were from Invitrogen. For Western blot assays performed with phosphorylated STAT antibodies (pSTAT1, pSTAT2, pSTAT3, pSTAT5, pSTAT6), membranes were stripped using Restore\textsuperscript{TM} Western Blot Stripping Buffer (Pierce) and re-blotted with antibodies to STAT1, STAT2, STAT3, STAT5, and STAT6 respectively, then stripped again and re-blotted with \( \beta \)-actin antibody (Santa Cruz Biotechnology, CA). Results were expressed as ratios of relative intensity of the target protein (phospho-STAT) to that of the internal standard, \( \beta \)-actin, or STAT. For experiments with inhibitors, HBMEC were exposed for 30 min to HIV-1 with or without FLUD (20\( \mu \text{M} \)), the JAK2 (AG490, 50\( \mu \text{M} \)), and JAK3 (WHI-P154, 30\( \mu \text{M} \)) inhibitors, and protein extracted and analyzed as above. AG490 and WHI-P154 were from Calbiochem (San Diego, CA). Controls consisted of untreated cells, and cells exposed to FLUD, AG490 and WHI-P145.

Statistical analysis

One- or two-way analysis of variance and the general linear models procedures were performed using the SAS program followed by Dunnet or Tukey’s Multiple Comparison tests. Both
statistical procedures test differences among several means for significance without increasing Type I error rate\textsuperscript{39}. The threshold for significance was 0.05.

RESULTS

**HIV-1 and HIV-infected MDM induce IL-6 expression in HBMEC**

We previously showed that HIV-1 upregulate mRNA for several pro-inflammatory cytokines in HBMEC, including IL-6\textsuperscript{33}. To determine whether increased IL-6 mRNA was associated with increased protein, we quantified IL-6 in cell culture media. Co-culture of HBMEC with infected MDM increased IL-6 secretion in HBMEC by 10-fold (229.2 ± 38 pg/ml) compared to HBMEC co-cultured with uninfected MDM (23 ± 3 pg/ml) (P<0.001, Fig. 1A). Endothelial-macrophage co-culture did not change MDM IL-6 levels even following HIV-1 infection (Fig. 1A). To determine whether the induced HBMEC IL-6 was caused by virus or by secretory products from infected MDM, we exposed endothelial cells to 0.01 infectious viral particles of HIV-1\textsubscript{ADA} for 2 to 24 h and quantified IL-6 levels in culture fluids. Each of three experiments was done in quadruplicate. A significant and time-dependent increase in HBMEC IL-6 was observed following exposure to HIV-1 (Fig. 1B). IL-6 levels in HBMEC exposed to HIV-1 virions were 3.5 ± 0.2, 30.3 ± 1.7, 40.1 ± 4.4, and 83.5 ± 13.0 pg/ml respectively after 2, 6, 12, and 24 h of viral exposure.

**STAT1 inhibition attenuates HIV-1-induced IL-6 expression**

As IL-6 signals principally through the JAK/STAT pathway, we determined whether STAT1 modulates HIV-1-induced IL-6 production in HBMEC. HBMEC were exposed to HIV-1\textsubscript{ADA} (MOI=0.01) for 3, 6, 12, and 24 h, or to HIV-1 with 20 µM FLUD for 12 and 24 h; and IL-6
levels in culture supernatant were quantified by ELISA. HIV-1 induced a time-dependent increase in IL-6 levels and FLUD significantly diminished IL-6 secretion (P<0.01, Fig. 1C). No IL-6 was detected in HBMEC exposed to conditioned media from uninfected MDM or in untreated HBMEC.

HIV-1 upregulates HBMEC IL-6 receptor expression

As IL-6 secretion was upregulated following HIV-1 exposure or co-culture with infected MDM (Fig. 1), we evaluated the expression of IL-6 receptor (IL-6R) in HBMEC. Immunofluorescence analyses demonstrated that HBMEC express the IL-6R-α and gp130 (α and β chains of the IL-6 receptor, Fig. 2). Next, we determined whether this receptor could be upregulated by exposure to HIV-1. Indeed, HIV-1 significantly increased the expression of both α and β subunits of the IL-6R (Fig. 2A). These observations were confirmed in two independent experiments using HBMEC from two different donors. We performed semi-quantitative analyses of IL-6 expression (percentage of immunostained area) using computer-assisted image analysis (Image-Pro® Plus, Media Cybernetics, Silver Spring, MD). Image-Pro® Plus software measures the fluorescence intensity and densitometry of immunostained cells and normalize each measurement to the cell’s size. The data showed that 30 min HIV-1 exposure increased IL6-R-α expression 6-fold (P<0.001) and gp130 expression 4-fold (P<0.05). Conditioned media from uninfected MDM did not change IL-6R staining (Fig. 2). Interestingly, HIV-1-induced up-regulation of IL6-R-α expression was transient and decreased after 30 min (Fig. 2B), while gp130 expression was sustained for up to 2h following HIV-1 treatment (Fig. 2C).

Interactions of HIV-1-infected macrophages with HBMEC activate STAT1 in HBMEC
Based on data obtained regarding the influence of IL-6 and HIV-1 on STAT pathways, we tested whether co-culture of HBMEC with HIV-infected MDM activates STAT1 in HBMEC. In these experiments, HBMEC in 0.4-µm culture inserts were co-cultured with HIV-infected MDM for 5 min to 24h; HBMEC protein was extracted and analyzed by Western blotting. Interaction of HBMEC with infected MDM induced phosphorylation of STAT1 at serine-727 (S727) from 5 min to 4h co-culture and also increased total STAT1 levels (Fig. 3A). No activation of STAT1 at tyrosine residues was detected.

**HIV-1 virions induce activation of STAT1 at serine-727 in HBMEC**

To evaluate the effects of direct HIV-1 exposure to HBMEC, HIV-1ADA was placed onto HBMEC for 5 min to 24h, followed by protein extraction and Western blot analysis. Direct exposure of HBMEC to HIV-1 phosphorylated STAT1 at S727, with maximal level of activation at 30 min and 1h (Fig. 3B). Increased phospho-STAT1 correlated with decrease in total STAT1, however, long-term HIV-1 exposure (12-24 h) increased total STAT1 levels (Fig. 3C). HIV-1-induced STAT1 activation was specific to serine residues, no activation at tyrosine residues was detected. Treatment of HBMEC with conditioned media derived from uninfected MDM (5 min to 24h) did not induce STAT1 phosphorylation or change in total STAT1 levels (Fig. 3D). To assess whether IL-6 can activate endothelial STAT1, HBMEC were treated with IL-6 (100 ng/ml) for 5 min to 24h, followed by protein extraction and Western blot analysis. IL-6 did not phosphorylate STAT1 at serine or tyrosine residues (Fig. 3E). Based on our data showing that FLUD prevent HIV-1-induced IL-6 expression (Fig. 1) and diminished HIV- and IL-6-induced monocyte transendothelial migration (Fig. 4A), we tested the effect of FLUD on HIV-induced STAT1 expression and activation. HBMEC were exposed for 2h or 30 min to HIV-1, FLUD
(20µM), or HIV+FLUD and extracted proteins analyzed as above. HIV-1 exposure increased expression of STAT1 and phospho-STAT1 (S727). FLUD significantly diminished HIV-1 induced STAT1 activation and expression at 2h (Fig. 3F) and 30 min (Fig. 3G).

**STAT1 modulates monocyte migration across *in vitro* BBB models**

To evaluate the biological significance of HIV-1-induced IL-6 upregulation, we explored whether HIV-1 and IL-6 could affect monocyte transendothelial migration. HIV-1 infection increased monocyte migration 4-fold as compared to uninfected monocytes. IL-6 increased transendothelial migration of uninfected monocytes by 4.3-fold and increased migration of HIV-infected monocytes by 7.4-fold (Fig. 4A) as compared to monocytes without IL-6 application. Since STAT1 mRNA is upregulated in HBMEC co-cultured with HIV-1-infected MDM and IL-6 signals through the JAK/STAT pathway, we tested whether the STAT1 inhibitor FLUD could prevent HIV-1- and IL-6-induced monocyte transmigration. FLUD significantly diminished HIV-1- and IL-6-induced monocyte migration in our *in vitro* BBB model (Fig. 4A).

**HIV-1 virions phosphorylates STAT1 and STAT3 at serine-727 in HBMEC, JAK-3 inhibitor diminished HIV-1-induced phosphorylation of STAT1 and STAT3.**

To determine whether HIV-1 activates other members of the STAT family in HBMEC, we tested the levels of phosphorylated STAT-2, STAT-3, STAT-5, and STAT-6 in HIV-exposed HBMEC. HIV-1 induced phosphorylation of STAT-1 and STAT-3 at S727; HIV-1 exposure did not induce phosphorylation of STAT-3 at tyrosine residues and did not induce phosphorylation of STAT-2, STAT-5, or STAT-6 (Fig. 4B,C). FLUD had no effect on HIV-1-induced STAT-3 phosphorylation, confirming previous findings that FLUD is a specific STAT1 inhibitor. To
determine which effector upstream of STAT-1 may be involved in HIV-1-induced BBB dysfunction, we analyzed STAT activation in HBMEC exposed to HIV-1 with or without the presence of the JAK-2 inhibitor (AG490, 50µM) and the JAK-3 inhibitor (WHI-P154, 30µM). The JAK-3 inhibitor diminished HIV-1-induced activation of STAT-1 and STAT-3 (Fig. 4C).

**Activation and overexpression of STAT1 and pro-inflammatory molecules in brain MV**

To determine whether our *in vitro* findings correlate with changes at the BBB of HIV-1-infected humans, we isolated and analyzed MV from brain tissues of five HIV-1,2 seronegative control subjects, four HIV-1 seropositive patients without evidence of HIVE (HIV+ no HIVE), and five patients with HIVE (HIV-1 dementia scale >1). Table 1 shows the clinical history, post-mortem interval (PMI) between the time of death and autopsy, and a summary of post-mortem findings for all 14 individuals. The age ranges were 32 to 46, 27 to 46, and 30 to 50 years respectively for seronegative, HIV+ no HIVE, and HAD patients. Their PMI were respectively 4 – 8.5 h, 2.7 – 11 h, and 6 – 21 h. HIVE and HAD diagnoses were performed by neuropathologists and neuropsychologists of the NNCT and the CNND rapid autopsy program. Plasma and cerebrospinal fluid (CSF) viral load (VL) and CD4 counts were available for donors P1 and P4, HAD1, HAD2 and HAD5. For patient P1 plasma VL was 684,444 copies/ml, CD4+ T-cell count was 13/mm³, and CSF VL was 2,300 copies/ml. For patient P4, plasma VL was 75,000 copies/ml and CD4 count was 13/mm³. For patient HAD1 plasma VL was 104,300 copies/ml, CD4 count was 8/mm³, and CSF VL was 14 copies/ml. CSF VL was 962 copies/ml for patient HAD2 while for patient HAD5, plasma VL was 570 copies/ml and CD4 count was 361/mm³.

MV were isolated from the brain cortex as described in the Method section. The vascular identity of isolated MV was confirmed by immunostaining and confocal microscopy for the TJ
protein claudin-5 and vWF. All MV samples stained positive for claudin-5 and vWF (Fig. 5). To assess changes in claudin-5 expression, we performed semi-quantitative analyses for all MV samples (percentage of immunostained area) using Image-Pro®Plus analysis. Claudin-5 levels were lower in MV from HIV+ no HIVE patients, compared to seronegative controls, but the difference was not statistically significant (Fig. 5B). Claudin-5 staining was significantly lower in MV from HAD patients when compared to MV from control or HIV+ no HIVE (P<0.001, Fig. 5B). While continuous TJ strands were found in MV from seronegative controls, HAD patients with neuropathological evidence of HIVE featured fewer TJ strands with more gaps, or total absence of TJ staining (e.g. patient HAD5 in Fig. 5A). Because MP migrating through the BBB can be attached to the brain endothelium, we performed double immunostaining of MV for human CD163 and vWF; we found no macrophage contamination in the isolated MV (Fig. 5C).

To determine whether STAT1 can modulate expression of TJ protein in the brain endothelium, we determined the expression of claudin-5, ZO-1, and ZO-2 in HBMEC exposed to HIV-1, with or without FLUD. HIV-1 decreased claudin-5, ZO-1, and ZO-2 expression in HBMEC. FLUD diminished viral-induced downregulation of claudin-5 and ZO-1 (Fig. 5D-F).

Our previous works demonstrated that co-culture of HBMEC with infected MDM induce transcriptional up-regulation of inflammatory molecules, including chemokines, cytokines, and IFN-inducible genes. To correlate these results with in vivo situation, we performed qRT-PCR with: i) HBMEC co-cultured with infected or non-infected MDM; and ii) brain MV from HIV+ no HIVE, HAD with HIVE, and seronegative control patients. Data showed similar patterns of HIV-1-induced transcriptional up-regulation of CXCL1, TNFAIP3, TNFSP15, SOD2, UBD, ISG15, and RelB in endothelial cell cultures and brain MV from HIV-1 infected humans (Fig. 6A). Both MV from HIV+ no HIVE and HAD HIVE patients showed transcriptional
upregulation of CXCL1, TNFAIP3, SOD2, UBD, ISG15, and RelB, compared to MV from seronegative patients, with levels of SOD2 and UBD mRNA higher in HAD HIVE than in HIV+ no HIVE (Fig. 6A). HAD HIVE patients showed increased levels of TNFSP15 mRNA, while levels in HIV+ no HIVE was similar to that of control seronegatives (Fig. 6A). Our data also demonstrated similar patterns of HIV-1-induced transcriptional up-regulation of STAT1 and IL-6 in endothelial cell cultures and in MV of infected humans (Fig. 6B-6E).

We used Western blotting and confocal microscopy to further analyze STAT1 expression in human brain MV. Significantly, both Western blot (Fig. 7A) and confocal microscopy (Fig. 7B) showed overexpression of STAT1 and phospho-STAT1 (S727) in brain MV of HIV-infected individuals when compared to MV obtained from seronegative control subjects. The highest expression and activation of STAT1 were observed in MV of HAD patients. Semi-quantitative analysis of immunostained MV showed a 4- and 4.8-fold increase in phospho-STAT1 in MV of HIV-1 seropositive subjects with and without HAD as compared to controls (P<0.001, Fig. 7C). Similarly, there was a 2.4- and 2.6-fold increase in STAT1 in MV of HIV-1 seropositive and HAD patients respectively as compared to MV from HIV-1,2 seronegative controls (P<0.01, Fig. 7D). Again STAT1 phosphorylation at tyrosine residues was not detected in MV samples. These observations confirmed that HIV-1 activates STAT1 specifically at serine residues.

**DISCUSSION**

In HIV-1-infected individuals, BBB damage impairs its ability to protect the CNS, enabling infected cells and toxins from the peripheral blood to reach the brain and elicit neuronal injury. Elucidating the mechanisms by which HIV invades the nervous system and induces neurological deficits and the signaling pathways responsible for BBB compromise are keys to better
understand HIV neuropathogenesis and offer opportunities to improve therapies to combat disease. The foundation for the work contained in this report comes from observations made by microarray analysis, which showed a prominent relationship between IL-6, STAT1 and other pro-inflammatory genes activated in HIV-1-exposed HBMEC\textsuperscript{33}. In the present study, we demonstrate that HIV-1 induce expression of IL-6 and IL-6R in HBMEC, and STAT1 mediates HIV-induced IL-6 expression and BBB dysfunction. Previous studies suggested that HIV-1 infection dysregulates the expression of IL-6 and IL-6R. HIV-infected individuals showed increased expression of IL-6 and IL-6R in monocytes, B and T cells\textsuperscript{45}. Monocytic cell lines expressing IL-6R secreted more IL-6, which upregulated HIV-1 expression, and antibodies to IL-6R inhibited IL-6-induced HIV-1 expression\textsuperscript{46}. This suggests that increased expression of IL-6 and IL-6R can potentiate viral-induced effects on the brain endothelium and contribute to HIV-induced BBB dysfunction. In fact, HIV-1 and IL-6 synergistically increase monocyte migration across \textit{in vitro} BBB models. Higher serum IL-6 in HIV-1-infected humans correlate with increased progression to AIDS and neuroAIDS\textsuperscript{47-49}. High levels of IL-6 are found in the CSF and blood cells of HAD patients and is associated with worse cognitive and motor performance\textsuperscript{50-53}.

The JAK/STAT pathway plays a prominent role in cytokine-mediated inflammatory responses, and STAT1 has been implicated in the pathogenesis of HIV-1 infection and disease progression\textsuperscript{29,30}. There is enhanced activation of STAT1 in T-cells from HIV-1-infected individuals\textsuperscript{29} and increased expression of STAT1 in brain tissues of HIV-1-infected mice\textsuperscript{54} and SIV-infected monkeys\textsuperscript{55}. Infection with HIV-1 or human T-lymphotropic virus-2 prime T-lymphocytes for STAT1 activation\textsuperscript{30}. The present study demonstrates that IL-6 is involved in HIV-induced endothelial cell dysfunction, and STAT1 mediates these effects. FLUD is an anti-inflammatory compound\textsuperscript{56} that is clinically approved and currently used in humans for the
treatment of hematologic malignancies\textsuperscript{57,58}, and also shows anti-viral effects in SIV-infected monkeys\textsuperscript{41}. FLUD has been shown to specifically induce loss of STAT1 mRNA and proteins, as well as loss of STAT1-mediated gene activation \textit{in vivo} and \textit{in vitro}\textsuperscript{40}. However, the precise mechanism of FLUD-mediated inhibition of STAT1 has not been elucidated. There is evidence that deletion of DNA methyltransferase in brain precursor cells results in DNA hypomethylation, enhanced phosphorylation of STAT1 and STAT3, and cell differentiation\textsuperscript{59}. Thus, if HIV-induced upregulation/activation of STAT1 involve demethylation of STAT1 gene, FLUD could prevent STAT1 activation by inhibiting viral-induced demethylation. There is also evidence that FLUD downregulates DNA synthesis by inhibiting the primase activity associated with DNA polymerase-alpha, thereby inhibiting RNA-primed DNA synthesis\textsuperscript{60}. Our future studies will further test these hypotheses to investigate the mechanisms of FLUD-mediated STAT1 inhibition in HIV-induced BBB dysfunction.

HBMEC and MV data presented in this study are significant; and to our knowledge, this is the first demonstration of STAT1 involvement in HIV-1-induced endothelial dysfunction and BBB injury. STAT1 and IL-6 mRNA were higher in MV from HIV+ no HIVE patients, compared to MV from HAD HIVE patients, while expression of STAT1 and phospho-STAT1 were higher in MV of HAD HIVE patients. This may suggest that during HIV-1 infection, transcriptional upregulation of pro-inflammatory cytokines and STAT1 precede cytokine expression, STAT1 activation, inflammation, and neuroAIDS. SOD2 and UBD mRNA increased in HIV-1 seropositive MV but the levels were much higher in MV from HAD patients, suggesting increased oxidative stress and impairment of the ubiquitin-proteasome system in HIVE. TNFSF15 is a potent inhibitor of endothelial cell proliferation that induce expression of pro-inflammatory cytokines, apoptosis, and senescence in endothelial cells\textsuperscript{61}. Increased levels of
TNFSF15 in HIV-exposed HBMEC and HAD HIVE MV further demonstrate vascular damage in HIVE.

In the JAK/STAT pathway, phosphorylated STATs form dimers and associates with the IFN-stimulated gene factor 3 gamma (ISGF3G) to form a complex transcription factor (ISGF3) that translocates to the nucleus, binds to the IFN-stimulated response element (ISRE) to activate the transcription of interferon or cytokine stimulated genes. We previously showed that HIV-1 induce transcriptional upregulation of ISGF3G and IRF7, both downstream effectors of STAT1. We demonstrate that STAT1 and STAT3 are the STAT family members activated by HIV-1, which suggests that in HIV-induced BBB dysfunction, the STAT dimers that translocates to the nucleus consist of STAT1-STAT3 heterodimers. The exact mechanism through which HIV-1 activates STAT1 and STAT3 remains to be elucidated. It is possible that HIV-1 upregulate STAT expression by downregulating DNA methyltransferase, since hypomethylation enhanced expression and phosphorylation of STAT1 and STAT3. STAT1 transactivating domain resides in the C-terminus. Phosphorylation of S727 increases the transcriptional activity of STAT1 dimers and greatly increases the ability of STAT1 to interact with ISGF3G. Mutation of the C-terminal S727 to alanine decreases the transcriptional activity of STAT1, and severely reduces STAT1 ability to induce anti-proliferative or viability effects. Furthermore, STAT1 transactivating domain and its phosphorylation at S727 are necessary for the complete induction of gene expression and establishment of anti-viral response to type I IFN.

The main features of the brain endothelium is the presence of TJ between endothelial cells, which restrict BBB permeability. HIV-1 and viral proteins disrupt HBMEC TJ proteins. In HIV and SIV encephalitis there is evidence that BBB perturbation is associated
with disruption of TJ and increased trafficking of HIV-infected MDM into the CNS\textsuperscript{16,68,69}. We now provide new insights into this seemingly complex process. Indeed, the evidence provided show significant decrease in claudin-5 and TJ strands in MV from autopsy brains of HAD patients, and clearly demonstrate that STAT1 plays a major role in HIV-induced inflammation and BBB damage. We further demonstrate decreased claudin-5, ZO-1, and ZO-2 expression in HIV-1-exposed HBMEC and show that FLUD diminished viral-induced downregulation of TJ proteins. Perhaps most importantly, our data suggests that inhibiting STAT1 activation could provide a unique therapeutic strategy to prevent HIV-1-induced BBB dysfunction and as such improve clinical outcomes in infected humans.

Acknowledgements

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Explanation of authors’ contribution

CA and BY performed research; HEG and YP provided assistance in data analysis and edited the paper; GDK designed and performed research, collected and analyzed data, and wrote the paper. All authors declare no competing financial interests.
References


45. van der Meijden M, Gage J, Breen EC, Taga T, Kishimoto T, Martinez-Maza O. IL-6 receptor (CD126’IL-6R’) expression is increased on monocytes and B lymphocytes in HIV infection. Cell Immunol. 1998;190:156-166.


Figure legends

Figure 1. STAT1 modulates HIV-1-induced upregulation of IL-6 expression in HBMEC. (A) Interaction of HBMEC with HIV-infected MDM induced IL-6 secretion in HBMEC. Co-culture of HBMEC with infected MDM increased IL-6 expression / secretion in endothelial cells by 10-fold (229.2 ± 38pg/ml) compared to endothelial cells co-cultured with non-infected MDM (23 ± 3pg/ml). Endothelial-MDM co-culture did not change MDM IL-6 levels regardless of HIV-1 infection. (B) Direct exposure of HBMEC to 0.01 infectious viral particles induced a time-dependent increase in IL-6 secretion. (C) Fludarabine (FLUD, 20µM) significantly diminished HIV-1-induced IL-6 secretion in HBMEC. Controls are untreated cells, NI-control represent HBMEC exposed for 24 h to conditioned media from non-infected MDM. For each experimental condition, the number of replicates n=6. (***P<0.001 compared to controls).

Abbreviations: TM: top media; BM: bottom media; CM: conditioned media; NI-MDM: non-infected monocyte-derived macrophage; HIV-MDM: HIV-infected monocyte-derived macrophage; MOI: multiplicity of infection.

Figure 2. HBMEC express the IL-6 receptor and HIV-1 exposure increase receptor expression. Exposure of HBMEC to HIV-1 for 30 min significantly increased the expression of both alpha (IL6-R-α, panels A and B) and beta (Gp130, panels A and C) subunits of the IL-6 receptor, and altered endothelial cell phenotype (panel A). HIV-1-induced up-regulation of IL6-R-α expression was transient and decreased after 30 min (panels A and B), while HIV-induced increase in Gp130 expression was sustained for up to 2h (panel C). Control represent untreated HBMEC; NI-control consisted of HBMEC exposed 1h to conditioned media from non-infected macrophages. Original magnification of images in panel A: x 200.
Figure 3. HIV-1 activates STAT1 at serine-727 in HBMEC, and FLUD inhibits HIV-induced STAT1 expression and activation. Co-culture of HBMEC with HIV-1-infected MDM (A), or direct exposure of HBMEC to infectious viral particles (B) activates STAT1 at S727. Long-term HIV exposure (12-24h) also increased total STAT1 levels in HBMEC (C). No activation of STAT1 at tyrosine residues was detected. No phosphorylation of STAT1 was observed in HBMEC exposed to conditioned media from non-infected MDM and there was no change in total STAT1 levels (D). Exposure of HBMEC to IL-6 did not phosphorylate STAT1 at serine or tyrosine residues; however, 12 to 24 h IL-6 exposure increased total STAT1 levels (E). The STAT1 inhibitor FLUD significantly diminished HIV-1-induced STAT1 activation and expression at 2h (F) and 30 min (G) exposure. (*P<0.05; **P<0.01, ***P<0.001).

Abbreviations: CM: conditioned media; C: control.

Figure 4. (A) Effect of FLUD on monocyte migration across in vitro BBB models in response to HIV-1 and IL-6. HIV-1 infection and IL-6 increased monocyte migration across the BBB and the STAT1 inhibitor (FLUD) significantly diminished HIV-1- and IL-6-induced monocyte migration (*P<0.05). NI represent migration of non-infected macrophages. (B, C) HIV-1 phosphorylates STAT1 and STAT3 at serine-727, and the JAK-3 inhibitor WHI-P154 diminished HIV-1-induced phosphorylation of STAT1 and STAT3. HIV-1 induced phosphorylation of STAT-3 at S-727 but did not phosphorylate STAT-5 or STAT-3 at tyrosine residues. FLUD had no effect on HIV-1-induced STAT-3 phosphorylation (panel B). The JAK-3 inhibitor (WHI-P154) significantly diminished HIV-induced serine phosphorylation of STAT1 and STAT3, while the
JAK-2 inhibitor (AG490) had no effect (C). No activation of STAT3 at tyrosine residues was detected. HIV-1 did not activate STAT-2, STAT-5 or STAT-6.

**Figure 5. Expression of TJ proteins in HBMEC and microvessels from autopsy brains of seronegative controls and HIV-1-infected humans.** (A) All MV samples stained positive for claudin-5. MV from seronegative control donors (e.g. patients N1 and N2) shows prominent and continuous strands of tight junction (TJ, arrows); TJ strands in MV from HIV-1 seropositive patients without HIVE (e.g. P1 and P2), and HAD HIVE patients (e.g. HAD2 and HAD5) were fewer and had more gaps (arrow-heads). Patient HAD5 shows very little TJ strands. (B) Computer-assisted semi-quantitative analyses of all MV samples show a significant decrease in claudin-5 expression in MV from HAD patients, compared to MV from HIV-1 seropositive patients without HIVE (HIV-POS), or seronegative controls (HIV-NEG). MV from HIV-1 seropositive patients without HIVE also showed a small (non-statistically significant) decrease in claudin-5 expression compared to seronegative controls. (C) Double immunostaining of MV for human CD163 (green) and vWF(red) showed that the isolated MV did not have macrophage contamination. (D-F) Exposure of HBMEC to HIV-1 (MOI: 0.01) for 24h decreased the expression of claudin-5, ZO-1 and ZO-2. Densitometry analysis showed that FLUD significantly diminished HIV-induced downregulation of claudin-5 (E) and ZO-1 (F). Original magnification of images in panels A and C: x 400.

**Figure 6. Quantitative RT-PCR of patients’ brain MV and HIV-1-exposed HBMEC.** (A) Data showed similar pattern of HIV-1-induced upregulation of mRNA for inflammatory cytokines, oxidative stress enzymes, ubiquitin-related genes, and the transcription factor RelB in
tissue culture and patients’ brain MV. Both co-culture of HBMEC with infected MDM (B) and direct exposure of HBMEC to infectious viral particles (C) induced transcriptional upregulation of STAT1. Data demonstrated similar pattern of HIV-1-induced transcriptional upregulation of STAT1 and IL-6 in endothelial cell cultures and in brain MV of infected humans (B-E). For co-culture experiments, controls are untreated endothelial cells. Each group (control, NI, and HIV) had 3 replicate samples; each sample was tested in triplicate and normalized to its GAPDH. (*P<0.05, **P<0.01, ***P<0.001). Abbreviations: NI: HBMEC co-cultured with non-infected MDM; HIV: HBMEC co-culture with HIV-infected MDM; HIV-neg: MV from HIV seronegative donors (donors N1 to N5); HIV-pos: MV from HIV+ no HIVE donors (donors P1 to P4); HAD: MV from HAD patients (donors HAD1 to HAD5).

Figure 7. Increased expression and activation of STAT1 in brain MV of HIV-1-infected humans. (A) Western blot analysis showed increased expression of STAT1 and phospho-STAT1 (S727) in brain MV of some HIV-1 seropositive patients and patients with HAD and HIVE. No phosphorylation of STAT1 at tyrosine residues was detected. (B - D) Confocal microscopy showed increased expression of STAT1 and phospho-STAT1 (S727) in brain MV of HIV-1 seropositive and HAD HIVE patients, compared to MV from HIV seronegative controls. Computer-assisted semi-quantitative analyses of all MV samples showed a significant increase in phospho-STAT1 (C) and STAT1 (D) in brain MV of both HIV+ no HIVE and HAD patients (***P<0.01; ***P<0.001). Original magnification of images in panel B x 400.
# Table 1. Clinical history of brain MV donors

<table>
<thead>
<tr>
<th>HIV-1 status</th>
<th>ID</th>
<th>Gender/Age (years)</th>
<th>PMI (h)</th>
<th>Neurocognition/Neuropathology</th>
<th>Other autopsy diagnosis</th>
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<tr>
<td>Neg</td>
<td>N1</td>
<td>M / 35</td>
<td>8.5</td>
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<td>Mild Alzheimer type 2 gliosis</td>
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<td>Neg</td>
<td>N2</td>
<td>N/A</td>
<td>N/A</td>
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<td>Neg</td>
<td>N3</td>
<td>F / 38</td>
<td>5.75</td>
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<tr>
<td>Neg</td>
<td>N4</td>
<td>M / 32</td>
<td>4.25</td>
<td>Normal / Not Significant</td>
<td>Cystic fibrosis, bronchopneumonia, respiratory and multiorgan failure</td>
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<tr>
<td>Neg</td>
<td>N5</td>
<td>F / 46</td>
<td>4</td>
<td>Normal / Not Significant</td>
<td>Congenital aortic stenosis with acute myocardial infarction, mild infarction. Two small subependymal microinfarcts in the brain</td>
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<td>2.75</td>
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<td>12</td>
<td>HAD / HIVE</td>
<td>N/A</td>
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<tr>
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<td>HAD5/D5</td>
<td>M / 47</td>
<td>11</td>
<td>HAD / HIVE</td>
<td>Anoxic-ischemic encephalopathy, microglial nodule encephalitis, chronic meningoencephalitis with microvascular damage</td>
</tr>
</tbody>
</table>

**Abbreviations.** Neg: HIV seronegative; Pos: HIV seropositive; ADC: AIDS dementia complex; HAD: HIV-associated dementia; HIVE: HIV encephalitis; M: male; F: female; ? or N/A – not available; PMI: post-mortem interval.
Figure 1

(A) IL-6 (pg/ml)

(B) IL-6 (pg/ml)

(C) IL-6 (pg/ml)

**P<0.01
***P<0.001
**P<0.05
*P<0.05

Time (HIV-1: MOI=0.01)
Figure 2

A

IL6-Rα

Control  NI-Control  HIV: 30 min  HIV: 1h  HIV: 2h

Gp130

B

C

IL6Rα (% control area)

*** P<0.001

GP130 (% control area)

* P<0.05

** P<0.01
Figure 4

A

Number of migrated monocytes (% of control)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Control</th>
<th>FLUD</th>
<th>IL-6</th>
<th>FLUD + IL-6</th>
<th>Control</th>
<th>FLUD</th>
<th>IL-6</th>
<th>FLUD + IL-6</th>
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<td></td>
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<tr>
<td>Non-infected monocytes</td>
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</table>

B

- pSTAT3 (Tyr705)
- pSTAT3 (S727)
- STAT-3
- pSTAT-5 (Tyr694)
- STAT-5
- Actin

HIV-1 (MOI: 0.01)
FLUD (20μM)

C

- pSTAT-1 (S727)
- STAT-1
- pSTAT-2 (Tyr690)
- pSTAT-5 (Tyr694)
- STAT-5
- pSTAT-6 (Tyr641)
- STAT-6
- pSTAT-3 (Tyr705)
- pSTAT-3 (S727)
- STAT-3
- Actin

HIV-1 (MOI: 0.01)
AG490 (50μM)
WHI-P154 (30μM)
Figure 5

A

Patient N1

Patient P1

Patient HAD2

Patient N2

Patient P2

Patient HAD5

B

Claudin-5 (% control area)

HIV-NEG  HIV-POS  HAD

P<0.001  P<0.001

C

HIV+ Patient

P2

Patient

HAD4

CD163

CD163 + vWF

D

Claudin-5
ZO-1
ZO-2
Actin

HIV-1 (24h)
FLUD (20 μM)

E

Claudin-5 / Actin

Control  HIV  HIV+FLUD

P<0.01  P<0.05

F

ZO-1 / Actin

Control  HIV  HIV+FLUD

P<0.001  P<0.001  P<0.01
Figure 6

(A) HBMEC Co-cultures

(B) STAT-1

(C) STAT-1

(D) STAT-1

(E) IL-6

For personal use only.
Figure 7

A

B

C

D

***P<0.001

**P<0.01

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STAT1 signaling modulates HIV-1-induced inflammatory responses and leukocyte transmigration across the blood-brain barrier

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