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Toll-Like Receptor-3 Mediates HIV-1-Induced Interleukin-6 Expression In The Human Brain Endothelium Via TAK1 And JNK Pathways: Implications For Viral Neuropathogenesis

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Abstract

HIV-1-associated neurocognitive impairment (HAND) is associated with blood-brain-barrier (BBB) inflammation, and inflammation involves toll-like receptors (TLRs) signaling. It is not known whether primary human brain microvascular endothelial cells (HBMEC), the major BBB component, express TLRs or whether TLRs are involved in BBB dysfunction and HAND. We demonstrate that HBMEC express TLR3, 4, 5, 7, 9, and 10, and TLR3 was the most abundant. HIV-1 and TLR3 activation increased endothelial TLR3 transcription and expression. HIV-1-positive human subjects showed significantly higher TLR3 expression in brain tissues and blood vessels, with higher TLR3 levels in subjects with HAND. HIV-1 and TLR3 activation increased endothelial IL6 expression by 6-to-127-fold ($P < 0.001$), activated c-jun(serine-63) and SAPK/JNK(Thr183/Tyr185). HIV-1 upregulated IL6 through interleukin-1 receptor-associated-kinase(IRAK)-1/4/TAK1/JNK pathways, via ATP-dependent JNK activation. TLR3 activation upregulated IL6 through TAK1/JNK pathways, via ATP-dependent or -independent JNK activation. HIV-1 and TLR3 activation also upregulated transcription factors associated with IL6 and TAK1/JNK pathways (Jun, CEBPA, STAT1). Blocking TLR3 activation prevented HIV-1- and TLR3 ligands-induced upregulation of these transcription factors, prevented IL6 transcription and expression, c-jun and JNK activation. HIV-1 and TLR3 ligands significantly increased monocytes adhesion and migration through the BBB, and decreased endothelial claudin-5 expression. Blocking TLR3 and JNK activation prevented HIV-1- and TLR3 ligands-induced claudin-5 downregulation, monocytes adhesion and transendothelial migration. These data suggests that viral immune recognition via endothelial TLR3 is involved in endothelial inflammation and BBB dysfunction in HIV/AIDS and HAND. Our data provides novel insights into the molecular basis of these HIV-1- and TLR3-mediated effects.

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Keywords

TLR3; HIV-1; blood-brain barrier; IL6; TAK1/MKK7/JNK signaling; human brain microvascular endothelial cells; claudin-5; monocytes

Background

Toll-like receptors (TLRs) are pattern-recognition receptors that detect invading pathogens via their pathogen associated molecular patterns (PAMP), and the resulting TLR/PAMP interaction triggers the innate immune system [1, 2]. Thus, TLRs play a major role in innate immunity. Thirteen different TLRs have been discovered so far, of which 11 (TLR1 to TLR11) are expressed in human cells [3, 4]. TLR1, 2, 4, 5, 6, and 10 are mostly expressed on the outer cell membranes, whereas TLR3, 7, 8, and 9 are mostly expressed on endosomal membranes [3, 4]. However, the localization of TLRs vary amongst cell types; TLR3 is often located intracellularly on endosomes in many cells, but in human astrocytes TLR3 is expressed both intracellularly and on the cell surface [5]. TLR3 can be activated by viral double-stranded RNA (dsRNA) [6], viral single-stranded RNA (ssRNA) [7], endogenous viral mRNA [8], and polyinosinic-polycytidylic acid (PIC), a synthetic analogue of viral dsRNA [9]. It has been demonstrated that viral dsRNA is a replication intermediate of several ssRNA viruses; and that dsRNA are produced during the course of replication of ssRNA viruses such as the respiratory syncytial virus, encephalomyocarditis virus and West Nile virus, and activate TLR3 [10–12].

Ligand-induced TLR activation can be beneficial since the TLR-ligand complex initiates a quick and protective innate immune response against invading pathogens [1, 2]. However, it can also be detrimental because uncontrolled and unregulated TLR activation can result in excessive inflammation, cell injury, and tissue damage [13–15]. In fact, TLR3 has been implicated in the pathogenesis of several viral infections, including HIV-1- and SIV-induced inflammation in lymph nodes and astrocytes [16, 17], HIV-1 transactivation, replication, and inflammation [18, 19].

Human brain microvascular endothelial cells (HBMEC) are a major component of the blood-brain barrier (BBB). Under normal physiological conditions, HBMEC functions as an interface between the blood and the brain parenchyma, strictly regulating influx of ion molecules and leukocytes into the central nervous system (CNS) [20, 21]. During HIV-1 infection, the BBB function is compromised, and this may enable the entry of free virus and/or HIV-infected mononuclear phagocytes into the brain, infection of resident CNS cells and inflammation, and these may contribute to the development of HIV-1 associated neurocognitive impairments (HAND) [21–24]. We previously showed that HIV-1-induced BBB dysfunction was associated with activation of pro-inflammatory cytokines and chemokines in HBMEC, and that IL6 and TLR signaling were major canonical pathways involved in this HIV-induced BBB inflammation [25, 26]. It is not known whether primary HBMEC express TLRs, and which of the 11 human TLRs may be involved in HIV-induced BBB dysfunction. In the present study, we identify the TLRs expressed in primary HBMEC. We show that HBMEC express TLR3, 4, 5, 7, 9, and 10, and TLR3 was the most abundant

TLR expressed in HBMEC. Ex-vivo studies using human brain tissues confirmed these findings and showed significantly higher TLR3 transcription and expression in brain tissues of HIV-1-infected subjects, mostly on the brain blood vessels; with much higher TLR3 levels in brain tissues of subjects with HAND or HIV encephalitis (HIVE). We also showed that exposure of HBMEC to HIV-1 or the TLR3 ligand PIC increased TLR3 levels and IL6 expression via Transforming growth factor- β activated kinase-1 (TAK1) and c-Jun N-terminal kinase (JNK) Pathways. Functional studies showed that both HIV-1 and PIC significantly increased monocytes adhesion and migration through *in vitro* BBB models, and decreased the expression of the endothelial tight junction protein claudin-5. The TLR3/dsRNA complex inhibitor, as well as JNK inhibitors blocked HIV-1- and PIC-induced claudin-5 downregulation, monocytes adhesion and transendothelial migration. These data suggest that viral immune recognition via endothelial TLR3 is involved HIV-1-induced endothelial inflammation and BBB dysfunction; and that HIV-induced inflammation, BBB dysfunction and HAND are associated with increased TLR3 expression in the CNS and brain endothelium.

Materials and Methods

Brain endothelial cells (EC) culture

Primary HBMEC were isolated from brain tissue obtained during surgical removal of epileptogenic cerebral cortex in adult patients, under an Institutional Review Board-approved protocol at the University of Arizona as described previously [27, 28]. Routine evaluation by immunostaining for von-Willebrand factor, *Ulex europaeus* lectin and CD31 showed that cells were >99% pure. Freshly isolated cells were cultured in collagen-coated flasks or 6-well culture plates using Dulbecco's Modified Eagle Medium (DMEM)/F12 (Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Atlanta Biologicals, Flowery Branch, GA, USA), supplemented with 10 mmol/l L-glutamine (Life Technologies), 1% heparin (Thermo Fisher Scientific, Pittsburgh, PA, USA), 1% endothelial cell growth supplement (ECGS; BD Bioscience, San Jose, CA, USA), 1% penicillin-streptomycin (Life Technologies), 1% fungizone (MP Biomedicals, Solon, OH, USA). Cells at passage 2 to 4 were used in this study.

Human brain tissues

Brain tissues (cortex) from HIV-1-seropositive patients with or without neurocognitive impairment, and HIV-1/2-seronegative controls were obtained from the National NeuroAIDS Tissue Consortium and the Department of Pharmacology and Experimental Neuroscience brain bank. The clinical histories of all brain tissue donors are detailed in Table 1.

RNA extraction and cDNA synthesis

Confluent HBMEC in six-well plates were treated with high molecular weight PIC (50 μ g/ml) (InvivoGen, San Diego, CA, USA) or HIV-1_{ADA} (multiplicity of infection, MOI: 0.001) for 2 to 24 hours. Controls consisted of untreated cells and cells treated with similar concentrations of PIC or HIV-1 in the presence of the TLR3/dsRNA Complex Inhibitor (TLR3.CI) (30 nM). Total RNA was extracted from HBMEC and human brain tissues using

the Trizol reagent (Life Technologies) according to the manufacturer's protocol. RNA was further cleaned using Total RNA cleanup kit (Qiagen, Valencia, CA, USA); RNA yield and quality were checked using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE) and for all samples absorbance ratio of 260/280 was 2. cDNA was generated from 1 µg RNA in a 20 µl reaction volume, using the Verso cDNA kit (Thermo Fisher, Waltham, MA, USA), according to the manufacturer's instructions. Reverse transcription was carried out for 30 min at 42°C.

Reverse transcription (RT)-PCR

Two microliters of the cDNA obtained was used for RT-PCR, in a 20 µl reaction volume containing 50 U/ml *Taq* DNA polymerase supplied in a proprietary reaction buffer (pH 8.5) (Promega, Madison, WI, USA), 400 µM each dNTP, 3 mM MgCl₂ (all from Promega), and 25 µM human TLRs (1–10) specific primers (InvivoGen) (Table 2). Positive controls consisted of PCR performed using 10 ng TLRs (1–10) cDNA provided by the manufacturer (InvivoGen). PCR was performed in a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA), using the following conditions: 95°C for 2 min, followed by 35 cycles of 95°C for 30 sec, 60°C for 30 sec, 72°C for 2 min and 72°C for 5 min, and overnight incubation at 16°C. For endogenous control, additional PCR were performed using GAPDH-specific primers (forward primer: 5'-CGAGATCCCTCCAAAATCAA-3'; reverse primer: 5'-TGTGGTCATGAGTCCTTCCA-3'). Ten microliters of each PCR products were analyzed by electrophoresis on a 2% agarose gel containing ethidium bromide, and the images were captured using a Gel Imaging system (G:BOX, Syngene, Frederick, MD, USA).

Real-time PCR

The cDNA generated as described above was diluted (1:10) in nuclease-free water. A 10 µl reaction mixture containing 2 µl of each diluted cDNA sample, 5 µl master mix (containing the polymerase enzyme, dNTPs and MgCl₂), 0.5 µl primer-probe mixture [containing 900 nM of each forward and reverse primer and 250 nM Taqman minor groove binder (MGB) probe], and 2.5 µl nuclease-free water was used for quantitative real-time PCR. Gene amplification was performed in a LightCycler® 480 II Real-Time PCR System (Roche, Indianapolis, IN) and the software monocolor hydrolysis probe universal cycling conditions (95 °C for 10 min, followed by 45 cycles of 95 °C for 10 sec, 60 °C for 30 sec, and 72 °C for 1 sec). Gene quantification was performed using the Ct method as described in the software user manual. All PCR primers and probes were obtained from Applied Biosystems and primer IDs were as follows: IL6 (Hs00985639), JUN (Hs99999141_s1), RELB (Hs00232399_m1), cAMP response element binding protein (CEBP)A (Hs00269972_s1), CEBPG (Hs01922818_s1), signal transducer and activator of transcription (STAT)1 (Hs00234829_m1), and TLR3 (HS01551078_m1). For endogenous control, each gene expression was normalized to the sample GAPDH (Hs99999905_m1).

Immunofluorescence and confocal microscopy

Sections (5 µM) were cut from each human brain tissue using a cryostat (Leica, Buffalo Grove, IL, USA), mounted on glass slides, fixed, permeabilized, and incubated for 1 hour in PBS containing 3% bovine serum albumin to block non-specific binding. Tissues were

incubated overnight with antibodies to TLR3 and glucose transporter-1 (GLUT1) (Abcam, Cambridge, MA, USA), followed by staining (1 hour in the dark at room temperature) with secondary antibodies coupled with Alexa Fluor-488 or -635 (Invitrogen, Waltham, MA, USA) at 1:500 dilutions. Stained tissues were washed in PBS, mounted in Prolong Gold anti-fade reagent containing DAPI (Molecular Probes, Grand Island, NY) and examined under an Olympus FV500-IX 81 confocal laser scanning imaging system. The triple laser lines excitation were 405-nm for nucleus-stains; 484-nm for TLR3, and 635-nm for GLUT1. A fourth laser line (excitation: 543-nm; emission: 595-nm) was used to detect auto-fluorescent pigments.

Protein extraction and Western blot analysis

Following cellular treatment, protein extraction, quantification, and Western blot analysis were performed as previously described [26, 28, 29]. Briefly, cells were lysed using the mammalian cell lysis buffer CelLytic M (Sigma, St Louis, MO, USA), and protein quantified using the bicinchoninic acid assay as we previously described [26, 28, 29]. Protein (35 µg) was fractionated in a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Membranes were blocked for 1 hour with SuperBlock T-20 (Pierce, Rockford, IL, USA) and blotted for 2 hours or overnight with monoclonal antibodies to TLR3, claudin-5 (Abcam), JNK, phospho-JNK, c-Jun, phospho-c-Jun (all from Cell Signaling Technology, Danvers, MA, USA). These primary antibodies were used at 1:1,000 dilutions. Membranes were then washed, blotted for 1 hour with horseradish peroxidase-conjugated secondary antibody, washed again, and visualized using the enhanced chemiluminescence (Pierce) and gel doc system (Syngene, Frederick, MD, USA). After each western blot experiment, membranes were stripped using the Restore Western Blot Stripping Buffer (Pierce) and re-blotted with β -actin antibody (Abcam) to confirm equal loading.

Interleukin-6 ELISA

Interleukin (IL)-6 expression and secretion was quantified using the BD OptEIA Human IL6 ELISA kit II (BD Biosciences, CA, USA) according to the manufacturer's protocol. Briefly, confluent HBMEC were treated with PIC (50 µl/ml), HIV-1_{ADA} (MOI: 0.001) and/or pharmacological inhibitors (Table 3) for 48 hours. Controls consisted of untreated cells and cells treated with the TLR3/dsRNA Complex Inhibitor (TLR3.CI) (30 nM). Following cell treatment, culture supernatants were collected and further centrifuged for 5 min at 2350 g to remove any cellular debris, and 100 µl of each sample used for IL6 ELISA according to manufacturer's instructions. Absorbance readings were obtained using SpectraMax M5 ELISA plate reader (Molecular Devices, Sunnyvale, CA, USA). For each experiment, IL6 standards provided with the kit were used to generate a standard curve and determine IL6 concentrations in each sample. Each experimental condition was tested in duplicate or triplicate.

Monocyte isolation and labeling

Human monocytes were obtained from HIV-1/2 and hepatitis-B seronegative donor leukopaks, and separated by countercurrent centrifugal elutriation as previously described [26, 28, 30]. Cells were identified as >98% pure monocytes by Wright staining and CD68

immunostaining (at 1:50 dilution) (Dako, Carpinteria, CA, USA). Monocytes were used for adhesion and transendothelial migration assays within 24 hours of elutriation. Freshly elutriated monocytes were washed twice with pre-warmed (37°C) PBS, re-suspended in pre-warmed PBS containing 5 μ M of 5-carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE) (Life Technologies); 1×10^6 cells / ml, and incubated at 37°C, 5% CO₂, for 15 min. Monocytes were then pelleted by centrifugation at 300 g for 10 minutes, re-suspended in pre-warmed media and incubated at 37°C, 5% CO₂, for 30 min. CFDA-SE-labeled monocytes were washed three times with pre-warmed serum-free media before use for adhesion and migration assays.

Monocyte adhesion and migration through *in vitro* BBB models

Adhesion and migration experiments were performed as we previously described [26, 28, 30]. Briefly, for adhesion assays, HBMEC were plated on 96-well collagen-coated black plates, and for migration assay, HBMEC were plated on collagen-coated FluoroBlok-tinted tissue culture inserts (3- μ m pore size; BD Biosciences). Confluent HBMEC were treated with PIC (50 μ l/ml), HIV-1_{ADA} (MOI: 0.001), and/or pharmacological inhibitors (Table 3) for 24 or 48 hours. Controls consisted of untreated HBMEC and cells treated with similar concentrations of pharmacological inhibitors for 24 or 48 hours. Following treatment, HBMEC were rinsed three times to remove HIV-1, PIC, or pharmacological inhibitors. For adhesion assays, HBMEC were exposed to 2.5×10^5 CFDA-SE-labeled monocytes and incubated at 37°C, 5% CO₂, for 15 min. After the 15 min incubation, HBMEC were washed 3 times with PBS and the number of adherent monocytes quantified by spectrophotometry (top readings, absorbance 494 nm; emission, 517 nm), with a standard curve derived from a serial dilution of a known number of CFDA-SE-labeled monocytes. For migration assays, following treatment and rinsing of HBMEC, 2.5×10^5 CFDA-SE-labeled monocytes were added to the upper chamber of the FluoroBlok inserts and allowed to migrate for 2 hours (37°C, 5% CO₂). The numbers of migrated monocytes were quantified by spectrophotometry (bottom readings, absorbance 494 nm; emission, 517 nm), with a standard curve derived from a serial dilution of a known number of CFDA SE-labeled monocytes.

Statistical analyses

Data were analyzed by t-test (two-tailed) for two-group comparisons and one- or two-way ANOVA followed by Tukey's multiple-comparisons tests using GraphPad Prism 5.0b. (GraphPad Software, La Jolla, CA, USA). Threshold of significance level was 0.05. Data are presented as means $\pm$ standard error of the mean.

Results

Expression of TLRs in primary HBMECs

Our previous studies showed that signaling via TLRs was a major canonical pathway involved in HIV-induced activation of pro-inflammatory cytokines and chemokines in HBMEC, and BBB inflammation [25]. We performed RT-PCR to investigate the expression of TLRs in HBMEC and their role in HIV-induced BBB inflammation. We show that primary HBMEC express six TLRs (TRL3, 4, 5, 7, 9, 10) mRNA (Fig. 1a). TLR3 was the

most abundant TLR expressed and no mRNA for TLR1, 2, 6, or 8 was detected (Fig. 1a). These results were confirmed by densitometry analyses of data from three independent experiments using HBMEC from three different human donors (Fig. 1b).

Exposure of HBMEC to HIV-1 or TLR3 ligands increased TLR3 transcription and expression

To determine whether HIV or TLR3 ligands can alter TLR3 expression on the brain endothelium, we assessed TLR3 mRNA and proteins levels in HBMEC exposed to HIV-1_{ADA} (MOI: 0.001) or PIC (25 to 75 µg/ml) for 0.5 to 24 hours. Exposure of HBMEC to HIV-1 or the TLR3 ligand PIC increased TLR3 transcription (Fig. 1c), and densitometry analyses of data from three independent experiments using HBMEC from three different human donors, confirmed these results (Fig. 1d). Exposure of HBMEC to HIV-1 or the TLR3 ligand PIC also increased TLR3 expression (Fig. 2). Initial reduction in TLR3 expression was observed at 30 min HIV-1 exposure, but TLR3 levels gradually increased thereafter (Fig. 2a), with time-dependent increase in TLR3 expression observed from 12 to 48 hours HIV-1 exposure (Fig. 2b). Exposure of HBMEC to PIC for 24 hours increased TLR3 expression, with maximal levels observed with 25 to 50 µg/ml PIC (Fig. 2c). Densitometry analyses of data from three independent experiments using HBMEC from three different donors, confirmed that HIV-1 and PIC increased TLR3 expression (Fig. 2).

Increased transcription and expression of TLR3 in brain tissues of HIV-1-infected humans

To determine whether HIV-1 infection alter TLR3 levels in the human brain, we analyzed brain tissues from 12 HIV-1/2-seronegative control subjects, 9 HIV-1-seropositive patients without evidence of HIVE or HAND, and 10 HIV-1-seropositive patients with HIVE and HAND. All brain tissues were from the cortical regions, with 28 of the 31 samples from the frontal cortex, 2 samples from the parietal cortex, and 1 sample from the temporal cortex. Table 1 shows the age, gender, clinical history, post-mortem interval (PMI) between the time of death and autopsy, and a summary of post-mortem findings for all 31 human subjects. No significant differences were detected in age and PMI between the seronegative, HIV-1-infected, or HIVE groups. For seronegative controls, HIV-1-infected, and HIVE patients, the age ranges in years (and means $\pm$ standard deviations) were respectively 32 to 72 (mean: 52 ± 13.4), 27 to 54 (mean: 41.78 ± 8.8), and 30 to 52 (mean: 40.6 ± 7.76). For seronegative controls, HIV-1-infected, and HIVE patients, the PMI ranges in hours (and means $\pm$ standard deviations) were respectively 3 to 8.5 (mean: 4.6 ± 1.6), 2.75 to 15 (mean: 8.5 ± 4), and 4 to 21 (mean: 9.45 ± 5.16).

Quantitative real-time PCR showed significant upregulation of TLR3 mRNA transcripts in brain tissues from HIV-1-infected humans, with the highest TLR3 mRNA levels in brain tissues of patients with HIVE/HAND (Fig. 3a,b). To further investigate the effects of HIV-1 infection on TLR3 expression in the human BBB *in vivo*, we analyzed brain tissue sections from seronegative controls, HIV-infected and HIVE patients by confocal microscopy. Data showed TLR3 expression on the brain blood vessels of seronegative controls (Fig. 3c–f, yellow arrows), HIV-positive (Fig. 3g–j, yellow arrows) and HIVE (Fig. 3k–n, yellow arrows) patients, with higher TLR3 expression in the blood vessels and tight junction strands of HIV+ and HIVE patients brains, as demonstrated by increased staining intensity. Brain

tissues from HIV-positive and HIVE patients also showed mononuclear phagocytes expressing TLR3 crossing the blood vessels (Fig. 3, orange arrows).

HIV-1 and TLR3 ligands upregulate IL6 expression in HBMEC

To determine whether PIC- and HIV-1-induced increase in TLR3 expression in HBMEC (Figs. 1 and 2) could be associated with increased BBB inflammation, we analyzed the effect of TLR3 ligands and HIV-1 on IL6 expression and secretion in HBMEC. Studies using HBMEC from three different human donors (Fig. 4a–c) showed that PIC at 0.5 to 75 $\mu\text{g/ml}$ increased IL6 expression and secretion in primary HBMEC by 6 to 127-fold (Fig. 4a–c, $P < 0.001$). Studies using HBMEC from the third donor showed that the TLR3/dsRNA complex inhibitor (TLR3.CI) blocked both PIC- and HIV-1-induced IL6 expression (Fig. 4c, $P < 0.001$).

HIV-1 and TLR3 ligands induced upregulation of mRNA transcripts for IL6 and transcription factors associated with the TAK1 and JNK pathways in HBMEC

To determine whether HIV-1- and PIC-induced IL6 expression was associated with increased IL6 transcription, we used real-time PCR to quantify IL6 mRNA in HBMEC exposed to HIV-1 (MOI: 0.001) and PIC (50 $\mu\text{g/ml}$) for 2 to 24 hours. Both PIC and HIV-1 induced a time-dependent increase in IL6 transcription in HBMEC, with the highest IL6 mRNA levels observed from 24 hours HIV-1 or PIC exposure (Fig. 5a). The IL6 promoter contains several cis-acting response elements that regulate its transcription and expression. These included the activator protein-1 (AP-1), NF κ B, STATs, and the cAMP response element binding protein (CREB/CEBP) [31–33]. Therefore, we analyzed the effects of HIV-1 and TLR3 ligands on the expression of these transcription factors. Compared to untreated controls both PIC and HIV-1 significantly increased JUN, RELB, CEBPA, CEBPG, and STAT1 transcription in HBMEC (Fig. 5b–f). PIC- and HIV-1-induced expression of these transcription factors was mostly time-dependent, with the highest mRNA levels observed at 24 hours exposure (Fig. 5b–f). Compared to untreated controls, exposure of HBMEC to PIC for 2 to 24 hours increased IL6, JUN, RELB, CEBPA, CEBPG, and STAT1 mRNA levels respectively by 9 to 108.6-fold (Fig. 5a), 3 to 4 fold (Fig. 5b), 1.7 to 29-fold (Fig. 5c), 2 to 4.2-fold (Fig. 5d), 1.97 to 6.26-fold (Fig. 5e), and 9 to 108.6-fold (Fig. 5f). Exposure of HBMEC to HIV-1 for 2 to 24 hours increased IL6, JUN, RELB, CEBPA, CEBPG, and STAT1 mRNA levels respectively by 27.8 to 150-fold (Fig. 5a), 4 to 10.78 fold (Fig. 5b), 13 to 54.78-fold (Fig. 5c), 1.92 to 12-fold (Fig. 5d), 3.65 to 14.8-fold (Fig. 5e), and 27.8 to 150-fold (Fig. 5f).

TLR3/dsRNA complex inhibitors blocked both TLR3 ligands- and HIV-1-induced upregulation of mRNA transcripts for IL6 and transcription factors associated with the TAK1 and JNK pathways

To confirm the involvement of TLR3 in HIV-1-induced upregulation of mRNA transcripts for IL6 and the transcription factors identified above (Fig. 5) we performed real-time PCR to quantify their mRNA levels in HBMEC exposed for 24 hours to HIV-1_{ADA} (MOI: 0.001) or PIC (50 $\mu\text{g/ml}$) with and without the presence of the TLR3/dsRNA complex inhibitors (TLR3.CI). Compared to untreated controls, exposure of HBMEC to HIV-1 increased the transcription of IL6, JUN, RELB, CEBPA, CEBPG, and STAT1 respectively by 264-, 11.2-,

24.93-, 10-, 9.6-, and 92.5-fold (Fig. 6). Exposure of HBMEC to PIC increased the transcription of IL6, JUN, RELB, CEBPA, CEBPG, and STAT1 respectively by 302.8-, 13.48-, 22.78-, 10.4-, 8.86-, and 91-fold (Fig. 6). The TLR3.CI blocked both HIV-1- and PIC-induced transcription of IL6 (Fig. 6a, $P<0.05$ and $P<0.01$), JUN (Fig. 6b, $P<0.001$), RELB (Fig. 6c, $P<0.001$), CEBPA (Fig. 6d, $P<0.05$), CEBPG (Fig. 6e, $P<0.01$), and STAT1 (Fig. 6f, $P<0.001$).

HIV-1 and TLR3 ligands induced phosphorylation of c-Jun and JNK in HBMEC

Because HIV-1 and TLR3 ligands upregulates the transcription factors associated with the JNK pathways (Figs. 5 and 6), we analyzed the effects of HIV-1 and PIC on c-Jun and JNK activation in HBMEC. Exposure of HBMEC to both PIC (Fig. 7a) and HIV-1 (Fig. 7b) induced the phosphorylation of c-Jun at serine-63 (Ser63). PIC- and HIV-1-induced c-Jun phosphorylation occurred as early as 5 min and gradually increased for up to 2 hours, with maximal phosphorylation observed at 2 hours (Fig. 7a and 7b). Both PIC (Fig. 7a) and HIV-1 (Fig. 7b) increased the phosphorylation of SAPK/JNK at Thr183/Tyr185; with PIC-induced SAPK/JNK phosphorylation occurring as early as 5 min following PIC exposure (Fig. 7a), and HIV-1-induced SAPK/JNK phosphorylation starting at 30 min following viral exposure (Fig. 7b). Repeated experiments using HBMEC from different donors confirmed these results, and showed that TLR3.CI inhibited both HIV-1- and PIC-induced phosphorylation of c-Jun, and also inhibited HIV-1- and PIC-induced phosphorylation of SAPK/JNK (Fig. 7c).

TAK1 and JNK pathways mediates HIV-1 or TLR3 ligands induced IL6 expression HBMEC

To determine whether blocking the pathways associated with transcription factors upregulated by TLR3 ligands and HIV-1 (Figs. 5 and 6) can alter PIC- and HIV-1-induced IL6 expression, we quantified IL6 expression and secretion in HBMEC exposed to PIC or HIV-1, in the presence or absence of specific inhibitors of effectors associated with those transcription factors. The ATP-competitive JNK inhibitor (420129), the JNK inhibitor 420119, and the TAK1/MKK7 inhibitor (5ZO) blocked PIC-induced IL6 expression and secretion (Fig. 8a), whereas the inhibitor of c-Jun/JNK complex (420130), the inhibitor of NF κ B transcriptional activation (481406), the inhibitor of AP-1 transcription (SR11302), and the IRAK1/4 inhibitor only partially reduced or had no major effect on PIC-induced IL6 expression (Fig. 8b). The ATP-competitive JNK inhibitor (420129), the TAK1/MKK7 inhibitor (5ZO), and the IRAK1/4 inhibitor blocked HIV-1-induced IL6 expression and secretion (Fig. 8c); whereas the JNK inhibitor 420119, the inhibitor of c-Jun/JNK complex (420130), the inhibitor of NF κ B transcriptional activation (481406), and the inhibitor of AP-1 transcription (SR11302) had no major effect on HIV-1-induced IL6 expression (Fig. 8c).

Effects of HIV-1 and TLR3 ligands on the blood-brain barrier properties and function

We previously demonstrated that HIV-1 and viral factors impair the BBB properties and function, and this was associated with decreased expression of tight junction proteins, increased adhesion and migration of human mononuclear phagocytes through the BBB [26, 29, 30]. To determine whether this involves TLR3, we assessed the effects on HIV-1 and TLR3 ligands on claudin-5 expression in primary HBMEC, and monocytes adhesion and

migration through *in vitro* BBB models. Exposure of HBMEC to HIV-1 and PIC (50 µg/ml) for 24 hours increased monocytes adhesion (by 4- and 3.5-fold respectively, Fig. 9a) and transmigration through the BBB (by 4.1- and 3.45-fold respectively, Fig. 9b) ($P<0.001$), and decreased claudin-5 expression in HBMEC by 3.77- and 2.85-fold respectively (Fig. 9c, $P<0.001$). Exposure of HBMEC to HIV-1 and PIC for 48 hours increased monocytes adhesion (by 5.86- and 1.83-fold respectively, Fig. 9d) and transmigration through the BBB (by 14- and 3.78-fold respectively, Fig. 9e) ($P<0.001$), and decreased claudin-5 expression in HBMEC by 2.43- and 1.32-fold respectively (Fig. 9f). PIC-induced monocyte adhesion and trans-endothelial migration decreased over time, with less adhesion and migration after 48 hours (Fig. 9d, e), compared to 24 hours PIC treatment (Fig. 9a, b). Similarly, 48 hours PIC treatment of HBMEC did not significantly alter claudin-5 expression (Fig. 9f), whereas 24 hours PIC treatment significantly decreased claudin-5 expression (Fig. 9c, $P<0.001$).

The TLR3.CI decreased HIV-1- induced monocytes adhesion (by 2.4 to 4.6-fold, Fig. 9a,d), BBB transmigration (by 3 to 4-fold, Fig. 9b, e) ($P<0.001$), and abrogated HIV-1-induced decrease in claudin-5 expression (Fig. 9c, f). TLR3.CI also decreased PIC-induced monocytes adhesion (by 1.4 to 2.4-fold, Fig. 8a, c), transmigration through the BBB (by 1.6 to 4-fold, Fig. 8b, d) ($P<0.001$), and abrogated PIC-induced downregulation of claudin-5 expression (Fig. 9c, f).

Data in Figs 5, 6, and 8 showed that the JNK pathways mediated HIV-1- and TLR3 ligands-induced inflammation. To determine whether this pathway was also involved in HIV-1- and PIC-induced alterations in BBB properties and function, we assessed the effects of an ATP-competitive JNK inhibitor (420129) on monocytes adhesion, BBB transmigration, and endothelial claudin-5 expression. This JNK inhibitor reduced HIV-1-induced monocytes adhesion (by 2- to 3.3-fold, Fig. 9a, d) and transmigration through the BBB (by 2- to 6-fold, Fig. 9b, e) ($P<0.001$). It significantly reduced HIV-1-induced decrease of claudin-5 expression (by 2.2- to 2.5-fold, Fig. 9c, f). This JNK inhibitor also reduced PIC-induced monocytes adhesion (by 1.7- to 2.5-fold, Fig. 9a, d), transmigration through the BBB (by 1.95- to 2.3-fold, Fig. 9b, e) ($P<0.001$), and also abrogated PIC-induced decrease in claudin-5 expression (Fig. 9c, $P<0.05$). Densitometry analyses of data from two independent experiments using HBMEC from two different donors, confirmed HIV-1 and PIC-induced downregulation of claudin-5 expression, as well as the protective effects of the TLR3.CI and JNK inhibitor (Fig. 9c, f).

Discussion

In HIV-infected individuals, the BBB is exposed to virions and viral products from the systemic circulation, and there is evidence that HIV-1 induced BBB injury enables viral entry into the brain, infection of resident CNS cells and HAND [21–24]. This HIV-1-induced BBB injury is associated with signaling via IL6 and TLRs [25], and the present study shows that primary HBMEC, the major component of the BBB, express TLR3, 4, 5, 7, 9, and 10. We also show that TLR3 was the most abundant TLR expressed in these cells. It has also been shown that the human cerebral endothelial cell line hCMEC/D3 express mRNA for TLR2, 3, 4, and 6 [34] and that primary rat cerebral EC express mRNA for TLR2, 3, and 6 [34]. The fact that TLR2 and 6 are expressed in the hCMEC/D3 cell line and

rat cerebral EC but not in primary HBMEC; and that TLR5, 7, 9, and 10 are expressed in primary HBMEC, but not in hCMEC/D3 or rat cerebral EC, shows heterogeneity in TLRs expression on blood vessels, with differential expression in primary brain EC compared to cell lines, and in EC from animals brain vessels compared to EC from human brain vessels. EC from other vascular beds also express TLRs. Human lung and dermal EC [35], as well as human choroidal and retinal EC [36] express TLR1, 2, 3, 4, 5, 6, and 9 [35, 36].

TLR3 can be activated by viral dsRNA [6], viral ssRNA [7], or by dsRNA generated by RNA polymerases as intermediates during replication of positive-sense ssRNA viruses [12, 37, 38]. HIV-1 is a positive-sense ssRNA virus; in HIV-infected subjects, the BBB is constantly exposed to virus from the peripheral blood, which could also activate TLR3. In the current study, we showed that exposure of HBMEC to HIV-1 or TLR3 ligands increased TLR3 transcription and expression. Significantly, we showed increased transcription and expression of TLR3 in brain tissues from HIV-1-infected humans, with higher TLR3 expression in blood vessels and tight junction strands of HIV+ and HIVE patients. This indicates that HIV-1 CNS infection is associated with increased TLR3 expression in the brain, and that HIV-induced encephalitis further increased TLR3 expression. Our data showing expression of TLR3 in mononuclear phagocytes on blood vessels suggest that increased TLR3 expression in HIV-1 infected subjects was associated with increased infiltration of blood mononuclear phagocytes into the CNS. These data demonstrates the involvement of TLR3 in HIV-1-induced BBB injury and HIVE, and are in agreement with other studies implicating TLR3 in HIV/AIDS pathogenesis. In fact, there is increased TLR3 expression in peripheral blood mononuclear cells of HIV-1 infected patients [39], with the highest TLR3 levels in cells from patients with advanced AIDS and higher viral load [40].

Human studies showed that increased plasma or serum IL6 levels in HIV-infected subjects is associated with higher viremia, faster progression to AIDS, worse clinical outcomes and high mortality [41–43]. We previously showed that HIV-infection and HAND was associated with increased expression of IL6 in human brain tissues and the BBB [25, 26, 28, 29]. Our current study shows that exposure of primary HBMEC to HIV-1 or TLR3 ligands increased IL6 transcription and expression. This involvement of TLR3 in HIV-mediated IL6 production and BBB inflammation was further confirmed by data showing that inhibitors of TLR3/dsRNA complex blocked both HIV-1- and PIC-induced IL6 transcription and expression. There is evidence that TLR3 activation of EC from other vascular beds also induce inflammation in humans. Exposure of human glomerular EC [44], lung EC [35, 45], dermal microvascular EC [35], choroidal and retinal EC [36], and coronary artery and umbilical vein EC [46] to PIC increased the expression of IL6 [35, 36, 44, 45], as well as IL8 and IP10 *in vitro* and *in vivo* [45, 46]; TNF α , IFN β , CCL2, CCL5, and CXCL10 [44].

The IL6 gene has functional cis-regulatory elements that contain binding sites for NF κ B, CEBP, AP-1, STATs, and CEBPs [31–33]. In the current study, we showed that in addition to increasing IL6 transcription and expression, exposure of HBMEC to HIV-1 or PIC significantly upregulated the transcription factors JUN, RELB, CEBPA, CEBPG, and STAT1. TLR3 dimerizes to form a signaling complex [47] and ligands bind to the TLR3 dimer via its N-terminal ectodomain [48]. Ligand binding results in a conformational change and recruitment of Toll/IL-1 receptor domain-containing adaptor inducing IFN- β (TRIF),

which then triggers a signaling cascade that results in expression of INF- β or proinflammatory cytokines [49]. Our data confirm the specificity of TLR3 pathways in PIC- and HIV-1-induced IL6 expression, as we demonstrate that both HIV-1- and PIC-induced IL6 expression and secretion could be blocked by an inhibitor of the TLR3/dsRNA complex (TLR3.CI) that specifically antagonize TLR3 signaling by blocking the binding of dsRNA to TLR3. TLR3.CI also blocked HIV-1- and PIC-induced upregulation of mRNA transcripts for IL6, JUN, RELB, CEBPA, CEBPG, and STAT1. c-Jun dimerizes with c-Fos to form AP-1 [50]; and c-Jun is activated through phosphorylation by the JNK pathways [50, 51]. We show that TLR3.CI also blocked both HIV-1- and PIC-induced phosphorylation of c-jun and JNK. These data further confirmed the involvement of JNK pathways downstream of TLR3 in HIV-1-induced BBB dysfunction.

HIV-1-induced and TLR3-mediated IL6 expression in HBMEC was blocked by 5ZO, an ATP-dependent and competitive inhibitor of TAK1 and the MAP kinases MKK7, MEK1 and ERK2; and was also blocked by 420129, an ATP-dependent and competitive JNK inhibitor. In the MAPK/JNK pathways, stress signal activates small GTPases such as Rac1 and Rho; activated GTPase then stimulate MAPKKK, which phosphorylates MKK4 or MKK7 [52–54]. Following activation, MKK4/7 phosphorylates and activates JNK. Activated JNKs form dimers, translocate to the nucleus, bind to the c-Jun transcriptional domain, and phosphorylate c-Jun at serine or threonine residues [52, 54]. Thus, JNK regulates c-Jun/AP-1 activity and it has been shown that phosphorylation of c-Jun at Ser63 or Ser73 increases the transcription of downstream target genes [50–53]. This was confirmed by our present data showing that exposure of HBMEC to HIV-1 and TLR3 ligands induced Ser63 phosphorylation of c-Jun and Thr183/Tyr185 phosphorylation of JNK, and that this was associated with increased IL6 expression. HIV-1- and PIC-induced IL6 expression was blocked by TLR3.CI and by pharmacological inhibitors of JNK and TAK1/MKK7, all downstream effectors of TLR3 signaling (Fig. 10). The MAP kinases TAK1, MKK7, MEK1, and ERK2 all contain a cysteine residue in their ATP-binding site; suggesting that 5ZO and JNK inhibitors may block IL6 expression by interfering with cysteine residues in the ATP-binding region of these kinases. This would suggest that a region on these kinases ATP-binding site is involved in HIV-1-induced and TLR3-mediated endothelial inflammation and BBB dysfunction. The fact that the ATP-dependent and competitive JNK inhibitor 420129 blocked both PIC- and HIV-1-induced IL6 expression and secretion while the non-ATP-dependent JNK inhibitor 420119 only blocked PIC and had no effect on HIV-1-induced IL6 expression suggests that TLR3-mediated IL6 expression can occur via ATP-dependent or-independent activation of JNK, whereas HIV-1-induced IL6 expression on the brain endothelium occur strictly via ATP-dependent activation of JNK.

Functional studies showed that HIV-1 and PIC decreased claudin-5 expression in HBMEC, significantly increased monocytes adhesion and migration through the BBB, and that the ATP-dependent JNK inhibitor 420129 and TLR3.CI blocked both PIC- and HIV-1-induced monocytes adhesion and trans-endothelial migration, and abrogated PIC- and HIV-1-induced claudin-5 downregulation. This suggests that both HIV-1 and TLR3 ligands use similar TAK1-and JNK-dependent pathways upstream to induce IL6 expression in HBMEC and BBB dysfunction (Fig. 10); and that this occurs through ATP-dependent mechanisms. Inhibitors of IRAK1/4, downstream effectors of TLR signaling, blocked HIV-1-induced IL6

expression, but only partially diminished PIC-induced IL6 expression. Activated JNK phosphorylates c-Jun, which dimerizes with c-Fos to form AP-1 [50, 51]. The inhibitor of c-Jun/JNK complex formation, the inhibitor of AP-1 transcriptional activity, and the inhibitor of NF κ B transcriptional activation had no major effect on HIV-1- or PIC-induced IL6 expression. This suggests that HIV-1-induced and TLR3-mediated IL6 expression & inflammation occurs principally via TAK1 and JNK pathways, not by affecting the c-Jun/JNK complex or AP1 transcriptional activity, nor by affecting NF κ B transcriptional activation. Antibodies to IL6 or IL6 receptors are clinically used for the treatment of inflammatory diseases [55–57]. Our current study provides novel insights into the molecular basis for HIV-1-induced and TLR3-mediated IL6 expression in HBMEC, BBB dysfunction and CNS injury. The data suggests that targeting TLR3, TAK1, and JNK signaling pathways could provide a therapeutic approach for preventing BBB inflammation, dysfunction and CNS injury in HIV/AIDS.

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Abbreviations

TLR	Toll-like receptor
PAMP	Pathogen associated molecular patterns
HIV	Human immunodeficiency virus
BBB	Blood-brain barrier
HBMEC	Human brain microvascular endothelial cells
EC	endothelial cells
CNS	Central nervous system
PIC	Polyinosinic-polycytidylic acid
ssRNA	single stranded ribonucleic acid
dsRNA	double stranded ribonucleic acid
TLR3.CI	TLR3/dsRNA complex inhibitor
GLUT1	Glucose transporter-1
MOI	Multiplicity of infection
kDa	kilo Dalton
bp	base pairs

RT-PCR	reverse transcription polymerase chain reaction
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
cDNA	Complementary DNA
IL6	interleukin-6
ATP	Adenosine triphosphate
CFDA-SE	5-carboxyfluorescein diacetate, succinimidyl ester
CREB/CEBP	cAMP response element binding protein
STAT-1	Signal transducer and activator of transcription 1
5ZO	5Z-7-oxozeaenol. Table 1 and Table 3 include additional list of abbreviations

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Fig. 1. TLR3 mRNA is the most abundant TLR transcript expressed on HBMEC, and HIV-1 and TLR3 ligands increased TLR3 transcription

(a): Semi-quantitative RT-PCR using primers specific for TLRs 1–10 showed mRNA for TLR3, 4, 5, 7, 9, and 10 in HBMEC, and TLR3 were the most abundant. **hTLR PCs**: human TLRs positive controls; RT-PCR was performed using 10 ng TLRs (1–10) cDNA from InvivoGen. (b): Densitometry analyses of RT-PCR data from three independent experiments using HBMEC from three different human donors. (c): Exposure of HBMEC to HIV-1_{ADA} (MOI: 0.001) or PIC for 24 hours increased TLR3 transcription, and densitometry analyses of data from three independent experiments using HBMEC from three different human

donors, confirmed these results (**d**). Additional controls consisted of RT-PCR with GAPDH primers (**a**, **c**). M: molecular weight marker; bp: base pairs; * $P<0.05$, *** $P<0.001$.

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Fig. 2. HIV-1 and TRL3 ligands increased TLR3 expression in HBMEC

(a): Exposure of HBMEC to HIV-1_{ADA} increased TLR3 expression, with higher levels observed from 12 hours post-exposure. (b): Exposure of HBMEC to HIV-1_{ADA} for 12 to 48 hours induced a time-dependent increase in TLR3 expression. (c): Exposure of HBMEC to PIC (for 24 hours) increased TLR3 expression, with higher levels observed with 25 to 50 μg/ml PIC. Additional controls consisted of Western blot analyses with β-actin antibodies; densitometry analyses of data from three independent experiments using HBMEC from three different human donors, confirmed that HIV-1 and PIC increased TLR3 expression (a-c). kDa: kilodalton. * $P < 0.05$, *** $P < 0.001$.

Fig. 3. Increased TLR3 expression in brain tissues and CNS blood vessels of HIV-1-infected humans

(a, b): Quantitative real-time PCR show increased TLR3 mRNA in brain tissues of HIV-1-positive individuals (HIV pos, donors P1 to P9), and infected patients with HAND or HIVE (donors D1 to D10), compared to seronegative controls (HIV Neg, donors N1 to N12).

* $P < 0.05$, *** $P < 0.001$. (c-n): Confocal microscopy of human brain tissues sections stained with monoclonal antibodies to TLR3 (green) and the endothelial glucose transporter-1 (GLUT1) (red). Images with (c, d, f, g, h, j, k, l, n) and without (e, i, m) DAPI staining shows TLR3 expression on blood vessels, including blood vessels tight junction strands

(yellow arrows). For HIV+ and HIVE patients, data also shows TLR3 expression on mononuclear phagocytes ingressing across the blood-brain barrier (orange arrows). Scale bar for all panels: 20 μ M.

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Fig. 4. HIV-1 and TLR3 ligands upregulate IL6 expression in HBMEC

Exposure of HBMEC to PIC [0.5 ng/ml to 5 μg/ml (**a**) or 10 to 75 μg/ml (**b**)] induced IL6 expression and secretion. Experiments in panels **a** and **b** were performed using endothelial cells from two different human donors. Experiments using HBMEC from a third donor (**c**) showed that exposure of HBMEC to PIC (25 to 50 μg/ml) or HIV-1_{ADA} (MOI: 0.001) induced IL6 expression and secretion; and the TLR3/dsRNA complex inhibitor (TLR3.CI) blocked both PIC- and HIV-1-induced IL6 expression (**c**). *** $P < 0.001$.

Fig. 5. HIV-1 and TLR3 ligands induce transcriptional upregulation of IL6 and transcription factors associated with the TAK1 and JNK pathways in HBMEC

Quantitative real-time PCR showed that exposure of HBMEC to PIC (50 μ g/ml) or HIV-1_{ADA} (MOI: 0.001) for 2 to 24 hours induce transcriptional upregulation of IL6 (a), JUN (b), RELB (c), CEBPA (d), CEBPG (e), and STAT1 (f). * P <0.05, ** P <0.01, *** P <0.001 compared to untreated controls. These are representative data from three independent experiments using HBMEC from three different human donors.

Fig. 6. The TLR3/dsRNA complex inhibitor blocked both TLR3 ligands- and HIV-1-induced transcriptional upregulation of IL6 and transcription factors associated with the TAK1 and JNK pathways

Quantitative real-time PCR showed that exposure of HBMEC to PIC (50 μ g/ml) and HIV-1_{ADA} (MOI: 0.001) for 24 hours induce transcriptional upregulation of IL6 (a), JUN (b), RELB (c), CEBPA (d), CEBPG (e), and STAT1 (f). The inhibitor of TLR3/dsRNA complex (TLR3.CI) blocked both PIC- and HIV-1-induced upregulation of IL6 (a), JUN (b), RELB (c), CEBPA (d), CEBPG (e), and STAT1 (f). * P <0.05, ** P <0.01, *** P <0.001. These are representative data from three independent experiments using HBMEC from three different human donors.

Fig. 7. HIV-1 and TLR3 ligands induced the phosphorylation of c-Jun and JNK in HBMEC
(a): Exposure of primary HBMEC to the TLR3 ligand PIC (50 µg/ml) induced the phosphorylation of c-Jun at Ser63, with maximal phosphorylation at 2 hours; increased c-Jun, and increased the phosphorylation of SAPK/JNK at Thr183/Tyr185. **(b):** Exposure of primary HBMEC to HIV-1_{ADA} (MOI: 0.001) induced the phosphorylation of c-Jun at Ser63 and SAPK/JNK at Thr183/Tyr185. **(c):** The inhibitor of TLR3/dsRNA complex (TLR3.CI) blocked PIC- and HIV-1-induced phosphorylation of c-Jun and SAPK/JNK. These are representative data from two independent experiments using HBMEC from two different human donors.

Fig. 8. The JNK and TAK1/MKK7 pathways mediate TLR3 ligands- and HIV-1- induced IL6 expression HBMEC

(a) The JNK inhibitor 420119, the ATP-competitive JNK inhibitor 420129, and the TAK1/MKK7 inhibitor 5ZO blocked PIC induced IL6 expression and secretion. (b): PIC induced IL6 expression was reduced by the inhibitor of AP-1 transcriptional activity SR11302, whereas the inhibitor of NFκB transcriptional activation 481406, the IRAK1/4 inhibitor, and the inhibitor of c-Jun/JNK complex 420130 had minimal to no effect. (c): The ATP-competitive JNK inhibitor 420129, the IRAK1/4 inhibitor, and the TAK1/MKK7 inhibitor 5ZO blocked HIV-1-induced IL6 expression and secretion; whereas the JNK inhibitor

420119, the inhibitor of c-Jun/JNK complex 420130, the inhibitor of $\text{NF}\kappa\text{B}$ transcriptional activation 481406, and the inhibitor of AP-1 transcriptional activity SR11302, had minimal to no effect. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

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Fig. 9. Effects of HIV-1 and TLR3 ligands on the blood-brain barrier properties and function
Exposure of HBMEC to HIV-1_{ADA} (MOI: 0.001) for 24 hours (**a, b, c**) or 48 hours (**d, e, f**) increased monocytes adhesion to HBMEC by 4 to 5.86-fold (**a, d**), increased monocytes transmigration through the BBB by 4 to 14-fold (**b, e**), and decreased claudin-5 expression by 2.43 to 3.77-fold (**c, f**). Exposure of HBMEC to PIC (50 µg/ml) for 24 hours (**a, b, c**) or 48 hours (**d, e, f**) increased monocytes adhesion to HBMEC by 1.83 to 3.56-fold (**a, d**), increased monocytes transmigration through the BBB by 3.45 to 3.78-fold (**b, e**), and decreased claudin-5 expression by 1.32 to 2.85-fold (**c, f**). The TLR3/dsRNA complex inhibitor (TLR3.CI) and the ATP-competitive JNK inhibitor (420129) significantly reduced HIV-1- and PIC-induced monocytes adhesion (**a, d**), transendothelial migration (**b, e**), and claudin-5 downregulation (**c, f**). **** $P < 0.001$. Controls consisted of untreated HBMEC, and HBMEC exposed only to TLR3.CI or the JNK inhibitor. These controls showed no alteration in BBB function. These are representative data from two independent experiments using HBMEC from two or three different human donors.

Fig. 10. Model illustrating the signaling pathways in HIV-1-induced and TLR3-mediated IL6 expression and BBB dysfunction

Arrows with solid blue lines indicate activation and direct signaling of HIV-1. Dotted blue lines indicated potential/alternative pathways. Arrows with solid black lines indicate activation and direct signaling of TLR3 ligands. The $\perp$ symbol indicates pharmacological inhibitors. Grey color rectangular boxes indicate compounds that inhibited both HIV-1- and TLR3 ligands-mediated effects. The blue rectangular boxes indicate compounds that inhibited HIV-1-mediated IL6 expression, but had minimal effects PIC-mediated effects. The brown color rectangular boxes indicate compounds that blocked or significantly diminished TLR3 ligands-induced IL6 expression but had no effects on HIV-1-mediated effects. The

black color boxes indicate compounds that had no effect on HIV-1 or TLR3 ligands-mediated effects. ↑ indicates an increase.

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Table 1

Clinical history of brain tissues donors

HIV-1 Status	ID	Gender/ Age (y)	PMI (h)	Neurocognition/ Neuropathology	Other autopsy diagnosis
Neg	N1	M/35	8.5	Normal/None	Mild Alzheimer gliosis
Neg	N2	N/A	N/A	Normal/None	N/A
Neg	N3	F/38	5.75	Normal/Not significant	Mild gliosis, lung and bile duct carcinoma
Neg	N4	M/32	4.25	Normal/Not significant	Cystic fibrosis, and multiorgan failure
Neg	N5	F/46	4	Normal/Not significant	Mild fibrosis, mild patchy gliosis
Neg	N6	F/49	4.5	Normal/Not significant	Hepatic cirrhosis, liver failure
Neg	N7	M/52	5.25	Normal/None	Hypertension, renal failure
Neg	N8	M/72	3	Normal/None	Hypertension, COPD
Neg	N9	M/64	3.3	Normal/None	Hepatic carcinoma
Neg	N10	M/56	3	Normal/Not significant	Lung adenocarcinoma, mild nonspecific cortical atrophy
Neg	N11	M/67	3.5	Normal/None	COPD, TB
Neg	N12	M/61	5.75	Normal/Not significant	Cardiomyopathy, mild gliosis
Pos	P1	?/46	2.75	Normal/None	N/A
Pos	P2	?/27	8	Normal/None	N/A
Pos	P3	?/37	5	Normal/None	N/A
Pos	P4	M/39	11	Normal/None	Minimal non-diagnostic abnormalities
Pos	P5	M/35	6.5	Normal/None	Non-Hodgkins lymphoma, AIDS
Pos	P6	M/54	6.5	Normal/None	AIDS
Pos	P7	M/48	15	Normal/None	Liver disease
Pos	P8	M/52	8	Normal/None	AIDS
Pos	P9	M/38	14	Normal	Pneumonia, hemophilia A, AIDS
Pos	HAND1/D1	M/30	6	HAND/HIVE	Minimal terminal anoxia
Pos	HAND2/D2	?/50	21	HAND/HIVE	N/A
Pos	HAND3/D3	?/39	12	HAND/HIVE	N/A
Pos	HAND4/D4	?/40	12	HAND/HIVE	N/A
Pos	HAND5/D5	M/47	11	HAND/HIVE	Encephalopathy, microglial nodule encephalitis, meningoencephalitis with microvascular damage

HIV-1 Status	ID	Gender/ Age (y)	PMI (h)	Neurocognition/ Neuropathology	Other autopsy diagnosis
Pos	HAND6/D6	M/40	5	HAND/HIVE	Non-Hodgkin's lymphoma, AIDS, lymphocytic meningitis
Pos	HAND7/D7	M/44	4	HAND/HIVE	Microglial nodules, astrogliosis, widespread gliosis, AIDS
Pos	HAND8/D8	M/52	5	HAND/HIVE	Atherosclerosis
Pos	HAND9/D9	M/34	11.5	HAND/HIVE	AIDS
Pos	HAND10/D10	M/38	7	HAND/HIVE	HIV encephalopathy / Leukoencephalopathy, AIDS

Neg indicates HIV seronegative; Pos, HIV seropositive; HAND, HIV-associated neurocognitive disorders; HIVE, HIV encephalitis; y, years; M, male; F, female; ? or N/A, not available; and PMI, postmortem interval; AIDS, acquired immunodeficiency syndrome; COPD, chronic obstructive pulmonary disease; TB, tuberculosis.

Table 2

TLR primers

Gene name	Invivogen catalog#	Amplicon size (bp)
Human TLR1	rtp-htlr1	706
Human TLR2	rtp-htlr2	657
Human TLR3	rtp-htlr3	602
Human TLR4	rtp-htlr4	550
Human TLR5	rtp-htlr5	493
Human TLR6	rtp-htlr6	435
Human TLR7	rtp-htlr7	387
Human TLR8	rtp-htlr8	335
Human TLR9	rtp-htlr9	312
Human TLR10	rtp-htlr10	214

Table 3

Description of pharmacological inhibitors used in the study

Inhibitor name	Catalog# / Abbreviation	Mechanism of action	Manufacturer
TLR3/dsRNA Complex Inhibitor	TLR3.CI	Acts as a direct, competitive and high affinity inhibitor of dsRNA binding to TLR3. Selectively antagonizes TLR3 signaling without affecting other TLRs.	Calbiochem- EMD Millipore
NFκB Activation Inhibitor	481406	Inhibitor of NFκB transcriptional activation	Calbiochem- EMD Millipore
SR 11302	SR11302	Inhibitor of activator protein-1 (AP-1) transcriptional activity	Tocris Biosciences
JNK Inhibitor II	420119	Selective and reversible inhibitor of <i>c</i> -Jun N-terminal kinase (JNK1, JNK2, and JNK3).	Calbiochem- EMD Millipore
JNK Inhibitor V	420129	Reversible and ATP-competitive inhibitor of <i>c</i> -Jun N-terminal kinase (JNK1, JNK2, and JNK3).	Calbiochem- EMD Millipore
JNK Inhibitor III	420130	Specifically disrupt <i>c</i> -Jun/JNK complex formation and the subsequent phosphorylation and activation of <i>c</i> -Jun by JNK	Calbiochem- EMD Millipore
IRAK-1/4 inhibitor	IRAK-1/4	Inhibitor of interleukin-1 receptor-associated kinases (IRAK)-1 and -4	Sigma-Aldrich
5Z-7-Oxozeaenol	5ZO	ATP-competitive irreversible inhibitor of TAK1 (MEKK7), MKK7, MEK1, and ERK2. Has no activity against other MAPK	Sigma-Aldrich

Abbreviations: TLR3: Toll-like receptor-3; JNK: *c*-Jun N-terminal kinase; dsRNA: double-stranded RNA; AP-1: activator protein-1; IRAK: Interleukin-1 Receptor-Associated Kinase; NFκB: nuclear factor kappaB; TAK1: Transforming growth factor- β activated kinase-1; MEKK7: MAP Kinase Kinase Kinase-7; MKK7: MAP Kinase Kinase-7; MEK1: MAP Kinase Kinase-1; ERK2: extracellular-signal-regulated kinase-2 / MAP Kinase-1