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Microbial molecule ingress promotes neuroinflammation and brain CCR5 expression in persons with HIV-associated neurocognitive disorders

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ABSTRACT

Background: Systemic inflammation accompanies HIV-1 infection, resulting in microbial translocation from different tissues. We investigated interactions between lentivirus infections, neuroinflammation and microbial molecule presence in the brain.

Methods: Brain tissues from adult humans with (n = 22) and without HIV-1 (n = 11) infection as well as adult nonhuman primates (NHPs) with (n = 11) and without (n = 4) SIVmac251 infection were investigated by RT-PCR/ddPCR, immunofluorescence and western blotting. Studies of viral infectivity, host immune gene expression and viability were performed in primary human neural cells.

Findings: Among NHPs, SIV DNA quantitation in brain showed increased levels among animals with SIV encephalitis (n = 5) that was associated with bacterial genomic copy number as well as CCR5 and CASP1 expression in brain. Microbial DnaK and peptidoglycan were immunodetected in brains from uninfected and SIV-infected animals, chiefly in glial cells. Human microglia infected by HIV-1 showed increased p24 production after exposure to peptidoglycan that was associated CCR5 induction. HIV-1 Vpr application to human neurons followed by peptidoglycan exposure resulted in reduced mitochondrial function and diminished beta-III tubulin expression. In human brains, bacterial genome copies (250–550 copies/gm of tissue), were correlated with increased bacterial rRNA and GroEL transcript levels in patients with HIV-associated neurocognitive disorders (HAND). Glial cells displayed microbial GroEL and peptidoglycan immunoreactivity accompanied by CCR5 induction in brains from patients with HAND.

Interpretation: Increased microbial genomes and proteins were evident in brain tissues from lentivirus-infected humans and animals and associated with neurological disease. Microbial molecule translocation into the brain might exacerbate neuroinflammatory disease severity and represent a driver of lentivirus-associated brain disease.

1. Introduction

Neurological disease is common during immunodeficiency-causing lentivirus (e.g., HIV, SIV, FIV) infections, apparent as damage to both the central and peripheral nervous systems and affecting over 25 % of persons with HIV-1 infection, including those receiving antiretroviral therapy (ART) (Saylor et al., 2016; Patrick et al., 2002). While the precise mechanisms underlying lentivirus-associated neurological disease remain uncertain, perturbation of the blood–brain barrier with neuroimmune activation, particularly induction of innate immune pathways, accompanied by neuronal damage underpin brain disease caused by lentivirus infections (Mallard and Williams, 2018; Power, 2018). Immune dysregulation during HIV-1 infection precedes development of AIDS, which is especially evident in gut-associated lymphoid...
tissue where mucosal CD4+ T-cells are depleted in the early acute phase of the infection (Guadalupe et al., 2003), with accompanying aberrant activation of CD8+ T-cells and B cells (Nilsson et al., 2007). The development of HIV-associated neurocognitive disorders (HAND) remains an ongoing challenge affecting 20–30 % of persons during HIV-1 infection despite receiving ART (Tu et al., 2020); (Sacktor et al., 2016). HIV-1 pathogenesis is driven by both the direct effects of the virus on infected cells together with chronic immune activation of uninfected proximal cells, coupled with injury or death of uninfected bystander cells, including neurons. A key component of this immune dysfunction is reduced barrier functions at various body sites including the gut, oral cavity, urogenital tract (Douek, 2007) that can result in release of microbial molecules into the circulation (Schröder and Backhed, 2016; Huang et al., 2019). Concurrent with immune dysregulation in the gut and other organs during lentivirus infections is an accompanying increase in bacterial translocation from mucosal tissues into circulation and transport to other sites in the body (Marchetti et al., 2008; Ancuta et al., 2008). Moreover, enhanced translocation by pathogenic, opportunistic or innocuous bacteria and their products into the blood amplify both local and systemic inflammation (Mourits et al., 2020). Whether the translocated bacteria successfully colonize other body sites resulting in a clinical infection, achieve subclinical colonization (Zhou et al., 2018) or are cleared by the immune system, their presence nonetheless represents a source of microbe-associated molecular patterns (MAMPs) that drive chronic inflammation (Brenchley et al., 2006). A collateral aspect of systemic inflammation resulting from microbiota egress into the circulation is modulation of the blood–brain barrier’s permeability (Braniste et al., 2014). Indeed, it is increasingly apparent that dispersal of the gut microbiota and microbial metabolites in the circulation can alter neurological development, function (as reviewed in (Vuong et al., 2017) as well as disease severity (Brown, 2019; Zhan et al., 2021; Hakopian et al., 2021).

Previous reports have described the presence of bacterial molecules including nucleic acids (e.g., DNA), glycoproteins (e.g., peptidoglycan) (Krüger et al., 1982; Schrijver et al., 2001; Gabanyi et al., 2022) and glycolipids (e.g., endotoxin) in the human brain including patients with HIV-1 (Branton et al., 2013), multiple sclerosis (Branton et al., 2016), and other neurological diseases such as Alzheimer’s Disease (Dominy et al., 2019) as well as non-human primates (NHPs) with and without HIV infection (Branton et al., 2013). The low quantities of bacterial nucleic acids in brain tissue, low ratio of bacterial ribosomal RNA to DNA, in addition to observed association of peptidoglycan (PGN) presence within phagocytic cells, implies that these bacterial molecules might not be produced by replicating or metabolically active bacteria. Instead, they could be derived from inert or partially degraded bacteria that have been trafficked in infiltrating phagocytes or directly translocated from the circulation, as recently reported (Gabanyi et al., 2022). In fact, bacterial antigens can be concentrated at the sites of active neuroinflammation, as reported for multiple sclerosis, enabled by proximal blood–brain barrier disruption (Branton et al., 2016). Given microbial molecules exert proinflammatory actions through defined pattern recognition receptors (PRRs), and can be detected in the brain, we hypothesized that bacterium-derived molecules might contribute to neuroinflammation and the development of neurological disease during lentivirus infections.

Using three experimental platforms, we investigated this hypothesis and showed that bacterial molecule abundance and actions promoted activation of innate immune pathways in the brain that were associated with neurological disease during lentivirus infections. SIV infection of NHPs represents the model that most closely recapitulates HIV-1 disease in humans. As in humans infected by HIV-1, SIV rapidly disseminates throughout the body, independent of route of infection, to infect tissue resident CCR5-immunopositive cells including microglia in the brain. In previous studies we demonstrated that in SIV-infected macaques lacking overt neurological disease there was a profile of bacterial phyla, similar to ART naïve HIV specimens (Branton et al., 2013). Herein, we extended these findings by examining a cohort of animals in which a subset displayed enhanced cerebrospinal fluid SIV load, associated neurobehavioral deficits and/or neuropathological features of lentivirus infection. These models disclosed a link between bacterial MAMP load and lentiviral neuropathology that was further examined in established primary human neural cell culture models of HIV neuropathogenesis (Doan et al., 2021; Jones et al., 2007; Rosales et al., 2015) and validated in a human clinical cohort.

2. Methods

2.1. Ethics statement

The use of autopsied human brain tissues was approved (Pro0002291) by the University of Alberta Human Research Ethics Board (Biomedical) and written informed consent was received for all samples. In addition, brain tissue from patients was obtained from the National NeuroAIDS Tissue Consortium (NNTC) collection, which undergoes oversight from institutional review boards at four clinic sites in the USA and contains assigned neurological diagnoses based on pre-mortem clinical, laboratory and neuropsychological assessments. Human fetal brain tissues were obtained from 15 to 22-week aborted fetuses that were collected with the written informed consent from the donor (Pro00027660), approved by the University of Alberta Human Research Ethics Board (Biomedical). Adult Indian rhesus macaques (Macaca mulatta) were housed at BPRC in accordance with the rules and regulations of the Netherlands Animal Care guidelines (Mothapo et al., 2017; Koopman et al., 2013), as approved by the Institutional Animals Care and Use Committee (DEC-BPRC) under protocol number DEC#635.

2.2. Animal housing and care

Animals were fed standard monkey chow diet, supplemented daily. Social enrichment was delivered and overseen by veterinary staff, and overall animal health was monitored daily. Animals were evaluated clinically. In the situation that an animal displayed persistent diarrhoea and showed more than a 10 % loss in body weight in combination with abnormal haematological parameters or if an animal was deemed to be in distress, it was immediately and humanely euthanized as per established institutional guidelines.

2.3. Animal infection and sample collection

Animals (n = 15) that were seronegative for SIV, simian T leukemia virus type 1 (STLV-1), simian type D retrovirus 1 (SRV-1), and herpes B viruses were infected intravenously with 10 MLD50 of pathogenic cell-free SIVmac251 stock (107 TCID50) SIVmac251 (Mothapo et al., 2017; Koopman et al., 2013). SIVmac251 was kindly provided by Dr C.J. Miller (University of California, Davis, USA). Animals were sacrificed at different time points post-infection. Necropsy including harvest of plasma, cerebrospinal fluid and brain was performed immediately after sacrifice. Animals were categorized into Neuro-SIV+ or non-Neuro-SIV+ groups based on having displayed one or more signs of neurological dysfunction (e.g., altered behaviors, wasting and movement abnormalities), the presence or absence of neuropathological lesions, coupled with a CSF viral load of greater than 1 million copies per milliliter.

2.4. RNA and DNA preparation

Total RNA and DNA were extracted from brains of HIV-infected and uninfected patients (midfrontal gyrus) and SIV-infected animals (parietal cortex, striatum and cerebellum), as previously described, using the RNeasy, DNeasy or All-prep kits (Qiagen Germantown MD, USA) according to manufacturer’s protocol (Mohammadzadeh et al., 2021).
2.5. Quantitative real time RT-PCR

cDNA was synthesized using DNase-treated RNA, random primers (Roche) and SuperScript II, reverse transcriptase (Invitrogen, Carlsbad, CA, USA) at 44 °C for 90 min according to the manufacturer’s instructions. cDNA was diluted 1:3 (adding 100 μl of ultrapure water to 50 μl of cDNA). Expression of human and macaque immune genes was normalized to GAPDH and quantified relative to the control group using verified primers (Table S1) as previously described (Mohammadzadeh et al., 2021).

2.6. Protein extraction and western blotting

Total protein was extracted from 20 mg of brain tissue (midfrontal gyrus) of uninfected individuals, HIV-infected patients without neurological disease and HAND patients by lysis in 20 volumes of RIPA buffer (AbCam, ab156034) with protease inhibitor cocktail (Millipore-Sigma, 539134) using a FastPrep24 (MPBiomedicals) homogenizer. The lysate was run on 4–20 % acrylamide gels (Biorad), transferred to nitrocellulose membranes and incubated with anti-CCR5 antibody (1/1000, Table S2) overnight at 4 °C, washed 3X, GHRP conjugated secondary antibodies were applied for 1 h, washed 3X with PBS-T and developed using pierce ECL-plus (ThermoFisher) and imaged (Cy2 channel GE ImageQuant 4000)) and normalized to β-actin (Table S2) intensity.

2.7. Droplet digital PCR analyses of viral RNA and total and integrated DNA

HIV-1 and SIV RNA were quantified using 5.0 μl of diluted cDNA as template. For the quantification of bacterial GroEL and total viral DNA, 300 ng of genomic DNA (gDNA) was used. For both HIV-1 RNA and total DNA quantification, HIV-1 pol-F and HIV-1 pol-R and for both SIV RNA and total DNA quantification, SIV-F and SIV-R primers were used (Table S1). The Bio-Rad QX200 droplet digital PCR system and QX200 ddPCR EvaGreen supermix were used for quantification as per manufacturer’s protocol, as previously described (Ferrucci et al., 2011). Droplets were generated from each reaction with a final volume of 20.0 μl using Bio-Rad QX200 Droplet Generator. PCR was performed using an S1000 thermal cycler (Bio-Rad). Using the following program: 10 min at 98 °C, 30 sec denaturation at 94 °C, 58 °C extension for 60 sec, and 10 min at 98 °C for a total of 40 cycles. After the cycling, droplets were analyzed using QX200 Droplet Reader. Raw data were analyzed with the QuantaSoft Analysis Pro software (QX200) by setting a common fixed fluorescence threshold intensity based on the non-template control. The number of template copies was then calculated based on the number of positive droplets detected in a corresponding channel and the number of total accepted droplets. Samples were quantified at least in duplicate. Template copies per sample were calculated averaging the overall replicate wells per sample. The values are reported as copies per gram of tissue with approximately 30 mg of tissue used for initial RNA and DNA extraction (Mohammadzadeh et al., 2021).

2.8. Cell cultures

Primary human fetal microglia (HFM) were infected and allowed to replicate in the presence or absence of PGN (Sigma, SMB00288) (Doan et al., 2021). Primary HFM were isolated from fetal brain tissues with a gestational age of 16 to 21 weeks, as previously described. The cells were washed twice and plated in vented Cellvivo T-75 tissue culture flasks (Sarstedt) and cultures were incubated at 5 % CO2 for two weeks. Cultures were maintained in MEM supplemented with 10 % FBS, 2 mM l-glutamine, 1 mM sodium pyruvate, 1 × MEM nonessential amino acids, 0.1 % dextrose, 100 U/ml penicillin, and 100 μg/ml streptomycin. After 2 weeks, cultures were gently rocked for 20 min to suspend the weakly adhering microglia in medium, which were then decanted, washed and plated. Purity of cultures was verified by immunofluorescence.

2.9. Production of viral stock

HIV-1 was produced by co-transfection of HIV-1NL4-3-ADA-Env and a VSV-G encoding plasmid into 8 × 10^6 293 T cells cultured in DMEM with 10 % FBS in a 10 cm tissue culture dish (ThermoFisher). Lipofectamine 3000 (Invitrogen, Carlsbad, CA) was used as transfection reagent according to manufacturer’s protocol (Mohammadzadeh et al., 2021). Six hours post transfection, the media was changed, and at 72 h post-transfection virus was harvested, cleared by centrifugation at 500 × g for 10 min, and then frozen at –80 °C. HIV-1 p24 was measured by ELISA and virus was titered using TZM-bl reporter cell line and the Reed and Muench method to determine TCID_{50} (Mohammadzadeh et al., 2021).

2.10. Effect of PGN on HIV infection of human fetal microglia (HFM)

HFM from three independent donors were used in three independent experiments. Cells were plated in parallel on 96 well tissue culture plates and 8 well chambered cover slips (Ibidi). A minimum of three technical replicates per assay per condition were used for each experiment. HIV infections were performed at an MOI of 0.1. Cells were washed 6 h after HIV exposure. Fresh culture media containing peptidoglycan (PGN) at a concentration of 100 μg/ml was added to the cells either 16 h prior to infection (PGN pre HIV) or for 16 h after infected cells were washed to remove unbound virus (PGN post HIV). All mock treatments were subjected to media change at the relevant time points. On Day 3 post-infection, supernatants were collected from all wells for HIV-1 p24 quantification by ELISA (ABL Inc.). Cells from the 96 well plates for RT-PCR and supernatants were collected and cells on coverslips were fixed for immunofluorescence imaging using antibodies described in Table S2.

2.11. Effect of PGN on Vpr toxicity

SK-N-SH human neuroblastoma cells (ATCC) were plated in 6 and 96 well plates, differentiated using dibutylryl cAMP (1 mM, Sigma) for 3 days and then exposed to recombinant baculavirus-derived Vpr (100 nM, x4h) (subtoxic dose) (Kinnakeet Biotechnology) with or without PGN (100 μg/ml) pre- or post-Vpr exposure (16 h) (Jones et al., 2007) with three technical replicates per condition in each format. Cell viability was measured in the 96 well plates using the Presto blue assay (Invitrogen, New York, USA) according to manufacturer’s instructions. The principle of the assay depends on viable cells maintaining a reducing environment within the cytosol of the cell. Presto blue contains resazurin, a non-toxic, cell permeable compound that is blue in color and virtually non-fluorescent in its oxidized state but is reduced to a compound that is red in color and highly fluorescent upon entering cells. Absorbance was measured on a Biotek Synergy HT plate reader at 570 nm. Background absorbance values at 600 nm were subtracted from the absorbance values at 570 nm. Following exposure, the cells cultured in 6 well plates were lysed, and the lysate pooled and run on 4–20 % acrylamide gels (Biorad), transferred to nitrocellulose membranes and exposed to primary an anti-beta-III-tubulin (Sigma-Aldrich), anti-DnA K (abcam, ab69617) or anti-GroEL (abcam, ab90522). overnight at 4 °C, washed 3X, CHRP conjugated secondary antibodies were applied for 1 h, washed 3X with PBS-T and developed using pierce ECL-plus (ThermoFisher) and imaged (Cy2 channel GE ImageQuant 4000). Beta-III-tubulin band intensity was quantified using Image studio Lite (LiCor) and normalized to β-actin (Santa Cruz, sc-47778) intensity. Each experiment was compared to the results using VPR (200 nM) (toxic dose). Each experiment was performed independently on three occasions.

2.12. Immunofluorescence imaging

Fixed tissues from uninfected [–] and HIV or SIV-infected brains were paraffin-embedded followed by sectioning and mounting (Doan et al., 2021).
et al., 2021). Slides were rehydrated and subjected to antigen retrieval by boiling in 10 mM sodium citrate, pH 6 before immunostaining. Tissues were labeled with primary antibodies described in Table S2 overnight at 4 °C. Autofluorescence was quenched using TrueBlack® Lipofuscin Autofluorescence Quencher (Biotium), followed by application of appropriate secondary antibodies (Doan et al., 2021). Images were acquired on a Wave Fx (Quorum technologies) spinning disk confocal microscope using Velocity (Perkin Elmer) acquisition and analysis software. All immunostaining was performed using at least two animals per group. Blinded assessment of frequency of immunolabeled proteins was performed on each slide.

2.13. Statistical analyses

One-way ANOVA and Tukey’s multiple comparison test were used for the analysis of brain viral load and neuroimmune responses. Statistical tests were applied using GraphPad Prism version 5 (GraphPad Software, San Diego California USA, www.graphpad.com).

3. Results

3.1. Neuroinflammation in SIV infection

Among uninfected (SIV−, n = 4) and SIVmac251-infected (n = 11) NHPs, a subset of SIV-infected animals (Neuro-SIV+[+], n = 5) displayed lower CD4+ T-cell levels in blood, higher virus levels in plasma and CSF with neurological signs (n = 3) and/or focal neuropathological lesions (n = 3), compared to individuals lacking neurological signs or neuropathology (NN-SIV+[+], n = 6) (Table 1). Viral quantitation in brain (frontal lobe) disclosed absent viral total DNA (Fig. 1A) and RNA (Fig. 1B) in SIV− animals while all Neuro-SIV+[+] animals showed detectable viral DNA and RNA in contrast to the NN-SIV+[+] group in which a subset displayed viral RNA and DNA in brain. Although CD3ε transcript levels, as an indicator of T-cell presence, were detectable in all NHPs although bacterial genome copy numbers per gram (tissue) were significantly increased in the Neuro-SIV+[+] group (p = 0.01) (Fig. 1B). To investigate if bacterial (glyco)proteins were also detectable in brains of NHPs, immunofluorescence was performed on frontal lobe sections revealing that bacterial DnA was observed in brains from each group (Fig. 2 vii-ix). DnA immunoreactivity was most evident in the Neuro-SIV+[+] group (Fig. 2Cix) and was associated with both astrocytes (GFAP) and microglia (iba-1) (Fig. 2Cxxii, arrow). Likewise, the bacterial cell wall glycoprotein, peptidoglycan, was also detected in all groups (Fig. 2C vii-ix, insets), albeit more apparent in the Neuro-SIV+[+] group, chiefly associated with Iba-1 immunopositive microglia.

### Table 1

<table>
<thead>
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<th>SIV− (n = 4)</th>
<th>NN-SIV+[+] (n = 6)</th>
<th>Neuro-SIV+[+] (n = 5)</th>
</tr>
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<tbody>
<tr>
<td>Sex (M:F)</td>
<td>1:3</td>
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| Duration of infection (mean months ± SD) | 7.25 ± 0.61 | 5.95 ± 0.87
| Brain lesions  | absent      | 1/6                | 3/5                  |
| Neurological signs | 2/6         | 3/5                |                      |
| Plasma RNA viral copies (mean log copies/ml ± SD) | 6.18 ± 4.50 | 7.42 ± 5.48
| CSF RNA viral copies (mean log copies/ml ± SD) | 4.68 ± 2.98 | 8.50 ± 6.60
| CD4+ T-cells (mean blood cells/µl ± SD) | 413 ± 3.3 | 234 ± 67

1 SIV-infected animals were assigned to the NN-SIV+[+] or Neuro-SIV+[+] groups based on the presence or absence of neurological signs, brain lesions, and CSF viral RNA LOAD (less or greater than 10⁶ copies/ml).  
2 N/A – not applicable.  
3 Mann-Whitney U test, *p < 0.05.

Given that viral RNA and DNA as well the macrophage-specific gene, CD68, were increased in a subset of SIV-infected animals, we verified these latter findings by showing that burden of Iba-1 immunoreactivity on microglia and trafficking macrophages was increased in frontal lobes of the Neuro-SIV+[+] group (Fig. 1Eii) compared to the SIV−[−] (Fig. 1Ei) and NN-SIV+[+] (Fig. 1Eii) groups. Similarly, SIV p27 immunoreactivity was most apparent in the Neuro-SIV+[+] (Fig. 1Evi) relative to the SIV−[−] (Fig. 1Eiv) and NN-SIV+[+] (Fig. 1Ev) groups, which was complemented by increased intensity of CCR5 immunoreactivity, co-localized with Iba-1, particularly in the Neuro-SIV+[+] group (Fig. 1Fiii). Thus, the morphological features in these samples recapitulated the molecular findings of increased virus and myeloid cell activation in the Neuro-SIV+[+] group.

3.2. Detection of microbial molecules in macaque brains

Earlier studies indicated that bacterial molecules including RNA, DNA, glycolipids, and glycoproteins were detectable in brains from humans, NHPs, and other species (Rosales et al., 2015). To determine if bacterial molecule burden was associated with the occurrence of neurological disease, we quantified bacterial nucleic acid levels and immunostained for conserved bacterium-encoded proteins. Bacterial 16s rRNA V3-V4 was detected in frontal lobes from all NHPs examined herein, showing a trend towards higher RNA burden in the Neuro-SIV+[+] group (Fig. 2A). The single copy bacterial chaperone gene GroEL was also detected in the brains of all NHPs although bacterial genome copy numbers per gram (tissue) were significantly increased in the Neuro-SIV+[+] group (p = 0.01) (Fig. 2B). To investigate if bacterial (glyco)proteins were also detectable in brains of NHPs, immunofluorescence was performed on frontal lobe sections revealing that bacterial DnA was observed in brains from each group (Fig. 2C vii-ix). DnA immunoreactivity was most evident in the Neuro-SIV+[+] group (Fig. 2Cix) and was associated with both astrocytes (GFAP) and microglia (iba-1) (Fig. 2Cxxii, arrow). Likewise, the bacterial cell wall glycoprotein, peptidoglycan, was also detected in all groups (Fig. 2C vii-ix, insets), albeit more apparent in the Neuro-SIV+[+] group, chiefly associated with Iba-1 immunopositive microglia.

3.3. Peptidoglycan expression drives HIV-1 replication in microglia

As the presence of bacterial antigens in brain tissues was associated with Iba-1+ cells, and increased bacterial nucleic acid load correlated with increased viral RNA and DNA quantities and CCR5 expression, we examined the direct impact of PGN exposure on HIV-1 production and CCR5 expression in primary human microglia. Microglia from different donors (n = 3) were infected with HIV-1NL4-3-ADE-Env [+] pseudotyped with a VSV-G encoding plasmid, together with PGN exposure for 16 h pre-infection or 16 h post-infection. Cells were subsequently stained with DAPI and co-immunolabeled for HIV-1 p24 and human CCR5 (Fig. 3A) or harvested for RNA preparation at 72 h post-HIV-1 infection, while culture supernatants were also collected in which HIV-1 p24 was measured. CCR5 immunolabeling of microglia was evident in all experimental conditions (Fig. 3i, iv, vii, x, xiii) while HIV-1 p24 immunoreactivity was detected only in HIV-infected cultures (Fig. 3ii, v, viii, xi, xiv). CCR5 transcript levels were significantly increased in the HIV-infected cultures with post-infection PGN exposure (p = 0.04) (Fig. 3B). CCR5 immunoreactivity was also significantly increased in cultures infected by HIV-1 and subjected to PGN exposure (pre-infection exposure p = 0.0073, post-infection exposure p = 0.0001) relative to uninfected HIV infected cells (Fig. 3C). Intracellular HIV-1 p24 immunoreactivity was apparent in HIV-infected microglia but pre- or post-infection PGN exposure increased HIV p24 intracellular immunoreactivity (Fig. 3D). Frequency of DAPI-stained nuclei did not differ between treatments (Supplementary Fig. 3). Analyses of HIV-1 p24 released in supernatants disclosed detectable p24 in all infected cultures
but it was significantly increased in supernatants from HIV-infected cultures with pre-infection PGN exposure (Fig. 3E). These data implied that PGN exposure promoted HIV-1 production.

3.4. Peptidoglycan promotes HIV-1 neurotoxicity

Considering the association between bacterial burden and severity of neurological disease in the NHP cohort, we examined the effects of PGN in a model of HIV-1 neurotoxicity mediated by the HIV-encoded protein, Vpr (Jones et al., 2007). Using a cultured human neuronal cell line (SK-N-SH) in the absence or presence of Vpr exposure (150 nM) (Fig. 4A), cellular viability was examined in conjunction with PGN application before or after Vpr exposure. Cell mitochondrial integrity was measured using a resazurin-based colorimetric assay (PrestoBlue) in which healthy mitochondria reduce resazurin (Zhang et al., 2004), resulting in media color change that is then measured by absorbance (Fig. 4B) following exposure of cells to PGN in the setting of pre- or post-Vpr application to cultures. These studies revealed that Vpr-exposed cells showed decreased absorbance, indicative of mitochondrial injury. While PGN alone exerted no effects on mitochondrial function, injury was statistically significantly worsened by application of PGN following Vpr exposure (p < 0.001). To examine cellular viability in the same model system, beta-III-tubulin immunoreactivity, indicative of neuronal structural integrity, was assessed by western blotting. These studies showed that Vpr application followed by PGN exposure markedly reduced beta-III-tubulin immunoreactivity, indicative of neuronal structural integrity, was assessed by western blotting. These studies showed that Vpr application followed by PGN exposure markedly reduced beta-III-tubulin immunoreactivity although matched beta-actin expression was preserved (Fig. 4C). Quantitation of western blots showing the ratio of beta-III-tubulin to beta-actin immunoreactivity (Fig. 4D) revealed that this effect was significant (p < 0.05) and recapitulated the findings from the resazurin staining studies (Fig. 4B). These observations suggested that while PGN had no effects per se on neuronal viability, in concert with Vpr, it amplified the neurotoxic actions of this HIV-encoded protein.
3.5. Viral load and neuroinflammation in human brain during HIV-1 infection

Frontal cortex from brains of autopsied uninfected (HIV[−], n = 10), HIV-infected with no neurological disease (NN-HIV[+], n = 10) or with HIV-associated neurocognitive disorders (HAND, n = 12) (Table 2) were investigated in terms of viral load, host neuroimmune responses as well as bacterial antigen burden. HIV-encoded RNA (Fig. 5A) and total DNA (Fig. 5B) were detected in a subset of both NN-HIV[+] and HAND groups without significant difference between groups but were not detected in the uninfected group. The NN-HIV[+] and HAND groups showed a trend toward higher CD3E levels (Fig. 5C) than in the uninfected group while CD68 (Fig. 5D) transcript levels were significantly increased in the HAND group (p = 0.003).

**TLR2** (Fig. 5E) and **CASP1** (Fig. 5F) were significantly increased in both the NN-HIV[+] (p = 0.036) and HAND (p = 0.023) groups while **IL1B** (Fig. 5G) and **GSDMD** (Fig. 5H) transcript levels displayed significantly increased expression in the HAND group (p = 0.024; p = 0.005). To extend these observations, western blotting of brain tissues from the HIV[−], NN-HIV and HAND groups was performed disclosing increased CCR5 immunoreactivity in the HAND group (Fig. 5I) that was confirmed by quantitative analyses of the CCR5 relative to matched beta-actin blots (p = 0.0253) (Fig. 5J). Thus, these studies indicated that host neuroinflammatory changes were apparent as expected in brains in the HAND group.

3.6. Increased bacterial burden in brain from people with HAND

In the present human cohort, neuroinflammation differed among clinical groups depending on the diagnosis with the HAND group. To examine the relationship between diagnosis and bacterial presence and abundance, bacterial 16s rRNA (V3-V4) (Fig. 6A) and **GroEL** (Fig. 6B) transcript levels were measured in all groups and showed that there were no group differences for both amplicons’ levels although all samples had detectable bacterial RNA (Fig. 6A and 6B). These latter analyses were extended by quantifying the genomic copy number of GroEL DNA as a measure of bacterial genome load (Fig. 6C); these analyses showed that GroEL copy number per gram of tissue ranged from 150 to 550 copies/gm depending on the group and was significantly higher levels in the HAND group compared to HIV[−] individuals (p < 0.05). GroEL DNA copy number was correlated with 16srRNA V3-4 levels (Fig. 6D),
Fig. 3. Exposure to PGN increases HIV-1 production in infected microglia. (A) Immunofluorescence showed increased intensity of CCR5 and HIV-1 p24 immunofluorescence in HIV-infected microglia exposed to PGN relative to uninfected, unexposed cells or cells exposed to only PGN. (B) sqRT-PCR of microglia-derived RNA revealed PGN exposure following HIV-1 infection significantly increased CCR5 mRNA expression. (C) CCR5 immunofluorescence was increased in cells exposed to PGN prior to or following HIV-1 infection relative to HIV infection alone. (D) Cell-associated HIV-1 p24 immunofluorescence was increased in cells exposed to PGN both before and after HIV-1 infection. (E) Prior exposure to PGN significantly increased HIV-1 p24 release as measured by p24 ELISA of cell culture supernatants collected 72 h post-HIV-1 infection. (Mean ± SEM; n = 3, donors; Kruskal-Wallis *p < 0.05, **p < 0.01, ***p ≤ 0.0001).
Fig. 4. PGN exposure potentiates HIV-1 Vpr-induced neurotoxicity. (A) Representative fluorescent images of cultured and differentiated human neuronal cells, immunolabeled for β-actin (green) and stained for DAPI (blue) in the absence (Vpr[−]) or presence (Vpr[+]) of soluble Vpr (150 nM). (B) Vpr exposure significantly reduced resazurin activity while exposure to PGN alone had no effects. However, cells exposed to PGN following Vpr exposure showed significantly less resazurin activity than cells exposed to Vpr alone. (C) Immunodetection of β-III tubulin was decreased by Vpr exposure with further reduced immunoreactivity when PGN exposure followed Vpr exposure, (D) as illustrated by graphic analyses. (Mean ± SEM for 3 independent experiments; ANOVA, Tukey’s post hoc * p < 0.05, **** p < 0.0001). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
but it was not associated with time to autopsy (Supplementary Fig. 4A), pre-mortem plasma (Supplementary Fig. 4B), CSF (Supplementary Fig. 4C) or autopsied brain (Supplementary Fig. 4D) HIV-1 pol RNA copy numbers per gram (tissue). Conversely, GroEL genomic DNA amplicon levels in brain were negatively correlated with Abstract Executive T-scores from HIV-infected patients (Supplementary Fig. 4E) while 16s rRNA V3-V4 levels in brain were also negatively correlated with Memory Domain T-score among HIV-infected patients (Supplementary Figure 24), based on pre-mortem neuropsychological testing. As infection is a key aspect of HIV-1 neuropathogenesis, the relationships between GroEL DNA copy number/gram and transcript levels for CASP1 (Fig. 6E) and GSDMD (Fig. 6F) as well as AIM2 (a dsDNA sensor, data not shown) levels were examined and found to show a positive and significant (p < 0.001) correlation with GroEL genomic copy number.

Given that bacterial RNA and DNA were detectable in all groups but were increased in the HAND group, bacterial antigens were probed with antibodies (Fig. 6G) to GroEL and PGN in (inset) in brain tissue sections from HIV[–] and HAND patients. These studies disclosed that GroEL immunoreactivity was detected in both HIV[–] and HAND groups (Fig. 6Gv and Gvi) and was co-localized with immunoreactivity for GFAP in astrocytes (Fig. 6Gvii and Gviii) and for Iba-1 in microglia/macrophages (Fig. 6Gviiii). Similarly, PGN immunoreactivity was present in both HIV[–] and HAND sections (Fig. 6Gv and Gvi, insets) that was co-localized with Iba-1 immunoreactivity (Fig. 6Gvii and Gviiii, insets). These studies indicated that bacterial molecule burden in the brain was associated with the neurological diagnosis, worsened neuropsychological performance, and neuroinflammation.

4. Discussion

To our knowledge, our study represents the first report of translocated bacterial proteins and genomes as direct contributors to neurological disease during viral infections of the brain. In both humans and NHPs infected with the lentiviruses, HIV-1 and SIV, respectively, microbial molecules (e.g., DnaK, GroEL, and peptidoglycan) were immunodetected in the brain, principally in microglia and astrocytes with increased bacterial burden in humans and animals displaying neurological disease (Fig. 7). Bacterial GroEL DNA quantitation showed increased levels that were correlated with CCR5 expression in the brain. Moreover, peptidoglycan exposure increased p24 production in HIV-infected cultured microglia, associated with CCR5 induction, while also accentuating cytotoxic actions of HIV-1 Vpr on human neurons. In human brains, bacterial RNA and DNA levels were correlated with neuroinflammatory responses although not with HIV-1 RNA or total DNA load in brain. These findings underscore the impact of microbial translocation into the brain during infections and its potential contribution(s) to the development of neurological disease.

As with HIV-1 infections, a subset of SIV-infected animals showed neurological complications manifested by behavioral changes as well as neuropathological findings at necropsy (Table 1). We analyzed tissues from SIV-infected animals using similar tools to those applied to the human brain samples and found that both viral load and inflammatory marker expression were elevated in the animals with neurological disease (i.e., Neuro-SIV[+]). These findings mirrored observations in humans with HAND, unlike the NN-HIV[+] group without neurological disease; not surprisingly, neuroinflammatory changes with accompanying cellular infiltration was associated with the development of neurological disease in both human and NHPs. However, viral burden was higher in the Neuro-SIV[+] group’s brains unlike the HAND group, likely because the HAND group had been exposed to ART pre-mortem, which diminished the differences in viral RNA and DNA between the HAND and NN-HIV[+] groups. However, recapitulating the human results, SIV-infected animals with neurological disease also displayed a higher burden of bacterium-encoded DNA and proteins.

Earlier studies have reported the presence of bacterial genomes and proteins in both blood (Potgieter et al., 2015; Castillo et al., 2019) and brain (Schrijver et al., 2001; Branton et al., 2016; Zhan et al., 2016; Visser et al., 2005) in the absence of disease. The source of these bacterial molecules was unclear, although the disruption of mucosal barriers in the gut, urogenital tract, mouth and perhaps lung are potential sources of microbiome-derived bacterial products, especially in the setting of an inflammatory disease such as AIDS. It is plausible that the bacterial protein or genome levels were higher in the Neuro-SIV[+] and HAND groups because of ongoing neuroinflammation driven by blood-derived leukocytes trafficking into the brain and at the same time serving as vehicles for bacterial molecules phagocytosed while in circulation. An alternative mechanism lies in the increased blood–brain barrier permeability reported for persons with HAND (Power et al., 1993), which could facilitate microbial molecule translocation into the brain parenchyma, leading neurobehavioral changes, as reported recently for radio-labeled peptidoglycan (Gabanyi et al., 2022). The convergence of microbial-associated molecular patterns (MAMPs) such as PGN or bacterial DNA/RNA with lentivirus-infected or activated leukocytes has the capacity to promote neuroinflammation and neurological disease sequelae. In fact, induction of molecular pathways in the present studies including the inflammasome-pyroptosis axis (e.g., caspase-1, gasdermin D) (Shenderov et al., 2013) and CCR5 are recognized downstream consequences of bacterial molecule engagement of pattern recognition receptors such PGLYRP2, NOD1/2 (Saha et al., 2009), AIM2 (Rae et al., 2011), or TLR2-4 (Uehori et al., 2005). Of note, we examined expression of all the endogenous peptidoglycan receptors in human brain and found very limited expression (data not shown), which underscored peptidoglycan’s other potential mechanisms of action (e.g., NOD1/2, AIM2 and TLR2-4). Enhanced expression of CCR5 in both SIV- and HIV-infected in vivo as well as in cultured microglia following HIV-1 infection, might reflect an evolutionary advantage for lentiviruses by promoting their infection through enhance co-receptor expression and ensuing production in the brain and perhaps other organs.

We previously reported that low levels of bacterial nucleic acids and

<table>
<thead>
<tr>
<th>Neuro-pathology.</th>
<th>No anomalies (n = 6), Anoxic-ischemic Encephalopathy (n = 2), Parenchyma hemorrhage (n = 2), non-diagnostic abnormalities (n = 2)</th>
<th>No anomalies (n = 3), Alzheimer Type 2 Gliosis (n = 4), Anoxic-ischemic Encephalopathy (n = 1), FocalInfaract (n = 3), Other Non-InfectionPathology (n = 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV[−] (n = 11)</td>
<td>No anomalies (n = 3), Alzheimers (n = 1), * Type 2 Gliosis (n = 1), secondary infection (n = 3), FocalInfaract (n = 1), * non-diagnostic abnormalities (n = 1)</td>
<td></td>
</tr>
<tr>
<td>HIV[+] (n = 10)</td>
<td>9:1 50.1 ± 5.4 1.33 ± 2.21 3.09 ± 3.7</td>
<td>10:2 40.2 ± 7.8</td>
</tr>
<tr>
<td>HAND (n = 12)</td>
<td>9:2 50.5 ± 10.4</td>
<td>10:2 40.2 ± 7.8</td>
</tr>
</tbody>
</table>

| Sex (M:F) | 9:2 | 10:2 |
| Age (yr.) (mean ± SD) | 50.5 ± 10.4 | 50.1 ± 5.4 |
| Plasma HIV-1 load (×10⁷ copies/ml) | N/A | 1.33 ± 2.21 |

| Plasma HIV-1 load | N/A | 1.33 ± 2.21 |

From Table 2, Human autopsy cohort including patients uninfected (HIV[−]), HIV-infected without neurological disease (NN-HIV[+]) or with HIV-associated neurocognitive disorders (HAND). | From SIV-infected animals using similar tools to those applied to the human brain samples and found that both viral load and inflammatory marker expression were elevated in the animals with neurological disease (i.e., Neuro-SIV[+]). These findings mirrored observations in humans with HAND, unlike the NN-HIV[+] group without neurological disease; not surprisingly, neuroinflammatory changes with accompanying cellular infiltration was associated with the development of neurological disease in both human and NHPs. However, viral burden was higher in the Neuro-SIV[+] group’s brains unlike the HAND group, likely because the HAND group had been exposed to ART pre-mortem, which diminished the differences in viral RNA and DNA between the HAND and NN-HIV[+] groups. However, recapitulating the human results, SIV-infected animals with neurological disease also displayed a higher burden of bacterium-encoded DNA and proteins.

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We previously reported that low levels of bacterial nucleic acids and
Fig. 5. HIV-1 quantities and neuroinflammatory responses in human brains. Three groups of patients were investigated: HIV-uninfected (HIV[−], n = 10); ART-treated HIV-infected without neurocognitive impairments (NN-HIV[+], n = 10) or with HAND (n = 12). HIV-1 pol RNA (A) and DNA (B) was quantified in the frontal neocortex by ddPCR. HIV-1 pol DNA was undetectable in all HIV[−] subjects and in several brain samples in the NN-HIV[+] group but present in all HAND patients without a statistically significant difference between the two HIV-infected groups. (C) CD3E transcript levels did not differ between groups but (D) CD68 mRNA expression was increased in the HAND group. (E) TLR2 and (F) CASP1 were increased in both HIV-infected groups while (G) IL1B and (H) GSDMD showed significantly elevated transcript levels in the HAND group. CCR5 immunoreactivity in brain was significantly elevated in HAND group (I, J). (ANOVA, *p < 0.05, **p < 0.01).
Fig. 6. Burden of bacterial molecules in brain is associated with HIV-1 encephalitis. (A) Bacterial 16s rRNA V3-V4 amplicon levels (A) as well as GroEL transcript levels (B) were measured by semi-quantitative RT-PCR, showing no differences in bacteria-encoded RNA levels associated with increasing brain disease. (C) Quantitation of GroEL genomic DNA showed significantly increased copy numbers per gram (tissue) in the HAND group. Bacterial 16s rRNA V3-V4 detection was significantly correlated with (D) GroEL gDNA amplicon levels, (E) CASP1, and (F) GSDMD transcript levels in brain. (G) Bacterial GroEL and peptidoglycan (PGN) immunoreactivity was apparent within glial cells in human brain. GFAP positive cells (red) are associated with GroEL (purple) and Iba-1 (inset, green) -labelled cells have extending processes towards (closed arrow) or have engulfed (open arrow) PGN positive (orange) bodies (Spearman’s correlation coefficient (r); Kruskal-Wallis *p < 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
peptidoglycan immunoreactivity were observed in the human and NHP brains post-mortem despite stringent efforts to minimize contamination, particularly in experiments performed to verify the findings of next generation sequencing (Branton et al., 2013). We postulated that these bacteria translocated into the brain via a Trojan horse mechanism and were likely dormant or non-viable, given the low bacterial copy numbers, but nonetheless could act as MAMPs driving neuroinflammation and exacerbating the primary effects of HIV-1 or SIV infections. In the present studies, bacterial burden was assessed in terms of both DNA and RNA levels by independent systems showing highly correlated results that are difficult to attribute solely to reagent contamination. Further supporting the specificity and significance of these findings were the associations between bacterial RNA and DNA burdens with neuroimmune responses in brains but the seeming lack of correlation with HIV-1 or SIV RNA and DNA quantities. In fact, neurocognitive impairment severity in HIV-infected humans was also associated with bacterial burden (Supplementary Fig. 4). While these correlations do not clarify cause and effect relationships, they point to associations between bacterial presence and immune or clinical outcomes that preclude reagent contamination or autopsy-related artefacts as sources of bacterial detection in brain. It is plausible that bacterial translocation provides MAMPs to pattern recognition receptors on brain parenchyma cells that drive neuroinflammation-enhancing neuropathology or that lentivirus-driven inflammation recruits phagocytic innate immune cells containing bacteria into the brain. What seems implausible is that multiple clinical and molecular determinants of lentivirus neuropathogenesis would be correlated with either post-mortem or procedural introduction of bacteria to the specimens. Nonetheless, considerable concerns have been raised regarding procedure or reagent contamination in sequence-based analyses (Salter et al., 2014). As such, we focused on validating the presence of bacteria in brain by multi-modal immune-detection of conserved bacterial proteins. Antibodies that detected GroEL, DnaK and PGN were tested for sensitivity of FFPE tissues from cases with pre-mortem diagnosis of bacterial meningoencephalitis demonstrating that both DnaK and GroEL were equally effective in this context but did not react to lysates from cultured human neural cells (Supplementary Fig. 2). It is important to note that while precautions were taken to reduce contamination during collection and manipulation of the tissue specimens, we cannot eliminate the possibility that the quantities of bacterial products observed here may reflect a post-mortem ‘bloom’ derived from even smaller numbers of bacteria that were present at the time of death. The contribution of bacterial replication after the death of the host is difficult to quantify. However, the correlation with clinical measures, but not times to autopsy, and the detection of bacterial genomes or proteins within apparent phagocytic vesicles argues against our findings being a strictly post-mortem phenomenon. Nonetheless, peptidoglycan can be shed and act as a signaling molecule independently of its role as the primary structural component of the bacterial cell wall (Tosoni et al., 2019). Thus, we also examined the expression of conserved bacterial proteins including GroEL and DnaK. The antibodies used for the present studies were reactive against several bacterial species. The antibodies to both GroEL and DnaK immunolabeled small cocci in brain tissues from patients or NHPs with bacterial meningoencephalitis with co-localization (Supplementary Fig. 2). As seen with anti-PGN immunolabeling, bacterial GroEL immunofluorescence was associated with glial cells, including microglia and astrocytes with more staining evident in HAND patients (Fig. 6). The presence of bacterial polymerases in the brain was a robust indicator that intact bacterial cells had traversed the blood–brain barrier. The primary brain-resident cells capable of productive HIV-1 infection are microglia although trafficking macrophages in the brain also show productive infection. In the present studies peptidoglycan potentiated HIV-1 replication in microglia, as suggested by increased p24 release in otherwise identical culture conditions. This outcome could be a consequence of upregulation of the co-receptor CCR5 among other factors. Whether through the actions of live bacteria or bacterial MAMPs, there was transcriptional induction associated with bacterial nucleic acid load in brain tissue including an increase in CCR5 expression; this could enhance HIV-1 infectivity in vitro with evidence of comparable changes in brains with high HIV-1, SIV and bacterial loads. This finding has important implications for HIV-1 persistence because microbial products translocating into the brain can potentiate HIV-1 replication (Annavajhala et al., 2020) and compound the already challenging issue of eradicating HIV-1 from the brain. The fact that peptidoglycan worsened Vpr-mediated neuronal damage (Ferrucci et al., 2011) is potentially important because it suggests that bacterial-derived MAMP exposure could be a driver of HIV-associated neurological disease. Collectively, these observations imply that bacteria-derived MAMP exposure either prior to or after HIV-1 infection could be a multi-faceted driver of neurological disease.

The present studies faced several challenges that included a lack of identification of the specific bacterial species translocating into the
brain. Previous studies from our group have highlighted proteobacteria as a predominant phylum detected in the brain although consistent results from attempts to isolate bacteria from human brains are lacking, possibility due to low levels of replication-competent bacteria in brain or sub-optimal culture conditions. Another shortcoming in the present studies was the uninfected control group brains; for the human studies, the subjects’ brain included those with other systemic disorders (e.g., stroke, drug overdose) and similarly, the uninfected control NHPs had been used in other (vaccine) studies that might influence microbial translocation into the brain. Future studies will need to delineate the exact bacterial species present in the brains together with their extra-nervous system organ sources (e.g., gut, mouth, lung, urogenital tract). Moreover, dissection of the individual neuroimmune pathways induced by translocated MAMPs warrants further investigation to discern specific biomarkers and future therapeutic targets.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data are available in the main text or the supplementary materials.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbbi.2022.09.019.

References


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