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Viral impact on CNS: mechanisms of immune dysfunction and cognitive decline

Edited by
Thomas A. Angelovich, Sonia Mediouni, Bruce James Brew, Melissa Jayne Churchill, Jacob D. Estes and Robyn Klein

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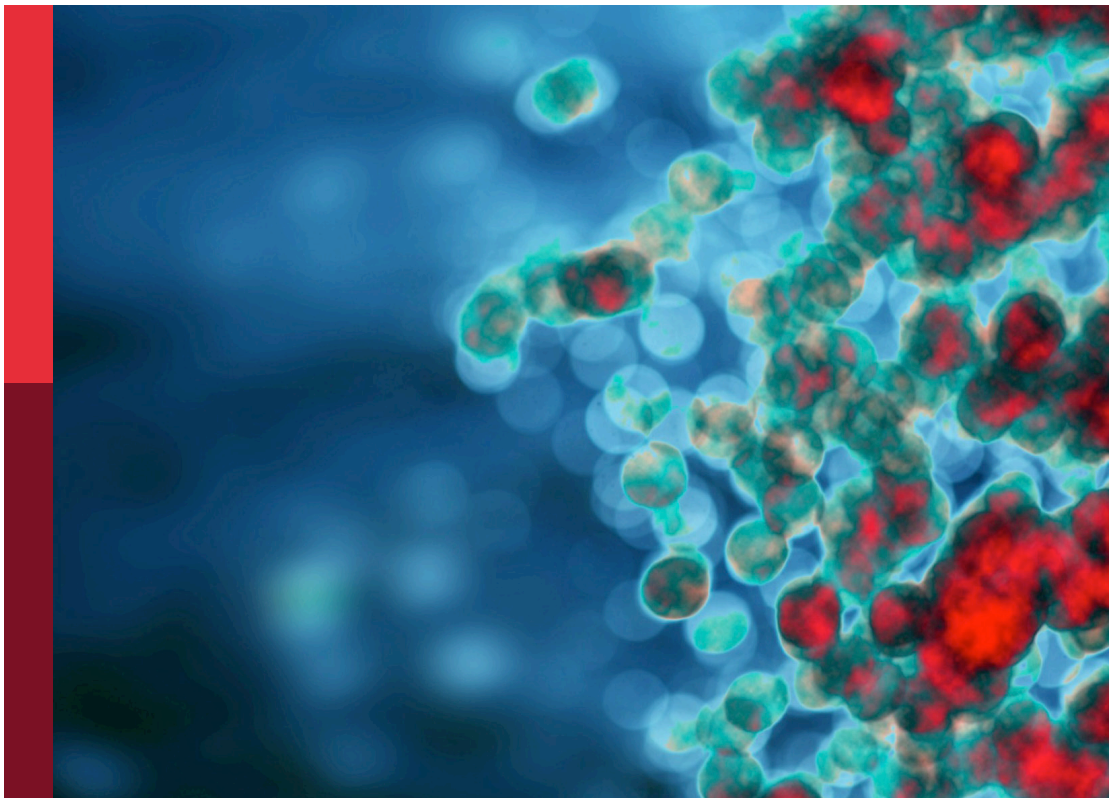

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Viral impact on CNS: mechanisms of immune dysfunction and cognitive decline

Topic editors

Thomas A. Angelovich — RMIT University, Australia
Sonia Mediouni — University of Florida, United States
Bruce James Brew — St Vincent's Hospital Sydney, Australia
Melissa Jayne Churchill — RMIT University, Australia
Jacob D. Estes — Oregon Health & Science University, United States
Robyn Klein — University of Western Ontario (Western University), Canada

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EDITED AND REVIEWED BY
Pietro Ghezzi,
Brighton and Sussex Medical School,
United Kingdom

*CORRESPONDENCE
Melissa J. Churchill
melissa.churchill@rmit.edu.au

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Editorial: Viral impact on CNS: mechanisms of immune dysfunction and cognitive decline

Thomas A. Angelovich[1,2,3], Sonia Mediouni[4], Robyn S. Klein[5], Jacob D. Estes[1,6], Bruce J. Brew[7,8] and Melissa J. Churchill[1,2,9]*

[1]ATRACT Research Centre, School of Health and Biomedical Sciences, Royal Melbourne Institute of Technology (RMIT) University, Melbourne, VIC, Australia, [2]Department of Infectious Diseases, The University of Melbourne at the Peter Doherty Institute for Infection and Immunity, Melbourne, VIC, Australia, [3]Life Sciences, Burnet Institute, Melbourne, VIC, Australia, [4]Department of Immunology and Microbiology, The Herbert Wertheim UF Scripps Institute for Biomedical Innovation & Technology, Jupiter, FL, United States, [5]Department of Microbiology & Immunology, University of Western Ontario, London, ON, Canada, [6]Vaccine & Gene Therapy Institute, Oregon Health & Science University, Beaverton, OR, United States, [7]Peter Duncan Neurosciences Unit, Departments of Neurology and Immunology St Vincent's Hospital, Darlinghurst, NSW, Australia, [8]Faculty of Medicine, University of New South Wales, Sydney, NSW, Australia, [9]Departments of Microbiology and Medicine, Monash University, Melbourne, VIC, Australia

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Editorial on the Research Topic
Viral impact on CNS: mechanisms of immune dysfunction and cognitive decline

Introduction

Recently, the contribution of viruses to neuropathology and cognitive decline has garnered significant interest with viral infection associated, at least in part, with the pathogenesis of dementia, multiple sclerosis and virus-specific cognitive impairment (1–4). Neuropathology can occur during acute, chronic and latent infection and, in some cases, even in the presence of antiviral therapy. However, the precise mechanisms by which specific viruses induce neuropathology and cognitive dysfunction remain unclear. This underscores the need to elucidate the underlying processes in order to develop effective therapeutic strategies. In this Research Topic, we have collated a series of manuscripts that assess the contribution of various viruses including Human Immunodeficiency Virus (HIV), Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and others, to neuroinflammation, neuropathology and cognitive disorders.

Summary of contributions

As mentioned above, the role of viruses in neuroinflammation and neuropathology has gained significant attention in recent years. Contributing to this Research Topic, Li and Wu performed a bibliographic analysis of research publications in the field and found an increasing trend in the number of publications and citations over the last 20 years. Naturally, the contribution of pathogens to neuropathology and/or cognitive disorders varies based on factors such as the viral replication cycle, pathogenesis, host responses, and environmental influences. In this Research Topic, Nisa Awan et al. reviewed current evidence regarding viruses contributing to neuropathology and cognitive disorders. They further described underlying processes and, importantly, current clinical trials and drug treatment studies aimed at limiting the impact of viruses on the brain.

Antiviral signaling and cellular activation in the brain are critical defense mechanisms against viral infection, both within the central nervous system and systemically. Several studies in this Research Topic evaluated cellular activation in the context of HIV, revealing notable changes in both *ex vivo* brain tissue and cerebrospinal fluid (CSF) from people with HIV (PWH) - a population that continues to experience neuropathology and, in some cases, cognitive disorders despite viral suppression with antiretroviral therapy (5). Chan et al. demonstrated that the frequency of activated CD4+ and CD8+ T cells increased over time in CSF early during primary HIV infection. While the level of CSF CD4+ T cell activation correlated with levels of CSF HIV RNA, levels of CSF CD8+ T cell activation or the frequency of monocyte subsets in CSF did not. Importantly, T cell activation remained elevated in the CSF following ART initiation, indicating a persistent state of cell activation in the CSF. In a separate study, Byrnes et al. (including members of the editorial team) also demonstrated persistent cell activation in frontal cortex tissue from ART-suppressed PWH. Interestingly, levels of microglial activation were associated with both intact and 5' defective HIV proviral DNA, supporting a relationship between HIV reservoirs in the brain and neuroinflammation. These findings suggest that ongoing immune activation, driven by viral reservoirs, may contribute to continued neuropathology in PWH despite successful systemic viral suppression.

While HIV is known to directly infect microglia and, to a lesser extent, astrocytes and pericytes, infection can impact surrounding cells including neurons, thereby contributing to neuropathology. Alternatively, signals from surrounding cells may exert protective effects that mitigate neuropathology. In a mini-review, Lopez and Brown described the role and impact of secreted phosphoprotein-1 (SPP1) on innate immune activation and inflammation in the brains of PWH through mammalian target of rapamycin (mTORC1/2) signaling and NLRP3 inflammasome activation to respond to neuronal injury, highlighting a potentially protective crosstalk mechanism between microglia and neurons. Therefore, therapeutic strategies that target sources of persistent inflammation, in combination with earlier treatment initiation, are likely to offer significant benefits in reducing HIV-associated neuropathology.

Importantly, factors beyond viral persistence and/or replication in the brain must also be considered when studying the neuroinflammation and neuropathology associated with viral infections. In a model of systemic viral infections, Li et al. demonstrated that intraperitoneal injection of polyriboinosinic: polyribocytidylic acid (poly I:C), a synthetic analog of viral double-stranded RNA, triggered neuroinflammation in rats. This was measured using [18F]DPA-714 positron emission tomography, supporting the role of peripheral immune activation on central neuroinflammatory processes. Additionally, illicit drug use and other modifiable factors may also exacerbate virus-mediated neuroinflammation. Miao et al. reviewed how methamphetamine use contributes to neuronal activation and persistent HIV in virally suppressed PWH.

A major limitation in studying neuroinflammation and neuropathology is the restricted accessibility of the brain, making direct cellular-level assessment extremely challenging. As a result, identifying predictive biomarkers of cognitive disease and pathology is essential. SARS-CoV-2 infection has been shown to induce long-term neurological effects in some individuals with levels of IL-1β in the brain associated with neuropathology and cognitive impairment (6, 7). In a study of individuals with Long COVID or recovered SARS-CoV-2 infection, Elahi et al. identified a positive correlation between levels of Galectin-9 and artemin with measures of cognitive deficit in people with Long-COVID and myalgic encephalomyelitis/chronic fatigue syndrome, suggesting potential clinical utility as prognostic biomarkers.

Models of viral infection of the brain are also essential tools in understanding the fundamental mechanisms of disease. Current approaches include cell coculture systems, organoids, animal models, and, in some cases, *ex vivo* human organotypic culture; each with distinct advantages and limitations. In a study by Govaerts et al., a mature human pluripotent stem cell (hiPSC)-derived neurospheroid model was used to investigate Varicella-zoster virus infection and antiviral evasion mechanisms. This study demonstrated that these neurospheroids could be infected with VSV and that infection suppressed antiviral signaling, highlighting the model's potential as a novel platform for studying Vesicular Stomatitis Virus-mediated immune responses.

Concluding remarks

In summary, this Research Topic offers new insights into the role of viruses as key drivers of neuroinflammation, neuropathology and cognitive impairment, helping to inform future therapeutic strategies.

Author contributions

TA: Writing – original draft, Conceptualization, Writing – review & editing. SM: Writing – original draft, Writing – review & editing. RK: Writing – original draft, Writing – review & editing. JE: Writing –

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Robyn Klein,
Washington University in St. Louis,
United States

REVIEWED BY
Candice Brinkmeyer-Langford,
Texas A and M University, United States
Wakiro Sato,
National Center of Neurology and
Psychiatry, Japan

*CORRESPONDENCE
Yilong Cui
cuiyl@riken.jp

†These authors have contributed equally to this work

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Regional neuroinflammation induced by peripheral infection contributes to fatigue-like symptoms: a [18F]DPA-714 positron emission tomography study in rats

Danxi Li[1,2,3†], Di Hu[1†], Yuta Ochi[1†], Wakiko Arakaki[4], Aya Mawatari[4], Mika Shigeta[1], Yuping Wu[1], Emi Hayashinaka[5], Hiroyuki Neyama[1], Tsuyoshi Tahara[1], Yasuhiro Wada[5], Feng Li[2], Hisashi Doi[4], Yasuyoshi Watanabe[5] and Yilong Cui[1*]

[1]Laboratory for Biofunction Dynamics Imaging, RIKEN Center for Biosystems Dynamics Research, Kobe, Japan, [2]Department of Chinese Medicine Diagnostics, School of Traditional Chinese Medicine, Beijing University of Chinese Medicine, Beijing, China, [3]Institute for Brain Disorders, Dongzhimen Hospital, Beijing University of Chinese Medicine, Beijing, China, [4]Laboratory for Labeling Chemistry, RIKEN Center for Biosystems Dynamics Research, Kobe, Japan, [5]Laboratory for Pathophysiological and Health Science, RIKEN Center for Biosystems Dynamics Research, Kobe, Japan

Introduction: A series of symptoms, including fever, widespread pain, fatigue, and even ageusia, have frequently been reported in the context of various infections, such as COVID-19. Although the pathogenic mechanisms underlying an infection causing fever and pain have been well established, the mechanisms of fatigue induced by infection in specific brain regions remain unclear.

Methods: To elucidate whether and how the peripheral infection cause fatigue via regional neuroinflammation, we performed a brain-wide investigation of neuroinflammation in a peripheral pseudoinfection rat model using [18F]DPA-714 positron emission tomography (PET) imaging analysis, in which the polyriboinosinic: polyribocytidylic acid (poly I:C) was intraperitoneally injected.

Results: Transient fever lasting for several hours and subsequent suppression of spontaneous activity lasting a few days were induced by poly I:C treatment. Significant increase in plasma interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)-α were observed at 2 and 4 h following poly I:C treatment. PET imaging analysis revealed that the brain uptake of [18F]DPA-714 was significantly increased in several brain regions one day after poly I:C treatment, such as the dorsal raphe (DR), parvicellular part of red nucleus (RPC), A5 and A7 noradrenergic nucleus, compared with the control group. The accumulation of [18F]DPA-714 in the DR, RPC and A5 was positively correlated with subsequent fatigue-like behavior, and that in the A7 tended to positively correlate with fever.

Discussion: These findings suggest that peripheral infection may trigger regional neuroinflammation, which may cause specific symptoms such as fatigue. A similar mechanism might be involved in COVID-19.

KEYWORDS

fatigue-like symptoms, regional neuroinflammation, peripheral infection, PET, sickness behavior

1 Introduction

Fatigue and other prolonged neuropsychiatric and physical manifestations caused by SARS-CoV-2 infection have received growing attention as the most frequently claimed post-COVID-19 sequelae and are becoming a serious global public health issue. In general, infections induced by various kinds of pathogens or pathogenic organisms are known to be associated with a series of symptoms including fever, widespread pain, and fatigue. The underlying mechanisms for infection-evoked fever and pain have been well investigated. In response to peripheral infection, prostaglandin E2 (PGE2) is produced and increases in the brain parenchyma, which activates PGE receptor 3 (EP3) receptors of thermoregulatory neurons in the preoptic area of the hypothalamus leading to fever (1, 2). Peripheral and central mechanisms, such as upregulation of the transient receptor potential family in afferent sensory neurons evoked by pro-inflammatory mediators have been proposed to be involved in infection-related pain (3, 4). Long-term debilitating fatigue and severe fatigue sensations have also been reported frequently in various infections. In 1985, there was an outbreak of illness characterized by chronic or recurrent debilitating fatigue linked to the Epstein–Barr virus in Nevada in USA. The illness was defined as chronic fatigue syndrome (CFS) by the Centers for Disease Control and Prevention and first described in a publication in 1988 (5). Thereafter, similar symptoms have been frequently reported in some virus infections, including coronavirus disease 2019 (COVID-19). The latest clinical studies in COVID-19 have mentioned that besides respiratory symptoms, fatigue is one of the most common (approximately 50%) typical clinical manifestations related to COVID-19, and might be observed as sequelae (6–8). However, the detailed mechanisms pointing to the involvement of infection in fatigue pathophysiology remain unclear, and conclusive evidence has yet to be demonstrated.

Recently, neuroinflammation has been proposed as a possible mechanism related to the development of fatigue. Neuroinflammation, an immune response in the central nervous system (CNS) whereby glial cells are activated, is known to be involved in a variety of CNS diseases. In a pioneering study, Nakatomi et al. (9) reported that widespread neuroinflammation, particularly in the hippocampus, amygdala, thalamus, and midbrain, correlated with the severity of symptoms in patients with CFS. Although peripheral infection has been reported to trigger inflammatory responses in the brain (10–12), the underlying mechanisms for fatigue involved in neuroinflammation in the specific brain regions remain unclear.

The regional neuroinflammation in the brain could be quantitatively evaluated by positron emission tomography (PET) imaging non-invasively using radiolabeled compounds targeting specific biomarkers of activated glial cells. [^{18}F]DPA-714 (N,N-Diethyl-2-(2-[4-(2-Fluoroethoxy)-Phenyl]-5,7-Dimethyl-Pyrazolo [1,5-a]Pyrimidin-3-yl)-Acetamide) has been developed and widely used for the quantitative assessment of neuroinflammation in diverse central nervous system diseases as a specific radioligand for the translocator protein 18 kDa, a reliable biomarker for activated microglia (13). To investigate whether and how the regional neuroinflammation is involved in peripheral infection induced fatigue-like symptoms, we induced a peripheral pseudoinfection in rats by intraperitoneal injection of polyriboinosinic: polyribocytidylic acid (poly I:C) (14). Using this animal model, we performed brain-wide quantitative evaluation of neuroinflammation using [^{18}F]DPA-714 PET imaging analysis and assessed the correlation between regional neuroinflammation and sickness behaviors, including fatigue.

2 Materials and methods

2.1 Animals and peripheral pseudoinfection generation

Forty-six male Sprague-Dawley rats (6 weeks old) were purchased from Japan SLC (Hamamatsu, Japan). Since data variability has been reported to be greater in the females than in males in rodents PET imaging study, only male rats was used (15). The rats were housed in a temperature- (23 ± 1°C), humidity- (60 ± 5%), and light- (lights on at 8:00 and off at 20:00) controlled environment. A standard laboratory diet and tap water were available *ad libitum*. For acclimation, rats were housed in the experimental room for at least 1 week before the week-long pre-level measurement of spontaneous activity, and randomly divided into saline- (control) and poly I:C-treated groups. A pseudo-viral infection in rats (8 weeks old) was induced by intraperitoneal injection of poly I:C (GE Healthcare Life Science, Buckinghamshire, UK), a synthetic double-stranded RNA which has been widely used to mimic peripheral viral infections, dissolved in saline at a dose of 10

mg/kg body weight between 10:00 and 11:00 in the morning (14, 16). In the control group, rats were injected with saline at analogous procedure. Body weight was measured in the morning every day from 3 days before to 4 days after the poly I:C injection. The experimental procedures in the present study were approved by the Institutional Animal Care and Use Committee of RIKEN, Kobe Branch, and were performed in accordance with the *Guide for the care and use of laboratory animals* (NIH publication No. 85-23, revised 2011).

2.2 Measurement of spontaneous activity

To quantitatively evaluate fatigue state, the spontaneous activity of each rat was recorded with an infrared beam sensor (NS-AS01; Neuroscience, Tokyo, Japan) prior to and following a poly I:C injection. The infrared beam sensor was placed 15 cm above the center of each cage, and the activities of rats housed in individual cages were measured. The level of night-time spontaneous activity was normalized by the mean value of the 3 days prior to poly I:C injection. The fatigue of rats was calculated by assessing night-time spontaneous activity, which was added up every 60 min and analyzed in Clock Lab (Neuroscience, Tokyo, Japan). In addition, the spontaneous activity in all rats used in [^{18}F]DPA-714 PET scan was also examined separately throughout the entire experimental period for the correlation analysis.

2.3 Body temperature measurement

Body temperature of rats was monitored using an implantable programmable temperature transponder (IPTT-300, Bio Medic Data Systems, Seaford, USA), which was implanted gently into the subcutaneous tissue between the scapulae of each rat under anesthesia (with a mixture of 1.5% isoflurane and nitrous oxide/oxygen 7:3) with a syringe-like action 7 days before intraperitoneal injection of poly I:C or saline. Temporal changes in the body temperature of the rats were measured wirelessly using an IPTT reader from 0 h (before injection) to 48 h following the poly I:C or saline injection.

2.4 Cytokine analysis

Besides the pre-injection levels (baseline), at 2 h, 4 h, 8 h, 24 h, and 48 h after poly I:C injection, rats were shortly anesthetized with a mixture of 1.5% isoflurane and nitrous oxide/oxygen (7:3), and blood samples were collected from an indwelling catheter in the tail vein implanted just before each sampling. Venous blood was centrifuged at 12,000 rpm for 10 min at 4°C and cytokine levels were measured on the resulting plasma. The cytokines interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)-α were simultaneously assessed using the Bio-Plex Pro Rat Cytokines Assay (Bio-Rad Laboratories, California, USA) (17). Since, the level of plasma cytokines remained stable following repeat measurements in satellite control rats (Supplemental Figure), the poly I:C induced temporal changes in plasma cytokines were compared with their own baseline (pre-injection level).

2.5 PET scanning

In the present study, [^{18}F]DPA-714 was synthesized as reported by Sydney group (18). The product was identified and purified using high-performance liquid chromatography on a COSMOSIL C18-AR-II column (10 × 250 mm, Nacalai, Kyoto, Japan). Molar activity ranged from 33 to 160 GBq/μmol. Radiochemical purity analyzed using HPLC exceeded 99%.

All PET scans were performed using a microPET Focus220 (Siemens, Knoxville, USA) designed for small laboratory animals. Both saline- and poly I:C-treated rats were anesthetized with 1.5% isoflurane and nitrous oxide/oxygen (7:3) and placed in a prone position in the PET scanner gantry. During the PET scan, the body temperature was maintained at 37°C using a small animal warmer connected to a thermometer (BWT-100A; Bio Research Center, Nagoya, Japan). A 45-min emission scan was performed immediately after the bolus injection of [^{18}F]DPA-714 (≈75 MBq per animal) via a cannula inserted into the tail vein; the energy window was 400-650 keV and the coincidence time window was 6 ns. Emission data were collected in list mode and sorted into dynamic sonograms (6 × 10 s, 6 × 30 s, 11 × 60 s, and 10 × 180 s, for a total of 33 frames). The acquired data were reconstructed by standard 2D-filtered back projection (FBP) (ramp filter, cutoff frequency at 0.5 cycles per pixel) for quantification, and by a statistical maximum a posteriori probability (MAP) algorithm (12 iterations with point spread function effect) for image registration.

2.6 Image analysis

PET images were co-registered to a magnetic resonance imaging (MRI) template which was placed in a Paxinos and Watson stereotactic space using the PMOD imaging processing software (version 3.6, PMOD Technologies, Zürich, Switzerland). Each FBP image was spatially smoothed using an isotropic Gaussian kernel (0.6-mm full width at half maximum) for enhancement of the statistical power. The radioactivity was normalized with cylinder phantom data and expressed as standardized uptake values (SUVs).

A voxel-based statistical analysis was performed using Statistical Parametric Mapping (SPM) 8 software (Welcome Department of Imaging Neuroscience, London, UK). A two sample *t*-test was used for estimating the statistical differences between groups. The statistical threshold was set to be $P < 0.005$ familywise error (FWE) with an extent threshold of 200 contiguous voxels.

2.7 Statistical analysis

All results are expressed as the mean ± SEM. All data were analyzed in SPSS (version 24.0, IBM, Armonk, USA). One-way

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analysis of variance (ANOVA) with Bonferroni's multiple-comparison procedure was used to assess changes in body temperature, cytokines, and spontaneous activity prior to and following poly I:C injection. Two-way repeated measures ANOVA with Bonferroni's multiple-comparison procedure was used to assess differences in body temperature and spontaneous activity between the two groups of rats. Pearson's test was used for correlation analysis of the accumulation of [^{18}F]DPA-714 in each brain region and fatigue-like behavior. Differences were considered statistically significant at $P < 0.05$.

3 Results

3.1 Poly I:C-induced symptoms and plasma cytokine elevation

The body weight of poly I:C-treated rats decreased approximately by 10% of the pre-level value the day after poly I:C injection, thereafter recovering gradually. The body temperature of rats in the poly I:C-treated group increased significantly again and reached a peak at 5 h ($P < 0.001$), following a significant increase as an acute stress response within the first hour after the poly I:C injection (Figure 1A).

To assess poly I:C-induced peripheral inflammatory responses, temporal changes in plasma cytokines were detected up to 48 h after the poly I:C injection (Figure 1B). Several pro-inflammatory cytokines were significantly elevated at early injection time points, as compared with the pre-level. Two hours after poly I:C injection, cytokines IL-1β ($P = 0.019$), IL-6 ($P = 0.004$) and TNF-α ($P < 0.001$) were significantly elevated. A significant elevation of IL-1β ($P = 0.002$) and IL-6 ($P = 0.006$) was observed until 4 h following the poly I:C injection.

3.2 Poly I:C-induced suppression of spontaneous activity

Fatigue can be assessed by changes in voluntary activity, known to be associated with motivation (14). To evaluate fatigue, night-

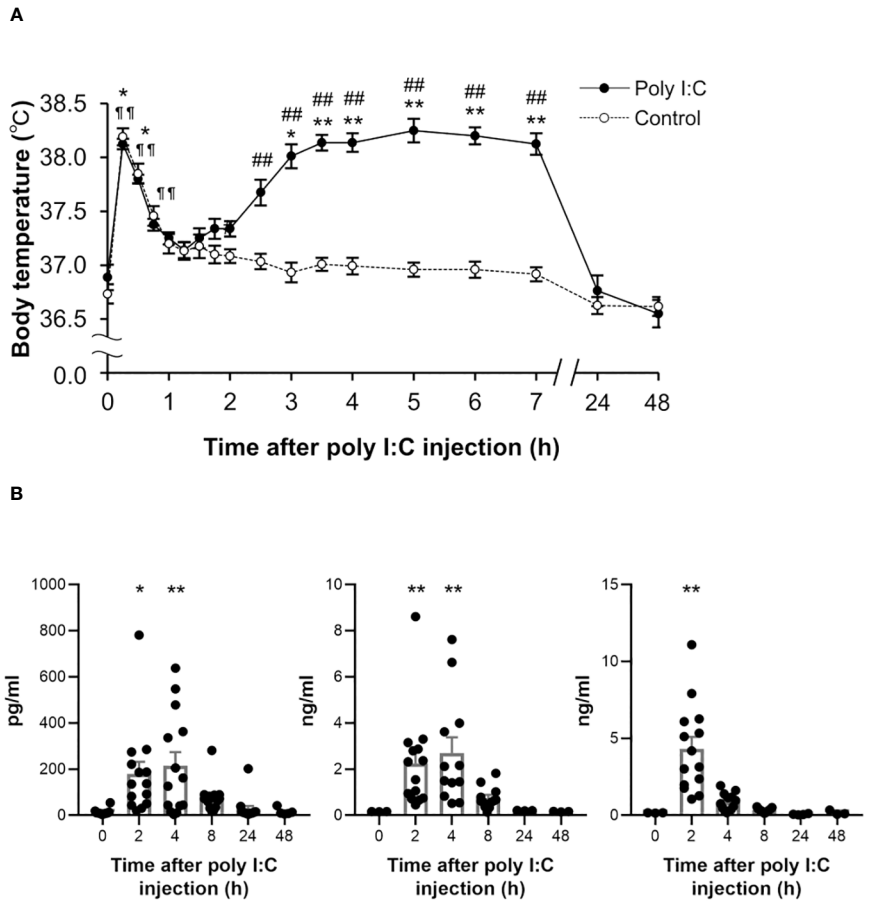

FIGURE 1
Temporal changes in body temperature and peripheral cytokines following a poly I:C or saline treatment. **(A)** Subcutaneous body temperature of rats from poly I:C (10 mg/kg) treated group (closed circles, $n = 8$) and control group (open circles, $n = 12$) up to 48 h after intraperitoneal injection with poly I:C or saline were plotted. $^{*}P < 0.05$, $^{**}P < 0.01$ for poly I:C-treated group, $^{¶¶}P < 0.01$ for control group vs. 0 h (before injection). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. control group. **(B)** Plasma IL-1β, IL-6, and TNF-α were detected at 2 h, 4 h, 8 h, 24 h and 48 h following poly I:C injection, as well as pre-injection (0 h). Each value represents the mean ± SEM, $n = 14$. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. pre-treated level. IL, interleukin; TNF, tumour necrosis factor.

time spontaneous activity in the home cage was investigated in both groups. As shown in Figure 2, the night-time spontaneous activity in the control group remained nearly stable throughout the experiment. However, the night-time spontaneous activity decreased significantly on the first night after the poly I:C injection (post day 1, $P < 0.001$). On the second night (post day 2), the night-time spontaneous activity sharply recovered to 78 ± 4% ($P < 0.001$) of its pre-level, and gradually returned to baseline level within 1 week. A significant difference in night-time spontaneous activity between the two groups was observed until day 5 post-injection ($P < 0.001$).

3.3 Peripheral infection-induced neuroinflammation

In order to confirm whether the peripheral infection would induce neuroinflammation in the brain, a PET scan with [^{18}F]DPA-714 was performed in rats from both groups 1 day after the poly I:C or saline injection. As shown in the representative PET images (Figure 3), [^{18}F]DPA-714 radioactivity was barely observed within the brain in the saline-injected rats, except in the choroid plexus in the cerebral ventricles and some surrounding circumventricular area. However, the radioactivity of [^{18}F]DPA-714 apparently increased throughout the brain regions after the poly I:C injection, especially in the mesencephalon and medulla, as well as in the cerebellum. A voxel-based statistical analysis showed that the accumulation of [^{18}F]DPA-714 significantly increased in the several brain regions following poly I:C injection, including the dorsal raphe (DR), parvicellular part of red nucleus (RPC), central medial thalamic nucleus (CM), parabrachial nucleus (PB), gigantocellular reticular nucleus (Gi), A5, A7, A11 nuclei, and so on (Figure 4 and Table 1).

3.4 Correlation between regional neuroinflammation and fatigue-like behavior

Finally, to assess whether and how those regional neuroinflammations cause peripheral infection-induced symptoms, we analyzed the correlation between the [^{18}F]DPA-714 accumulation in all the brain regions showing significant increment and the fever or fatigue-like behavior. The correlation analysis revealed that the [^{18}F]DPA-714 accumulation in the DR, RPC and A5 positively correlated with the persistent fatigue severity defined by decrease in spontaneous activity from day 2 to day 5 following the poly I:C injection (Figures 5A–C). Moreover, a tendency towards a positive correlation of the [^{18}F]DPA-714 accumulation with body temperature was observed in the A7 noradrenergic nucleus (Figure 5D).

4 Discussion

In this study, we demonstrated that regional neuroinflammation caused by peripheral infection could be involved in fatigue and related symptoms, such as fever. Here, we provide lines of evidence that 1) transient fever and suppressed spontaneous activity lasting a few days were observed after an intraperitoneal injection of poly I:C, which has been widely used for induction of pseudoinfection; 2) an increased accumulation of [^{18}F]DPA-714 was found in widespread brain regions 1 day after treatment with poly I:C; 3) a voxel-based statistical analysis showed that a significant increment of [^{18}F]DPA-714 accumulation in the brain regions was closely related to fatigue-like behavior. Indeed, the accumulation of [^{18}F]DPA-714 in the DR, RPC, and A5, was positively correlated with fatigue severity, and

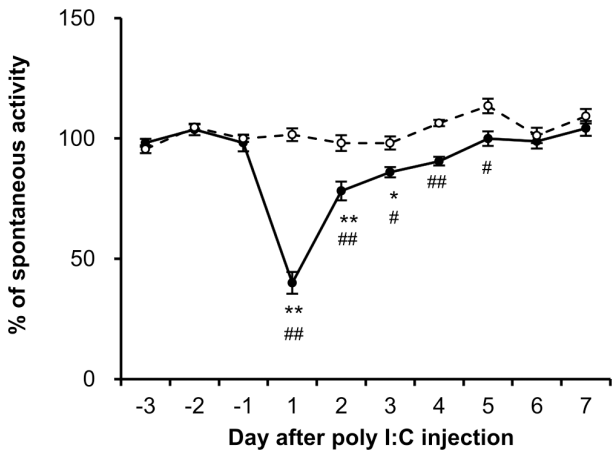

FIGURE 2
Dynamics of night-time spontaneous activity induced by poly I:C intraperitoneal injection. The spontaneous activity of each rat from control (open circles, $n = 6$) and poly I:C-treated (closed circles, $n = 6$) groups was recorded from 3 days prior to injection, and the percentage of night-time spontaneous activity was normalized by the mean value over the course of the 3 days (-3 to -1). Each value represents the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. pre-injection level. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. control group.

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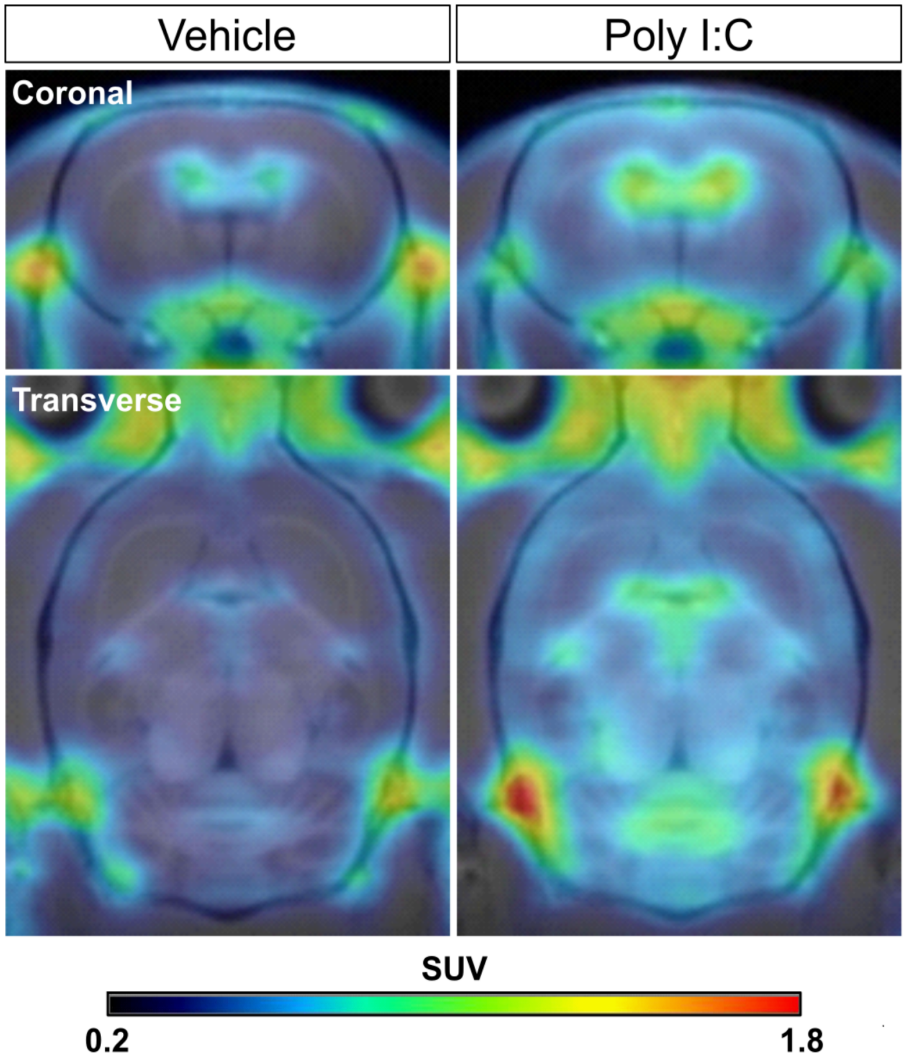

FIGURE 3
Representative [^{18}F]DPA-714 PET images co-registered with an MRI template in the saline- and poly I:C-treated rats. The coronal and transverse views of representative PET images were shown. PET scan with [^{18}F]DPA-714 was performed in rats from both groups at 24 h after poly I:C or vehicle (saline) injection. PET images were reconstructed with a MAP algorithm and summed from 5 to 45 min following a [^{18}F]DPA-714 bolus injection. MAP, statistical maximum a posteriori probability.

that in the A7 tended to positively correlate with fever. To our knowledge, this is the first brain-wide investigation to determine the region specific neuroinflammation induced by peripheral infection that may relate to fatigue and specific related symptoms.

Pro-inflammatory cytokines, including IL-1β, are known to activate the primary afferent nerve terminal or IL-1 receptors present on perivascular macrophages and endothelial cells, resulting in neuroinflammation following peripheral infection (19–21). An increase in plasma IL-1β concentrations was observed, suggesting that these two signaling pathways may represent pathways for conveying immune signals from the periphery to the brain, in the present study.

The main finding of the present study is that regional neuroinflammation in several brain regions may relate to the pathophysiology of fatigue-like symptoms following peripheral infection, such as the DR, RPC, and A5. Since a PET imaging technique provides a non-invasive approach for the quantitative evaluation of neuroinflammation *in vivo*, the association of regional neuroinflammation with consequent behavioral changes may be observed in the same animal. In the present study, we found that the peripheral infection-induced regional neuroinflammation in the DR was positively correlated with the subsequent fatigue-like symptoms. Functional alternations in the brain serotonergic system have long been implicated in fatigue development and sensation (22). It has been suggested that dysfunction of the serotonergic system could represent an underlying mechanism involved in chronic/pathogenic fatigue (23). In exercise-induced acute/physiological fatigue, the increased biosynthesis and release of serotonin (5-HT) in several brain regions have been reported to be involved in fatigue sensations (23, 24). In contrast, selective

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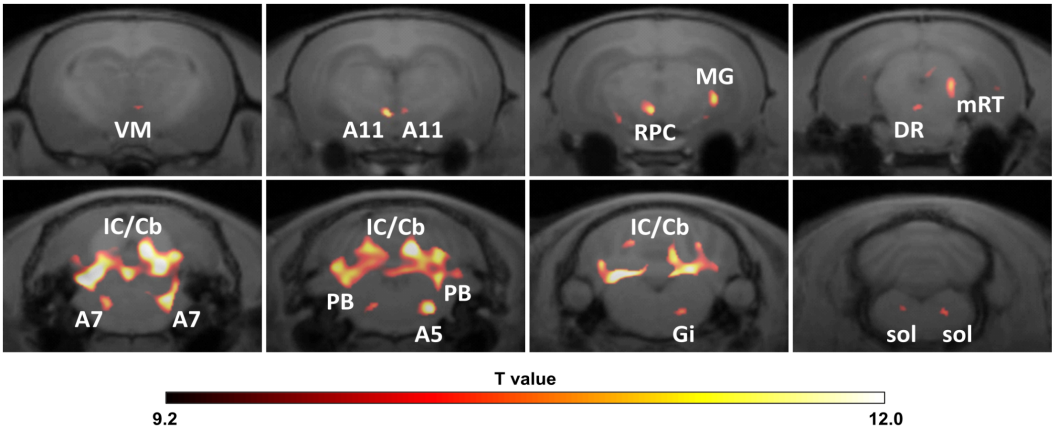

FIGURE 4
Significant increment of regional neuroinflammation following peripheral pseudoinfection. Images were obtained by voxel-based statistical comparison of [^{18}F]DPA-714 accumulation in entire brain regions between vehicle (saline) (n = 8) and poly I:C (n = 8) injected rats and co-registered with an MRI template. The T value of 9.14 was used as the threshold corresponding to the $P < 0.005$ FWE threshold. The right side of images corresponds to the right hemisphere. A5, A5 noradrenergic nucleus; A7, A7 noradrenergic nucleus; A11, A11 region; DR, dorsal raphe nucleus; Gi, gigantocellular reticular nucleus; IC/Cb, inferior colliculus/cerebellum; mRT, mesencephalic reticular formation; MG, medial geniculate nucleus; PB, parabrachial nucleus; RPC, parvicellular part of red nucleus; sol, nucleus tractus solitarius; VM, ventromedial thalamic nucleus.

TABLE 1 Brain regions of significantly increased [^{18}F]DPA-714 accumulation following peripheral pseudo infection.

		T value	Volume
Brain regions	Laterality	(peak)	(mm^3)
Ventromedial thalamic nucleus, VM	R	9.88	0.16
A11 dopaminergic nucleus, A11	L/R	12.02/10.04	0.5/0.19
Red nucleus, parvicellular part, RPC	L	11.76	0.45
Medial geniculate nucleus, MG	R	11.46	1.39
Mesencephalic reticular formation, mRT	R	10.7	0.68
Dorsal raphe nucleus, DR		10.09	0.17
Dorsolateral periaqueductal gray, DLPAG	R	10.42	0.77
Hippocampus, HC	L	10.5	0.63
Precuneiform area, PrCnF	R	10.8	0.6
Subiculum, transition area, STr	R	10.9	0.37
Entothinal cortex, Ent	R	11.4	0.61
Parasubiculum, PaS	L	10.4	0.34
Cuneiform nucleus, CnF	R	11.31	0.58
Parabrachial nucleus, PB	L/R	12.37/11.4	0.74/0.36
A7 noradrenergic nucleus, A7	L/R	9.83/11.76	0.27/1.25
Pontine reticular nucleus, oral part, PnO	L	9.29	0.96
A5 noradrenergic nucleus, A5	R	12.46	0.98
Gigantocellular reticular nucleus, Gi	R	10.82	0.67
nucleus tractus solitarius, Sol	L/R	9.93/11.56	0.13/0.75
Inferior colliculus/Cerebellum, IC/Cb		13.34	29.58

Vehicle (Saline) (n = 8) versus Poly I:C (10 mg/kg) (n = 8). Height threshold: T = 9.14 with an extent threshold of 200 contiguous voxels, p < 0.005 Familywise Error (FWE) corrected.

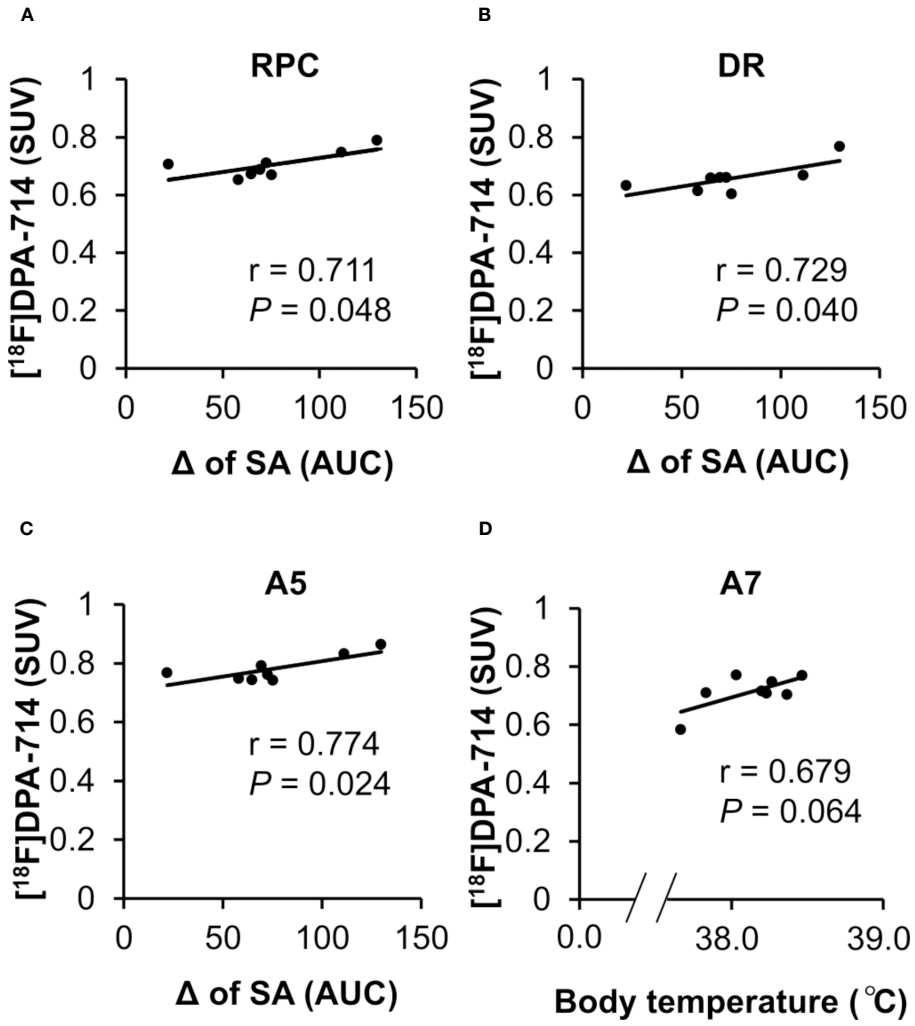

FIGURE 5
Correlation between regional neuroinflammation in brain areas and fatigue-like behavior. **(A–C)** The correlation between neuroinflammation in the RPC, DR and A5 and night-time spontaneous activity. The positive correlation between the accumulation of [^{18}F]DPA-714 in the RPC, DR, and A5 at 24 h after poly I:C injection with a prolonged decrease in night-time spontaneous activity from day 2 to day 5 following poly I:C injection. The Pearson coefficient value (r) is shown for each relation. **(D)** The correlation between neuroinflammation in the A7 and body temperature. The tendency towards a positive correlation between the accumulation of [^{18}F]DPA-714 in the A7 at 24 h following poly I:C injection and an elevated body temperature following poly I:C injection. The Pearson coefficient value (r) is shown for the relation. A5, A5 noradrenergic nucleus; A7, A7 noradrenergic nucleus; DR, dorsal raphe nucleus; RPC, parvicellular part of red nucleus; SA, night-time spontaneous activity.

serotonin reuptake inhibitors, which result in an increase in extracellular serotonin concentration, have been demonstrated to be effective for some patients with CFS. A gene polymorphism analysis in CFS patients by our group demonstrated that the frequency of longer (L or XL) allelic variants of the 5-HT transporter (5-HTT) promoter region was significantly increased compared to that in controls, pointing to elevated 5-HTT expression and low levels of extracellular 5-HT concentrations in CFS patients (25). Moreover, clinical studies have also demonstrated that the upregulation of 5-HTT and consequent reduction of extracellular 5-HT levels were observed in IFN-α and IFN-γ therapies to treat various forms of cancer and hepatitis C, in which patients often complain of serious tiredness (26, 27). These observations suggest that the dysfunction of serotonergic system could represent an underlying mechanism involved at least in chronic/pathogenic fatigue. Along with the fact that neuroinflammation is known to induce dysfunction or decline in regional neural activity (28), the results in the present study suggest that regional neuroinflammation in the DR probably cause fatigue-like behavior via functional changes in the serotonergic system. In addition, neuroinflammation in the RPC and the A5 noradrenergic nucleus were also positively associated with fatigue-like behavior. Recently, a positive correlation has been reported between the magnitude of atrophy in the superior cerebellar peduncle (Scp) which envelops and traverses the RPC at all rostrocaudal leves, and fatigue severity in multiple sclerosis patients (29), and such volumetric variation in the Scp was then considered as an early structural change preceding fatigue development (30). Overall, these observations suggest that regional neuroinflammation in these brain areas could be a plausible mechanism underlying

peripheral infection-induced fatigue-like symptoms. Incidentally, chronic fatigue has been reported to be one of most frequently reported symptoms following COVID-19 infection, in which the elevation of IL1-family cytokines was also observed (7, 31), suggesting that a similar mechanism underlying neuroinflammation in multiple brain regions might be involved in such fatigue evoked by COVID-19.

In the present study, we also found that neuroinflammation in several other brain regions, including the A7, A11, CM, PB, and Gi, was significantly increased, but was not correlated with fatigue-like behavior. The tendency towards a positive association between [^{18}F] DPA-714 accumulation in the A7 and fever was observed following poly I:C treatment. Peripheral infection-induced PGE2 in A7 has been reported to suppress the inhibitory innervation of the A7 noradrenergic nucleus to the rostral medullary raphe (RMR), resulting in fever (32). In the present study, although the correlation was weak owing to the mismatched [^{18}F]DPA-714 PET scan timing, it indicated that neuroinflammation in A7 may be implicated in fever (Figure 1A). Taken together, these results suggested that the peripheral infection-induced diverse symptoms were probably attributed to regional neuroinflammation in specific brain areas.

In conclusion, in the present study, we performed a brain-wide investigation to provide prospective evidence of the brain regions of peripheral infection-induced neuroinflammation. We also demonstrated the effect of regional neuroinflammation to fatigue and specific related symptoms. Future research is needed to further clarify the multiple interactions of these symptoms, which will aid in the development of more effective treatment strategies based on anti-inflammatory effects to address all fatigue related symptoms.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by Institutional Animal Care and Use Committee of RIKEN, Kobe Branch. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

DL: Formal Analysis, Investigation, Writing – original draft, Writing – review & editing. DH: Formal Analysis, Investigation, Writing – original draft, Writing – review & editing. YO: Formal Analysis, Investigation, Writing – original draft, Writing – review & editing. WA: Methodology, Resources, Writing – original draft. AM: Methodology, Resources, Writing – original draft. MS: Investigation, Writing – original draft. YWu: Investigation, Writing – original draft. EH: Investigation, Writing – original draft. HN: Investigation, Writing – original draft. TT: Investigation, Writing – original draft. YWad: Data curation, Methodology, Writing – original draft. FL: Formal Analysis, Writing – review & editing. HD: Methodology, Resources, Writing – original draft. YWat: Formal Analysis, Supervision, Writing – review & editing. YC: Conceptualization, Formal Analysis, Investigation, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1261256/full#supplementary-material

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EDITED BY
Wassim Elyaman,
Columbia University, United States

REVIEWED BY
Kristen E. Funk,
University of North Carolina at Charlotte, United States
Marta Olah,
Columbia University, United States

*CORRESPONDENCE
Amanda M. Brown
abrown76@jhmi.edu

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Microglial- neuronal crosstalk in chronic viral infection through mTOR, SPP1/OPN and inflammasome pathway signaling

Catalina Argandona Lopez[1] and Amanda M. Brown[1,2]*

[1]Division of Neuroimmunology, Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, MD, United States, [2]Division of Neuroimmunology, Department of Neurology and Neuroscience, Johns Hopkins University School of Medicine, Baltimore, MD, United States

HIV-infection of microglia and macrophages (MMs) induces neuronal injury and chronic release of inflammatory stimuli through direct and indirect molecular pathways. A large percentage of people with HIV-associated neurologic and psychiatric co-morbidities have high levels of circulating inflammatory molecules. Microglia, given their susceptibility to HIV infection and long-lived nature, are reservoirs for persistent infection. MMs and neurons possess the molecular machinery to detect pathogen nucleic acids and proteins to activate innate immune signals. Full activation of inflammasome assembly and expression of IL-1β requires a priming event and a second signal. Many studies have demonstrated that HIV infection alone can activate inflammasome activity. Interestingly, secreted phosphoprotein-1 (*SPP1*/OPN) expression is highly upregulated in the CNS of people infected with HIV and neurologic dysfunction. Interestingly, all evidence thus far suggests a protective function of *SPP1* signaling through mammalian target of rapamycin (mTORC1/2) pathway function to counter HIV-neuronal injury. Moreover, HIV-infected mice knocked down for *SPP1* show by neuroimaging, increased neuroinflammation compared to controls. This suggests that *SPP1* uses unique regulatory mechanisms to control the level of inflammatory signaling. In this mini review, we discuss the known and yet-to-be discovered biological links between *SPP1*-mediated stimulation of mTOR and inflammasome activity. Additional new mechanistic insights from studies in relevant experimental models will provide a greater understanding of crosstalk between microglia and neurons in the regulation of CNS homeostasis.

KEYWORDS

neuroimmunology, neuroinflammation, microglia, latency, human immunodeficiency virus, neurodegeneration, neurological disorders, integrins

Introduction

Neurologic and gait disturbances were hallmark features of HIV-1 disease in the 1980s demonstrating the profound negative impact of the virus on central nervous system (CNS) functioning (1). The clinical manifestations of NeuroHIV can include cognitive impairment, depression, anxiety, and deficits in fine motor movements (2, 3). Comprehensive neuropsychological testing is used to identify people with HIV-associated neurocognitive disorder, now more generally known as NeuroHIV, to reflect the changing clinical spectrum of neurologic and psychiatric co-morbidities (4–6). Seminal neuropathology studies on HIV-infected post-mortem human brain tissue identified brain microglia and macrophages (MMs) as the predominant cellular targets of the virus (7–10). Through different mechanisms, HIV-infected monocytes, T-cells, and viral particles cross the blood-brain-barrier, which itself becomes impaired (11–14). Targeted antiretroviral therapies (ART), first introduced in 1996, were highly effective at blocking virus replication and sparing CD4+ T-cell death and immune system dysfunction (15). Many ART regimens reach pharmacological levels in the CSF; however, whether inhibitory concentrations reach regions in the brain parenchyma, where HIV-infected MMs reside, remains unclear (16–18). Additionally, yolk sac-derived microglia are relatively long-lived cells with a turnover of many months, and their capacity for self-renewal provides a sanctuary for HIV in brain tissue (19–21). Even under conditions of low-level HIV gene expression, immune activation in the form of increased circulating pro-inflammatory cytokines and immune markers are present in people with HIV on ART (22, 23).

HIV encodes nine genes that co-opt intrinsic immune cell pathways normally used for growth, metabolism and homeostasis (24, 25). Innate immune signaling is an early detection system meant to thwart pathogen replication by activating the release of inflammatory molecules that, in turn, prime adaptive immunity (26–28). HIV-1 binds to CD4 and chemokine receptors, in a process that initiates fusion of the viral and plasma membranes (Figure 1). Neurons express chemokine receptors that support neuronal development and maturation, but not CD4, and therefore, do not allow HIV entry (33). Viral fusion is followed by the release and trafficking of the preintegration complex (PIC) to the nucleus (Figure 1). The PIC uncoating process within the nucleus was first shown for primary human macrophages years ago (29, 31), but only recently confirmed for T-cells (34). This mechanistic detail has important implications for understanding whether HIV can delay detection by nucleic acid sensors that activate Toll-like receptor (TLR) signaling (24, 30). Importantly, in MMs, virus is packaged in vesicular bodies and buds from the plasma membrane in contrast to the cytopathic release of viral particles from T-cells (35, 36).

Microglia not only protect the brain from pathogens and injury, but also serve critical roles in maintaining neuronal viability, proper synaptodendritic function and integrity in development and over the lifespan (20, 21, 37–39). Understanding the mechanisms by which

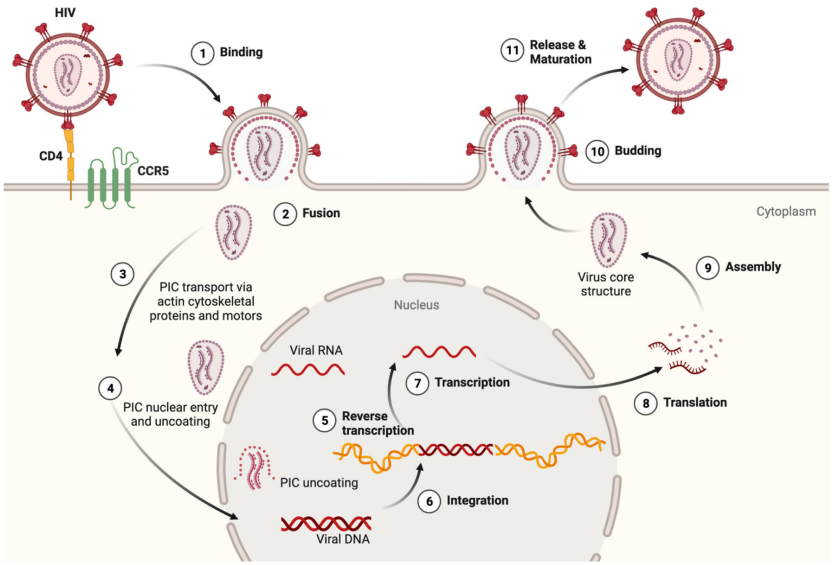

FIGURE 1
HIV lifecycle and relevance to inflammasome activation. (1) At target cell plasma membrane domains, HIV envelope protein gp120 trimer (red) binds to the CD4 receptor (yellow). Conformational alterations expose binding surfaces for coreceptor CCR5 on the Env trimer (green). (2) Fusion of Env with the cell plasma membrane is followed by uncoating and release of the preintegration complex (PIC) which contains a few molecules of reverse transcriptase, integrase and two copies of HIV RNA (vRNA) (29–31). Should the integrity of the PIC be compromised, viral RNA and proteins could be detected by innate immune sensors and thus initiate an inflammasome priming. (3) The actin cytoskeleton and specific microtubule motors transport the PIC to the nucleus (32). (4) The PIC can enter the nucleus in several ways including direct nuclear import and induced invaginations (24, 30). Degradation of the nucleoprotein coat would expose vRNA outside the nucleus, and provide another opportunity to activate innate antiviral responses. After reverse transcription (5), integration (6), transcription (7), and translation (8), viral proteins, vRNA, and (9) certain host proteins assembled at the inner plasma membrane surface. This mobilizes cytoskeletal proteins and molecular forces that facilitate budding (10), (11) release and maturation of new viral particles (32). Macrophages and microglia unlike T-cells are much more resistant to the cytopathic effects of HIV replication and therefore undergo innate immune activation in a sustained fashion. The figure was created with BioRender.

HIV-1 affects microglial innate immune function is key to addressing the brain as a source of pathologic neuroinflammation correlated with neurological and psychiatric comorbidities (18, 23, 40, 41). Below, we discuss what is known about HIV activation of the inflammasome, particularly as it relates to microglia and neurons and the expression of specific pro-inflammatory cytokines that remain elevated in people with NeuroHIV. We then discuss another innate sensor, secreted phosphoprotein-1 (or osteopontin, *SPP1*/OPN), and its intersection with the mammalian target of rapamycin pathway (mTOR) and potentially the inflammasome to provide a unifying view of putative mechanistic connections and cell-type dependent crosstalk between the pathways.

HIV activation of inflammasome signaling in the CNS

As the exploration of inflammasome function has progressed, NLRP3 is implicated in a variety of neurodegenerative diseases, including NeuroHIV (42–48). The inflammasome is a multiprotein complex involved in the immune and inflammatory response. Different inflammasomes types exist in the nucleotide-binding oligomerization domain, Leucine-rich-containing proteins (NLR) family (49). However, all inflammasomes contain key components including: NALP/NLR protein, PYCARD/ASC (Apoptosis-associated speck-like protein containing a CARD), and an enzyme responsible for pro-inflammatory cytokine activation (50–52). The NLRP3 inflammasome complex interacts with caspase-1 to activate IL-1β and IL-18 (53, 54). Both are pro-inflammatory cytokines that play various roles throughout the body. In microbial infections, the increase in IL-1β secretion is responsible for recruiting innate immune cells. In neurodegenerative diseases, IL-1β levels increase in response to microglial activation and neuronal injury (55, 56). IL-18 induces IFN production in T-cells and natural killer cells, promotes the production of other cytokines, and is suggested to exacerbate demyelination and cellular infiltration (44, 57).

NLRP3 inflammasome assembly needs two signals: a priming and an activating signal (58–60). Of the many ways to prime the inflammasome, the most studied route is through NFkappaB-dependent signals (Figure 2). Many ligands can prime the NLRP3 inflammasome, including lipopolysaccharide (LPS) and TLR inducers like dsRNA (59–61). During reverse transcription, dsRNA can be detected by intracellular, endosome-bound TLR3 (Figure 2) (32, 65). TLR3 ligand binding activates ERK 1/2, MAPK, and NFkappaB-pathways, promoting gene transcription (62). Interestingly, the HIV transactivator of transcription (Tat) protein alone can prime and activate the inflammasome complex (Figure 2) (66). Various ligands such as, ATP, nigericin, aggregated proteins, reactive oxygen species (ROS), and HIV viral proteins activate the NLRP3 inflammasome (46, 49, 58, 61, 66–69). These signals allow for the recruitment of additional proteins like NLRP3, ASC, and caspase-1 that are necessary for oligomerization and subsequent cleavage and maturation of cytokines (62). Caspase-1 also cleaves gasdermin D, leading to cell membrane pore formation, and a type of pro-inflammatory cell death known as pyroptosis (Figure 2) (62).

The NLRP3 inflammasome is robustly expressed in microglia (42, 59). However, whether the same is true for neurons is less well known. Interestingly, neurons undergoing pyroptosis have been documented (70–72). This is important since pyroptosis is strongly associated with NLRP3 inflammasome activation (73–77). The NLRP1 and AIM2 inflammasome complexes of cortical neurons have been the most investigated (70–72). Recently, studies reported that dopaminergic neurons express NLRP3 throughout the progression of Parkinson's disease (45, 47). However, activation of NLRP3 in microglia contributes to demyelination through IL-1β and IL-18 secretion (44). HIV-positive individuals have increased caspase-1, IL-1β, and IL-18 levels, suggesting NLRP3 inflammasome activation systemically and in the CNS (78–80). Given the association between neurologic disorders, neuroinflammation, and the activation of the NLRP3 inflammasome in microglia and neurons, the potential for crosstalk between these cells is expected.

HIV induced inflammasome activation and mTOR signaling in NeuroHIV

There is renewed interest in mTOR signaling in HIV infection as new roles for this pathway have emerged. Early studies implicated a role for mTORC signaling in promoting virus replication (81–83). Most recently, mTORC-regulated mechanisms in HIV escape from latency in T-cells (84), autophagy (85), apoptosis (86), and the homing of intestinal CCR6+CD4+ T-cells (87) have been reported. Interestingly, in efforts to identify new candidate genes involved in latent HIV infection, a role for pro-inflammatory cytokines and signaling pathways regulated by secreted phosphoprotein-1/osteopontin (*SPP1*/OPN) were discovered (88). The mTOR pathway is composed of two structurally distinct, multi-subunit protein complexes, mTORC1 and mTORC2 that receive signals about a cell's metabolic status to fine tune growth and repair processes through activation of relevant transcriptional programs (89, 90). HIV-positive individuals have dysregulated autophagy, indicating upregulated levels of mTOR activity (91). Increases in mTOR activity are associated with reactive microglia, neuronal damage, neurodegeneration, and memory deficits, all characteristics of NeuroHIV (63, 92). Although scarcely investigated, evidence of a regulatory relationships between mTOR and NLRP3 in immune cells and neurons have been reported. Studies have shown that downregulating mTOR activity reduces NLRP3 activation (93–96). With reduced mTOR activity, autophagy removes detrimental pro-inflammatory stimuli, including ROS. Indeed, ROS activates the NLRP3 inflammasome and has been associated with NeuroHIV (97, 98). Another study found that inhibition of mTORC1 leads to decreased secreted IL-1β, indicating post-transcriptional effects on NLRP3 activation (94). A similar regulatory relationship was observed with *in vitro* and *in vivo* NLRP3 knock-out studies in which mTOR activity decreased (93, 99). In macrophages an interaction between NLRP3 and mTOR was found, indicating a

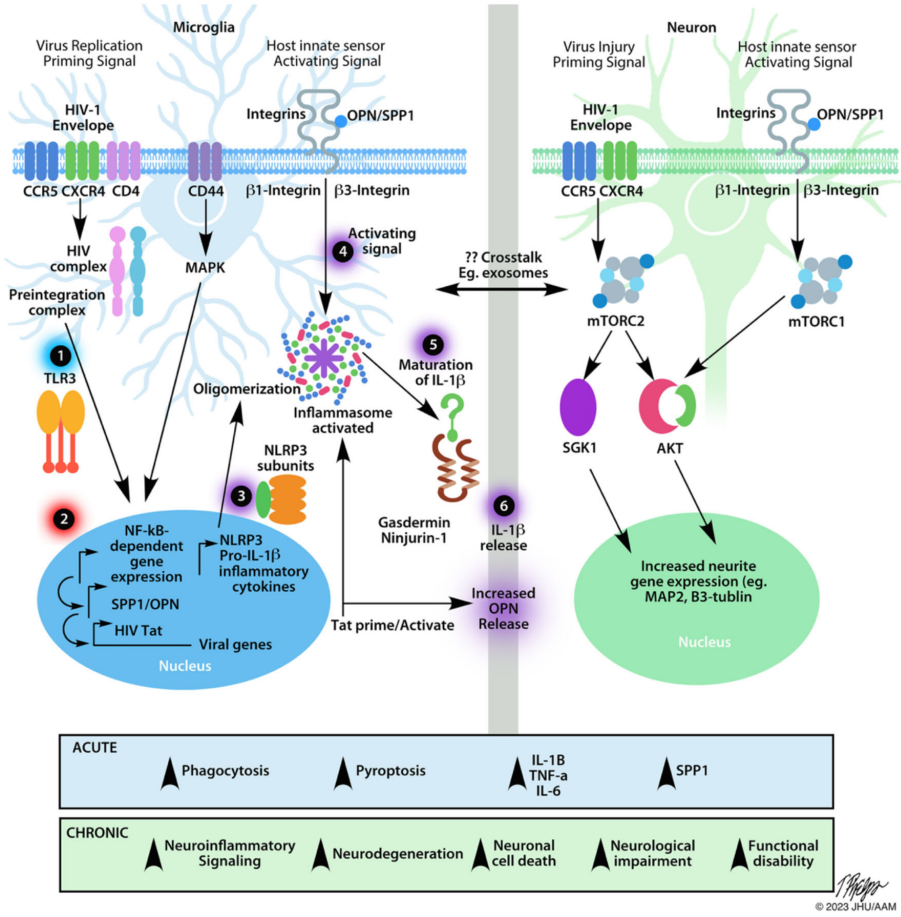

FIGURE 2
Schematic of the effects of HIV infection on microglia and neurons. The NLRP3 inflammasome is a multi-protein complex implicated in many neurodegenerative diseases including HAND. After HIV crosses the blood-brain barrier, it can bind to CD4+ cells, such as microglia, initiating the fusion of the virus to the plasma membrane, ultimately allowing HIV to enter the cell. After infiltrating the cell, many different aspects can affect the transcriptional activity of microglia via the NF-$\kappa\beta$ pathway. Microglia. Step 1 indicates the first step required for inflammasome assembly: the priming step. Many stimuli can prime the NLRP3 inflammasome, including dsRNA for endosome-bound TLR3 (58, 59, 61, 62). Priming of the inflammasome leads to the localization of NF-$\kappa\beta$ into the nucleus, indicated by Step 2. Along with host gene transcription, proteins like HIV TAT can be transcribed, which can act to prime/activate the inflammasome. Step 3 indicates the availability of the NLRP3 subunits necessary for the inflammasome to be oligomerized such as the NLRP3 protein, apoptosis-associated speck-like protein (ASC), and pro-caspase-1 (62). Step 4 indicates the activating step in NLRP3 inflammasome activation. Various stimuli, such as extracellular TAT protein, can trigger the activating signal. After receiving an activating signal, the NLRP3 inflammasome can begin its oligomerization and become functional. Pro-interleukin enzymes are recruited to be cleaved into their mature forms. For the NLRP3 inflammasome, IL-1B and IL-18 are cleaved by Caspase-1 and released, as shown by Steps 5, 6. The release of NLRP3-associated pro-inflammatory cytokines occurs via pores formed in the cell membrane. Caspase-1 will also cleave gasdermin D, leading to pyroptosis (62). The release of cytokines and viral proteins can then exacerbate local inflammation, leading to the recruitment of more immune cells and can affect other cell types, such as neurons. Neurons. Considering that HIV is unable to infect neurons directly, there are many examples of HIV-induced neuronal damage. One major contributor is the HIV-1 gp120 (Env). This protein can bind to CXCR4 and CCR5 receptors, expressed on neurons. HIV-1 Env has been shown to damage synaptic connections in cortical neurons when bound to CXCR4 via mTORC2 (63). When neurons were co-treated with HIV-1 Env and OPN/SPP1. Neurons showed signs of activated mTORC1/mTORC2 pathways, suggesting a regulatory feedback loop. Along with the required β1 and β3 integrin receptors, which OPN/SPP1 binds to, OPN/SPP1 acts as a neuroprotective modulator that promotes neurite growth in cortical neurons (β1 integrin) and regulates post-synaptic dendritic spine density in hippocampal neurons (β3 integrin) through mTORC1 (63, 64). Despite the protective effects of OPN/SPP1, over time, HIV-infected individuals present with neuronal degradation. Crosstalk. HIV-infected microglia have increased levels of NLRP3 activity, leading to pyroptosis and the release of highly pro-inflammatory cytokines. Given the role of IL-1β in inflammation, it is important to consider the various impacts it can have on the local microenvironment. Surrounding cells will respond to the inflammatory signal, such as upregulating SPP1/OPN. Regardless of the intent to reduce neuroinflammation, we see that HIV-infected individuals continue having low levels of chronic inflammation while on antiretroviral treatment. When looking at the acute effects, there is an increase in microglial phagocytosis, pyroptosis, pro-inflammatory cytokines, and OPN/SPP1 secretion. Chronically, we begin to see prolonged neuroinflammatory signaling, neurodegeneration, neuronal cell death, neuronal impairment, and functional disability, indicating the urgency to understand better the mechanisms of disease progression, cellular interactions, and regulation of neuroinflammatory pathways in HIV infection.

direct protein-protein interaction and communication between both pathways (93). Lastly, IL-1β can activate mTOR in T-cells and in hippocampal neurons further illustrating NLRP3 cell-specific- and cell-to-cell communication pathways and functional outcomes like neuroinflammation (100–102). The emerging relationships between NLRP3, mTOR, and HIV infection becomes more interesting when considering the function of additional innate immune sensors like *SPP1*/OPN.

Innate signaling pathways collide: SPP1/OPN and mTOR activation in NeuroHIV

The term neuroinflammation, as it is currently understood, broadly signifies a mix of innate and adaptive responses of resident brain- and circulating immune cells that, if left unregulated, can have damaging short- and long-term consequences (103). In this regard, chronic expression of proinflammatory molecules leads to over activation of the immune system and accumulation of damage and disability with time. Secreted phosphoprotein-1 (*SPP1*/OPN), by virtue of its modular domain structure, is a multifunctional phosphoprotein implicated in several neurodegenerative diseases (104–110). The expression of *SPP1*/OPN is markedly elevated in the CNS of humans and non-human primate models of HIV infection (109, 110). However, more recent findings with humanized mice and positron emission tomography neuroimaging demonstrate that *SPP1*/OPN expression is required to downregulate the microglial inflammatory response (111). How exactly *SPP1*/OPN modulates the HIV-induced inflammatory response in the brain is not yet understood. However, in cultured primary human macrophages, HIV replication and NF-$\kappa\beta$ activity is increased in the presence of *SPP1*/OPN (110). The degree of neuroinflammation correlated with the extent of HIV replication only in humanized mice expressing *SPP1*/OPN (111). Neurons cannot be infected with HIV due to their lack of the CD4 receptor, however the presence of certain chemokine coreceptors like CCR5 or CXCR4 makes them vulnerable to excitotoxicity, degeneration and death after binding interactions with HIV Gp120 (33, 112). However, treatment of neurons with recombinant OPN protects hippocampal post-synapses from synaptodendritic injury, and the structural integrity of cortical axons and dendrites via mTORC1/mTORC2 activation (Figure 2) (63, 64). Therefore, in NeuroHIV, increased expression of *SPP1*/OPN is largely neuroprotective.

The intersection of SPP1/OPN, mTOR and inflammasome signaling in neurodegenerative disorders

We first hypothesized that the overexpression of *SPP1*/OPN in individuals with NeuroHIV was harmful, but as discussed above the findings thus far point to a neuroprotective function. While there is increasing evidence of linkages between neurodegeneration and cellular repair processes involved in resolving neuronal injury and neuroinflammation, significant gaps in our understanding of the molecular mechanisms remain. *SPP1*/OPN was identified as a highly-expressed transcript that clustered with a collection of genes termed "disease-associated microglia (DAM) (113–115). Recent studies by Rentsendorj et al., and Qiu et al., beautifully demonstrate using the ADtg and 5XFAD mouse models for AD, respectively roles for specific populations of *SPP1*+/- expressing monocytes, resident microglia and/or macrophages in the phagocytosis of amyloid and speculate about a role for inflammasome signaling (116, 117). In contrast, in a slow-progressing model of AD (App^{NL-F} knock-in reporter mice), *SPP1*+ macrophages and microglia associated with brain blood vessels and those located in the hippocampus were responsible for pathologic microglia-synapse destruction (118, 119). In another example of neuroprotection, regulatory T-cells localized in the brain several weeks after stroke express *SPP1*/OPN and, through a microglial-β1-integrin-dependent manner, foster repair of white matter axonal damage (120). In a model of glaucoma, a protective role for SPP1/OPN was found (117). Interestingly, in an ischemia model, intranasal delivery of a *SPP1*/OPN peptide suppressed microglial activation and the release of pro-inflammatory cytokines IL-1β and IL-6, an indication of reduced NLRP3 activity (121). To further support this idea, Zhang et al. demonstrated that *SPP1*/OPN negatively regulates the NLRP3 inflammasome in ischemic infarction (122). Lastly, in a MS model, NLRP3 knockout, as well as one of its components ASC, reduced mRNA SPP1/OPN expression in splenic CD4+ T cells (123). Whether this same relationship exists in the CNS is unknown, though it is possible that NLRP3 priming lead to NF-kappaB transcription of *SPP1/OPN*. Given its neuroprotective function, a negative feedback loop may be in place to prevent chronic inflammation via continuous NLRP3 activation. Importantly, as more details on the molecular mechanisms of *SPP1*/OPN function continue to emerge, the information will help provide a more complete understanding of the correlative findings of clinical studies (124) and toward the design of possible efficacious therapeutic interventions.

Over the last several years, understanding of the direct role of glycolytic metabolism on effector immune cell functions has greatly increased (125–128). As such, there are opportunities for pathogens to alter and/or harness signaling dynamics that feed directly into the mTOR pathway (129–132). Tissue macrophages and microglia assume a variety of activation states in response to local cues, and downstream stimulation of mTOR signaling is implicated in their M2- (anti-inflammatory) or M1-polarization (proinflammatory), respectively (133). Interestingly, inhibition of inflammasome activation is protective against disease progression in a mouse model of multiple sclerosis. In this regard, rapamycin, an immunosuppressive agent, was shown to block antigen presentation by dendritic cells and inflammatory signaling by microglia (133, 134).

The homeostatic balance of the immune system is maintained through direct and indirect interactions and with soluble factors acting locally and over long distances (refs). HIV infection disrupts and hijacks the important cell-to-cell communication network. The virus infects T-cells and MMs robustly and astrocytes in a limited fashion (35), and cells located nearby initiate a signaling cascade that amplifies locally, and recruits additional immune cells from a distance. This idea of cellular crosstalk was investigated by Wang and Gabuzda, who saw that direct contact between neurons and microglia was not necessary for neuronal damage (135). The same study also found that activated astrocytes promoted HIV replication in microglia. In this regard, as discussed above, mTOR signaling in cultured cortical neurons preserves structural integrity, however increased mTOR activity can also be detrimental to cells of the brain (133, 134, 136). Cortical neurons, as well as infected microglia may, in turn, be upregulate and secrete OPN/SPP1 to reduce the inflammatory response by inactivating the NLRP3 inflammasome

in microglia, and promoting neuronal survival through mTOR activity. Decreased mTOR activity in astrocytes is primarily beneficial, but negatively affects their ability to differentiate (133, 134, 136). In oligodendrocytes, decreased mTOR activity impairs their differentiation and myelination functions (133, 134, 136). The release of damage signals and proinflammatory molecules from impaired glial cells, activates immune cells and neurons thus amplifying a neuroinflammatory response. An example being the rapid release of IL-1β and IL-18 from microglial pyroptosis (Figure 2). We emphasize the importance of considering that homeostasis in chronic low-level HIV infection is tightly regulated via crosstalk between different cells through secreted pro- and anti-inflammatory cytokines/chemokines. The delicate balance, or lack thereof, of a cellular local environment, can act to exacerbate or ameliorate neuroinflammation. Indeed, HIV utilizes these delicate communication pathways to promote an optimal environment for replication.

Given that microglia have receptors for OPN, it's possible that signaling by cortical OPN/SPP1 via mTOR acts on microglia to reduce the inflammatory response and increase transcriptional programs involved in preserving neuronal function. Given their opposing, yet collaborative, roles in inflammation, it is important to investigate the relationship between *SPP1*/OPN, mTOR, and NLRP3 in HIV-induced neuroinflammation and NeuroHIV. In this regard, more research is needed to get a better understanding of the molecular and cellular mechanisms that take place in chronic HIV infection. Doing so would allow us to understand better how HIV manipulates the host's protective measures, allowing for better treatments aimed to improve the host response to latent HIV infection, guiding us toward a solution to eliminate HIV-associated neuroinflammation and cognitive deficits.

Discussion

There is a greater appreciation that during development and adulthood, dynamic homeostatic regulation of the brain's neural network is intertwined with and dependent on crosstalk and connectivity with glial. Disruption of the integrity of the brain, as seen in viral infection, leads to activation of what are meant to be protective responses, resulting in a neuroinflammatory response involving resident brain cells and immune sentinels that conduct tissue-level surveillance. As reviewed herein, innate immune signaling, including mTOR, SPP1/OPN, and NLRP3 inflammasome activation, is initiated to monitor and/or alter cell metabolic state, stimulate repair, migration, and other immune effector processes. Given that several myeloid and glial cells and cofactors can contribute and stimulate autocrine and paracrine feedback and feed-forward looping, how are the outputs integrated to restore homeostatic levels of regulation and surveillance? Deeper insight into the physiological, cellular, and molecular mechanisms will help to advance the development of effective interventions to help those suffering from neurological and neuropsychiatric comorbidities related to chronic over-activated innate immune responses in the central nervous system.

Author contributions

AB: Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing. CA: Conceptualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Andrew MacLean,
Tulane University, United States

REVIEWED BY
Fabrícia Lima Fontes-Dantas,
Rio de Janeiro State University, Brazil
Ahmed Nadeem,
King Saud University, Saudi Arabia

*CORRESPONDENCE
Minghua Wu
wuminghua554@aliyun.com

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Potential value and research frontiers of virus in neuroinflammation: a bibliometric and visualized analysis

Danyang Li[1,2] and Minghua Wu[1,2]*

[1]Hunan Cancer Hospital/the Affiliated Cancer Hospital of Xiangya School of Medicine, Central South University, Changsha, Hunan, China, [2]The Key Laboratory of Carcinogenesis of the Chinese Ministry of Health, The Key Laboratory of Carcinogenesis and Cancer Invasion of the Chinese Ministry of Education, Cancer Research Institute, School of Basic Medicine, Central South University, Changsha, Hunan, China

Background: Neuroinflammation represents the immune response of the central nervous system to nerve injury, infection, toxin stimulation, or autoimmunity and is implicated in a wide range of neurological disorders. Viruses play a pivotal role as extrinsic biological drivers in neuroinflammation; however, numerous aspects remain unexplored. In this study, we employed bibliometric analysis to assess the current status of viral research in neuroinflammation and anticipate future research directions and emerging trends.

Methods: Conduct a comprehensive search for scholarly publications within the Web of Science Core Collection database, with search terms on neuroinflammation and virus. Apply Microsoft Excel Office, Hiplot, R (version 4.3.1), VOSviewer (version 1.6.20) and CiteSpace (6.2.R6, advanced) software for the bibliometric analysis and visualization.

Results: A total of 4230 articles and reviews on virus and neuroinflammation were identified, demonstrating a consistent upward trend over time. The United States was the country that contributed the most publications. Approximately 22274 authors from 4474 institutions contributed to the research. Johns Hopkins University leads with the highest number of publications and citations. The top three authors with the most published articles on this field are Power, C., Lane, T. E., and Buch, S. The Journal of Neuroinflammation is the most authoritative choice for researchers. The main research focuses in this field include multiple sclerosis, Parkinson's disease, blood-brain barrier, COVID-19, Alzheimer's disease, gene therapy. In recent years, stress have emerged as hot keywords, particularly depression, human immunodeficiency virus-associated neurocognitive disorders, blood-brain barrier, gut microbiota related directions, indicating a potential shift in research focus.

Conclusion: Research on the virus and neuroinflammation has attracted increasing attention in the past decade. European and American countries have been pivotal in conducting research on virus and neuroinflammation, while China has produced a significant number of publications, its impact is

still limited. Stress is likely to emerge as the next area of focus in this field. The association and regulation between viral infection and psychiatric disorders are not fully understood, and further research is needed to explore the role of neuroinflammation caused by different types of viral infection and psychiatric disorders.

KEYWORDS

bibliometric analysis, neuroinflammation, virus, psychiatric disorders, VOSviewer, CiteSpace, hotspots

1 Introduction

Neuroinflammation is an immune response activated by glial cells in the central nervous system (CNS), typically occurring in response to nerve injury, infection, toxin stimulation, or autoimmunity (1). This response is implicated in nearly all neurological disorders. Under normal physiological conditions, neuroinflammation can effectively clear or inhibit harmful substances, aid the body's defense against pathogen invasion, and maintain internal homeostasis (2). However, excessive activation of neuroinflammation upon stimulation can lead to neuronal damage and exacerbate disease progression (3). Persistent inflammatory responses can activate glial cells (primarily microglia and astrocytes) within the CNS that serve functions such as immune surveillance and danger signal recognition while playing a pivotal role in maintaining CNS homeostasis and mediating neuroinflammatory responses (1, 4). Furthermore, recent studies have highlighted the significant contribution of peripheral systemic inflammation (5). For instance, disruption of tight junctions or endothelial cell damage impairs blood-brain barrier (BBB) function allowing infiltration of peripheral proinflammatory factors, immune cells, and harmful substances into the brain parenchyma (6, 7). Additionally, peripheral nerves transmit inflammatory signals from periphery to brain through receptors for inflammatory factors. These inflammatory factors and signals can directly or indirectly activate microglia and astrocytes leading to an inflammatory response within the CNS (8). Currently, the significance of neuroinflammation in the field of neurology is increasingly evident. Apart from well-established neuroinflammatory disorders like multiple sclerosis (MS), neuroinflammation also assumes a pivotal role in numerous ostensibly non-inflammatory neurological conditions, including Alzheimer's disease (AD), Parkinson's disease (PD), schizophrenia, stroke, and Glioblastoma (9–13).

Due to the concept of "neuroinflammation", the brain is no longer considered a closed area. There is a bidirectional and complex interaction between the nervous system and the immune system, which plays a pivotal role in perceiving internal and external environmental stimuli as well as maintaining physiological homeostasis. Due to the prolonged parasitic nature of viruses within the human body, their wide-ranging infectivity, and limited targeted treatment options in certain cases, research on virus infection and neuroinflammation has progressively emerged as a field of interest among neuropathology researchers. The earliest studies in this field focused on viral meningitis and viral encephalitis caused by enterovirus, mumps virus, herpes simplex virus, and adenovirus (14, 15), and the incidence of both is increasing year by year, with high mortality and disability rates. Due to the varying regions of brain inflammation invasion, distinct clinical manifestations arise which can lead to different degrees of neurological sequelae. This poses a significant threat to human health while also resulting in substantial economic losses. Recent investigations have gradually unveiled that viral infections substantially elevate the risk of neurodegenerative diseases such as AD and PD. Multi-omics studies have identified elevated genomic DNA levels of human herpesviruses 6A and 7 within the brains of AD patients compared with cognitively normal controls; moreover, viral abundance correlates with transcriptomic features associated with amyloid-β (Aβ) processing (16, 17). Furthermore, amidst the outbreak of Corona Virus Disease 2019 (COVID-19), research has demonstrated persistent inflammatory responses within the brain tissue of individuals with mild COVID-19 infection; additionally, COVID-19 infection significantly heightens the risk for psychiatric disorders (18). Although there is ample evidence supporting the crucial role of viral infections in neuroinflammatory disorders' development and progression, research on non-inflammatory neurological disorders mainly focuses on clinically relevant studies. Therefore, conducting an extensive investigation into neuroinflammation induced by viral infections will enhance our understanding of their involvement in non-inflammatory neurological disorders and refine our comprehension of pathological processes underlying CNS diseases, ultimately suggesting personalized treatment strategies for these diseases.

Bibliometrics emerged in the early 20th century and became an independent discipline in 1969, which has been widely used in literature analysis (19). It is a quantitative method used to describe and analyze the dynamics and progress of a certain discipline or research field. In the analysis process, detailed information such as countries, institutions, journals, authors, keywords, and references can be obtained (20). With the assistance of computer technology, the results of literature analysis can be visually represented to explore information relationships effectively.

To date, despite the extensive research conducted on the relationship between viruses and neuroinflammation, there remains a lack of clarity regarding the overall research trends in this area. In order to address this gap, this study aims to comprehensively explore the research trends and emerging hotspots concerning viruses and neuroinflammation from a bibliometric perspective, thereby providing guidance for future investigations into non-inflammatory neurological disorders caused by viral infections.

2 Materials and methods

2.1 Data source and search strategy

As a high-quality digital literature resource database covering various fields, Web of Science (WOS) has been accepted by many researchers and is the most suitable database for bibliometric analysis (21, 22). We conducted a comprehensive search for scholarly publications within the WOS Core Collection (WOSCC) database. The search strategy was set as the following: TS = ("neuroinflammation" OR "neurogenic inflammation" OR "nerve inflammation" OR "neural inflammation" OR "brain inflammation" OR "neural inflammatory" OR "neuritis" OR "nervous inflammatory" OR "CNS inflammation" OR "central nervous system inflammation" OR "neuroimmune" OR "neuroimmunology" OR "microglia activation") AND TS = (*virales OR *viridae OR *virinae OR *virus* OR "virus" OR "viruses" OR "virological" OR "viral"). The retrieved data were collected on January 31, 2024, to avoid any potential deviation due to daily updates. The publications included in this study are categorized as either Article or Review Article and are written in the English language. A total of 4230 records were exported as the format of "plain text file", and then recorded as "full record and cited references". The flowchart of the study is shown in Figure 1.

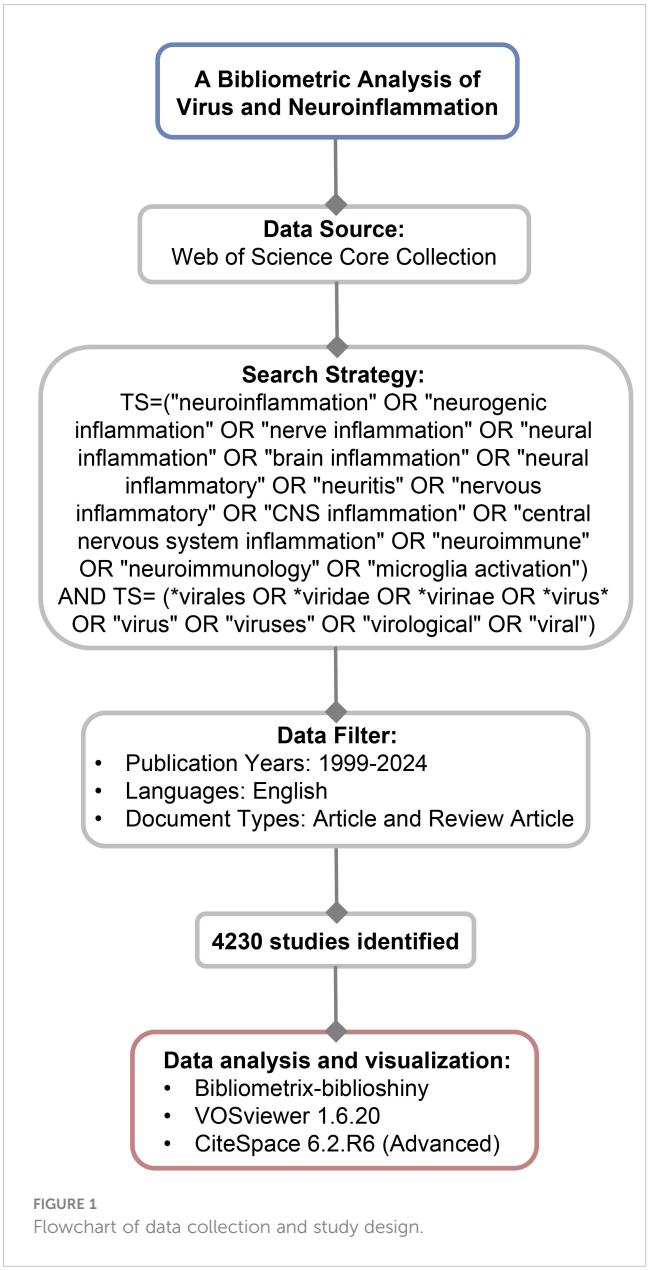

FIGURE 1
Flowchart of data collection and study design.

2.2 Data analysis and visualization

This study used Microsoft Excel Office, Hiplot, R (version 4.3.1), VOSviewer (version 1.6.20), and CiteSpace (6.2.R6, advanced) as the software tools for organizing, analyzing and visualizing data.

Microsoft Excel Office was used in this study to organize relevant data including the number of publications and citation. Hiplot (https://hiplot.com.cn) was used in this study to map the global geographic distribution.

Bibliometrix package in R software provides a set of tools for quantitative research in bibliometrics and scientometrics (23). And biblioshiny is a shiny app that presents the web interface of bibliometrix, which supports scholars to easily use the main features of bibliometrix (24). The tool can perform data importing, conversion to data frame collection and data filtering. Additionally, it can analyze and visualize the data based on three different level metrics (sources, authors and documents) and three structures of Knowledge (conceptual structure, intellectual structure and social structure). In this study, it was used to summarize the overview of the data and convert it into a dataset that can be used for R analysis and analyze the annual number of documents and citations.

VOSviewer is a free Java-based software developed in 2009 by van Eck and Waltman of Leiden University in the Netherlands (25). VOSviewer uses a data standardization method based on probability theory to analyze each information of the literature and visualize the results, which has been applied to research in many fields. VOSviewer provides three kinds of visualization views: network visualization, overlay visualization, and density visualization, which have the advantages of beautiful images and easy interpretation. The subject terms and keywords obtained from the database can be used to describe the research status and internal correlation in this field through cooperative network analysis, co-word analysis and cluster analysis. Therefore, VOSviewer was used to conduct a statistical analysis of relevant research countries,

institutions, journals, authors, keywords, and references, summarize hot research, and look forward to the research frontier.

CiteSpace is developed by Professor Chaomei Chen of Drexel University in the United States (26). It is mainly used for literature review and summary, and for visual quantitative analysis of current data. It is a widely used bibliometric analysis tool for citation space. In addition to having the same functions as VOSviewer, CiteSpace can also extract information from titles, keywords and abstracts and generate cluster labels according to log-likelihood rate (LLR), latent semantic indexing (LSI) and mutual information (MI) algorithms (27). In addition, it can also find the research hotspots and heat in different periods through the emergence of keywords, so as to analyze the development trend of the field. VOSviewer was used in this study to perform the dual-map overlay of journals, the cluster analysis, timeline visualization and burst analysis of references and keywords.

This study followed the guideline for reporting bibliometric reviews of the biomedical literature (BIBLIO) (28).

3 Results

3.1 Global trend in publication outputs and citations

The 4230 publications utilized in this study were written by 22274 authors from 4474 institutions in 106 countries, published in 1046 journals, and cited 191063 references from 13107 journals. Figure 2 illustrates the annual publication volume and annual citation frequency of related articles from 1999 to the January 2024. Overall, there has been a consistent upward trend in the number of annual publications pertaining to viruses and neuroinflammation, with a notable surge observed since the year 2021. The year with the highest number of publications is 2022, with 559 articles. With the increase of the number of publications, the annual citation frequency of relevant literature showed a rugged upward trend. The number of citations in 2022 was as high as 9780. This indicates that the research concerning virus and neuroinflammation has garnered substantial attention and emerged as a prominent area for investigation within recent years.

3.2 Distribution of countries/regions

There were 106 countries involved in publications on the virus and neuroinflammation. The geographical distribution of these countries is shown in Figure 3A, and the volume of publications is represented by color variation, which shows that the countries involved in this field are mainly distributed in North America, East Asia, and Europe. Figure 3B shows the international cooperation relationships in the research on virus and neuroinflammation. The connections between nodes reflects the collaborative relationship between individual countries and regions, and the thickness of the links is positively correlated with the depth of collaboration (29). It is worth noting that the links between countries/regions are mainly concentrated between the United States and other countries, whereas research collaborations among other countries were scattered. To further analyze the highly productive countries in this field, Table 1 shows the top 10 countries/regions in terms of number of publications, and Figure 3C visualizes the geographical distribution and collaboration. The United States emerged as the leading contributor with 1,908 articles published, followed by China with 649 articles and Germany with 325 articles. In terms of citations received, the United States garnered the highest count with 79247 citations, followed by Germany with 13055 citations and the United Kingdom with 12287 citations. Although United States has the highest number of publications, the average number of

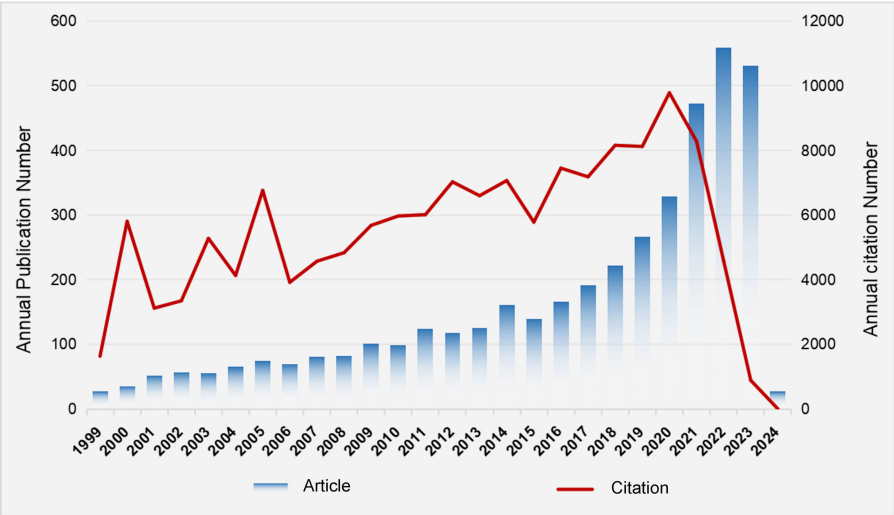

FIGURE 2
Trends in the growth of publications and the number of citations. The number of publications and citation frequency for each year from 1999 to the January 2024 showed the steady growth trend.

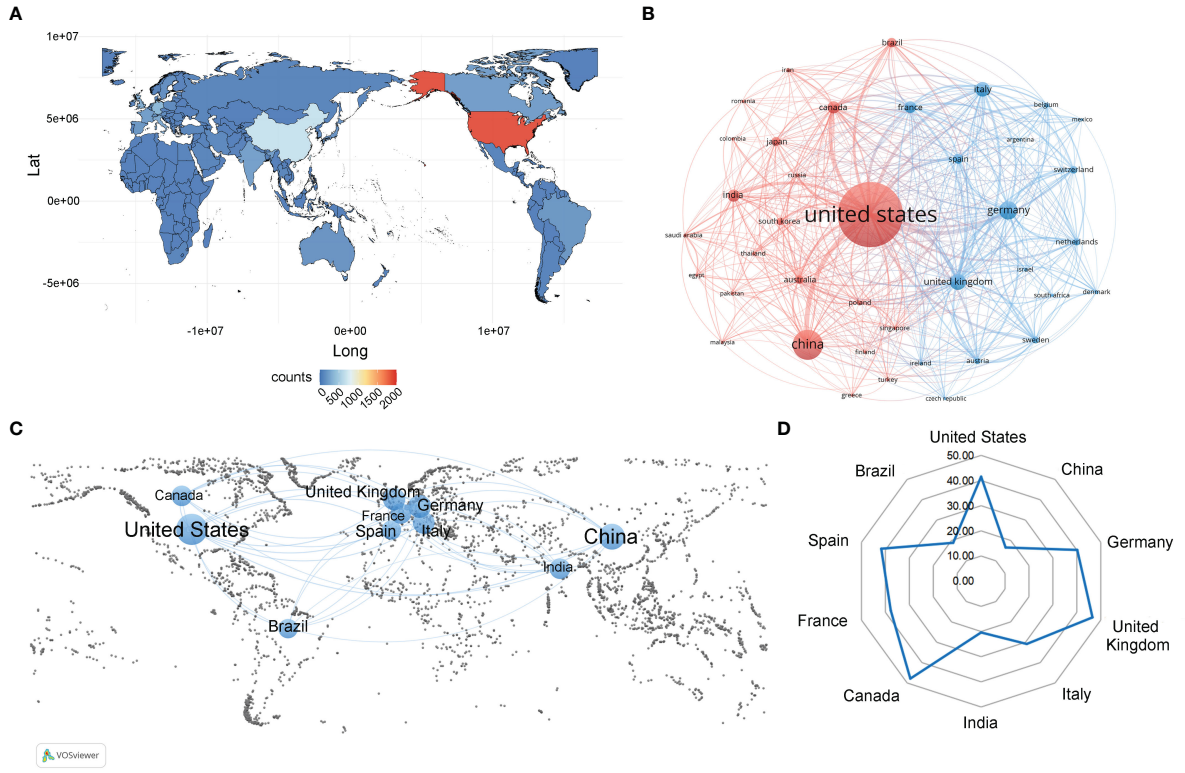

FIGURE 3
Analysis of country/region. **(A)** Geographical distribution of global output. The volume of publications is represented by color variation. **(B)** Visual cluster analysis of cooperation among countries. The nodes of different colors represent the countries/regions with different clusters, and the thickness of the lines indicates how closely countries cooperate. **(C)** Geographical distribution and the co-authorship network of the top 10 productive countries. **(D)** Radar chart of the average cited frequency of the top 10 productive countries.

citations per article (N = 41.53) is lower than that of Canada (N = 47.86), the United Kingdom (N = 46.54) and Spain (N = 41.74). At the same time, although China has a high number of publications and citations, its average number of citations per article (N = 16.55) is the lowest among the 10 countries, indicating that the academic influence of Chinese scholars is low, and it is still necessary to publish higher quality, more innovative and widely recognized academic papers in the professional field (Figure 3D).

3.3 Analysis of institutions and authors

Approximately 22274 authors from 4474 institutions contributed to the research on virus and neuroinflammation. Table 2 and Figure 4A show the citations and the average number of citations per article of the top 10 institutions in terms of publication volume. Johns Hopkins University leads with the highest number of publications and citations, whereas University of California, San Francisco has the highest average

TABLE 1 The top 10 most productive countries.

Rank	Country	Counts	Citations
1	United States	1908	79247
2	China	649	10739
3	Germany	325	13055
4	United Kingdom	264	12287
5	Italy	236	7278
6	India	189	3851
7	Canada	182	8711
8	France	177	6682
9	Spain	135	5635
10	Brazil	132	2490

TABLE 2 The top 10 most productive institutions.

Rank	Institutions	Counts	Citations
1	Johns Hopkins University	102	6027
2	Harvard Medical School	66	1708
3	University of Pennsylvania	60	2228
4	University of Nebraska Medical Center	59	2053
5	University of California, San Francisco	48	3066
6	University of California, San Diego	47	2777
7	University of California, Irvine	44	1791
8	Mayo Clinic	43	1567
9	University of Minnesota	42	1779
10	University of California, Los Angeles	41	1545

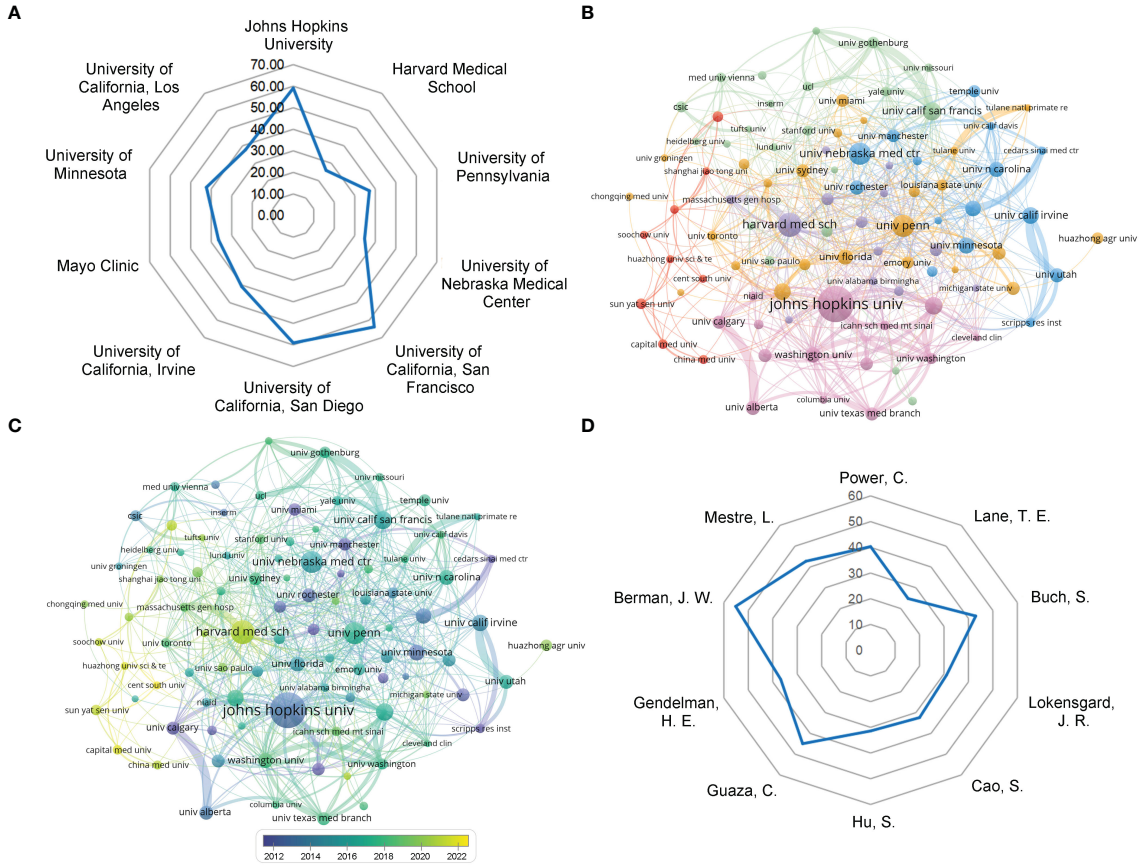

FIGURE 4
Analysis of institutions and authors. **(A)** Radar chart of the average cited frequency of the top 10 institutions. **(B)** Visual cluster analysis of cooperation among institutions. The nodes of different colors represent the institutions with different clusters, and the thickness of the lines indicates how closely institutions cooperate. **(C)** Timeline visualization of cooperation among institutions. The different colors represent the time at which the institution began the relevant study. **(D)** Radar chart of the average cited frequency of the top 10 authors.

number of citations. Also, the top 10 institutions were in the United States, which demonstrated the high interest of the United States institutions in the study of virus and neuroinflammation and highlighted their important position and contribution to this field of research. To further investigate collaboration between institutions, we performed a co-authorship analysis of all publications. It is showed that the division of institutions into 6 clusters based on an occurrence frequency and cooperative relationships (Figure 4B). The institutions with close collaboration were mostly in the United States, further signifying the robustness of related research in the United States. Among them, Johns Hopkins University has the strongest collaboration with other institutions. According to Figure 4C, the United States institutions such as Johns Hopkins University have published relevant articles since around 2012. The institutions from China (the red cluster in Figure 4B) have been active in the virus and neuroinflammation research in recent years (yellow color).

The top three authors with the most published articles on the virus and neuroinflammation are Power, C. from the University of Alberta, Lane, T. E. from University of Utah Salt Lake City, and Buch, S. from the University of Nebraska Medical Center (Table 3). Berman, J. W. from the Albert Einstein College of Medicine has the highest average number of citations (Figure 4D). Her research focuses on the mechanisms of human immunodeficiency virus (HIV) infection and BBB penetration (30, 31). Supplementary Figure 1 shows the maps of cooperation between authors; the minimum number of papers per author was set as 7. Of the remaining 130 authors, there were few links, indicating inadequate collaboration between research teams/laboratories conducting relevant research in the field.

3.4 Distribution of journals

The articles on virus and neuroinflammation research were published across 1046 journals. Table 4 displays the top 10 journals with the greatest number of publications and their recent impact factor (IF). The Journal of Neuroinflammation leads with 201 articles, followed by Journal of Neurovirology with 103 articles, and Frontiers in Immunology with 96 articles. According to the density map of the journals, the journals that published articles on this field were mainly divided into four categories: neuroimmunology, neurology, virology and comprehensive (Figure 5A). And in recent years, articles in this field tend to be published in comprehensive journals (Figure 5B). The dual-map overlay of journals indicates the position of the research topic in the

TABLE 3 The top 10 most productive authors.

Rank	Author	Counts	Affiliations	Citations	Average Citation/Publication
1	Power, C.	28	University of Alberta	1128	40.29
2	Lane, T. E.	27	University of Utah Salt Lake City	670	24.81
3	Buch, S.	21	University of Nebraska Medical Center	903	43
4	Lokensgard, J. R.	20	University of Minnesota	622	31.1
5	Cao, S.	20	Huazhong Agricultural University	648	32.4
6	Hu, S.	19	University of Minnesota	597	31.42
7	Guaza, C.	19	Consejo Superior de Investigaciones Científicas	854	44.95
8	Gendelman, H. E.	19	University of Nebraska Medical Center	698	36.74
9	Berman, J. W.	18	Albert Einstein College of Medicine	993	55.17
10	Mestre, L.	17	Consejo Superior de Investigaciones Científicas	727	42.76

mainstream research subject classification, showing the cited trajectory as well as the change of research center. Each dot on the graph represents a journal, with the citing chart on the left and the cited chart on the right. The length of the ellipse indicates the number of authors, the width of the ellipse indicates the number of publications, and the trajectory indicates the relationship between interdisciplinary classifications. Citing journals are mainly from MOLECULAR, BIOLOGY, IMMUNOLOGY, MEDICINE, MEDICAL, CLINICAL, and NEUROLOGY. The cited journals are mainly from MOLECULAR, BIOLOGY, GENETICS, PSYCHOLOGY, EDUCATION, and SOCIAL (Figure 5C).

3.5 Analysis of research hotspots

3.5.1 Most cited articles and cited authors

Table 5 shows the top 10 most cited articles on the virus and neuroinflammation. All of them were published in journals classified as Q1 and with great influence. The most frequently cited article is " Neurologic Manifestations of Hospitalized Patients with Coronavirus Disease 2019 in Wuhan, China " which focuses on the neurologic manifestations of patients with COVID-19 (32). In general, research in this field mainly focused on HIV-associated neurocognitive disorders (HAND) before 2020, and COVID-19 and neuroinflammation after 2020. In addition, there are 283 cited authors, and we listed the top 10 based on different countries (Table 6). The number of cited authors was ranked by country as follows: the United States, China, Germany, Canada, South Korea, India, Spain, etc.

TABLE 4 The top 10 most productive journals.

Rank	Journal	Counts	Citations	IF
1	Journal of Neuroinflammation	201	6090	9.3
2	Journal of Neurovirology	103	2514	3.2
3	Frontiers in Immunology	96	1936	7.3
4	Journal of Neuroimmunology	89	2270	3.3
5	Journal of Virology	83	2671	5.4
6	Brain Behavior and Immunity	77	2705	15.1
7	Plos One	73	3265	3.7
8	Journal of Neuroimmune Pharmacology	67	2367	6.2
9	International Journal of Molecular Sciences	58	807	5.6
10	Frontiers in Neurology	58	690	3.4

3.5.2 Co-cited references and references bursts

Co-citation analysis is a method to measure the relevance between academic papers, which can not only reflect the influence of articles in the field, but also reflect the hot research directions in the field. We further constructed a network visualization of co-cited references (Figure 6A) and conducted cluster analysis, which led to the identification of 19 cluster modules (Figure 6B). The nominal terms of these clusters are extracted from the keywords of the cited articles by the latent semantic indexing (LSI) algorithm. By studying the references corresponding to each node category, the mainly intellectual base of current research on virus and neuroinflammation was summarized as follows: Cluster 0 (COVID-19): studies that investigated the neurological manifestations and neuro-immune interactions in patients with COVID-19; Cluster 1 (central nervous system): studies that investigated the immune resistance of the central nervous system under viral infection; Cluster 2 (Alzheimer's disease): studies that investigated the mechanism by which viral infection produces inflammatory triggers leading to Alzheimer's disease; Cluster 3 (multiple sclerosis): studies that investigated the mechanism of virus and neuroimmune in the occurrence and development of multiple sclerosis. Furthermore, we visualized the clustering timeline (Figure 6C) and identified multiple sclerosis, mu-opioid receptors, adenoviral vectors, arachidonic acid, T cells and nerve growth factor as the focus of early research on virus and neuroinflammation. With the outbreak of COVID-19, related topics in this field such as cytokine storm, chronic infection,

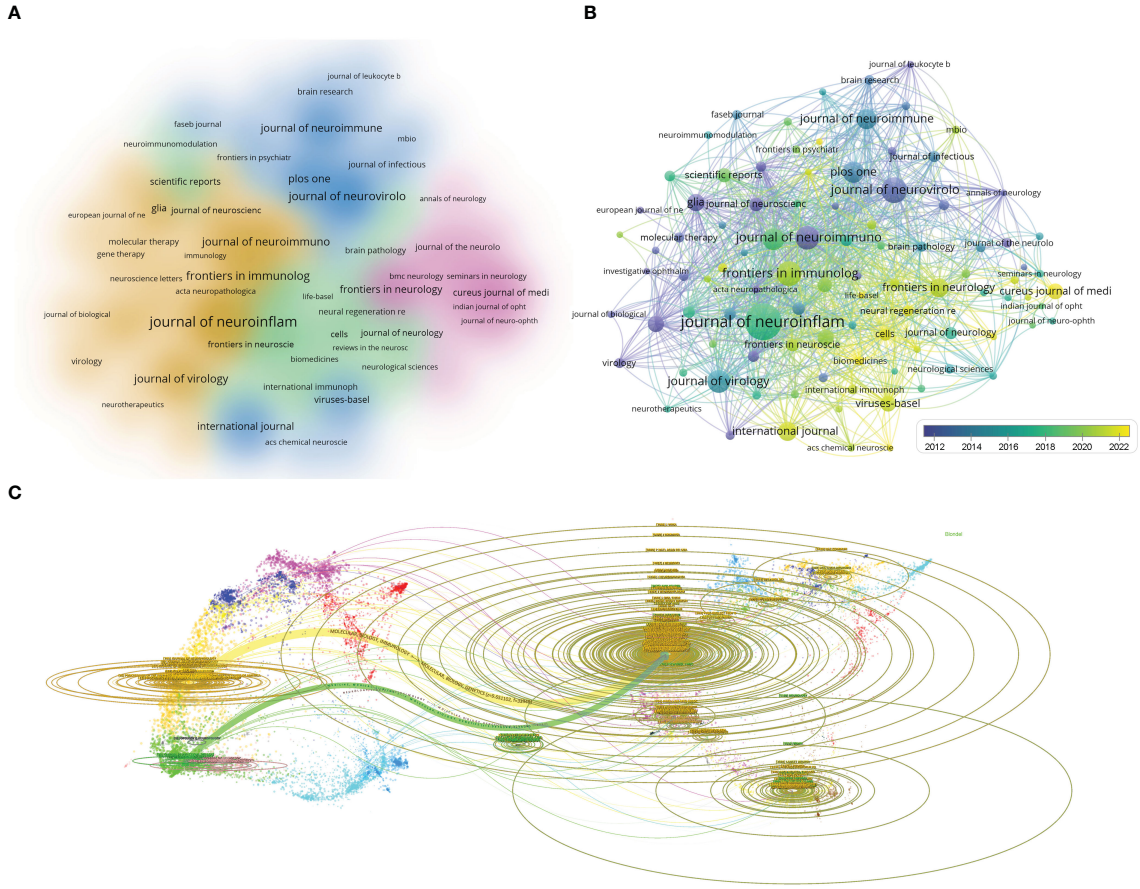

FIGURE 5
Analysis of journals. **(A)** Visual cluster analysis among journals. The density map of different colors represent the journals with different clusters. **(B)** Timeline visualization among Journals. The different colors represent the time when the journal began to publish relevant studies. **(C)** The dual-map overlap of journals. Citing journals are on the left, cited journals are on the right, and colored paths indicate citation relationships.

central nervous system, and neuromyelitis optica have been pushed to a research climax. Figure 6D shows the top 20 references with the strongest citation bursts. The strongest citation burst was for the 2010 article " HIV-associated neurocognitive disorders persist in the era of potent antiretroviral therapy: CHARTER Study " (33). This article has an intensity of 24.53. In recent years, the cited literature mainly studies neurological symptoms of patients with COVID-19 and reveals neuroinflammation with distinct microanatomical microglia-T-cell interactions (34).

TABLE 5 The top 10 most cited references.

Rank	First author	Journal	DOI	Year	IF(2023)
1	Mao, L.	JAMA Neurology	DOI 10.1001/jamaneurol.2020.1127	2020	29.0
2	Heaton, R. K.	Neurology	DOI 10.1212/wnl.0b013e318200d727	2010	9.9
3	Antinori, A.	Neurology	DOI 10.1212/01.wnl.0000287431.88658.8b	2007	9.9
4	Matschke, J.	Lancet Neurology	DOI 10.1016/s1474-4422(20)30308-2	2020	48.0
5	González-Scarano, F.	Nature Reviews Immunology	DOI 10.1038/nri1527	2005	100.3
6	Meinhardt, J.	Nature Neuroscience	DOI 10.1038/s41593-020-00758-5	2021	25.0
7	Kaul, M.	Nature	DOI 10.1038/35073667	2001	64.8
8	Hoffmann, M.	Cell	DOI 10.1016/j.cell.2020.02.052	2020	64.5
9	Helms, J.	New England Journal of Medicine	DOI 10.1056/nejmc2008597	2020	158.5
10	Moriguchi, T.	International Journal of Infectious Diseases	DOI 10.1016/j.ijid.2020.03.062	2020	8.4

TABLE 6 The top 10 most cited authors from each country.

Country	Author	Counts	Citations	Average Citation/ Publication
United States	Sacktor, N.	5	1053	211
	Harms, A.S.	8	1045	131
	Standaert, D.G.	8	1004	126
	Volsky, D.J.	7	858	123
	McArthur, J.C.	11	1214	110
	Valcour, V.	5	505	101
	Brundin, P.	5	470	94
	Smeyne, R.J.	5	457	91
	Gendelman, H.E.	7	635	91
	Nath, A.	6	541	90
Germany	Arbusow, V.	7	634	91
	Strupp, M.	8	654	82
	Gerhauser, I.	5	312	62
	Tumani, H.	7	401	57
	Brandt, T.	10	461	46
	Bauer, J,	8	361	45
	Chhatbar, C.	6	267	45
	Strupp, M.	14	597	43
	Kaeufer, C.	5	213	43
	Kalinke, U.	7	281	40
Canada	Power, C.	8	619	77
	Ellestad, K.K.	5	367	73
	Maingat, F.	7	349	50
	Silva, C.	6	297	50
	Branton, W.G.	7	309	44
	Power, C.H.	28	1128	40
	Noorbakhsh, F.	5	191	38
	Hollenberg, M.D.	5	183	37
	Cohen, E.A.	7	243	35
	Zhu, Y.	5	149	30
China	Huang, Y.L.	6	450	75
	Yao, H.H.	8	456	57
	Zhu, B,B,	6	339	57
	Chen, S.Y.	5	277	55
	Raung, S.L.	6	289	48
	Song, Y.F.	6	273	46
	Ou, Y.C.	7	318	45
	Cui, M.	10	448	45
	Chen, C.J.	8	336	42
	Liao, S.L.	8	336	42
India	Periyasamy, P.	8	399	50
	Basu, A.	17	765	45
	Vrati, S.	6	210	35
	Banerjee, A.	6	185	31
	Mishra, R.	6	149	25
	Banerjea, A.C.	5	105	21
	Das sarma, J.	17	331	19
	Jha, N.K.	5	52	10
Spain	Hernangomez, M.	5	289	58
	Docagne, F.	8	435	54
	Mecha, M.	13	660	51
	Guaza, C.	19	854	45
	Mestre, L.	17	727	43
	Feliu, A.	10	416	42
	Carrillo-Salinas, F.J.	5	199	40
South Korea	Suk, K.	5	124	25
	Kim, S.B.	7	166	24
	Kim, J.H.	7	157	22
	Uyangaa, E.	7	149	21
	Choi, J.Y.	9	177	20
	Eo, S.K.	9	177	20
	Kim, K.	8	145	18
	Park, S.Y.	6	97	16
	Hossain, F.M.A.	5	78	16
	Patil, A.M.	7	102	15

3.5.3 Keywords analysis of research hotspots

Keywords summarize the core and essence of a paper, and research hotspots in a scientific field can be found through keyword co-occurrence analysis. VOSviewer was used to draw the keyword co-occurrence network view, and 69 key keywords with a frequency greater than or equal to 80 were selected for visualization (Figure 7A). The frequency of keywords was positively correlated with the size of the circle node. Based on the co-occurrence network, the research strategy in this field is to explore the

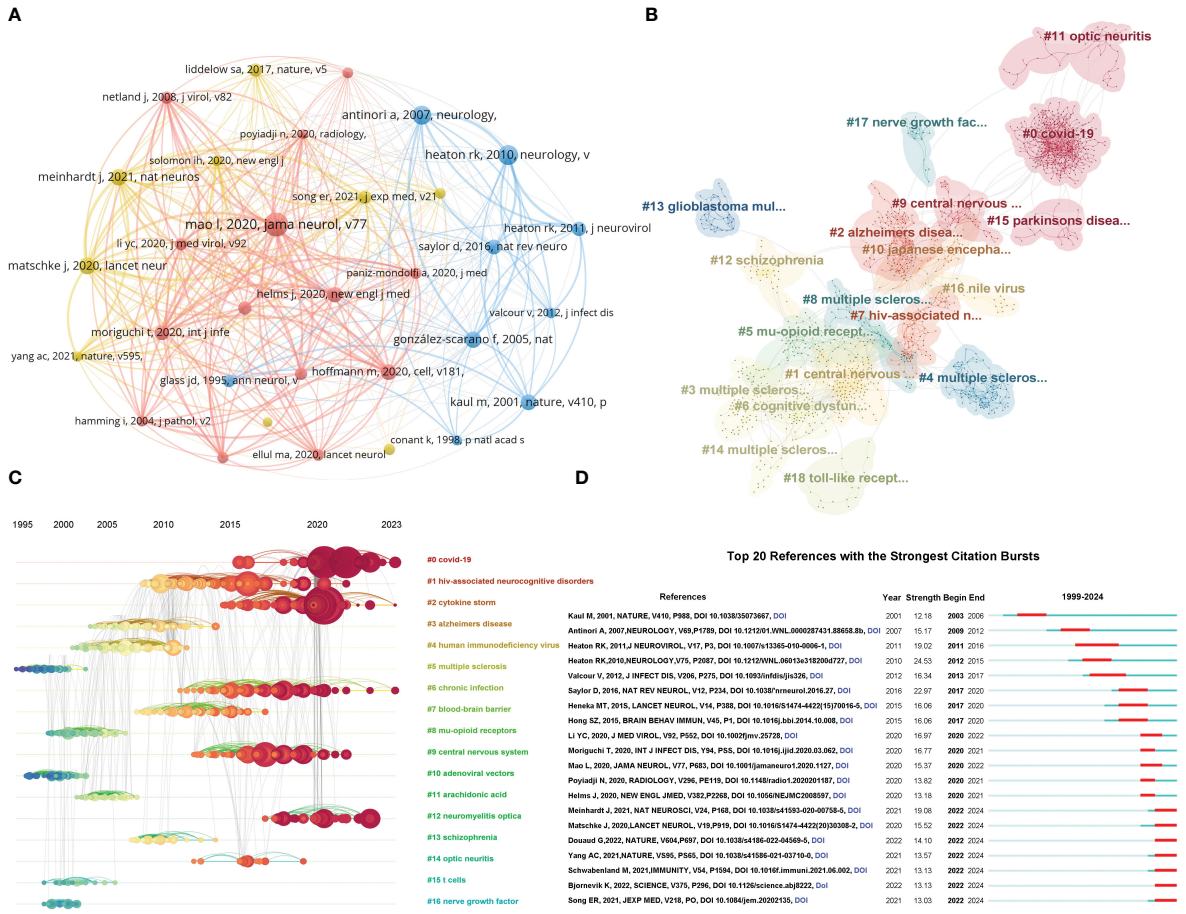

FIGURE 6
Analysis and network visualization of reference co-citation. **(A)** Visual analysis of co-cited references. The nodes of different colors represent the co-cited references with different clusters. **(B)** Cluster analysis of co-cited references. The nominal terms of these clusters are extracted from the keywords of the cited references by the LSI algorithm and clustered into 19 clusters. **(C)** Timeline distribution of the clusters. Each horizontal line represents a cluster. The smaller the number is, the larger the cluster, with #0 being the largest cluster. Nodes size reflects co-citation frequency, and the links between nodes indicate co-citation relationships. Nodes occurrence year is the time when they were first co-cited. **(D)** The top 20 references with the strongest citation bursts.

mechanism by which viral infection promotes neuroinflammation in the central nervous system by triggering inflammatory factors such as NF-κB/TNF-α, and then leading to diseases at the *in vitro* and *in vivo* levels. Then we performed cluster analysis through CiteSpace using the same strategy as the co-cited references and obtained 9 clusters, which were multiple sclerosis, 1-associated myelopathy, Parkinson's disease, neuroinflammation, blood-brain barrier, covid-19, Alzheimer's disease, gene therapy and tumor necrosis (Figure 7B). These findings highlight the key research topics in this field. Furthermore, we also conduct a timeline visualization analysis of keywords, which can show the dynamic evolution path of the research hotspots represented by the keywords, and explore the time characteristics and the rise and fall process of the research field reflected by the aggregation of hot keyword research (Figure 7C). From the evolution of keywords, large-scale studies in multiple sclerosis, neuroinflammation, 1-associated myelopathy, central nervous system, and Alzheimer's disease were conducted in this field as early as 1999. In the following research showed an annual average, maternal immune activation and cytokine storm intensively entered the research field in 2020. In order to better understand the research hotspots of sudden outbreaks in the field of viruses and neuroinflammation, we conducted a burst analysis on keywords. Figure 7D shows the top 25 keywords with the strongest citation bursts. It can be seen that "covid-19" has the highest burst strength and may still have high intensity research in 2024. At the same time, we also noticed that "stress" from 2021 and "neuroinflammation" from 2022 have become the recent research upsurge, indicating that viruses, stress and neuroinflammation may become the new research hotspot in this field.

3.6 Virus, neuroinflammation and psychiatric disorders

Based on the above findings and the global mental health reconstruction project in the context of COVID-19, we would like to take a deeper look at the current research on virus, neuroinflammation, and psychiatric disorders. According to the above search strategy, we added some conditions "TS= (mental* OR

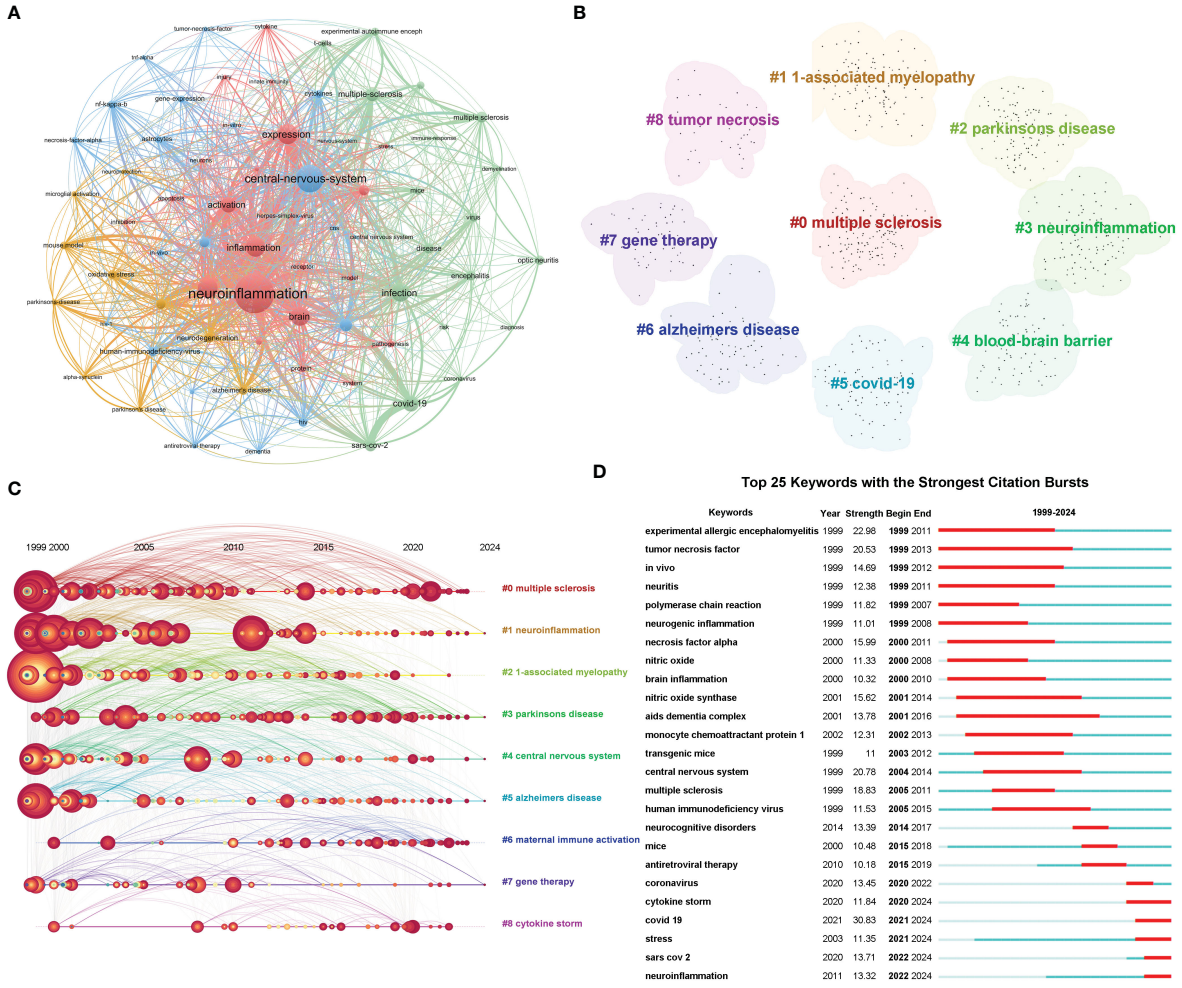

FIGURE 7
Analysis and network visualization of the research hotspots. **(A)** Network visualization map of keywords co-occurrence. The nodes of different colors represent the keywords with different clusters. **(B)** Cluster Analysis of keywords. The nominal terms of these clusters are extracted from the keywords of the publications by the LSI algorithm and clustered into 9 clusters. **(C)** Timeline distribution of cluster analysis of keywords. Nodes occurrence year is the time when they first appeared. **(D)** The top 25 keywords with the strongest citation bursts.

psychiatr* OR neuropsych* OR depressi* OR MDD OR anxi* OR "bipolar disorder" OR mania OR manic OR "mood disorder" OR "affective disorder" OR "feeding and eating disorder" OR anorexia OR "eating disorder" OR "neurocognitive disorders" OR "neurodevelopmental disorder" OR "personality disorder" OR schizophren* OR schizoaffect* OR psychotic OR psychosis OR "sleep wake disorders" OR "trauma and stressor related disorders" OR post-traumatic* OR PTSD OR autis* OR "attention deficit" OR ADHD OR "obsessive compulsive" OR OCD). After screening, 697 articles related to virus, neuroinflammation, and psychiatric disorders were included. In terms of global trends in publication output, the number of annual publications in this field has shown a steady growth trend, with a sharp increase since 2021 (Figure 8A). The United States leads with the highest number (n=366), followed by China (n=97) and the United Kingdom (n=52) (Figure 8B). According to overlay visualization of the Journal in which the article was published, the most articles in this field were published on The Journal of Neuroinflammation, and Molecular Psychiatry and Viruses-Basel has gained popularity for submissions in recent years (Figure 8C). Cluster analysis, timeline visualization, and burst analysis of keywords revealed that recent studies focused on depression, covid-19, HIV-associated neurocognitive disorders, neurofilament light chain, blood-brain barrier, hypercytokinemia, maternal immune activation, gut microbiota, stress, neuropathology and other directions, indicating that these areas are potential future research frontiers in the field of the virus, neuroinflammation and psychiatric disorders (Figures 8D–F).

4 Discussion

4.1 General information

This study conducted a comprehensive literature search of virus and neuroinflammation publications from January 1, 1999, to January 31, 2024, in the WOSCC database. After excluding publications that did not meet the predefined inclusion criteria,

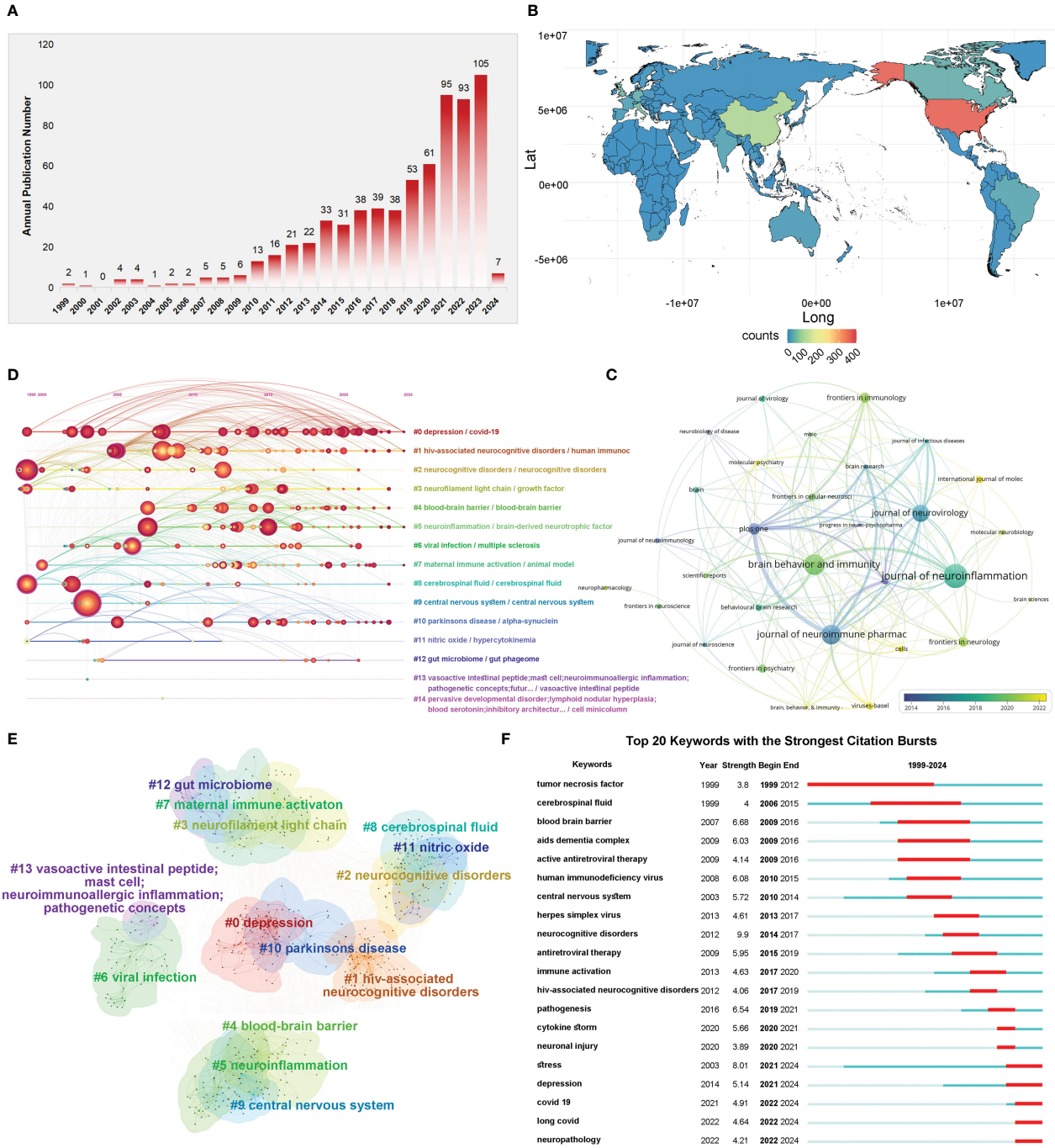

FIGURE 8
Analysis and network visualization of research on viruses, neuroinflammation, and psychiatric disorders. **(A)** Trends in the growth of publications. **(B)** Geographical distribution of global output. **(C)** Timeline visualization among Journals. The different colors represent the time when the journal began to publish relevant studies. **(D)** Timeline distribution of cluster analysis of keywords. Nodes occurrence year is the time when they first appeared. **(E)** Cluster Analysis of keywords. The nominal terms of these clusters are extracted from the keywords of the publications by the LSI algorithm and clustered into 13 clusters. **(F)** The top 20 keywords with the strongest citation bursts.

this bibliometric analysis encompassed a total of 4230 English articles. The annual publication trend exhibited a period of stability (prior to 2020) followed by rapid growth (from 2021 onwards), indicating the emerging prominence of virus and neuroinflammation research as a burgeoning field. The surge in research activity can be attributed to the heightened recognition of viral involvement in CNS-related disorders, which has been further accentuated by the COVID-19 outbreak-induced alterations in neural biochemical functions.

The bibliometric analysis revealed that the relevant publications were mainly published by corresponding authors from the United States, China, Germany, the United Kingdom, Canada, and Italy. This national disparity can be attributed to the strong correlation between academic capacity and a country's economic situation.

With robust government economic support, science and technology will continue to innovate and advance. Since the Renaissance, European scientists have embarked on exploring the fundamental structure and function of the nervous system, its interplay with psychology, as well as treatments for neurological disorders. In recent times, researchers in Germany, France, the United Kingdom, and the United States have employed cutting-edge imaging techniques such as electron microscopy to unravel the intricacies of nervous system architecture and functionality. In contemporary society, luminaries like Georg Nagel, Karl Deisseroth, Edward Boyden, Armbruster Roth et al., have pioneered optogenetics and chemogenetics respectively to precisely modulate neuronal activity. By integrating these approaches with brain-computer interfaces (BCIs), a deeper comprehension of distinct neuron roles in behavior modulation, learning processes,and memory formation has been achieved. Government investment in healthcare alongside scientific advancements serve as pivotal indicators of medical research output; factors that may contribute to why the United States leads in terms of publication quantity and citation impact. Whether analyzing the collaboration of countries, institutions, or authors on viral and neuroinflammation research also gravitates towards the United States—a testament to its remarkable contributions within this academic domain.This observation further underscores that other nations,institutions,and scholars should foster international cooperation to augment their influence.

Among the top 10 authors, three authors, Power C, Lane TE, and Buch s, have made the most outstanding contributions in this field. Professor Berman JW from the Albert Einstein College of Medicine is the author with the highest average number of citations per article (N= 55.17). His research focuses on the increased sensitivity of HIV infection of $CD14^+CD16^+$ monocytes to CCL2 and the expression of CCR2, JAM-A, and ALCAM on $CD14^+CD16^+$ monocytes promote the entry of HIV-infected and uninfected $CD14^+CD16^+$ monocytes into the brain (35). This in turn leads to ongoing neuroinflammation that occurs during HIV pathogenesis. Meanwhile, his findings indicate that CCR2 on $CD14^+CD16^+$ monocytes is a novel peripheral blood biomarker of HAND (30). Despite the significant contributions made by numerous authors in this field, it remains imperative to enhance collaboration among international and domestic institutions and laboratories in order to expedite the resolution of major scientific challenges and collectively achieve groundbreaking advancements from scratch. The majority of the top 10 journals that exhibit high research activity in viruses and neuroinflammation are published in the United States and Europe. The Journal of Neuroinflammation emerged as the journal with the highest number of publications, whereas Plos One garnered the highest number of average citations. In contrast, although China and India are also major contributors to research in this field, there is a lack of Asian publishers. This highlights the significance for Asia to establish its own foundations and develop internationally influential journals. Figure 5C demonstrates the widespread recognition and study of viruses in both basic and clinical research on neuroinflammation, which holds great importance for diagnosing and treating CNS-related diseases.

4.2 Hotspots and frontiers

The analysis of highly cited articles and frequent keywords provides valuable insights into influential findings, which can effectively guide future research directions in a specific field. In this study, we employed key co-occurrence analysis to identify the primary research focuses and emerging trends in virus-related neuroinflammation, as well as to elucidate the evolution and dynamics of its thematic structure.

Analysis of the references showed that 8 of the top 10 most cited articles were clinical trial articles and two reviews. These publications primarily focused on HAND, HIV-associated dementia (HAD), and COVID-19, reflecting the relatively concentrated research directions of virus and neuroinflammation, and their close clinical relevance provides scientific significance for basic research.

Keywords analysis of research hotspots showed that research on virus and neuroinflammation were mostly related to neurodegenerative diseases. Neurodegenerative diseases are characterized by the degeneration of various brain regions; however, they all exhibit two common features: neuroinflammation and an epidemiological association with viral infection. MS is a chronic inflammatory demyelinating disease of the central nervous system, in which the immune system plays a crucial role in its pathogenesis (36). Nerve fibers are enveloped by myelin sheaths, and the immune system erroneously targets these sheaths, resulting in nerve function impairment and subsequent disruption of normal nerve signal transmission, leading to associated symptoms (37). Viral infection is recognized as one of the primary triggers for MS development. Researchers have observed a 32-fold increase in MS risk following Epstein-Barr virus (EBV) infection (38). It was found that high levels of $EBNA_{386-405}$ specific antibodies and $GlialCAM_{370-389}$ derived from the CNS cross-react due to their similar structure, thus provoking the immune system to attack the body's own nervous system and causing MS (39). Researchers have identified certain genetic factors and specific EBV variants that influence MS development by significantly attenuating immune responses against autoreactivity, thus promoting disease progression (39). Furthermore, AD and PD are also research hotspots in this field. In a study by Cairns DM, it was found that Varicella zoster virus (VZV) infection of cells quiescently infected with herpes simplex virus type 1 (HSV-1) causes reactivation of HSV-1 and consequent AD-like changes, including Aβ and P-tau accumulation (40). Moreover, the N protein of SARS-CoV-2 can interact with α-synuclein to accelerate the formation of amyloid deposits (41). Additionally, COVID-19-induced systemic inflammatory response or toxicity may exacerbate symptoms in elderly or severe PD patients; however, current clinical evidence does not establish a causal relationship between inflammation and PD (42). Since its first reported case in 2019, research on SARS-CoV-2 has remained active. The inflammatory response triggered by the virus can indirectly harm the nervous system, potentially leading to encephalitis, acute myelitis, cerebrovascular diseases, demyelinating diseases of the central nervous system, epilepsy, and other neurological disorders (43, 44). In conclusion, viral infection-

induced neuroinflammation is involved in an increasing number of neurological diseases, thereby establishing a solid foundation for subsequent investigations. However, there remains a paucity of clinical studies targeting viral genes or proteins as therapeutic interventions for related neurological diseases. Consequently, the future focus in this field lies in the implementation of precise and efficacious personalized treatments that specifically target viruses to address neuroinflammation-related disorders.

4.3 Summary and prospects

In summary, the involvement of immune cells and disruption of the BBB are crucial steps in the virus-induced neuroinflammatory process. Virus infection typically results in an upregulation of chemokines (CCL2 and CCL5) and alterations in tight junction protein expression, thereby modifying endothelial cell function and increasing vascular permeability. This allows for direct viral penetration across the initial barrier of the CNS. Simultaneously, viruses can also infect white blood cells (monocytes and macrophages), enabling them to transport pathogens across the BBB, leading to sustained infection of macrophages and microglia within the central nervous system. Prolonged viral infection induces a shift in microglial phenotype from a neuroprotective state to a neurodegenerative state, characterized by production of neurotoxins and activation of immune cells. Furthermore, upon invasion into the CNS, viruses induce accumulation of immune cells such as $CD8^+$ cells as well as pro-inflammatory cytokines like IFN-γ and TNF-α. These factors can exacerbate inflammation within the CNS, ultimately contributing to onset of neurological diseases (Figure 9).

With the advancement of research on viral infection-induced neuroinflammation in nervous system diseases, novel research directions in this field have gained prominence. In recent years, the investigation of neuroinflammation caused by viral infection in psychiatric disorders has emerged as a primary area of study, garnering significant attention from researchers. Particularly since the onset of COVID-19, an emerging acute infectious disease capable of inducing not only systemic symptoms but also psychiatric disorders (45). The largest study to date on the health of COVID-19 patients, which encompassed data from over 236,000 individuals afflicted with COVID-19, has been published online in The Lancet Psychiatry (46). Findings revealed that within six months of contracting COVID-19, 33.62% of patients received a diagnosis for a neurological or psychiatric disorder, with 12.84% being newly diagnosed cases. Among critically ill patients, 46.42% were diagnosed with neurological or mental disorders, and among them, 25.79% were newly diagnosed cases. Anxiety (17.39%) and mental disorders (1.40%) emerged as the most prevalent mental health conditions observed in this cohort. Notably, individuals affected by COVID-19 exhibited a 44% higher risk of developing neurological and psychiatric conditions compared to those infected with influenza and a 16% higher risk compared to those suffering from respiratory infections (46). These results indicate that COVID-19 confers an elevated susceptibility to neurological and psychiatric disorders when contrasted against influenza and

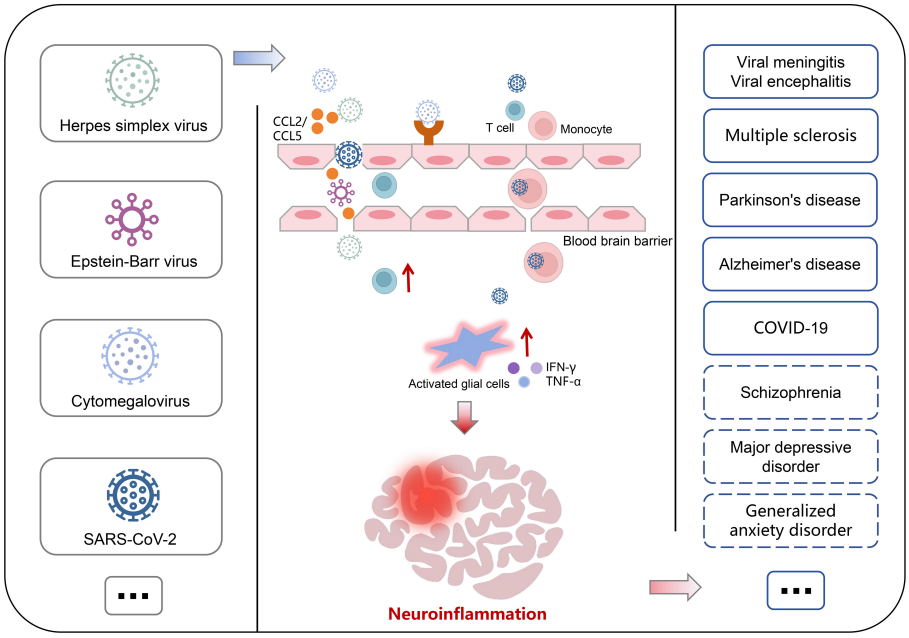

FIGURE 9
Association of virus and neurological disorders. The virus can directly traverse the endothelial cells of blood vessels and exploit the "Trojan horse" mechanism to breach the blood-brain barrier, thereby inducing an upregulation of immune cells and pro-inflammatory factors, facilitating excessive activation of glial cells for mediating neuroinflammation, consequently leading to neurological disorders. The contribution of this process in the pathogenesis of mental illnesses remains to be elucidated.

respiratory infections alike. Furthermore, depression represents a significant public health concern; recent reports suggest its association with disease progression as well as increased complications arising from specific viral infections such as SARS-CoV-2 and HIV infection while also rendering individuals more susceptible to viral infections (47, 48). revealed a decrease in the expression of Abelson helper integration site 1 (AHI1) in peripheral blood mononuclear cells (PBMC) and macrophages from individuals with major depression (MDD), resulting in impaired antiviral immune response (49). The hypothesis of inflammation in the development of schizophrenia suggests that maternal exposure to infections such as influenza virus, Toxoplasma, herpes simplex virus, measles virus, rubella virus during the first and second trimesters of pregnancy is considered an important risk factor for adult-onset schizophrenia (50). Furthermore, dormant human cytomegalovirus (CMV) infection may be associated with mood disorders, suicidal behavior, and neuroinflammation (51, 52). A higher proportion of individuals with psychiatric disorders were found to be CMV seropositive compared to controls. Interestingly, CMV seropositivity was also linked to increased microglial activity, suggesting that CMV-associated neuroinflammation could potentially contribute to psychiatric disorders (51). These studies collectively suggest that neuroinflammation caused by viral infection not only plays a role in neuroinflammatory diseases, but also may have a potential role in non-inflammatory neurological diseases. In this bibliometric analysis, although the number of such studies is limited, they have been increasing steadily over the years. The majority of publications focus on relevance studies; therefore, future research should aim to investigate specific mechanisms in greater detail. Moreover, considering that psychiatric disorder is a multifactorial and highly heterogeneous disease, it would be worthwhile to explore whether personalized antiviral treatment could serve as a potential therapeutic approach.

5 Limitations

This study represents the pioneering attempt to investigate the evolving trends and potential research frontiers on virus-induced neuroinflammation, employing a bibliometric approach. Nevertheless, certain limitations should be acknowledged in this investigation. Specifically, only English articles and reviews from the WOSCC database were included with a literature collection cut-off date of January 31, 2024, potentially excluding relevant publications. Moreover, it is worth noting that an article's impact can be influenced by its publication duration; thus, some recently published high-quality articles might have been overlooked due to their low citation frequency. However, these limitations are unlikely to alter the fundamental trends elucidated in this paper.

6 Conclusion

Through comprehensive bibliometric analysis in virus and neuroinflammation, this study evaluates the publications information of different years, countries, institutions, authors, and journals and analyzes the theme development and future research hotspots. The findings indicate a growing research interest in the field. European and American countries have played a pivotal role in conducting extensive studies on this topic; however, China has also made significant contributions despite its limited impact thus far. Our findings reveal that multiple sclerosis, Parkinson's disease, blood-brain barrier, COVID-19, Alzheimer's disease, neurocognitive disorders, gene therapy, depression, maternal immune activation, gut microbiota, and stress are the primary focus and hot spots of this field. However, the majority of previous studies have primarily focused on investigating the association between viruses and neuroinflammatory diseases. Only a limited number of epidemiological studies have suggested that viral infections may also contribute to non-inflammatory neurological disorders, such as psychiatric conditions. By examining current research trends and emerging areas of interest in the field of viruses and neuroinflammation, this study aims to provide valuable insights into understanding the mechanisms underlying neuroinflammation induced by different types of viruses in relation to the onset and progression of psychiatric disorders.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

DL: Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. MW: Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1390149/full#supplementary-material

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EDITED BY
Prasun K. Datta,
Tulane University, United States

REVIEWED BY
Santosh Kumar,
University of Tennessee Health Science Center (UTHSC), United States
Jennifer Kelschenbach,
Icahn School of Medicine at Mount Sinai, United States
Jerel Adam Fields,
University of California, San Diego, United States

*CORRESPONDENCE
Genmeng Yang
1223984885@qq.com
Juan Li
121093258@qq.com
Xiaofeng Zeng
zxf2004033@163.com

†These authors have contributed equally to this work and share first authorship

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Mechanisms and treatments of methamphetamine and HIV-1 co-induced neurotoxicity: a systematic review

Lin Miao[1†], Haowei Wang[1†], Yi Li[1†], Jian Huang[1], Chan Wang[1], Hanxin Teng[2], Lisha Xu[2], Xue Yang[2], Yunqing Tian[1], Genmeng Yang[1*], Juan Li[2*] and Xiaofeng Zeng[1*]

[1]NHC Key Laboratory of Drug Addiction Medicine, School of Forensic Medicine, Kunming Medical University, Kunming, China, [2]Department of Pathogen Biology and Immunology, School of Basic Medical Science, Kunming Medical University, Kunming, China

Combination antiretroviral therapy (cART) has dramatically reduced mortality in people with human immunodeficiency virus (HIV), but it does not completely eradicate the virus from the brain. Patients with long-term HIV-1 infection often show neurocognitive impairment, which severely affects the quality of life of those infected. Methamphetamine (METH) users are at a significantly higher risk of contracting HIV-1 through behaviors such as engaging in high-risk sex or sharing needles, which can lead to transmission of the virus. In addition, HIV-1-infected individuals who abuse METH exhibit higher viral loads and more severe cognitive dysfunction, suggesting that METH exacerbates the neurotoxicity associated with HIV-1. Therefore, this review focuses on various mechanisms underlying METH and HIV-1 infection co-induced neurotoxicity and existing interventions targeting the sigma 1 receptor, dopamine transporter protein, and other relevant targets are explored. The findings of this review are envisaged to systematically establish a theoretical framework for METH abuse and HIV-1 infection co-induced neurotoxicity, and to suggest novel clinical treatment targets.

KEYWORDS

methamphetamine, psychostimulant, HIV-1, neurotoxicity, programmed cell death, blood-brain barrier, sigma 1 receptor

1 Introduction

Acquired immunodeficiency syndrome (AIDS) is a highly infectious disease caused by infection with the human immunodeficiency virus (HIV). Most people with AIDS are infected with HIV-1, which is the most prevalent and more virulent subtype. It has been demonstrated that HIV-1 may trigger HIV-associated neurocognitive disorder (HAND) by inflicting damage to the neurovascular units (NVU), blood-brain barrier (BBB) dysfunction, and inflammatory response (1). It has been shown that the interplay

between diverse HIV-1 viral proteins (Tat, Vpr, and gp120) and neural cells may be linked to the pathogenesis of HIV-1-induced neurotoxicity, which results in cellular injury and anomalous alterations in the CNS (2). However, the precise mechanism of action is not yet well defined. Despite a significant reduction in mortality rates among HIV-1 patients due to combination antiretroviral therapy (cART), chronic viral infections continue to persist in the brain (3). Moreover, it has been shown that long-term cART may disrupt mitochondrial function, affect metabolism, and even cause neurotoxicity and fatal complications (4, 5). Therefore, we need to explore safer and more effective ways for HIV treatment.

The global issue of substances misuse significantly affects the social welfare and public health. The World Drug Report 2024 by the United Nations Office on Drugs and Crime (UNODC) indicates that 13.9 million individuals engaged in substances injections in 2022, and about one in eight injecting substances abusers will be living with HIV-1 (6). Methamphetamine (METH), a highly addictive psychostimulant, is widely abused and can lead to severe health risks following withdrawal (7, 8). Prolonged METH use has also been reported to negatively impact memory, learning, and cognitive function, leading to symptoms such as paranoia, insomnia, irritability, hallucinations, and delusions (9). In severe cases, METH abuse may contribute to the development of neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD). And yet, there are currently no the US Food and Drug Administration (FDA) -approved drugs for the treatment of METH-induced neurotoxicity (10).

METH abuse is often associated with HIV-1 infection through needle sharing and high-risk sexual activity among users. HIV-1-infected individuals who abuse METH exhibit higher viral loads and more severe cognitive dysfunction (11). Concurrently, studies have shown that the pathways of neurotoxicity can be induced by METH and HIV are somewhat similar. For instance, they can cause mitochondrial dysfunction and altered metabolic pathways (12–14), which in turn can lead to programmed cell death and neuroinflammatory (15–17). However, the specific mechanisms underlying this synergistic neurotoxicity are not fully understood and effective treatments are lacking. Therefore, this paper reviews the mechanisms of synergistically induced neurotoxicity by METH and HIV-1 and proposes potential therapeutic targets.

2 Potential mechanisms of neurotoxicity co-induced by METH and HIV-1

2.1 Oxidative stress and endoplasmic reticulum stress

Oxidative stress, a harmful result of free oxygen radicals in the body, is commonly associated with the processes of ageing and disease (18). Endoplasmic reticulum stress (ERS) is characterized by an atypical accumulation of unfolded or misfolded proteins, resulting from an increased demand for properly folded proteins in response to external stimuli, ultimately leading to ER dysfunction (19). The interaction between ERS and oxidative stress was apparent. The disruption of redox homeostasis in the endoplasmic reticulum caused by oxidative stress has been shown to significantly affect endoplasmic reticulum function, leading to endoplasmic reticulum signaling activation, which could result in ERS development and could produce high levels of reactive oxygen species (ROS), thereby exacerbating oxidative stress (20).

METH has been demonstrated to damage dopaminergic neurons through oxidative stress processes. Upon entering the neuron, METH displaces dopamine (DA) from vesicles, leading to an increase in intracellular and synaptic gap DA levels, which results in elevated auto-oxidation and DA metabolism, leading to higher ROS production (21). The resulting damage to cellular proteins, lipids, and DNA leads to a loss of cellular function and subsequent neurotoxicity, which is further exacerbated when METH and HIV-1 act together (22). Within the human innate immune macrophage cell line system, METH induces significant cellular ROS production, thereby activating the expression of interleukine-1β (IL-1β) and tumor necrosis factor-alpha (TNF-α) in a dose-dependent manner. Additionally, METH-induced oxidative stress is further exacerbated by the presence of HIV-1 Tat protein (23). Combined exposure to METH and HIV-1 synergistically induces oxidative stress by activating transient receptor potential melastatin 2 (TRPM2) channels in endothelial cells, thereby resulting in ROS production, which further exacerbates the toxic effects on the nervous system by compromising the antioxidant defenses of catalase (CAT), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) (1). TRPM2 channels can mediate oxidative stress by activating NLRP3 inflammasome and microglia or inducing TNF-α production, triggering autophagy in human cerebrovascular pericytes and damaging BBB (24).

It was also found that METH caused a significant increase in ERS-related proteins Bip, ATF-6, ATF-4, eIF2α, and CHOP in C57BL/6 mice, where memory loss and cognitive impairment were improved when treated with taurine, an ERS inhibitor, suggesting that METH-induced memory loss was associated with ERS (25).Abnormal mitochondrial function is a significant contributor to oxidative stress (26). Sirtuins (SIRTs) are NAD^+-dependent deacetylases involved in mitochondrial biogenesis, protein responses, and intrinsic apoptosis. The activation of SIRTs has been reported to exert a neuroprotective effect in conditions such as AD and PD (27). In HIV-1 disease models, the miR-505/SIRT3 axis was implicated in mitochondrial oxidative stress, associated with the induction of the HIV-1 Tat protein-mediated microglia senescence phenotype, leading to elevated superoxide production within mitochondria (28). Consequently, HIV-1 Tat protein may induce oxidative stress and ERS by inhibiting SIRT1 and SIRT3, thereby diminishing their neuroprotective properties (28, 29). Mitochondria-associated endoplasmic reticulum membranes (MAMs) represent direct contact sites between the ER and mitochondria, functioning as critical platforms for the coordination of essential cellular processes, including mitochondrial dynamics and calcium homeostasis (30). Notably, the strategic targeting of MAMs to modulate astrocyte mitochondrial function holds potential as a promising approach to enhance the metabolic and antioxidant

coupling between astrocytes and neurons, thereby promoting neuronal resilience against CNS pathologies (30). It has also been shown that METH and HIV-1-induced astrocyte excitatory amino acid transporter-2 dysfunction can be restored by maintaining calcium homeostasis (31).

Regulation of endoplasmic reticulum-related signaling pathways is critical for cellular homeostasis (19). Results of a study evaluating the effects of 7-day HIV-1 infection and chronic METH exposure on the endoplasmic reticulum and mitochondria showed that the expression level of ERS-related inositol-requiring protein 1α (IRE1α) was significantly upregulated (32). These results imply that ERS is implicated in the synergistic effect of HIV-1 and METH on the induction of neurocognitive impairment. Nonetheless, moderate ERS is beneficial for the body, but excessive ERS can cause neurocognitive dysfunction; thus, both oxidative stress and ERS are important factors in neurotoxicity caused by METH and HIV-1 (Figure 1).

2.2 Programmed cell death

Presently, the prevalent types of programmed cell death include apoptosis, pyroptosis, autophagy, necrosis, and ferroptosis (33). The molecular mechanisms underlying these forms of cell death are not self-contained, and recent findings indicate an intricate interplay between them, collectively promoting nerve cell death (Figure 2) (34).

2.2.1 Autophagy

Autophagy is a crucial physiological mechanism for preserving cellular homeostasis and energy conservation. This process is primarily initiated by the activation of ULK1, Atg13, and FIP200 complexes via the PI3K/Akt/mTOR pathway, which then triggers the commencement of autophagic program (35). The acceleration of cell death resulting from dysregulation of autophagy occurs when the body is overstimulated. Autophagy dysfunction has been identified as a contributing factor to mental disorders and synaptic damage in degenerative diseases (36). Meanwhile, autophagy is the primary pathway for the degradation of long-lived proteins and organelles and is closely associated with survival, death, and neuron transformation (33). The formation of double-membrane vesicles, known as autophagosomes, is facilitated by the copolymerization of a series of Atg complexes that can capture organelle fragments in the cytoplasm (37). The completion of autophagy depends on the correct formation, maturation, and fusion with lysosomes of autophagosomes.

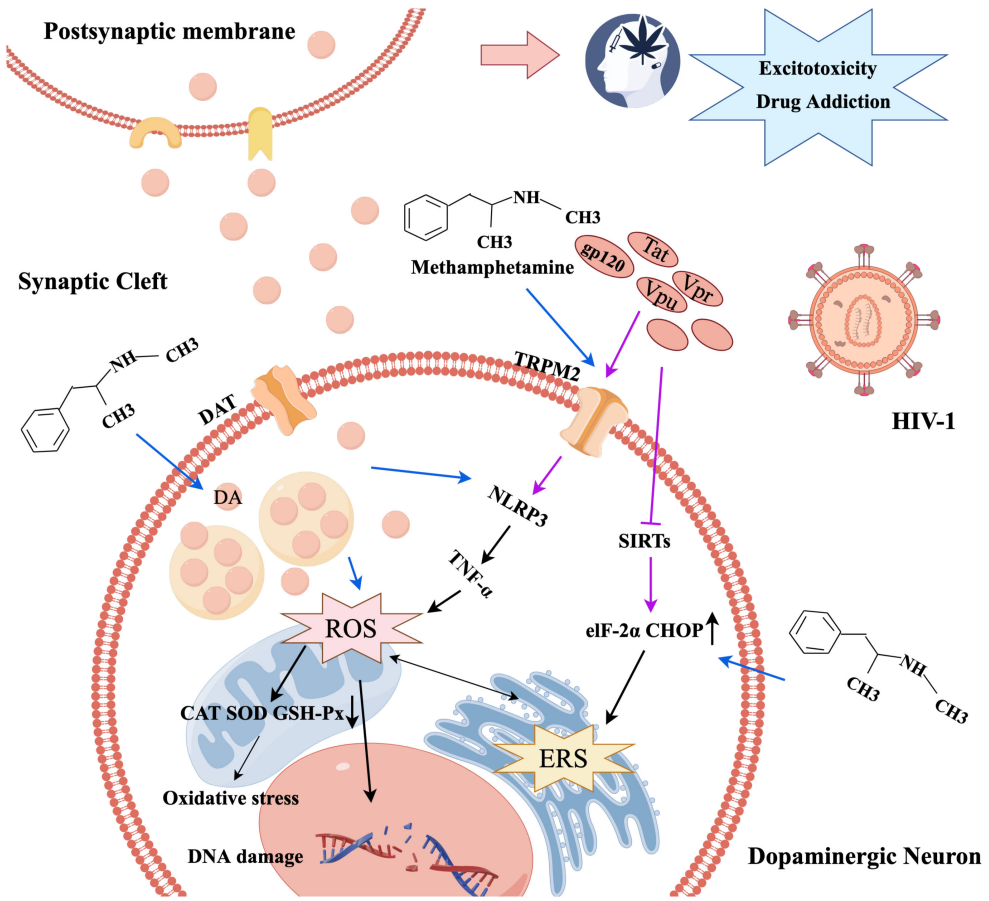

FIGURE 1
The mechanism diagram of oxidative stress and endoplasmic reticulum stress caused by METH and HIV-1.

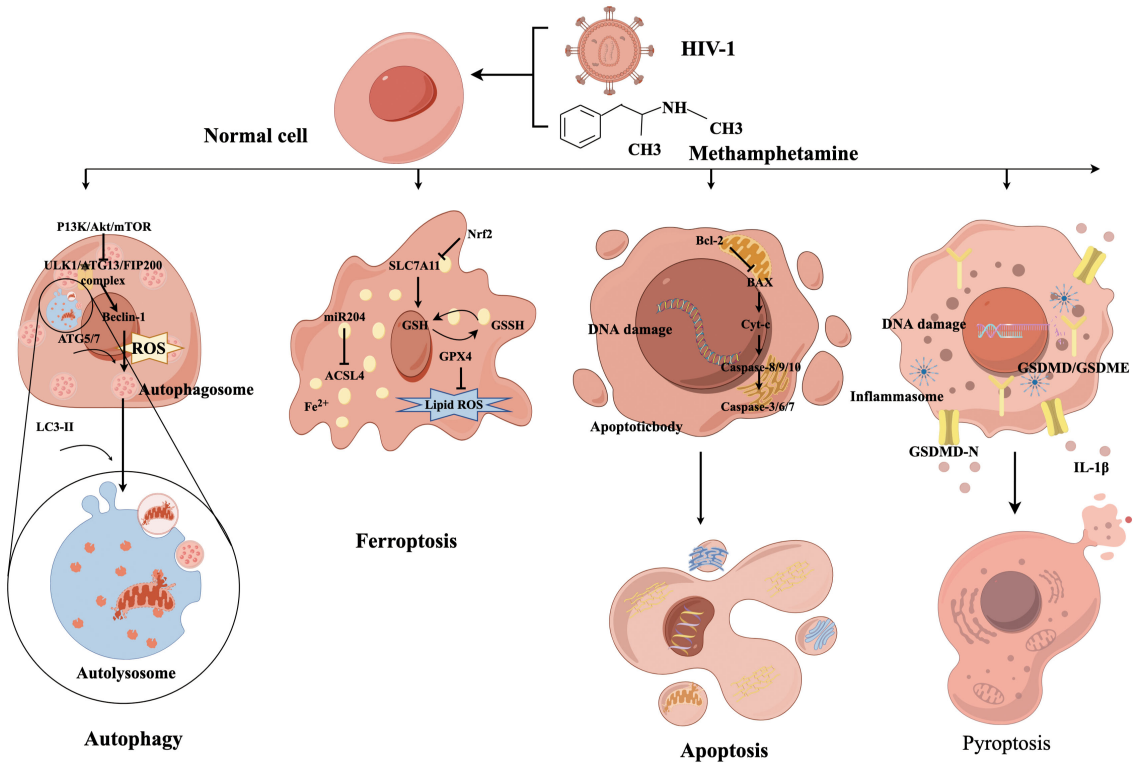

FIGURE 2
The mechanism diagram of programmed cell death caused by METH and HIV-1.

The specific role of autophagy in the body is twofold. On the one hand, the potential for the accumulation of misfolded proteins following HIV-1 infection to induce ERS, autophagy can effectively remove these toxic proteins, thereby reducing ERS and inhibiting the progression of HAND. On the other hand, an autophagic imbalance may result in the build-up of harmful substances in neurons (38). Similarly, METH enhanced HIV-1 gp120-mediated autophagy via Beclin-1 and Atg5/Atg7, thereby exerting a protective effect on astrocytes (39). Recent research on the gut-brain axis has revealed that the METH-abused BALB/c mouse model with continuously increasing multiple doses can induce neurotoxicity and enteritis and alter the gut microbiota and fecal metabolites, which is closely associated with autophagy (40). Our previous study revealed that HIV-1 Tat protein can promote autophagy in nerve cells in a concentration-dependent manner, and the induction of autophagy by HIV-1 Tat protein was found to decrease cellular activity, signifying its detrimental impact on the nervous system (41).

METH abuse has been observed to result in elevated systemic inflammation and CNS impairment in HIV-1-infected individuals. METH was found to inhibit macrophage phagocytosis of aggregated amyloid-β, increase total ROS, and dysregulate the autophagic process in HIV-1-infected human macrophages (42). Furthermore, previous research demonstrated that the combined effects of HIV-1 Tat protein and METH can induce autophagy in primary tree shrew midbrain neuronal cells through mTOR signaling and the Atg5/Atg7 pathway (43). The combined impact of METH and HIV-1 Tat proteins results in a significant increase in LC3-II expression and a blockade of autophagic flow in primary human neuronal cells, resulting in increased mitochondrial DRP1-dependent breakdown or rupture and neuronal degenerative changes by inhibiting mitochondrial autophagy (44). These findings suggest that the combined action of METH and HIV-1 Tat proteins induces autophagy, thereby contributing to neurotoxic damage.

2.2.2 Ferroptosis

Ferroptosis is a recently discovered iron-dependent form of programmed cell death distinct from apoptosis, necrosis, and autophagy (45). Although Ferroptosis was initially proposed in 2012, prior research has identified irregularities in iron metabolism within the CNS (46). Ferroptosis is characterized by unique morphological and biochemical features, including depletion of intracellular GSH and inactivation of GSH-Px4, resulting in the accumulation of lipid peroxidation (34). Ferroptosis induction is closely associated with oxidative stress, a phenomenon that METH contributes to through various pathways, ultimately affecting neurocognitive function. Many studies have implied the importance of maintaining normal intracellular iron levels for proper development and functioning of the CNS. Consequently, Ferroptosis may be pivotal in METH-induced neurotoxicity.

Several studies have established a correlation between ferroptosis and various neurodegenerative disorders such as PD, AD, and HAND (47). Neurons and glial cells exhibit elevated susceptibility

to ferroptosis and neurodegeneration, with microglia being the most iron-laden cells. Iron-activated microglia may participate in the aberrant elimination of neurons and synapses, thereby exacerbating ferroptosis-induced neurodegeneration (48). Researchers found, as early as the 1990s, that ferritin concentrations increased, and iron-mediated oxidative stress was evident as AIDS progressed (49). According to a recent clinical study, elevated concentrations of iron transport proteins in the cerebrospinal fluid are associated with the incidence of HAND (50). The underlying mechanism of HAND may be attributed to HIV-1-related proteins inducing ferroptosis, where HIV-1 Tat protein specifically triggers ferroptosis in microglia by suppressing miR-204 expression, which subsequently upregulates ACSL4 expression, leading to increased lipid peroxidation. This process ultimately results in microglial activation and secretion of proinflammatory cytokines, culminating in neuroinflammation and neurotoxicity (48).

SLC7A11 is a crucial cystine/glutamate transporter protein constituent that expedites GSH synthesis and impedes ferroptosis (51). Our research team discovered that METH and HIV-1 Tat proteins can collaboratively instigate ferroptosis in BV2 cells, which is antagonized by transcription factor NF-E2-related factor (Nrf2) by regulating SLC7A11, thus providing a theoretical basis for investigate the synergistic effects of METH and HIV-1 induced neurotoxicity (17). However, the precise mechanisms underlying ferroptosis are poorly understood and warrant further investigation.

2.2.3 Apoptosis

Apoptosis is among the earliest identified forms of programmed cell death mechanisms (52). Unlike necrosis, apoptosis is an activecell death process that does not involve rupture of the cell membrane. It is often characterized by cell shrinkage, nuclear breakage, budding of the cytoplasmic membrane, and formation of apoptotic vesicles (53). Apoptosis has been a research hotspot for METH and HIV-1 induced neurotoxicity.

Apoptosis can be divided into an initiation phase and an execution phase, where the former is generally mediated by caspases 8, 9, and 10 and the latter by caspases 3, 6, and 7 (54). Among them, caspases 8 and 10 are initiators of exogenous apoptosis, and caspase 9 is an initiator of endogenous apoptosis. The activation of caspase-3 heralds the beginning of the apoptotic process and is considered a key enzyme that leads to apoptosis. A variety of proteins play equally important roles in the development of apoptosis. Bcl-2 and Bcl-XL act as potent apoptosis inhibitors to prevent apoptosis. Factors such as Bak, Bad and Bax promote apoptosis. In addition, P53 and Cyt-c are also involved in apoptosis development (55).

As previously mentioned, autophagy may have deleterious effects, resulting in autophagic cell death, and multiple intermediates of programmed cell death engage in intricate interactions (56). In an *in vitro* astrocyte experiment, the combined effects of METH and gp120 were observed, where inhibition of autophagy resulted in significant astrocyte apoptosis, indicating that autophagy plays a protective role against apoptosis caused by METH and gp120 (39). Similarly, another study found that gp120 and METH-induced oxidative stress through the cytochrome (CYP) P450 and NADPH oxidase (NOX) pathways result in astrocyte apoptosis (57), implying that inhibiting these two pathways could significantly help suppress the apoptosis of astrocytes. These findings demonstrated that METH and gp120 can induce apoptosis in several ways. In addition to gp120, a significant increase in TUNEL-staining-positive cells was also found in the hCMEC/D3 model of human microvascular endothelial cells following the combined action of HIV-1 Tat protein and METH (24). This indicates that both METH and multiple viral proteins of HIV-1 can induce apoptosis and that efficacious interventions are currently unavailable.

2.2.4 Pyroptosis

Pyroptosis is a mode of inflammatory cell death activated through two main pathways: gasdermin D (GSDMD)-dependent activation regulated by caspase-1/4/5/11 and GSDME-dependent activation regulated by caspase 3 (58). Activated caspases cleave the hinge region between the N- and C-terminal domains of GSDMD or GSDME to recognize and bind to phospholipid molecules in the cell membrane, This results in the formation of pores or perforations within the cell membrane, leading to alterations in cellular osmolarity and ultimately cell membrane rupture and cell death (59).

Substances misuse has been shown to accelerate neurological symptoms in HIV-1-infected patients by facilitating HIV-1 entry into the brain and triggering an immune response, thereby mediating neuroinflammation and pyroptosis through the activation of microglia and the release of neurotoxins (60). Studies have disclosed that METH can induce GSDME-dependent cell death of hippocampal neurons through the ERS pathway (61). In addition, manifestations of cognitive impairment and changes in the NLRP1/Caspase1/GSDMD signaling pathway were found in an *in vivo* model of METH administration in rats (62). The use of aspirin and NLRP1 siRNA significantly attenuated METH-induced cognitive deficits while decreasing the activities of NLRP1 and cleaved caspase-1, IL-1β, and TNF-α, providing further evidence of complex programmed cell death mechanisms. Similarly, one study reported HIV-1 gp120-induced NLRP3-dependent pyroptosis and IL-1β production in microglia (63). Long-term administration of MCC950 (an NLRP3 inhibitor) to gp120 transgenic mice attenuated neuroinflammation and neuronal death, promoted neuronal regeneration, and restored impaired neurocognitive functions (64).

All the aforementioned findings suggest a link between METH and HIV-1 and pyroptosis. Indeed, a recent study showed that METH enhanced HIV-1 gp120-induced activation of microglia and NLRP3 inflammasome, while elevating GSDMD and GSDMD-N expression (65). Thus, activation of microglia-mediated neuroinflammation and pyroptosis might be one of the HAND etiological factors. Nevertheless, the investigation into METH and HIV-1 acting synergistically in inducing pyroptosis is still in its infancy. Further research is required to elucidate the interrelationships between pyroptosis and other mechanisms.

2.3 Neuroinflammation

Neuroinflammation is an important mechanism of neurotoxicity that has been closely associated with the activation

of inflammasomes, glial cells, and cytokines (9, 66–68). The activation of microglia and astrocytes is a crucial component of the synergistic induction of neuroinflammation by METH and HIV-1. It has been shown that METH can activate inflammatory cells such as astrocytes and microglia, leading to the secretion of TNF-α and IL-1β inflammatory factors (23). Upon HIV-1 infection of human microglia, pro-IL-1β is secreted at 4 h, and mature IL-1β is secreted at 24 h, accompanied by caspase-1 activation, initiating the inflammatory process (69). Furthermore, HIV-1 infection induces a notable release of proinflammatory cytokines, namely IL-1β and IL-18. Exposure of monocytes to HIV-1 Tat has been observed to cause IL-1β release, which can lead to neuronal cell death and HAND through a direct or indirect proinflammatory mechanism (70). Similarly, the severity of this inflammatory response is elevated in HIV-1 patients with a history of METH abuse (71).

At present, five primary types of inflammasomes, namely NLRP3, NLRP1, NLRC4, IPAF, and AIM2, have been extensively studied and are known to play crucial roles in the onset and progression of various diseases (72). In a study involving METH abuse in rats, elevated NLRP1 activity was observed in the hippocampus, and NLRP1 inhibition significantly ameliorated METH-induced cognitive impairment (62). Moreover, inhibition of the NLRP3 inflammasome has the potential to mitigate motor impairments and cerebellar alterations induced by METH administration (68). Similarly, HIV-1 activates inflammasomes through diverse mechanisms, resulting in immune cell death and associated pathological changes (73). This shows a notable correlation between inflammasomes and neuroinflammation induced by METH and HIV-1.

The findings of our study indicated that microglia in the prefrontal cortex of METH abusers with concurrent HIV-1 infections exhibited significant activation (74). Additionally, METH and HIV-1 led to the activation of NLRP3 inflammasomes and caused neuroinflammation, as well as neuronal apoptosis in the prefrontal cortex and hippocampus (63). These results have suggested that neuroinflammation in HIV-1-infected individuals with METH abuse may contribute to neuronal apoptosis and consequent damage to the nervous system. Neuroinflammation involves a multifaceted interplay of mechanisms, including apoptosis and oxidative stress (75). Therefore, investigating the interplay between neuroinflammation and other mechanisms is a promising area for future research.

2.4 Blood-brain barrier damage

The BBB serves as a critical interface between the brain and the peripheral circulation and plays a vital role in maintaining homeostasis within the CNS (76). The neurovascular unit (NVU), which comprises endothelial cells, pericytes, astrocytes, microglia, neurons, and basement membranes, is the main functional unit of the blood-brain barrier (77). To access the CNS, both METH and HIV-1 must traverse the BBB. It has been established that the benzene ring and methyl group in the chemical structure of METH render it lipid-soluble and capable of traversing cell membranes and even the BBB, thereby exerting an effect on the central nervous system. HIV-1 may gain access to the central nervous system by infecting endothelial cells or mononuclear macrophages via a "Trojan horse" mechanism (78). Notably, HIV-1 infection and METH abuse synergistically impairs the BBB (79).

Disruption of BBB results in infiltration of various plasma components, and immune factors into the CNS, thereby inducing neuroinflammation and ultimately leading to toxic damage to the nervous system (80). The mechanisms of neuroinflammation and BBB damage caused by METH and HIV-1 are summarized in Figure 3. METH affects CNS homeostasis by impairing pericytes and astrocytes and disrupting tight junction proteins, such as occludin and claudins (81). Recent research has demonstrated that METH exposure induced neuroinflammation and BBB damage through neuronal-derived alpha-nucleoprotein transfer to astrocytes, as evidenced by the co-culturing of neurons with astrocytes (76). Upon entry into the BBB, HIV-1-infected cells secrete a range of HIV-1-associated viral proteins, including Tat, gp20, and Vpr. Notably, the HIV-1-associated viral proteins can exert disruptive effects on the BBB through various mechanisms (82), such as specifically enhancing vascular permeability, suppressing the expression of tight junction proteins, inducing a reduction in the expression of claudin-5 and laminin, and stimulating the expression of matrix metallopeptidase 9 (MMP9) and matrix metallopeptidase 2 (MMP2), ultimately resulting in the cleavage of tight junction proteins (83, 84). However, the mechanism of injury associated with HIV-1 Tat protein-induced BBB damage is multifaceted and not isolated. Despite extensive research, complete elucidation of the mechanism of injury remains elusive.

In our previous series of experiments, we found that ginsenoside Rb1 and gastrodin could alleviate the synergistically induced BBB damage by METH and HIV-1 Tat protein to a certain extent (85, 86). However, the neurotoxic effects caused by METH and HIV-1 are likely to arise from the interaction of various cellular and molecular mechanisms, and more in-depth studies, as well as additional clinical testing, may be required to achieve a therapeutic cure (22). Furthermore, the BBB presents significant challenges for future pharmacological interventions. Consequently, enhancing the ability of drugs across the BBB remains a prominent research focus and obstacle.

3 Potential therapeutic targets for neurotoxicity co-induced by METH and HIV-1

Despite the significant extension of life expectancy for individuals with HIV-1 through combination antiretroviral therapy (cART), chronic infections persist in the brain, and HIV-1-related neurocognitive impairment remains a formidable, incurable challenge (87). To date, the FDA has not approved any effective treatments for METH-induced neurotoxicity (88). Nevertheless, researchers continue to explore potential therapeutic agents for the neurotoxic effects induced by METH and HIV-1. The potential drugs that may treat METH and HIV-1-induced

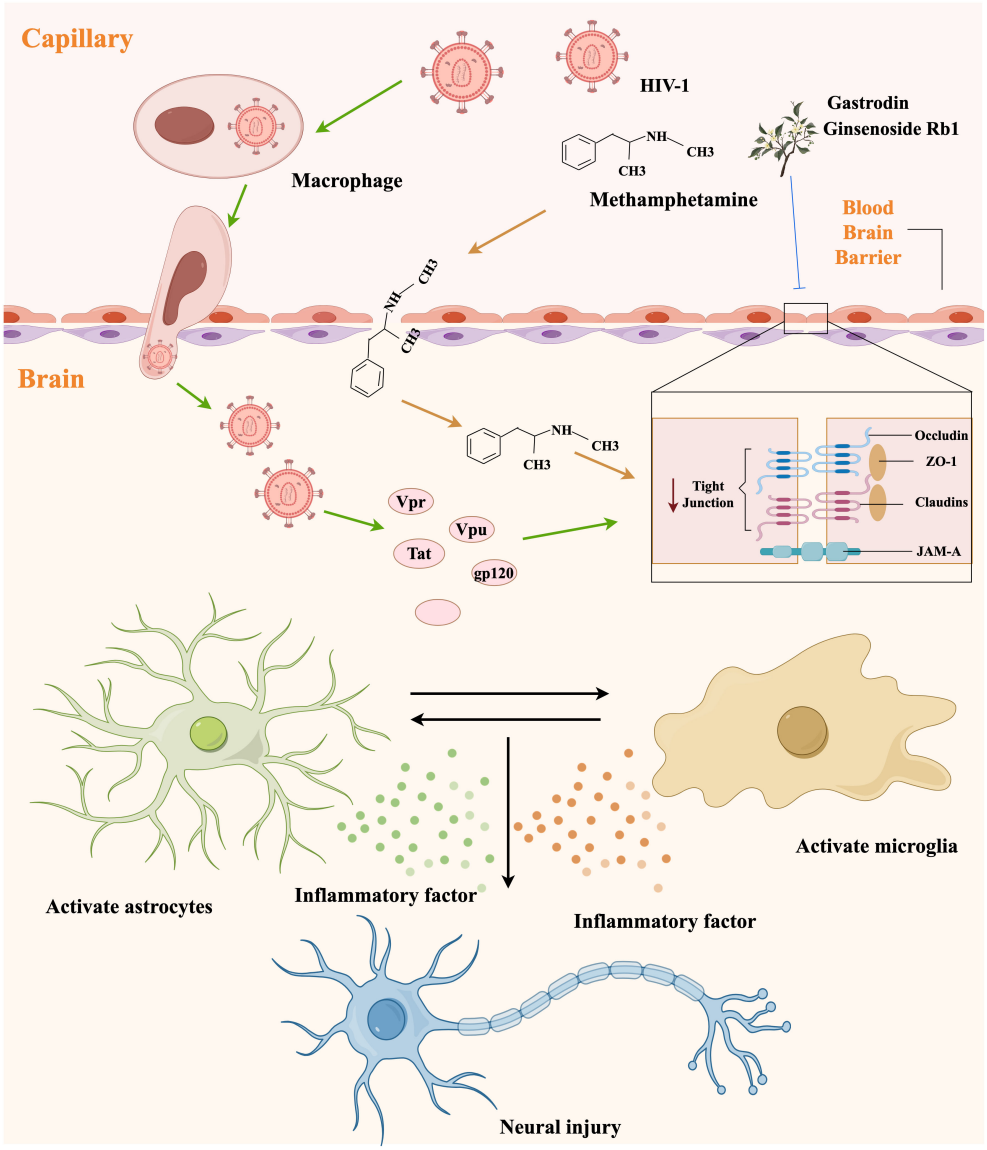

FIGURE 3
The mechanism diagram of neuroinflammation and blood-brain barrier damage caused by METH and HIV-1.

neurotoxicity are summarized in Table 1. For instance, ginsenoside Rb1 has shown promise in mitigating METH-induced neurotoxicity through the NR2B/ERK/CREB/BDNF pathway (89). Gastrodin, another agent, has demonstrated to attenuate METH-induced autophagy and apoptosis (41, 90). However, these agents have not yet provided a complete solution to the problem. Therefore, in this section, we offer a thorough overview of the targets for potential treatment of the neurotoxicity induced by METH and HIV-1 (Figure 4).

3.1 Sigma 1 receptor

Initially, Martin et al. proposed that the sigma 1 receptor (σ1R) was a new subtype of opioid receptor that facilitates specific effects induced by benzomorphans (109). Subsequent research has revealed that σ1R, an endoplasmic reticulum chaperone protein, is modulated by various ligands and serves as a potential therapeutic target for psychostimulant addiction (92). The σ1R has multiple functions within the cell, and its activation or inhibition may have different effects on the cell, depending on the specific biological context and pathological conditions (110).

A recent study suggests that supplementation with short-chain fatty acids can improve anxiety and depression-like behaviors induced by METH, through the activation of the σ1R/BDNF/TrkB pathway in the hippocampus of mice (13). Studies have found that HIV-1 gp120 can induce neuronal apoptosis and neuronal generation, while the 4-phenyl-1-(4-phenylbutyl) piperidine (PPBP), σ1R agonist, can weaken the neurotoxicity of gp120 (95). It suggests a protective role for σ1R. However, BD1047, a σ1R antagonist, has been found to mitigate METH-induced mortality and reduce seizures and convulsions in Swiss-Webster

TABLE 1 The potential drugs that may treat METH and HIV-1-induced neurotoxicity.

Agent	Cell/Animal Type	Curative effect	Mechanisms	Ligand
Ginsenoside Rb1	SH-SY5y cells Rats	Regulated METH-induced neurotoxicity and METH-induced CPP through the NR2B/ERK/CREB/BDNF regulatory pathway. Protect the BBB against the toxic effects of HIV-1 Tat and METH.	Apoptosis BBB damage	METH (89) METH+HIV-1 Tat (86)
Gastrodin	SH-SY5y cells Rat primary cortical neurons Rats Tree shrews Human brain capillary endothelial cells	Enhances the expression of tight junction proteins. Exhibit an anti-autophagic effect on the inhibition of the METH-induced Beclin-1 protein expression, partly via the AKT/mTOR. Regulation of cAMP/PKA/CREB signaling pathway and upregulates the expression of BDNF. Attenuate the effects of METH-induced CPP in rats by regulating the PKA/CREB signaling pathway.	Apoptosis BBB damage Autophagy	METH (41, 90, 91) METH+HIV-1 Tat (85)
MCC950	C57BL/6 mice Rats Primary microglia	Inhibiting NLRP3 alleviated the above-mentioned motor deficits and cerebellar pathologies. Inhibited pyroptosis and release of inflammatory factors.	Neuroinflammation Pyroptosis	METH (68) METH+HIV-1 gp120 (63)
Sigma 1 receptor antagonist	PC12 cells Rats Swiss-Webster mice	Prevents METH-induced sensitization. Mitigate METH-induced mortality and reduce seizures and convulsions. Attenuates conditioned place preference in male rats and viability in PC12 cells through the Sigma1R/AKT/GSK3β/CREB signaling pathway.	Sensitization	METH (92–94)
Sigma 1 receptor agonist	Primary cortical neuronal C57BL/6 mice Sigmar1$^{-/-}$ mice	SCFAs supplementation optimized METH-induced microbial dysbiosis, ameliorated colonic inflammation, and repressed anxiety- and depression-like behaviors. Protecting neurons from gp120 invasion by regulating the expression of bcl-2.	Cognitive disorders Apoptosis	METH (13) HIV-1 gp120 (95)
Nrf2 agonist	BV2 cells SH-SY5y cells Primary microglia Primary hippocampal neurons Nrf2-KO C57BL/6J mice Autopsied brain tissues of METH-abusing and/or HIV-1 infection	Reduced the level of oxidative stress in the organism, thereby attenuating the synergistic induction of autophagy by METH and HIV-1 Tat protein. Nrf2 antagonizes BV2 cell ferroptosis induced by METH and HIV-1 Tat through regulation of SLC7A11. TREs may exert potent neuroprotective effect via activation of both ERK and Nrf2 pathways. Preserves neuronal cells from METH-induced neurotoxicity by upregulating HO-1 expression through the Nrf2 and PI3K/Akt/mTOR signaling pathways. Resveratrol might effectively prevent memory impairment via the interaction with Keap1, activation of the Keap1-Nrf2 pathway, and inhibition of DNA damage and apoptotic responses post METH exposure.	Autophagy Ferroptosis Oxidative stress Apoptosis	METH (96–98) METH+HIV-1 Tat (17, 74)
Chaihu-jia-Longgu-Muli decoction	Rats	Prevent the development of METH-induced withdrawal symptoms in rats	Sensitization Neuroinflammation	METH (99)
DA receptor blocker	C57BL/6 mice	Attenuate METH-induced CPP in mice	Sensitization	METH (100)
DAT mutants	PC12 cells HEK293 cells	Mutating these residues attenuates this inhibitory effect by disrupting the Tat-hDAT interaction	/	HIV-1 Tat (101)
PPAR-γ agonists	Human cerebral microvascular endothelial cells (hCMEC/D3) U937 cells FVB/NJ wild-type or MMP-9-knockout mice Rats C57BL/6 mice BALB/c mice Rat astrocytes and microglia	Prevented the expression of behavioral sensitization to METH challenge on withdrawal day 7. Treatment with ibuprofen, a commonly used nonprescription NSAID, can attenuate METH-induced neurotoxicity and microgliosis. Suppression of the inflammatory response in brain tissue. Against HIV-1-induced MMP-9 expression in brain endothelial cells.	Sensitization Neuroinflammation Oxidative stress BBB damage	METH (102, 103) HIV-1 (104, 105)
Insulin	Autopsied brain tissues, Primary human microglia Primary human neurons Cats(FIV^{+})	Reduced glial cell activation decreased CXCL 10 and IL-6 transcript levels and improved memory and motor function	Neuroinflammation	HIV-1 (106)

(Continued)

TABLE 1 Continued

Agent	Cell/Animal Type	Curative effect	Mechanisms	Ligand
miR-204 mimics	HIV-1 transgenic (Tg) rats Wild-type rats The human frontal cortex brain sections Mouse primary glia Adult microglia from the wild-type and HIV-1 Tg rats	Reduced the expression of ACSL4 while inhibiting HIV-1 Tat-mediated ferroptosis and the release of proinflammatory cytokines	Neuroinflammation Ferroptosis	HIV-1 Tat (48)
miRNA-505 inhibitor	Human brain tissues HIV-1 transgenic rats Mouse primary microglial	Upregulated the expression of SIRT3 and mitochondrial antioxidant enzymes, with a concomitant decrease in microglial senescence	Oxidative stress	HIV-1 Tat (105)
Anti-miR-143 lentivirus	Human brain microvascular endothelial cells (HBMECs) C57BL/6 mice PUMA KO mice	Prevented the BBB damage induced by METH	BBB damage	METH (107)
Ibudilast (miR-29a inhibitor)	Macaques Rats The NR-9460 microglial cell line The NR-19980 microglial cell line RAW 264.7 macrophages	Attenuated inflammation and rescued synaptodendritic injury	Neuroinflammation	METH (108)
miR-34a inhibitor	TZM-bl cells	Upregulation of protein expression of SIRT1	Neuroinflammation	HIV-1 Tat (29)

mice (93). Prior research also has demonstrated that cannabidiol can attenuate METH-induced conditioned place preference in rats through the σ1R/AKT/GSK-3β/CREB signaling pathway (94). The incidence of HIV-1 infection in quiescent $CD4^+$ T cells is typically low; but even minor alterations in these cells can result in increased susceptibility to HIV-1 infection. Similarly, studies have indicated that the utilization of stimulants, such as cocaine, can enhance the likelihood of HIV-1 infection in quiescent $CD4^+$ T cells through σ1R activation (111, 112). These findings suggest that σ1R may have damaging effects on the organism. In summary, σ1R plays a complex role in regulating homeostatic processes and may serve as a potential target for future therapeutic interventions.

3.2 Transcription factor NF-E2-related factor 2

Nrf2 is a significant transcriptional regulator with anti-inflammatory and antioxidant properties and a safeguarding effect on cellular survival (113). Under normal physiological conditions, Nrf2 is predominantly located in the cytoplasm and associates with Keap1 to form a complex. Upon exposure to stimuli such as ROS, Nrf2 disengages from Keap1 and translocates into the nucleus, where it interacts with antioxidant response elements and triggers the expression of antioxidant enzymes (114). When the extent of damage surpasses its regulatory capacity, the Keap1/Nrf2 pathway can have deleterious effects on the body, exacerbating oxidative stress.

The potential of targeting Nrf2 to treat METH and HIV-1-induced neurotoxicity has been extensively studied. Research has demonstrated that the endogenous antioxidant pathway, mediated by Nrf2, can mitigate METH-induced neurological dysfunction resulting from oxidative stress (96, 97). Resveratrol, a natural polyphenol, has been extensively studied for treating neurodegenerative diseases (115). Numerous studies have shown that multiple exposures to METH significantly impair cognitive function, leading to long-term memory deficits (116), whereas resveratrol may be effective in preventing memory impairment through interaction with Keap1, activation of the Keap1-Nrf2 pathway, and inhibition of DNA damage and apoptotic responses after METH exposure (98). Additionally, Nrf2 has also been reported to regulate iron homeostasis *in vivo* (117). It has been shown that Nrf2 can respectively prevent ferroptosis and autophagy induced by METH and HIV-1 Tat proteins by regulating the SLC7A11 and Nrf2/NQO1/HO-1 signalling pathways (17, 74). Consequently, further investigation of the functional role of Nrf2 provides great value for the treatment of METH and HIV-1-induced neurotoxicity.

3.3 Dopamine transporter protein

DA is crucial for regulating various physiological processes, such as reward, motivation, movement, working memory, and cognition (118). Dopaminergic neurons contain a transmembrane protein, DAT, which primarily promotes reuptake of DA released into the synaptic cleft (119).

Acute or single high dose METH abuse is indicated to cause a decrease in the amount of DAT and affect DA reuptake. This causes an increase in extracellular concentrations of DA, which in turn leads to the euphoria effect (120). However, the reduction in DAT can persist for up to 3 years after METH discontinuation. Prolonged

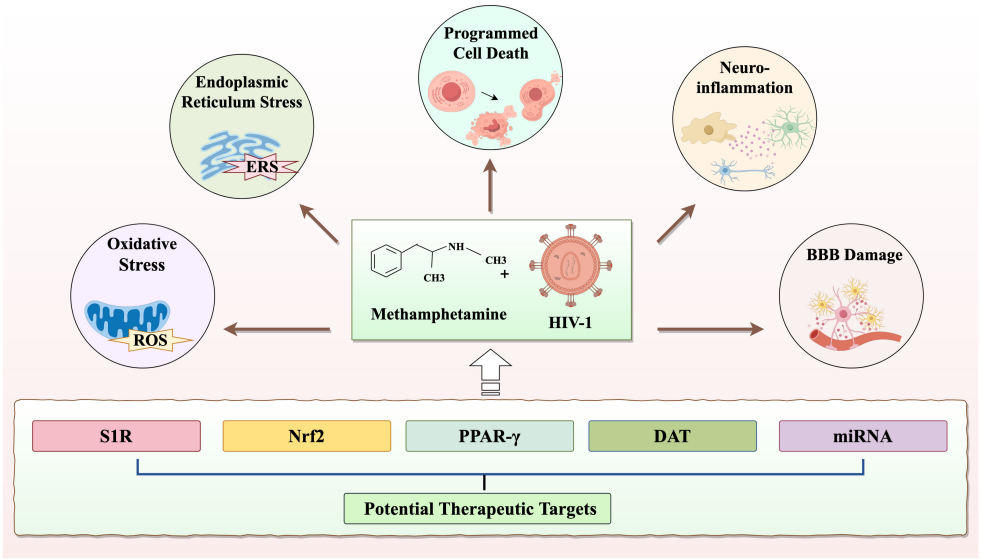

FIGURE 4
The neurotoxicity and therapeutic targets co-induced by METH and HIV-1.

or repeated METH administration can lead to a decrease in DA levels (121). The administration of traditional Chinese medicines, such as Chaihu-jia-Longgu-Muli decoction and levo-tetrahydropalmatine, has been demonstrated to regulate the levels of DA intracellular and extracellular, thereby mitigating the neurotoxicity associated with METH (99, 100). It has been posited that long-term HIV-1 viral protein exposure leads to a decreased dopaminergic state, which continues to persist despite the advent of cART (122).. Therefore, HIV-1 treatment should prioritize the restoration of DA function. The HIV-1 Tat protein plays a crucial role in the onset of HAND by binding to DAT, impeding DA transmission, and inducing neurotoxicity (123). Research has demonstrated that the HIV-1 Tat protein inhibits DA uptake by directly interacting with the human-dopamine transporter protein (hDAT) through mutation of crucial hDAT residues, namely D-H547, D-Y88, and D-D206, which are anticipated to participate in the binding process. This inhibition can be attenuated by disrupting the Tat-hDAT interaction via mutations in these residues (101). In the context of the combined influence of METH and HIV-1, the HIV-1 Tat protein has been observed to intensify the sensitizing impact of METH by altering the DA function (124). These findings indicate that extrasynaptic DA levels and DATs are crucial elements to focus on in managing neurotoxicity caused by METH and HIV-1, potentially paving the way for novel avenues in interventional research.

3.4 Peroxisome proliferator-activated receptor gamma

Peroxisome proliferator-activated receptor gamma (PPAR-γ) belongs to the nuclear hormone receptor superfamily of ligand-activated transcription factors that govern various functions, such as lipid metabolism and inflammation (125). Additionally, PPAR-γ plays a crucial anti-inflammatory role in brain injury and neurodegenerative diseases, is implicated in the pathogenesis of several diseases, including diabetes, cancer, and obesity, and is a promising therapeutic target for CNS disorders (126).

Activation of PPAR-γ results in its translocation to the nucleus, increasing anti-inflammatory gene expression and decreasing microglia/macrophage activation, ultimately downregulating proinflammatory factors (127). Behavioral sensitization, an experimental model of psychostimulant psychosis, is induced by repeated administrations of psychostimulants and has linked to brain inflammation. Notably, PPAR-γ activation attenuated METH-induced behavioral sensitization, potentially due to its anti-inflammatory properties (102). After the three-day METH injection, a gradual decrease in PPAR-γ expression was observed. However, subsequent treatments with aspirin and ibuprofen gradually reversed the DAT expression reduction, microglial activation, and PPAR-γ expression. These findings suggest a correlation between the reduction of PPAR-γ and METH-induced neurotoxicity, implying that the PPAR-γ agonistic effect of ibuprofen may underlie its ability to reverse neurotoxicity (103).

HIV-1 infection results in a range of neurological dysfunctions that entail activating glial cells and releasing inflammatory factors. These events lead to the occurrence of lethal toxic effects on nerve cells (104). Insulin treatment has been observed to upregulate PPAR-γ expression in HIV-1-infected primary microglia (106). Furthermore, HIV-1 primarily infects the CNS by disrupting the BBB. A reduction in PPAR-γ impedes MMP-9 and MMP-2 activity in human monocytes infected with HIV-1, leading to BBB impairment (105). Inflammation is a common factor in both METH and HIV-1 induced neurotoxicity, and the anti-inflammatory properties of PPAR-γ provide compelling evidence for synergistic therapeutic investigation of these conditions.

3.5 miRNA

The emergence of diverse genetic testing technologies, including transcriptome sequencing, has increased the interest in the *in vivo* function of non-coding RNAs (128), including rRNA, tRNA, snRNA, snoRNA, and microRNA. Recent extensive research has established that miRNAs can contribute to disease pathogenesis through various mechanisms and serve as viable targets for therapeutic drugs (129). This section provides a concise summary of the involvement of miRNAs in METH and HIV-1-induced neurotoxicity.

The pivotal function of miRNAs is contingent on their target genes, as established in the literature. Extensive studies have supported an important role for miRNAs in neurotoxicity induced by METH and HIV-1 (48, 130, 131). Specifically, METH induces the upregulation of miRNA-143 via σ1R, resulting in detrimental effects on human brain endothelial cells and disruption of tight junction proteins, ultimately compromising BBB integrity. Notably, silencing of miRNA-143 has been shown to confer protection to the BBB against METH-induced damage (107). Studies have demonstrated that chronic administration of METH leads to an increase in the expression of exosome-secreted miRNA-29a. However, ibudilast, an anti-inflammatory drug, can reduce the secretion of extracellular vesicles and subsequently downregulate miRNA-29a, mitigating synaptic damage (108). HIV-1 neurotoxicity is closely linked to its infectious capacity, and various stimuli can alter miRNA expression *in vivo*, thereby influencing HIV-1 replication. Activated CD4+ T cells can exhibit high levels of miRNA-132, the first miRNA discovered to enhance HIV-1 replication (132). Furthermore, the elevation of miR-34a by HIV-1 Tat protein impeded SIRT1, consequently triggering neuroinflammation (29). These results highlight the importance of miRNA-based gene therapy in addressing the neurotoxicity of both METH and HIV-1, as these miRNAs have been shown to be stably expressed *in vivo*.

4 Discussion

The excitotoxicity induced by METH leads to an elevated likelihood of engaging in risky sexual behavior and unhygienic injection practices among substance abusers, thereby increasing the risk of HIV-1 acquisition. This risk is further exacerbated in individuals with co-infection of HIV-1. Currently, there are no pharmacological interventions specifically targeting METH-induced neurotoxicity, with only symptomatic treatments available based on clinical presentation. Antiretroviral therapy can effectively manage HIV-1, but it does not completely remove the virus from the brain. In addition, a variety of complementary therapies can be employed, including psychotherapy, lifestyle changes, and social support. The above treatments primarily relieve clinical symptoms without preventing disease progression. Therefore, researchers are trying to find effective drugs that can treat the neurotoxicity they co-cause by exploring the common targets of METH and HIV-1. In this review, we have provided a comprehensive and up-to-date overview of the mechanisms and targets currently under investigation for METH and HIV-1-induced neurotoxicity, drawing on published literature and recent reports.

We have identified that σ1R, NRF2, DAT and PPAR-γ are all involved in METH and HIV-1 co-induced neurotoxicity (13, 17, 125, 133). Many preclinical studies have suggested that modulation of these targets may have significant therapeutic effects on METH and HIV-1 co-induced neurotoxicity. This provides new perspectives and potential breakthroughs for such future therapeutic strategies. However, future research should encompass more in-depth experiments and foster multidisciplinary communication to identify precise biomarkers. These biomarkers are instrumental for the early detection and classification of METH and HIV-1 co-induced neurotoxicity, thereby facilitating the development of personalized treatment plans. Meanwhile, we also found that METH and HIV-1 co-induced neurotoxicity has been significantly altered at the transcriptional level, and we briefly summarized some aberrant miRNAs, such as miRNA-143, miRNA-29a and miRNA-132 (108, 131, 134). The researchers have found that targeting these miRNAs could also ameliorate METH and HIV-1 co-induced neurotoxicity, providing a new strategy for treatment. However, the clinical application of miRNA therapy continues to face a lot of challenges, including issues of drug delivery, specificity, and safety, which need to be addressed through further research and clinical trials.

We believe that there are numerous unanswered questions for researchers to explore regarding the neurotoxicity associated with METH and HIV-1 co-exposure. Specific questions include: I. How METH affects HIV-1 latency and reactivation. II. The role of environmental factors in exacerbating or mitigating the neurotoxicity. III. The effects of simultaneous use of different drugs on the co-induced neurotoxicity of METH and HIV-1. IV. The specific roles of cellular populations and neural circuits in the neurotoxicity induced by the co-exposure to METH and HIV-1. Meanwhile, we should not overlook the impact of the social environment on patients. We must enhance public awareness of substance use disorder and HIV-1 infection through education and prevent such issues at their root. It is crucial to provide emotional support and practical assistance to individuals affected by HIV-1 and METH use, to promote societal understanding and acceptance of them, thereby reducing their psychological barriers to seeking help and improving their quality of life. In conclusion, we hope more researchers will focus on exploring treatments for METH and HIV-1-induced neurotoxicity.

5 Conclusion

The review provided an overview of the distinct mechanisms underlying neurotoxicity induced by METH and HIV-1, emphasizing their interplay. These mechanisms include oxidative stress, endoplasmic reticulum stress, neuroinflammation, programmed cell death, and blood-brain barrier damage. Additionally, potential targets for treating METH and HIV-1-induced neurotoxicity were discussed. The objective of this review

was to establish a theoretical foundation for understanding METH and HIV-1-induced neurotoxicity and to suggest potential avenues for clinical research and treatment.

Author contributions

LM: Conceptualization, Writing – original draft. HW: Conceptualization, Writing – original draft. YL: Conceptualization, Writing – original draft. JH: Methodology, Supervision, Writing – review & editing. CW: Methodology, Supervision, Writing – review & editing. HT: Formal Analysis, Visualization, Writing – review & editing. LX: Formal Analysis, Visualization, Writing – review & editing. XY: Formal Analysis, Visualization, Writing – review & editing. YT: Formal Analysis, Visualization, Writing – review & editing. GY: Funding acquisition, Supervision, Writing – review & editing. JL: Funding acquisition, Supervision, Writing – review & editing. XZ: Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Michael H. Lehmann,
Ludwig Maximilian University of Munich, Germany

REVIEWED BY
Paul R. Kinchington,
University of Pittsburgh, United States
Abraam Yakoub,
Harvard Medical School, United States

*CORRESPONDENCE
Peter Ponsaerts
peter.ponsaerts@uantwerpen.be

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Varicella-zoster virus recapitulates its immune evasive behaviour in matured hiPSC-derived neurospheroids

Jonas Govaerts[1,2,3], Elise Van Breedam[1], Sarah De Beuckeleer[1,4,5], Charlotte Goethals[1], Claudio Peter D'Incal[6], Julia Di Stefano[1], Siebe Van Calster[1], Tamariche Buyle-Huybrecht[1,2,3], Marlies Boeren[1,2,3], Hans De Reu[1,7], Søren R. Paludan[8], Marc Thiry[9], Marielle Lebrun[10], Catherine Sadzot-Delvaux[10], Helena Motaln[11], Boris Rogelj[11,12], Johan Van Weyenbergh[13], Winnok H. De Vos[4,5], Wim Vanden Berghe[6], Benson Ogunjimi[3,14,15,16], Peter Delputte[2,17] and Peter Ponsaerts[1,7]*

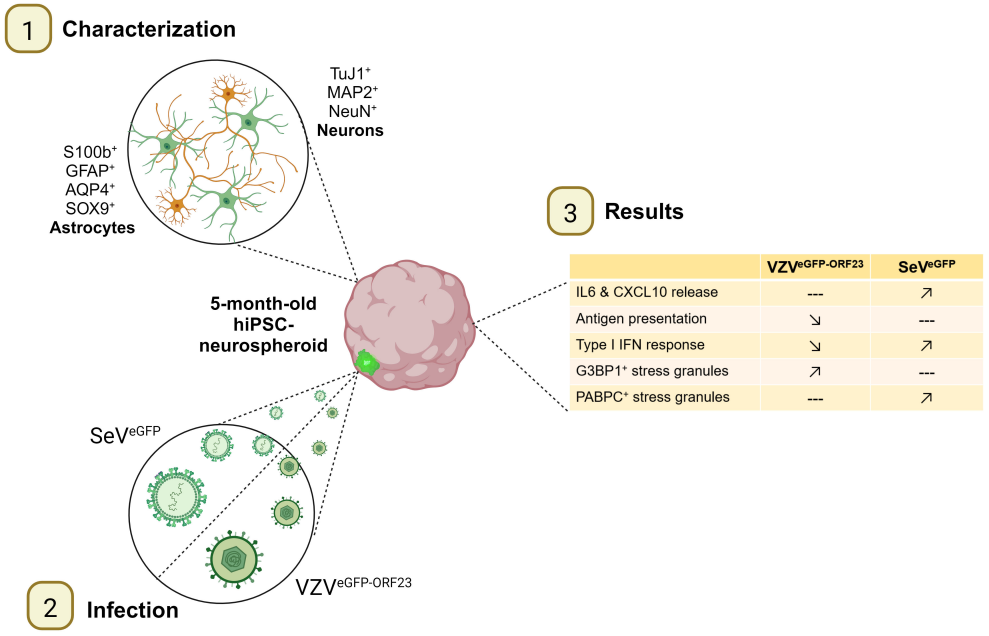
[1]Laboratory of Experimental Hematology (LEH), Vaccine and Infectious Disease Institute (Vaxinfectio), University of Antwerp, Antwerp, Belgium, [2]Laboratory of Microbiology, Parasitology and Hygiene (LMPH), University of Antwerp, Antwerp, Belgium, [3]Antwerp Center for Translational Immunology and Virology (ACTIV), Vaccine and Infectious Disease Institute (Vaxinfectio), University of Antwerp, Antwerp, Belgium, [4]Laboratory of Cell Biology and Histology, Antwerp Center for Advanced Microscopy, Department of Veterinary Sciences, University of Antwerp, Wilrijk, Belgium, [5]μNEURO Research Centre of Excellence, University of Antwerp, Wilrijk, Belgium, [6]Cell Death Signaling – Epigenetics Lab, Department of Biomedical Sciences, University of Antwerp, Antwerp, Belgium, [7]Flow Cytometry and Cell Sorting Core Facility (FACSUA), University of Antwerp, Antwerp, Belgium, [8]Department of Biomedicine, Aarhus University, Aarhus, Denmark, [9]Laboratory of Cell and Tissue Biology, GIGA-Neurosciences, Cell Biology L3, University of Liège, Liege, Belgium, [10]Laboratory of Virology and Immunology, GIGA-Infection, Inflammation and Immunity, University of Liège, Liège, Belgium, [11]Department of Biotechnology, Jozef Stefan Institute, Ljubljana, Slovenia, [12]Faculty of Chemistry and Chemical Technology, University of Ljubljana, Ljubljana, Slovenia, [13]Department of Microbiology, Immunology and Transplantation, Laboratory of Clinical and Epidemiological Virology, Rega Institute, KU Leuven, Leuven, Belgium, [14]Antwerp Unit for Data Analysis and Computation in Immunology and Sequencing (AUDACIS), Antwerp, Belgium, [15]Centre for Health Economics Research and Modelling Infectious Diseases (CHERMID), Vaccine and Infectious Disease Institute (Vaxinfectio), University of Antwerp, Antwerp, Belgium, [16]Department of Paediatrics, Antwerp University Hospital, Antwerp, Belgium, [17]Infla-Med, University of Antwerp, Antwerp, Belgium

Varicella-zoster virus (VZV) encephalitis and meningitis are potential central nervous system (CNS) complications following primary VZV infection or reactivation. With Type-I interferon (IFN) signalling being an important first line cellular defence mechanism against VZV infection by the peripheral tissues, we here investigated the triggering of innate immune responses in a human neural-like environment. For this, we established and characterised 5-month matured hiPSC-derived neurospheroids (NSPHs) containing neurons and astrocytes. Subsequently, NSPHs were infected with reporter strains of VZV ($VZV^{eGFP\text{-}ORF23}$) or Sendai virus (SeV^{eGFP}), with the latter serving as an immune-activating positive control. Live cell and immunocytochemical analyses demonstrated $VZV^{eGFP\text{-}ORF23}$ infection throughout the NSPHs, while SeV^{eGFP} infection was limited to the outer NSPH border. Next, NanoString digital transcriptomics revealed that SeV^{eGFP}-infected NSPHs activated a clear Type-I IFN response,

while this was not the case in VZV$^{eGFP\text{-}ORF23}$-infected NSPHs. Moreover, the latter displayed a strong suppression of genes related to IFN signalling and antigen presentation, as further demonstrated by suppression of IL-6 and CXCL10 production, failure to upregulate Type-I IFN activated anti-viral proteins (Mx1, IFIT2 and ISG15), as well as reduced expression of CD74, a key-protein in the MHC class II antigen presentation pathway. Finally, even though VZV$^{eGFP\text{-}ORF23}$-infection seems to be immunologically ignored in NSPHs, its presence does result in the formation of stress granules upon long-term infection, as well as disruption of cellular integrity within the infected NSPHs. Concluding, in this study we demonstrate that 5-month matured hiPSC-derived NSPHs display functional innate immune reactivity towards SeV infection, and have the capacity to recapitulate the strong immune evasive behaviour towards VZV.

KEYWORDS

human iPSc, neurospheroids, varicella-zoster virus, type-I interferon signalling, antigen presentation, stress granules, structural integrity



GRAPHICAL ABSTRACT
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Highlights

- Characterisation of 5-month-old hiPSC-derived neurospheroids (NSPHs).
- Varicella zoster virus and Sendai virus infection of NSPHs.
- Molecular and cellular profiling of innate immunity in NSPHs.
- Viral immune evasion and stress granule formation in NSPHs.

1 Introduction

Varicella-zoster virus (VZV) is a highly species-specific human neurotropic alphaherpesvirus that, due to its long co-evolutionary

history with the human host, has developed a wide range of immune evasion mechanisms preventing early host immune activation (1, 2). Although VZV has a high transmission rate, resulting in the natural infection of most children before the age of 10 and the typical varicella (chickenpox) disease phenotype, complications can be unpredictable (3, 4). While infection is usually self-limiting, VZV remains in a latent state in the neurons of the sensory ganglia. Reactivation of VZV causes herpes zoster (HZ), which is characterised by a more localised, painful skin rash with blisters. In addition, about 20% of HZ patients suffer from debilitating, long-lasting pain known as postherpetic neuralgia (PHN) (5). Neurological complications in the central nervous system (CNS) can also occur (4). Here, the most common manifestation of VZV infection is vasculopathy, which manifests as headaches, cognitive decline and/or focal neurological deficits (6). In rare cases, VZV infection can lead to even more severe neurological complications such as meningitis, encephalitis, cerebellitis and myelopathy (4, 6). The most common hallmarks underlying these pathologies include persistent inflammation and/or virus-induced hypercoagulability (6). Although VZV-related CNS infections are treatable with intravenous acyclovir therapy and corticosteroids, the wide spectrum of possible CNS complications is not necessarily accompanied by a VZV-associated rash, potentially leading to mis- or non-diagnosis (6). Consequently, this may lead to long-lasting CNS dysfunction and emphasizes the need for a better understanding of VZV neuropathogenesis in the CNS. A specific focus on the neuro-immune mechanisms of disease and/or protection could advance both preventive and therapeutic strategies to avoid or efficiently cure severe neurological complications following VZV infection of CNS tissue.

Even though there is an unmet need for studying VZV neuropathology, its exclusive human tropism limits profound cellular studies due to the scarcity of human CNS tissue available for research. To cope with this, recent advances in cell and developmental biology have led to the establishment of self-organising, stem cell-derived 3D cultures containing a mixture of different CNS cell types (7, 8). These structures are often referred to as neurospheroids (NSPHs) or brain organoids, and have demonstrated improved differentiation capabilities, time-dependent maturation, and superior functionality of neural cell types as compared to classically used (stem) cell line-derived 2D cultures (9–12). In the context of viral infection, stem cell-derived brain organoids have already been applied to investigate neurodevelopmental and neuro-immune consequences for neurotropic viruses of significant public health concern, such as - but not limited to - Zika virus (ZIKV) (13, 14), herpes simplex virus 1 (HSV1) (13, 15), and more recently severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (16–18). However, to our knowledge, no stem cell-derived NSPH models have been used to study VZV neuro-immune interactions (7, 19, 20). Continuing on previously reported 2D human pluripotent stem cell derived neuronal and neuro-immune co-culture models studying VZV infection (5, 21–25), in this study we aim to investigate whether a productive VZV infection is immunologically recognised within a neural-like NSPH environment.

This research question directly relates to our preceding studies whereby innate immune recognition of VZV was investigated in a 2D human (h)iPSC-derived compartmentalised neuronal model (25). Although hiPSC-derived neurons are able to adequately respond to IFNα signalling and suppress a productive VZV infection upon IFNα treatment, they do not mount a Type-I IFN response themselves upon VZV infection, and as such are unable to autonomously suppress a productive VZV infection (25). Subsequently, we hypothesised that co-culture of VZV-infected hiPSC-derived neurons with isogenic hiPSC-derived macrophages, as an immune-competent bystander population, could control a productive VZV infection. However, even though hiPSC-derived macrophages were demonstrated to be fully immune competent, no Type-I IFN response was mounted upon VZV challenge, and no suppression of productive VZV infection within co-cultured neurons was seen (5). Within the CNS, additional cells such as astrocytes and microglia play an important role in immune surveillance and may thus be able to control a productive VZV infection (26). Given the functional similarities between microglia and macrophages, and based on our preceding work, we do not expect microglia to be able to mount a strong Type-I IFN response upon VZV recognition. Therefore, in this study we focused on the potential role of astrocytes as bystander immune cells to control VZV infection.

Based on previous reports demonstrating that astrocyte maturation is highly essential for shifting their functional role from support in brain development to support in brain homeostasis and immunity (9, 11), we here opted to investigate cellular responsivity to VZV infection in 5-month matured hiPSC-derived NSPHs. Furthermore, infection of NSPHs with a murine Sendai virus, which is expected to trigger a strong Type-I IFN response, was applied as positive control to allow better understanding of the immune evasive behaviour of VZV in a human neural-like environment (25).

2 Materials and methods

2.1 NSPH generation, differentiation, and maturation

A previously established and characterised self-renewing hiPSC-derived neural stem cell line (hiPSC-NSC) was cultured as described in Van Breedam et al. (27). For the initial generation of NSPHs, hiPSC-NSCs were harvested and seeded at a density of 1.6 x 10^4 cells per well in an ultra-low attachment (ULA) 96-well plate (Costar, 7007) in 100µL of complete neural expansion medium [cNEM, consisting of 1:1 Neurobasal medium (Gibco, 21103-049): Advanced DMEM/F12 (Gibco, 12634-010) supplemented with 1x neural induction supplement (Gibco, A16477-01) and 1% Penicillin/Streptomycin solution (Gibco, 15070-063)]. NSPH cultures were maintained in a humidified cell culture incubator at 37°C and 5% CO_2. On the third day post-seeding, 100µL of cNEM was added and from this point onwards, the cultures were kept under constant orbital shaking at 88 rpm. Partial (50%) medium changes were then performed every 2-3 days until day 14. At this

point, NSPHs were transferred to ULA 6-well plates (Corning, 3471) with 5-6 NSPHs per well in 3 mL cNEM. Partial (50%) medium changes were performed every 2-3 days throughout the culture period until the age of 5 months.

2.2 Propagation of VZV$^{eGFP-ORF23}$ in ARPE19 cells

The human retinal pigment epithelial cell line ARPE19 (ATCC, CRL-2302) was used for the propagation of the VZV$^{eGFP-ORF23}$ strain, as previously described (25, 28). In this pOka-derived recombinant VZV strain, the minor capsid protein ORF23 is fused with the enhanced green fluorescent protein (eGFP). Following VZV$^{eGFP-ORF23}$ propagation in ARPE19 cells, infected cells were harvested and cryopreserved in 900µL cARPE19 medium [90% DMEM/F12 (Gibco, 11320-074) + 10% FBS (Gibco, 10270-106)] + 100µL DMSO (Sigma, D2650) at a concentration of 1.0 x 10^6 cells per vial. After thawing, VZV$^{eGFP-ORF23}$ titres in plaque-forming units (PFU) were determined using an infectious foci assay in ARPE19 cells, according to previously described procedures (25, 28, 29).

2.3 Generation and propagation of VZV$^{ORF65-tdT-66}$ in ARPE19 cells

The VZV WT-tdTomato (VZV$^{ORF65-tdT-66}$) strain, based on the pOka-derived recombinant VZV strain, was generated by cloning the tdTomato-tag in the intergenic region between ORF65 and 66 under the control of the SV40 early promotor, according to previously described procedures (28). This newly generated VZV$^{ORF65-tdT-66}$ was maintained in ARPE19 cells as described above.

2.4 Generation of ARPE19-eGFP cells

ARPE19 cells stably expressing the eGFP-reporter protein were generated following transduction with a Lentiviral vector (LVv) encoding eGFP and the hygromycin resistance protein (pCHMWS-eGFP-IRES-Hyg^r, kindly provided by the Leuven Viral Vector Core, LVVC, Molmed, KU Leuven, Leuven, Belgium) (25). Stable transduction and selection were performed as described previously (30). Stable expression of eGFP was confirmed by both fluorescence microscopy and flow cytometric analysis. ARPE19-eGFP cells were cryopreserved in 900µL cARPE19 medium + 100µL DMSO at a concentration of 1.0 x 10^6 cells per vial.

2.5 Pro-inflammatory stimulation of NSPHs

For pro-inflammatory stimulation, NSPHs were treated with a combination of 27.5 µg/mL ATP (Avantor, ICNA0219461301), 1 µg/mL LPS (Sigma, L7895), 10ng/mL IL1β (Immunotools GmbH, 11340012), 50ng/mL IFNγ (Immunotools GmbH, 11343534) and 10ng/mL TNFα (Immunotools GmbH, 11343013) for 72 hours, after which the cell culture supernatant was collected and frozen at -80°C for subsequent analysis.

2.6 VZV$^{eGFP-ORF23}$ and VZV$^{ORF65-tdT-66}$ infection of NSPHs

For infection of NSPHs, VZV$^{eGFP-ORF23}$-infected ARPE19 cells were thawed and used directly as a vehicle for cell-associated VZV$^{eGFP-ORF23}$ infection. In this study, 3.25 x 10^3 PFU were added per NSPH, corresponding to 16.25 x 10^3 VZV$^{eGFP-ORF23}$-infected ARPE19 cells. As negative control for VZV$^{eGFP-ORF23}$ infection in downstream experiments, NSPHs were stimulated with ARPE19-eGFP cells. For this, an equal amount of 16.25 x 10^3 ARPE19-eGFP cells were added to the NSPHs. Medium changes were performed at 3dpi (80%), 5dpi (50%) and 7dpi (50%), thereby removing VZV$^{eGFP-ORF23}$-infected ARPE19 and ARPE19-eGFP cells. Supernatant collected at 3, 5 and 7dpi was centrifuged to remove debris and frozen at -80°C for downstream analysis. Similarly, VZV$^{ORF65-tdT-66}$ infected ARPE19 cells were used directly as a vehicle for cell-associated VZV $^{ORF65-tdT-66}$ infection of NSPHs, as described above.

2.7 SeV^{eGFP} infection of NSPHs

Commercially available eGFP-labelled Sendai Virus (SeV^{eGFP}) was obtained from ViraTree (S124). For NSPH infection, 3.25 x 10^3 PFU were added per NSPH, with medium changes performed at 3dpi (80%), 5dpi (50%) and 7dpi (50%), thereby removing remaining free SeV^{eGFP}. Supernatant collected at 3, 5 and 7dpi was frozen at -80°C for downstream analysis.

2.8 Single cell dissociation of NSPHs

NSPHs were dissociated into a single cell suspension using a commercially available Papain/DNase-I dissociation kit (Worthington, 9035-81-1) following a procedure described by Barbar et al., 2020 (31, 32), with minor modifications. Briefly, 2-4 NSPHs were transferred into a single 24-well and mechanically broken into smaller pieces using sterile tweezers. Next, Papain/DNase-I solution (1 mL) was added and NSPH pieces were gently triturated 3 times. Subsequently, NSPH pieces were incubated for 45 minutes on an orbital shaker (88 rpm at 37°C), gently triturated 10 times, re-incubated for 15 minutes under orbital shaking and gently triturated again 10 times. The NSPH dissociation process was then stopped by transferring the dissociated cell suspension to an ovomucoid inhibitor solution (1,2 mL + 2 mL Earle's medium). Finally, cells were centrifuged and resuspended in PBS.

2.9 Flow cytometry analysis of NSPHs

For flow cytometry analysis, the single cell populations obtained from dissociated NSPHs were immediately co-stained with a

phycoerythrin (PE)-labelled anti-human CD49f antibody (1:20, BD Biosciences, 555736) and a LIVE/DEAD™ Fixable Aqua Dead near-IR Cell Stain (Invitrogen, L34976), according to previously described procedures (33). Flow cytometric analysis was performed using a BD FacsLyric™ analytical flow cytometer (BD Biosciences) and data were analysed using FACSuite v1.5 (BD Biosciences) and FlowJo (10.8.1) software.

2.10 Cryosectioning of NSPHs

NSPHs were fixed individually in 1,5 mL of 4% paraformaldehyde solution [PFA in phosphate-buffered saline (PBS)] for 150 min at room temperature (RT). Following two wash steps with 1,5 mL of PBS, NSPHs were stored at 4°C in PBS with 0.01% sodium azide. Following overnight dehydration in 20% sucrose solution (in dH_2O), NSPHs were embedded in TissueTek-OCT (VWR) for cryosectioning in a NSPH array setup, as previously optimised by us (27). 10 to 20 µm thick frozen sections were prepared using an NX70 cryostar cryostat (Thermo Scientific) and collected on glass slides coated with poly-L-lysine (Sigma) and stored at -20°C before further processing.

2.11 Immunofluorescent staining of NSPH cryosections

For immunofluorescence staining, sections were rehydrated with PBS for 5-10 minutes and permeabilised for 30 minutes using 0,1% (v/v) Triton X-100 (Sigma) in Tris-buffered saline (TBS), both at RT. Next, NSPH sections were blocked with a solution of TBS supplemented with 20% serum of the corresponding secondary antibody host species or with 1% bovine serum albumin (BSA) for 1 hour at RT. Next, NSPH sections were incubated overnight at 4°C with primary antibodies diluted either in 10% (m/v) milk solution (Sigma) in TBS or in 3% BSA in TBS. After washing with TBS and a subsequent 1-hour incubation with the secondary antibodies in the dark, slides were washed again and counterstained with DAPI (1 µg/mL, Sigma) for 10 minutes at room temperature. After a final washing step with distilled water, sections were mounted in ProLong R Gold antifade reagent (Thermo Fisher). The used primary and secondary antibodies, as well as their final working concentrations and combinations applied, are provided in Table 1 (primary antibodies) and Table 2 (secondary antibodies).

TABLE 1 List of primary antibodies used for immunocytochemistry and flow cytometry.

	Antibody	Host	Source	Final concentration
A	TuJ1	Mouse	R&D Systems (MAB1195)	2 µg/mL
B	MAP2	Chicken	Abcam (ab5392)	1,33 µg/mL
C	NeuN	Guinea pig	Merck Millipore (ABN90P)	1,25 µg/mL
D	GFAP	Rabbit	Abcam (ab7260)	1 - 10 µg/mL
E	S100b	Rabbit	Abcam (ab52642)	5 µg/mL
F	AQP4	Rabbit	Merck Millipore (HPA014784)	3 µg/mL
G	CD49f	Rat	BioLegend (313602)	2 µg/mL
H	CD49f-PE	Rat	BD Biosciences (555736)	NA - 1:20 dilution
I	SOX9	Rabbit	Abcam (ab5535)	0,85µg/mL
J	MX1	Rabbit	Abcam (ab95926)	150 ng/mL
K	IFIT2	Rabbit	Invitrogen (16870404)	0,675 µg/mL
L	ISG15	Rabbit	Proteintech (15981-1-AP)	1,125 µg/mL
M	CD74	Mouse	Invitrogen (15207077)	1,25 µg/mL
N	HLA-DR-PE	Mouse	BioLegend (307605)	NA - 1:200 dilution
O	G3BP1	Mouse	Proteintech (66486-1-lg)	4 µg/mL
P	PABPC	Rabbit	Proteintech (10970-1-AP)	5 µg/mL

NA, not available.

2.12 Microscopy and image analysis

Live cell images of control and virus infected NSPHs at different time points during culture were captured with a Zeiss Axio Observer.Z1 inverted fluorescence microscope using a N-Achroplan 5x (NA 0,13) objective. Immunofluorescence images of stained NSPHs were acquired using an Olympus BX51 fluorescence microscope equipped with an Olympus DP71 digital camera and using a UPlanFLN 10× (NA 0,30), UPlanFLN 20× (NA 0,50) or PlanC 40× (NA 0,65) dry objective. High-resolution images were acquired using the Nikon CSU-W1 SoRa confocal microscope using a Plan Apo 10x air objective (NA 0.45), Plan Apo 40x air objective (NA 0.95) and Plan Apo 60x water objective (NA 1.2) in normal confocal mode and NIS-Elements software (Nikon). Fiji image analysis freeware was used for image processing and analysis (http://fiji.sc). Briefly, images of stained NSPHs were manually delineated selecting two regions of interest (ROIs): (i) a 200 µm viable border of the NSPH (ROI(i), region of interest), and (ii) the necrotic core of the NSPH (ROI(ii), region of background fluorescence). Results indicating specific immunofluorescence signal of a given marker are presented as mean fluorescence intensity ROI(i)/ROI(ii).

2.13 Transmission electron microscopy

$VZV^{eGFP\text{-}ORF23}$-infected and control NSPHs were fixed in 2.5% glutaraldehyde in Sorensen's buffer 0.1 M solution (pH 7.4) at RT for 10 min before being moved 4°C for 2 h. After three washes in Sorensen's buffer, samples were post-fixed for 60 min in 2% osmium tetroxide and dehydrated through a graded ethanol–propylene oxide series then embedded in epoxy resin. The resin was then polymerised at 60°C for 72 h. Ultrathin Sections (60–80

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TABLE 2 List of secondary antibodies used for immunocytochemistry.

Antibody	Host	Conjugation	Source	Final concentration	In combination with
Anti-rabbit	Goat	Texas red	Abcam (ab6719)	5 µg/mL	D, E, F, K, L, J
Anti-rat	Goat	AF555	Invitrogen (a21434)	4 µg/mL	G
Anti-mouse	Goat	AF555	Invitrogen (a21425)	2 µg/mL	A, M
Anti-guinea pig	Donkey	Cy3	Jackson ImmunoResearch (706–165-148)	7,5 µg/mL	C
Anti-chicken	Donkey	Cy3	Jackson ImmunoResearch (703-166-155)	1,5 µg/mL	B
Anti-mouse	Goat	AF555	Invitrogen (a21127)	2 µg/mL	O
Anti-rabbit	Goat	AF647	Invitrogen (a21245)	2 µg/mL	P
Anti-rabbit	Goat	FITC	Jackson ImmunoResearch (111-096-114)	7,5 µg/mL	D, I
Anti-chicken	Donkey	FITC	Jackson ImmunoResearch (703-096-155)	3,75 µg/mL	B

nm) were cut using a diamond knife (Diatome) mounted in an ultramicrotome (Ultracut S Leica) and contrasted in the dark for 15 min in uranyl acetate solution, and for 15 min in lead citrate solution. For ultrastructural analyses, random fields of these samples were examined under a Jeol TEM JEM-1400 transmission electron microscope at 80 kV, and random fields were photographed using an 11-megapixel camera system (Quemesa, Olympus).

2.14 Analysis of cytokine secretion

Concentrations of interleukin 6 (IL-6), CXCL10, interferon alpha-2 (IFN-α2) and interferon beta (IFN-β) in cell culture supernatant were determined using commercially available ELISA MAX™ Deluxe Sets (BioLegend, 430504, 439904, 446404, 449504), according to the manufacturer's instructions.

2.15 Haematoxylin-eosin staining

A haematoxylin and eosin (H&E) staining was performed on cryosections of NSPHs using Carazzi's haematoxylin (0.1% (m/v) dissolved in 1:4 distilled water:glycerol 85% containing 105 mM $KAl(SO_4)_2.12\ H_2O$, 0.9 mM KIO_3 Sigma) and eosin Y (1% (m/v) in distilled water, Sigma), as previously described (27). In short, cryosections were stained for 2 min. with Carazzi's haematoxylin and washed for 5 min. with running water. After 5 min. of eosin Y staining, slides were dipped 5 times in distilled water and subsequently dehydrated by 95% ethanol (2 min.), 100% ethanol (2 min.) and xylene (10 min.) and finally mounted using ProLong® Gold anti-fade reagent (Thermo Fisher).

2.16 Nanostring digital transcriptomics

At 7 days post stimulation/infection, NSPHs were washed with ice cold PBS, snap frozen in liquid nitrogen and stored at -80°C. RNA extraction was performed on 2 pooled NSPHs of each condition (three samples per condition) using the RNeasy Mini Kit (Qiagen, 74104) according to the manufacturer's protocol. RNA concentration was determined with the Qubit RNA Broad Range Assay Kit (Invitrogen, Q10211) and the 260/280 ratio, indicative of RNA purity, was checked using NanoDrop™ 2000/2000c Spectrophotometer (Thermo Scientific, ND-2000). The RNA samples were used for digital transcriptomic analysis (nCounter, NanoString Technologies) on a nCounter® MAX Analysis System, as previously described for other viral infections (34–37). Briefly, RNA extracts were hybridised to ± 600 unique capture/reporter pairs (50bp each) targeting 585 immune transcripts and 15 housekeeping genes, as defined in the Human Immunology V2 nCounter® panel, as well as 6 positive and 8 negative control probes (all from NanoString). Results were sequentially corrected for background (negative control probes), technical variation (positive control probes) and RNA content (housekeeping genes) using nSolver 4.0 (NanoString), followed by differential gene expression (DGE) analysis and gene set enrichment (GSE) analysis (based on GO-terms) using Omics Playground (BigOmics Analytics). The original NanoString digital transcriptomics data are available in the GEO database via accession number GSE273529.

2.17 Data representation and statistical analysis

Box/dot plots representing cytokine production and quantification of ICC markers were created with GraphPad Prism v.8.2.1 software. Statistical analyses were carried out using JMP® Pro Version 16 statistical software. All data were modelled using a linear mixed-effects model, accounting for the repeated measures, i.e. independent experiments and/or repeated measurements for each observation. *Post-hoc* analyses for linear mixed-effects models were carried out with Tukey's HSD correction for multiple comparisons. A p-value <0,05 was considered statistically significant. All other statistical analyses were performed directly within nSolver 4.0 (NanoString) and Omics Playground (BigOmics Analytics) software.

3 Results

3.1 Longitudinal characterisation of NSPH differentiation

To visualise NSPH differentiation longitudinally, NSPHs were harvested after 1, 2, 3 and 5 months of culture and analysed by ICC (Figure 1). Following the culture method applied, NSPHs increase in size up to 3 months in culture after which growth stabilised at a diameter of approximately 2-3 mm. Although ICC for neuron-specific proteins (Figure 1A) demonstrated the early presence of Tuj1^{+} neurons in 1-month-old NSPHs, expression of the more mature neuronal marker MAP2 was only detected in NSPHs from the age of 2 months. However, due to the increasing size of the NSPHs, a large TUNEL^{+} core of dead cells becomes more pronounced within the NSPHs from 3 months of age onwards. Nevertheless, 5-month-old NSPHs clearly displayed the presence of matured Tuj1^{+}MAP2^{+}NeuN^{+} neurons in the viable border. In contrast to the early appearance of developing neurons, only a small population of GFAP^{+} astrocytes was detected at earliest after 2 months of culture, and this specifically at the outer border of the NSPH (Figure 1B). Upon continued NSPH culture, this immature astrocyte population colonised the viable NSPH border and matured into GFAP^{+}S100b^{+}AQP4^{+}SOX9^{+} astrocytes between the 3rd and the 5th month of age, giving rise to a matured bi-partite (neurons + astrocytes) hiPSC-derived NSPH model that will be used throughout this study.

3.2 5-month-old NSPHs are susceptible to VZV$^{eGFP\text{-}ORF23}$ infection

At the age of 5 months, NSPHs were inoculated with cell-associated VZV$^{eGFP\text{-}ORF23}$, as described in detail in the Materials and Methods section. Following 7 days of infection, widefield live cell fluorescence microscopy revealed a widespread bright eGFP signal from inoculated NSPHs as compared to control NSPHs (Figure 2A). Next, flow cytometry of single cell populations dissociated from control and VZV$^{eGFP\text{-}ORF23}$-infected NSPHs was performed to determine the proportion of VZV$^{eGFP\text{-}ORF23}$-infected astrocytes (CD49f^{+}, Figures 2B, C) and neurons (CD49f$^{-/low}$, Figure 2C) within NSPHs. We found that both the CD49f^{+} cell population (comprising astrocytes) and the CD49f^{-} cell population (comprising neurons) were highly susceptible to VZV$^{eGFP\text{-}ORF23}$ infection (Figure 2C), with - within the viable cell population recovered - 80,5 +/- 1,3% of neurons and 94,2 +/- 1,2% of

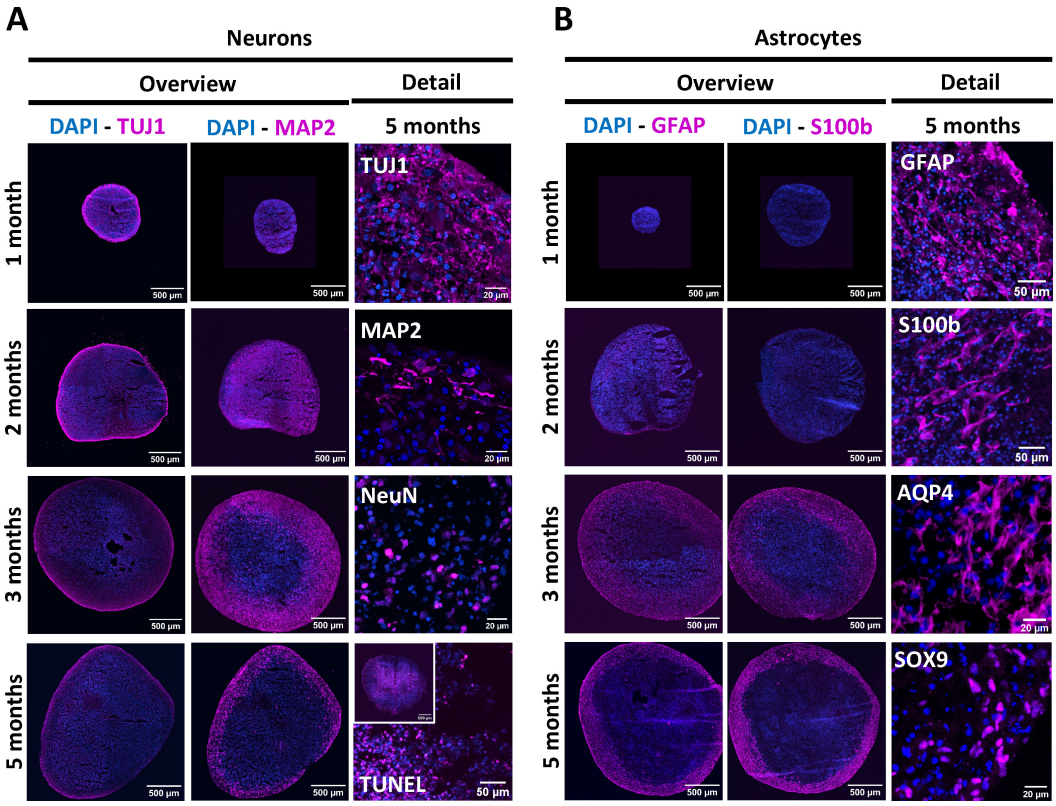


FIGURE 1
Longitudinal characterisation of NSPH differentiation. **(A)** Representative images of NSPHs at the age of 1, 2, 3 and/or 5 months immunolabelled for the neuronal markers Tuj1 (magenta), MAP2 (magenta) and NeuN (magenta), and the late-stage apoptosis TUNEL staining (magenta), as indicated. **(B)** Representative images of NSPHs at the age of 1, 2, 3 and/or 5 months immunolabelled for the astrocyte markers GFAP (magenta), S100b (magenta), AQP4 (magenta) and SOX9 (magenta), as indicated. Nuclei are labelled with DAPI (blue). Scale bars of 20, 50 and 500 µm are indicated on the images.

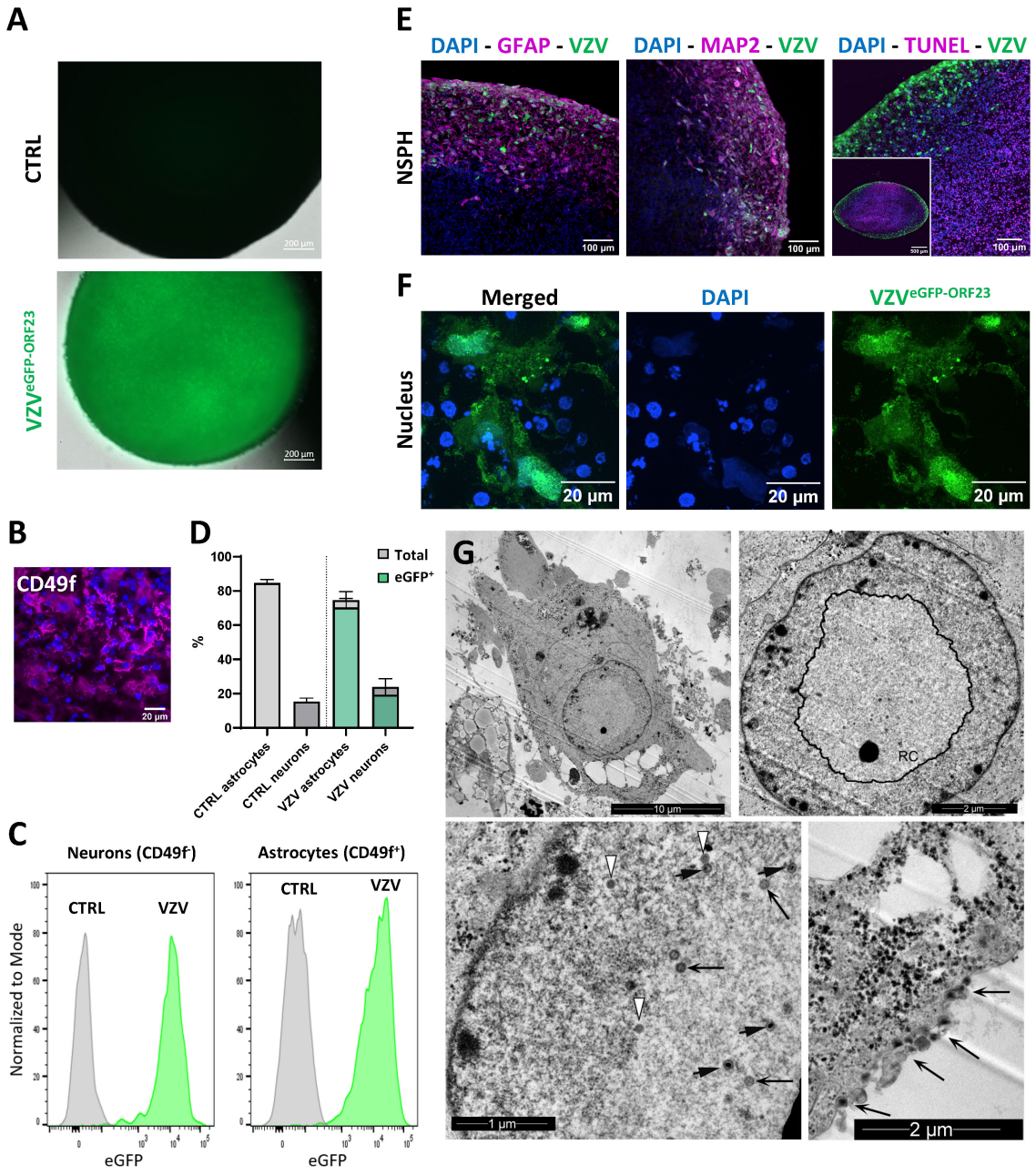


FIGURE 2
VZV$^{eGFP-ORF23}$ infection of 5-month-old NSPHs. **(A)** Representative live cell fluorescence image of uninfected NSPHs and VZV$^{eGFP-ORF23}$ infected NSPHs at day 7 post-infection. Scale bars of 200 µm are indicated on the images. **(B)** Representative image of a NSPH stained with the extracellular astrocyte-enriched CD49f marker (magenta) and nuclei labelled with DAPI (blue). Scale bar of 20µm indicated on the image. **(C)** Representative flow cytometric analysis showing histogram overlay of CD49f$^-$ neurons and CD49f$^+$ astrocytes obtained from dissociated uninfected NSPHs (CTRL) and VZV$^{eGFP-ORF23}$ infected NSPHs (VZV) at day 7 post-infection. **(D)** Bar graph indicating the average percentage of astrocytes and neurons measured in uninfected NSPHs (CTRL) and VZV$^{eGFP-ORF23}$ infected NSPHs (VZV), as well as the average % of astrocytes and neurons displaying eGFP-ORF23 fluorescence in VZV$^{eGFP-ORF23}$ infected NSPHs (n=3 for CTRL and n=3 for VZV). Error bars indicate standard deviation (SD). **(E)** Representative images of VZV$^{eGFP-ORF23}$ infected NSPHs (green) at the age of 5 months immunolabelled for GFAP (magenta), MAP2 (magenta) and TUNEL (magenta), as indicated. Nuclei are labelled with DAPI (blue). Scale bars of 100 and 500 µm are indicated on the images. **(F)** Representative images of the cytoplasmic and nuclear localisation of VZV$^{eGFP-ORF23}$ (green) in infected NSPHs at the age of 5 months. Nuclei are labelled with DAPI (blue). Scale bars of 20 µm are indicated on the images. **(G)** Representative transmission electron microscopy (TEM) images of VZV$^{eGFP-ORF23}$ infected NSPHs at the age of 5 months. Upper left image showing an overview TEM image of a VZV$^{eGFP-ORF23}$ infected cell. Upper right image showing the nuclear replication complex (RC). Lower left image showing different maturation stages of the VZV virion, with white arrows indicating procapsids, long black arrows indicating capsid B and short black arrows indicating capsid C Lower right image showing viral particles egressing at the cell surface (long black arrows). Scale bars of 1, 2 and 10 µm are indicated on the images.

astrocytes being infected (Figure 2D). Additionally, ICC analysis of cryosections of VZV$^{eGFP\text{-}ORF23}$-infected NSPHs (Figure 2E; Supplementary Figure 1) confirmed widespread eGFP-ORF23 expression throughout the entire viable border of the VZV$^{eGFP\text{-}ORF23}$-infected NSPHs, consisting of both astrocytes (GFAP staining) and neurons (MAP2 staining), but not in the necrotic core of the NSPHs (TUNEL staining). Higher magnification confocal images (Figure 2F) revealed both nuclear and cytoplasmic localisation of the eGFP-ORF23 fluorescent signal (green), indicative of a productive VZV infection. The latter was further confirmed using TEM, showing the VZV$^{eGFP\text{-}ORF23}$ replication complex (RC) in the nucleus (Figure 2G, upper left and upper right panel), with the presence of different viral capsid structures (procapsids, capsid B and capsid C) in- and outside of the RC (Figure 2G, lower left panel), as well as egressing viral particles at the cell surface (Figure 2G, lower right panel). In summary, these results demonstrate the susceptibility of bi-partite hiPSC-derived NSPHs to VZV$^{eGFP\text{-}ORF23}$ infection.

3.3 Matured NSPHs are immunosensitive, but do not secrete a selected panel of pro-inflammatory cytokines following VZV$^{eGFP\text{-}ORF23}$ infection

Given the high infectivity of NSPHs by VZV$^{eGFP\text{-}ORF23}$, and in line with our preceding studies demonstrating the lack of neuronal innate immune signalling towards VZV (5, 25), we here questioned whether astrocytes could immunologically sense the presence of a productive VZV$^{eGFP\text{-}ORF23}$ infection. To investigate this, 4 experimental conditions were included: (a) control NSPHs, (b) NSPHs inoculated with eGFP$^+$ control ARPE19 cells (= control for condition c), (c) NSPHs inoculated with VZV$^{eGFP\text{-}ORF23}$-infected ARPE19 cells, and (d) NSPHs infected with an SeV^{eGFP}, serving as a positive control for induction of Type-I/II IFN response. At first, NSPH infectivity with VZV$^{eGFP\text{-}ORF23}$ and SeV^{eGFP} was monitored by live cell fluorescence microscopy over 7-days (Supplementary Figure 2; Figure 3A). This revealed successful NSPH infectivity by VZV$^{eGFP\text{-}ORF23}$ and SeV^{eGFP}, as demonstrated by a gradual increase in green fluorescent signal. Inoculation of NSPHs with eGFP$^+$ control ARPE19 cells only resulted in the appearance of a few eGFP$^+$ foci resulting from ARPE19 cells adhering to the NSPHs. Subsequent ICC analysis (Figure 3B) however revealed a different infectivity pattern whereby VZV$^{eGFP\text{-}ORF23}$ displayed widespread distribution within the viable NSPH border, while SeV^{eGFP} only displayed infectivity in the outer layer of the NSPHs. Next, we investigated the capacity of NSPHs to respond immunologically to VZV$^{eGFP\text{-}ORF23}$ or SeV^{eGFP} infection. Although NSPHs secreted high levels of IL-6 and CXCL10 following 3 days of stimulation with a pro-inflammatory cocktail consisting of ATP, LPS, IL-1β, IFNγ and TNFα (Figure 3C), no significant release of IL-6 and CXCL10 in the NSPH cell culture supernatant could be detected following VZV$^{eGFP\text{-}ORF23}$ infection over the 3 measured timepoints (3-, 5- and 7-days post-stimulation) (Figure 3D). In contrast, SeV^{eGFP} infection resulted in a significant release of IL-6 and CXCL10 (Figure 3D), albeit at ten-fold lower levels as compared to stimulation with a pro-inflammatory cocktail (Figure 3C). Additionally, we investigated the secretion of IFN-α2 and IFN-β at the same timepoints following VZV$^{eGFP\text{-}ORF23}$ and SeV^{eGFP} infection. However, for both VZV$^{eGFP\text{-}ORF23}$ and SeV^{eGFP}, infected NSPHs did not secrete detectable levels of IFN-α2 and IFN-β (data not shown). Concluding, 5-month-old NSPHs are immune responsive to pro-inflammatory stimulation and SeV^{eGFP}-infection, as demonstrated by the secretion of IL-6 and CXCL10, but do not secrete pro-inflammatory cytokines following VZV$^{eGFP\text{-}ORF23}$-infection, at least not for the panel applied in this study.

3.4 Molecular immune profiling of VZV$^{eGFP\text{-}ORF23}$ and SeV^{eGFP}-infected NSPHs

Given the observation that SeV^{eGFP}, but not VZV$^{eGFP\text{-}ORF23}$, induced cytokine release by infected NSPHs, we performed a multiplex human immunology NanoString gene expression analysis on the 4 experimental conditions described above to gain a broader insight into the immune signalling pathways triggered upon viral infection. DGE analysis between SeV^{eGFP} infected NSPHs and control NSPHs revealed strong upregulation of gene transcripts related to the Type-I interferon response, such as IFIT2, BST2, MX1, IFITM1 and STAT1 (Figure 4A, indicated in red on the volcano plot), as well as a moderate upregulation of gene transcripts related to MHC class I antigen presentation, such as HLA-A, HLA-B and HLA-C (Figure 4A, indicated in blue on the volcano plot). Consequently, GSE analysis (GO terms) revealed positive enrichment scores (meaning activation) for pathways related to antigen processing and presentation, Type-I IFN signalling and cellular defence against viral infection (Figure 4A, GSE table), albeit only the latter two being significant (based on the meta.q value). In contrast, DGE analysis between NSPHs inoculated with VZV$^{eGFP\text{-}ORF23}$-infected ARPE19 cells and NSPHs inoculated with eGFP$^+$ control ARPE19 cells revealed a significant downregulation of gene transcripts related to the Type-I interferon response, such as IFI35, IFIT2 and IFITM1 (Figure 4B, indicated in red on the volcano plot), and gene transcripts related to MHC class II antigen presentation, such as HLA-DPA1, HLA-DRA, HLA-DRB1, HLA-DMB and CD74 (Figure 4B, indicated in blue on the volcano plot). Next, GSE analysis (GO terms) revealed negative enrichment scores (meaning suppression) for pathways related to antigen processing and presentation and lysosomal functioning (Figure 4B, GSE table), albeit only the latter being significant (based on the meta.q value). Of note, GSE analysis of ARPE19-stimulated NSPHs vs. control NSPHs revealed no significant differences caused by the stimulation of the NSPHs with the ARPE19 vehicle cells (data not shown). Concluding, these results suggest that VZV$^{eGFP\text{-}ORF23}$ infection in NSPH, besides not triggering and even actively suppressing a Type-I IFN response, also interferes with antigen processing and presentation processes.

3.5 VZV$^{eGFP\text{-}ORF23}$ infection of NSPHs interferes with Type-I IFN response and antigen presentation

To validate the observed alterations in molecular signalling pathways, ICC analyses were performed on cryosections obtained

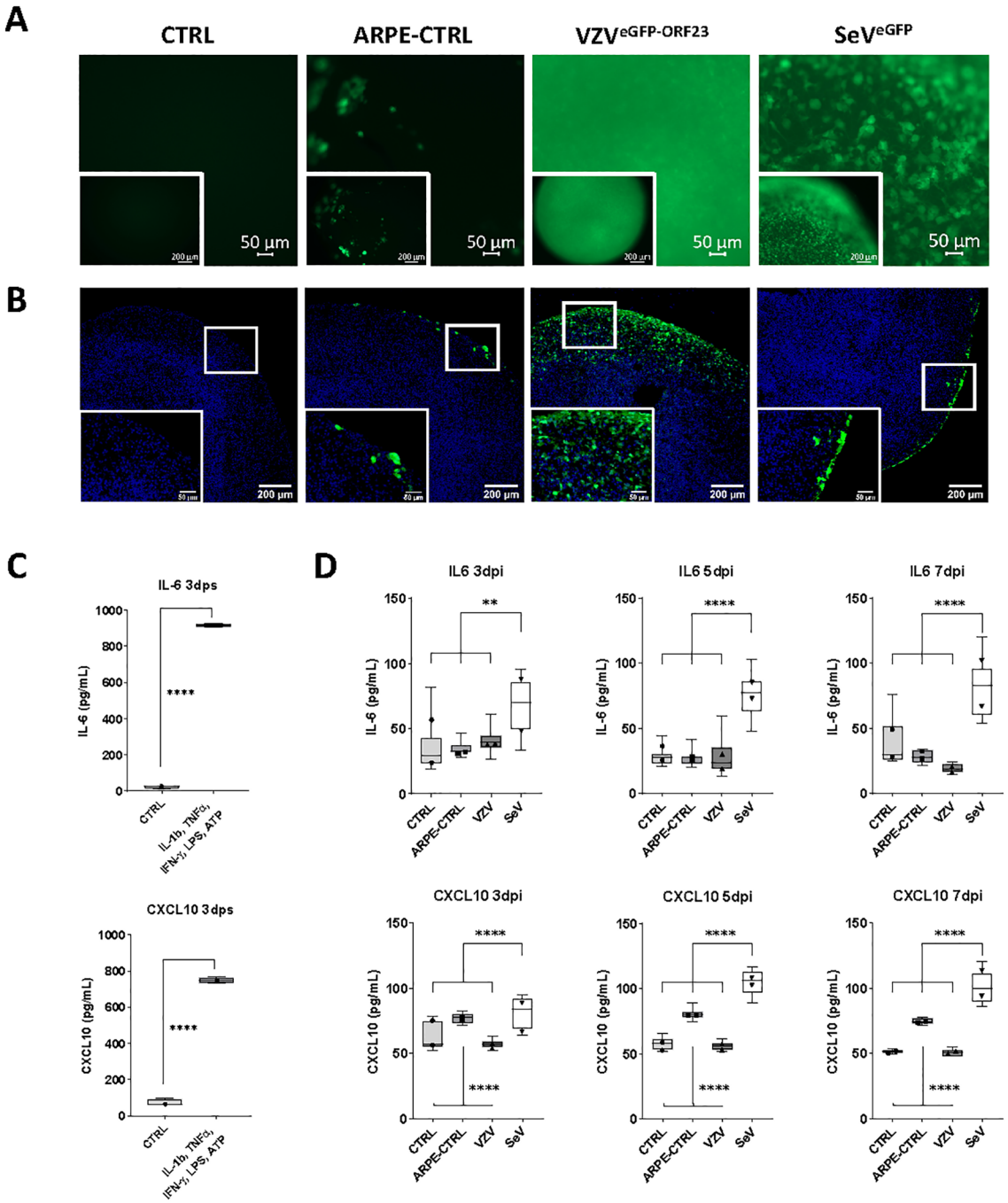
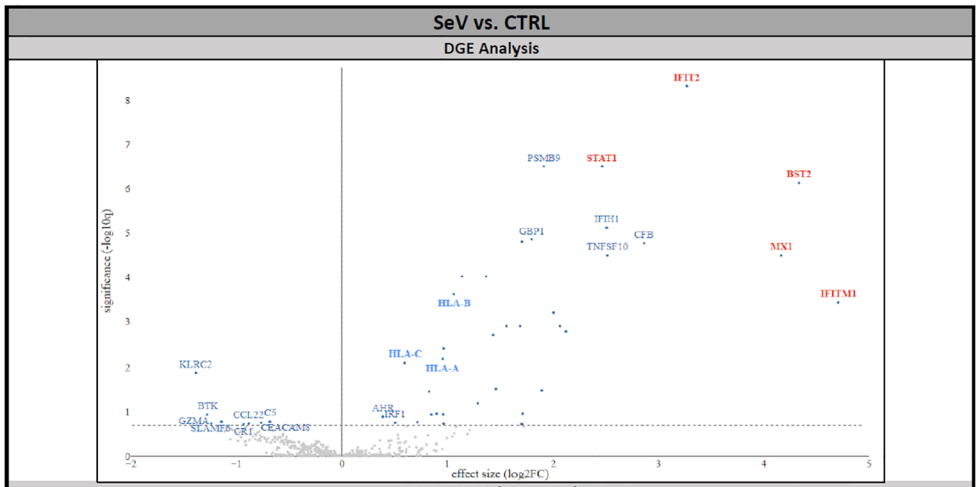


FIGURE 3
Immune responsiveness of VZV$^{eGFP\text{-}ORF23}$- and SeV^{eGFP}-infected NSPHs. **(A)** Representative live cell fluorescence image of 5-month-old control NSPHs (CTRL), NSPHs inoculated with eGFP+ control ARPE19 cells (ARPE-CTRL, green), NSPHs inoculated with VZV$^{eGFP\text{-}ORF23}$-infected ARPE19 cells (VZV$^{eGFP\text{-}ORF23}$, green), and NSPHs infected with SeV^{eGFP} (SeV^{eGFP}, green) at day 7. Scale bars of 50 and 200 µm are indicated on the images. **(B)** Representative images of 5-month-old control NSPHs (CTRL), NSPHs inoculated with eGFP+ control ARPE19 cells (ARPE-CTRL, green), NSPHs inoculated with VZV$^{eGFP\text{-}ORF23}$-infected ARPE19 cells (VZV$^{eGFP\text{-}ORF23}$, green), and NSPHs infected with SeV^{eGFP} (SeV^{eGFP}, green) at day 7. Nuclei are labelled with DAPI (blue). Scale bars of 50 and 200 µm are indicated on the images. **(C)** Boxplots showing IL-6 and CXCL10 cytokine secretion (in pg/mL) by 5-month-old control NSPHs (CTRL, n=4) and NSPHs stimulated with IL-1b, TNF-α, IFN-γ, LPS and ATP (n=4) at day 3 post-stimulation (dps). **** p<0,0001. Error bars indicate standard deviation (SD). **(D)** Combined box/dotplots showing IL-6 and CXCL10 cytokine secretion (in pg/mL) derived from 2 independent experiments for5-month-old control NSPHs (CTRL, n=8 for Exp1, n=4 for Exp 2), NSPHs inoculated with eGFP+ control ARPE19 cells (ARPE-CTRL, n=8 for Exp1, n=4 for Exp 2), NSPHs inoculated with VZV$^{eGFP\text{-}ORF23}$-infected ARPE19 cells (VZV, n=8 for Exp1, n=4 for Exp 2), and NSPHs infected with SeV^{eGFP} (SeV, n=8 for Exp1, n=4 for Exp 2) at 3-, 5- and 7-days post-infection (dpi). The mean of each individual experiment is given as a dot within the boxplot. ** p<0,01. **** p<0,0001.

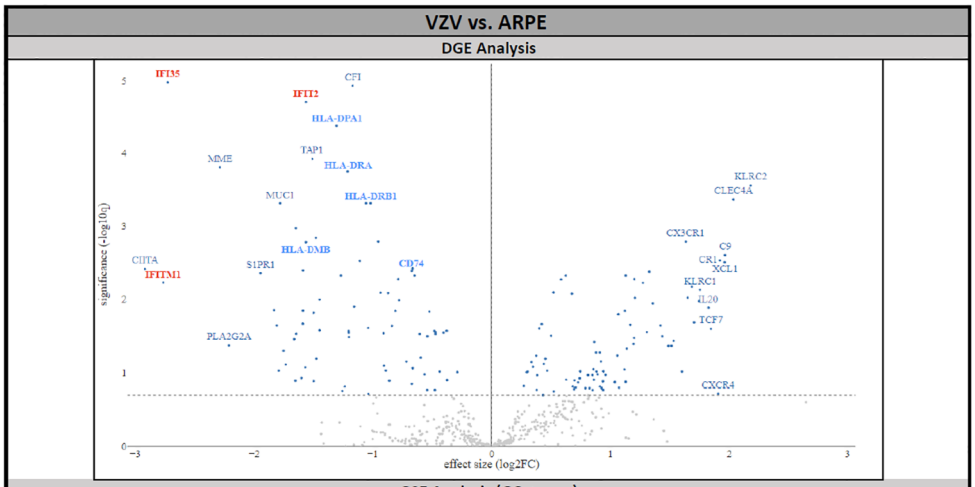
A



GSE Analysis (GO-terms)

id	term	score	logFC	meta.q
GO:0002479	antigen processing and presentation of exogenous peptide antigen via MHC class I	3.282	0.3921	0.0985
GO:0071357	cellular response to type I interferon	2.883	1.0265	0.0032
GO:0060337	type I interferon signaling pathway	2.761	1.0265	0.0032
GO:0051607	defense response to virus	2.168	0.8679	0.0126
GO:0140546	defense response to symbiont	2.075	0.8369	0.0139

B



GSE Analysis (GO-terms)

id	term	score	logFC	meta.q
GO:0019886	antigen processing and presentation of exogenous peptide antigen via MHC class I	-4.051	-0.6344	0.0771
GO:0005765	lysosomal membrane	-3.933	-0.6223	0.0394
GO:0098852	lytic vacuole membrane	-3.821	-0.9348	0.0196
GO:0002495	antigen processing and presentation of peptide antigen via MHC class II	-2.446	-0.6344	0.0771

FIGURE 4
Human Immunology NanoString gene expression analysis of VZV$^{eGFP\text{-}ORF23}$- and SeV^{eGFP}-infected NSPHs. Differential gene expression (DGE) analysis and gene set enrichment (GSE) analysis. **(A)** NSPHs infected with SeV^{eGFP} (SeV, n=3) vs. uninfected control NSPH (CTRL, n=3). **(B)** NSPHs inoculated with VZV$^{eGFP\text{-}ORF23}$-infected ARPE19 cells (VZV, n=3) vs. NSPHs inoculated with eGFP+ control ARPE19 cells (ARPE, n=3). Genes related to the Type-I IFN pathway are marked in red and genes related to the MHC antigen presentation pathway are marked in blue on the DGE volcano plot. The activation score, LogFC and meta.q significance values for the top-ranked GO terms from the GSE analysis are provided for **(A, B)**.

under the 4 experimental NSPH conditions described above. First, examination of downstream proteins of the Type-I Interferon pathway (Supplementary Figure 3; Figure 5A) revealed significant upregulation of MX1, IFIT2 and ISG15 at the protein level in SeV^{eGFP}-infected NSPHs, but not in VZV$^{eGFP\text{-}ORF23}$-infected NSPHs. Second, investigation of proteins related to MHC Class II antigen processing and presentation (Supplementary Figure 3; Figure 5B), confirmed downregulation of the invariant chain protein CD74 in VZV$^{eGFP\text{-}ORF23}$-infected NSPHs as compared to control NSPHs. However, at this stage of analysis, this did not yet result in a significantly lower expression of HLA-DR. Concluding, these results confirm that VZV$^{eGFP\text{-}ORF23}$ interferes with Type-I IFN response and antigen presentation in a human neural-like environment.

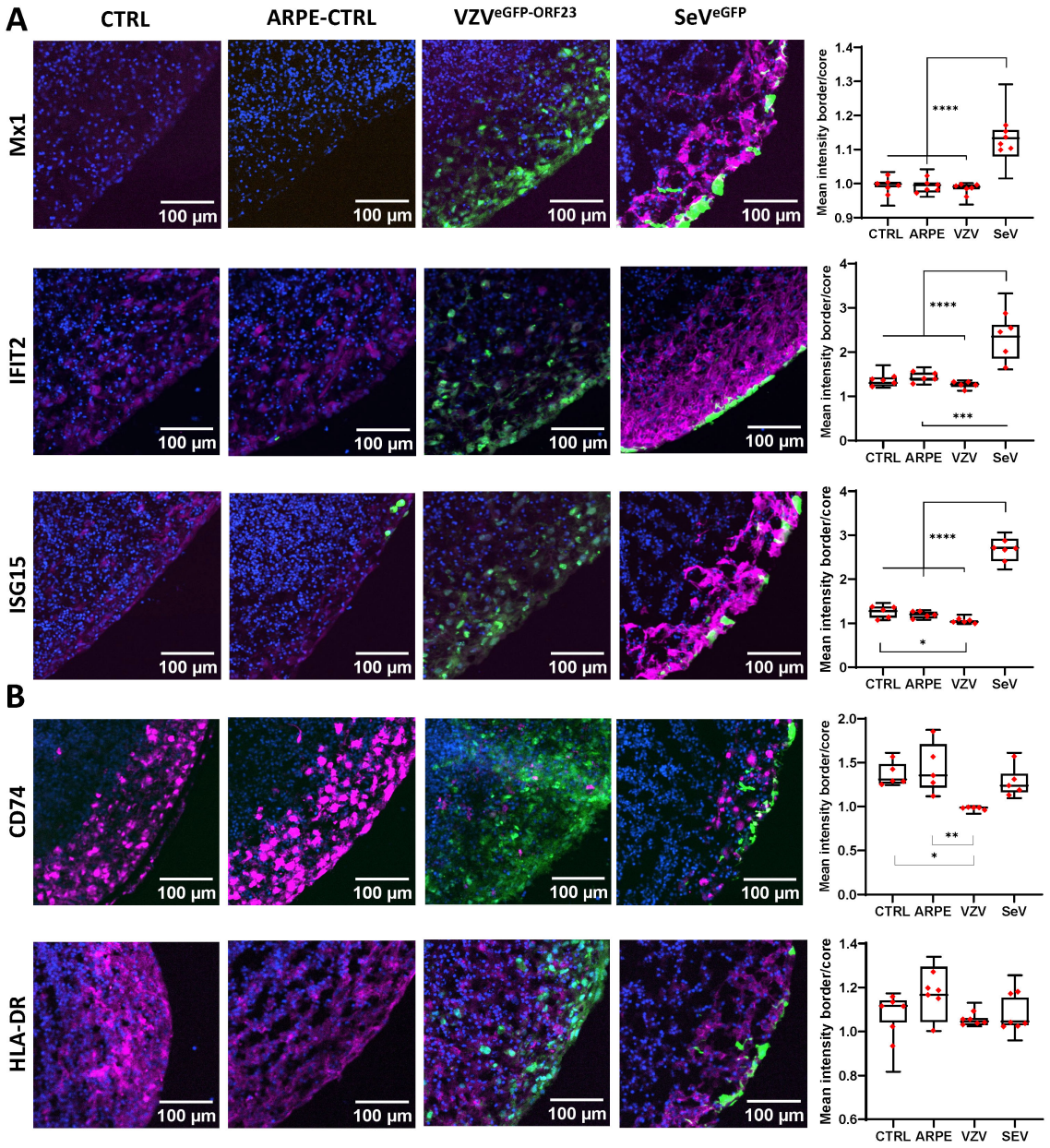


FIGURE 5
VZV$^{eGFP-ORF23}$ and SeV^{eGFP}-infected NSPHs display opposite effects on protein expression related to Type-I interferon response and antigen presentation pathway. Representative immunofluorescence images for 5-month-old control NSPHs (CTRL, n=6), NSPHs inoculated with eGFP+ control ARPE19 cells (ARPE-CTRL, green, n=6), NSPHs inoculated with VZV$^{eGFP-ORF23}$-infected ARPE19 cells (VZV$^{eGFP-ORF23}$, green, n=6), and NSPHs infected with SeV^{eGFP} (SeV^{eGFP}, green, n=6), immunolabelled for **(A)** Type-I interferon response markers MX1, IFIT2 and ISG15, and **(B)** antigen presentation pathway markers HLA-DR and CD74. All in magenta. Nuclei are labelled with DAPI (blue). Scale bars of 100 µm are indicated on the images. Combined box/dotplots showing signal quantification. The mean value for each individual NSPH analysed is given as a dot within the boxplot. * p<0,05. ** p<0,01. *** p<0,001. **** p<0,0001.

3.6 VZV$^{eGFP-ORF23}$- and SeV^{eGFP}-infection of NSPHs induces stress granule formation

Even though VZV$^{eGFP-ORF23}$ can evade innate immune signalling in NSPHs, it is highly unlikely that a productive VZV$^{eGFP-ORF23}$ infection has no consequences in infected cells. We questioned whether a cellular stress response was induced in VZV$^{eGFP-ORF23}$- and SeV^{eGFP}-infected NSPHs upon prolonged viral challenge of a neural-like environment. Hereto, ICC analyses were performed at 7 days post-infection for 2 well-described protein components of stress granules (SGs), namely G3BP1 (G3BP stress granule assembly factor 1) and PABPC1 (poly(A) binding protein cytoplasmic 1). While no SG formation was observed in control NSPHs and NSPHs inoculated with eGFP$^+$ control ARPE19 cells (Figure 6A), within VZV$^{eGFP-ORF23}$-infected NSPHs we observed a strong induction of G3BP1$^+$ SG formation in VZV$^{eGFP-ORF23}$-infected cells (Figure 6B). In contrast, SeV^{eGFP}-infected cells in the NSPH induce the formation of PABPC1$^+$ SGs, but not G3BP1$^+$

SGs, in infected cells (Figure 6C). Concluding, these results indicate a clear difference in the type of SGs formed following prolonged viral presence in our NSPH model for both types of viruses. Furthermore, the formation of SGs following VZV$^{eGFP\text{-}ORF23}$ infection of the NSPHs is a clear indication of induced cellular stress upon prolonged VZV$^{eGFP\text{-}ORF23}$ presence, even though further downstream, VZV still manages to affect innate immune signalling and antigen-processing machinery.

3.7 VZV$^{ORF65\text{-}tdT\text{-}66}$ affects cellular integrity in infected NSPHs

Further documenting the cellular stress VZV induces in infected NSPHs, we performed a final experiment in which NSPHs were infected with cell-associated VZV$^{ORF65\text{-}tdT\text{-}66}$ (Figure 7A). At first, comparison of Haematoxylin-Eosin (H&E)-stained slides from control and VZV$^{ORF65\text{-}tdT\text{-}66}$ infected NSPHs already indicates increased structural degradation inside the VZV$^{ORF65\text{-}tdT\text{-}66}$ infected NSPHs, as well as a clear disruption of the outer border of the NSPH (Figure 7B). Although subject to further investigation, additional stainings for the neuronal marker MAP2 and the astrocyte marker GFAP indicate that the morphology and/or integrity of both NSPH cell types is severely affected following infection with VZV$^{ORF65\text{-}tdT\text{-}66}$ (Figure 7C).

4 Discussion

While several human pluripotent stem cell (PSC)-derived *in vitro* models have been developed and successfully applied to demonstrate neuronal susceptibility to VZV infection, and to investigate subsequent downstream cell- and/or virus-specific intracellular events (5, 21–25), to date little is known about the behaviour of VZV in a multicellular context, especially including cell types involved in innate immunity (38). This approach is highly important as during primary infection, as well as during secondary infection following reactivation from latency, innate immune signalling is the first line of VZV's recognition by the host's immune system that can be activated either by the infected cell itself or by surrounding bystander cells (39–41). In the CNS, besides microglia, astrocytes are in close contact with neurons too, and as such most likely one of the first cells to come into contact with viral particles released from infected neurons (42–45). As mentioned before, there are very few studies concerning VZV neuro-immune biology in primary human brain tissue. A preceding study by Bubak and colleagues has already shown that VZV alters morphology and suppresses pro-inflammatory cytokines in primary human spinal cord and hippocampal astrocytes during VZV infection *in vitro*, and these events may as such represent an immune evasion strategy during VZV myelopathy and encephalopathy (46). While this study already provided valuable insights into (the absence of) an anti-viral astrocyte response upon VZV challenge, the use of human post-mortem isolated and cultured astrocytes may not fully recapitulate *in vivo* physiological responses due to the absence of important astrocyte-neuron interactions.

Mixed human neuron-astrocyte cultures are difficult to establish in a 2D context due to differences in growth factor requirements for both cell types and the time needed to regain physiological rest and functional maturity upon plating of individual cell populations (11, 47). For this reason, PSC-derived NSPHs have gained significant importance in the study of virus infections of the CNS as they have been able to overcome some of the major limitations of the preceding preclinical models (7, 8, 19, 48–50). Nevertheless, Depla and colleagues recently pointed out that heterogeneity in organoid generation protocols, as well as their age/maturation at the time of infection, may explain confounding results (7). With a specific focus on studying astrocyte-mediated anti-viral immune responses, it is of utmost importance to allow for time-dependent maturation of the astrocyte-lineage cells in human NSPHs from an embryonic-like developmental stage to a stage resembling at least late-foetal/early-postnatal or even adult human astrocytes, which requires a prolonged maturation period (9, 11). Based on these studies by Sloan et al. (11) and Gordon et al. (9), we decided to define the appearance of AQP4 as a marker for mature(d) adult-like astrocytes, which appeared in this study - with the hiPSC-line and culture protocol used - highly expressed in 5-month-old NSPHs. Therefore, in this study NSPHs were allowed to differentiate/mature for 5 months after which they displayed a stable composition of neurons and astrocytes expressing a clear set of markers associated with matured neurons and astrocytes (Figure 1) (9, 11, 31, 51). Furthermore, immune-competence of our cultured NSPHs was demonstrated by release of the pro-inflammatory cytokines IL-6 and CXCL10 following pro-inflammatory cocktail stimulation, previously defined by us (52) and others (31, 46, 53, 54) to demonstrate astrocyte reactivity.

Upon initial VZV$^{eGFP\text{-}ORF23}$ infection experiments of 5-month matured NSPHs, we observed VZV$^{eGFP\text{-}ORF23}$ spreading rapidly throughout the viable area of the NSPHs, infecting both neurons and astrocytes (Figure 2). In agreement with the preceding study by Bubak et al. (46), we here demonstrate that immune-competent NSPHs, with their immune-responsiveness being demonstrated by cytokine production following pro-inflammatory cocktail stimulation or following SeV^{eGFP}-infection, are not triggered to release the pro-inflammatory cytokines IL-6 and CXCL10 upon VZV $^{eGFP\text{-}ORF23}$-infection (Figure 3). These observations in our 5-month matured NSPH model, wherein we initially hypothesised VZV immune recognition to be coordinated by astrocytes, thus recapitulate the findings of Bubak et al. (46) that also astrocytes – even within a more neural-like context - become subjected to the extensive repertoire of immune evasion strategies of VZV (1, 55–57). A major advantage of this NSPH approach – in contrast to scarce human material – is the (theoretically) unlimited amount of neural-like tissue that can be generated and used for research purposes. As shown in this study, a multiplex human immunology NanoString gene expression analysis (Figure 4), and subsequent histological confirmation (Figure 5), could easily be performed and demonstrated the ability of VZV$^{eGFP\text{-}ORF23}$ to interfere with Type-I interferon response as well as antigen presentation pathways in matured hiPSC-derived NSPHs. Even though this was highly expected based on published literature reviews regarding HSV and VZV immunobiology (1, 38, 58–62), the multicellular context of hiPSC-derived NSPH models allows for single model validation strategies in a human neural-like context.

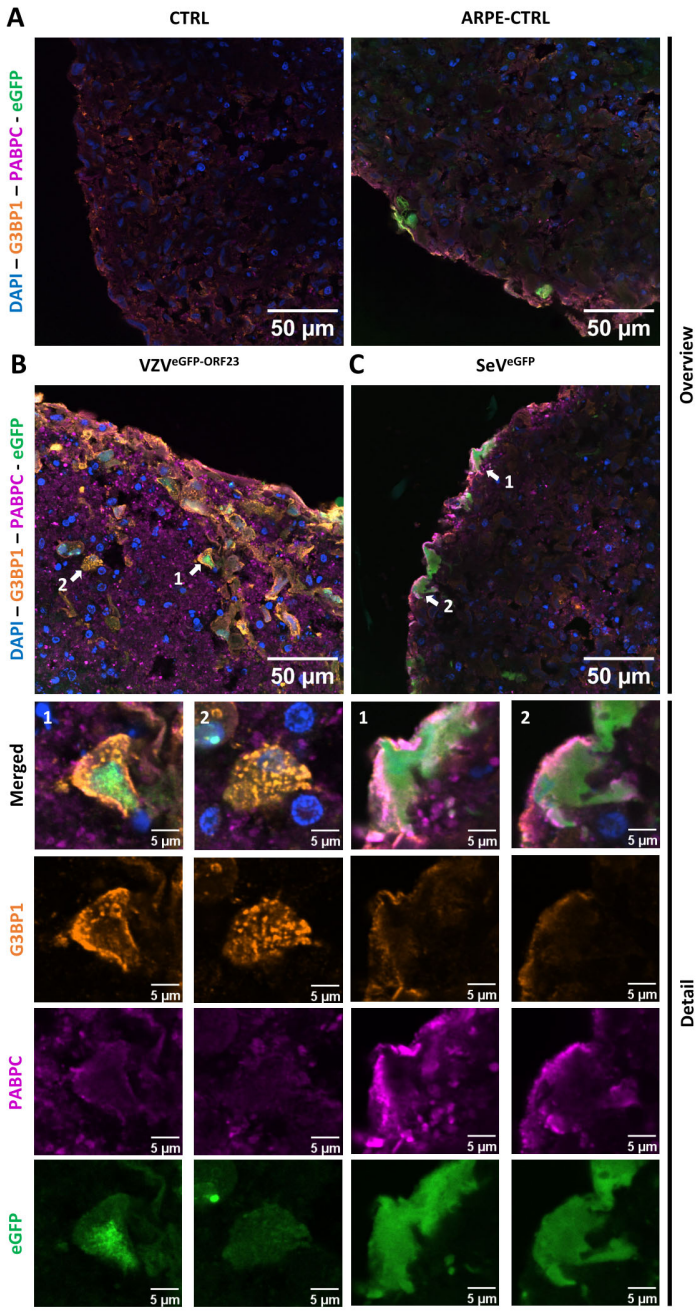


FIGURE 6
Formation of stress granules in VZV$^{eGFP\text{-}ORF23}$ and SeV^{eGFP}-infected NSPHs. Representative overview immunofluorescence images of control NSPHs [**(A)**, top left], NSPHs inoculated with eGFP+ control ARPE19 cells [**(A)**, top right, ARPE-CTRL], NSPHs inoculated with VZV$^{eGFP\text{-}ORF23}$-infected ARPE19 cells [**(B)**, top left, VZV$^{eGFP\text{-}ORF23}$], and NSPHs infected with SeV^{eGFP} [**(C)**, top right, SeV^{eGFP}], immunolabelled for G3BP1 (orange) and PABPC1 (magenta). Insets of virus-infected cells are depicted by white arrows on the overview image [**(B, C)**, top] and shown below the corresponding overview images [**(B, C)**, bottom]. Nuclei are labelled with DAPI (blue). Scale bars of 50 (overview) and 5 (detail) µm are indicated on the images.

Noteworthy, next to IL-6 and CXCL10, we also investigated whether NSPHs could be triggered to release IFN-α2 and IFN-β upon infection with VZV$^{eGFP\text{-}ORF23}$ or SeV^{eGFP}, as a sign of intrinsic anti-viral response. Our observation that no Type-1 IFNs were detected in the culture supernatant in VZV$^{eGFP\text{-}ORF23}$ and SeV^{eGFP} infected NSPHs at day 3, day 5 or day 7 post-infection, is in fact also in line with our multiplex human immunology NanoString gene expression analysis, whereby no significant upregulation of IFN-α2 and IFN-β mRNA was noted (Supplementary Table 1). Although not yet investigated in detail by us for hiPSC-derived astrocytes and/or NSPHs as a whole, we did report previously that hiPSC-derived neurons, even though they can respond to exogenous IFN-α, they cannot produce IFN-α themselves even after stimulation with strong synthetic inducers (25). Based on literature reports, we

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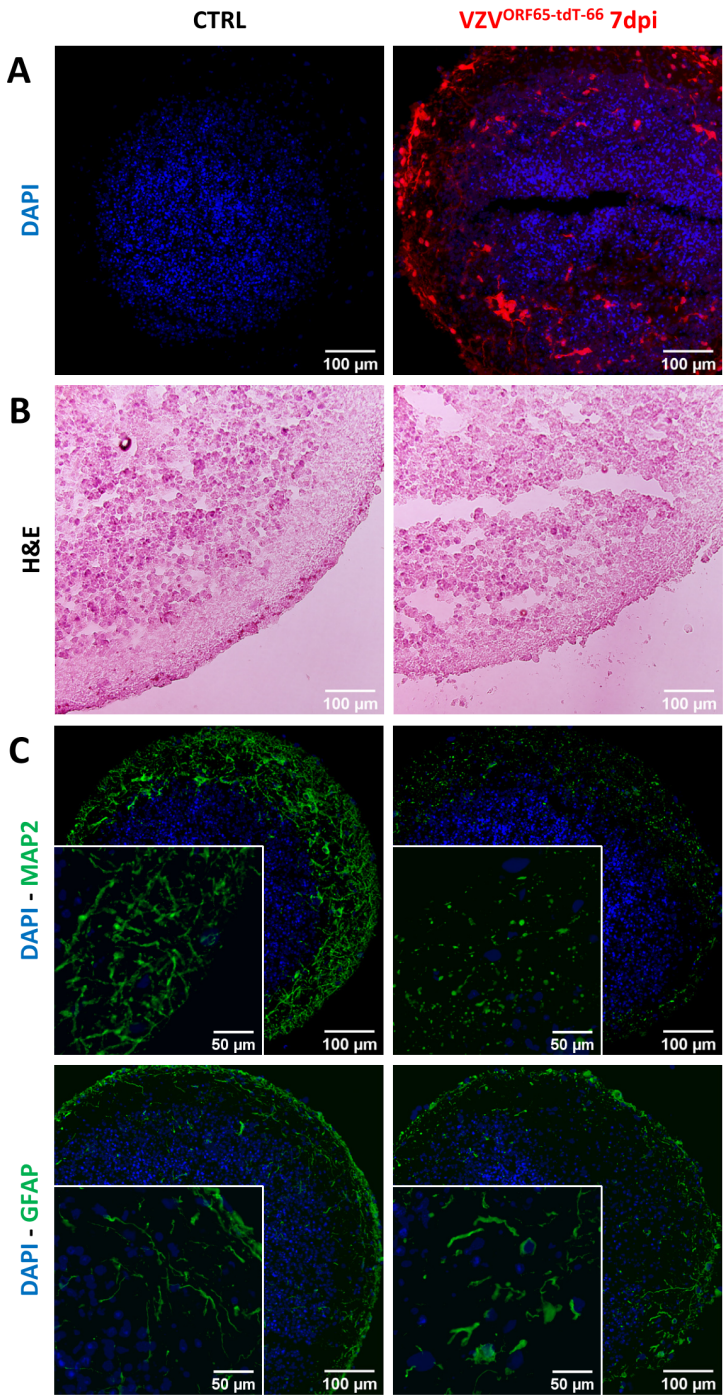

FIGURE 7
VZV$^{ORF65\text{-}tdT\text{-}66}$ affects cellular integrity in infected NSPHs. **(A)** Representative images of cryosections of control (CTRL) and VZV$^{ORF65\text{-}tdT\text{-}66}$ infected NSPH at day 7 post-infection (dpi), in which nuclei are labelled with DAPI (blue) and infection with VZV$^{ORF65\text{-}tdT\text{-}66}$ is shown in red (direct tdTomato fluorescence signal). **(B)** Representative images Haematoxylin-Eosin stained cryosections of control (CTRL) and VZV$^{ORF65\text{-}tdT\text{-}66}$ infected NSPH at 7 dpi. **(C)** Representative immunofluorescence images of control (CTRL) and VZV$^{ORF65\text{-}tdT\text{-}66}$ infected NSPH at 7 dpi, immunolabelled for the neuronal marker MAP2 (green, top panel) and astrocyte marker GFAP (green, bottom panel). Nuclei are labelled with DAPI (blue). Scale bars of 100 (overview) and 50 (detail) µm are indicated on the images.

hypothesised this to be an intrinsic defence mechanism of the brain against IFN-α mediated neuronal damage. As such, it is also not unexpected that no IFN-α2 and IFN-β was detected upon SeV^{eGFP} infection of NSPHs.

An interesting finding from our approach relates to the invariant chain protein CD74, which – besides stabilising newly generated MHC class II molecules - promotes the intracellular trafficking of empty MHC class II proteins from the endoplasmic

reticulum (ER) via the Golgi Apparatus towards late-stage phagolysosomes for subsequent MHC class II antigen-loading (38). Although interference with CD74 has been observed for HSV-1 (38, 63), our new study now also ascribes this immune evasion mechanism also to VZV. In addition, although not further investigated in this study, our GSE analysis (Figure 4) also revealed a significant downregulation of signalling pathways related to lysosomal membranes in VZV$^{eGFP\text{-}ORF23}$-infected NSPHs. Indeed, several herpesviruses have developed strategies to escape autolysosomal degradation, although there is an increasing body of evidence suggesting complex and sometimes opposing effects of autophagy in the context of VZV infection (64–66), with one recent study suggesting a neuro-protective role for autophagy in VZV-infected neurons (67). Clearly, this is a topic requiring further research.

Regarding the methodological level of NSPH research, we acknowledge several improvements that ultimately need to be integrated, one of which is the inclusion of autologous macrophages and/or microglia (68). Hereto, several protocols have been developed to generate hiPSC-derived NSPH models containing isogenic microglia (69, 70). However, based on our preceding research whereby isogenic hiPSC-derived macrophages were added in co-culture with hiPSC-derived peripheral nervous system (PNS)-like neurons (5), we do not expect them to play a direct role in counteracting a productive VZV-infection, not in the PNS nor in the CNS. As hypothesised before, macrophages/microglia, and potentially even astrocytes, may play an important role in linking innate and adaptive immunity. We suggest that, both in PNS and CNS, respectively macrophages and microglia/astrocytes, will be subject to inhibition of Type-I IFN signalling, but may still play an important role, albeit (partially) suppressed, in primary/secondary T-cell activation (5, 38, 71, 72). As shown in this study, the antigen-presentation machinery becomes subject to downregulation by VZV$^{eGFP\text{-}ORF23}$ infection but does not seem to be fully absent as it may require a certain time to fully downregulate HLA-DR expression (Figures 4, 5). To investigate the latter, it would be highly interesting to generate iPSC-lines from both primed (naturally or vaccinated) and naïve healthy individuals, whereupon VZV-infected hiPSC-derived NSPHs can be co-cultured with autologous peripheral blood mononuclear cells (PBMC). In this context, we have recently demonstrated that not only a lower VZV-specific T-cell receptor (TCR) diversity, but also reduced functional TCR affinity for VZV-specific proteins in HZ patients, leads to lower T cell activation and consequently affects the susceptibility for viral reactivation (73). While such studies are currently not feasible due to financial constraints, it is plausible that reduced TCR diversity and/or affinity may aid to susceptibility for VZV-induced CNS neuropathology. Likewise, with the knowledge that several mutations are known to be involved in increased VZV-associated neuropathology (41, 74), in time the use of patient-derived iPSC and autologous PBMC may need to be explored to gain a better understanding of VZV-associated neuropathology and/or potential neuroprotective interventions.

Still under continuous development by many research groups, another limitation of most of the current NSPH models, including ours, is the lack of vascularisation. While on one hand micro-vascularisation may prevent the occurrence of TUNEL^{+} necrotic cells in the NSPH core (Figure 1) (75, 76), in the context of VZV-associated neuropathology it may more readily be considered as a way to model VZV vasculopathy and subsequent CNS infection. Specifically for modelling the latter, one may not need a fully vascularised and perfused NSPH model, but rather an external endothelial cell layer, similar to existing blood-brain-barrier (BBB) models (77). Again, these approaches may need to be performed in an autologous experimental setup, as discussed above.

Nevertheless, we would like to emphasise the future importance of multicellular NSPH models, especially as they allow the study of cellular stress in a 3D neural-like context. This is exemplified by our staining for SGs, a stress-induced membraneless organelle, in which over 140 cytoplasmic proteins can intertwine with cytoplasmic mRNAs, translation initiation components and proteins affecting mRNA function (78, 79). While the initial stage of SG formation is considered to be protective, as they allow the cell to control energy consumption in favour of cell survival, their long-term persistence and/or reduced clearance may lead to the activation of cell death processes, as is the case in the pathogenesis of many neurodegenerative diseases (80). In this study, we observed a marked increase in G3BP1^{+} cytoplasmatic aggregates in VZV$^{eGFP\text{-}ORF23}$-infected NSPHs (Figure 6), suggesting active cellular stress upon sustained NSPH infection (i.e. 7 days post-infection). Although further comparative studies between HSV- and VZV-infections in NSPHs are required, the induced formation of G3BP1 + SGs as such is a novel finding for VZV and has not previously been observed for wild-type HSV (81), although this may be cell-type and/or context dependent, and more specifically in our study the chosen (late) timing of analysis following initial infection. Finnen and colleagues did observe G3BP1+ SGs in cell lines infected with an engineered mutant HSV-2 strain, however, these SGs were not observed following infection with wild-type HSV-2 (82). On the other hand, we observed induction of PABPC1+ SG formation in SeV^{eGFP}-infected NSPHs (Figure 6), highlighting a clear difference between the two viral infections in terms of the types of SGs induced in the NSPH model. In contrast to VZV, no G3BP1+ SG formation was observed in the SeV^{eGFP}-infected NSPHs, which may be consistent with previous studies by Iseni and colleagues indicating that SeV inhibits SG formation by investigating another SG component, TIAR1, which was not tested in our case (81, 83). Like TIAR, G3BP1 is also a primary SG nucleating protein involved in phase 2 of SG assembly (81), further suggesting interference by SeV during SG formation. Although not the initial aim of this study, our NSPH model may thus become an interesting future tool to study SG formation and/or resolution in a more complex *in vitro* human neural-like environment upon viral infection and/or other neuropathologies.

5 Conclusion

In this study we have demonstrated that matured 5-month-old hiPSC-derived NSPHs, containing neurons and astrocytes, are immune competent, susceptible to viral infection, and most importantly, able to recapitulate VZV- and SeV-specific innate immune signatures. In this model, we demonstrate that VZV evades innate neuro-immune recognition by suppressing the

Type-I IFN and antigen presentation pathways, in contrast to SeV. Furthermore, even though VZV is highly immune evasive, NSPHs do suffer from long-term cellular stress upon infection. This NSPH model is therefore well suited to study viral neuro-immune responses and evasion strategies in a human CNS-like environment.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: GSE273529 (GEO).

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used.

Author contributions

JG: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. BO: Conceptualization, Methodology, Writing – review & editing, Funding acquisition, Supervision. PD: Conceptualization, Methodology, Writing – review & editing, Funding acquisition, Resources, Supervision. PP: Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Funding acquisition, Resources, Supervision. EVB: Methodology, Investigation, Formal analysis, Writing – review & editing. SDB: Methodology, Investigation, Formal analysis, Writing – review & editing. CDI: Methodology, Investigation, Formal analysis, Writing – review & editing. JDS: Methodology, Investigation, Formal analysis, Writing – review & editing. SVC: Methodology, Investigation, Writing – review & editing. TBH: Methodology, Writing – review & editing. MB: Methodology, Writing – review & editing. HDR: Methodology, Investigation, Formal analysis, Writing – review & editing. MT: Methodology, Investigation, Formal analysis, Writing – review & editing. ML: Methodology, Investigation, Formal analysis, Writing – review & editing, Funding acquisition. HM: Methodology, Investigation, Formal analysis, Writing – review & editing. JVW: Methodology, Investigation, Formal analysis, Writing – review & editing. WDV: Methodology, Writing – review & editing, Resources. WVB: Methodology, Formal analysis, Writing – review & editing, Resources. CG: Investigation, Formal analysis, Writing – review & editing. SP: Writing – review & editing, Funding acquisition. CSD: Writing – review & editing, Funding acquisition. BR: Writing – review & editing, Funding acquisition, Resources.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1458967/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Single channel images of Figure 2E. Representative images of 5-month-old VZV$^{eGFP\text{-}ORF23}$ infected NSPHs as seen in Figure 2E, but depicted individually as single-channel images of the nuclei labelled with DAPI (blue), the VZV$^{eGFP\text{-}ORF23}$ infection (green) and the stainings (magenta). Scale bars of 100 µm are indicated on the images.

SUPPLEMENTARY FIGURE 2
Longitudinal imaging of VZV$^{eGFP\text{-}ORF23}$ and SeV^{eGFP} infection in NSPHs. Representative live cell fluorescence image of 5-month-old control NSPHs (CTRL), NSPHs inoculated with eGFP+ control ARPE19 cells (ARPE-CTRL, green), NSPHs inoculated with VZV$^{eGFP\text{-}ORF23}$-infected ARPE19 cells

(VZV$^{eGFP\text{-}ORF23}$, green), and NSPHs infected with SeV^{eGFP} (SeV^{eGFP}, green) at day 3, 5 and 7. Scale bars of 50 and 200 µm are indicated on the images.

SUPPLEMENTARY FIGURE 3
VZV$^{eGFP\text{-}ORF23}$- and SeV^{eGFP}-infected NSPHs display opposite effects on protein expression related to Type-I interferon response and antigen presentation pathway – Overview images. Representative images for 5-month-old control NSPHs (CTRL), NSPHs inoculated with eGFP+ control ARPE19 cells (ARPE-CTRL, green), NSPHs inoculated with VZV$^{eGFP\text{-}ORF23}$-infected ARPE19 cells (VZV$^{eGFP\text{-}ORF23}$, green), and NSPHs infected with SeV^{eGFP} (SeV^{eGFP}, green), immunolabelled for the Type-I interferon response markers MX1, IFIT2 and ISG15, and the antigen presentation pathway markers HLA-DR and CD74. All in magenta. Nuclei are labelled with DAPI (blue). Scale bars of 500 µm are indicated on the images.

SUPPLEMENTARY TABLE 1
Complete gene expression list Human Immunology V2 nCounter® panel. Gene expression list showing 585 immune transcripts, 15 housekeeping genes, 6 positive and 9 negative control probes for the 4 conditions 'CTRL', 'ARPE-GFP', 'VZV$^{eGFP\text{-}ORF23}$' and 'SeV^{eGFP}'. The included gene expression data was sequentially corrected for background, technical variation and RNA content, as described in M&M section 2.16.

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EDITED BY
Thomas A. Angelovich,
RMIT University, Australia

REVIEWED BY
Farzana Bhuyan,
National Institute of Allergy and Infectious Diseases (NIH), United States
Carlos Manuel Zapata-Martín del Campo,
National Institute of Cardiology Ignacio Chavez, Mexico

*CORRESPONDENCE
Shokrollah Elahi
elahi@ualberta.ca

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Exploring the role of galectin-9 and artemin as biomarkers in long COVID with chronic fatigue syndrome: links to inflammation and cognitive function

Shokrollah Elahi [1,2,3,4,5,6]*, Maryam Rezaeifar [1], Mohammed Osman [2,3,7] and Shima Shahbaz [1]

[1]School of Dentistry, Division of Foundational Sciences, Edmonton, AB, Canada, [2]Li Ka Shing Institute of Virology, Edmonton, AB, Canada, [3]Women and Children Health Research Institute, Edmonton, AB, Canada, [4]Cancer Research Institute of Northern Alberta, Edmonton, AB, Canada, [5]Glycomics Institute of Alberta, Edmonton, AB, Canada, [6]Alberta Transplant Institute, Edmonton, AB, Canada, [7]Department of Medicine, Division of Rheumatology, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, AB, Canada

This study aimed to assess plasma galectin-9 (Gal-9) and artemin (ARTN) concentrations as potential biomarkers to differentiate individuals with Long COVID (LC) patients with myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) from SARS-CoV-2 recovered (R) and healthy controls (HCs). Receiver operating characteristic (ROC) curve analysis determined a cut-off value of plasma Gal-9 and ARTN to differentiate LC patients from the R group and HCs in two independent cohorts. Positive correlations were observed between elevated plasma Gal-9 levels and inflammatory markers (e.g. SAA and IP-10), as well as sCD14 and I-FABP in LC patients. Gal-9 also exhibited a positive correlation with cognitive failure scores, suggesting its potential role in cognitive impairment in LC patients with ME/CFS. This study highlights plasma Gal-9 and/or ARTN as sensitive screening biomarkers for discriminating LC patients from controls. Notably, the elevation of LPS-binding protein in LC patients, as has been observed in HIV infected individuals, suggests microbial translocation. However, despite elevated Gal-9, we found a significant decline in ARTN levels in the plasma of people living with HIV (PLWH). Our study provides a novel and important role for Gal-9/ARTN in LC pathogenesis.

KEYWORDS

long COVID, chronic fatigue syndrome, galectin-9, artemin, microbial translocation, HIV

Introduction

Long COVID (LC) is a major global health concern that has impacted the quality of life of millions of individuals. It is a multisystemic condition with incidence rates ranging from 10 to 70% depending on the studied cohorts and the time of screening (1–3). LC can be observed in any age group, but it appears to be more common between the ages of 30 and 50 years. Additionally, it is frequent to observe more LC cases in those with mild acute COVID-19 disease (1, 4). This suggests that LC can occur regardless of the severity of acute disease and in the absence of other co-morbidities. Notably, women are disproportionality impacted, with more severe symptoms associated with LC and more frequently than men (4, 5).

While LC improves over time in some patients, others continue to experience LC symptoms even for years. Unfortunately, a subset of patients exhibits the most debilitating form of LC, myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS), and this might be lifelong in a subset of them (1, 6). LC patients experience a wide range of symptoms, such as cardiovascular and thrombotic diseases, cerebrovascular disease, ME/CFS, dysautonomia, autoimmune conditions, cognitive impairment, etc.

Nevertheless, the molecular mechanism underlying the most post-acute disease symptoms, including ME/CFS remains not fully understood. Several potential mechanisms have been proposed, including metabolomic and immune dysregulation, chronic inflammation, dysregulated hematopoiesis, hypoxia, viral persistence, autoimmunity, alteration of microbiota and gastrointestinal inflammation, endothelial dysfunction, and impaired signaling in the brainstem and/or vagus nerve (1, 4, 7–12). Although fatigue is one of the main symptoms in this group, the major symptom observed in a subset of LC patients is cognitive impairment, or brain fog, which has been linked to autonomic nervous system dysfunction (13). Given the blood-brain barrier (BBB) disruption, neurological symptoms (e.g. brain fog) observed in LC patients are likely due to sustained systemic inflammation and BBB dysfunction (14). Indeed, a peripheral immune-mediated response to viral antigens and neuroinflammation might indirectly result in neurocognitive symptoms (15). Mechanistically, it is known that in some cases circulating pathogen associated patterns (PAMPs) can reach the CNS and choroid plexus (CP) and the circumventricular reigns (16). For example, peripheral administration of LPS and/or inflammatory cytokines are associated with impaired learning skills in animal models (17, 18). Therefore, it is possible to speculate that an unresolved localized or systemic pro-inflammatory state contributes to CNS symptoms in LC patients. In this scenario, residential cells in the CNS are exposed to systemically produced pro-inflammatory cytokines, chemokines, and other damage-associated molecules (DAMPs), ultimately resulting in neurological symptoms, fatigue, and neurocognitive impairment in a subset of LC patients.

Galectin-9 (Gal-9) is a β-galactosidase binding lectin with diverse immunomodulatory properties (19). It is widely abundant in immune and non-immune cells and binds to different receptors, such as TIM-3, PD-1, PDI, IgE, CD44, CD45, and CD3 among others (20–24). The concentration of soluble Gal-9 is reported to be elevated in the plasma, saliva, and synovial fluids in HIV and other inflammatory conditions (25–27). Gal-9, as a DAMP, has been reported to play a predominant role in cytokine release storm in the acute SARS-CoV-2 infection (28). Most recently, we reported elevated levels of Gal-9 in the plasma of LC patients with ME/CFS (29). In particular, our findings supported a positive correlation between the plasma Gal-9 levels with CRP, MIP-1β, IL-10, and VCAM-1 in these patients (29). Of note, persistent gastrointestinal (GI) symptoms are reported in acute phase of COVID-19 disease and LC patients (30, 31). This may, in part, suggest the presence of compromised GI mucosal integrity in LC patients. Thus, plasma soluble CD14 (sCD14) and intestinal fatty acid binding protein (I-FABP) are considered reliable biomarkers for assessing GI permeability and epithelial integrity (32, 33). However, the association of these biomarkers with Gal-9 has not been documented in LC. Moreover, elevated Gal-9 expression across brain tissues is reported to be associated with neuropathology and cognitive impairment in HIV-infected individuals (34).

Additionally, we have reported the elevation of artemin (ARTN), a neurotrophic factor, in the plasma of LC patients with ME/CFS (29). ARTN interacts with its receptor GFRα3 and its co-receptor RET affecting cell growth and differentiation (35). Notably, the role of ARTN in neuropathic pain has been the subject of debate (35, 36). We previously reported that systemic ARTN levels were positively correlated with the cognitive impairment and pain scores in LC patients (29). Therefore, in the current study, we decided to determine whether plasma Gal-9 levels have any association with cognitive impairment scores in LC patients with ME/CFS, as reported in people living with HIV (PLWH) (34, 37). Given that compromised gut integrity and microbial translocation are contributing factors to chronic inflammation commonly observed in PLWH (32, 38), due to this similarity, we decided to examine whether this is the case in LC patients by measuring LPS-binding protein (LPS-BP). Finally, considering the association of plasma ARTN levels with cognitive impairment scores in LC patients with ME/CFS (29) and reported HIV-associated neurocognitive disorder (34), we quantified this neurotrophic factor in the plasma of PLWH and compared them with values of the other groups. Taken together, our findings suggest that plasma Gal-9 and ARTN levels, with high sensitivity and specificity, can differentiate LC from the recovered group. Furthermore, we observed a positive correlation between plasma Gal-9 with inflammatory biomarkers such as sCD14, I-FABP, LPS-BP, serum amyloid A (SAA), and IP-10 in LC patients. Notably, we found that Gal-9 was positively correlated with cognitive failure score in LC patients as reported in PLWH (37, 39). Despite the reported elevation of Gal-9 in the plasma of PLWH, these individuals had significantly lower levels of ARTN compared to healthy individuals and LC patients.

Methods

LC cohort

The first cohort (discovery) comprised 44 LC patients (median age 51.5±13.1, 11 males and 33 females) and 24 SARS-CoV-2 infected individuals who had recovered (R) from the disease without any obvious symptoms and complications (median age 50.5 ± 13.3, 6

males and 18 females). All were infected with the original Wuhan SARS-CoV-2 strain. All study subjects were recruited approximately 12 months (371±19 days LC vs. 368±6.2 days R) after the onset of SARS-CoV-2 infection as reported elsewhere (29). We utilized a set of well-defined validated clinical questionnaires developed by CDC and WHO (40, 41) for the diagnostic of LC patients with ME/CFS. To determine the applicability of our findings to another cohort, we established a validating cohort. The validating cohort consisted of 34 LC patients (median age 48 ± 9.8, 9 males and 25 females) and 34 recovered individuals (median age 45 ± 11.39, 10 males and 24 females) from SARS-CoV-2 infection without any symptoms. The infection was confirmed by PCR in both cohorts. This validating cohort was infected mainly with the Delta/or Omicron variants. Similar to the discovery cohort, they were recruited approximately 12 months (435±89 days LC vs. 415±40 days R) after the onset of acute disease, as reported elsewhere (29).

Study participants were age-and sex-matched, and considering that the majority of our patients had a mild acute infection, confounding health conditions were not common. All study subjects (LC and R) in the first cohort were SAS-CoV-2 vaccine-naïve but 67.3% of LC and 73.5% of R were vaccinated in the validating cohort. All of our LC patients in both cohorts met the criteria for categories I, II, III, IV, V, and VI associated with ME/CFS as we previously reported (29). Those LC patients who did not meet the criteria established by CDC and WHO (40, 41) were excluded from the study. Considering that a subset of PLWH presents cognitive impairments, we decided to compare our findings with a cohort of PLWH.

HIV cohort

Our HIV group comprised 63 individuals on antiretroviral therapy (ART) form the Northern Alberta HIV cohort as reported elsewhere (24, 42). We also recruited 25 healthy controls who were serologically negative for HIV, hepatitis C virus (HCV), and hepatitis B virus (HB) for comparison.

Ethics statement

The COVID-19-related study was approved by the Human Research Ethics Board (HREB) at the University of Alberta (protocol # Pro00099502). Similarly, the HIV-related study was approved by the HREB (protocol # Pro00070528) and HCs (protocol # Pro00063463). A written informed consent form was obtained from all participants.

Cytokine and chemokine multiplex analysis and ELISA assays

Frozen plasma samples stored at -20/80°C were thawed and centrifuged for 15 min at 1500g followed by dilution in PBS for quantifying cytokine/chemokine and other soluble analytes. The concentration of cytokines and chemokines was quantified using the V-PLEX Neuroinflammation panel 1 kit (K15210D-1) from Meso Scale Discovery (MSD) (28, 43). Additionally, the plasma was subjected to ELISA assays for Gal-9 (R&D, DY 2045), ARTN (R&D, DY 2589), LPS-Binding protein (DY870-05), FABP-1 (R&D, Z-001), and sCD14 (DC140), as we have reported elsewhere (21, 28, 43).

Statistical analysis

Spearman correlation was used to measure association between two variables. The non-parametric Mann-Whitney U test for two groups or One-way ANNOVA was used when more than two groups were compared. Measures are expressed as mean ± SEM, and a P-value < 0.05 was considered to be statistically significant.

Results

Recently, we reported elevated levels of Gal-9 and ARTN in the plasma of LC patients with ME/CFS (29). To further investigate whether plasma Gal-9 and/or ARTN concentrations could serve as surrogate biomarkers to differentiate LC patients from recovered (R) and/or HCs, we conducted additional analysis. Using the receiver operating characteristic (ROC) curve, we calculate the optimal cut-off value of Gal-9 to distinguish LC patients from HCs. The ROC curve point with the best sensitivity/specificity indicated that a plasma Gal-9 level greater that 1725 pg/ml separates LC from HCs with 97% sensitivity and 100% specificity (Figure 1A). To identify LC patients from the R group, we determined a Gal-9 cut-off value of greater than 2779 pg/ml, achieving a sensitivity of 78.5% and specificity of 100% (Figure 1B). This finding was confirmed in our validating cohort, where a cut-off value of greater than 1702 pg/ml/ml differentiated LC patients from the R group with 82.35% sensitivity and 95% specificity (Figure 1C). These observations suggest that the plasma Gal-9 is a sensitive screening biomarker for discriminating LC patients with ME/CFS from both R and HCs.

In addition to our previous findings of a positive correlation between plasma Gal-9 and CRP, VCAM-1, MIP-1β, and IL-10 (28, 44), we observed similar correlations between plasma Gal-9 and SAA and IP-10 levels in our discovery (Figures 1D, E) and validating LC cohorts, respectively (Figures 1F, G).

Activated innate immune cells (e.g. neutrophils and monocytes) can shed Gal-9 (20, 28). Given the elevated sCD14 in the acute phase of COVID-19 disease (44) and in LC patients (29), our observations suggest that Gal-9 may contribute to the elevation of sCD14 in the plasma of LC patients. As such, we found a moderate positive correlation between plasma Gal-9 and sCD14 concentrations in LC patients across both discovery and validating cohorts (Figures 2A, B). Furthermore, with the elevation of the I-FABP in LC patients' plasma (29), we observed a positive correlation between I-FABP and Gal-9 levels in both cohorts (Figures 2C, D). Measurement of LPS-binding protein levels revealed a significant increase in LC patients compared to the R group (Figure 2E). Given the association of Gal-9 with HIV neuropathology and cognitive deficits (34, 37, 45), we explored

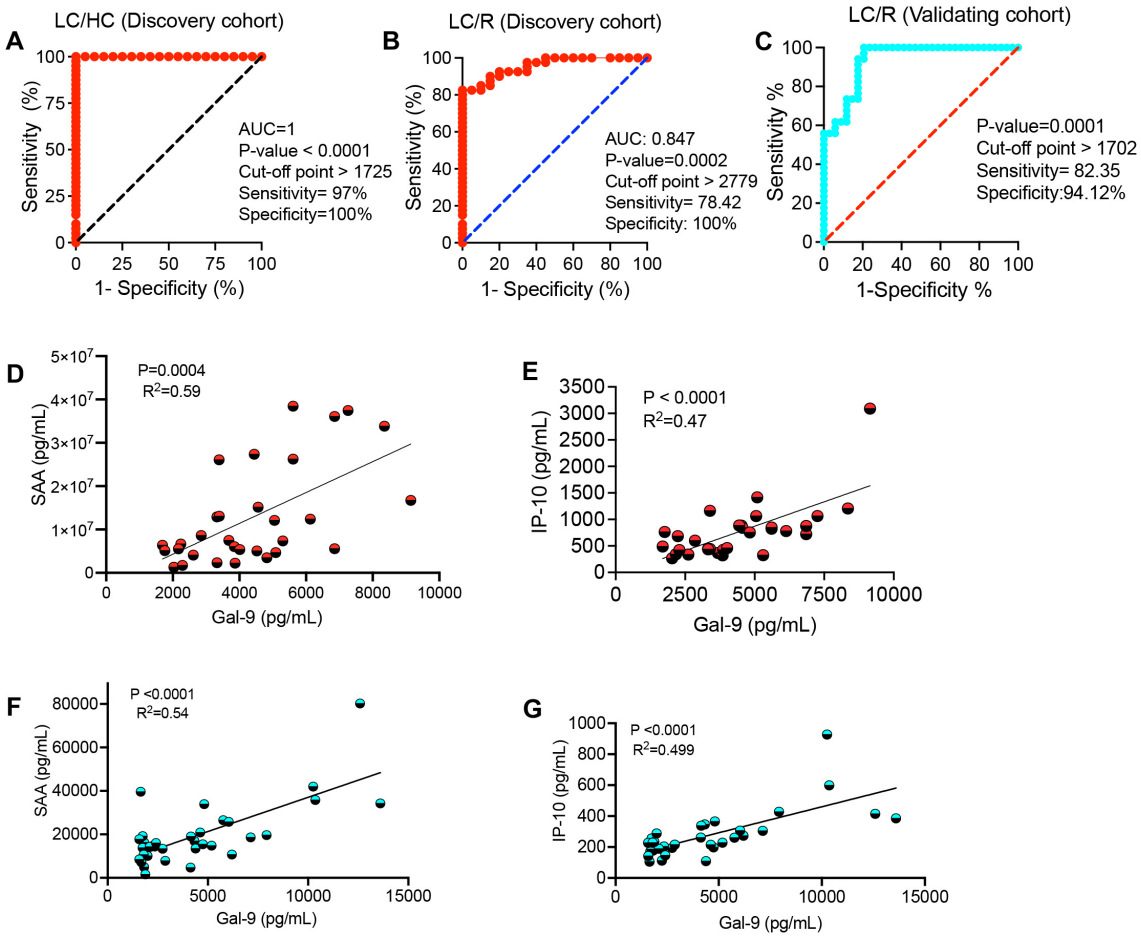

FIGURE 1
The diagnostic value of galectin-9 (Gal-9) in Long-COVID patients. **(A)** The receiver operating characteristic (ROC) curve for Gal-9 in LC versus healthy controls (HC) in the discovery cohort. **(B)** The ROC curve for Gal-9 in LC versus recovered individuals (R) in the discovery cohort. **(C)** The ROC curve for Gal-9 in LC versus R in the validating cohort. **(D)** The correlation between plasma Gal-9 and SAA, and **(E)** IP-10 levels in LC patients of the discovery cohort. **(F)** The correlation between plasma Gal-9 and SAA, and **(G)** IP-10 levels in LC patients of the validating cohort.

whether plasma Gal-9 is linked to cognitive impairments in LC patients. Our analyzes showed a positive correlation between Gal-9 levels and impaired cognitive function scores in both LC cohorts with ME/CFS (Figures 2F, G). However, we did not find any association between Gal-9 levels and other symptoms such as pain severity or widespread pain scores in LC patients (data not shown). Considering the elevated plasma ARTN levels in LC patients (29), we evaluated the diagnostic value of ARTN concentrations as a non-invasive biomarker in these patients. A cut-off value of greater than 2813 pg/ml plasma ARTN distinguished LC from HCs with 79% sensitivity and 82% specificity in our discovery cohort (Figure 3A). Similarly, a cut-off value of greater than 2780 pg/ml plasma ARTN differentiated LC from R with 79% sensitivity and 83% specificity in the discovery cohort (Figure 3B). Notably, in the validating cohort, a cut-off value of greater than 2855 pg/ml ARTN reliably differentiated LC patients from the R group with 100% sensitivity and 70% specificity (Figure 3C). The diagnostic values of both Gal-9 and ARTN were supported by a moderate and positive correlation between their plasma concentrations in both LC cohorts (Figures 3D, E).

Given the elevated plasma Gal-9 levels in PLWH (21, 24), we examined whether ARTN levels were also increased in HIV infection. Surprisingly, we found a significant reduction in ARTN levels in PLWH compared to HCs (Figure 3F). Although ARTN is a member of the TGF-β superfamily (46), its plasma levels were sharply elevated in PLWH compared to HCs (Figure 3G). In contrast, TGF-β levels were significantly reduced in LC patients (29). Despite the commonality of chronic immune activation and immune dysregulation in both HIV and LC subjects, ARTN levels were substantially lower in PLWH compared to LC patients (Figure 3F).

Discussion

This study aimed to explore the potential of plasma Gal-9 and ARTN levels as surrogate biomarkers for distinguishing LC patients from HCs and R individuals. The ROC curve analysis revealed that the plasma Gal-9 and ARTN serve as effective biomarkers with high sensitivity and specificity in differentiating LC patients from Rs and HCs. This suggests that Gal-9 and ARTN hold promise as non-

invasive screening biomarkers for identifying LC individuals experiencing ME/CFS. Whether these biomarkers are associated with idiopathic ME/CFS requires further investigation.

The reproducibility of our findings in a separate cohort strengthens the evidence that plasma Gal-9/ARTN concentrations could be valuable tools in distinguishing LC patients with ME/CFS.

The observed positive correlations between plasma Gal-9 levels and inflammatory markers such as SAA and IP-10 are consistent with our previous observations during acute SARS-CoV-2 infection and in LC patients (28, 29, 44). This reaffirms the role of Gal-9 as a potential indicator of immune dysregulation in both acute and prolonged phases of COVID-19 disease. However, elevated plasma Gal-9 levels have been reported in various pathological conditions such as HIV infection, virus-associated solid tumors, chronic lymphocytic leukemia, hepatitis C infection, autoimmune hepatitis, influenza infection, and other inflammatory conditions (21, 22, 25, 26, 47–49). Therefore, it is important to consider that other chronic inflammatory conditions may also influence Gal-9 levels in plasma when evaluating this lectin in LC study subjects. Furthermore, it is important to note that Gal-9 interacts with various receptors such as TIM-3, CD45, CD44, CD3, PDI, and PD-1. The biological consequences of these interactions vary significantly depending on the target cell, the expression level of the corresponding receptor, and the microenvironment. For instance, interactions of Gal-9:TIM-3 and Gal-9:PD-1 are associated with CD8+ T cell exhaustion (22, 50, 51). In contrast, Gal-9:CD3 interaction enhances TCR signaling in T cells (20, 52). Similarly, while Gal-9:CD44 interaction promotes NK cell effector functions under physiological conditions, it impairs their cytotoxic capabilities in chronic conditions (23, 53).

Our investigation into potential sources of elevated Gal-9 in LC patients revealed a positive and moderate correlation with plasma sCD14 levels. This association with sCD14 implies that activated innate immune cells (e.g. neutrophils and monocytes) likely shedding this lectin (20, 28) may contribute to the elevated Gal-9 levels in LC patients. In agreement, blocking CD14 has resulted in a substantial reduction in neutrophil abundance in the lung and peripheral blood and subsequently reduced organ dysfunction due

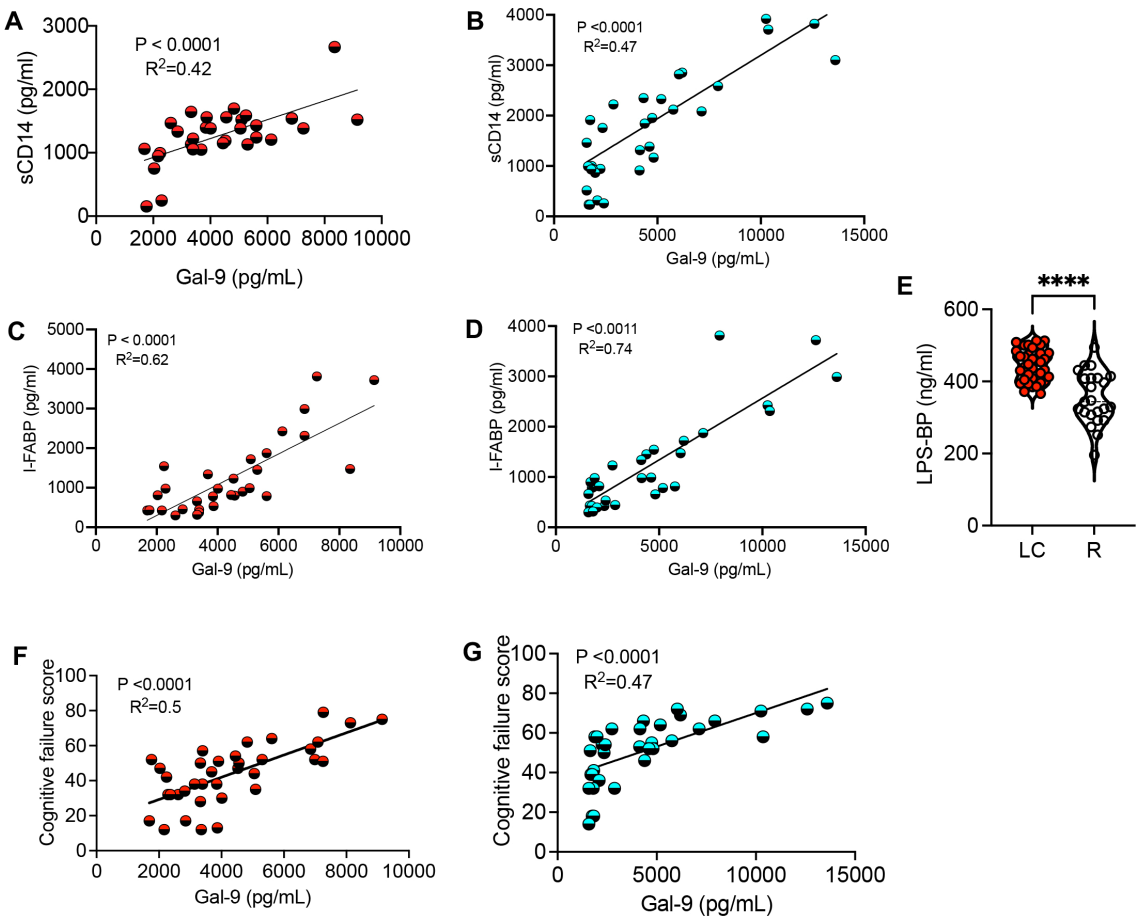

FIGURE 2
The correlation of Gal-9 with inflammatory and cognitive impairment in LC patients. **(A)** The correlation between plasma sCD14 and Gal-9 in the discovery, and **(B)** validating cohort. **(C)** The correlation between plasma I-FABP and Gal-9 in the discovery, and **(D)** validating cohort. **(E)** Detected concentrations of LPS-binding protein (LPS-BP) in the plasma of LC versus R in both cohorts. **(F)** The correlation between plasma Gal-9 and cognitive failure score in the discovery, and **(G)** validating cohort. The symbol **** shows a p value less than 0.0001 or P < 0.0001.

to inflammation in COVID-19 patients (54). Given the importance of plasma concentrations of sCD14 in conjunction with I-FABP in compromised intestinal permeability (33, 55), we found a substantial correlation between Gal-9 with sCD14 and I-FABP in LC patients. Moreover, the elevation of LPS-binding protein in the plasma of LC patients further supports the complex interplay between immune activation, compromised GI integrity, and metabolic pathways in LC. The elevation of LPS in the plasma of PLWH is considered as a marker of microbial translocation associated with increased sCD14 and chronic immune activation (56). These observations suggest that compromised intestinal barrier integrity during the early phase of infection or persistent SARS-CoV-2 replication in the GI tract contributes to sustained immune activation and dysregulation in LC patients. This hypothesis is further supported by shedding of fecal SARS-CoV-2 RNA in patients up to 7 months post-acute disease (57).

Therefore, persistent residual viral replication in LC patients, similar to HIV, or the presence of SARS-CoV-2 viral antigens in tissues (e.g. gut) (12), can result in chronic immune activation. Consequently, the pro-inflammatory response and metabolomic alterations (4, 29) may promote the Warburg effect in LC patients. We suggest that the chronic inflammatory state may promote mitochondrial dysfunction in LC patients. The observed reduction in ATP plasma levels in LC patients (4) suggests the potential effects of persistent viral replication/innate immune activation on the glycolytic and mitochondria pathway in LC. It is noteworthy that the Warburg effect may subsequently downregulate T and B cell effector functions, as reported in cancer patients (58). For example,

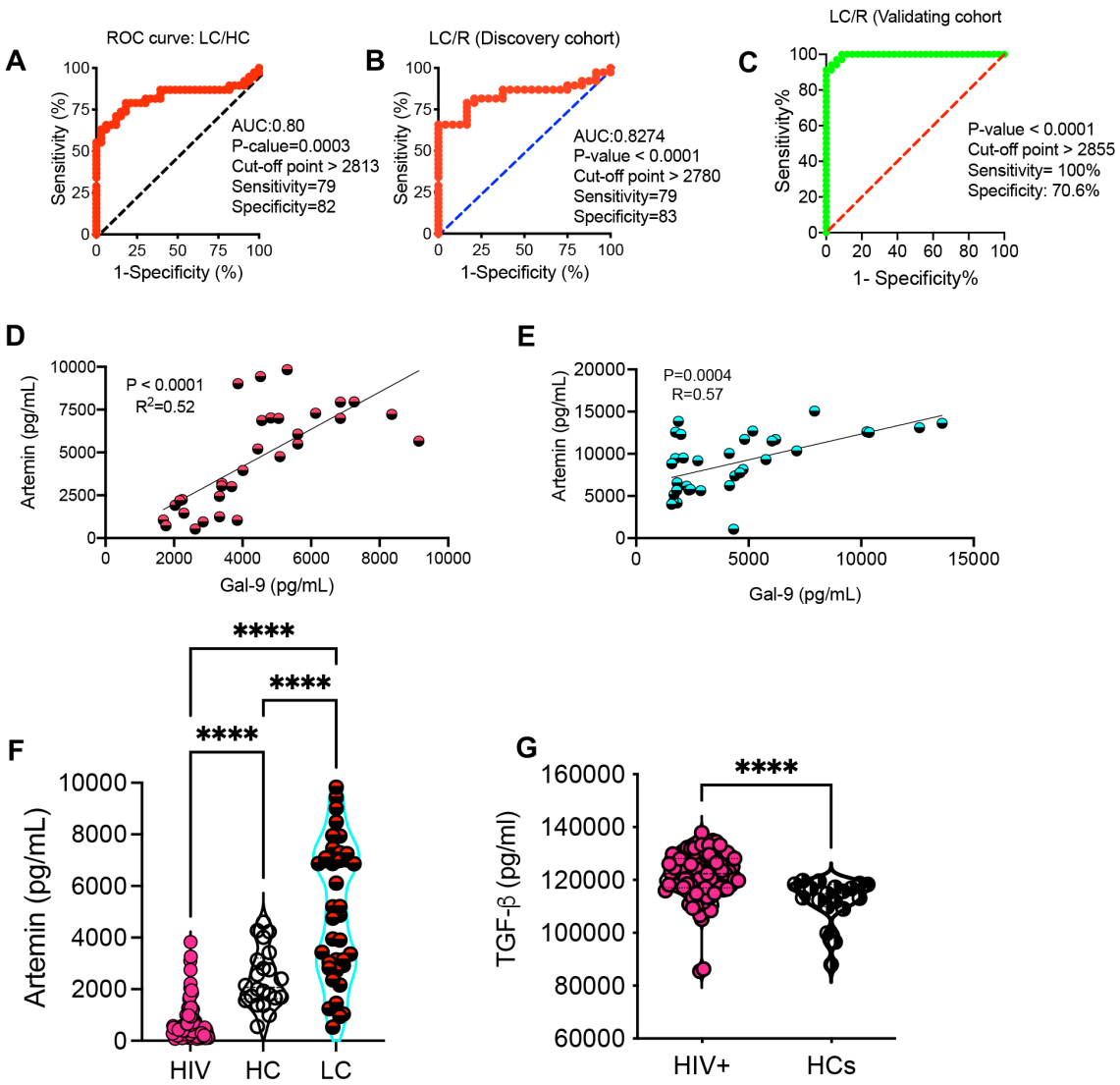

FIGURE 3
The diagnostic value of artemin (ARTN) in Long-COVID patients. **(A)** The ROC curve for ARTN in LC versus HCs in the discovery cohort. **(B)** The ROC curve for ARTN in LC versus the R group in the discovery cohort. **(C)** The ROC curve for ARTN in LC versus R in the validating cohort. **(D)** The correlation between plasma Gal-9 and ARTN in LC patients of the discovery, and **(E)** validating cohort. **(F)** Detected ARTN levels in plasma samples from HIV-infected individuals, HCs and LC patients of both cohorts. **(G)** TGF-β levels in plasma samples from HIV-infected individual versus HCs. The symbol **** shows a p value less than 0.0001 or P < 0.0001.

enhanced tryptophan metabolism and increased kynurenine metabolites can impair B cell effector functions (59) and also contribute to musculoskeletal symptoms observed in LC patients (60). Additionally, elevated Gal-9 may enhance SARS-CoV-2 entry in a glycan-dependent manner, as reported via enhanced binding of the spike protein with ACE2 (61). Likewise, enhanced SARS-CoV-2 infection by Gal-3, Gal-8, and Gal-9 has been reported (44). However, the effect of Gal-9 on HIV infection is receptor-dependent. For instance, Gal-9, via interaction with TIM-3, reduces HIV infection, and via interaction with PDI enhances HIV infection in CD4+ T cells (21, 62). Notably, our study unveils a potential link between Gal-9 levels and cognitive function in LC patients as documented in PLWH (37, 39). The positive correlation with cognitive failure scores suggests that Gal-9 may contribute to cognitive impairment in individuals with LC. Whether this effect is indirect through the elevation of pro-inflammatory cytokines/chemokines or direct needs to be determined. It has been reported that Gal-9 acts as an astrocyte-microglia signaling molecule, enhancing cytokine production (e.g. IL-6 and TNF-α) (63). Moreover, elevated levels of plasma and cerebrospinal fluids (CSF) Gal-9 are correlated with cognitive impairments in PLWH (64), further supporting its potential role in cognitive deficits in other viral infections, such as LC. Of note, the potential impact of ART on Gal-9 and other inflammatory biomarkers in PLWH should be considered. Therefore, further studies beyond HIV and LC are needed to evaluate the potential impact of elevated plasma Gal-9 on cognitive functions in other pathological conditions. Nevertheless, the source of Gal-9 needs to be determined, as immune and non-immune cells in the periphery and CNS can express and secrete this lectin (20, 28, 63). Although Gal-9 could originate from the periphery, diffusion between the plasma and CNS may occur due to the disruption of the BBB (65). Furthermore, the positive correlation between Gal-9 with ARTN concentrations, previously associated with cognitive failure and pain symptoms (29), adds another layer to the intricate network of molecular interactions in LC. GFRα3, the major ARTN receptor, is highly expressed in sensory and sympathetic ganglia of the peripheral nervous system (46) but not in immune cells (our unpublished observations). Similarly, ARTN is observed in human tissues (e.g.

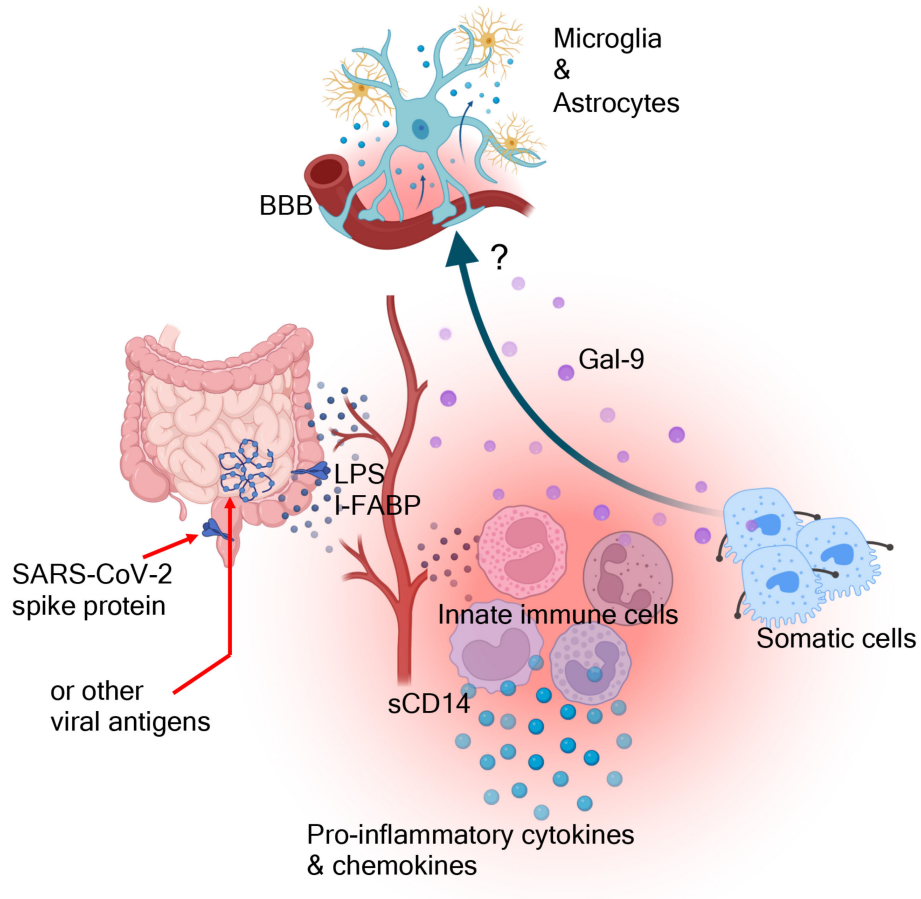

FIGURE 4
The proposed graphic summary. The gastrointestinal involvement during acute SARS-CoV-2 infection and the possible persistence of viral antigen/replication beyond the acute phase result in compromised gut barrier integrity. This, subsequently, leads to the translocation of microbial by-products (e.g. LPS) into the blood circulation. This leads to the activation of innate immune cells and the release of variety of pro-inflammatory cytokines and chemokines. This inflammatory cascade may result in immune/non-immune cell apoptosis and the release of damage-associated molecular patterns (e.g. Gal-9). Gal-9 may influence the activation/deactivation of different immune cells and ultimately may directly/indirectly influence the effector functions of microglia and astrocytes.

kidney and lung), Schwann cells, and upregulated after nerve injury, which implies glia are the main source of this neurotrophic factor (46). Therefore, it is so complex to delineate the role of ARTN in this context. On one hand, we have shown that the plasma ARTN concentrations are positively associated with cognitive impairments and pain scores in LC patients (29). On the other hand, ARTN might function as a compensatory mechanism to repair neural damage in LC patients by ongoing inflammation. Alternatively, we have reported that the expanded CD71+ erythroid cells (CECs) (66, 67) in the peripheral blood of LC patients by secretion of ARTN may play a role in this scenario (29), as reported by CECs in animal cancer models (68). However, it is unclear whether Gal-9 directly or indirectly influences ARTN expression in tissues. Therefore, the correlation of Gal-9 with ARTN merits further investigations.

Despite a previous report that Gal-9 levels were elevated in the plasma and CSF of PLWH (45), we found a significant reduction in the plasma ARTN concentrations in PLWH compared to HCs and LC patients. This observation indicates that Gal-9 does not have ever an absolute control over ARTN in PLWH. The expansion of CECs in the peripheral blood of PLWH (69) alongside the reduction in ARTN, contradicts their role as a major source of this neurotrophic factor. This discrepancy between SARS-CoV-2 and HIV might be explained by differential viral pathogenicity, chronicity, or other unknown mechanisms. Notably, recent studies have indicated the persistent SARS-CoV-2 RNA in LC patients up to several months post the onset of acute disease (12, 70). Nevertheless, our studies were performed 12 months after the acute SARS-CoV-2 infection, and at this stage, we are unaware of viral persistence in our LC cohort. Alternatively, the elevation of ARTN levels likely reflects a response to chronic inflammation, immune dysregulation, and nerve damage, as the body attempts to repair neuronal damage in LC patients. In contrast, reduced ARTN levels in HIV are likely due to the overall impaired neuroimmune responses and potential direct effects of the virus or inflammation on ARTN production. Therefore, further studies are required to determine other ARTN sources, such as endothelial cells and peripheral neurons, in LC patients with ME/CFS (64).

We propose that compromised gut barrier integrity occurs in a subset of SAR-CoV-2 infected individuals at the onset of acute disease or alternatively due to persistent viral replication and/or the presence of viral antigens in the gut of LC patients (Figure 4). This leads to microbial translocation to the periphery, which subsequently results in the activation of innate immune cells. This is supported by the elevation of LPS-BP, I-FABP, and sCD14 in the plasma of LC patients. Activated innate immune cells secrete pro-inflammatory cytokines and chemokines, contributing to systemic inflammation in LC patients (Figure 4). Additionally, Gal-9, released by immune and non-immune cells due to its immunomodulatory properties, exacerbate immune dysregulation. Finally, elevated plasma Gal-9 and pro-inflammatory biomarkers may directly or indirectly influence the CNS due to BBD disruption in LC patients (Figure 4). In conclusion, our findings highlight the potential of plasma Gal-9 and/or ARTN as sensitive biomarkers for identifying and stratifying LC individuals with ME/CFS. The correlations with inflammatory markers, immune activation, and cognitive impairment underscore the multifaceted nature of Gal-9 in LC, providing valuable insights for future research and potential therapeutic interventions. However, we are aware of several study limitations. This is a descriptive study, and further investigations are needed to understand how Gal-9 and/or ARTN affect cognitive impairments. Additionally, similar studies in larger cohorts, particularly, longitudinal studies are needed to confirm our findings. The heterogeneity of LC patients in terms of clinical presentations should be considered in future studies, given that the majority of our LC patients had a mild acute infection. Considering the differential immunological effects of SARS-CoV-2 strains, such as the Wuhan strain compared to the Delta and Omicron variants (44), it is important to examine how these variants might differentially impact LC syndrome. Our discovery cohort consisted of individuals infected with the Wuhan strain, while our validating cohort primarily included subjects infected with the Delta and Omicron variants. Therefore, future studies should account for the potential differential effects of SARS-CoV-2 variants of concerns. Finally, given the chronic systemic immune dysregulation/activation and potential cross-talk between HIV and SARS-CoV-2 (71), it would be informative to determine whether PLWH are more prone to have worst outcome when they become infected by SARS-CoV-2 virus or to developing LC syndrome.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by The Human Research Ethics Board (HREB) at the University of Alberta. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

SE: Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft. MR: Formal analysis, Investigation, Writing – review & editing. MO: Data curation, Funding acquisition, Resources, Writing – review & editing. SS: Data curation, Formal analysis, Investigation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Bruce James Brew,
St Vincent's Hospital Sydney, Australia

REVIEWED BY
Christina K. Psomas,
Hôpital Européen Marseille, France
Farzana Bhuyan,
National Institute of Allergy and Infectious Diseases (NIH), United States

*CORRESPONDENCE
Serena Spudich
serena.spudich@yale.edu

†These authors have contributed equally to this work and share first authorship

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Longitudinal CNS and systemic T-lymphocyte and monocyte activation before and after antiretroviral therapy beginning in primary HIV infection

Phillip Chan[1,2†], Xiang Li[1†], Fangyong Li[3], Brinda Emu[4], Richard W. Price[5] and Serena Spudich[1,2*]

[1]Department of Neurology, Yale University School of Medicine, New Haven, CT, United States, [2]Yale Center for Brain and Mind Health, Yale University School of Medicine, New Haven, CT, United States, [3]Yale Center for Analytical Sciences, Yale University School of Medicine, New Haven, CT, United States, [4]Department of Medicine, Division of Infectious Diseases, Yale School of Medicine, New Haven, CT, United States, [5]Department of Neurology, University of California, San Francisco, San Francisco, CA, United States

Background: Trafficking of immune cells to the central nervous system is hypothesized to facilitate HIV entry and immune-induced neuronal injury and is mediated by surface proteins such as chemokine receptors and α4 integrin. We longitudinally assessed immune cell activation and surface marker expression in cerebrospinal fluid (CSF) and blood and their relationship with CSF HIV RNA beginning during primary HIV infection (PHI) before and after antiretroviral therapy (ART).

Methods: Longitudinal paired blood and CSF were obtained in ART-naïve PHI (<12 month since infection) participants; some independently initiated ART during follow up. Multiparameter flow cytometry of fresh samples determined activation (% $CD38^+HLADR^+$) and chemokine receptor expression (% $CCR5^+$ and $CXCR3^+$) on $CD4^+$ and $CD8^+$ T cells, and subtype and α4 integrin expression (% and mean fluorescence intensity (mfi) of $CD49d^+$) on monocytes. HIV RNA was quantified by PCR. Analyses employed Spearman correlation, within-subject correlation, and linear mixed models.

Results: 51 participants enrolled at a median 3.2 months post HIV transmission with 168 total visits (113 pre-ART, 55 post-ART) and a median of 6.5 months of longitudinal follow up (range 0-40). In pre-ART PHI, frequencies of activated CD4+ and CD8+ T cells were much higher in CSF than in blood, with levels similar to ART-naïve people with chronic HIV infection. Both CSF CD4+ and CD8+ T cell activation increased longitudinally prior to initiation of ART. In multivariate analysis, CSF CD4+ but not CD8+ T cell activation independently predicted CSF HIV RNA. Neither CSF monocyte subtypes or α4 expression correlated with CSF HIV RNA. Blood monocyte α4 MFI correlated with CD4+ and CD8+ T cell activation ($p<0.05$). Following ART initiation, blood but not CSF T cell activation declined with days on treatment (slope=-0.06, $p=0.001$). During ART, blood and CSF monocyte α4 MFI correlated with T cell activation ($p<0.05$).

Conclusions: In untreated early infection after PHI, immune activation increases over time, and CSF CD4+ T cell activation but not monocyte activation correlates

with CSF HIV RNA. Intrathecal T cell activation does not decline during early follow up on ART. Immunomodulating therapies may be needed to prevent neuronal injury and HIV neuroinvasion during early HIV.

KEYWORDS

primary HIV infection, antiretroviral therapy, cerebrospinal fluid, T-lymphocyte, monocyte

Introduction

HIV is a multi-organ disease that involves the central nervous system (CNS) (1). Prior investigation confirms that HIV invades the CNS within days after transmission (2–4) and establishes infection of cells residing in the CNS within the first year after acquisition and that evolves throughout untreated infection (5–7). This local infection of CNS cells persists despite antiretroviral therapy (ART) (8–12). Both systemic and intrathecal immunologic responses are readily observed during Fiebig I-V acute HIV infection (AHI) (13), including immune cell activation and elevation of immune activation markers in both blood and cerebrospinal fluid (CSF) compartments (4, 14). While these findings demonstrate the onset of HIV neuropathogenesis during AHI, studies during primary HIV infection (PHI), defined as within the first year of infection following acquisition, highlight that several adverse CNS events may happen after AHI and during the early phases of infection. Sequencing studies have revealed the development of compartmentalized quasispecies in the CSF during PHI, but not AHI (5, 15–17), suggesting the establishment of local viral replication within the CNS during this period. Abnormal levels of CSF neurofilament light chain (NFL), a protein biomarker reflective of active injury to neurons, has been detected in up to 40% of CSF samples collected from untreated people with HIV (PWH) during PHI, in association with neuroimaging evidence of reduced neuronal integrity (18). Moreover, soluble markers of immune activation and blood brain barrier compromise are both elevated during early infection and increase in the first few years following initial HIV infection in the absence of treatment with ART (19, 20). Diffusion tensor imaging (DTI) further reveals that white matter and grey matter structural changes manifest in the brain during PHI (21, 22) and that regional volumes in the brain decrease over time with duration of infection after PHI (23). These findings suggest that PHI is a highly evolutionary period of HIV neuropathogenesis, during which timely ART initiation may alter the trajectory of neurological outcomes.

It remains unclear how immune cell states in the blood and CSF compartments evolve and contribute to CNS HIV infection during PHI prior to and after ART. These include cells that are known to be pathogenically important in HIV, such as CD4+ T-cells, monocytes, and cytotoxic CD8+ T-cells. The former two are susceptible to HIV infection and contribute to HIV replication, while the latter are responsible for limiting HIV replication and at the same time associating with adverse inflammation. Recent studies have suggested that CD4+ T cells may be target cells of infection not only in the periphery but also in the CNS during acute HIV, and may importantly contribute to establishment and maintenance of CNS HIV reservoirs (24–27). To date, evaluating the natural course of immunological changes during PHI and early HIV infection has posed significant challenges. First, identifying HIV infection within the first year after transmission is difficult, as PWH are mostly asymptomatic during the period. Second, as immediate ART upon HIV diagnosis provides the best outcomes for PWH (28), observational studies deferring ART initiation are no longer feasible.

Leveraging a prospective study of paired blood and CSF samples collected before guidelines for implementation of immediate ART, this analysis aimed to examine longitudinal changes in CD4+ and CD8+ T-cell and monocyte phenotypes in the respective compartments beginning during PHI both prior to and following ART initiation. Comparison participants included cross-sectionally collected paired samples from pre-ART PWH with chronic HIV infection (CHI, defined as known HIV diagnosis for at least three years) and people without HIV (PWoH). Laboratory evaluations on paired samples included measurements of activated CD4+ and CD8 + T-cells frequency, monocyte subpopulation composition, and alpha-4 (α4) integrin expression in monocytes, which signifies monocytes' potential trafficking capacity across compartments via cell adhesion and transmigration. Finally, potential correlations between T-cell activation and monocyte subpopulations, both before and after ART initiation, were explored.

Materials and methods

Study design and participants

PHI study participants were enrolled in the Primary Infection Stage CNS Events Study (PISCES) cohort study conducted in San Francisco between 2005 and 2014, including 51 pre-ART PHI participants and 11 age and gender matched PWoH. In addition, data collected using identical methods from 32 initially pre-ART CHI participants enrolled in a separate observational study in San Francisco was included for comparison. In most participants, PHI

was confirmed by documentation of a negative HIV test within the previous 12 months followed by a positive test, where estimated date of infection was designated 14 days prior to the onset of an acute retroviral syndrome, or in asymptomatic participants as the halfway point between the last negative and first positive test. In a minority of participants, recent infection was confirmed by a participant reporting a recent exposure, an acute retroviral like syndrome, and evidence of infection within the past six months by a diluted (less sensitive) HIV antibody test, as described in previous reports (20, 29). All PHI participants were ART-naïve at baseline and were followed at 6 weeks, and then every 6 months thereafter. As PISCES was established before guidelines recommended immediate ART initiation for all PWH regardless of CD4+ T-cell levels, the timing of ART initiation among PHI participants varied individually, depending on decisions made by the participants and their clinicians. The settings resulted in the collection of serial CSF samples from PHI participants both before and after ART. Compared to longitudinal CSF samplings among participants with PHI, CHI and PWoH participants served as study controls and underwent cross-sectional CSF sampling (29). Informed consent was obtained from all participants. The PISCES study protocol was approved by the University of California San Francisco Committee on Human Research (H9133-26278).

Sample collection & laboratory procedures

Paired blood and CSF samples were obtained from all study participants at baseline, and from PHI participants during follow-up visits. Blood CD4+ and CD8+ T-cell count, CSF white blood cell (WBC) count, protein and albumin were measured using fresh samples. Blood and CSF HIV-1 RNA were measured in previously frozen (-70°C) cell free samples, using the ultrasensitive Amplicor HIV Monitor (version 1.5; Roche Molecular Diagnostic Systems, Branchburg, NJ) assay (29).

Flow cytometry

Paired blood and CSF samples were prepared as previously described (30, 31). Briefly, multiparameter flow cytometry was performed on fresh samples of whole blood and the cellular component of CSF (separated by centrifugation from the cell free fraction) to assess the percentage of activated $CD4^+$ and $CD8^+$ T-cells in the samples, based on CD38 and HLA-DR co-expression (32). Blood and CSF monocytes were classified by CD14 and CD16 expression, while expression of α4 integrin was measured as the percentage of $CD49d^+$ monocytes and the mean/median fluorescence intensity (MFI) of CD49d staining.

Since low monocyte percentages in CSF precluded splitting CSF cell pellet samples, we chose to examine a subset of samples for T-cell flow cytometry markers including analysis of CCR5+ and CXCR3+ expression in T-cells (Panel 1), and in 2008, flow cytometry antibody-dye panels were switched in order to study monocyte markers including expression of α4 integrin in monocytes (Panel 2). Participants already enrolled by 2008 continued to have longitudinal samples analyzed using Panel 1 while newly enrolling participants in 2008 and thereafter had samples analyzed using Panel 2 (Supplementary Tables S1, S2). Comparison of the 2 panels showed differences in CSF CD4+ T-cell activation and CSF monocyte activation, hence data from the two panels were analyzed separately throughout this study. Panel 1 data was used for baseline and longitudinal analyses of T-cells. Panel 2 data was used for analyses of monocytes and for comparison of monocyte alpha-4 integrin expression with T-cell activation.

Monoclonal antibodies included CD3, CD4, CD8, CD38, HLA-DR, CCR5, CXCR3, CD45, CD14, CD16, and CD49d conjugated to allophycocyanin (APC), phycoerythrin (PE) peridinin chlorophyll protein (PerCP), fluorescein isothiocyanate (FITC), and tandem conjugations with cyanine (ACP-Cy7, PE-Cy7) and Texas Red (PE-Texas Red). Blood samples were stained with fluorescence-minus-one controls in which one antibody was omitted; an unstained control and single-stained samples were also prepared as compensation controls. Samples were run on a FACS DIVA (BD Biosciences, San Jose, CA) and flow cytometry data analyzed with FlowJo (TreeStar, Ashland, OR).

Statistical analyses

Participant characteristics were summarized as frequency and percentage, or median and interquartile range (IQR) as appropriate. Chi-square test for categorical variables or Kruskal-Wallis test with *post hoc* testing using Dunn's multiple comparisons for continuous variables was performed to compare groups. Longitudinal data was analyzed using linear mixed models, which accounts for correlations among repeated assessments within same individual using the effect of random intercept. Further, Sobel's test was used for mediation analysis, to examine whether the increase in CSF CD8+ cells from the increase of CSF HIV RNA level was medicated by the increase of CSF CD4+ cells. Statistics were performed and graphics generated using IBM SPSS Statistics 25 (IBM, Armonk, NY) or Prism 7 (GraphPad Software Inc, La Jolla, CA). Statistical significance was $p<0.05$, two-sided.

Results

Participant characteristics

All 51 PHI participants were male, with a median age of 36 (IQR 31-45) years at study baseline (i.e., 1st CSF sampling, pre-ART). Please see Table 1 for demographic information. The estimated duration of HIV infection was 3.2 (IQR 2.4-5.6) months. At baseline, plasma and CSF HIV RNA were 4.37 (IQR 3.80-4.86) and 2.31 (IQR 1.69-3.10) log_{10}cps/ml, while blood CD4+ and CD8+ T-cell levels were 581 (IQR 429-738) and 985 (IQR 691-1336) cells/μl.

PHI participants were longitudinally followed for a median duration of 6.5 (IQR 0-22.7) months. Including the baseline visit,

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TABLE 1 Characteristics of primary infection participants.

	Primary HIV (n=51)
Age (years)	36 (31, 45)
Male, n (%)	51 (100)
Baseline Visit	
Estimated time post HIV transmission (months)	3.2 (2.4, 5.6)
CD4+ count (cells/μl)	581 (429, 738)
CD8+ count (cells/μl)	985 (691, 1336)
Plasma HIV RNA (log_{10} copies/ml)	4.37 (3.80, 4.86)
CSF HIV RNA (log_{10} copies/ml)	2.31 (1.69, 3.10)
CSF WBC count (cells/mm^3)	6 (2, 11)
CSF protein (mg/dL)	41.0 (35.5, 51.3)
Longitudinal Analysis	
Follow-up duration (months)	6.5 (0, 22.7)
Total number of study visits	168
Sample included in longitudinal Analysis	
Without ART	113
With ART initiation	55
Estimated duration of HIV infection at ART initiation (months)	6.2 (2.1, 16.0)

Median (interquartile range) shown unless otherwise indicated.

they contributed to 168 visits with paired blood and CSF sampling. Two longitudinal analyses were constructed according to their ART status. The first one examined the immunological changes based on samples collected from PHI participants without ART initiation. The analysis included 113 paired samples, including 21 PHI participants who underwent repeated CSF sampling. The second longitudinal analysis examined the immunological changes after ART based on 55 paired samples. In the analysis, all baseline samples were collected pre-ART, whereas all subsequent samples were collected post-ART, contributed by 11 PHI participants. Of note, all post-ART samples donors achieved and maintained plasma HIV suppression during longitudinal sample collection.

T-cell activation in blood and CSF samples at baseline

Figure 1 compares the percentages of activated $CD4^+$ and $CD8^+$ T-cells in blood and CSF samples across the three participant groups: pre-ART PHI at baseline, pre-ART CHI and PWoH. In blood, both PHI and CHI participants exhibited higher percentages of activated CD4+ and CD8+ T-cell than PWoH ($p<0.001$). Percentages of activated CD4+ and CD8+ T-cells in blood did not differ statistically between PHI and CHI participants.

In CSF, CHI but not PHI participants demonstrated greater percentages of activated CD4+ and CD8+ T-cell than PWoH ($p=0.02$). However, percentages of activated CD4+ and CD8+ T-cells in CSF did not differ statistically between PHI and CHI participants. We interpret these data as suggesting that PHI participants had levels of T-cells activation intermediate between those of CHI and PWoH.

Monocyte subpopulation and α4 integrin expression in blood and CSF samples at baseline

Figure 2 displays the monocyte subpopulations in blood and CSF samples across the three participant groups, revealing no significant differences in the percentages of non-classical (CD14dim CD16+), intermediate (CD14+ CD16+), and classical (CD14+ CD16-) monocytes in either blood and CSF between groups. However, α4 integrin expression in monocytes, as determined by the percentage of monocytes expressing α4 integrin and MFI-based α4 integrin expression intensity differed by HIV status (Figure 3). Specifically, compared to PWoH, PHI and CHI participants exhibited higher α4 integrin MFI in blood monocytes ($p=0.001$ and $p=0.005$) and a higher percentage of α4 integrin expression in CSF monocytes ($p=0.008$ and $p=0.03$).

Longitudinal immunological changes in blood and CSF samples in untreated PHI participants

CD4+ and CD8+ T-cell activation

Figure 4 illustrates longitudinal percentage changes in activated CD4+ and CD8+ T-cells in blood and CSF samples, based on 113 follow-up samples collected from 21 PHI participants who had not initiated ART during the period. In the blood, activated CD4+ T-cells increased by 0.03% per week ($p=0.01$), while activated CD8+ T-cells did not show a significant increase ($p=0.34$). In CSF, activated CD4+ T-cells increased by 0.10% per week longitudinally ($p=0.005$), while activated CD8+ T-cells increased by 0.11% per week ($p=0.005$). These findings highlight a greater increase in activated CD4+ and CD8+ T-cells in the CSF compared to plasma during this period. Additionally, activated T-cells were more frequently observed in the CSF than in plasma, up to a three-fold difference.

Monocyte subpopulation and α4 integrin expression

Compared to the significant increases in the percentages of activated CD4+ and CD8+ T-cells in blood and CSF samples, mixed model analysis did not reveal significant changes in the composition of intermediate, classical, and non-classical monocyte subpopulations in either sample over time. Furthermore, metrics of α4 integrin expression in blood and CSF monocytes, whether by expression percentage or by MFI, did not statistically differ over time.

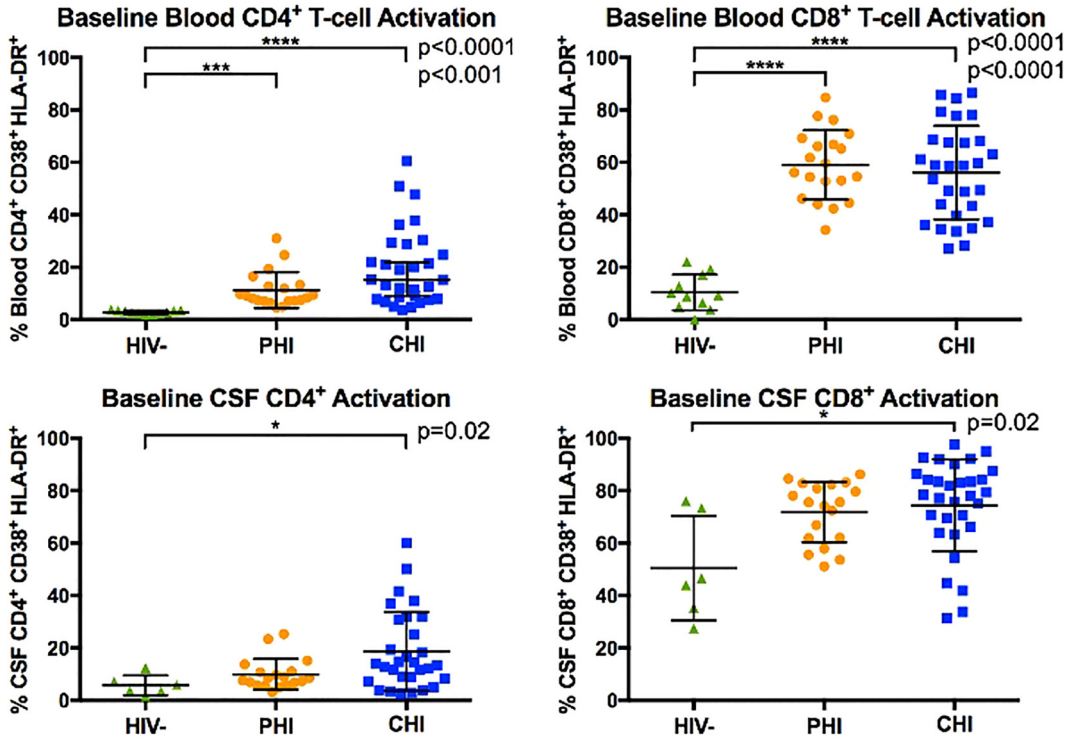

FIGURE 1
Comparison of blood and CSF CD4$^+$ and CD8$^+$ T-cell activation (% CD38$^+$ HLA-DR$^+$) between HIV-negative, PHI, and CHI participants at baseline visit. Horizontal line indicates the group mean and error bars indicate standard deviation. * p< 0.05, *** p< 0.001, **** p< 0.0001.

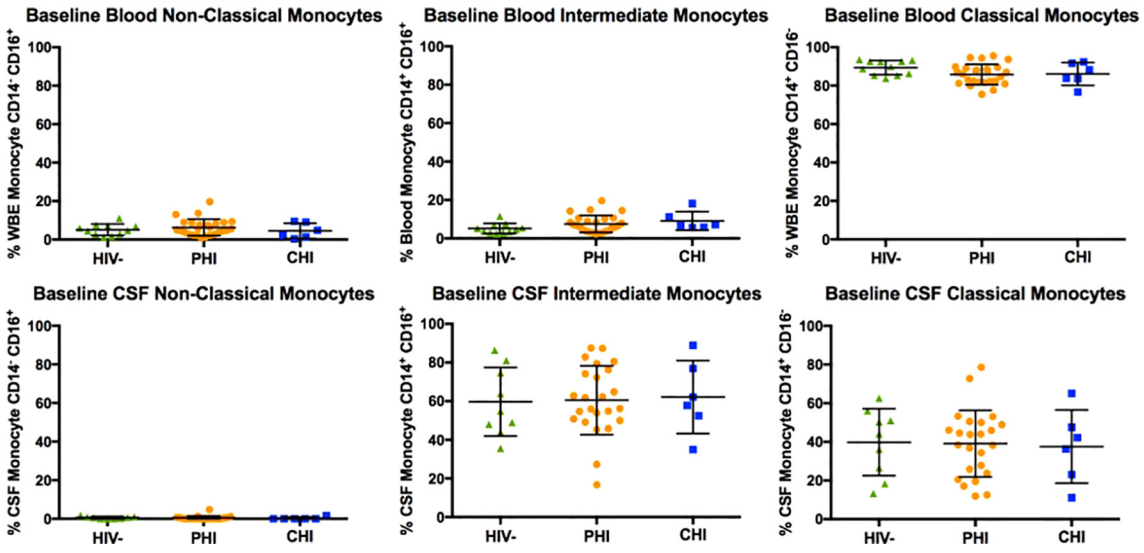

FIGURE 2
Baseline comparison of non-classical (CD14^{dim} CD16$^+$), intermediate (CD14$^+$ CD16$^+$), and classical (CD14$^+$ CD16$^-$) monocyte subtypes in blood and CSF of HIV-negative, PHI, and CHI participants.

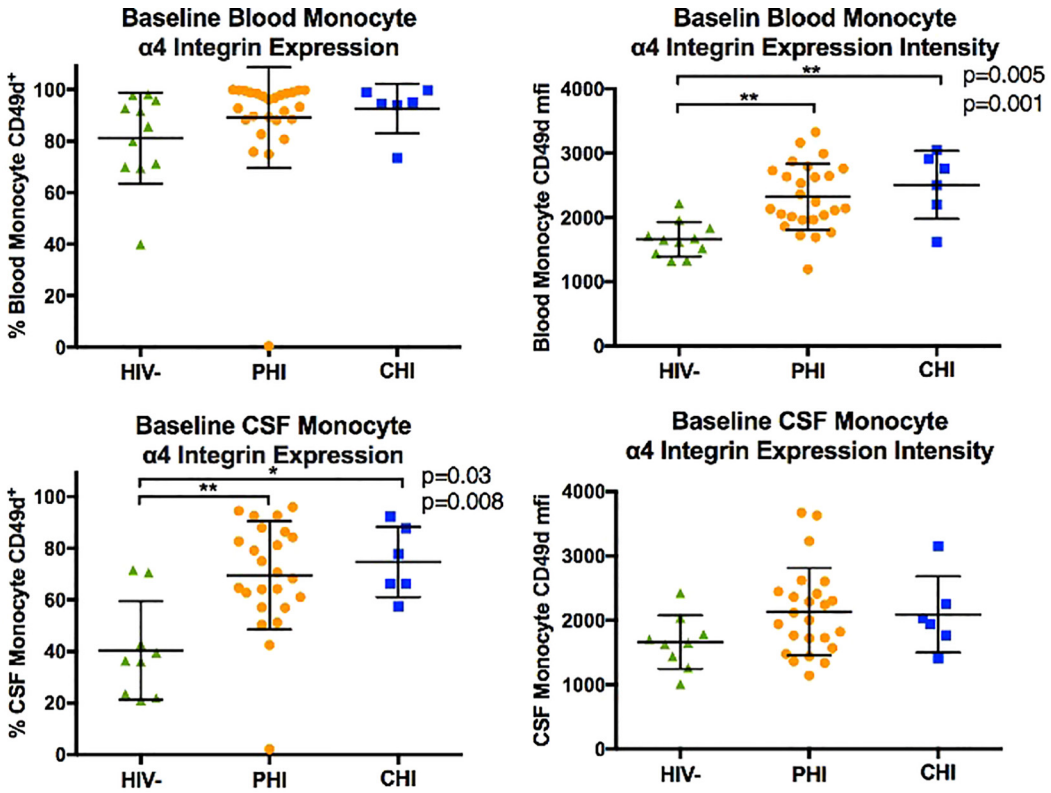

FIGURE 3
Comparison of monocyte α4 integrin expression (in % CD49d$^+$ monocytes and CD49d mean fluorescence intensity, MFI) in blood and CSF of HIV-negative, PHI, and CHI participants at baseline. * p< 0.05, ** p< 0.01.

Longitudinal immunological changes in blood and CSF samples following ART initiation

CD4+ and CD8+ T-cell activation

Figure 5 illustrates the longitudinal changes in CD4+ and CD8+ T-cell activation in blood and CSF samples following ART initiation, based on 55 paired samples from 11 PHI participants. Following ART initiation, activated CD4+ T-cell level did not significantly decline in blood (p=0.08), while activated CD8+ T-cell level declined at a rate of 0.42% per week (p<0.001). No statistically significant decline in activated CD4+ or CD8+ T-cells was observed in CSF samples after ART.

Monocyte subpopulation and α4 integrin expression

Following ART initiation, neither blood nor CSF samples exhibited a significant change in the composition of monocyte subpopulations. Additionally, α4 integrin expression in monocytes in blood and CSF remained generally unchanged, except a reduction in MFI-based α4 integrin expression intensity in blood monocytes over time (p=0.03).

Correlations between HIV RNA, T-cell activation and monocyte subpopulations in CSF

Potential association between CSF HIV RNA, CD4+ and CD8+ T-cell activation and metrics of monocytes in the CSF samples were explored using a mixed model longitudinal data analysis (Table 2). Before ART initiation, percentages of activated CD4$^+$ T-cells (p<0.001) and activated CD8$^+$ T-cell (p=0.013) in CSF correlated with CSF HIV RNA levels in univariate analyses. In multivariate analyses, CSF CD4+ T-cell activation (p=0.005), but not CD8+ T-cell activation (p=0.45), remained independently associated with CSF HIV RNA, demonstrating significant mediation effects that explained 69% of the association in the predictive model (Supplementary Figure S1). In contrast, neither the percentages of monocyte subpopulations nor the metrics of α4 integrin expression in CSF correlated with CSF HIV RNA pre-ART in the univariate analysis (not shown).

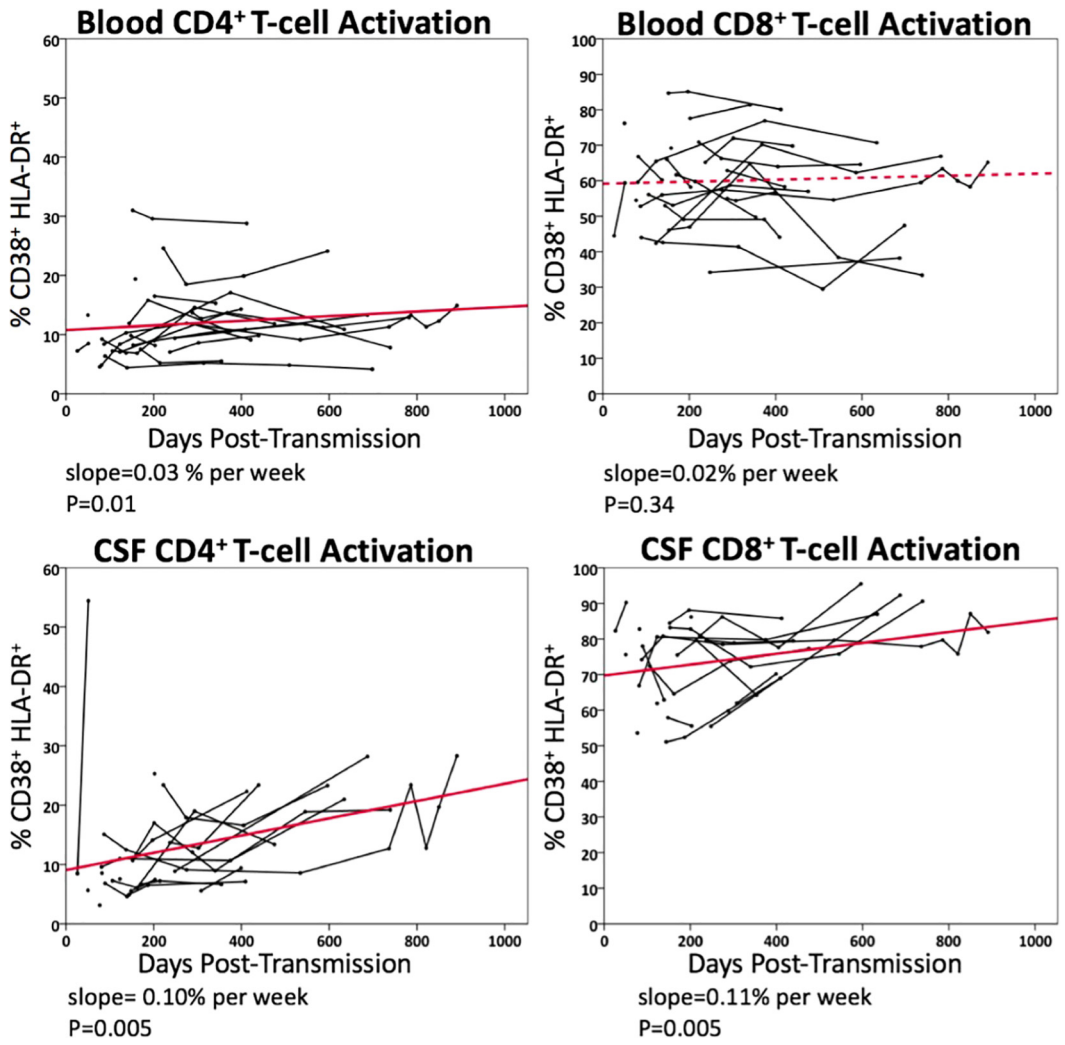

FIGURE 4
Longitudinal changes in blood and CSF CD4$^+$ and CD8$^+$ T-cell activation (% CD38$^+$HLA-DR$^+$) in untreated PHI participants (n=21). Data points from the same participant over time are connected. Solid line represents significant regression slope and dashed line non-significant regression slope.

Correlations between T-cell activation and measures of monocytes before and after ART initiation

Potential correlations between CD4+ and CD8+ T-cell activation and metrics of monocytes in the blood and CSF samples were explored in pre-ART and post-ART settings. Prior to ART initiation, correlations between monocyte metrics and T-cell activation were generally absent in both blood and CSF samples, except positive but modest correlations between monocyte α4 integrin MFI and the frequencies of activated CD4+ T-cells (b=0.004, p=0.02) and activated CD8+ T-cells (b=0.008, p=0.01) in the blood (Supplementary Table S3).

In blood, following ART initiation, the frequency of intermediate monocytes increased with the percentages of activated CD4+ T-cells (b=0.22, p=0.02) and CD8+ T-cells (b=1.4, p<0.001), while the frequency of classical monocytes was negatively associated with the percentages of CD4+ T-cells (b=-0.19, p=0.03) and CD8+ T-cells (b=-1.14, p=0.001) (Supplementary Table S4). Additionally, the frequency of α4 integrin expression and α4 integrin MFI in monocytes exhibited opposite directions of correlation with CD4+ and CD8+ T-cells activation in blood. The former was negatively associated with the frequencies of activated CD4+ T-cells (b=-0.21, p=0.007) and CD8 T-cells (b=-0.69, p=0.02), while the latter was positively associated with the frequencies of activated CD4+ T-cells (b=0.005, p=0.01) and CD8 + T-cells (b=0.018, p=0.01). In CSF, none of the monocyte parameters were associated with T-cell activation, except a positive and modest correlation between α4 integrin MFI and frequencies of activated CD4+ T-cells (b=0.014, p=0.005).

Discussion

Leveraging a longitudinal collection of paired blood and CSF samples since PHI diagnosis, this study aimed to investigate the evolution of immune cell activation in blood and CSF compartments during early HIV infection, before and after ART

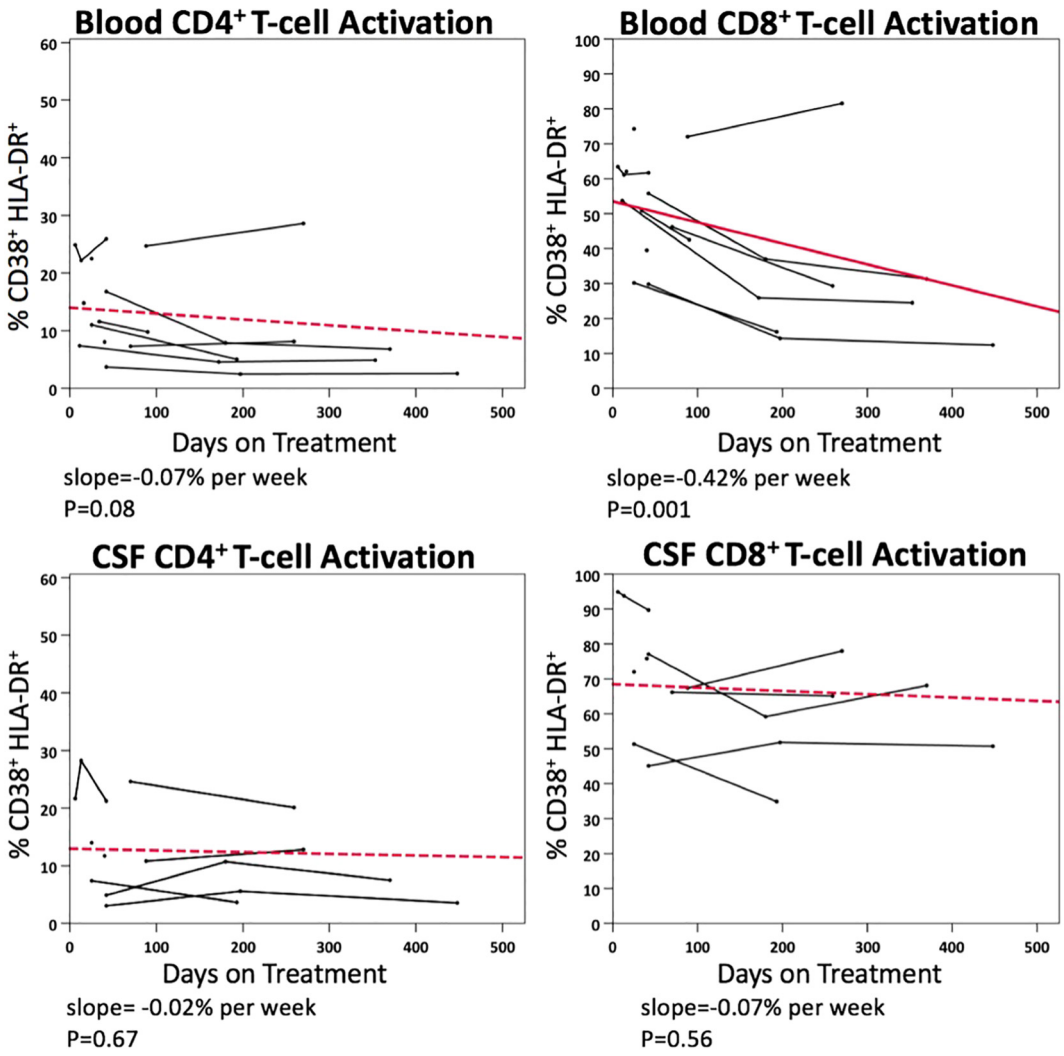

FIGURE 5
Longitudinal changes in blood and CSF CD4+ and CD8+ T-cell activation (% $CD38^+$ $HLA\text{-}DR^+$) in PHI participants (n=11) after ART.

initiation. Previous studies suggest that immune activation in the CSF compartment occurs following HIV CNS invasion, defined as detection of HIV RNA in CSF. In one study, CSF pleocytosis occurred following the detection of HIV RNA in CSF (33). Another study reported a significant increase in activated CD8+ T-cells in CSF during Fiebig stage III of AHI, though no statistical difference was found between the percentages of activated CD8+ T-cells in CSF from Fiebig stages I-II of AHI and PWoH (14). Importantly, while there was a notable rise in the percentage of activated CD8+ T-cells in CSF during Fiebig stage III of AHI, the level of activated CD8+ T-cells remained lower than those seen in PWH with untreated CHI (14).

T-cell activation in PHI before and after antiretroviral therapy during PHI

In the current study, measurements of activated CD4+ and CD8+ T-cells in the paired samples revealed similar frequencies of activated CD4+ and CD8+ T-cells in blood between untreated PHI and CHI participants. However, levels of CD4+ and CD8+ T-cell activation in CSF during PHI were intermediate between those of CHI and PWoH, without reaching statistical significance compared to either group. During longitudinal follow-up prior to initiation of ART, PHI participants exhibited progressive increases in activated CD4+ and CD8+ T-cells in the CSF, but not in the blood. These findings align with published studies indicating that T-cell activation in the CSF is relatively delayed compared to activation in the systemic circulation, and data from this cohort that soluble immune activation markers continue to increase over time during early HIV untreated infection (20).

In this study, following ART initiation, levels of T-cell activation declined in both blood and CSF compartments. However, only the decline of activated CD8+ T-cell in blood reached statistical significance, whereas the corresponding decline in CSF was relatively modest (0.42% vs 0.07% per week). While the lack of significant change could be related to small sample size, the findings may indicate that T-cell activation in the CSF, and potentially

TABLE 2 CSF CD4+ and CD8+ T-cell activation (% $CD38^+$ $HLA\text{-}DR^+$) as univariate and multivariate predictors of CSF viral load (log_{10} CSF HIV RNA).

Univariate Analysis			
Variable 1	Variable 2	Regression Coefficient	p-value
CSF $CD4^+$ T-cell activation	CSF HIV RNA (log_{10} copies/mL)	0.0436	0.0003
CSF $CD8^+$ T-cell activation	CSF HIV RNA (log_{10} copies/mL)	0.0274	0.013
CSF $CD8^+$ T-cell activation	CSF $CD4^+$ T-cell activation	0.496	<0.0001
Multivariate Analysis			
Variable 1	Variable 2	Regression Coefficient	p-value
CSF $CD4^+$ T-cell activation	CSF HIV RNA (log_{10} copies/mL)	0.0383	0.005
CSF $CD8^+$ T-cell activation		0.0088	0.45

*T-cell activation = % $CD38^+$ $HLA\text{-}DR^+$

intracerebral inflammation, requires a prolonged duration to normalize if it ever reaches levels comparable to those in PWoH. In ART-naïve PWH with CHI, especially in those with advanced immunodeficiency and HIV encephalitis, a protracted course of decay in HIV RNA in the CSF compared to blood is frequently observed (34), potentially contributing to a slower course of decline in activated CD8+ T-cells in the CSF. However, this phenomenon would not be applicable to the current study, as all PHI participants commenced ART before the development of advanced immunodeficiency or overt neurological manifestations.

Monocyte subpopulation and α4 integrin expression in PHI before and after antiretroviral therapy

Monocytes may be susceptible to HIV infection and contribute to long-term viral reservoirs as tissue macrophages (35). Monocytes can be classified into three subtypes: $CD14^{dim}CD16^+$ non-classical monocytes, $CD14^+CD16^+$ intermediate monocytes, and $CD14^+$ $CD16^-$ classical monocytes. In PWH, various monocytes measures have been associated with adverse CNS outcomes. For instance, higher levels of circulating intermediate monocytes and intact HIV reservoir in monocytes in blood were associated with worse cognitive function in virally-suppressed women with HIV (36, 37), whereas non-classical monocyte levels in blood were negatively correlated with cerebral small vessel disease and cognitive performance (38). Additionally, monocyte activation markers, such as sCD14 and sCD163, are frequently elevated in PWH with cognitive impairment compared to those without (39).

Prior studies of AHI and PHI have highlighted elevations of monocyte activation markers in blood and CSF shortly after HIV acquisition (3, 4). However, there has been less investigation into changes in monocyte subpopulations and their expression of trafficking markers in both compartments. α4 integrin, also known as very late antigen 4 (VLA-4) or CD49d, is a subunit of the transmembrane integrin protein that mediates immune cell adhesion to endothelial cells, facilitating their extravasation from the blood into other tissue compartments including the CNS (40, 41). In a prior study, application of anti-α4 antibody that blocks monocyte/macrophage trafficking to the brain and gut in late Simian immunodeficiency virus (SIV) infection reduced and stabilized neuronal injury in non-human primate model (42). However, the potential benefit of alpha-4 blocker on direct virologic control in the SIV model remains unclear (43, 44).

In the current study, the composition of monocyte subpopulations did not differ by HIV status in either compartment when assessed cross-sectionally. Additionally, no significant changes in the composition of monocyte subpopulations were observed in blood and CSF samples during the longitudinal follow-up of pre-ART PHI participants. The findings contrast with a previous study that investigated the composition of blood monocyte subpopulations in PWH. That study reported elevated levels of intermediate monocytes and reduced levels of classical monocytes in blood samples from pre-ART PWH with Fiebig stages III-V AHI and CHI (45). The findings from that study suggest that similar changes are likely to occur during PHI, as it is chronologically positioned between AHI and CHI. To date, the understanding on the impacts of HIV on compartmental monocyte subpopulations remains limited, with challenges including the confounding effects from co-infections and chronic conditions. For instance, syphilis infection (46) and the use of methamphetamine (47), which are not uncommon in PWH, can individually affect the composition of monocyte subpopulations in the blood. Another study reported differing composition of monocyte subpopulations in blood and CSF between HIV-subtypes (48).

Compared to monocyte subpopulation composition, the expression of α4 integrin in monocytes differed between PWH and PWoH. Pre-ART PHI and CHI participants showed higher levels of α4 integrin MFI in blood monocytes and a greater percentage of α4 integrin expression in CSF monocytes than PWoH. Moreover, this elevated α4 integrin expression in blood and CSF monocytes persisted over time in PHI participants, though without significant progression. Our exploratory analysis further suggests that α4 integrin expression in monocytes is associated with T-cell activation, particularly in the blood compartment and after ART initiation. Future studies should investigate the role of α4 integrin expression in monocytes in residual immune activation in PWH on stable ART.

This study has limitations. The longitudinal analysis was based on a relatively small sample size of paired blood and CSF samples from people with PHI, with variation in the timing of ART initiation. In addition, longitudinal sampling of participants with CHI was not included, limiting our ability to compare the impact of early versus late ART initiation on T-cell activation and monocyte subpopulations. The duration of HIV infection was estimated through combined sequential HIV-related serological changes and clinical history acquisition, however accurate estimation is often challenging and exact durations are not available. The impacts of other potential modifiers on study outcomes in the participants who started ART, such as CNS-penetrating efficacy of ART and the

degree of immune recovery (e.g., individual improvement in CD4+ T-cell counts), were not determined because of the small sample size in this category (n=11). Finally, this analysis did not assess associations between cellular immune measures and clinical outcomes such as neuropsychological performance or daily function.

Conclusions

This study of paired CSF and blood samples from young male participants initially studied in the first year of HIV acquisition and followed longitudinally pre and post-ART demonstrates a compartmentalized immune response in the CNS as compared to blood throughout early infection and treatment. While we observed a delay in emergence of T-cell activation in the CNS compartment compared to during untreated PHI, overall we found a higher frequency of CD8+ T cell activation in the CSF, and increasing frequencies of CD4+ and CD8+ T cell activation in the CSF during early infection prior to ART. This did not contemporaneously follow responses in blood, where CD4+ T cell activation but not CD8+ T cell activation mildly increased during this period. Our study also observed a slower decline of CD8+ T-cell activation in the CSF compared to blood after ART initiation, indicating the presence of persistent intrathecal immune activation despite ART initiation during early stages of HIV infection. Finally, frequency of CSF CD4+ T-cell activation but not CD8 + T-cell activation or monocyte sub-phenotypes was independently associated with CSF HIV RNA prior to ART, suggesting that in the early stages of infection, T cell infection is an important determinant of viral replication within the CNS. Compartmentalized cellular CNS immune activation occurs and progresses during early HIV infection and is not immediately ameliorated by ART.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by The University of California San Francisco Committee on Human Research (H9133-26278). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

PC: Formal Analysis, Visualization, Writing – original draft, Writing – review & editing. XL: Formal Analysis, Visualization, Writing – original draft, Writing – review & editing. FL: Data curation, Formal Analysis, Methodology, Supervision, Visualization, Writing – original draft. BE: Data curation, Formal Analysis, Investigation, Supervision, Writing – review & editing. RP: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – review & editing. SS: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Robyn Klein,
University of Western Ontario, Canada

REVIEWED BY
Felix Ngosa Toka,
Ross University School of Veterinary Medicine, Saint Kitts and Nevis
Abraam Yakoub,
Harvard Medical School, United States

*CORRESPONDENCE
Jun Xu
neurojun@126.com

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Underestimated virus impaired cognition-more evidence and more work to do

Maher Un Nisa Awan[1], Faisal Mahmood[2], Xiao-bin Peng[1,3], Fenshuang Zheng[4] and Jun Xu[1,5]*

[1]Department of Neurology, The Affiliated Hospital of Yunnan University, Kunming, China, [2]Central Laboratory, Liver Disease Research Center and Department of Infectious Disease, The Affiliated Hospital of Yunnan University, Kunming, China, [3]School of Medicine, Yunnan University, Kunming, Yunnan, China, [4]Department of Emergency, The Affiliated hospital of Yunnan University, Kunming, China, [5]Department of Neurology, Beijing Tiantan Hospital, Capital Medical University, Beijing, China

Neurodegenerative disorders (NDs) are chronic neurological diseases that can be of idiopathic, genetic, or potentially infectious origin. Although the exact cause of neurodegeneration is unknown, it might be result of a confluence of age, genetic susceptibility factors, and environmental stresses. The blood-brain barrier shields the brain from the majority of viral infections, however neurotropic viruses are able to breach this barrier and infect central nervous system. Growing research points to a possible connection between viruses and neurodegenerative diseases, indicating that virus-induced neuroinflammation and disruption of neuronal protein quality control may play a role in the initial stages of disease progression. The diagnosis and treatment of NDs are urgent and challenging. Even though there is limited clinical evidence to support the use of antiviral medications and their dose regimens within the central nervous system (CNS), with the exception of acyclovir, they are currently utilized to treat various viral CNS infections. Understanding the neuropathogenesis of viral CNS infection may help with targeted diagnosis and treatment plans by focusing on the molecular mechanisms of the CNS. It may also be helpful in the search for new antiviral drugs, which are crucial for better managing these neurotropic viral infections. This review focuses on new findings linking viral infection to NDs and explores how viral modifications of cellular functions can impact the development of neurodegeneration and will also explore the therapeutic potential of antiviral drugs in NDs.

KEYWORDS

neurodegeneration, virus, antiviral drugs, impaired cognition, dementia

Introduction

Neurodegenerative diseases (NDs) are chronic degenerative disorders of the central nervous system (CNS) that are characterized by the chronic and progressive loss of the structure and function of neurons (1). Millions of people worldwide are impacted by them, making them the fourth most common cause of mortality in developed nations.

Furthermore, their influence is growing in developing countries. With an increasing lifespan, it is expected that the incidence rate will rise. Even with extensive investigation, most NDs' basic root causes are still poorly understood (1, 2). Numerous intracellular mechanisms, such as apoptosis, inefficient axonal transport, mitochondrial malfunction, and protein degradation, are linked to neurodegenerative diseases (3). The etiology of numerous neurodegenerative illnesses has also been linked to long-term viral infections, malnutrition, exposure to heavy metals in the environment, autoimmune reactions, vascular disorders, head trauma, brain fluid buildup, and alterations in neurotransmitter concentrations (2, 4, 5). Viral infections can infiltrate the immune system and other organ systems, resulting in a variety of symptoms (6).

The majority of NDs have a pathogenic connection to the accumulation and aggregation of cellular proteins (7, 8). Notably, dementia with Lewy bodies, multiple systems atrophy (MSA), and Parkinson's disease (PD) have all been associated with α-synuclein (α-syn) aggregates (9). Alzheimer's disease (AD) patients also have extracellular amyloid-β (Aβ) plaques and intraneuronal tangles of hyperphosphorylated tau in their brains (10). Like prions, these pathogenic proteins can aggregate and form pathogenic plaques, which leads to the eventual development of NDs (11, 12). A significant contributing component to these processes is an imbalance in the cellular mechanisms that control the creation of misfolded proteins and their breakdown, or protein homeostasis (13). The potential for viral infections to significantly disrupt protein homeostasis makes cells more vulnerable to protein misfolding (14). Moreover, maintaining protein homeostasis may benefit from the release of pro-inflammatory cytokines and chemokines in response to a virus (15). Up-regulation of pro-inflammatory cytokines plays a dual role in neurodegeneration and neuroprotection. Activated microglia can cause harm by releasing pro-inflammatory cytokines such IL-1β, IL-6, and TNF-α, which affect surrounding brain tissue.

Therefore, it is believed that viruses, particularly neurotropic viruses, play a part in the genesis of various NDs. Table 1 lists the several viruses that are believed to be involved in NDs.

Viruses in neurodegeneration

It is likely that aging, genetic vulnerability, and environmental stressors all play a part in this process, even if the precise etiological reasons of NDs are still not entirely understood. There is mounting evidence that suggests viral infections, especially neurotropic viruses, may play a factor in the onset and progression of depressions that are not diagnosed. The progressive loss of cognitive, motor, and behavioral abilities is a hallmark of neurodegenerative illnesses like AD, PD, and amyotrophic lateral sclerosis (ALS) (53). Despite early assumptions that neuroinflammation results from neurodegeneration, further studies have demonstrated that neuroinflammation can both cause and accelerate the development of NDs. The hypothesis that neuroinflammation causes neurodegeneration was reinforced by genome-wide association studies (GWAS) that identified immune-related genes, including as CD33 and TREM2, as risk factors for AD (54). Additionally, it has been suggested that neuroinflammatory processes are largely influenced by the ε4 allele of the apolipoprotein E gene (APOE ε4), which is the most powerful genetic risk factor for AD and accounts for around 10–20% of the risk of late-onset illness (55). These genetic factors increase the risk of NDs, but they are not sufficient to cause the condition on their own. There is increasing evidence that viruses and neurodegenerative illnesses are associated (56–59). Virus-induced neuroinflammation and disruption of neuronal protein quality control may also be involved in the early phases of illness development (60). Viruses can begin and/or aggravate degenerative processes because they have the capacity to take over the host cell's internal machinery and induce inflammation. Viral infections can stimulate astrocytes and microglia or allow peripheral immune cells to invade the central nervous system, which can result in neuroinflammation (61). Certain viruses can disrupt neuronal activities, cause neuronal death, or trigger lytic egress from infected neurons, all of which can lead to neurodegeneration. Numerous negative outcomes are brought on by CNS viral infections, such as elevated morbidity and mortality as well as mild to severe neurological aftereffects, shown in Figure 1. Viral infections have a wide range of impact on neuronal dysfunction, including promoting chronic inflammation, inducing cellular oxidative stress, impairing mitophagy, interfering with mitochondrial dynamics, enhancing metabolic rewiring, altering neurotransmitter systems, and inducing misfolded and aggregated pathological proteins linked to neurodegenerative diseases. These pathogenetic processes cause a multifaceted brain injury that results in neuronal and brain dysfunctions. By interfering with the immune system, it can either directly or indirectly induce encephalitis (62). Neurotropic viral infections have an impact on a multitude of factors related to neuronal dysfunction. These include the induction of misfolded and aggregated pathological proteins linked to neurodegenerative diseases, the promotion of chronic inflammation, the induction of cellular oxidative stress, the impairment of mitophagy, the interaction with mitochondrial dynamics, the enhancement of metabolic rewiring, the modification of neurotransmitter systems (63). A complex brain injury brought on by these pathogenetic mechanisms gives rise to specific brain and neuronal dysfunction (64). Understanding the molecular mechanisms behind the neurophathogenesis associated with viral infection-induced neurodegeneration could lead to the development of efficient prophylactic, therapeutic, and preventive measures against CNS virus infections.

Molecular mechanisms associated with viral infection-related neurodegeneration

Viruses can directly cause neuronal dysfunction through their cytolytic effects, and they can also indirectly cause neuronal degeneration through a variety of mechanisms, including the

TABLE 1 Viruses in Neurodegeneration.

Neurodegenerative disorders (NDs)	Virus	References
Parkinson's disease (PD)	Coxsackievirus B3 (CVB3)	(16, 17)
	Human Immunodeficiency Virus (HIV)	(18)
	Influenza A virus (IAV)	(19, 20)
	West Nile Virus (WNV)	(21, 22)
	Western equine virus (WEV)	(23, 24)
	Hepatitis C virus (HCV)	(25–28)
	Hepatitis B virus (HBV)	(28, 29)
	Japanese encephalitis virus (JEV)	(30, 31)
	Herpes simplex virus (HSV)	(32)
	Varicella-Zoster Virus (VZV)	(33)
	Epstein-Barr virus (EBV)	(34, 35)
Alzheimer's disease (AD)	Herpes simplex virus (HSV)	(36, 37)
	Human immunodeficiency virus (HIV)	(18, 38, 39)
	Human Herpesvirus (HHV)	(40)
	Hepatitis B virus (HBV)	(41, 42)
	Hepatitis C virus (HCV)	(41–44)
	Epstein-Barr virus (EBV)	(35, 45)
	Varicella Zoster Virus (VZV)	(46, 47)
Amyotrophic lateral sclerosis (ALS)	Enteroviruses (EVs)	(48)
	Herpes simplex virus (HSV)	(49)
(Multiple sclerosis) MS	Epstein-Barr virus (EBV)	(50)
	HSV	(51)
Vascular dementia	VZV	(52)

expression of viral genes that disrupt the host's immune system and cellular functions, bystander inflammatory responses, or apoptosis (65). Herpes simplex virus (HSV; family Herpesviridae) and human immunodeficiency virus (HIV; family Retroviridae) are two examples of viruses that exhibit oxidative stress and cause latent or delayed infections. Microglia and brain cells were found to produce intracellular ROS in response to HSV-1 infection. In cultured mouse neural cells, HSV-1 infection results in oxidative stress and triggers the production of bioactive lipid peroxidation byproducts, MDA/hydroxyalkenals (HAEs), which are essential for viral replication (66). A number of HIV-1 component proteins, through various processes, increase the formation of ROS in neural cells, including neurons, microglial cells, and astrocytes. ROS generation and substantial DNA damage are induced by the HIV-1 transactivator of transcription (Tat) protein (67). Nitroxidative stress marker proteins, including cytochrome P450-2E1 (CYP2E1), iNOS, and NADPH oxidase, are found to be elevated in the brains of HIV-1 transgenic rats. Neuronal cell death in HIV-1 transgenic rats was linked to markedly increased hippocampal levels of activated caspase-3 and BCL2 associated X (BAX) in the HIV-1 model. In conjunction with the activation of MAPK pathways mediated by ERK and JNK and the reduction of B-cell lymphoma 2 (BCL-2) expression, HIV-1 gp120 protein causes death in neurons and microglial cells (68). In neurons and glial cells, JEV (family Flaviviridae) infection raises the concentrations of superoxide anions (O2.-), nitric oxide (NO), and peroxynitrite (OONO-) (69). Neuronal cells infected with other members of the Flaviviridae family, such as West Nile virus (WNV) (70) and dengue virus type 2 (DENV-2), also showed excessive O2.- production during viral infection (71, 72), which resulted in host cell apoptosis.

The coronavirus is the largest kind of RNA virus, human proteins that interact with SARS-CoV-2 proteins have also been implicated in a number of biological processes linked to aging and neurodegenerative diseases, including lipid metabolism, responses to oxidative stress, and problems with protein homeostasis and mitochondrial function (73). Due to immune-response dysregulation and the effect of COVID-19-related discomfort on cognitive performance, people with AD seem to be at a higher risk of experiencing severe COVID-19 outcomes. COVID-19-induced systemic inflammation may be a factor in neurodegeneration and cognitive impairment.PD patients have a higher case fatality rate during COVID-19 infections, but the underlying mechanisms remain unclear. Additional research is required to determine whether the diseases share any pathophysiological pathways or risk factors. Akinetic-rigid parkinsonism that develops after severe COVID-19 instances begs the question of how the virus affects dopamine pathways. Due to respiratory muscle involvement and heightened vulnerability to respiratory problems during the pandemic, ALS patients face challenges. In COVID-19 cases, some genetic variants associated with familial ALS, like C9orf72 repeat expansions, may affect the severity of the disease (74). A study revealed that Intranasal infection of C57BL/6J mice with the SARS-CoV-2 Beta strain causes Ly6Chi monocyte infiltration of the central nervous system and activation of microglia. SARS-CoV-2, but not H1N1 influenza virus, raises brain IL-1β levels and causes IL-1R1-mediated loss of hippocampus neurogenesis, resulting in post-acute cognitive impairments. Vaccination with a low dosage of adenoviral-vectored spike protein suppresses hippocampus synthesis of IL-1β during breakthrough SARS-CoV-2 infection, resulting in neurogenesis loss and memory impairments (75, 76).

Influenza virus, belonging to Orthomyxoviridae family, which are negative sense, single-stranded, segmented RNA viruses. Influenza A virus was found to be present in substantia nigra pars compacta (SNpc) from postmortem PD brain sections. Neuroinflammation and the influenza A virus's function in PD pathogenesis were convincingly demonstrated by the colocalization of influenza A and immune cells with caspase-cleaved Beclin-1 within the SNpc. It has been shown that the H5N1 influenza virus

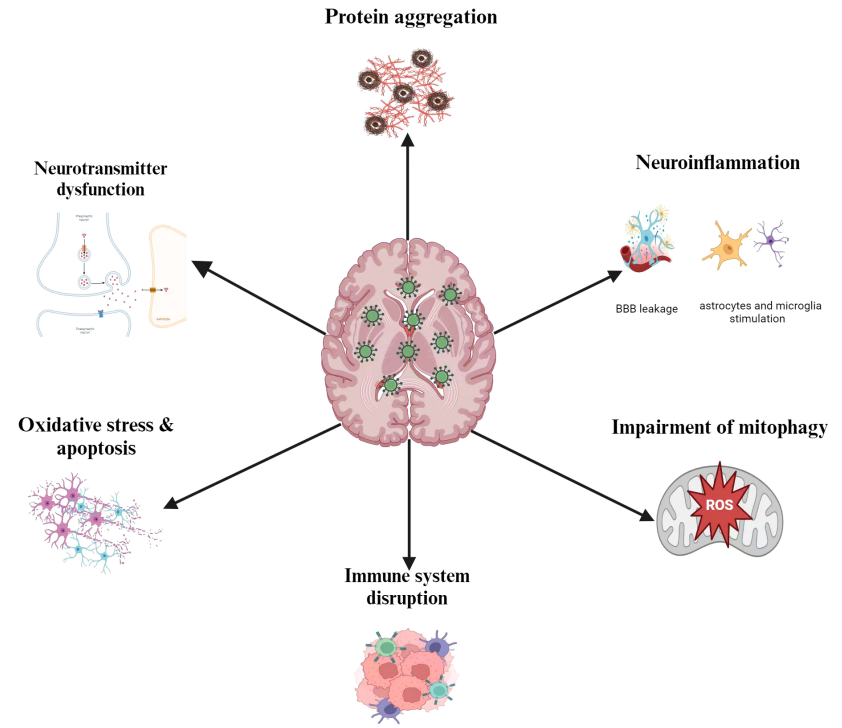

FIGURE 1
Molecular mechanisms adapted by viruses in causing Nds.

enhances α-synuclein phosphorylation and aggregation as it moves from the peripheral nervous system into the central nervous system (77).

Emerging RNA viruses that target the CNS cause cognitive consequences in survivors. Studies in people and animals infected with WNV, a re-emerging RNA virus linked to learning and memory disorders, demonstrated microglial-mediated synapse destruction in the hippocampus. Furthermore, CNS-resident memory T (TRM) cells activate microglia, which limits synapse regeneration and causes spatial learning deficits in WNV-recovered animals (78). Innate immune responses to emerging RNA viruses are becoming recognized as having substantial implications to neurologic sequelae, including memory impairments. Using a recovery model of WNV encephalitis it was found that, while macrophages deliver the antiviral and anti-neurogenic cytokine IL-1β during acute infection; viral recovery is associated with continued astrocyte inflammasome-mediated production of inflammatory levels of IL-1β, which is maintained by hippocampal astrogenesis via IL-1R1 signaling in neural stem cells (NSC). As a result, the absence of IL-1 signaling in NSC prevents abnormal astrogenesis, implying that only freshly produced astrocytes cause neurotoxicity by blocking synapse repair and enhancing spatial learning deficits (79, 80). In mice recovering from WNV or ZIKV infection, T cell-derived interferon-γ (IFN-γ) signaling in microglia causes spatial-learning defects through virus-target-specific mechanisms. Recovery from WNV infection resulted in presynaptic termini elimination with no repair, while recovery from ZIKV resulted in extensive neuronal apoptosis with loss of postsynaptic termini (81, 82).

Viral hepatitis B and C neurological impairment

Systemic parenteral hepatitis is characterized by a wide range of neurological issues and symptoms caused by several immune illnesses (6). Pathological processes are caused by viral agents replicating within and outside of brain. Depending on the degree, neurological problems brought on by acute or chronic viral hepatitis may arise from the brain, spinal cord, or peripheral nervous system. From subclinical alterations to neurocritical situations, these symptoms can occur (83, 84). Viral particles' direct neurotoxic effects on brain cells as well as the indirect effects of viruses' influence on the immune system or from the use of antiviral medication are the causes of these disorders (85). Identifying the key neurological symptoms of individuals with viral hepatitis is critical for neurologists who treat these patients on a regular basis. This will make it easier to guarantee the quick implementation of diagnostic and treatment plans (83, 84).

Nevertheless, in the last few years, a growing body of research has investigated the relationship between the Hepatitis C virus and dementia (41, 86, 87). The mechanism underlying the emergence of dementia in viral hepatitis C patients is still unclear (41). Hepatitis viruses may be able to directly infect endothelial cells and get

through the blood-brain barrier to reach the central nervous system The component molecules that viruses release during replication are known as pathogen-associated molecular patterns (PAMP). When the central nervous system is damaged in inflammatory infections, inflammatory mediators such TNF-α, IFN-γ, IL-1β, IL-6, IL-18, and chemokines are produced that promote neuronal death (88).

Parkinson's disease pathogenesis in viral hepatitis is associated with the ability of hepatitis viruses to replicate in brain macrophages and microglial cells as well as their capability to pass the blood-brain barrier. Pro-inflammatory cytokines and chemokines are released more frequently as a result, which damages neurons and eventually results in their death (89, 90). Moreover, recent studies on rats have shown that the hepatitis virus depletes dopaminergic neurons in rodents' brains (90, 91). Numerous studies have demonstrated that individuals with chronic viral hepatitis are more likely to develop PD (27, 29). Thus, a major population-based study conducted in Taiwan with 49,967 individuals who had viral hepatitis C revealed that this patient group is more prone to Parkinson's disease than those who had no history of viral hepatitis (92). Previous studies have found similar results showing a considerable increased risk of Parkinson's disease in individuals with viral hepatitis; nevertheless, to obtain more reliable data, the authors recommend doing further large-scale studies (28, 29, 93). Dementia, particularly Alzheimer's disease, has been linked to HCV infection (94). According to a recent study, treating HCV infection with direct-acting antivirals (e.g., ledipasvir/sofosbuvir, elbasvir/grazoprevir, and glecaprevir/pibrentasvir) dramatically lowers the risk of death in individuals with dementia associated with AD (95). Furthermore, viruses play a significant role in the development of AD through promoting the accumulation of amyloid-β (Aβ) peptides in the brain (96). Previous research has shown that the blood-brain barrier permeability, which controls HCV infection and activity in the central nervous system, is influenced by the ApoE level, which is also strongly linked to the neuropsychiatric symptoms experienced by HCV-infected individuals (96, 97). Although evidence suggests that HCV infection is linked to CNS impairment, it is unclear if any HCV infection promotes AD etiology. Observational studies can be difficult to understand as the results may have been impacted by reverse causality and confounding factors.

Human immunodeficiency virus type 1

In elderly HIV-1-positive patients receiving highly active antiretroviral therapy, age-related AD-like illness may be more likely to occur due to neurocognitive impairments associated with Aβ deposition and hyperphosphorylated Tau (98). HIV multiplies and contributes to neurodegeneration by affecting brain energetics at the cellular level, causing changes in overall brain metabolic homeostasis. Even though immunological dysfunction and dysregulation are typically attributed to the underlying pathophysiology of HIV infection, cognitive impairments associated with the virus have long been recognized. The spectrum of progressive neurological effects of infection includes asymptomatic neurocognitive impairments (ANI), moderate neurocognitive disorders (MND), and the more severe HIV-associated dementia (HAD) (99). According to estimates, 20–50% of HIV-positive individuals suffer from certain cognitive dysfunctions; these conditions are collectively known as HIV-associated neurological disorders (HAND). Functional status assessments and neuropsychological tests are used in the diagnosis of several disorders (100). HIV infection in the CNS is associated with activation of microglia and astrocytes, as well as the production of inflammatory and neurotoxic insults, all of which contribute to the neurodegeneration and cognitive impairment characteristic of HAND disease. Macrophages and microglia can release pro-inflammatory cytokines such as TNFα, IFNα, IL6, IL8, and IL1β, as well as chemokines such as CCL2, CCL5, and MIP-1β. These indications point to the presence of cellular reservoirs in the CNS established within 3 to 5 days of HIV-1 infection, which include three types of long-lived infected cells: astrocytes, monocyte lineage cells, and microglial cells (101). HIV enters the brain through infected CD4+ macrophages and lymphocytes, which permits the virus to transmigrate to the CNS's perivascular spaces without being noticed by the immune system (102). The molecular and cellular mechanisms underpinning HIV-associated cognitive dysfunctions (HAND) are poorly understood, despite the prevalence of these disorders. These pathways are thought to combine the neurotoxic effects of HIV-associated proteins, indirect host factor involvement, and direct viral infection of CNS cells (103). Notably, it has been shown that the HIV viral proteins Tat and gp120 both increase viral entry into the central nervous system and modify the integrity of the blood-brain barrier. HIV transactivator of transcription, or Tat, is a viral regulatory protein that initiates viral transcription and is among the first HIV proteins to be generated upon infection (104).

Moreover, HIV-RNA in the cerebrospinal fluid (CSF) and viral replication in the CNS can occur in non-viremic people receiving combined antiretroviral therapy, a condition that can cause neurological harm like cognitive decline (105, 106). Despite a decrease in the occurrence of these disorders throughout the era of combined antiretroviral medication, the frequency of minor to severe HAND remains high, even in those who get sufficient treatment (100, 107). Neopterin levels in the CSF in HIV patients with viral suppression can actually be high (108). Neopterin is associated with both cognitive decline and phagocyte activity, suggesting a potential role for CNS phagocytes in neuronal damage and degeneration. CNS phagocytes express neurodegeneration associated molecules and are located topographically in inflammatory foci rich in reactive astrocytes. Neurodegenerative phagocytes appose neurons and consume synaptic material. Aberrant phagocyte activation may be responsible for the cognitive abnormalities seen in HAND. A notable histological characteristic of HAND is synaptic degeneration (109, 110). While persistent chronic inflammation is thought to contribute to cognitive decline, the molecular basis of CNS immune activation in the context of HAND remains little known. Because the population of HIV-positive people is aging, it is imperative to understand the

processes behind these synaptic alterations in HIV in order to find new therapy targets to stop cognitive decline in HAND and other disorders (111).

Influenza virus

Flu and neuropsychiatric disorders include encephalopathy, delirium, convulsions, and confusion are well-establishedly linked (112). Influenza infections during pregnancy have also been linked to a higher chance of schizophrenia or bipolar illness in the child (113). Numerous studies suggest that the neurological effects of influenza are caused by neuroinflammatory insult, which is primarily immune-mediated rather than the result of direct viral invasion of the CNS (114). Studies on animals have raised the possibility of a link between influenza and AD. In particular, these investigations have revealed increased microglial activity in the mouse hippocampal region, a place critical for the formation of new memories and an early stage in the pathophysiology of AD due to loss of neuronal cells (115). A follow-up study on mice was able to demonstrate a connection between influenza-induced hippocampus neuroinflammation and cognitive impairment (114).

There has been speculation of an infectious etiology, and some research has linked certain diseases to PD (116, 117). Whether influenza and Parkinson's disease or parkinsonism are related has been debated for decades (118, 119). Influenza has been implicated in an outbreak of postencephalitic parkinsonism that happened from 1916 and 1930, right before and after the 1918 influenza pandemic (120, 121). The connection between influenza and Parkinson's disease and parkinsonism has been extensively studied, and some of the results suggest that infections may be the root cause of some cases (91, 122). Neurotropic influenza-A virus-infected mice exhibit activation of microglia, inflammatory responses, and inclusions of α-Synuclein in dopaminergic neurons in an experimental setting (19). The primary protein component of Lewy bodies and Lewy neurites, α-syn, was in fact produced by dopaminergic cells expressing the H1N1 influenza virus, but not tau or Transactive response DNA binding protein 43 kDa (TDP-43) (123).

SARS-CoV-2

Multiple sclerosis (MS), AD, and PD are neurodegenerative illnesses that are increasingly thought to be comorbidities in SARS-CoV-2-infected patients (124). Age dependence and co-morbidities like obesity, diabetes, and cardiovascular problems are among the many parallels between COVID-19 and PD. Furthermore, it is possible that COVID-19 will influence PD patient treatment practices and vice versa (125). Other common COVID-19 traits, such as fever, tension, and anxiety, may also negatively impact tremor, gait, and dyskinesias in PD, in addition to impairing the efficiency of L-Dopa (124). The functional relationship between AD and COVID-19 is becoming more and more evident. Like other neurodegenerative diseases, AD is considered a co-morbidity with COVID-19, meaning that having one condition usually makes the other worse (126). Neurodegeneration and neurocognitive impairment are associated with both situations with the buildup of amyloid precursor protein (APP) and activation of N-methyl-D-aspartate (NMDA)receptors. Furthermore, because these disorders share proinflammatory signaling cascades, neuronal cell death and dysfunction in both circumstances have been linked to microglial-mediated responses (127).

One of the largest RNA viruses is the SARS-CoV-2 virus. With the help of a complex array of accessory and nonstructural proteins, the virus is able to elude the innate immune system and replicate, translate, and exocytose as a fully functional virion. The single-stranded RNA that encodes 29 proteins includes the spike protein, which has the essential domains needed for binding to Angiotensin-converting enzyme 2 (ACE2). Furthermore, the possibility that these proteins have a role in the metabolic and molecular pathways of neurodegeneration is starting to gain more attention. Viruses or necessary protein components can be transported by extracellular vesicles to neurons in the substantia nigra, human cortical astrocytes, and microglia in addition to being directly absorbed by brain endothelium. This facilitates the faster formation of pathogenic fibrils (128). Liquid condensate can be produced by the intrinsically disordered SARS-CoV-2 nucleocapsid protein, which can even create harmful heteropolymers with RNA-binding proteins associated with neurodegenerative disease, such as TDP-43, fused-in sarcoma (FUS), and heterogeneous nuclear ribonucleoprotein A1 (hnRNP1A). More transmissible but less severe than the initial strain, the SARS-CoV-2 virus is continually evolving in response to the immune pressure imposed by very efficient vaccinations. Its potential long-term impacts on the brain system may therefore be a legacy of a global health crisis far more grave than acute disease (129). More severe SARS-CoV-2 and IAV infections are significantly correlated with aging-related proteostasis degradation in older people. A growing body of research indicates that the SARS-CoV-2 infection affects cognitive function over the long term and may eventually result in neurodegenerative diseases like AD (129–131). A number of pathways have been suggested, which are not mutually exclusive, while research to identify the exact mechanism(s) by which SARS-CoV-2 attacks the neurological system, both acutely and chronically, is underway (132, 133).

Herpes simplex virus-1

Lifelong latent infections in sensory neurons are brought on by neurotropic herpesviruses. HSV-1 is a periodically reactivating virus that can enter the brain and cause encephalitis or create CNS latency. Many studies link AD and HSV-1. In fact, HSV-1 seropositivity appears to increase the risk of AD (134), and HSV-1 DNA can be detected in Aβ plaques (135). In animals and cellular models, reactivation of repeated HSV-1 infections results in the accumulation of hyperphosphorylated Tau and the AD biomarkers Aβ over time (136).

The ε4 genotype of APOE is a known risk factor for AD. In animal models, apoE ε4 appears to allow HSV1 latency in the brain

much more and is more effective than apoE ε3 in promoting viral colonization of the brain following acute HSV1 infection (137). It was demonstrated that apoE ε4 was more common in the brains of AD patients who were HSV1-positive than HSV1-negative, and in those who had recurrent cold sores than in those who did not (138). These findings suggest that individuals with the apoE ε4 allele may be more susceptible to HSV's effects on the brain.

Human herpesvirus 6

HHV6 belongs to the β herpesvirus subfamily, which consists of two distinct species. It damages nerve cells and has been connected to a number of neurological disorders. The olfactory route allows HHV6 to enter the brain (139). In addition to AD, HHV6 is frequently seen in older, healthy brains. The HHV6 IgG antibodies reactivity of AD patients were significantly lower than that of normal controls. Although HHV6 might be linked to the genesis of AD, these findings might potentially point to a causal relationship or an opportunistic participant in neurodegeneration (140). A multiscale network analysis that includes late-onset AD-associated viromes and integrated genomic, transcriptomic, proteomic, and histological data from four distinct brain regions in human post-mortem tissue was used to demonstrate that AD patients had greater levels of HHV6A and human herpesvirus 7 than controls (141). There are regulatory relationships between viral abundance and APP metabolism modulators, including HHV-6A's activation of APBB2, APPBP2, BIN1, BACE1, CLU, PICALM, and PSEN1. This suggests that specific virus species can cause neuropathology and Alzheimer's disease (142).

Other viruses involved in neurodegeneration

Recent research has unequivocally shown that a history of Epstein-Barr virus (EBV) infection is associated with a higher risk of developing multiple sclerosis (MS) (143). A motor neuron disease called ALS damages brain and spinal cord nerves. A build-up of RNA-binding proteins such as FUS or TDP-43, along with cytoplasmic mislocalization, are indicative of both frontotemporal dementia and ALS. An underlying viral infection that is ordinarily epigenetically repressed and incapable of replication is up-regulated in individuals with ALS (144). Enteroviruses in the brains and cerebrospinal fluid of individuals with ALS are a topic of discussion (48). However, mice infected with two enteroviruses developed an accumulation of TDP-43 and persistent inflammation (145). Mice infected with Theiler's murine encephalitis virus (TMEV) developed an ALS-like phenotype with TDP-43 and FUS inclusions in their cytoplasm, which affected their motor neurons and glial cells (146). The Japanese encephalitis virus (JEV) can infect humans and cause Japanese encephalitis, which has a high death rate in severe cases and leaves 30 to 50 percent of survivors with severe, permanent neurological or mental repercussions (147). Increased production of reactive oxygen species (ROS) from JEV infection intensifies the death of neurons brought on by both mature and replication-incompetent viruses (148). Increased ROS production and decreased membrane fluidity in JEV-infected neuronal cells lead to serious cytopathic effects, which ultimately cause neuronal cell death (149). Neuronal cells infected with other members of the Flaviviridae family, such as West Nile virus (WNV) (70) and dengue virus type 2 (DENV-2) (150), also showed excessive O2.- production during viral infection, which resulted in host cell apoptosis. Different viruses adapt different routes to enter the CNS and causes neurodegeneration explained in Figure 2.

The Venezuelan equine encephalitis virus (VEEV) causes serious neurological abnormalities in 4–14% of patients, and fatal encephalitis in 1% of cases (151). Upon infection with VEEV, astrocytoma U87MG cells exhibit an abrupt rise in ROS levels (152). Deadly rabies virus (RABV) attacks the central nervous system (CNS), leading to encephalitis and ultimately mammalian death. Research has shown that RABV infection results in increased ROS production in mouse neuroblastoma cells (153). Inducing oxidative stress is a crucial role of the RABV viral component (154). Infection with the deadly RABV causes changes in cellular gene expression. RABV, like other neurodegenerative diseases, may be involved in neuronal death due to an imbalance in Ca2+ homeostasis. Due to the role of calcium homeostasis in dysregulation in neurodegenerative diseases and other pathophysiology, there is reason to assume that neurons that contain certain intracellular calcium-binding proteins have a greater capacity to buffer calcium, and therefore would be more resistant to degeneration (155). Oxidative stress is a major factor in the pathogenesis of neurodegeneration in viral infections of the central nervous system, as evidenced by elevated levels of free radicals and lipid peroxidation caused by neurotrophic viruses. Table 2 lists the numerous population-based investigations that were carried out to determine the role of viruses in neurodegenerative diseases.

Possible mechanism of viral pathogenesis, inflammation and neurodegeneration

Neurotropic viruses are a type of newly and re-emerging infections that specifically target and damage the integrity of the CNS (159, 160). There are several distinctive ways in which they can enter the CNS, leading to a range of neurological symptoms (161). Viruses have a particular method in which they first enter the peripheral nervous system before migrating into the CNS via axon fibers (162). Neurotropic viruses employ a variety of techniques in addition to exploiting the peripheral nervous system to bypass host barrier defenses and directly infiltrate the central nervous system. For instance, immune cells like macrophages, monocytes, and dendritic cells can become infected by the Zika virus (ZIKV), human cytomegalovirus (HCMV), and human immunodeficiency virus (HIV), which then function as carriers to move the virus into the CNS (161, 163, 164). Moreover, it has been shown that viral

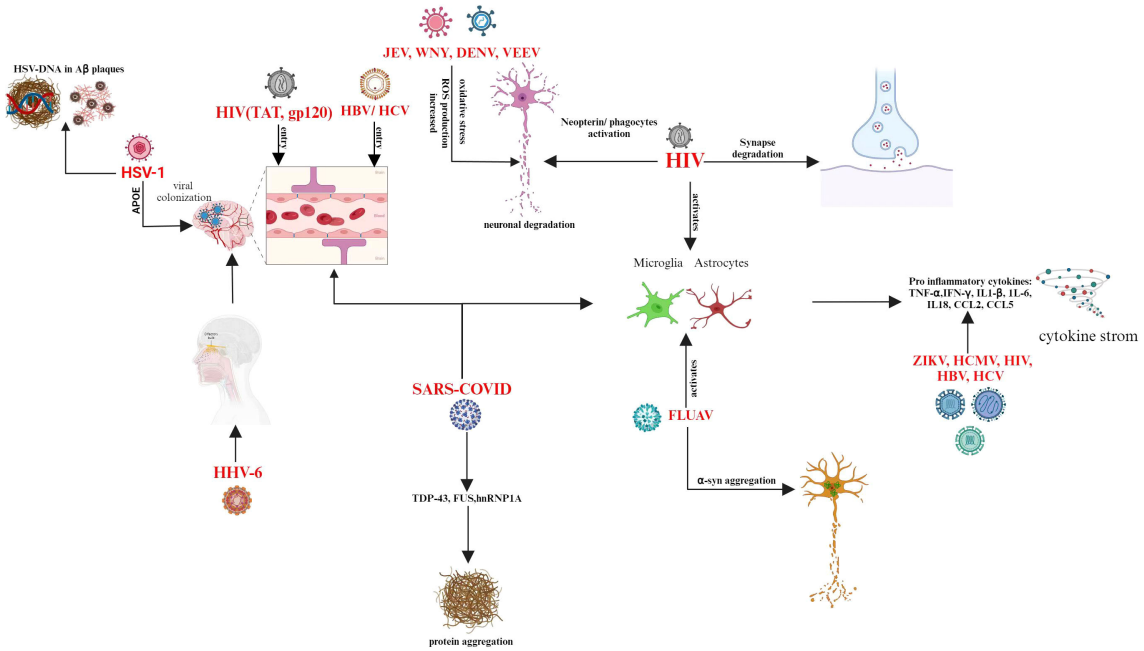

FIGURE 2
Entry routes of different viruses to infiltrate the CNS and induces neurodegeneration.

infections stimulate the production of chemokines and pro-inflammatory chemicals like TNF-α, CCL2, CCL5, IL-6, and IL-8, which can trigger a cytokine storm. The systemic pro-inflammatory state impairs the blood-brain barrier, allowing more pro-inflammatory cytokines and viruses to enter the CNS. The cytokine storm at the nervous system level can cause neuronal death, activation of microglia, synaptic plasticity impairment, and neurotransmission dysfunction (161, 165). Viruses have the ability to activate astrocytes and microglia (166, 167), cause neuroinflammation (167), oxidative stress (168), immunological responses (159), protein aggregation (169), and upset the balance of microbes in the gut (170) after they have entered the central nervous system. Accumulating data has revealed a bidirectional relationship between the gut microbiome and CNS, known as the "microbiota-gut-brain axis." Early microbiome changes were observed in preclinical Alzheimer's disease (AD) and prodromal Parkinson's disease (PD) patients (171, 172). These processes have the capacity to both initiate and exacerbate NDs.

Risk factors were recently analyzed with publicly available datasets from two large-scale population-based studies, UK Biobank and FinnGen. The UK Biobank contained twenty-two of the forty-five significant correlations between viral infections and NDs that were discovered in FinnGen. It's interesting to see that the strongest hazard ratio was associated with viral encephalitis and AD (57). Additionally, utilizing virome analysis, nine viruses were shown to be present in various CNS brain tissues in patients with PD, with PD patients showing greater positive frequencies of viruses than patients in the control group (173). Remarkably, evidence from recent studies provide credence to the hypothesis that persons with viral illnesses may be less likely to develop NDs if they receive immunizations or antiviral drugs (123, 174). When considered collectively, these results provide credibility to the theory that viral infections raise the chances of NDs.

Viruses have evolved unique defense methods to evade host defense reactions. These mechanisms include autophagy disruption and additional interference with host antiviral signaling triggered by viral infection (175). In Table 3 various mechanisms are summarized through which viruses cause neurodegeneration in AD and PD. Although some illnesses interfere with specific signaling pathways to prevent autophagosomes from fusing with lysosomes or lysosomal breakdown, autophagosomes can also serve as reproduction sites for viruses as they infect a host (202–204). Activation of autophagy by various viruses, including flaviviruses and enteroviruses, can promote virus spread by assembling and releasing infectious particles through autophagic vacuoles. In certain viral infection cases, such as poliovirus and coronavirus infection, autophagy induction by infected cells promotes the generation of double-membrane vesicles to enhance viral replication (205).

Antiviral therapies in ND's

Antivirals could be interesting alternative drug options for treating NDs. In cell culture, antivirals were able to decrease HSV-1-induced production of Aβ and phosphorylated Tau (206) Acyclovir, penciclovir, and foscarnet are anti-HSV1 antiviral medications that decreased Aβ and P-tau accumulation along with HSV1. The antiviral-induced decrease in Aβ is attributable to the reduced number of new viruses, and hence the reduction in viral spread. Since antiviral agents reduce greatly Aβ and P-tau accumulation in HSV1-infected cells, they would be suitable for

TABLE 2 Prospective cohort studies for involvement of viruses in NDs.

Virus	ND	Source	Year	References
HCV	PD	Taiwan National Health Insurance Research Database	2016	(92)
HCV/HBV	PD	Community-based integrated screening program in Taiwan	2015	(156)
Cytomegalovirus	PD	UK Biobank	2024	(157)
HBV/HCV	Dementia	Korean National Health Insurance Service	2021	(41)
HSV	AD/Dementia	Vasculature in Uppsala Seniors (PIVUS) cohort	2024	(37)
HSV/VZV	Dementia	Korean National Health Insurance Service	2017	(47)
HCV	Multiple sclerosis (MS)	Neurology department at Ain Shams University Hospital, Egypt	2023	(158)

TABLE 3 Mechanisms adopted by viruses causing neurodegeneration in PD and AD.

Neurodegenerative disorder	Virus	Pathogenesis	Markers	References
PD	CVB3	cause neurons to develop α-syn-associated inclusion bodies, which may serve as a PD trigger	Elevated α-syn expression α-syn fibrils in damaged mitochondria	(16)
	HBV/HCV	Invade the central nervous system Dopaminergic neuron death	Elevated levels of TNF-a, IL-6, and IL-1b, IL-8, IL-29, IL-22	(176, 177)
	IAV	Increased mRNA levels of CD36, CD68, C1QA, and C3, together with a changed expression pattern of major histocompatibility complex classes I and II, CD80, and F4/80, indicating evolving synaptic pruning	Increased levels of IL-6 and IFN-γ, TNF	(114)
	West Nile virus (WNV)	Abnormalities in the basal ganglia, thalamus, and pons, mostly bilaterally, evident in T2 and DWI sequences	Damage to the substantia nigra Secretion of α-syn	(178–180)
	HIV	Dopaminergic basal ganglia damage Neuroinvasion	Tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β production	(181, 182)
	JEV	Profound gliosis in the substantia nigra pars compacta (SNpc), similar to that seen in PD lower dopamine and norepinephrine levels in JEV-infected rats Dopaminergic and norepinephrinergic system impairment	Lower CSF concentrations of dopamine, norepinephrine and homovanillic acid	(30, 183, 184)
	IAV H5N1	Blocking protein degradation pathways Blocking of autophagosome formation and inhibition of autophagic flux	α-syn phosphorylation and aggregation	(19, 123)
AD	HHV-6A HHV-6B	Dysregulation of autophagy in neurons astrocytoma cells Neuroinflammation	Aβ deposition Increasing beta-amyloid and tau	(14, 134, 185–188)
	HIV,	Synaptic deficits Trojan horse mechanism	Aβ1–42 dysregulated Amyloid plaques in the CSF and blood	(189, 190)
	HCMV	Neuroinvasion	Aβ production Astrocyte reactivity	(191, 192)
	HHV-6/7	Neuroinflammation	Elevated tau, ApoE, and Aβ1–42 protein expression	(186, 193, 194)
	HSV-1	Accelerated Aβ deposition Gliosis Cognitive dysfunction	triggers the phosphorylation of Tau by activating protein kinase A (PKA) and glycogen synthase kinase 3β (GSK3β)	(195–197)

(Continued)

TABLE 3 Continued

Neurodegenerative disorder	Virus	Pathogenesis	Markers	References
			Initiate the translation of β-site amyloid precursor protein cleaving enzyme 1 (BACE1) and the buildup of Aβ by activating RNA-activated protein kinase (PKR).	
	Hepatitis viruses (HBV, HCV)	Infect endothelial cells directly and enter the central nervous system across the blood-brain barrier	Elevated levels of TNF-α, IFN-., IL-1ß, IL-6, IL-18, IL-10, IL-12 Elevated tau and amyloid beta-peptide levels	(41, 88)
	HHV-6A HHV-6B	Dysregulation of autophagy in neurons astrocytoma cells Neuroinflammation	Aβ deposition Increasing beta-amyloid and tau	(14, 185, 186)
PD/AD	SARS-CoV-2	Viral invasion Immune-mediated inflammation Endothelial dysfunction	Aggregation of Aß, α-syn, tau, and TDP-43	(198–201)

treating AD with great advantage unlike current AD therapies, only the virus, not the host cell, would be targeted (206). Ribavirin is a low-molecular-weight nucleoside analogue and inhibitor of inosine monophosphate dehydrogenase that functions as a broad-spectrum antiviral drug against a variety of DNA and RNA viruses. Ribavirin is approved in the United States for the treatment of RSV infections and, when combined with interferon, for hepatitis C virus infections (207). However, studies have shown that, as compared to a placebo, oral ribavirin formulations do not improve virologic response or the treatment of chronic hepatitis C. As a result, ribavirin is not permitted for use as a monotherapy for hepatitis C (208). Moreover, despite divergent opinions in the literature, ribavirin has been shown to be efficient against HSV both on its own and in combination with acyclovir, where it has been shown to augment the effects of acyclovir (209). Activity of ribavirin against EV has been demonstrated *in vitro* (210). Hepatitis C, RSV, and HSV are among the infectious diseases that ribavirin effectively treats; AD has been connected to several of these infections (87, 209). In a clinical trial, the Apovir group's CSF biomarker levels showed a decrease in Aβ42 over the duration of treatment (86).

The main antiviral drug used to treat HSV1 infections is called acyclovir (ACV); as expected, ACV dramatically reduces the number of HSV1 and the levels of Aβ and P-tau in HSV1-infected cells in culture (206). P-tau production is reliant on HSV1 replication and eventually drops to zero. Antibody formation is significantly decreased, but it depends, at least partially, on a previous phase of the cycle. Lower viral DNA replication is probably the cause of this decrease in viral dissemination. These results suggest that ACV might be helpful in the management of AD (211). Individuals who test positive for HSV have a higher likelihood of cognitive impairment, and antiviral drugs have a potent anti-HSV infection impact. Recent studies employing databases incorporating electronic health information have shown that HSV infections increase the risk of dementia, but antiviral medication treatment lowers this risk. In a trial including schizophrenia, the generic antiviral drug valacyclovir showed better memory improvement than a placebo (212). It has also been shown that acyclovir administration prevents HSV-1-induced neuronal death (213). When dexamethasone and acyclovir were given together, the impairments in spatial cognition were lessened. Together with microglia activation, this combination also decreased the levels of neuroinflammation markers as TNF-α and IL-6 (214). However, these effects happen only when acyclovir and dexamethasone are administered simultaneously.

Antiviral medication significantly reduces the risk of Parkinson's disease in patients with viral hepatitis (25, 215). *In vitro* models showed that the anti-influenza drug oseltamivir phosphate inhibited the aggregation of α-synuclein caused by H1N1 (123). Antiviral medication has demonstrated promise in reducing the likelihood of HCV infection, which is a risk factor for PD. In patients, the incidence of PD with persistent HCV infection appeared to be lower when treated with interferon-based antiviral therapy (216). Anti-HIV drug maraviroc specifically inhibited CCR5, ameliorating tauopathies and Huntington's disease (HD) in model mice (217).

Ever since the initial appearance of the acute respiratory coronavirus SARS-CoV-2, scientists have been searching for novel antiviral medications and repurposing those that have demonstrated efficacy against other coronaviruses. antiviral medication that could be applied in case of COVID-19 outbreak. PD, AD, and fatigue associated with multiple sclerosis have been shown to benefit from amantanes such as amantadine, rimantadine, and memantine. These conditions are all known comorbidities associated with COVID-19. Additionally, basic pharmacological studies conducted *in vitro* and *in vivo* have shown that amantadine can inhibit SARS-CoV-2 by down-regulating host-cell proteases, which impairs the release of the viral genome into the host cell, and by acting as an NMDA receptor antagonist, which prevents the acute lung injury and respiratory distress that are hallmarks of COVID-19 (124). Antiviral drugs like oseltamivir, which are frequently prescribed to treat influenza, have been demonstrated to significantly enhance parkinsonism and increase dyskinesia (218).

Antiviral drugs are now being tested for the treatment of ALS. Combination antiretroviral therapy lowers transcript levels of the

HERV-K subtype HML-2, that has been demonstrated to be elevated in ALS (219). A Phase IIa clinical trial including ALS patients found that antiretroviral medication (effective against HERV-K HML-2) indicates a trend toward delayed disease progression in patients with virological response to the treatment (220). Even though the results were encouraging, more randomized controlled trials (RCTs) are now required to assess any potential advantages for NDs.

Additionally, the potential antiviral properties of bioflavonoids produced from Ginkgo biloba leaves, such as ginkgetin, isoginkgetin, and ginkgolic acid, were investigated. These substances have a well-established antiviral profile from earlier research (221). Ginkgetin has been shown to effectively block the synthesis of viral proteins and impede the replication of HSV-1, HSV-2, and the human cytomegalovirus (222). The important significance that traditional Chinese medicine plays in treating COVID-19 aftereffects has been acknowledged. Research has demonstrated that chalcones and flavonoids can prevent neurodegeneration, prolonged COVID-19 illness, and SARS-CoV-2 infection (223). The bioactive constituents of Ginkgo biloba extract, ginkgolides and bilobalide (BB), have demonstrated neuroprotective effects in AD via pathways including anti-excitotoxicity, anti-inflammatory, and anti-oxidative properties. Furthermore, by blocking the major protease of SARS-CoV-2, ginkgolides and BB may also have antiviral effects against COVID-19. But whether pure ginkgolides or BB are given over an extended period of time at potentially therapeutic doses is actually beneficial or harmful for treating COVID-19 and AD is still up for debate (223).

Different medications have demonstrated promise in alleviating the long-term clinical symptoms of COVID-19 and neurodegenerative disorders, despite the fact that there is presently no standardized treatment for COVID-19. One way to lessen the harmful impact on nerve cells is to either preserve internal Ca2+ homeostasis or prevent the long-term inflow of Ca2+ ions. By inhibiting the extrasynaptic N-methyl-D-aspartate receptors, N-methyl-D-aspartate antagonists such as amantadine and memantine can do this by reducing the long-term Ca2+ ion influx that contributes to neuronal excitotoxicity. Amantadine is an antiviral medication that has been demonstrated to help patients with PD with their altered motor behavior. It may also help with persistent fatigue. However, memantine might aid in the improvement of cognitive deficiencies. Overlooking these issues may result in neuronal death and the associated functional deficits (224). To ascertain the effectiveness and comprehend the molecular underpinnings of these drugs' anti-coronavirus activity or inhibitory potential, more *in vitro* and *in vivo* research are required. Different antiviral drugs are in trials for neurodegenerative disorders explained in Table 4.

AAV gene therapy

In recent years, adeno-associated virus (AAV) has become the main vector for CNS gene therapy. AAV has already shown promising results in the clinic for a range of CNS ailments, including neuromuscular diseases, lysosomal storage disorders, and illnesses that are intractable with medicine. Gene therapy uses DNA or RNA as a pharmacological agent to produce gene products that permanently mute, repair, or modify endogenous genes. One "one-and-done" treatment method that can cross the blood-brain barrier is gene therapy (227) help prevent the long-term progression of neurological diseases (6). In recent years, gene therapies—like AAV-based therapy—have progressed from being the exclusive focus of preclinical research to being an effective form of treatment (228). AAV has the advantages of immunological privilege, high delivery efficiency, and specialized tissue or cell tropism in the CNS.

Regarding AAV-based gene therapy, the most clinically studied CNS condition is PD. PD is currently being studied using three different methods: glutamate decarboxylase (GAD)-inhibited glutamine synthesis as a neurotransmitter; aromatic amino acid decarboxylase, AADC-induced dopamine production; and glial cell line-derived neurotrophic factor (GDNF) in the substantia nigra to protect nigral neurons. However, the majority of AAV-based treatments are unable to treat pathologically complex diseases (229). To treat PD, AAV-based gene therapy vectors can increase dopamine levels in target cells (230). In PD primate model, intrastriatal infusion of an AAV vector containing the human aromatic l-amino acid decarboxylase (hAADC) gene results in robust gene expression (231). Alternatively, an AAV-based α-synuclein expression vector (AAV-PHP.B-GBA1) can be injected intravenously (IV) into the target neural parenchyma as an alternative to the more common injection of the mouse forebrain in PD gene therapy. Because of this, the vector was able to enter the brain parenchyma and propagate throughout it. This allowed the vector to target the central and peripheral nervous systems globally and restored animal behavior by reducing synucleinopathy (232).

More than one hundred clinical trials have involved Alzheimer's patients. Other than immunotherapy, there is currently no medication that can impede the progression of Alzheimer's disease in those with cognitive impairments. However, AAV-based gene therapy continues to be ineffective. The only experiment that was successfully completed used AAV2-driven nerve growth factor to reverse basal cholinergic neuronal dysfunction. Ten patients with mild-to-moderate AD were treated in a Phase I clinical trial with bilateral stereotactic injections of AAV2-nerve growth factor into the Meynert nucleus basalis without the use of immunosuppressive drugs (233). This medicine worked effectively, was safe, and was well tolerated. No side effects were reported. Another trial, a Phase II trial, used a higher dose, although the treatment and placebo groups' outcomes in terms of brain metabolic or cognitive performance did not vary statistically (234). The autopsy results of the three cases showed that stereotactically injected AAV2 did not reach the nucleus basalis of Meynert due to restricted AAV2 diffusion; hence, no reliable conclusions could be drawn (235). Three other therapeutic modalities are the subject of clinical investigation, the results of which have not yet been made public: Intravenous or intrathecal telomerase (hTERT) delivery to lengthen telomeres; brain-derived

TABLE 4 Clinical trials of antiviral drugs against NDs.

ND	Virus	Anti-viral Drugs	Clinical trial	References
AD	Pleconaril (active on enteroviruses) ribavirin (active on several viruses)	Apovir	Phase IIa	(86)
AD	HSV	Valacyclovir	Phase II	https://clinicaltrials.gov/study/NCT03282916
PD	Influenza	Amantadine	completed	https://clinicaltrials.gov/study/NCT00632762
AD	HSV-1	Penciclovir		(206)
PD	HCV	Interferon-α		(216)
PD	HCV	Interferon-free direct-acting antiviral (DAA) therapy with ledipasvir (LDV) plus sofosbuvir (SOF)		(225)
ALS	HIV/AIDS	Combination Antiretroviral Therapy (Triumeq)	Phase IIa	https://clinicaltrials.gov/study/NCT02868580
Schizophrenia	HSV-1	Valaciclovir (pro-drug of acyclovir)	Phase II	(226), https://clinicaltrials.gov/study/NCT02008773
AD	HSV-1	Acyclovir		(214)
AD	HBV/HIV	Lamivudine/3TC	Phase I Phase II	https://clinicaltrials.gov/study/NCT04552795
Mild Cognitive Impairment	HBV/HIV	Lamivudine/3TC	Phase II	https://clinicaltrials.gov/study/NCT06519357
Amyotrophic Lateral Sclerosis (ALS)	HIV	Antiretroviral regimen approved to treat HIV	Phase I	https://clinicaltrials.gov/study/NCT02437110
Multiple Seclerosis (MS)	Epstein-Barr virus (EBV)	Famciclovir	Phase II	https://clinicaltrials.gov/study/NCT05283551
PD	HBV/HIV	Tenofovir Disoproxil Fumarate	Phase I	https://clinicaltrials.gov/study/NCT06356662

neurotrophic factor administered via parenchymal delivery to minimize neuronal loss and promote synaptic reconstruction; and intra-CSF delivery of apolipoprotein E2 to restore protein expression in patients homozygous for apolipoprotein E4 (235).

Numerous novel issues highlight the need for further research, especially in the areas of safe delivery methods, well-understood immunological systems, cost-effective production procedures, targeted vectors, and further immune system suppression strategies. To extend AAV-based gene therapy from monogenic disorders to other diseases, we need to understand the whole phenotypic range of each disease and find objective biomarkers to capture the essential features of the condition. Ongoing research on the imaging of viral vectors is necessary to monitor the pharmacokinetics of viruses.

Conclusion

CNS infection diagnosis and therapy are difficult but essential. There are either none or very few antiviral medications on the market now for treating viral infections of the central nervous system. A viral infection causes an imbalance between free radicals and antioxidants, which increases oxidative stress within cells and causes neuronal cells to undergo programmed death through apoptosis. In order to interfere with mitophagy and mitochondrial dynamics in their hosts, viruses work with the recycling machinery of the cell.

Viral disturbance of mitochondrial homeostasis alters neuronal metabolism and consequently affects brain function. When neurotropic viruses enter the brain, specific brain functions are harmed, neurotransmitter systems are changed, and pathological

signs of NDs appear. An understanding of the neuropathogenesis of viral CNS infection may help in the creation of more efficient diagnosis and treatment plans by focusing on the molecular mechanisms underlying CNS infection. It might also be helpful in the search for new antiviral drugs, which are necessary to treat these neurotropic viral infections in an efficient manner.

Author contributions

MA: Writing – original draft, Writing – review & editing. FM: Formal analysis, Investigation, Writing – review & editing. PX: Investigation, Writing – review & editing. JX: Conceptualization, Resources, Supervision, Validation, Writing – review & editing. FZ: Writing – review & editing, Funding acquisition.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The author(s) declare that no Generative AI was used in the creation of this manuscript.

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Glossary

AAV	Adeno-Associated Virus
ACE2	Angiotensin-Converting Enzyme 2
ACV	Acyclovir
AD	Alzheimer's disease
ALS	Amyotrophic Lateral Sclerosis
ALS	Amyotrophic Lateral Sclerosis
ANI	Asymptomatic Neurocognitive Impairments
ApoE	Apolipoprotein E
APOE ε4	Apolipoprotein E ε4 allele
APP	Amyloid Precursor Protein
Aβ	Amyloid-β
BACE1	β-Site Amyloid Precursor Protein Cleaving Enzyme 1
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
CVB3	Coxsackievirus B3
DAA	Direct-Acting Antiviral
DENV-2	Dengue Virus Type 2
EBV	Epstein-Barr virus
EBV	Epstein-Barr Virus
EVs	Enteroviruses
GAD	Glutamate Decarboxylase
GDNF	Glial Cell Line-Derived Neurotrophic Factor
GSK3β	Glycogen Synthase Kinase 3β
GWAS	Genome-Wide Association Studies
HAD	HIV-Associated Dementia
HAND	HIV-Associated Neurological Disorders
HBV	Hepatitis B virus
HCMV	Human Cytomegalovirus
HCV	Hepatitis C virus
HHV	Human Herpesvirus
HHV6	Human Herpesvirus 6
HIV	Human Immunodeficiency Virus
HSV	Herpes simplex virus
HSV-1	Herpes Simplex Virus-1
IAV	Influenza A virus
IV	Intravenously
JEV	Japanese encephalitis virus
JEV	Japanese Encephalitis Virus'
LDV	Ledipasvir
MND	Moderate Neurocognitive Disorders
MS	Multiple sclerosis
MSA	Multiple Systems Atrophy
NDs	Neurodegenerative diseases
NMDA	N-methyl-D-aspartate
PD	Parkinson's disease
PKA	Protein Kinase A
PKR	RNA-Activated Protein Kinase
RABV	Rabies Virus
ROS	Reactive Oxygen Species
SNpc	Substantia Nigra Pars Compacta
SOF	Sofosbuvir
TMEV	Theiler's Murine Encephalitis Virus
VEEV	Venezuelan Equine Encephalitis Virus
VZV	Varicella-Zoster Virus
WEV	Western equine virus
WNV	West Nile Virus
WNV	West Nile virus
ZIKV	Zika virus
α-syn	α-synuclein.

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EDITED BY
Pei-Hui Wang,
Shandong University, China

REVIEWED BY
Christina K. Psomas,
Hôpital Européen Marseille, France
Weili Kong,
Gladstone Institutes, United States
Narendran Annadurai,
University of Nebraska Medical Center, United States

*CORRESPONDENCE
Melissa J. Churchill
melissa.churchill@rmit.edu.au

†These authors have contributed equally to this work and share senior authorship

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Neuroinflammation associated with proviral DNA persists in the brain of virally suppressed people with HIV

Sarah J. Byrnes[1], Janna Jamal Eddine[1], Jingling Zhou[1], Emily Chalmers[1], Emma Wanicek[1], Narin Osman[1], Trisha A. Jenkins[1], Michael Roche[1,2], Bruce J. Brew[3,4], Jacob D. Estes[1,5], Thomas A. Angelovich[1,2,6†] and Melissa J. Churchill[1,7,8*†]

[1]ATRACT Research Centre, School of Health and Biomedical Sciences, RMIT University, Melbourne, VIC, Australia, [2]Department of Infectious Diseases, The University of Melbourne at the Peter Doherty Institute for Infection and Immunity, Melbourne, VIC, Australia, [3]Department of Neurology and Immunology, Peter Duncan Neuroscience Unit, St Vincent's Hospital, University of New South Wales, Darlinghurst, NSW, Australia, [4]University of Notre Dame, Sydney, NSW, Australia, [5]Vaccine & Gene Therapy Institute, Oregon Health & Science University, Beaverton, OR, United States, [6]Life Sciences, Burnet Institute, Melbourne, VIC, Australia, [7]Department of Microbiology, Monash University, Melbourne, VIC, Australia, [8]Department of Medicine, Monash University, Melbourne, VIC, Australia

Despite viral suppression with antiretroviral therapy (ART), people with HIV (PWH) continue to exhibit brain pathology, and ~20% of individuals develop HIV-associated neurocognitive disorders. However, the state of cellular activation in the brain of virally suppressed (VS) PWH and the impact of local viral reservoirs on cellular activation are unclear. Using multiplex immunofluorescence imaging, here, we demonstrate that the frontal cortex brain tissue from both non-virally suppressed (nVS; n=17) and VS PWH (n=18) have higher frequencies of astrocytes and myeloid cells expressing interferon-inducible Mx-1 and proinflammatory TNFα relative to HIV-seronegative individuals ($p<0.05$ for all). The frequency of TGF-β1+ cells were also elevated in the brain tissue from both nVS and VS PWH, which may support active immunoregulatory responses despite ART. Importantly, the frequency of Mx1+ myeloid cells correlated with levels of total HIV DNA and intact and 5′ defective HIV proviral DNA ($p<0.05$ for all) in the brain of VS PWH. These findings demonstrate that cell activation persists in the brain of VS PWH and is associated with HIV DNA in the brain, which may contribute to neuropathology.

KEYWORDS

HIV, brain, neuroinflammation, reservoirs, microglia, astrocytes

1 Introduction

Although sustained treatment with antiretroviral therapy (ART) suppresses HIV plasma viremia, which limits the risk of acquired immunodeficiency syndrome or viral transmission, virally suppressed people with HIV (VS PWH) continue to have chronic tissue damage and an elevated risk of developing comorbidities and long-term non-AIDS-related pathology (1–5). Specifically, VS PWH have a higher incidence of brain atrophy (6), reduced synaptic density (7), and elevated clinical neurometabolites associated with cellular activation than age-matched people without HIV with approximately 20% of VS PWH developing neurocognitive disorders (3, 6, 8). The mechanisms driving neuropathology and/or cognitive disorders are unclear; however, viral persistence in the central nervous system (CNS) and peripheral tissues, and ongoing neuroinflammation and systemic inflammation penetrating the brain are all thought to play fundamental roles (3, 9).

We and others have recently demonstrated that a reservoir of HIV DNA persists in the brain tissue of VS PWH, primarily in the frontal cortex (10–13). Levels of intact and defective HIV proviral DNA present in the CNS did not differ between VS and non-virally suppressed (nVS) PWH, demonstrating that ART does not reduce the size of the viral reservoir in the frontal cortex, which may impact cell activation.

Chronic HIV infection is associated with heightened measures of neuroinflammation and immune activation as primarily measured by surrogate markers in plasma and/or cerebrospinal fluid (CSF) (14–16). Studies, including our own, have utilized models of chronic ART-treated simian immunodeficiency virus (SIV) infection to further demonstrate chronic immune activation in the brain parenchyma at a cellular level (17–19). Specifically, we found heightened type I interferon (IFN), oxidative stress, and transforming growth factor (TGF-β1) signaling pathways in the frontal cortex of SIV+ non-human primates (NHPs) despite long-term viral suppression with ART (18). Whether these markers of immune activation are similarly elevated in the CNS of VS PWH, and importantly, the role of viral persistence in the brain on cellular activation is unclear.

In this study, cellular activation in the frontal cortex of the brain from VS PWH was measured using quantitative spatial multiplex immunofluorescence imaging of the autopsy brain tissue. The relationship between the local viral reservoir in the brain and neuroinflammation were examined to further understand the mechanisms driving neuroinflammation in VS PWH.

2 Materials and methods

2.1 Cohort

Formalin-fixed paraffin-embedded (FFPE) and matched fresh frozen human autopsy frontal cortex tissue from PWH and HIV-seronegative individuals were generously provided by the National NeuroHIV Tissue Consortium (NNTC, USA, https://nntc.org). The median (IQR) post-mortem interval (PMI) was 8.50 (5.75–16.5) h. Exclusion criteria included extended post-mortem interval (>27 h), any known co-infections, and comorbidities associated with the brain or vascular system. Tissue was not specifically anatomically matched within the frontal cortex. Clinical information including ART regimen, CD4+ T cell counts, plasma, and CSF viral loads were provided unless stated (Table 1). CNS penetrance scores were calculated as previously described (20).

2.2 Quantification of cellular immune activation in human brain tissue

Multiplex fluorescent immunohistochemistry was performed as previously described (21) with the following amendments: FFPE tissue was deparaffinized and rehydrated prior to antigen retrieval and hydrogen peroxide treatment. Tissues were then incubated in the first primary antibody, either Mx1 (1:100; 2 h; cat: MABF958; Merck, Rahway, NJ, United States), TNFα (1:100; 2 hours; cat: ab1793; Abcam), or TGF-β1 (1:50; overnight; cat: ab215715; Abcam, Waltham, MA, United States). Primary antibody was detected with the anti-rabbit/mouse polymer HRP-conjugated system (cat: DET-HP1000; EMD Millipore, Burlington, MA, United States). Opal fluorophore (opal 570; 1:200; cat: FP1488001KT; Akoya, Marlborough, MA, USA) was used to visualize the first primary antigen. To remove residual antibody for the next round of antigen detection, each slide was boiled for 20 min in citrate (pH6) retrieval buffer and left to cool at room temperature. This method was repeated for the second primary antibody (CD68; 1:200; 2 h; cat: M0814; DAKO, Jena, TH, Germany) and the third primary antibody (GFAP; 1:5,000; 2 h; cat: Z0334; DAKO, Jena, TH, Germany) and visualized with Opal 650 (1:200; cat: FP1496001KT; Akoya, Marlborough, MA, USA) and Opal 520 (1:200; cat: FP1487001KT), respectively. Nuclei were counterstained with DAPI (1:750; cat: 94774; DAKO, Jena, TH, Germany), and lipofuscin was quenched with True Black (1:20 in 70% ethanol; 30 s; cat: 23007; Biotium, Freemont, CA, USA). Slides were rinsed in H_2O and mounted with Fluoromount G (cat: 495802; Invitrogen, Carlsbad, CA, USA). Mounted slides were dried overnight and scanned at 20× magnification (Axioscan 7; Zeiss, Oberkochen, BW, Germany). Images were analyzed as a whole or stratified into gray and white matter using HALO AI 3.6 software (Indica Labs, Albuquerque, NM, USA). The percentage of positive and colocalized cells were quantified using HighPlex FL v4.2 with the frequency of marker-positive cells expressed relative to the number of nuclei per tissue section.

2.3 HIV DNA quantification in frontal cortex tissue

Genomic DNA was extracted from homogenized fresh frozen frontal cortex brain tissue (~10 mg pieces) and intact (Ψ+ and *env* +), 3′ defective (Ψ+ and *env*–) and 5′ defective (Ψ– and *env*+) HIV DNA standardized to RPP30 was quantified using the intact proviral DNA assay (QX200; BioRad, Hercules, CA, USA), as previously described (10).

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TABLE 1 Clinical parameters.

ID	Age	Sex (% male)	Plasma VL	CSF VL	CD4	ART	CPE score	VS (years)	Average T-Score
HIV seronegative									
HIV -ve 1	51	F	–	–	–	–		–	–
HIV -ve 2	61	M	–	–	–	–		–	–
HIV -ve 3	[a]	M	–	–	–	–		–	–
HIV -ve 4	39	M	–	–	–	–		–	–
HIV -ve 5	[a]	F	–	–	–	–		–	–
HIV -ve 6	[a]	F	–	–	–	–		–	–
Median (IQR)	51 (39-61)	50%							
Non-virally suppressed									
nVS PWH 1	56	M	61,223	[a]	24	3TC/ABC, ATV, BIC/FTC/TFV	13	–	34.6
nVS PWH 2	43	M	64	[a]	110	DRV, EFV/TFV, RTV	8	–	45.9
nVS PWH 3	57	F	576,000	10,005	98	None	–	–	39.6
nVS PWH 4	39	F	2,857	[a]	757	None	–	–	40.1
nVS PWH 5	48	M	750,000	[a]	2	None	–	–	[a]
nVS PWH 6	38	F	39,184	[a]	1	None	–	–	[a]
nVS PWH 7	56	F	35,723	[a]	73	BIC/FTC/TFV	9	–	43.1
nVS PWH 8	47	F	367,620	314	2	None	–	–	54.8
nVS PWH 9	62	M	222,840	501	8	3TC, ABC, D4T	7	–	26.2
nVS PWH 10	37	M	287,947	220	3	3TC, D4T, EFV	7	–	34.1
nVS PWH 11	49	M	750,000	1,101	3	None	–	–	50.4
nVS PWH 12	40	F	157,009	408	5	EFV/FTC/TDF	7	–	[a]
nVS PWH 13	55	F	17,387	UD	8	None	–	–	36.1
nVS PWH 14	42	M	688	[a]	441	3TC/ABC	5	–	61
nVS PWH 15[b]	35	M	2,827	78	211	3TC, D4T, IDV	7	–	43.9
nVS PWH 16[b]	46	M	17,500	[a]	[a]	[a]	[A]	–	[a]
nVS PWH 17[b]	57	F	730,085	[a]	25	None	–	–	34.6
Median (IQR)	47 (40–56)	52.9%	61,223 (17,387–367,620)	361 (184.5–651)	16 (3–101)		7 (7–8.25)		40.1 (35.6–45.9)
Virally suppressed									
VS PWH 1	67	M	UD	UD	355	3TC/ABC, ATV, RTV	8	7.32	28.9
VS PWH 2	46	M	UD	[a]	61	DRV, EFV/TFV, RTV	8	3.76	46.4
VS PWH 3	57	M	UD	[a]	48	3TC/ZDV, EFV	9	0.75	39.4
VS PWH 4	59	F	UD	[a]	328	EFV/TFV, RGV	7	2.47	[a]
VS PWH 5	39	M	UD	[a]	112	DRV, EFV/TFV, RTV	8	1.46	47.5
VS PWH 6	62	M	UD	UD	274	3TC, ABC, RGV	8	13.6	[a]
VS PWH 7	64	F	UD	[a]	537	3TC/ZDV, EFV	9	2.89	38.1
VS PWH 8	50	M	UD	105	113	ATV, EFV/TFV, RTV	7	5.30	[a]

(Continued)

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TABLE 1 Continued

ID	Age	Sex (% male)	Plasma VL	CSF VL	CD4	ART	CPE score	VS (years)	Average T-Score
Virally suppressed									
VS PWH 9	52	M	UD	UD	417	EFV/FTC/TDF	7	6.04	54.1
VS PWH 10	64	M	UD	[a]	798	3TC, ABC, EFV	8	2.54	39.5
VS PWH 11	63	M	UD	UD	140	3TC, EFV/TFV	6	9.15	[a]
VS PWH 12	66	M	UD	UD	527	EFV/FTC/TDF	7	10.0	28.6
VS PWH 13	58	M	UD	[a]	119	3TC, EFV/TFV	6	1.42	39.1
VS PWH 14	62	M	UD	UD	133	FTC/TDF, NVP	8	7.75	[a]
VS PWH 15	58	F	UD	[a]	155	3TC/ABC, DTG	9	1.46	39.8
VS PWH 16	62	M	UD	[a]	172	DTG, FTC/TDF	8	3.93	[a]
VS PWH 17	52	M	UD	[a]	383	3TC, D4T, EFV	7	2.58	[a]
VS PWH 18	61	F	UD	[a]	25	3TC/ABC, ATV, RTV	8	6.89	41.6
Median (IQR)	60 (53.3–62.8)	77.8%	49 (40–50)	50 (50–55)	163 (114.5–376)		8 (7–8)	3.8 (2.49–7.21)	39.6 (38.6–44)

3TC, lamivudine; ABC, abacavir; ATV, atazanavir; BIC, bictegravir; CD, cluster of differentiation; CI, cognitive impairment; CPE, central nervous system penetration effectiveness; CSF, cerebrospinal fluid; D4T, stavudine; DRV, darunavir; DTG, dolutegravir; EFV, efavirenz; F, female; FTC, emtricitabine; IDV, indinavir; M, male; IQR, interquartile range; nVS, non-virally suppressed; NVP, nevirapine; RGV, raltegravir; RTV, ritonavir; TDF, tenofovir disoproxil fumarate; TFV, tenofovir; UD, undetectable (<60 HIV RNA copies/m:L); VL, viral load; VS, virally suppressed; ZDV, zidovudine. Cognitive impairment: T score >40.
[a]Missing data.
[b]Non-virally suppressed individuals with HIV associated encephalitis.

2.4 Statistics

All statistical analysis was completed using GraphPad Prism software (version 10.2.2 Windows). Comparisons between groups were made using non-parametric Kruskal–Wallis tests with Dunn's *post-hoc* analysis for multiple comparisons; median and interquartile ranges are shown. Spearman's correlations were performed on log transformed data; rho (ρ) and p-values are shown.

3 Results

To characterize the cellular environment and HIV viral reservoir in the brain of nVS and VS PWH, matched fresh frozen and formalin-fixed paraffin-embedded (FFPE) frontal cortex tissue was obtained from nVS (n=17), VS PWH (n=18), and HIV-seronegative controls (HIV−; n=6) from the National NeuroHIV Tissue Consortium (NNTC, USA; https://nntc.org/; Table 1). Viral suppression was defined by >1.4 years of undetectable HIV RNA copies/mL in plasma. One participant (VS PWH 3) under this threshold was included, as they had long-term suppression (5.35 years) prior to two viral load tests <650 copies/mL, 0.75 years prior to death. This individual had two undetectable viral load tests prior to death, one of which was the day prior to death and a CD4 T-cell count in range of the virally suppressed group. PWH who were treated with ART but did not meet the criteria of undetectable viral loads as described above were collated in the nVS group for analysis. VS PWH were suppressed for a median of 3.8 years, were generally older than both nVS PWH (median age, 60 vs. 47 years; p<0.001) and seronegative individuals (median age, 60 vs. 51, p>0.05), and were predominantly men (77.5%). The average T score measure of cognitive impairment did not differ between VS and nVS groups (median T score: VS, 39.6 vs. nVS, 40.1; p>0.05). Three nVS PWH were classified with HIV encephalitis by expert neuropathologists at the NNTC.

3.1 Elevated reactive astrocytes persist in the frontal cortex of the brain in virally suppressed PWH

To assess immune activation in the frontal cortex of the brain from nVS and VS PWH, multiplex immunofluorescence imaging specific for type I IFN responses, proinflammatory NF-κB-mediated signaling and immunoregulatory TGF-β1 signaling were performed. Representative images of immunofluorescence staining are shown in Figure 1. Myeloid cells were defined as $CD68^+$ and astrocytes were defined as glial fibrillary acidic protein (GFAP) positive, which are upregulated during cell activation (22, 23). Due to regional differences in cell composition and function between white matter and gray matter in the frontal cortex, each region was annotated and assessed individually and a combined total value. The frequency of $CD68^+$ myeloid cells (consisting of both microglia and perivascular macrophage) did not differ between HIV-seronegative, nVS PWH, and VS PWH when examined in total frontal cortex, and in the white matter and gray matter alone (Supplementary Figures S1A–C). While the frequency of astrocytes in the gray and white matter were similar between groups

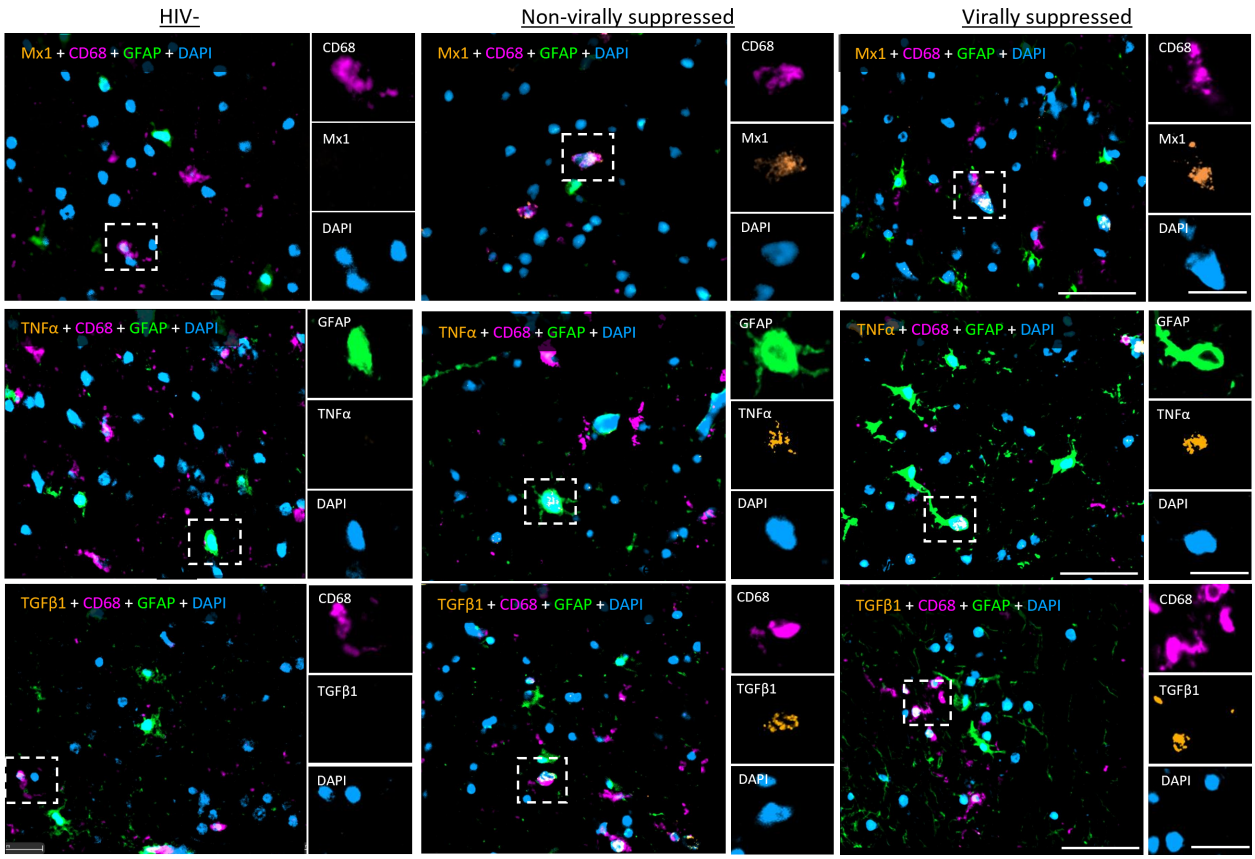

FIGURE 1
Representative images of multiplex immunohistochemistry of human frontal cortex tissue from PWH. Representative images of fluorescent IHC staining for Mx1, TNFα, and TGF-β1 (orange) multiplexed with CD68 (magenta), GFAP (green), and DAPI (blue) performed on the frontal cortex tissue of the brain from non-virally suppressed and virally suppressed PWH or HIV-seronegative (HIV–). Images shown at 30x magnification: scale bars: 50 and 20 μm (inset).

(HIV-seronegative, VS PWH, and nVS PWH), there was a higher frequency of astrocytes in the total frontal cortex for nVS PWH relative to HIV-seronegative controls (Supplementary Figures S1D–F).

3.2 Virally suppressed PWH harbors a state of cellular activation in the frontal cortex of the brain despite ART

Multiplex immunofluorescence staining for CD68, GFAP, Mx1, tumor necrosis factor alpha (TNFα), and TGF-β1 was performed to assess whether cellular activation is present in the frontal cortex of nVS and VS PWH. A higher frequency of cells expressing the anti-viral IFN-inducible protein Mx1 was present in total frontal cortex tissue from nVS PWH relative to HIV-seronegative individuals (Figure 2A). When stratifying by region, the frequency of $Mx1^+$ cells were elevated in both white and gray matter from nVS PWH ($p<0.05$ for both). However, $Mx1^+$ cells were elevated in gray matter from VS PWH (Figures 2B, C), suggesting that activation was localized to gray matter during viral suppression. Total frontal cortex tissue from both nVS and VS PWH also exhibited a higher frequency of cells expressing proinflammatory TNFα relative to HIV-seronegative individuals (Figure 2D). The frequency of $TNFα^+$ cells was elevated across white matter when analyzed separately for both nVS and VS PWH ($p<0.05$ for all; Figure 2E). In the gray matter, TNFα was elevated in VS PWH ($p<0.05$), and an increasing trend was present in nVS PWH ($p=0.066$; Figure 2F), indicating widespread production of TNFα within the frontal cortex of the brain from PWH.

Interestingly, a higher proportion of cells expressing the immunoregulatory cytokine TGF-β1 was observed in the total frontal cortex tissue of both nVS and VS PWH compared to HIV-seronegative individuals ($p<0.05$, Figure 2G), which may indicate active immunoregulatory responses. Regional analysis identified an increasing trend of $TGF\text{-}β1^+$ cell frequency in nVS and VS PWH in the white matter (nVS $p=0.054$, VS $p=0.055$; Figure 2H). In gray matter, TGF-β1 was increased in VS PWH ($p<0.05$) and was higher in nVS PWH; however, this did not reach significance ($p=0.071$; Figure 2I). Importantly, the frequency of immune markers did not correlate with age (Supplementary Table S1). Together, these observations demonstrate that a heightened state of cellular activation and reciprocal regulatory responses exists in the brain of PWH, which persists despite viral suppression with ART.

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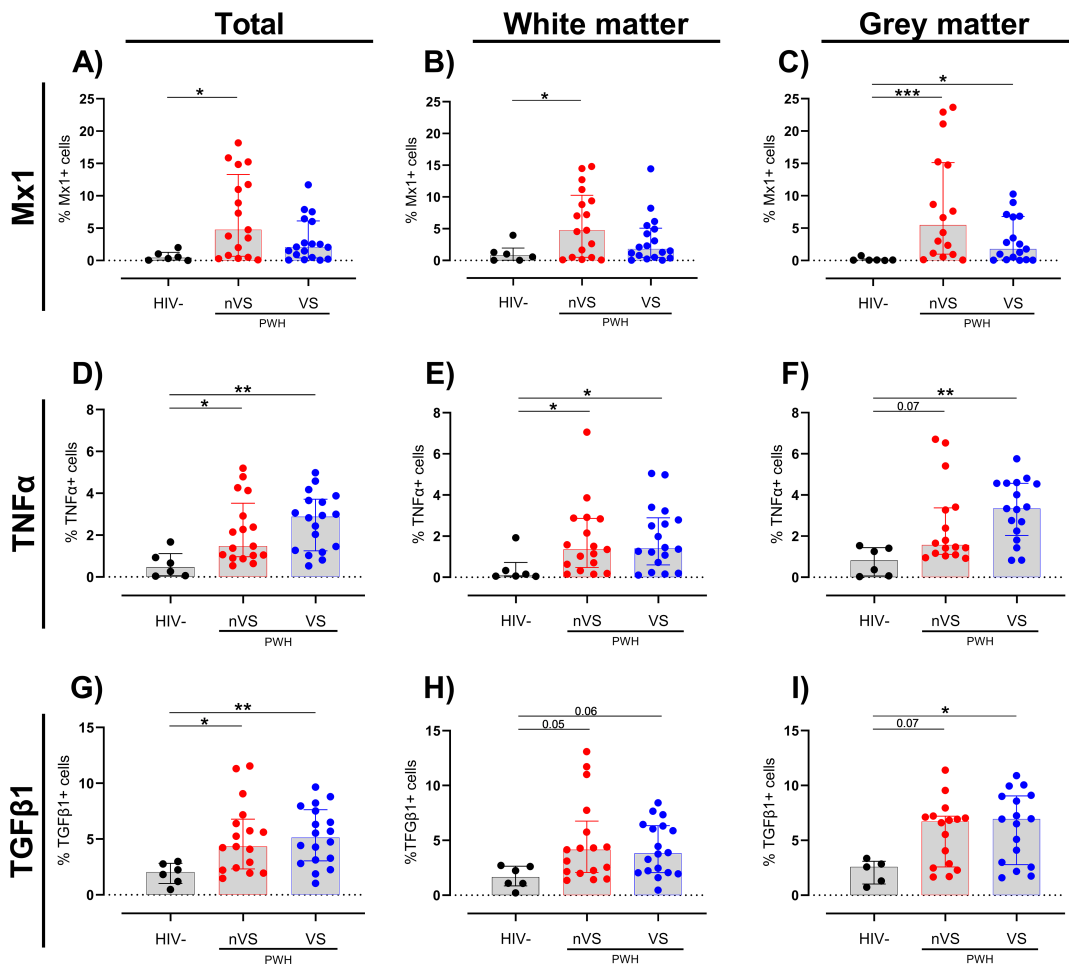

FIGURE 2
The frequency of cells expressing immune activation and immunoregulatory proteins are elevated in the frontal cortex from non-virally suppressed and virally suppressed people with HIV. The frequency of cells expressing **(A–C)** Mx1, **(D–F)** TNFα, and **(G–I)** TGF-β1 in the total (white + gray matter) and white and gray matter tissue alone of the frontal cortex from non-virally suppressed PWH (nVS; n=16–17), virally suppressed PWH (VS PWH; n=17–18), and HIV seronegative controls (HIV–; n=5–6) as measured by multiplex immunofluorescence. Data unavailable for gray matter for n=1 HIV-seronegative controls (TGF-β1 only), n=1 VS PWH and n=1 nVS PWH due to no gray matter region present in the tissue section provided. Median and interquartile ranges shown. Comparisons made using Kruskal–Wallis test with Dunn's *post-hoc* tests (*p<0.05; **p<0.01; ***p<0.001).

3.3 Activated myeloid cells and astrocyte phenotypes contribute to neuroinflammation in PWH

To further define the cellular origins of immune activation in the frontal cortex of nVS and VS PWH, colocalization analysis was performed using HALO imaging analysis software. Specifically, cell-marker-positive cells (i.e., either $CD68^+$ myeloid cells or $GFAP^+$ astrocytes) expressing a particular activation marker were enumerated and expressed as a percentage of cell-marker-positive cells. In the total frontal cortex and white matter, nVS PWH had a higher frequency of myeloid cells expressing Mx1 relative to HIV-seronegative individuals ($p<0.05$ for both; Figures 3A, B). Additionally, the frequency of $Mx1^+$ myeloid cells were higher in both nVS and VS PWH in the gray matter ($p<0.05$ for both; Figure 3C). Additionally, the frequency of $TNF\alpha^+$ myeloid cells were elevated in total frontal cortex from both nVS and VS PWH relative to HIV-seronegative individuals ($p<0.05$ for both; Figure 3D), supporting heightened cell activation leading to broad proinflammatory cytokine production in the frontal cortex that is not restored with ART treatment. The frequency of $TNF\alpha^+$ cells were also elevated across white and gray matter for both groups ($p<0.05$ for all; Figures 3E, F), indicating a higher frequency of TNFα-producing myeloid cells across all regions of the frontal cortex in PWH. The frequency of myeloid cells producing TGF-β1 was elevated in both nVS and VS PWH ($p<0.05$ for both; Figure 3G). A higher frequency of TGF-β1 expressing myeloid cells were observed in nVS PWH in the white matter ($p<0.05$), and an increasing trend was present in VS PWH ($p=0.058$; Figure 3H). In the gray matter, only nVS PWH displayed a trend of elevated TGF-$\beta1^+$ myeloid cells ($p=0.061$; Figure 3I).

Similar to observations for myeloid cells, an increased trend of $Mx1^+$-activated astrocytes was present in total frontal cortex tissue of nVS PWH ($p=0.054$; Figure 4A). Sub-analysis by region demonstrated that while there were no significant changes between groups in the white matter (Figure 4B), nVS PWH exhibited a higher frequency of $Mx1^+$ astrocytes in gray matter compared to HIV-

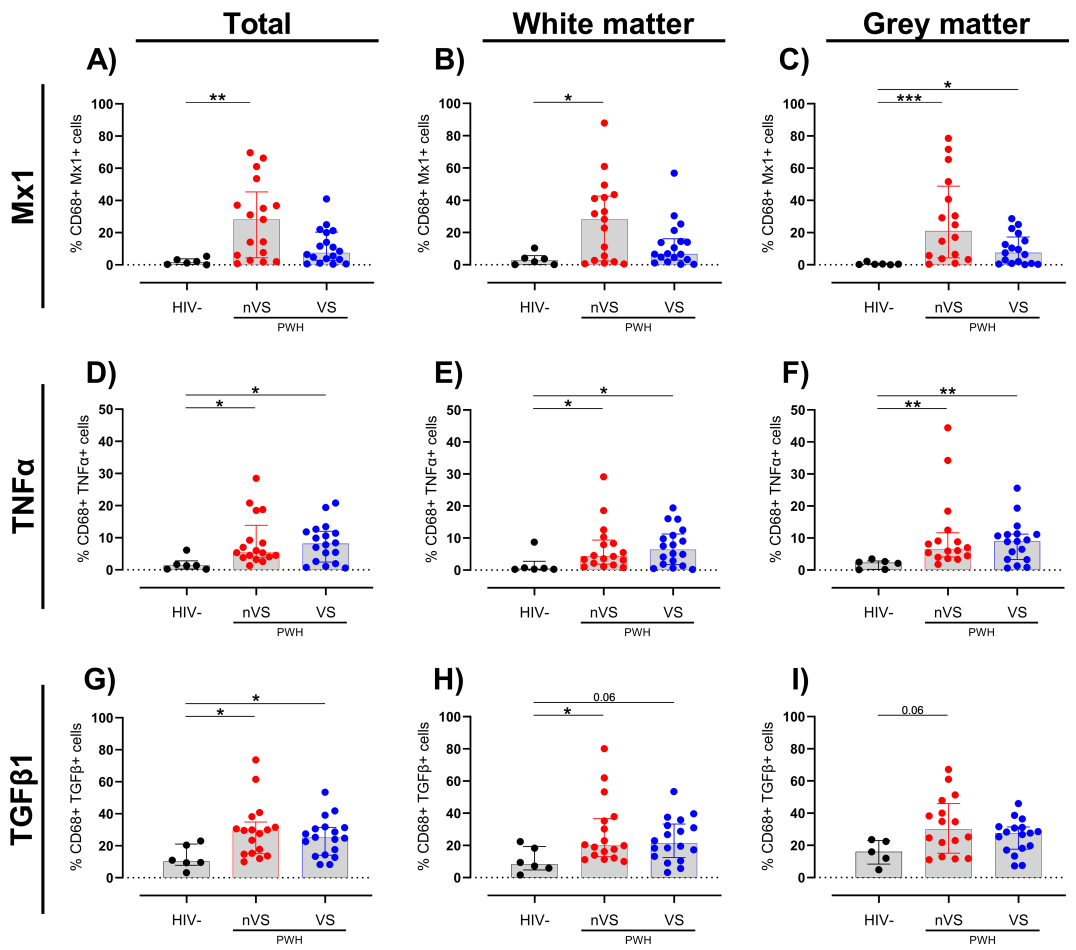

FIGURE 3
Non-virally suppressed and virally suppressed PWH have a higher frequency of myeloid cells expressing inflammatory markers in the frontal cortex. Frequency of **(A–C)** Mx1, **(D–F)** TNFα, and **(G–I)** TGF-β1 colocalized with CD68$^+$ myeloid cells as a percentage of total CD68$^+$ cells in total frontal cortex tissue, white matter or grey matter of non-virally suppressed (nVS; n = 16-17), virally suppressed (VS; n = 17–18) people with HIV and HIV-seronegative controls (HIV−; n = 5–6). Data unavailable for gray matter for n=1 HIV seronegative controls (TGF-β1 only), n=1 VS PWH, and n=1 nVS PWH due to no gray matter region present in the tissue section provided. Median and interquartile ranges shown. Comparisons made using Kruskal–Wallis test with Dunn's *post-hoc* tests (*p<0.05; **p<0.01; ***p<0.001).

seronegative individuals (p<0.05; Figure 4C). Additionally, a trend of increased frequency of Mx1+ astrocytes was present between VS PWH and HIV-seronegative individuals (p=0.060; Figure 4C), indicating that the gray matter is a site of elevated Mx1-expressing astrocytes in PWH.

An increase in TNFα-expressing astrocytes was observed in VS PWH (p<0.05; Figure 4D) relative to HIV-seronegative individuals in total frontal cortex tissue. In contrast to a consistently increased prevalence of TNFα-expressing myeloid cells across total, white, and gray matter in nVS and VS PWH (Figures 3D–F), a higher frequency of TNFα$^+$ astrocytes in nVS and VS PWH were found in the white matter (p<0.05 for both; Figure 4E), while TNFα$^+$ astrocytes were only elevated in VS PWH in the gray matter (p<0.05; Figure 4F).

Finally, the incidence of TGF-β1$^+$ astrocytes were significantly higher in total frontal cortex tissue from VS PWH (p<0.05; Figure 4G), and a trend to a higher frequency was observed in nVS PWH (p=0.062) relative to HIV-seronegative individuals. Following sub-region analysis, a higher frequency of TGF-β1$^+$ astrocytes was also observed in VS PWH (p<0.05; Figure 4H), and a trend was present in nVS PWH (p= 0.073) relative to HIV-seronegative individuals in the white matter. Similarly, the frequency of TGF-β1 + astrocytes were increased in both nVS and VS PWH compared to HIV-seronegative individuals (p<0.05 for both; Figure 4I).

3.4 Neuroinflammation in the frontal cortex of VS PWH is associated with total and 5′ defective HIV DNA in the brain

To determine whether cellular activation in the brain was associated with the level and type of HIV proviral DNA in the frontal cortex of PWH, the levels of intact, 3′ defective and 5′ defective HIV DNA were quantified by the intact proviral DNA assay (IPDA), and correlation analysis with measures of cellular activation was performed. Similar to our previous findings (10), the frontal cortex of all PWH in both nVS and VS groups contained similar levels of total HIV DNA (Supplementary Figure S2A). Intact proviral DNA was also present in the frontal cortex of both nVS and VS PWH

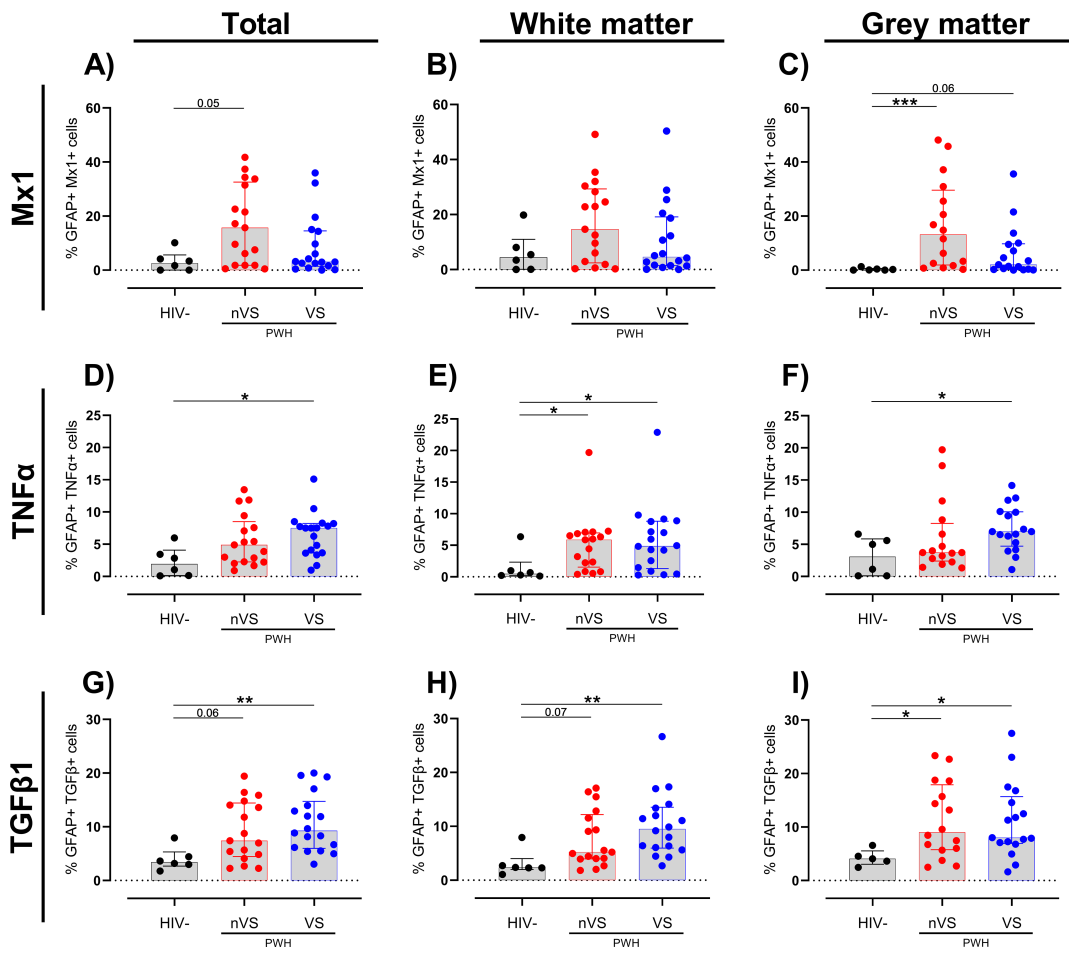

FIGURE 4
A higher frequency of activated astrocytes persists in the frontal cortex tissue from non-virally suppressed and virally suppressed people with HIV. Frequency of **(A–C)** Mx1, **(D–F)** TNFα, and **(G–I)** TGF-β1 colocalized with GFAP as a total of GFAP$^+$ cells in white and gray matter in non-virally suppressed (nVS; n=16–17), virally suppressed people with HIV (VS HIV; n=17–18) and HIV-seronegative controls (HIV-; n=5-6) as measured by multiplex immunofluorescence. Data unavailable for gray matter for n=1 HIV seronegative controls (TGF-β1 only), n=1 VS PWH, and n=1 nVS PWH due to no gray matter region present in the tissue section provided. Median and interquartile ranges shown. Comparisons made using Kruskal–Wallis test with Dunn's *post-hoc* tests (*p<0.05; **p<0.01; ***p<0.001).

(Supplementary Figure S2B) at similar levels, confirming that the brain tissue from both nVS and VS PWH harbored a reservoir of intact proviral HIV DNA that did not differ between groups. The level of total HIV proviral DNA was associated with Mx1$^+$ myeloid cells in the total frontal cortex tissue and also in white and gray matter regions alone when analyzed separately (p<0.05 for all, Table 2). Total HIV DNA was also associated with levels of Mx1$^+$ astrocytes in gray matter (ρ=0.507, p=0.040) and total frontal cortex tissue (ρ=0.510, p=0.039), with a trend observed in white matter (ρ=0.461, p=0.054; Table 2), supporting a relationship between levels of HIV proviral DNA and Mx1-expressing cells in the frontal cortex of VS PWH. Sub-analysis for intact, 3′ defective and 5′ defective proviral DNA, as measured by IPDA, demonstrated that levels of intact HIV proviral DNA correlated with those of total Mx1$^+$ cells in total frontal cortex and gray matter (p<0.05 for both), Mx1+ myeloid cells in all regions (p<0.05 for all) and Mx1$^+$ astrocytes in both total frontal cortex and gray matter (p<0.05 for both; Table 2). Importantly, levels of 5′ defective HIV DNA were associated with Mx1$^+$ myeloid cells in total frontal cortex, white and gray matter, and GFAP+ cells in total and white matter tissue (p<0.05 for all, Table 2), supporting an association between 5′ defective HIV proviruses in the brain of ART-suppressed PWH with Mx1-expressing myeloid cells and astrocytes in the frontal cortex tissue. Conversely, levels of 3′ defective HIV DNA did not correlate with Mx1, TNFα, or TGF-β1 expression in either white or gray matter (p>0.05 for all; Table 2). Total or intact HIV proviral DNA did not correlate with TNFα or TGF-β1 expression (p>0.05 for all; Table 2), indicating an alternative viral independent driver of cells expressing these proteins in VS PWH. Together, these findings indicate that the level of total and specifically 5′ defective HIV DNA in the frontal cortex tissue was associated with activation of anti-viral type I IFN signaling in VS PWH.

4 Discussion

Despite viral suppression with ART, PWH continue to develop comorbidities at a greater rate than seronegative individuals that

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TABLE 2 Association between HIV DNA in the frontal cortex and neuroinflammation in virally suppressed PWH.

	Mx1+ cells		TNFα+ cells		TGF-β1+ cells		Mx1+ myeloid		Mx1+ GFAP	
Grey matter	Rho	p-value	Rho	p-value	Rho	p-value	Rho	p-value	Rho	p-value
Intact HIV DNA	**0.577**	**0.017**	0.009	0.975	−0.221	0.391	**0.575**	**0.018**	**0.618**	**0.010**
3' defective HIV DNA	0.113	0.666	−0.382	0.131	−0.284	0.268	0.098	0.708	0.115	0.660
5' defective HIV DNA	0.463	0.063	−0.213	0.410	−0.306	0.231	**0.538**	**0.021**	0.466	0.062
Total HIV DNA	0.463	0.063	−0.086	0.744	−0.297	0.247	**0.523**	**0.026**	**0.507**	**0.040**
White matter										
Intact HIV DNA	0.418	0.084	0.187	0.458	−0.007	0.977	**0.522**	**0.026**	0.385	0.115
3' defective HIV DNA	0.212	0.399	−0.300	0.226	−0.269	0.280	0.152	0.548	0.189	0.453
5' defective HIV DNA	**0.562**	**0.015**	0.110	0.663	−0.152	0.548	**0.651**	**0.003**	**0.523**	**0.026**
Total HIV DNA	**0.498**	**0.035**	0.148	0.559	−0.189	0.453	**0.606**	**0.008**	0.461	0.054
Total Frontal Cortex										
Intact HIV DNA	**0.550**	**0.018**	0.090	0.722	−0.107	0.673	**0.572**	**0.013**	**0.488**	**0.040**
3′ defective HIV DNA	0.168	0.505	−0.432	0.073	−0.313	0.206	0.127	0.625	0.218	0.499
5′ defective HIV DNA	**0.554**	**0.017**	−0.051	0.842	−0.199	0.428	**0.510**	**0.039**	**0.507**	**0.040**
Total HIV DNA	**0.525**	**0.025**	−0.007	0.977	−0.222	0.376	**0.512**	**0.038**	**0.510**	**0.039**

Mx1, MX dynamin like GTPase; TGF-β1, transforming growth factor beta; TNFα, tumor necrosis factor alpha.
p-value and rho determined by non-parametric Spearman correlation ($p<0.05$ statistically significant).
Bold values indicate significance ($p<0.05$); underlined values indicate trend ($p>0.05$, <0.1).

significantly impact health and daily function. Neurocognitive issues and neuropathologies affect ~20% of VS PWH, reportedly leading to social withdrawal, reduction in cognitive function, and ability to perform daily tasks including using the internet, driving, and health navigation (24–26). The mechanisms causing disease are unclear but possibly relate to HIV persistence both in the brain and peripheral tissues that are associated with chronic inflammation that may lead to cellular activation. Utilizing a cohort of well-defined human autopsy brain tissue from nVS and VS PWH, we characterized cell activation and its relationship to local viral reservoirs in the brain. Specifically, a higher frequency of astrocytes and myeloid cells harboring activated IFN pathways and production of TNFα and TGF-β1 were observed, demonstrating an activated inflammatory state in the brain. Importantly, we identified that levels of total, intact, and 5′ defective proviruses in the frontal cortex of PWH were associated with increased measures of cellular activation.

In this study we observed an elevated frequency of Mx1-expressing cells in the gray matter of the frontal cortex of VS PWH relative to HIV-seronegative controls, which recapitulates our findings from ART-suppressed SIV-infected NHPs (18). Mx1 production is triggered via the Stat1 pathways following activation of IFNα/β receptors (IFNAR) by type I IFNs (i.e., IFNα/β) (27). Therefore, HIV infection in the brain results in IFN production leading to augmented expression of IFN stimulated genes (including Mx1) in infected and surrounding cells resulting in an elevated state of cellular activation and cognitive function. Type I IFNs including IFNα and β are traditionally associated with anti-viral-mediated effects both *in vitro* and *ex vivo*, and chronically elevated IFN signaling is also associated with the pathogenesis of some neuroinflammatory conditions (28, 29). Furthermore, a series of recent studies using human brain organoid models also observed increased Mx1 and interferon signaling in response to HIV infection despite ART suppression (30–32), supporting our *ex vivo* observations. *In vivo* studies using IFNAR knockout rodent models of neuroHIV have demonstrated an improved effect on cell damage and behavioral measures after knocking down IFN signaling (33), supporting a deleterious role of heightened interferon activation in the brain. Given the role of IFN signaling in mediating viral infection, uncontrolled peripheral viremia in nVS PWH may have a greater impact on the level of Mx1-expressing cells in the brain than local viral persistence alone, particularly in myeloid cells in nVS PWH. These findings support the further assessment of the impact of chronic IFN signaling on brain cells and cognitive outcomes in PWH.

Significantly, we found that the levels of the Mx1 in frontal cortex tissue correlated with levels of total, intact, and 5′ defective HIV proviruses, supporting a mechanism by which the local inflammatory environment influences viral persistence likely through elevated levels of cellular activation. Intact proviruses contain the necessary viral machinery for persistence, viral replication and, in the absence of ART, the generation of infectious virions as demonstrated by *ex vivo* studies from the brain tissue of both NHPs and humans (34). Defective proviruses, as characterized in this study by the IPDA, have been shown to represent replication incompetent viruses that may retain transcriptional activity and even produce select viral proteins that can contribute to cellular activation (35–37). 5′ defective viruses identified by Bruner et al. were defined as being

predominantly intact and predicted to be capable of producing the majority of HIV proteins, albeit at a lower level than intact virus (35). Therefore, the association between 5′ defective proviruses in the frontal cortex and Mx1 is unlikely driven by viral replication and instead may reflect the generation of viral transcripts and possible low-level production of viral proteins (37, 38), although these were not specifically assessed in this study. A study of defective proviruses missing the 5′ untranslated region demonstrated that 5′ defective proviruses were able to generate viral transcripts and proteins by using alternative reading frames (39), supporting a role of 5′ defective proviruses in modulating neuroinflammation. Therefore, our *ex vivo* findings from a cohort of autopsy brain tissue support the need for future studies to define the mechanisms governing cell activation in response to 5′ defective proviral DNA in the brain.

VS PWH had a higher level of TNFα-producing cells in the brain, which extends on previous observations in nVS PWH (40, 41), highlighting that even in the presence of ART, a higher proportion of TNFα-producing myeloid cells persists in the CNS. TNFα is a broadly proinflammatory cytokine known to activate and recruit microglia and astrocytes via TNF receptor 1 to sites of inflammation (42, 43). Plasma levels of TNFα are associated with adverse disease progression in untreated PWH (44), and plasma levels of soluble TNF receptor I (a surrogate measure of TNF-α) remain elevated in the plasma of virally suppressed PWH and are associated with HIV mortality independent of CD4+ T-cell counts (45, 46). A previous study in nVS PWH with dementia identified elevated levels of TNFα expression in the brain and astrogliosis (41, 47); however, we did not observe an association between levels of TNFα and the frequency GFAP$^+$ astrocytes in our cohort. Myeloid cells were the major producer of TNFα in VS PWH, as was previously observed in nVS PWH (40), and levels appeared to be distributed throughout both white and gray matter of the brain. While the frequency of total myeloid cells did not differ between groups, the frequency of cells expressing TNFα and Mx1 were higher in the brain of PWH, potentially reflecting cell activation rather than greater cellular recruitment. Similarly, the level of cells expressing the astrocyte activation marker GFAP+ and specifically GFAP+ cells expressing TNF-α were also higher in both white and gray matter of VS PWH, supporting activation of astrocytes throughout the frontal cortex of the brain. Given the role of astrocytes in maintaining homeostasis in the brain, chronic activation of astrocytes may lead to cell dysfunction and/or adverse cognitive outcomes in the brain of PWH. Furthermore, although neurons are not directly infected by HIV, astrocyte and microglial activation may have deleterious bystander effects on neurons through both release of viral proteins including tat and nef or through generation of proinflammatory cytokines (31, 48). We have previously demonstrated that HIV genomes and the viral protein p24 were detected in myeloid cells in the brain that may drive cellular activation (10, 21). However, here, we did not find an association between the levels of the viral reservoirs (cell associated HIV DNA) and measures of TNFα$^+$ myeloid cells. These findings may suggest that TNFα production in the brain is not solely due to local viral persistence in the brain; however, additional studies are required to define the mechanistic drivers of TNFα in the brain of virally suppressed PWH.

Interestingly, expression of TGF-β1 was higher in the frontal cortex of VS and nVS PWH, when compared to seronegative controls, reflecting our previous findings in SIV-infected ART-suppressed NHPs (18), and supports heightened immunoregulatory signaling in the brain during chronic HIV. TGF-β1 is a multifunctional immunosuppressive cytokine that can impact cell activation, proliferation, and apoptosis and has been demonstrated to activate astrocytes and promote glial scar formation in the brain following secretion by activated myeloid cells (49). The formation of fibrotic scars in the CNS has been implicated in neurological disorders and vascular dementia (50). Similarly, in lymph node tissue, TGF-β1-mediated fibroblast activation and the resulting fibrosis may facilitate local HIV replication and persistence and inflammation (51, 52). Furthermore, TGF-β1 has been shown to control HIV reservoir size, at least in the gut, via targeting cell proliferation and inducing apoptosis (53). Heightened TGF-β1$^+$ cells in the brain of nVS and VS PWH are also possibly a result of a direct response to heightened cellular activation and the proinflammatory environment observed in both nVS and VS PWH. While we did not observe an association between HIV proviral DNA and TGF-β1 expression, other factors including localized viral protein expression (54), systemic inflammation, and gut damage as seen in NHP models (18) may contribute to elevated TGF-β1 expression in the brain. The impact of gut dysfunction on neuroinflammation could not be assessed here but should be considered in specialized studies.

Associations between the level of proviral genomes and measures of neuroinflammation were limited to Mx1, with no correlation found between total, intact, or 3′ or 5′ defective HIV DNA and TNFα or TGF-β1. Therefore, it is important to recognize that other factors peripheral to the brain may contribute to neuroinflammation such as chronic gut damage, immune activation, and systemic inflammation, which are hallmarks of chronic HIV that are not resolved by ART and are associated with mortality (45). Measures of peripheral inflammation have also been associated with adverse cognitive performance (55–57), and we have previously demonstrated in SIV-infected NHPs a role for gut damage in contributing to neuroinflammation, supporting the contribution of other factors external to the brain on these parameters (18). It is also possible (and likely) that different combinations of ART regimens may have varying impacts on viral suppression and neuroinflammation in the brain. However, this study was not designed to address the impact of specific regimens, and this question may be better addressed in well-controlled SIV-infected NHP studies. Therefore, future studies in larger cohorts of both PWH and/or NHPs and organoid/organotypic brain tissue models are required to delineate alternate mechanisms and widespread impacts of both defective proviral HIV DNA and systemic inflammation on neuroinflammation.

Our study has significant clinical implications regarding the state of cellular activation and inflammation in the brain of VS PWH. We

found no association between measures of cellular activation or viral reservoir with age, drug penetrance into the CNS as determined by CNS penetrance score (20), or CD4+ T-cell count (Supplementary Table S1), potentially indicating that the markers of cellular activation and inflammation measured were not related to these clinical parameters. This is important, as biological age is associated with cellular and structural changes in the brain including cellular activation (58). Instead, in our cohort, we identified that both local (and likely peripheral) HIV reservoirs and related systemic inflammation are contributors to cellular activation in the brain. Understanding changes in cellular activation in the brain of VS PWH may inform targeted therapeutic approaches to limit neuroinflammation and anti-viral signaling in the brain. Furthermore, targeting HIV DNA and/or related transcription of intact and even 5′ defective viruses by transcriptional inhibitors or other novel targets silencing the viral reservoir may assist in limiting cellular activation in brain parenchyma. Therefore, approaches either directly or indirectly targeting cellular activation and inflammation in the brain may be required to improve brain health and function.

Together, in this study, we demonstrate the presence of chronic immune activation in the brain of VS PWH and a relationship between proviral HIV in the brain, including specifically 5′ defective proviruses, which may contribute to cellular activation and an underlying pathology in the brain that is not abrogated by ART. These findings offer key evidence for ongoing immune activation and viral persistence, highlighting a need to better understand defective proviruses as a contributor to neuroinflammation and cognitive disorders, which must be considered to improve brain health and cognitive function in VS PWH.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by RMIT University Human Ethics Research Committee. The studies were conducted in accordance with the local legislation and institutional requirements. The ethics committee/institutional review board waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin because Samples were provided from the National NeuroHIV Tissue Consortium biobank with ethics approval. No participants were specifically recruited for this study.

Author contributions

SB: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. JE: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. JZ: Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Writing – review & editing. EC: Data curation, Formal analysis, Investigation, Writing – review & editing. EW: Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. NO: Formal analysis, Writing – review & editing. TJ: Formal analysis, Writing – review & editing. MR: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Writing – review & editing. BB: Formal analysis, Investigation, Writing – review & editing. JE: Conceptualization, Formal analysis, Funding acquisition, Investigation, Resources, Supervision, Writing – review & editing. TA: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. MC: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2025.1570692/full#supplementary-material

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