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Long COVID and brain inflammation: unravelling mechanisms and potential therapies

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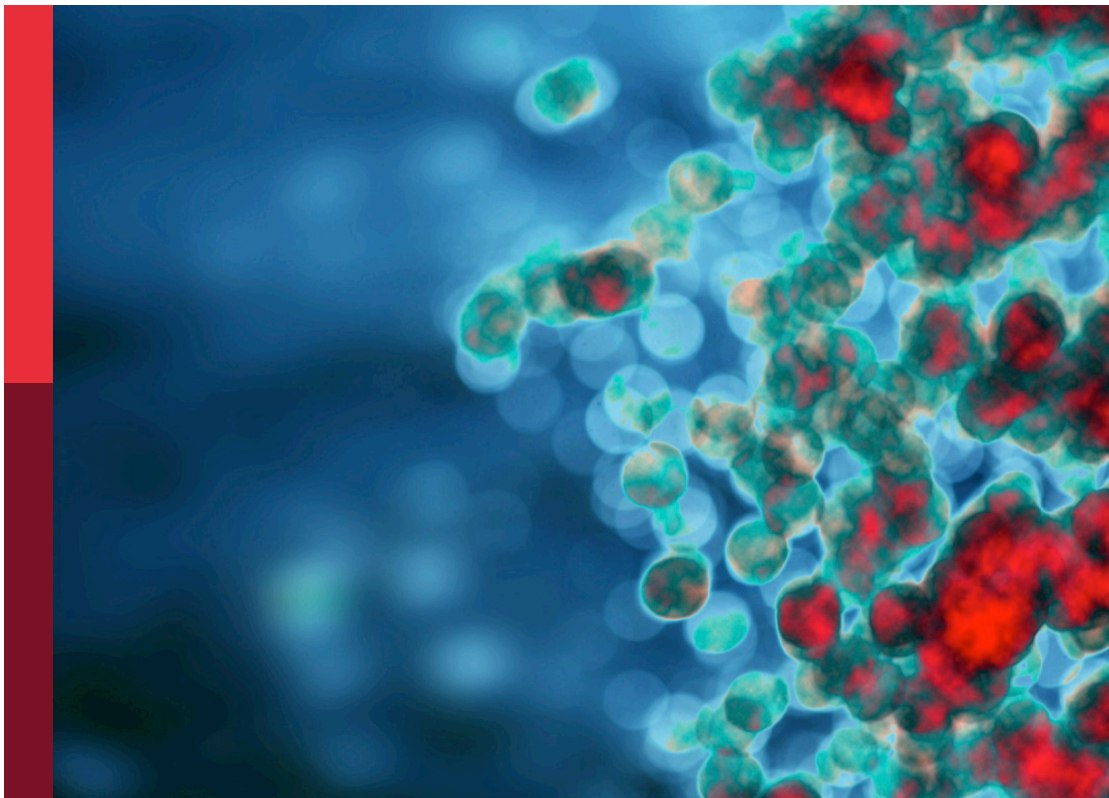

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Long COVID and brain inflammation: unravelling mechanisms and potential therapies

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Editorial: Long COVID and brain inflammation: unravelling mechanisms and potential therapies

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Editorial on the Research Topic

Long COVID and brain inflammation: unravelling mechanisms and potential therapies

Patients with Long COVID often experience persistent brain-related symptoms, including brain fog, mood changes, and dizziness (1–3), likely driven by neuroinflammation even after the virus is cleared. Imaging studies have shown structural and functional changes that indicate ongoing inflammation in the Long COVID brain. The biological mechanisms underlying these symptoms are still not fully understood, and research continues to identify effective therapies to improve both the physical and mental health of those affected (2).

In this Research Topic, we explore research on Long COVID and its investigation through preclinical animal models.

Missailidis et al. conducted RNA-Seq analysis of peripheral blood mononuclear cells (PBMCs) from individuals with Long COVID and those who had fully recovered COVID-19. Their findings revealed upregulation of ICOS and S1PR1, suggesting a persistent pro-inflammatory state (Figure 1A), as these genes are involved in immune cell survival and signaling. They also observed downregulation of LILRB1 and LILRB2, highlighting immune dysregulation as a distinguishing feature of Long COVID.

Lee et al. analyzed the expression of long noncoding RNAs (lncRNAs) in the brains of COVID-19 patients, identifying hundreds of differentially expressed lncRNAs compared to age- and sex-matched uninfected controls. Many of these lncRNAs correlated with cognitive decline and increased inflammation, aligning with cognitive dysfunction observed in Long COVID. These findings suggest a potential role for lncRNAs in the neurological effects of COVID-19, highlighting a key area for further research.

Noonong et al. proposed that mitochondrial dysfunction may play a crucial role in Long COVID, linking it to diabetes and oxidative stress (Figure 1A). Their hypothesis highlights mitochondria's role in inflammation and metabolic homeostasis, suggesting broader systemic implications for viral recovery and chronic symptoms.

Hanafy and Jovin emphasized the role of chronic inflammation in exacerbating neurological symptoms commonly seen in patients with Long COVID, such as brain fog, extreme fatigue (asthenia), and depression- a condition they refer as "Brain FADE syndrome" (Figure 1A). They advocate for an integrated treatment approach that targets both inflammatory pathways and associated mental health challenges.

Gu et al. investigated sex differences in COVID-induced autoimmunity and neurological effects, highlighting how female sex hormones and X-chromosome factors may increase women's susceptibility to Long COVID (Figure 1B). They proposed that COVID-19 may disturb immune tolerance, leading to autoantibodies infiltration into the central nervous system. They also suggested that COVID-19 could disrupt the female microbiome, contributing to neural damage, including demyelination, neuroinflammation, and neurodegeneration. Understanding these sex differences could help develop strategies to mitigate COVID-related neurological injuries.

Bustamante et al. explored risk factors for developing Long COVID, including a history of severe illness and intensive care. Using preclinical animal models, they examined the involvement of the nervous system in inflammation through the psychoneuroimmunoendocrine axes. They proposed that Long COVID involves peripheral and central sensitization, leading to dysregulation and chronic inflammation, and discussed therapeutic strategies to modulate these inflammation responses.

Dai et al. reviewed the utility of preclinical animal models in understanding Long COVID. They highlighted key features replicated in these models, including lung fibrosis, hyperglycemia, and neurological sequelae, while acknowledging limitations such as restricted genetic diversity and challenges in modeling Long COVID pathology. To improve translational relevance, they proposed incorporating genetically diverse populations, conducting longitudinal studies, and aligning animal findings with clinical data.

Singh et al. investigated the Long COVID implications of the Delta variant using K18-hACE2 mice. They found robust inflammatory responses linked to neuropsychiatric symptoms and

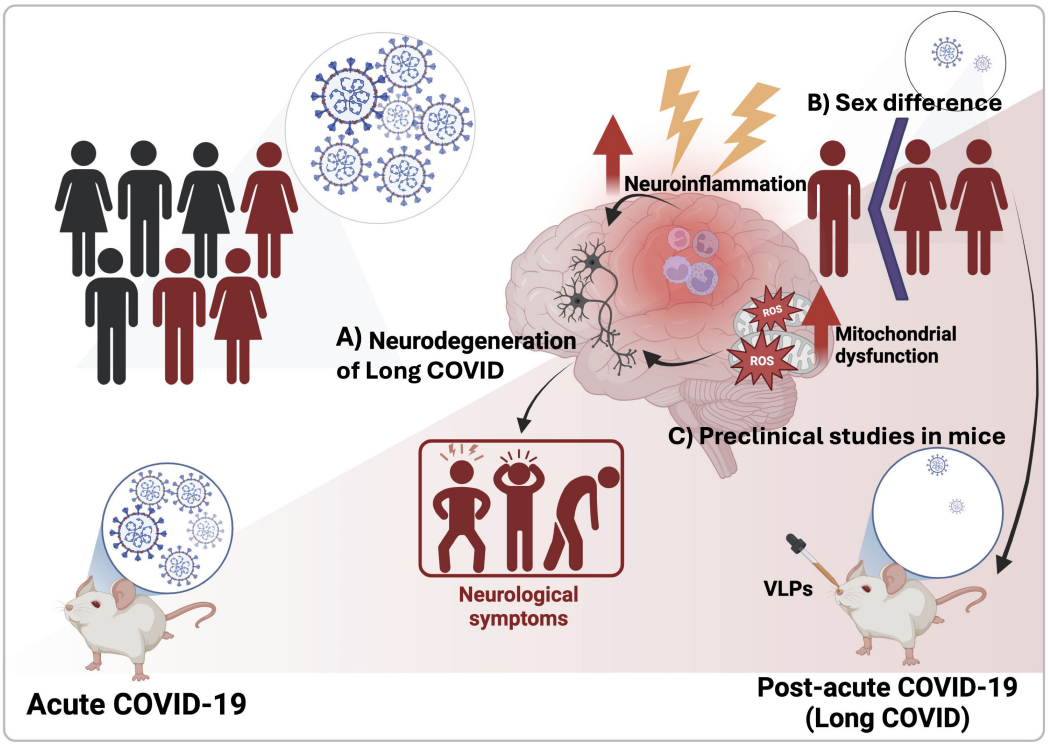

FIGURE 1
Neurological consequences of Long COVID. **(A)** In Long COVID, persistent neuroinflammation and mitochondrial dysfunction are implicated in ongoing neurodegeneration. **(B)** Certain populations, particularly females, are more susceptible due to hormonal and immune factors. **(C)** Preclinical models, including mice exposed to virus-like particles (VLPs), are being used to investigate the effects of Long COVID on the nervous system. These studies have linked neuroinflammation to behavioral disruptions, highlighting the need for further research into the neurological impacts of viral infections, particularly in Long COVID.

motor behavior changes during acute infections, with persistent immune activation post-infection. Post-acute infection, the brain showed no detectable viral RNA and minimal residential immune cell activation in surviving mice. However, transcriptome analysis revealed persistent activation of immune pathways, including humoral responses, complement, phagocytosis, along with gene expression linked to ataxia telangiectasia, impaired cognitive function, and neuronal dysfunction. Surviving mice exhibited strong neutralizing antibodies against both Delta and Omicron variants, months after the infection.

O'Niel et al. used virus-like particles (VLPs) expressing SARS-CoV-2 structural proteins (nucleocapsid (N), membrane (M), envelope (E) and spike (S), in human apolipoprotein E (apoE)-targeted replacement mice (Figure 1C). The study found apoE isoform-dependent effects on behavioral measures, with E2 mice more affected than E3 or E4 mice, despite E2 being linked to a lower Alzheimer's disease risk. VLPs also caused behavioral and circadian disruptions independent of apoE isoform, even in the absence of viral replication. Increased susceptibility in E2 mice was associated with elevated hippocampal CCL11, similar to CCL11 elevations seen in humans with cognitive symptoms after COVID-19 exposure. The authors emphasize the need for further research to better understand and treat neurological conditions associated with viral infections, especially as many continue to struggle with Long COVID.

The ongoing research discussed in this Research Topic reveals significant progress toward understanding the biological mechanisms of Long COVID. As we continue to uncover the complexities of the condition, further preclinical and clinical studies are critical for improving the well-being and brain function of those affected by Long COVID and other related neurological conditions.

Author contributions

FS: Writing – original draft, Writing – review & editing. JR: Writing – original draft, Writing – review & editing. KA: Writing – review & editing.

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Neurological risks of COVID-19 in women: the complex immunology underpinning sex differences

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The COVID-19 pandemic has uncovered many mysteries about SARS-CoV-2, including its potential to trigger abnormal autoimmune responses. Emerging evidence suggests women may face higher risks from COVID-induced autoimmunity manifesting as persistent neurological symptoms. Elucidating the mechanisms underlying this female susceptibility is now imperative. We synthesize key insights from existing studies on how COVID-19 infection can lead to immune tolerance loss, enabling autoreactive antibodies and lymphocyte production. These antibodies and lymphocytes infiltrate the central nervous system. Female sex hormones like estrogen and X-chromosome mediated effects likely contribute to dysregulated humoral immunity and cytokine profiles among women, increasing their predisposition. COVID-19 may also disrupt the delicate immunological balance of the female microbiome. These perturbations precipitate damage to neural damage through mechanisms like demyelination, neuroinflammation, and neurodegeneration – consistent with the observed neurological sequelae in women. An intentional focus on elucidating sex differences in COVID-19 pathogenesis is now needed to inform prognosis assessments and tailored interventions for female patients. From clinical monitoring to evaluating emerging immunomodulatory therapies, a nuanced women-centered approach considering the hormonal status and immunobiology will be vital to ensure equitable outcomes. Overall, deeper insights into the apparent female specificity of COVID-induced autoimmunity will accelerate the development of solutions mitigating associated neurological harm.

KEYWORDS

COVID-19, women, autoimmunity, neuroinflammation, X-chromosome

1 Introduction

The COVID-19 pandemic, caused by the novel coronavirus SARS-CoV-2 has been associated with autoimmune responses in some patients. Autoimmunity arises when the immune system loses tolerance to self-antigens and produces autoantibodies attacking host tissues (1). Both cellular and humoral autoimmune reactions have been described in COVID-19 patients (2, 3). For example, anti-interferon autoantibodies capable of impairing antiviral responses are detected in approximately 10.2% of severe COVID-19 cases (4). Additionally, SARS-CoV-2 binding to tissue antigens may induce cross-reactivity of immune cells and subsequent autoimmune damage in organs like the Livers and nervous system (5–7). Understanding the autoimmune aspects of COVID-19 is crucial, as they may exacerbate disease severity and cause prolonged symptoms in recovered patients.

Emerging evidence suggests that COVID-19 can trigger autoimmune responses through several mechanisms. Viral infections often provoke autoimmunity via molecular mimicry, wherein viral antigens resemble self-antigens (8). SARS-CoV-2 proteins may share sequences or structures with host proteins, leading to cross-reactivity of antibodies or T cells (5). Additionally, the severe inflammation and cytokine storm induced by COVID-19 may cause the breakdown of self-tolerance. Elevated levels of cytokines like IL-6, IL-17, and TNF-α can stimulate auto-reactive lymphocytes (9–11). SARS-CoV-2 infection can also prompt neutrophil extracellular trap (NET) formation and release of danger-associated molecular patterns (DAMPs), further enhancing immune dysregulation (12, 13). Identifying the pathways leading to autoimmunity following COVID-19 is imperative to improve understanding and management of the disease.

Autoimmune responses have been shown to impact and damage both the central and peripheral nervous systems. Autoimmune diseases like multiple sclerosis, Guillain-Barre syndrome, involve neural inflammation, demyelination, and neurodegeneration (14, 15). Autoantibodies can also directly attack neurons and synaptic connections, disrupting neural signaling (16). Moreover, increasing evidence indicates that acute infections can also elicit autoantibodies to cause cross-reactivity against the nervous system (17). Therefore, the autoimmune reactions associated with COVID-19 infection may similarly lead to neurological damage. Elucidating how autoimmunity induced by SARS-CoV-2 affects the nervous system is critical for managing neurological sequelae.

COVID-19 may disproportionately impact the female nervous system. While there is no evidence that women infected with SARS-CoV-2 have higher rates of acute neurological complications like stroke, female sex has been identified as an independent risk factor for developing long COVID syndromes (18, 19). Additionally, neuropsychiatric symptoms such as anxiety and depression are commonly reported neurological manifestations of long COVID, with a higher proportion of women experiencing these symptoms compared to men (19). The mechanisms underlying this female propensity for neuro-COVID sequelae are still unclear. However, sex differences in immune responses and hormonal influences may contribute to increased susceptibility of the female nervous system to long-term damage mediated by COVID-19. Moreover, autoimmune diseases like multiple sclerosis and rheumatoid arthritis that affect the nervous system are more prevalent in women (20–22).

2 Autoimmunity and nervous system

Contemporary research has uncovered dynamic interplays between the central nervous system (CNS) and the immune system, challenging the erstwhile notion of CNS immune privilege (23). Specific niches such as the choroid plexus, meninges, and perivascular spaces, along with the meningeal lymphatic system and skull microchannels, facilitate ongoing communication between the brain and the immune system, essential for CNS maintenance, function, and repair (23, 24). The blood-brain barrier (BBB), constituted by tightly connected brain endothelial cells, still plays a crucial role in limiting the entry of pathogens and activated immune cells into the CNS, thereby protecting neurons (25). In parallel, intrinsic brain cells like microglial cells and astrocytes monitor pathogen invasion and tissue damage, initiating moderate neuroinflammatory responses when necessary (26). This nuanced immune interaction forms a balanced immune environment essential for maintaining CNS homeostasis (27). However, infections have the potential to disrupt the balanced immune interaction by introducing inflammatory factors from outside the CNS, which might alter the permeability of the BBB, potentially allowing peripheral immune cells to enter the CNS (28, 29). The influx of inflammatory cells and cytokines into brain tissue can activate glial cells and trigger neuroinflammation (28). This may lead to autoantigen exposure and loss of immune tolerance, which in turn produces autoreactive T cells and antibodies that attack neural tissues. Particularly, the interaction between SARS-CoV-2 and the CNS could exacerbate these responses, potentially leading to severe neurological complications.

2.1 Mechanisms of autoimmunity against the nervous system

Molecular mimicry, a well-documented mechanism for infection-induced autoimmunity, arises from amino acid sequence or structural similarities between pathogen components and host proteins, leading to cross-reactivity of antibodies and T cells (8). This has been demonstrated for several neurotropic viruses, wherein immune cells primed by the virus later erroneously recognize and attack similar epitopes on nervous system antigens (30, 31). Analogously, molecular mimicry between SARS-CoV-2 and neuronal proteins is hypothesized to facilitate COVID-19 associated autoimmune neuropathology. The S1 protein of the SARS-CoV-2 spike has been found to share sequence homology with a number of CNS proteins, though cross-reactivity remains to be confirmed experimentally (32).

The severe inflammation and cytokine storm associated with severe COVID-19 may also promote autoimmune reactivity against the nervous system. Elevated levels of pro-inflammatory cytokines like IFN-γ, TNF-α, IL-6 and IL-17 can activate self-reactive T cells and B cells that have escaped tolerance mechanisms (33, 34). Numerous neurologic autoimmune diseases demonstrate associations with such pro-inflammatory cytokines. For instance, IFN-γ and IL-17 have been implicated in multiple sclerosis, with therapeutic blockade of these cytokines conferring benefits (35–37). Dampening the excessive inflammation in critical COVID-19 cases may help mitigate collateral autoimmune damage to the nervous system.

Cross-reactivity of immune cells primed by SARS-CoV-2 with CNS antigens provides another avenue for COVID-19 associated autoimmunity. The virus binding to ACE2 receptors on the surface of nervous system cells may prompt the formation of anti-SARS-CoV-2 antibodies or T cells capable of recognizing similar host cell surface features (32). Additionally, damage and release of sequestered CNS proteins due to viral infection can expose neo-epitopes and trigger new autoreactive clones (8, 38). For instance, immune responses targeting the virus nucleocapsid protein were found to also cross-react with host small nuclear ribonucleoprotein particles in the brain (39, 40). Identifying such potentially cross-reactive immune targets would enable a more accurate evaluation of autoimmune risk following COVID-19.

2.2 Nervous system dysfunction caused by autoimmunity

Autoimmune reactions can trigger neuroinflammation that impairs nervous system function. Infiltration of autoreactive T cells and autoantibodies activating microglia can establish chronic inflammatory foci in the brain and spinal cord (41). Enhanced levels of inflammatory cytokines disrupt neuronal signaling and alter neurotransmitter levels, while also weakening the blood-brain barrier (42, 43). Similar neuroinflammation is thought to underlie some neurological symptoms of long COVID, as autoimmunity triggered by SARS-CoV-2 persists even after viral clearance (44). Imaging studies in these patients have revealed microstructural changes in brain regions that regulate emotion, memory and cognition - aligning with symptoms like brain fog.

Demyelination due to autoimmune targeting of myelin sheaths is another major mechanism of nervous system damage. Destruction of myelin insulation around axons by autoantibodies and autoreactive lymphocytes leads to slow nerve conduction and neurological deficits (45, 46). Demyelinating diseases like multiple sclerosis and acute disseminated encephalomyelitis often follow viral or bacterial infections, via mechanisms like molecular mimicry (47). Although demyelination has not yet been specifically examined in neuro-COVID, it represents a plausible pathological consequence of COVID-19 induced autoimmunity. Demyelinating autoantibodies or T cells arising from cross-reactivity with SARS-CoV-2 components could manifest in neurological sequelae like fatigue, numbness and nerve pain.

Autoimmune-mediated neurodegeneration is characterized by progressive loss of neurons and neural networks. The binding of autoreactive antibodies to neurons, prion-like misfolding of proteins triggered by autoimmunity, and indirect damage via inflammation can all promote neurodegeneration (48, 49). Viral infections are often considered triggers for such pathology, as seen in HIV-associated dementia and post-encephalitic Parkinsonism (50, 51). Though not yet conclusively demonstrated, the long-term persistence of inflammation and autoimmunity associated with COVID-19 raises concerns about the risk of insidious neurodegenerative changes. Monitoring biomarkers and imaging indicators of neurodegeneration will be important among recovered COVID-19 patients, especially those reporting neurological complaints.

2.3 Glial cell and vascular damage induced by autoimmunity

Autoimmune responses triggered by COVID-19 may also contribute to nervous system injury by targeting glial cells and blood vessels. Autoantibodies binding astrocytes and oligodendrocytes can disrupt the homeostasis and function of these glial cells, which support and interact with neurons (52). Anti-endothelial cell autoantibodies can promote apoptosis of vascular endothelial cells lining the blood-brain barrier, leading to microhemorrhages and facilitating neuroinflammation, and although this increase is uncommon in infected patients in the acute phase of COVID-19, it cannot be ruled out in long COVID (53). A study indicates patients with COVID-19 associated encephalopathy were found to have autoantibodies binding components in the brain, though specific targets remain unclear (54). Further research is still needed to confirm and characterize autoimmune mediated damage to glial cells, neurons and the vasculature in SARS-CoV-2 infection. Understanding the mechanisms of COVID-19 elicited autoimmunity warrants urgent investigation to guide the management of neurological sequelae.

3 COVID-19, autoimmunity and female specificity

3.1 Sex differences in COVID-19-induced autoimmunity

Emerging evidence indicates that women exhibit distinct autoimmune responses following COVID-19 compared to men. Female COVID-19 patients were found to have higher frequencies of various autoantibodies like antiphospholipid antibodies (55). Furthermore, women tend to have more robust antibody reactions to the SARS-CoV-2 spike protein antigen compared to males (21, 56). These early findings suggest that female-specific factors may promote augmented autoimmunity following COVID-19 infection. Understanding the mechanisms underlying this sex bias could have implications for the diagnosis, monitoring, and

management of neurological sequelae that may persist longer in women.

3.2 Possible mechanisms of female specificity

Sex hormones like estrogen, progesterone and testosterone can modulate immune responses and may contribute to the heightened autoimmunity seen in female COVID-19 patients. Estrogen tends to promote more vigorous humoral immunity and antibody production compared to androgens like testosterone (57). The cyclic fluctuations in estrogen and progesterone levels over the menstrual cycle also drive periodic changes in immune activity (58). Therefore, hormonal differences in females may create a pro-inflammatory cytokine milieu and enhanced B cell responses conducive to developing autoreactive antibodies after viral infections like COVID-19 (21, 22). Further research is required to delineate the complex interplay between sex hormones and autoimmunity following SARS-CoV-2 infection.

The increased copy number of X chromosomes in females has also been implicated in the sexual dimorphism of autoimmunity. Several immune-related genes are located on the X chromosome, including TLR7 involved in antiviral responses (59–61). Higher expression of such genes in females due to X-chromosome mosaicism may promote stronger inflammatory reactions to viruses like SARS-CoV-2 (62, 63). Additionally, X chromosome inactivation is a complex process that generally helps achieve dosage compensation between XX females and XY males (64, 65). However, this process is imperfect and can lead to minor differences in gene expression between sexes (66). These subtle expression differences likely contribute to increased autoimmunity in females (66), the potential mechanisms underlying female-specific risks of COVID-19 induced autoimmunity show in Figure 1. These X-linked effects likely interact with hormonal influences to create a female-specific risk profile for developing autoantibodies and dysregulated immunity after COVID-19 infection.

Differences in the gut and reproductive tract microbiota between males and females could also help explain the increased COVID-19 associated autoimmunity in women. The female microbiome exhibits a greater abundance of certain bacteria that shape immune function (67). Dysbiosis of the female microbiota is also associated with higher risks of autoimmunity (68). For instance, bacterial genera such as *Alistipes*, *Akkermansia*, *Eggerthella*, *Blautia*, *Pseudoflavonifractor*, *Anaerotruncus*, and *Clostridium*, among others, are found to be more prevalent in females (69). Specifically, *Akkermansia muciniphila* and *Eggerthella lenta* have been implicated in the modulation of immune responses, with the former potentially having negative effects on certain neurological/autoimmune diseases like Multiple Sclerosis (70), and the latter promoting Th17 cell activation, thus exacerbating colitis and being enriched in various autoimmune diseases including Inflammatory Bowel Disease (IBD) (71, 72). Similarly, *Anaerotruncus colihominis* has been associated with the severity of Experimental Autoimmune Encephalomyelitis (EAE) in mice, a model for Multiple Sclerosis (73). The perturbation of the delicate balance of microflora by SARS-CoV-2 infection in susceptible individuals (74, 75), could further exacerbate this scenario, potentially leading to heightened autoimmune pathology preferentially in females (74, 76). Further characterization of the sexual dimorphism in microbiota composition and function could elucidate the role of the microbiome in the sex-biased autoimmune outcomes of COVID-19 observed clinically.

3.3 Observed neurological symptoms in COVID-19 female patients

A recent prospective cohort study of patients with mild COVID-19 found that female patients reported more neurologic and neuropsychiatric symptoms, like cognitive deficits, headaches, and hyposmia compared to males (77). However, the study did not compare COVID-19 patients to a control group without COVID-19, thus it remains unclear whether the observed sex differences represent increased neuro-autoimmunity, specifically caused by COVID-19 in women (77). Several factors like autoantibody-mediated microvascular damage may underlie the sex differences in neurological manifestations of COVID-19 (78), a retrospective study also found female patients had a higher frequency of certain neurological post-COVID symptoms, though mechanisms need further study (79). More research is still needed to elucidate the pathological mechanisms and determine if female sex is truly a risk factor for neurological sequelae after COVID-19 infection (77).

4 Perspective and future directions

The apparent female predisposition for neurological complications and autoimmunity following COVID-19 highlights the need to incorporate sex-based analyses into ongoing and future studies. All aspects of COVID-19 research should include female-specific cohorts to delineate differences in disease course, pathogenesis, and outcomes between the sexes (80). Mechanistic studies should aim to uncover the immunological, hormonal, genetic, and microbial factors driving the distinct female neuro-COVID manifestations (21). Such research efforts will enable more precise clinical monitoring, prognostication, and management tailored to female patients during acute infection and through long COVID (81). Overall, intentionally embracing female inclusivity and sex-comparisons in the basic, translational and clinical science of COVID-19 will ensure equitable biomedical progress for women.

The sex-specific characteristics of COVID-19 autoimmunity and neurological sequelae warrant the development of tailored therapeutic strategies for women. Hormonal modulation to stabilize immune dysregulation in female patients represents one approach (82). Personalized immunomodulators based on a woman's menstrual cycle stage or menopause status may also

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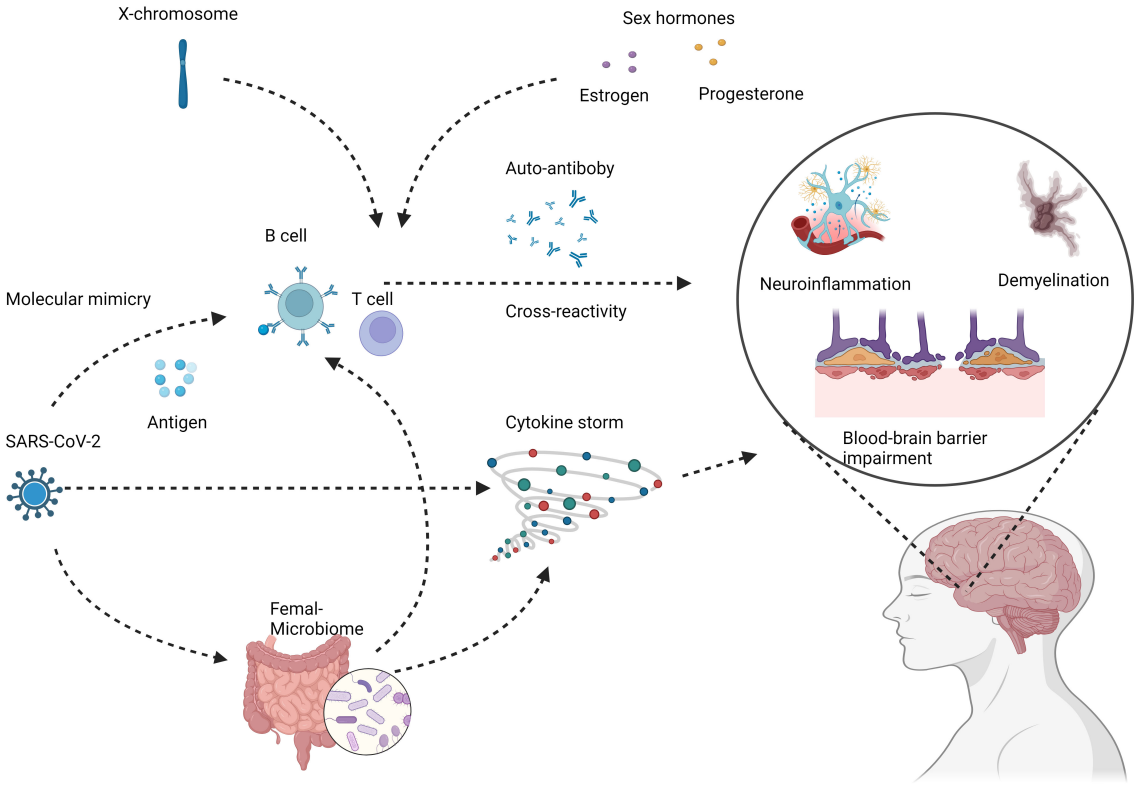

FIGURE 1
Potential mechanisms underlying female-specific risks of COVID-19 induced autoimmunity. COVID-19 infection can lead to loss of immune tolerance and production of autoreactive immune cells and antibodies through mechanisms like molecular mimicry and cytokine storm. These aberrant immune responses can target components of the nervous system, including neurons, glial cells, and the blood-brain barrier. The subsequent autoimmune attack manifests as neuroinflammation, demyelination, and neurodegeneration, potentially increasing the risks of chronic neurologic conditions like multiple sclerosis. Female COVID-19 patients demonstrate distinct immune dysregulation features such as higher autoantibody levels, skewed cytokine profiles, and enhanced B cell reactivity. Contributing factors likely involve sex hormones, X chromosome effects, and microbiome alterations. These specifically predispose women to COVID-induced autoimmunity that damages the nervous system. Created with BioRender.com.

prove beneficial (83, 84). Additionally, gut microbiome modification and pre/probiotics could help counteract any COVID-19 induced dysbiosis known to enable autoimmunity, which appears to preferentially affect the female microbiota (85, 86). Repurposing approved treatments for autoimmune illnesses with female predominance may provide faster solutions. Ultimately, any immunomodulatory or neuroprotective approaches to managing long COVID should consider female-specific metrics and mechanisms given the apparent sexual dimorphism of the underlying pathology.

The care of female COVID-19 patients should account for their potentially heightened risk of autoimmune-mediated neurological sequelae (87). Clinicians should maintain a high index of suspicion for neuropsychiatric symptoms and autoimmunity in women after COVID-19 (88). Extended monitoring for signs of neuroinflammation, subclinical autoantibodies and thrombotic markers may enable earlier intervention (89). Telemedicine can enhance access to appropriate care while remote patient-reported tracking of symptoms enables personalized evaluation. Overall, a nuanced clinical approach conscious of sex differences will be key to improving long-term outcomes in female COVID-19 survivors.

5 Conclusion

In summary, COVID-19 can elicit autoimmune responses that appear to disproportionately affect the female nervous system. The infection triggers elevated pro-inflammatory cytokines, autoreactive lymphocytes, and autoantibodies that can bind to or cross-react with neural antigens (17). Elevated cytokines, autoreactive immune cells, and autoantibodies triggered by the infection could potentially bind neurons and glial cells, leading to damage like neuroinflammation and demyelination (90, 91). Female-specific factors such as hormones, X chromosome effects, and microbiota composition likely interact to heighten COVID-induced autoimmunity (22). Elucidating the sex differences in COVID-19 pathogenesis and neurological outcomes remains an urgent priority. Characterizing the responsible mechanisms will pave the way for potential immunomodulatory treatments and female-centered clinical monitoring to improve long-term neural health after COVID-19.

COVID-19 can trigger autoimmune responses that damage the nervous system (92). Some early studies have reported more neurological symptoms in female COVID-19 patients compared

to males (93–95). Mechanisms like molecular mimicry and cytokine storm may enable COVID-autoimmunity (32). Female-specific factors including hormones and X-chromosome effects likely contribute (22). Understanding female-specific risks is vital to guide monitoring and therapies for neuro-COVID (96). Overall, the perspective emerging from early COVID-19 studies indicates that the interplay between SARS-CoV-2 infection and autoimmunity may pose a particularly insidious threat to the female nervous system. Further research intentionally examining female specificity in pathogenesis and outcomes will be crucial going forward.

Looking ahead, further investigation of sex-specific neurological effects of COVID-19 is imperative. Longitudinal studies tracking female patients will uncover the longer-term autoimmune impacts of the illness. Mechanistic research should delineate the contributions of hormonal fluctuations, X chromosome effects and immunological factors underlying the female predisposition (22). Multidisciplinary collaborations between neuroscientists, immunologists and clinicians will be key to unraveling the complex pathogenesis (97). Ultimately, insights from sex-based analyses can inform prognostic models to identify women at heightened risk of neurologic sequelae post-COVID. They may also guide clinical decision-making on the optimal timing and choice of immunomodulatory interventions to mitigate chronic neurologic damage in the expanding population of female COVID-19 survivors. An intentional focus on addressing female-specific risks will accelerate progress in improving the lives of women affected by this devastating illness.

Data availability statement

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

Author contributions

JG: Writing – original draft, Writing – review & editing. JZ: Writing – original draft, Writing – review & editing. QL: Writing – original draft, Writing – review & editing. SX: 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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Mitochondrial oxidative stress, mitochondrial ROS storms in long COVID pathogenesis

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Significance: This review discusses the coronavirus disease 2019 (COVID-19) pathophysiology in the context of diabetes and intracellular reactions by COVID-19, including mitochondrial oxidative stress storms, mitochondrial ROS storms, and long COVID.

Recent advances: The long COVID is suffered in ~10% of the COVID-19 patients. Even the virus does not exist, the patients suffer the long COVID for even over a year, This disease could be a mitochondria dysregulation disease.

Critical issues: Patients who recover from COVID-19 can develop new or persistent symptoms of multi-organ complications lasting weeks or months, called long COVID. The underlying mechanisms involved in the long COVID is still unclear. Once the symptoms of long COVID persist, they cause significant damage, leading to numerous, persistent symptoms.

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Future directions: A comprehensive map of the stages and pathogenetic mechanisms related to long COVID and effective drugs to treat and prevent it are required, which will aid the development of future long COVID treatments and symptom relief.

KEYWORDS

COVID-19, oxidative stress, mitochondrial ROS storms, long Covid, mitochondria

1 Introduction

Coronavirus disease 2019 (COVID-19) was first reported in Wuhan, China, in late December 2019. After the emergence of SARS-CoV-2 infections in December 2019 (1), the detailed symptoms were introduced in February 2020 by Huan et al. (2), Chen et al. (3), and Chan et al. (4). Lu et al. (5) concluded that the 2019-nCoV was a new human-infecting beta-coronavirus, sufficiently differing from SARS coronavirus (SARS-CoV). The COVID-19 pandemic has spread in the whole world. Yang et al. (6) compared the time course of the 2003 SARS pandemic and the 2020 novel coronavirus epidemic in China; the two diseases followed a similar course of events, although the number of cases was relatively limited in China during the 2003 SARS pandemic–the pneumonia outbreak associated with a new coronavirus of probable bat origin (7). SARS-CoV 2, Pangolin-CoV, SARS-CoV, Middle East respiratory syndrome CoV, and Bat-CoV viruses evolve quickly (8, 9). The first symptom reported for COVID-19 was pneumonia (10). This represents COVID-19 infection started from a respiratory tract infection that included fever, dizziness, and cough (11). Several variants of COVID-19, namely Alpha, Beta, Gamma (12), Delta (12–14), and Omicron (15, 16), have resulted in subsequent outbreaks in many countries worldwide. Despite improvements in the management of COVID-19, severe infection cases and COVID-19-related fatalities still occur. Presumed hospital-related transmission of COVID-19 was suspected in 41% of patients, 26% of patients received ICU care, and mortality was 4.3% (17). In addition, there is substantial concern regarding a complication known as long COVID-19.

Sudreres et al. (18) analyzed data from 4,182 COVID-19 cases and reported that the number of long COVID-19 cases was 558 (13.3%) participants reporting symptoms lasting ≥28 days, 189 (4.5%) for ≥8 weeks, and 95 (2.3%) for ≥12 weeks. They also reported that long COVID is characterized by symptoms of fatigue, headache, dyspnea, and anosmia and is likely associated with factors such as increasing age, increasing body mass index, and female sex (18). Moreover, patients experiencing more than five symptoms during the first week of illness were more likely to experience long COVID (odds ratio = 3.53 [2.76–4.50]) (18). A recent report by the Center for Disease Control and Prevention (CDC) National Center for Health Statistics (19), announced that new data from the Household Pulse Survey indicate that more than 40% of adults in the United States reported having COVID-19 in the past. Nearly one in five of those (19%) are currently still having symptoms of long COVID. These findings highlight the importance of investigating the cause of long COVID and developing potential treatments.

In this review, we focus on long COVID, a pathophysiological condition characterized by the recurrence of symptoms weeks or months after traces of the COVID-19 virus disappear. Despite abundant data on long COVID, its underlying causes and effective treatments remain unknown. This review focuses on the underlying cause of long COVID and its occurrence, providing essential insights into understanding long COVID.

2 COVID-19 and diabetes mellitus

2.1 The influence of DM on COVID-19 infection

Following the declaration of COVID-19 as a worldwide pandemic, patients with COVID-19 and DM were more likely to develop severe or critical disease conditions with more complications. The results of the meta-analysis showed that DM seemed to contribute to an increased mortality risk among hospitalized patients with COVID-19 compared to those without DM (Table 1, Supplementary Figure 1) (20–26). Hussain et al. (20) reported a significantly higher risk of intensive care unit (ICU) admission in patients with COVID-19 and DM compared to those without DM, with a pooled risk ratio of 1.88 (1.20–2.93%), $p < 0.006$, as well a significantly higher mortality risk, with a pooled risk ratio of 1.61 (95% confidence interval: 1.16–2.25%), $p = 0.005$. Shang et al. (21) reported that these patients had higher and more severe COVID-19 infection rates than those without DM, at 21.4% and 10.6%, respectively ($p < 0.01$), and were associated with an increased mortality risk (28.5 vs. 13.3%, respectively; $p < 0.01$; odds ratio: 2.14). Based on the data in Table 1, an *in silico* analysis of the overall meta-analysis results was performed. The forest plot of the pooled case mortality ratio in patients with COVID-19 and DM is shown in Supplementary Figure 1. Among a total of 4,450,522 patients with COVID-19, the average mortality ratio for patients

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TABLE 1 Total numbers of patients, infection rate, infection rate (DM), infection rate (COVID-19), intensive care unit (ICU) admission rate, case mortality ratio (DM vs. others) of patients with COVID-19 and diabetes mellitus (DM) in seven aricles (20–26) and the average 1.67.

Total no. of patients	Infection rate (DM)	Infection rate (COVID-19)	ICU admission rate ratio (DM vs. Others)	Case mortality ratio (MR;DM vs. others) 95% confidence interval (CI)	Reference
23,007 patients	15% (95% CI: 12–18%), $p < 0.0001$		1.88 (1.20–2.93), $p = 0.006$	Ratio: 1.61 (95% CI: 1.16–2.25), $p = 0.005$	Hussain et al., 2020 (20)
31,067 patients		DM 21.4%, Non-DM 10.6% ($p < 0.01$)		Ratio: 2.21 (95% CI: 1.83–2.66, I^2 50%), $p < 0.01$ (28.5% vs. 13.3%), $P < 0.01$	Shang et al., 2020 (21)
45,775 hospitalized COVID-19 patients	20% (95% CI: 15.0–25.0; I^2 = 99.3%)			Ratio: 1.82 (95% CI: 1.25–2.39), DM 20.0% (95% CI: 15.0–26.0; I^2 96.8%), Non-DM 11.0% (95% CI: 6.0–16.0; I^2 99.3%)	Saha et al., 2021 (22)
18,506 patients	20%			Ratio: 1.65 (95% CI 1.35–1.96; I^2 77.4%), $p < 0.001$	Palaiodimos et al., 2021 (23)
35,486 patients	17[15;19]%			5867 deaths (16.53%), Ratio: 1.85 (95% CI: 1.36–2.51) $p < 0.01$	Corona et al., 2021 (24)
25,934 patients	16.9% (n = 4381)			Ratio: 1.83 (95% CI: 1.61 - 2.05), (DM vs. Non-DM: 22.14% vs. 12.81%) $p < 0.05$	Gupta et al., 2021 (25)
4,270,747 COVID-19 patients and 43,203,759 controls.				Ratio: (risk ratio, 1.66; 95% CI 1.38; 2.00) $p < 0.0001$	Ssentonga et al., 2022 (26)
Total 4,450,522 patients		Average of DM patients with COVID-19: 18.9%		Average ratio of mortality ratio for DM patients: 1.67	

with DM was 1.67 on average (Table 1) (20–26). Moreover, hyperglycemia strongly predicts poor prognosis in patients with COVID-19 (27). Paul et al. (28) discussed the effects of oxidative stress management on alleviating COVID-19 symptoms in patients with DM as a comorbidity. It is considered that both COVID-19 and DM are oxidative diseases, so the patients receive oxidative stress synergistically.

2.2 DM and long COVID

Steenblock et al. (29) suggested the increased risk for people with diabetes in the acute phase of COVID-19, and this patient group seemed to be more often affected by long COVID and experience more long-term consequences than people without diabetes. However, the mechanisms behind these discrepancies between people with and without diabetes concerning COVID-19 are not entirely understood yet (30). Furthermore, Xie et al. (31) examined the risk of diabetes following COVID-19 infection and described that In the post-acute phase of the disease (in long COVID phase). Xie and Al-Aly compared with the contemporary control group, people with COVID-19 exhibited an increased risk of incident diabetes (31). Rizvi et al. (32) suggested that COVID-19 virus directly attacks the beta cells of islets by binding with ACE2. The other factors of overactivated inflammation, such as elevation in neutrophils, IL-6, and CRP, and imbalanced immunoreaction, such as reduction in lymphocytes, monocytes, CD4+ and CD8+ T cells cause insulin insufficient synthesis and systemic insulin resistance. These situations cause impaired glucose regulation and new-onset diabetes (32). Bramante et al. (33) described that outpatient treatment with metformin reduced long COVID incidence by about 41%, with an absolute reduction of 4.1%, compared with placebo. Overall, 93 (8.3%) of 1126 participants reported receipt of a long COVID diagnosis by day 300. The cumulative incidence of long COVID by day 300 was 6.3% (95% CI 4·2-8·2) in participants who received metformin and 10.4% (7.8-12.9) in those who received identical metformin placebo (hazard ratio [HR] 0.59, 95% CI 0.39-0.89; p=0·012). Metformin has clinical benefits when used as outpatient treatment for COVID-19 and is globally available, low-cost, and safe.

V'kovski et al. (34) described an essential understanding of SARS-CoV-2 infection throughout the intracellular viral life cycle. Mitochondria could be involved in the intracellular viral life cycle.

3 Prognosis for severity of COVID-19

The prognosis of severe COVID-19 cases is essential. Rizzi et al. (35) summarized the most promising biomarkers to predict the severity of COVID-19. Those are IP10 (36), Gas6 (37–39), serum SARS-CoV-2 nucleic acid (RNAaemia) (40), and Calcitonin Gene-Related Peptide (CGRP) plasma levels (41). Chen et al. (40) described that RNAaemia is closely related to IL-6. Therefore, IL-6 could also be an essential factor in predicting the severity of COVID-19.

4 COVID-19 virus influences mitochondria of the infected patient

Jackson et al. explained the mechanisms of SARS-CoV-2 entry into host cells; the binding of the spike (S) protein to its receptor, angiotensin-converting enzyme 2 (ACE2), and subsequent membrane fusion (42). It is shown that an association with COVID-19 causes redox imbalance or oxidative stress (43, 44). Viral infections alter mitochondrial dynamics at various levels and impact mitochondrial functioning (45). Upon SARS-CoV-2 entry, the RNA genome is released, and translated, and the resulting structural and non-structural proteins interact with mitochondrial components. Then, SARS-CoV-2 escape from mitochondria-mediated innate immune response and establish its infection (46). SARS-CoV-2 may manipulate mitochondrial function indirectly, first by ACE2 regulation of mitochondrial function, and once it enters the host cell, open-reading frames (ORFs) such as ORF-9b can directly manipulate mitochondrial function to evade host cell immunity and facilitate virus replication and COVID-19 disease. Manipulation of host mitochondria by viral ORFs can release mitochondrial DNA (mtDNA) in the cytoplasm, activate mtDNA-induced inflammasome, and suppress innate and adaptive immunity (47). The viruses may induce mtDNA degradation, alter mitochondrial metabolic pathways, impact mitochondrial membrane potential, and modify the mitochondrial intracellular number and distribution, thereby influencing apoptosis, mitochondrial homeostasis, or evade mitochondrial antiviral signals (48–50). Ajaz et al. investigated functional mitochondrial changes in live peripheral blood mononuclear cells (PBMCs) from patients with COVID-19 and subsequent changes in the inflammatory pathways. They demonstrated mitochondrial dysfunction, metabolic alterations with an increase in glycolysis, and high levels of mitokine in PBMCs from patients with COVID-19. They found that levels of fibroblast growth factor 21 mitokine correlate with COVID-19 disease severity and mortality (51)..

Mitochondria appear to be important in COVID-19 pathogenesis because of its role in innate antiviral immunity, as well as inflammation (52). Mitochondrial antiviral signaling protein (MAVS) is an innate immune adaptor on the outer mitochondrial membrane that acts as a switch in the immune signal transduction response to viral infections. Increased aerobic glycolysis provides material and energy for viral replication upon viral infection. MAVS is the only protein specified downstream of retinoic acid-inducible gene I (RIG-I) that bridges the gap between antiviral immunity and glycolysis. MAVS binding to RIG-I inhibits MAVS binding to Hexokinase (HK2), thereby impairing glycolysis (53). In contrast, excess lactate production inhibits MAVS and the downstream antiviral immune response, facilitating viral replication (53, 54).

SARS-CoV-2 RNA enters macrophages, MAVS and mitofusin 1 and 2 causing mitochondrial dysfunction and the subsequent increase in ROS generation and mt-DNA into the cytosol. This causes the activation and recruitment of NLR family pyrin domain containing 3 (NLRP3). Wu et al. have reported that MAVS mediates NF-κB and type I interferon signaling during viral infection and is also required to activate the NLR family pyrin domain containing 3 (NLRP3) that triggers an immune response (55).. Apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) protein (56), and Caspase-1, which assemble to create the NLRP3 inflammasome (57). The activated NLRP3 inflammasome cleaves the cytokines Pro-IL-1B and Pro-IL-18 into their mature and biologically active forms (IL-1B and IL-18), thus exacerbating the inflammation state (58). Moreno Fernández-Ayala suggested that chronic inflammation caused by mitochondrial dysfunction is responsible for the explosive release of inflammatory cytokines causing severe pneumonia, multi-organ failure, and finally death in COVID-19 patients (59).

SARS-CoV-2 enters the cells, and the RNA and RNA transcripts capture the mitochondria, and disrupt the mitochondrial electron transport chain (60). Prasada Kabekkodu et al. suggested that SARS CoV proteins localize in the mitochondria, increase reactive oxygen species (ROS) levels, perturbation of Ca2+ signaling, changes in mtDNA copy number, mitochondrial membrane potential (MMP), mitochondrial mass, and induction of mitophagy (61). Guarnieri et al. suggested that after the COVID-19 virus infection, there was a systemic host response followed by viral suppression of mitochondrial gene transcription and followed by induction of glycolysis (62). Even when the virus was cleared, mitochondrial function in the heart, kidney, liver, and lymph nodes remained impaired, leading to severe COVID-19 pathology (62). Miller et al. reported that SARS-CoV-2 did not dramatically regulate (1) mtDNA-encoded gene expression or (2) MAVS expression, and (3) SARS-CoV-2 downregulated nuclear-encoded mitochondrial (NEM) genes related to cellular respiration and Complex I (63). Bhowal et al. reported that open reading frames (ORFs) of COVID-19, ORF-9b, and ORF-6 impair MAVS protein and suppress innate antiviral response activation (64).

Duan et al. found significant changes in mitochondrion-related gene expression, mitochondrial functions, and related metabolic pathways in patients with COVID-19, analyzing RNA-sequencing dataset of lung tissue and blood from COVID-19 patients (65). Yang et al. exhibited that SARS-CoV-2 membrane protein (M protein) could induce mitochondrial apoptosis pathway via B-cell lymphoma 2 (BCL-2) ovarian killer (BOK) without BAK and BAX, thus exacerbating SARS-CoV-2 associated lung injury in vivo (66).

5 Long COVID

5.1 Long COVID as a well-developed feature

COVID-19 is a significant pandemic resulting in substantial mortality and morbidity worldwide. Of the individuals affected, approximately 80% had mild-to-moderate disease, and among those with severe disease, 5% developed critical illness (67). A few of those who recovered from COVID-19 developed persistent or new symptoms lasting for weeks or months; this is called "long COVID," "long haulers," or "post-COVID syndrome" (68, 69). Nguyen et al. (70) reported the long-term persistence of dyspnea in patients with COVID-19.

Long COVID was defined by Crook et al. (71), and published on May 5, 2020, in BMJ Opinion, where he shared his experience of seven weeks on a "roller coaster of ill health" following COVID-19 (72). Long COVID is now recognized in the National Institute for Health and Care Excellence guidelines on managing the long-term effects of COVID-19 (73). Datta et al. (74) define patients with long COVID or long haulers as individuals with ongoing symptoms of COVID-19 that persist beyond four weeks from the initial infection.

5.2 Long COVID symptoms

Long COVID is a debilitating illness in at least 10% of severe SARS-CoV-2 infections (75). COVID-19 is now recognized as a multi-organ disease with a broad spectrum of manifestations. As for post-acute viral syndromes, there is an increasing number of reports of persistent and prolonged effects following acute COVID-19. There are currently no validated effective treatments for long COVID (75). Common symptoms of long COVID include fatigue, shortness of breath, cough, joint pain, chest pain, muscle aches, and headaches (76). Patient advocacy groups, many members of which identify as long haulers, have contributed to recognizing post-acute COVID-19 (77). In the absence of a virus in patients after COVID-19 infection, long COVID causes symptoms similar to those of myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) (78–80). Linhoff et al. reviewed recent data on Long-COVID and Long-COVID-related fatigue (LCOF), focusing on cognitive fatigue (81). Regarding long COVID pathological co-factors, Bellan et al. (82) described that the condition of proinflammatory cytokines in patients can be essential. Explanations for "long COVID" include immune imbalance, incomplete viral clearance, and potentially even mitochondrial dysfunction (83). Of note, oxidative stress might be an underlying cause of long COVID (84).

5.3 Long COVID and mitochondria

Lactic acid, lactate/pyruvate ratio, ornithine/citrulline ratio, and arginine were identified as the most relevant metabolites for distinguishing long COVID patients even two years after acute COVID-19 infection (85). Long COVID causes mitochondrial dysfunction, redox state imbalance, impaired energy metabolism, and chronic immune dysregulation.

Carpenè et al. (86) demonstrated that blood lactate levels were higher in severe cases of non-survivor patients with COVID-19 than in non-severe survivor cases, as shown in Supplementary Figure 2 (87–96). Figure 2 shows the blood lactate levels in coronavirus disease 2019 (COVID-19) survivors vs. non-survivors taken from the results of references 87-96, suggesting that the blood lactate levels in COVID-19 non-survivors are significantly higher than the survivors. The results of Supplementary Figure 2 show that in long COVID patients, intracellular energy production tends to use glycolysis rather than using mitochondrial oxidative phosphorylation. The meta-analysis showed that lactate dehydrogenase (LDH) was also increased in patients with COVID-19 and associated with relatively poor outcomes (97). Lactate dehydrogenase is markedly elevated in plasma and strongly associated with mortality in severe COVID-19 (98). This finding is consistent with the potential explanations for "long COVID," which include mitochondrial dysfunction (83). Vitamin D is an immunomodulatory hormone with proven efficacy against various upper respiratory tract infections; it can inhibit hyperinflammatory reactions and accelerate the healing process in affected areas, especially lung tissue. Moreover, vitamin D deficiency is associated with the severity and mortality of COVID-19 cases, with a high prevalence of hypovitaminosis D found in patients with COVID-19 and acute respiratory failure (76). Antonelli et al. (99) described that among Omicron cases, 4.5% of

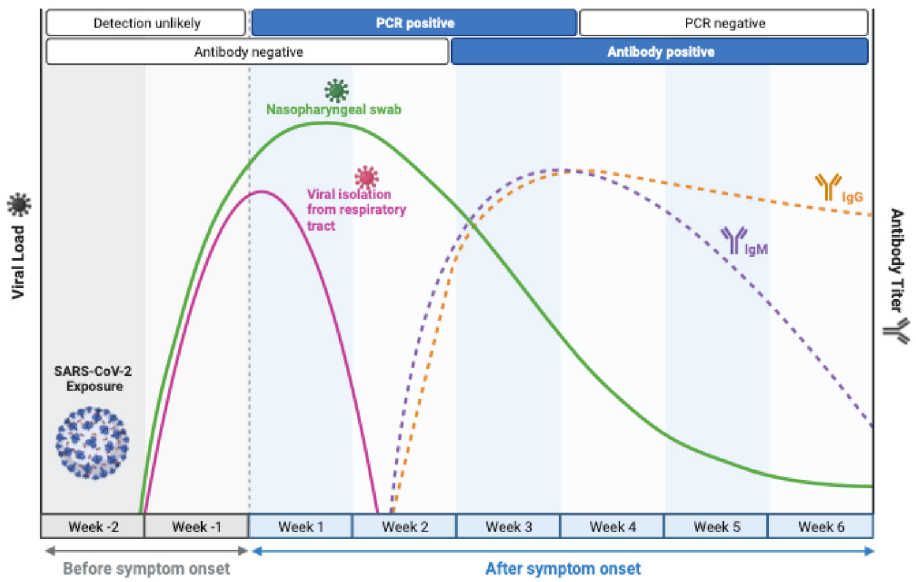

FIGURE 1
Timelines of long COVID. Long COVID-19 is defined as persistent symptoms and or delayed or long-term complications beyond 4 weeks from the onset of symptoms. (Figure 1 adapted from BioRender).

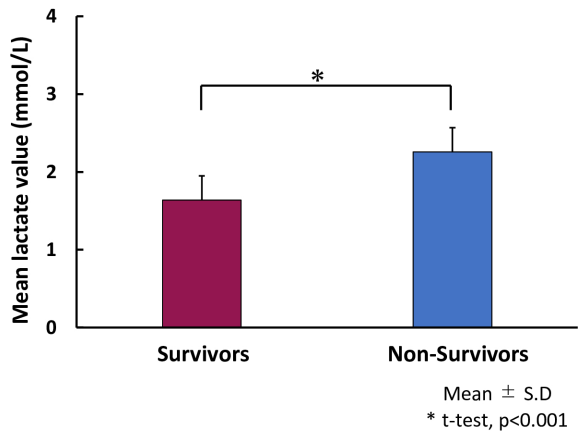

FIGURE 2
Lactate levels in coronavirus disease 2019 (COVID-19) survivors vs. non-survivors. Bar graph shows mean lactate levels in COVID-19 survivors (red bar) and non-survivors (blue bar). The blue dot is an outlier. Remended from Carpenè et al. (2021) (86) the and articles 87–96.

people experienced long COVID, whereas 10.8% experienced long COVID following Delta variant infection. Hernández-Aceituno et al. (100) described that the ongoing symptomatic COVID (4–12 weeks), post-COVID-19 (> 12 weeks with symptoms), and long COVID cases were less frequent in Omicron cases, compared with Alpha or Delta cases. These findings suggest that patients infected with the Omicron variant are less likely to experience long COVID.

Antonelli et al. (99) also described that after infection with Omicron or Delta variants, less than three months after vaccination, the long COVID odds ratio increases compared to the "3 to 6 month" and "prior to 6-month" groups. Vaccinated individuals are occasionally diagnosed with COVID-19, which is known as a breakthrough SARS-CoV-2 infection (BTI). Al-Aly et al. (30) showed that in long COVID, six months after infection, people with BTI exhibited a higher risk of death and incident post-acute sequelae, including cardiovascular, coagulation, hematologic, gastrointestinal, kidney, mental health, metabolic, musculoskeletal, and neurologic disorders. These results were consistent when compared against the historical and vaccinated controls. Long COVID is a debilitating syndrome that often includes persisting respiratory symptoms and, to a lesser degree, abnormalities in lung physiology (100). Respiratory features of long COVID may decrease over time, yet resolution is not achieved in all cases.

We have previously published that impairments of the electron transport chain and mitochondrial DNA damage increase ROS production, and so-called mitochondria caused oxidative damage (101). COVID-19 might influence mitochondrial function and induce mitochondrial damage, especially in the mitochondrial electron transport chain, and may cause mitochondrial oxidative damage.

Emerging evidence suggests that COVID-19 highjacks mitochondria of immune cells replicates within mitochondrial structures, and impairs mitochondrial dynamics, leading to cell death. Increasing evidence suggests that mitochondria from COVID-19-infected cells are highly vulnerable, and vulnerability increases with age (102). The relationship between long COVID and mitochondria has been focused on. First, after infection of COVID-19, the localization of the virus should be focused. Wu et al.

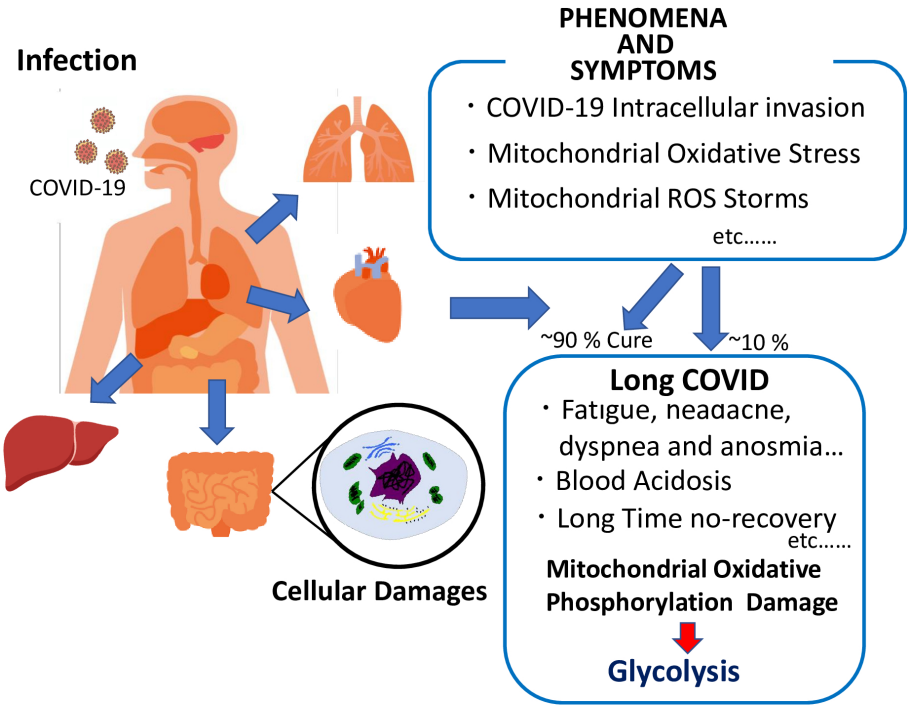

FIGURE 3
Schematic representation of COVID-19 phenomena and symptoms, and long COVID characteristics.

performed computational modeling of SARS-CoV-2 viral RNA localization across eight subcellular organelles: endoplasmic reticulum (ER) membrane, Nuclear lamina, Mito matrix, Cytosol, Nucleolus, Nucleus, Nuclear pore, and Mitochondria outer membrane. We compare hundreds of SARS-CoV-2 genomes to the human transcriptome and other coronaviruses and perform systematic sub-sequence analyses to identify the responsible signals. Using state-of-the-art machine learning models, we predict that the SARS-CoV-2 RNA genome and all sgRNAs are the most enriched in the host mitochondrial matrix (103). Interestingly, Padhaan et al. described that the severe acute respiratory syndrome coronavirus 3a protein activates the mitochondrial death pathway through p38 MAP kinase activation in 2008 (104). Cumpstay proposed the anti-ROS agents as the treatment tool against COVID-19, a redox disease (105). Chen et al. proposed possible pathogenesis and prevention of Long COVID considering SARS-CoV-2-induced mitochondrial disorder (106). Therefore, the most likely COVID-19 goes to mitochondria after the infection into the cells of the host patients and the severe ROS generation from mitochondria that destroys mitochondria and mitochondrial DNA, consequently less oxidative phosphorylation and shift to glycolysis, long COVID symptoms.

In conclusion, we summarized the mode of spread, clinical symptoms, infection route, and intracellular signaling of COVID-19, as well as the combination of COVID-19 and diabetes, COVID-19 intracellular invasion, including mitochondrial oxidative stress, mitochondrial ROS storm that destroys mitochondria and electron transport chain (ETC), and causes long COVID (summarized in Figure 3). We highlight that the mitochondria might be involved in the pathogenesis of long COVID and symptom manifestation. A comprehensive map of the stages and pathogenetic mechanisms related to the disease and effective drugs to treat and prevent long COVID are urgently required, warranting further investigation on long COVID treatments and symptom relief strategies.

Author contributions

KN: Investigation, Methodology, Validation, Writing – original draft. MC: Data curation, Validation, Writing – original draft. SiS: Data curation, Investigation, Validation, Writing – original draft. MKo: Data curation, Formal Analysis, Investigation, Validation, Visualization, Writing – review & editing. RH: Methodology, Validation, Writing – original draft. KB: Investigation, Validation, Writing – review & editing. CN: Investigation, Validation, Writing – original draft. AT: Investigation, Validation, Writing – review & editing. WK: Data curation, Investigation, Validation, Writing – original draft. MI: Data curation, Investigation, Validation, Writing – review & editing. FK: Investigation, Validation, Writing – review & editing, Supervision. MKu: Data curation, Investigation, Validation, Writing – review & editing. YK: Data curation, Methodology, Validation, Writing – review & editing. HI: Data curation, Funding acquisition, Methodology, Validation, Writing – review & editing. TK: Data curation, Investigation, Validation, Writing – review & editing. SuS: Data curation, Methodology, Validation, Writing – review & editing. VS: Data curation, Methodology, Validation, Writing – review & editing. LU: Data curation, Methodology, Validation, Writing – review & editing. TI: Data curation, Methodology, Validation, Writing – review & editing. VN: Data curation, Methodology, Validation, Writing – original draft. HI: Methodology, Validation, Writing – review & editing, Conceptualization, Investigation, Supervision, Writing – original draft. JT: Supervision, Writing – review & editing, Data curation. HM: Conceptualization, Data curation, Formal Analysis, 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1275001/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Case mortality ratio (DM vs. others) of patients with COVID-19 and diabetes mellitus (DM) in seven articles (20–26) and the average 1.67.

SUPPLEMENTARY FIGURE 2
Lactate levels in coronavirus disease 2019 (COVID-19) survivors vs. non-survivors. The forest plot shows significant differences in mean lactate levels between COVID-19 survivors and non-survivors ($p < 0.01$). Blue-squared box, effect estimate; Green diamond, the pooled effect estimate; Gray vertical line, no effect line; Red vertical line, the pooled effect estimate; N, number of individuals; SD, standard deviation; Mean Diff, mean difference. Remended from Carpenè et al. (2021) (86) and (87–96).

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Brain FADE syndrome: the final common pathway of chronic inflammation in neurological disease

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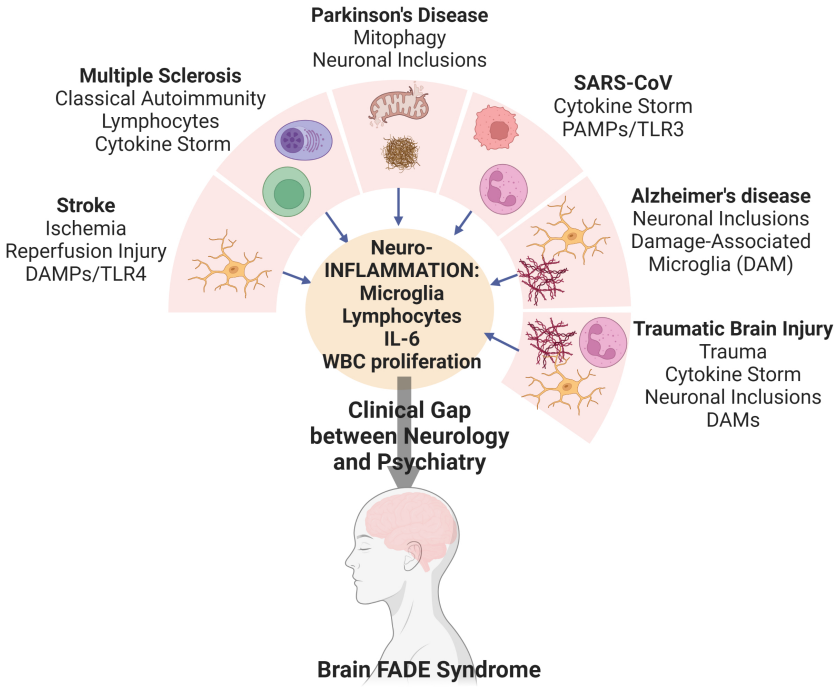
Importance: While the understanding of inflammation in the pathogenesis of many neurological diseases is now accepted, this special commentary addresses the need to study chronic inflammation in the propagation of cognitive Fog, Asthenia, and Depression Related to Inflammation which we name Brain FADE syndrome. Patients with Brain FADE syndrome fall in the void between neurology and psychiatry because the depression, fatigue, and fog seen in these patients are not idiopathic, but instead due to organic, inflammation involved in neurological disease initiation.

Observations: A review of randomized clinical trials in stroke, multiple sclerosis, Parkinson's disease, COVID, traumatic brain injury, and Alzheimer's disease reveal a paucity of studies with any component of Brain FADE syndrome as a primary endpoint. Furthermore, despite the relatively well-accepted notion that inflammation is a critical driving factor in these disease pathologies, none have connected chronic inflammation to depression, fatigue, or fog despite over half of the patients suffering from them.

Conclusions and relevance: Brain FADE Syndrome is important and prevalent in the neurological diseases we examined. Classical "psychiatric medications" are insufficient to address Brain FADE Syndrome and a novel approach that utilizes sequential targeting of innate and adaptive immune responses should be studied.

KEYWORDS

microglia, IL-6, PASC, vagus, inflammation, depression, COVID, stroke



GRAPHICAL ABSTRACT
Brain FADE Syndrome. Pictorial representation of the issues that patients face from disease pathogenesis to finding the correct type of physician to deal with Brain FADE Syndrome.

1 Introduction

The role of inflammation whether via damage-associated microglia, increased blood brain barrier permeability, or increased complement activity has a critical role in the pathogenesis of both cerebrovascular and neurodegenerative diseases (1). The brain, long thought an immunologically privileged organ, is now seen as immunologically active as the spleen with pseudo germinal centers, and resident innate and adaptive immune cells. With this understanding, the role of neuroinflammation has come to the forefront as an underlying etiology of neurological disease; however, the "psychiatric endpoints" have not received their due recognition.

While the neurological diseases of *depression, fatigue, and brain fog* affect a majority of patients with stroke, Alzheimer's, Parkinson's, multiple sclerosis, traumatic brain injury, and "long COVID," the role of chronic inflammation in mediating these very important neurological diseases have not been well-studied. We named this combined endpoint of depression, fatigue and brain fog from chronic inflammation as Brain FADE syndrome; cognitive Fog, Asthenia, and Depression Related to Inflammation. We posit that chronic inflammation is the final common pathway that connects all neurological diseases, regardless of initial insult, to Brain FADE Syndrome.

2 Stroke

Estimates of Brain FADE post-stroke vary from 20-70%, based on a conglomerate of all of these symptoms (2). While active attempts to uncover the individual contributions of fatigue, fog, and depression in classical autoimmune diseases like multiple sclerosis or novel diseases like Long Covid are underway (3, 4), the endpoint is generally combined in stroke as depression. The FLAME trial was the first randomized placebo-controlled trial (RCT) that indirectly addressed Brain FADE after stroke (5). While the hypothesis of using fluoxetine in stroke was based on increasing excitatory neurotransmitters to promote neurogenesis, fluoxetine is more commonly used in the treatment of depression (6). In this small study of 118 patients both depression and motor recovery were significantly improved by fluoxetine treatment. Due to the impressive effects of fluoxetine, but the small sample size, an attempt to replicate the findings in large, collaborative studies was performed: the FOCUS, EFFECTS, and AFFINITY trials (7–9). The FOCUS trial was an RCT that enrolled approximately 3,000 stroke patients in the UK and found no difference in functional outcome, and interestingly, the treated arm saw less depression, but an increase in bone fractures. Similar studies were carried out in Sweden with the EFFECTS trial and AFFINITY between Australia, Vietnam, and New Zealand. Both RCTs found similar results to FOCUS in that there was no change in functional outcome, with a decrease in new depression; however, both trials also demonstrated more falls, more bone fractures, and more seizures.

Perhaps the reason that fluoxetine not only failed to improve motor outcomes, but was potentially harmful causing seizures, falls, hyponatremia, and fractures lies in the underlying pathophysiology of stroke. Fluoxetine does not address the underlying inflammatory

etiology of chronic stroke symptoms, and potentially exacerbates the vascular etiology based on known mechanisms of this selective serotonin reuptake inhibitor. A focus on anti-inflammatory signal transduction may prove beneficial in the development of novel treatments for Brain FADE Syndrome.

The best studied "anti-inflammatory" agents in stroke with any data on depression and fatigue are the NSAIDs and statins. While SPARCL revealed no significant change in functional stroke outcomes, subsequent analysis of these data and others revealed mixed effects of statins on depression and fatigue (10). In a retrospective study in the Danish population, a study of over 300,000 people, the use of NSAIDs or ASA revealed a decreased risk of early depression after stroke, with the opposite effect on chronic depression. Conversely, the opposite temporal relationship was found with statin use alone (11). Another database study out of Taiwan with 11,000 patients demonstrated an increase in depression with statin use, while a smaller prospective study showed a slight decrease in depression (12, 13). Interestingly, the strongest link between depression, inflammation and statins is via the interleukin 6 (IL-6) pathway. Increases in IL-6 and IL-18 have been associated with depression after stroke, but no relationship to statins were drawn in these studies (14, 15). A weak causal relationship was established with a prospective study involving 400 Taiwanese patients indicating that statin-treated patients produced less IL-6 and had lower rates of depression, supporting an inflammatory hypothesis for depression.

The first anti-inflammatory RCTs in ischemic stroke were the SAINT trials where the free radical scavenger NXY-059 was used (16, 17). While SAINT I showed efficacy of the scavenger at reducing disability at 90 days, the repeat trial with twice as many patients failed to reproduce these effects. After initial evaluations in young, healthy male animals, further studies should be performed in females, aged animals, and animals with comorbid conditions such as hypertension, diabetes, and hypercholesterolemia. Although STAIR criteria for optimal research design in pre-clinical studies had recently been established, the pre-clinical studies did not include females, aged animals, and animals with comorbid conditions such as hypertension, diabetes, and hypercholesterolemia (18, 19). These are some of the reasons that NXY-059 may have failed. Importantly, NXY-059 may have influenced Brain FADE, but these outcomes were not measured.

The ESCAPE NA1 trial was another RCT with a multitude of pre-clinical studies supporting the concept that interfering with the interaction between post-synaptic density 95 and the NMDA receptor would prevent both the neurotoxicity induced by calcium influx as well as that from NOS II activation (20–22). The ESCAPE NA1 trial also failed to demonstrate any neuroprotection, yet again, no mention of any of the components of Brain FADE in the outcomes measured in the RCT or tested in the pre-clinical studies. While the preclinical studies gained much fame, the extent to which they were replicated in females, aged, and comorbid mice may have also lead to issues with the translation to human stroke.

A more direct approach studying the relationship between stroke and inflammation was taken in the ACTION trial where an inhibitor of leukocyte-endothelial interaction, effective in the treatment of multiple sclerosis, was tested (23). Although the natalizumab trial did not find any significant effects of infarction volume or functional outcome, depression and fatigue were not analyzed. The failure of ACTION may have many components, including timing and dosage, but the most striking difference is duration of treatment. Patients in ACTION received 1 dose of the drug within 9 hours of stroke, whereas in RCTs involving MS patients, the drug was given once per month for over 2 years (24, 25). Furthermore, natalizumab seemed to have remarkable efficacy in reducing fatigue and depression in MS patients; however, these endpoints were not studied in ACTION (26–29).

The newest anti-inflammatory agent to be bridged from bench to the bedside is a Toll-like receptor 4 (TLR4) aptamer. The toll like receptors are evolved to prevent prokaryotic attack of eukaryotic cells. Canonical TLR ligands are known as pathogen associated molecular patterns (PAMPs) that recognize and respond to specific common antigens on viruses, fungi, or bacteria. Similarly, danger associated molecular patterns (DAMPs) are endogenous molecular patterns that signal damage to the body like heme or mitochondrial membrane fragments (30, 31). Although the ligand to TLR4 in ischemic stroke is not as well-established as in hemorrhagic stroke, conditional and whole body knockouts of TLR4 in all models of stroke have demonstrated profoundly neuroprotective effects (32–36).

To translate this understanding to human ischemic stroke, aptamers or short sequences of nucleic acids that bind to inhibit and TLR4 have been developed. Aptamers, in theory, should result in a reduced inflammatory response due to lack of protein antigens and sequences that are not recognized as DAMPs by other toll like receptors. Preliminary studies demonstrate that the drug is safe and more importantly, Brain FADE is being investigated as an endpoint in future clinical trials (36–38).

Thus, future investigations into reducing neuroinflammation should include Brain FADE as an endpoint. Even if the pharmaceutical intervention does not show efficacy against "functional" outcome, as perceived by the physician, treating Brain FADE may improve the patient's perception of the outcome. Ultimately, that is the goal of any physician-patient interaction. This concept of the patient's opinion of a functional outcome is not entirely novel and has been used in novel metrics such as the utility-weighted modified Rankin Score (39).

3 Multiple sclerosis

Multiple sclerosis, on the other hand, is the archetypal model of cerebral inflammation, and synonymous with neuroimmunology. As such, it is not surprising the Brain FADE has been studied extensively; fatigue (40), fog also known as "cog fog" (41), and depression (42) are estimated to affect approximately 37–78% (43), 34–65% (44), and 5–59% (45) of patients, respectively. Of note, not only have each of these elements of Brain FADE syndrome been individually studied in MS, but the idea that they are part of the final common pathway due to the inflammation in MS, and are observed in aggregate, has also been studied in such detail that 252 original research articles have been published on the topic (46, 47). A small

study of 13 MS patients where half of them were treated with cyclosporine found an association where those treated had fewer cytotoxic $CD8^+$ and more $CD4^+$ T cells with less depression (48). Another small study examined MS patients with pre-existing depression versus those without depression. While they did not find difference in $CD8^+$ T cell populations, the CD8 cells in depressed patients produced more TNF-α and interferon (IFN)-γ. This association held even when the authors controlled for disease modifying therapy and current functional status (49). This study as well another small study measured serum TNF-α and IFN-γ and found they were associated with chronic fatigue as well (50). Another small study of 47 MS patients found that IL-6 in CSF was independently associated with depression and fatigue. Furthermore, in a larger study with 249 healthy controls, 108 MS patients without depression, and 42 patients with MS and depression; serum IL-6 was highest in those with MS and depression. Interestingly, serum IL-6 is also a marker of depression in the general population, further supporting the idea that inflammation may be final common pathway to Brain FADE syndrome (51, 52). Finally, in small study of cognitive fog, there seemed to be an inverse relationship between T cell production of IFN-γ and fog; however, this study was not controlled for IFN-β therapy, the most common MS therapy, which is known to lower levels of IFN-γ-producing CD8+ T cells (49, 53). Despite the fact that every RCT in MS tests an immunomodulator only one of them used Brain FADE as an endpoint: Satralizumab (humanized antibody against IL-6) (54). The Satralizumab trial enrolled 83 patients with neuromyelitis optica, a type of MS. The authors found the Satralizumab arm had a reduced relapse rate, but no change in fatigue or depression. While a negative study for Brain FADE, at least this study provides definite causal evidence for the lack of involvement of IL-6 in fatigue and depression in this type of MS. More studies like the Satralizumab trial are required to determine causal roles for the many immunomodulatory drugs used to treat MS and Brain FADE. Taking a lesson from stroke, with utility weighted mRS, and the Patient Reported Outcome Measurement Information System (PROMIS) used to define postacute sequelae of SARS-CoV-2 infection (PASC); the patient's input is critical to understand the relative importance of motor function versus Brain FADE.

4 Parkinson's disease

Neurodegenerative diseases such as Parkinson's disease have chronic inflammation that has been established in the glia (microglia, astrocytes, and neurons) of the brain in preclinical models, and serum inflammatory markers such as IL-2, IL-6, and TNF-α have been associated with depression and fatigue (55–58). Despite this association and the fact that over 50% of PD patients display some element of Brain FADE; no large scale, anti-inflammatory, RCTs have taken place in Parkinson's disease to address this issue (59). 3 small trials have aimed to address inflammation through a variety of mechanisms. Azathioprine (AZA-PD) is phase II RCT that was begun in 2020 and aims to enroll 60 PD patients to assess gait and ataxia as primary outcomes along with inflammatory mediators as secondary outcomes, but not depression, fatigue, or fog (60). Similarly, a an RCT with the iron chelator, deferiprone, did not show any improvement in cognition or gait, but Brain FADE was not investigated (61). Finally, a general bcr-abl kinase inhibitor, nilotinib, was tried based on MPTP, pre-clinical models of PD that showed increased TREM-2 expression on microglia and decreased α-synuclein. TREM-2 is celebrated marker of microglial function that induces clustering of microglia around protein inclusions and is critical in the signal transduction required for the phagocytosis of these inclusions (62). The phase II study showed increased TREM-2 expression on CSF myeloid cells, increased serum dopamine, and decreased serum α-synuclein. While the study was preliminary, it remains to be seen if Brain FADE will be studied in future trials with the tyrosine kinase inhibitor (63).

5 Long COVID

When discussing Brain FADE syndrome, the infection of over half a billion people in the recent pandemic with SARS-CoV-2 (COVID-19) must not be overlooked (64). About 6% of patients with COVID-19 have symptoms that do not resolve for months or years, hence Long COVID (65). SARS-CoV-2 is a single-stranded positive-sense RNA virus with spike projections that emerge from the virions' surface, a characteristic of the Coronaviridae family. Infection with coronavirus yields a significant inflammatory response stemming from both the innate TLR3 and cGAS-STING pathways, as well as the adaptive cytotoxic $CD8^+$ T cells (66). Specific to SARS-CoV-2 are 4 critical structural elements of the virus: the spike, membrane, envelope, and nucleocapsid proteins. SARS-CoV-2 most likely infects patients through the cribriform plate and olfactory nerve, thus explaining the common symptoms of anosmia and ageusia. The olfactory nerve, or cranial nerve I, is the only cranial nerve that does not synapse before it enters the brain parenchyma. Given this method of infection, it is not surprising that SARS-CoV-2 would present with acute neurological symptoms like anosmia, headache, encephalitis, and ischemic stroke, or chronic ones such as impaired executive functions and fatigue (67).

Moreover, the fact that steroids proved to be such an effective treatment in COVID-19-induced acute respiratory distress syndrome (ARDS), provides a causal relationship between the inflammation and ARDS (68, 69). Long term outcomes after COVID infection, even those patients not ventilated, demonstrates a Brain FADE syndrome called postacute sequelae of SARS-CoV-2 infection (PASC), which affects 10%. A recent study of almost 14,000 patients that survived COVID, utilized the Patient Reported Outcome Measurement Information System (PROMIS) database to describe what has previously been called "long COVID." Interestingly, patients defined PASC by 3 main criteria: fatigue, fog, and post-exertional malaise (4).

The main hypothesis put forth that might explain the persistent inflammation leading to Long COVID is persistence of the virus, found in the brain, gut, and lung parenchyma for up to 230 days after initial infection (70). Furthermore, the virus can persist in

tissues despite negative whole blood and nasopharyngeal PCR tests. One team found increased SARS-CoV-2 proteins in extracellular vesicles derived from neurons and astrocytes circulating at higher levels in PASC patients than those that had completely recovered (71). These circulating proteins could have the ability to activate classic innate immune pathways in both macrophages and neutrophils to initiate anti-viral responses through TLR3 and the cGAS/STING pathway, both which heavily activate interferons and contribute to cytokine storm pathology (72). The persistent viral load and inflammation can be sensed by the vagus nerve, which has thousands of afferent projections from the organs in the body. This visceral inflammation is sensed by the vagus nerve and results in a sickness behavior like PASC due to glial activation and neuroinflammation (73–76). This was also demonstrated in a murine model of bacterial pneumonia where pulmonary inflammation resulted in the activation of nociceptive afferent neurons, a branch of the vagus nerve. Subsequently, vagal efferents to the lung suppressed neutrophil and T cell responses resulting in lethal pneumonia (77).

Besides the visceral sickness mediated by the vagus nerve that can lead to PASC or Brain FADE, a more direct pathophysiology exists based on spike protein binding affinity for amyloid, synuclein, and tau proteins. These protein inclusions in neurons and the cerebral interstitial fluid are thought to be involved in Alzheimer's and Parkinson's and Traumatic Brain Injury. Lending credence to this hypothesis, one group found that the incidence of new onset of Alzheimer's diagnoses was significantly increased in older adults in the year following COVID infection Another autopsy found increased amyloid accumulation in the brains of patients with severe COVID illness younger than 60 years old (78, 79).

Further studies into these Brain FADE syndromes, and especially the brain-lung relationship via the vagus nerve are necessary in order to appropriately address and treat the chronic inflammatory component, possibly by vagotomy or vagus nerve stimulator.

6 Alzheimer's disease

In Alzheimer's disease (AD) the natural course of the disease leads to fog, but not necessarily fatigue and depression which can affect upwards of 40%. AD has been studied extensively with respect to pre-clinical models and clinical trials, but without significant emphasis on depression and fatigue as an endpoint. Attempts at treating cyclo-oxygenase induced inflammation in the aged population failed to reduce depression (80, 81). Alzheimer studies are unique in that a multitude of anti-inflammatory agents are being tested, ranging from herbal remedies like resveratrol to bromodomain epigenetic proteins (BET) to Apoε mimetics (82–84). Of these studies, only the MARBLE study, using an Apo mimetic, is specifically studying depression and fatigue in perioperative cognitive dysfunction. So even in Alzheimer's disease where depression can be seen in 90% of the population and fatigue and sleep disorders in 70%, these are not primary outcomes that are studied as a result of chronic inflammation (85).

7 Traumatic brain injury

Finally, traumatic brain injury (TBI), is perhaps the only disease, where Brain FADE syndrome is synonymous with the post-traumatic stress disorder (PTSD) moniker, apart from the etiologies that lead to these syndromes. While PTSD follows TBI, the mechanism by which one causes the other is not defined; as opposed to Brain FADE syndrome, where the mechanism is hypothesized to be chronic inflammation, regardless of the initial insult. In civilian populations, the frequency of PTSD is 18.6% after 12 months. In military populations, the frequency is reported to be upwards of 48.2%; however, this depends on the war as well as the variety of prior descriptions of this syndrome including, shell shock, disordered action of the heart, effort syndrome, effects of Agent Orange, and Gulf War syndrome to name a few (86, 87). From the TRACK-TBI study of civilians, frequencies of PTSD and MDD were relatively common, with 6-month rates ranging from 9% to 19% (88). Moreover, this study defined the population at greatest risk for PTSD and MDD after mild TBI: less education, being black, self-reported psychiatric history, and injury resulting from assault or other violence. These critical epidemiological studies are exactly what is needed in every neurological disease to carefully define risk factors for Brain FADE syndrome so that the chronic inflammation component can be studied in these at-risk populations.

Similar to previous neurological diseases, very few RCTs have been conducted to address Brain FADE syndrome or PTSD in TBI patients. Unlike other neurological disease, chronic inflammation is recognized as a critical factor in PTSD, as a tertiary phase of injury (89). The MRC Crash trial TBI patients were treated with high dose steroids for 48 hours and showed no improvement in outcomes (90). Consistent with the concept of Brain FADE and tertiary injury after TBI, low dose steroids have shown to improve PTSD (91, 92). Progesterone and erythropoietin both failed to improve outcomes in TBI, even though both showed reproducible, marked effects in rodent models of TBI (93, 94). Mifepristone, on the other hand, was specifically targeted against PTSD in war fighters, but also failed to show any efficacy (95). While all these therapies circuitously address Brain FADE syndrome or PTSD in TBI, a small, open label trial using etanercept, a soluble antibody to TNF-α, showed significant improvement across all domains of Brain FADE syndrome (96). Further RCTs that specifically target chronic inflammation are necessary. By and large the translational validity of treatment in TBI from rodent to human has been dismal. This may be due to several factors, among the most important could be that quadrupeds have a thicker cortex that is in line with their brainstem, whereas humans have a thinner cortex that is perpendicular to their brainstem with much more with matter, making diffuse axonal injury frequent. Furthermore, any TBI model requires sedation before the impact, the most common being ketamine or isoflurane, both of which have neuroprotective effects (89). The variety of traumatic brain injury mechanisms in humans are also difficult to replicate from improvised explosive devices, to boxing, to high-speed motor vehicle accidents, to violent attacks from our own species. Because so much of TBI is not only the mechanism of injury, but the circumstances surrounding the

trauma; it is not surprising that researchers cannot capture this with a simple controlled cortical impact model. Even if the "meta-data" surrounding the trauma, and the mechanism of injury could be accurately replicated, the measurement of fatigue, fog, and depression would prove difficult in a rodent. Future models of traumatic brain injury need to be applied to one mechanism of injury that most closely resembles a CCI, such as blunt force trauma to the head, and only large mammals capable of easily displaying depression or fatigue should be used as a first step toward a "win" in TBI therapeutics.

8 Conclusion

The role of inflammation as well as targeting inflammation alone may be a critical factor for the treatment of virtually all neurological disease, but these classical "psychiatric endpoints" need to be embraced by neurologists and neuroscientists, both in the lab and the clinic. A focus on the final common pathway will likely involve microglia, Toll like receptors, T lymphocytes, and IL-6. The approach to Brain FADE syndrome needs to be similar to that seen in oncology, where multiple targets are chosen along a common pathway, such as the treatment of HER2-positive breast cancer. Here, combination therapies attack the cancer along multiple pathways to enhance efficacy, manage resistance, and minimize side effects. Monoclonal antibodies against the HER2 protein inhibit growth of the tumor through this pathway; trastuzumab, targets one part of the HER2 protein and pertuzumab another. With growth inhibited, "-cidal" agents that kill cancer cells are then used, like paclitaxel. Finally, if the cancer is estrogen receptor positive, an aromatase inhibitor like tamoxifen can be used to maximize inhibition of all trophic pathways for the cancer (97).

As the tissue resident macrophage of the brain, microglia and its response to PAMPs and DAMPs will likely be critical in phase I of a pharmaceutical attack, such as a TLR4 aptamer. However, there are pathways that will allow for autonomous activation of lymphocytes a sustained immune response, independent of antigen presenting cell activation. Targeting T cells (Teriflunomide) and IL-6 (Satralizumab) would allow for a phase II disruption, hopefully impeding the recruitment of adaptive immunity to the propagation of chronic inflammation. Drug combination regimens are critical to the treatment of virtually every cancer; the application of a multi-targeted, phased approach could yield amazing results against Brain FADE syndrome and beyond.

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KH: Writing – original draft, Writing – review & editing. TJ: Conceptualization, 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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Cognition-associated long noncoding RNAs are dysregulated upon severe COVID-19

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Severe COVID-19 leads to widespread transcriptomic changes in the human brain, mimicking diminished cognitive performance. As long noncoding RNAs (lncRNAs) play crucial roles in the regulation of gene expression, identification of the lncRNAs differentially expressed upon COVID-19 may nominate key regulatory nodes underpinning cognitive changes. Here we identify hundreds of lncRNAs differentially expressed in the brains of COVID-19 patients relative to uninfected age/sex-matched controls, many of which are associated with decreased cognitive performance and inflammatory cytokine response. Our analyses reveal pervasive transcriptomic changes in lncRNA expression upon severe COVID-19, which may serve as key regulators of neurocognitive changes in the brain.

KEYWORDS

noncoding RNAs, lncRNAs, cognitive decline, COVID-19, frontal cortex

Introduction

Neurological symptoms including cognitive decline have been reported in individuals with COVID-19 (1–4). We and others have shown that severe COVID-19 induces widespread changes in protein-coding gene expression in the human frontal cortex (5, 6). However, the brain-related effects of COVID-19 on other RNA species such as long noncoding RNAs (lncRNAs), which may have widespread regulatory roles on transcriptional states despite lacking protein-coding potential (7), remain unclear. LncRNAs, which range from 200 base pairs to hundreds of kilobases, are a relatively understudied class of transcriptional regulators, often acting as scaffolds to recruit transcription factors and effectors to their target genes (8). Their target genes may reside near its gene locus (regulation in *cis*) or across the genome (regulation in *trans*) to regulate transcription (9–11). LncRNAs are expressed at different levels across brain areas and have

been linked to synaptic plasticity, memory, and multiple brain disorders (7, 10, 12–17). Due to their potential roles in transcriptional regulation, we sought to determine the breadth of lncRNA changes upon COVID-19.

Results

We analyzed our previously described total RNA-seq datasets (5), comprising of frontal cortex from 22 individuals with COVID-19 (23-84 years old) and 22 uninfected age- and sex-matched controls (± 2 years) (Figure 1A), to annotate both protein-coding and noncoding RNA genes (Figure 1B). Differential expression analysis revealed significantly increased (557) and decreased (269) expression levels of noncoding RNAs (ncRNAs) including numerous lncRNAs (long intergenic ncRNAs, antisense RNAs, and processed pseudogenes) associated with COVID-19 (Figures 1B, C; Supplementary Table). Clustering analysis using transcript abundances of significant differentially-expressed (DE) ncRNAs yielded a separation of COVID-19 cases from controls

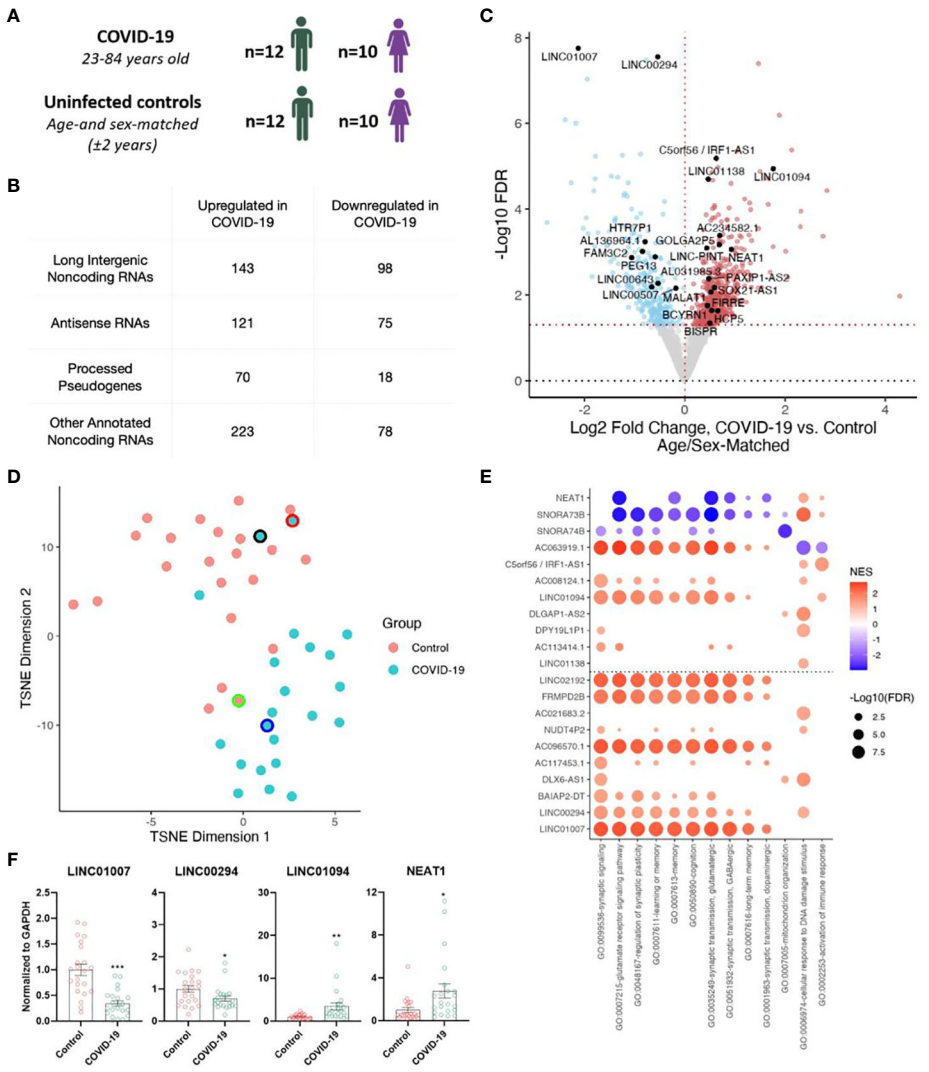

FIGURE 1
Severe COVID-19 changes the expression of long non-coding RNAs (lncRNAs) in the human frontal cortex. **(A)** Age and sex of individuals with COVID-19 and uninfected age/sex-matched control (± 2 years) groups (n=22/group) analyzed in this cohort; for further details see Mavrikaki et al. (5). **(B)** Tabulation of differentially expressed RNA species identified in our sequencing study. **(C)** Volcano plot showing the differentially expressed non-coding genes in the frontal cortex of COVID-19 cases versus age/sex-matched controls (n=22/group). Red points, significantly upregulated genes among COVID-19 cases (false discovery rate/FDR < 0.05). Blue points, significantly downregulated genes among COVID-19 cases. Black points, highlighted significant genes with corresponding gene symbols. **(D)** T-distributed stochastic neighbor embedding (TSNE) analysis of frontal cortex of COVID-19 cases and uninfected age/sex-matched controls, using significant differentially expressed noncoding RNA (ncRNA) expression levels as features. *Black border*, 23-year-old asymptomatic COVID-19 male. *Red border*, 62-year-old COVID-19 female individual with comorbid epilepsy. *Blue border*, 84-year-old COVID-19 female individual with comorbid Alzheimer's Disease (AD). *Green border*, uninfected age/sex-matched control for the COVID case with comorbid AD. n=22/group. **(E)** Guilt-by-association-based Gene Ontology (GO) biological pathway analysis of top differentially expressed ncRNAs and NEAT1, a lncRNA involved in cognitive processes (18). **(F)** Validation of sequencing data using qRT-PCR. n=22/group. Two tailed unpaired t-test, *$p<0.05$, **$p<0.01$, ***$p<0.001$. LINC01007 t(42)= 5.377, p=0.000003, LINC00294 t(42)= 2.224, p=0.0316, LINC01094 t(42)= 2.844, p=0.0069, NEAT1 t(42)= 2.583, p=0.0134.

(Figure 1D). Interestingly, the top downregulated lncRNA, LINC01007, and one of the top upregulated lncRNAs LINC01094 upon COVID-19 were previously reported to follow a similar trend as in the brains of aged individuals and Alzheimer's Disease (AD) patients (19, 20). Additional lncRNAs previously linked to brain aging and AD such as NEAT1, LINC00643, LINC00507, and MALAT1, were also identified (18, 19).

To better understand the roles of the differentially expressed noncoding RNAs in COVID-19, many of which have no known functional roles, we performed guilt-by-association pathway analysis for the top and bottom 10 COVID-19-regulated ncRNAs as well as NEAT1, a well-studied lncRNA involved in brain aging (19) and cognitive function (18). For each ncRNA we ranked the coexpression of protein-coding genes across the transcriptome-profiled samples from The Cancer Genome Atlas (TCGA), spanning multiple tissue samples and genetic backgrounds, and tested for pathway enrichment using these protein-coding gene rankings (21, 22). This analysis implicated many of these lncRNAs in pathways associated with cognitive function (e.g., memory and learning) (23–30) (Figure 1E). Finally, we validated the decreased expression of LINC01007 and LINC00294 and increased expression of LINC01094 and NEAT1 by qRT-PCR (Figure 1F; Supplementary Figure 1). We selected these genes because (1) LINC01007, LINC00294, and LINC01094 are among the top 10 up/down COVID-regulated genes, with LINC01007 and LINC00294 as the two most significantly downregulated lncRNAs, (2) LINC01007 and LINC01094 have been previously associated with aging (19), and (3) NEAT1, also a significantly upregulated lncRNA, is well-established as a regulator of cognitive function (18). Critically, overexpression of NEAT1 impairs cognitive function, whereas knockdown of NEAT1 improves memory in mice (18), in support of a functional role for NEAT1 upregulation in COVID-19-associated cognitive decline.

Next, we sought to evaluate whether the differential expression of these COVID-19-regulated ncRNAs was also associated with poor cognitive performance in humans. We utilized previously published cognitive and transcriptomic data, obtained from the same individuals, in the context of the ROSMAP cohort (31, 32). After splitting those cases (n=633: 406 females and 227 males) by median Mini-Mental State Examination (MMSE) score (high cognitive performance: ≥25, 207 females and 129 males, total 336; low cognitive performance: <25, 199 females and 98 males, total 297) and performing gene expression analysis, we found 1,307 downregulated ncRNAs and 1,322 upregulated ncRNAs in individuals with low cognitive performance (Figure 2A; Supplementary Table). The larger sample size of the ROSMAP cohort, in comparison to the COVID-19 cohort, likely contributes to increased statistical power and a greater number of significant differentially expressed ncRNAs in the ROSMAP cohort. By Gene Set Enrichment Analysis (GSEA) analysis, we found that ncRNAs associated with severe COVID-19 were also associated with low cognitive performance (Figures 2B, C). Moreover, the similarities in ncRNA expression profiles due to COVID-19 and poor cognitive performance are maintained in COVID-19 relative to control cases with history of intensive care unit or ventilator (ICU/VENT) treatment (n=9) (5), in support of potential roles for ncRNAs in COVID-19-induced cognitive changes independent of ICU/VENT-associated treatment (Figure 2D).

Finally, as circulating inflammatory factors have been suggested to affect neurological states in COVID-19 (33), we utilized previously published total RNA sequencing data and assessed ncRNA expression changes in primary human neurons upon cytokine treatment (Figure 2E; Supplementary Table). We found 19 ncRNAs differentially expressed by at least one of IFNB, IFNG, or TNFA that are also differentially expressed in both severe COVID-19 and poor cognition (Figure 2F). Of these overlapping genes, LINC01094, NEAT1, and LINC00643 have been previously linked to brain aging and AD (19). Interestingly, loss of NEAT1 not only improves cognitive function (18), but also reduces inflammatory response (34). To obtain further insights into the effects of lncRNAs on protein-coding gene expression, we assessed whether the cognate sense genes (IRF1, PAXIP1, SOX21) of the three significant antisense lncRNAs (C5orf56/IRF1-AS1, PAXIP1-AS2, SOX21-AS1), which often transcriptionally regulate their corresponding sense gene (11), are also significant following cytokine treatment. We found that all three protein-coding genes follow similar expression patterns as the lncRNAs in our *in vitro* neuron datasets (Supplementary Figure 2). Of note, IRF1 is also significantly differentially expressed in both COVID-19 and ROSMAP comparisons. This gene is well-implicated in interferon regulation (35–37) and COVID-19 response (37, 38), in support of a role for IRF1-AS1 in the disease. Our analyses highlight the potential for lncRNAs as therapeutic targets to modulate neuroinflammation and mitigate associated cognitive deficits (15).

Discussion

Given the cognitive decline reported in patients with milder COVID-19 (2), it is tempting to speculate that similar lncRNA expression changes might be found in milder COVID-19 cases. We note, however that our analysis is limited primarily to severe COVID-19 cases due to the availability of relevant specimens. Although we are not statistically powered to make comparisons in milder cases or in asymptomatic individuals with COVID-19, we have included one individual with asymptomatic COVID-19 in our analysis. We found that the ncRNA expression profile from this individual is more representative of control individuals rather than those with severe disease (Figures 1D, 2C).

In summary, we have identified widespread expression changes of numerous lncRNAs in the brain due to severe COVID-19 that are also associated with poor cognition. We link a number of these lncRNAs to transcriptomic changes in neurons upon inflammatory cytokine stimulation. As COVID-19 is associated with cognitive decline (2, 3), our findings suggest key roles for lncRNAs in cognitive decline in individuals with severe COVID-19 and support the idea that inflammation-associated lncRNAs may be targeted to alleviate cognitive deficits observed in COVID-19.

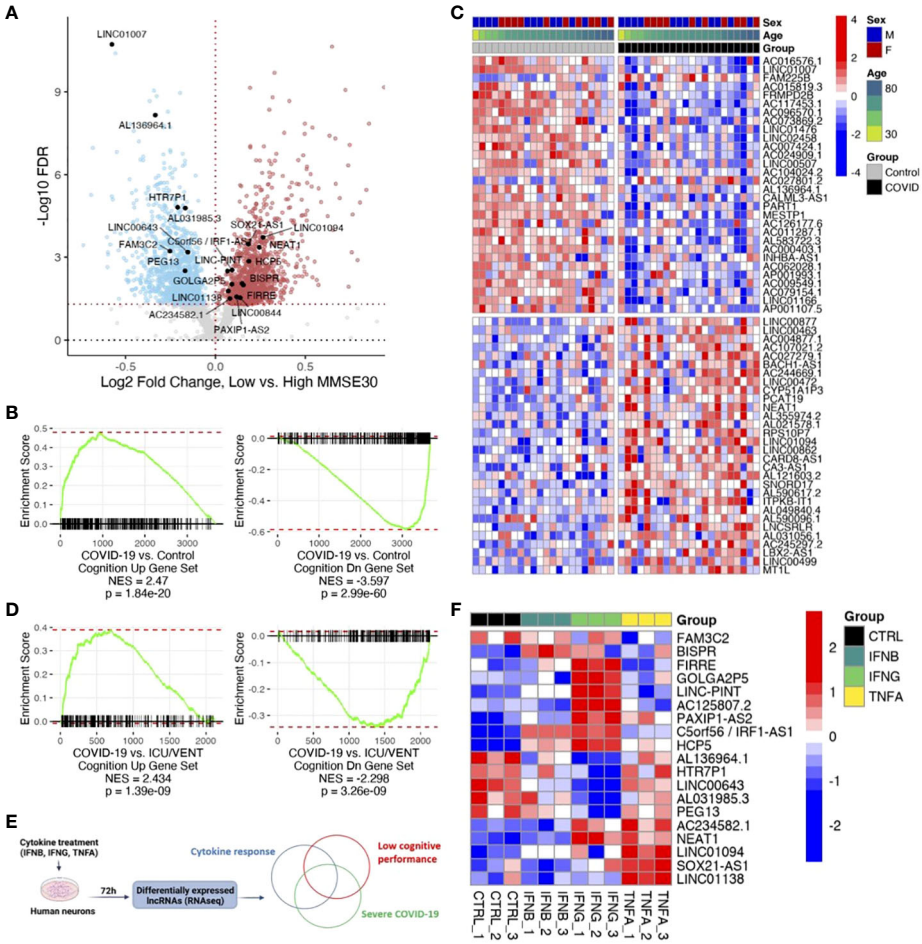

FIGURE 2
COVID-19 is associated with low cognitive performance-related noncoding RNAs (ncRNAs). **(A)** Volcano plot showing low cognitive performance-related ncRNAs identified in the ROSMAP cohort. Red points, significantly upregulated genes among individuals with low cognitive performance; MMSE scores <25 (false discovery rate/FDR < 0.05). Blue points, significantly downregulated genes with low cognitive performance; MMSE scores <25. Black points, highlighted significant genes with corresponding gene symbols (High MMSE 207 females and 129 males, total 336; Low MMSE 199 females and 98 males, total 297). **(B)** Gene set enrichment analysis (GSEA) of COVID-19-regulated ncRNAs using low cognitive performance-associated ncRNAs as gene sets. DEG ranks were assigned by signed -log10 FDR from frontal cortex transcriptome of COVID-19 versus transcriptome of age/sex-matched control (n=22/group). NES, normalized enrichment score. p, two-tailed GSEA p-value. **(C)** Heatmap of expression values (COVID-19 cohort) of top 30 upregulated ncRNAs and top 30 downregulated ncRNAs overlapping between COVID-19 and low cognitive performance-related ncRNAs. Red represents increased expression; Blue represents decreased expression. **(D)** GSEA of COVID-19-regulated ncRNAs using low cognitive performance-associated ncRNAs as gene sets. DEG ranks were assigned by signed -log10 FDR from frontal cortex transcriptomes of COVID-19 (n=22) versus transcriptomes of uninfected controls with ICU/VENT history (n=9). NES, normalized enrichment score. p, two-tailed GSEA p-value. **(E)** Schematic of *in vitro* cytokine treatment experiment and analytical approach. **(F)** Heatmap of expression values (*in vitro* human neurons) of significant ncRNAs overlapping between COVID-19, cognition, and cytokine response. IFNB: 1ng/ml^{-1}; IFNG: 1µg/ml^{-1}; TNFA: 100ng/ml^{-1}. Red represents increased expression; Blue represents decreased expression.

Materials and methods

Human biospecimen annotation and RNA-seq library preparation

In this study, we analyzed our previously described total RNA-seq datasets (5). In that cohort, frozen COVID-19 frontal cortex specimens were collected following a protocol for waived consent for the use of excess tissue, approved by the Mass General Brigham Institutional Review Board. Frozen control frontal cortex specimens were obtained from the NIH NeuroBiobank and the NIH HBCC. Clinical features of the COVID-19 cohort have been previously described in Mavrikaki et al. and included 22 cases with pre-mortem or peri-mortem positive testing for SARS-CoV-2 by nasopharyngeal swab qPCR (COVID-19 group) with mean age 61.91 ± 3.1 years (12 males and 10 females), age/sex-matched (± 2 years) uninfected controls without any known psychiatric or neurological disease with mean age 61.86 ± 3.1 years, and an independent group of 9 uninfected cases with history of ICU or ventilator treatment (ICU/VENT) with mean age 57 ± 6.98 years (6 males and 3 females) (5). Total RNA from those samples was extracted using Trizol and phase separation. 450 ng of RNA for the frontal cortex specimens and 80 ng of RNA for the human primary neurons was used for library preparation (5). Libraries were prepared using the KAPA RNA HyperPrep kit with RiboErase (HMR; Roche; #08098131702) following the manufacturer's recommendations, pooled together (4 runs), and processed for sequencing using NovaSeq 6000 (5). Total

RNA-seq data for the COVID-19 cohort and *in vitro* neuron experiment are available at the Gene Expression Omnibus (GEO) with accession number GSE188847.

RNA-seq alignment and quantification

Raw. fastq sequencing files were aligned to Ensembl v104 using salmon v1.4.0, combining both protein-coding (cdna.all.fa) and noncoding RNA (ncrna.fa) sequences. Annotated gene biotypes were obtained from the Ensembl v96 release (April 2019), as distinction between antisense, processed pseudogene, and long intergenic noncoding RNA were not included in further Ensembl updates. Gene-level abundances were determined using tximport v1.18.0, and differential expression analysis was performed with DESeq2 v1.30.1 using lfcShrink to stabilize variance. Preprocessed ROSMAP gene abundances from n=633 (High MMSE 207 females and 129 males; Low MMSE 199 females and 98 males) and corresponding MMSE cognitive data were obtained from https://www.synapse.org/#!Synapse:syn8691134 and https://www.synapse.org/Portal.html#!Synapse:syn3157322, respectively (39), and differential expression analysis was performed with DESeq2 v1.30.1.

Pathway analyses

Guilt-by-association pathway analysis of lncRNAs was performed as follows. First, Pearson correlations between the expression levels (log2 transcripts per million + 1) of candidate lncRNAs and those of all protein-coding genes were determined across 9,830 patient transcriptome samples generated as part of The Cancer Genome Atlas Research Network: https://www.cancer.gov/tcga. Protein-coding genes ranked by correlation with each tested lncRNA were used as input for gene set enrichment analysis (fgsea v1.16.0), using gene sets of previously identified Gene Ontology pathways (5). In addition to NEAT1, the top and bottom 10 differentially expressed lncRNAs as ranked by FDR in the COVID-19 cohort and detected in the TCGA dataset were tested for pathway analysis (one snoRNA and one lncRNA were not detected).

Association testing between COVID-19 and ROSMAP cohorts was performed as follows. Signed -log10 FDRs from COVID-19 vs. Control or COVID-19 vs. ICU/VENT comparisons were used to rank ncRNA genes for gene set enrichment analysis via fgsea v1.16.0, filtering out genes with an FDR < 0.5. Cognition-associated gene sets were collated from ROSMAP Poor vs. Normal MMSE comparisons, using significant (FDR < 0.05) ncRNAs. Ensembl gene IDs were used for gene matching in this analysis.

R scripts, reference files, and realigned RNA-seq files used for these analyses are available at https://github.com/jonathandlee12/covid19-brain-lnc. All other reference datasets are either publicly available or will be provided upon reasonable request to the corresponding authors.

qRT-PCR

A total of 400 ng RNA from each sample was used for cDNA synthesis, and qRT-PCR for orthogonal validation was performed and analyzed as previously described in Mavrikaki et al. (5). GAPDH (Qiagen; QuantiTect primer assay: QT00079247) and RPS18 (Qiagen; QuantiTect primer assay: QT00248682) were used for normalization. Primers for LINC01007 (#qhsaLED0063333), LINC01094 (#qhsaLED0101136), and NEAT1 (#qhsaLED0134812) were purchased from Bio-Rad. Primers for LINC00294 were TGTGTTGTCCTCCAGAATCG (forward) and CCAACCAAGAGCCAACAAAG (reverse) (40), and were synthesized by IDT. Data were analyzed according to the $2^{-\Delta\Delta Ct}$ method (41).

Transcriptomic data analysis of cytokine-treated neurons

We reanalyzed previously published total RNA-seq data of primary human neurons (ScienCell Research Laboratories, 1520-5) treated with different cytokines (5) which are available on the GEO with accession number GSE188847. Primary neurons were treated with IFN-β (1 ng ml^{-1}), IFN-γ (1 μg ml^{-1}), TNF (100 ng ml^{-1}) or nuclease-free water (control) for 72 h, and RNA was extracted using Trizol/phase separation, and 80ng of RNA was processed for total RNA-seq.

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: GSE188847 (GEO).

Ethics statement

The studies involving human specimens were approved by Mass General Brigham Institutional Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements. Controls were obtained from the NIH Neurobiobank and the NIH HBCC as de-identified samples.

Author contributions

JL: Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. IS: Resources, Writing – review & editing. FS: Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing. MM: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, 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.

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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.2024.1290523/full#supplementary-material

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A murine model of post-acute neurological sequelae following SARS-CoV-2 variant infection

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Viral variant is one known risk factor associated with post-acute sequelae of COVID-19 (PASC), yet the pathogenesis is largely unknown. Here, we studied SARS-CoV-2 Delta variant-induced PASC in K18-hACE2 mice. The virus replicated productively, induced robust inflammatory responses in lung and brain tissues, and caused weight loss and mortality during the acute infection. Longitudinal behavior studies in surviving mice up to 4 months post-acute infection revealed persistent abnormalities in neuropsychiatric state and motor behaviors, while reflex and sensory functions recovered over time. In the brain, no detectable viral RNA and minimal residential immune cell activation was observed in the surviving mice post-acute infection. Transcriptome analysis revealed persistent activation of immune pathways, including humoral responses, complement, and phagocytosis, and gene expression levels associated with ataxia telangiectasia, impaired cognitive function and memory recall, and neuronal dysfunction and degeneration. Furthermore, surviving mice maintained potent systemic T helper 1 prone cellular immune responses and strong sera neutralizing antibodies against Delta and Omicron variants months post-acute infection. Overall, our findings suggest that infection in K18-hACE2 mice recapitulates the persistent clinical symptoms reported in long-COVID patients and provides new insights into the role of systemic and brain residential immune factors in PASC pathogenesis.

KEYWORDS

SARS-CoV-2 variant, post-acute sequelae, long COVID, inflammatory responses, CNS inflammation

1 Introduction

Acute COVID-19 infection typically lasts 4 weeks from symptomatic onset and results in diverse clinical outcome ranging from mild to severe pneumonia, life-threatening multiorgan failure, and death (1, 2). An estimated 30% to 50% of COVID-19 survivors suffer from a post-viral syndrome known as long-COVID [post-acute sequelae of COVID-19 (PASC)], which encompasses ongoing persistent symptoms and complications beyond the initial 4 weeks of infection (3, 4). The key features of long-COVID include neurological symptoms, such as fatigue, cognitive dysfunction (brain fog, memory loss, and attention disorder), and sleep disturbances (5–7). Psychiatric manifestations (anxiety and depression) are also common (8, 9). Although the severity of acute infection is considered as one major risk factor for developing PASC (10, 11), increasing evidence suggests that long-COVID also occurs in people with non-symptomatic or non-hospitalized status during acute infection (12, 13). PASC has posed a significant threat to the global healthcare system. Nevertheless, our current understanding of its underlying mechanisms is limited.

Acute SARS-CoV-2 infection has been studied in various animal models, including hamsters, ferrets, non-human primates (NHPs), rats, and mice (14, 15). Mice are easier to work with, and most amenable to immunological manipulation. Angiotensin-converting enzyme 2 (ACE2) is the cell entry receptor for SARS-CoV-2 (16), and mouse ACE2 shows key differences from human ACE2 (hACE2); thus, wild-type mice present challenges for infection with human SARS-CoV-2 variants. To overcome this challenge, delivery of adeno-associated virus (AAV)-mediated expression of hACE2 into the respiratory tract or use of the mouse-adapted SARS-CoV-2 (SARS-CoV-2 MA strain or CMA strain), which incorporates key mutations that allows the virus to utilize mouse ACE2 for entry into cells in immunocompetent mice, results in a productive infection with mild acute respiratory distress syndrome (17–19). K18-hACE2 transgenic mice express the hACE2 protein under the human keratin 18 (K18) promoter, which confers efficient transgene expression in airway epithelial cells. Acute SARS-CoV-2 infection in K18-hACE2 transgenic mice induces weight loss, interstitial pneumonitis, encephalitis, and death (20–23). In this study, to investigate SARS-CoV-2 variant-induced PASC, we infected K18-hACE2 mice with the Delta variant and performed longitudinal behavior analysis for up to 4 months post-acute infection. Mice surviving acute Delta variant infection displayed persistent abnormalities in neuropsychiatric state and motor behavior post-acute infection. Although surviving mice developed and maintained potent Th1-prone cellular and antibody responses in the periphery, transcriptome analysis suggested persistent activation of immune pathways, cognitive dysfunction and neuronal dysfunction in the CNS for months post-acute infection.

2 Materials and methods

Viruses: SARS-CoV-2 Delta and Omicron B.A.2 strains were obtained from the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) at the University of Texas Medical Branch (UTMB) and were amplified twice in Vero E6 cells as described previously (24).

2.1 SARS-CoV-2 infection in mice and ethics statement

Six- to 8-week-old K18-hACE2 mice (Jackson Lab, stock #034860) were bred and maintained at UTMB animal facility. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC # 1412070) at UTMB. In order to study virus-induced PASC, mice were infected intranasally (i.n.) with sublethal doses [600 and 800 plaque-forming units (PFU) of SARS-CoV-2 Delta or Omicron B.A.2 strain, respectively. based on Singh et al. unpublished data]. Infected mice were monitored twice daily for morbidity and mortality. In some experiments, on 3 days, 6 days, 7 days, 8 days, 9 days, 1 month, 2 months, and 4 months post-infection (pi), mice were transcardially perfused with PBS to remove blood. Hemi brains and left lungs were collected in 4% paraformaldehyde for histopathology and immunofluorescence staining studies. The right, superior, and middle lobes of mouse lung, hemi brains, kidney, and liver tissues were collected in Trizol for RNA extraction used for viral RNA and cytokine analysis. The inferior lobes of right lung and hemi brains (in some experiments) were homogenized in 1 mL of PBS and viral titers in the tissue homogenates were further determined by plaque assays as PFU per lobe of lung (19).

2.2 Quantitative PCR

The sequences of the primer sets and PCR reaction conditions were described previously (25–27) and in Supplementary Table S1. Tissues were resuspended in TRIzol for RNA extraction according to the manufacturer's instructions (Thermo Fisher Scientific). RNA concentration and purity were determined by using WPA Biowave DNA Spectrophotometer. Complementary (c) DNA was then synthesized by using a qScript cDNA synthesis kit (Bio-Rad). Expression of SARS-CoV-2 S gene and mouse inflammatory cytokine and chemokine genes (IL-1β, IL-6, TNF-α, CCL2, CCL5, CCL7, CXCL10, and CCL11) were measured by quantitative PCR (Q-PCR) using the CFX96 real-time PCR system (Bio-Rad). PCR cycling conditions were as follows: 95°C for 3 min, 45 cycles of 95°C for 15 s, and 60°C for 1 min. Gene expression was calculated using the formula $2^{-[Ct(\text{target gene})-Ct(\beta\text{-actin})]}$ as described before (28).

2.3 RNA-seq, gene set enrichment analysis, and Cytoscape analysis

RNA was extracted from brain tissues as described above and 1–3 μg of RNA of each sample was used for RNA-seq analysis. The RNA quality was verified by the Next-Generation Sequence (NGS) core laboratory using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and an Agilent Bioanalyzer 2100 (Agilent

Technologies, Santa Clara, CA). PolyA+ RNA was purified from ~100 ng of total RNA and sequencing libraries were prepared with the NEBNext Ultra II RNA library kit (New England Biolabs) following the manufacturer's protocol. Libraries were pooled and sequenced on an Illumina NextSeq 550 High Output flow cell with a paired-end 75 base protocol. Pathway enrichment analysis was performed using gene set enrichment analysis (GSEA) version 3.0 (29, 30). Specifically, analysis parameters were set to 2,000 gene set permutation and gene set size limit 15–500. Primary gene sets investigated were obtained from the David Bader lab (http://download.baderlab.org/EM_Genesets/). GSEA FDR $Q < 0.05$ cutoff was applied to examine enriched gene sets in our dataset. Cytoscape Enrichment map tool was used to visualize results from the GSEA. GSEA output files were uploaded in the Enrichment Map app of Cytoscape and FDR Q value cutoff was set to 0.01. The AutoAnnotate Cytoscape app was used to define the clusters automatically. The RNA-seq data in this study were deposited in NCBI's Gene Expression Omnibus (GEO Series accession number GSE260625). The sequence can be found at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE260625.

2.4 Plaque assay

Vero E6 cells were seeded in six-well plates and incubated at 37°C. Tissue homogenates were serially diluted (10-fold) in DMEM with 2% FBS and 0.2 mL was used to infect cells at 37°C for 1 h. After incubation, samples were overlaid with MEM (Gibco) with 8% FBS and 1.6% agarose (Promega). After 48 h, plates were stained with 0.05% neutral red (Sigma-Aldrich) and plaques were counted to calculate virus titers expressed as PFU/mL (24, 31).

2.5 Histopathology studies

As previously described with some modifications (19), left lung and hemi brain tissues were collected and fixed in 4% paraformaldehyde for a minimum of 3 days at 4°C. The fixed tissues were then transferred and submerged in 10% buffered formalin (Fisher, Waltham, MA). Preparation of paraffin-embedded tissue blocks followed by cutting 10-µm tissue sections and hematoxylin and eosin (H&E) staining were performed by the Histopathology Laboratory Core at UTMB. Unstained tissue sections were also prepared for immunofluorescence staining.

2.6 Behavioral studies

At 1, 2, 3, and 4 months pi, all surviving animals underwent neurological assessments using a modified SmithKline Beecham, Harwell, Imperial College, Royal London Hospital phenotype assessment (SHIRPA) protocol (32, 33). Mice were also weighed at the above time points to confirm their growth. For the modified SHIRPA assessment, each mouse was placed in a transparent cylindrical viewing jar and observed for 5 min. Observations in body position, spontaneous activity, respiration rate, tremor, and defecation were noted. Subsequently, the mouse was transferred to an open field arena at which time transfer arousal and gait were noted. Following transfer, the mice were allowed to freely move around the open field arena for 30 s and the number of times that all four limbs crossed into new quadrants was counted to evaluate locomotor activity. For the next 5 min, gait, eye opening, piloerection, pelvic elevation, tail position, and touch escape were observed in the open field arena. Furthermore, tail lifting was performed to evaluate trunk curl and visual placing followed by assessment of reach touch, grip strength, whisker response, palpebral reflex, and ear twitch above arena. Additional behaviors of each mouse, such as fear, biting, irritability, and aggression, were observed throughout the procedure. Based on the observation, scores were provided (0, 1, 2, or 3); 2 was considered normal behavior, and any score outside of 2 was considered abnormal behavior. Each parameter assessed by SHIRPA was grouped into five functional categories (see Table 1). To measure motor coordination, a parallel rod floor test was performed. Briefly, mice were placed in the center of the cage that was covered with horizontal rods for 2 min. Foot/paw slips were counted and recorded manually.

2.7 Immunofluorescence staining

Formalin-fixed, paraffin-embedded tissue blocks were cut into 10-µm sections, which were deparaffinized with xylene and rehydrated with ethanol/water. To detect Iba1 and GFAP expression, tissue sections were exposed to anti-Iba1 rabbit antibody (FUJIFILM Wacko, 013-27691, 1:300 dilution) and anti-GFAP mouse antibody (Sigma, G3893, 1:300 dilution) overnight at room temperature, respectively. After washing with phosphate-buffered saline (PBS), slides were exposed to goat anti-rabbit Alexa Fluor Plus 555 (Thermo Fisher Scientific, A32732, 1:1,000 dilution) and goat anti-mouse Alexa Fluor 488 (Thermo Fisher Scientific, A32723, 1:1,000 dilution) secondary antibodies for 1 h at room temperature. For nuclei staining, we used 4′,6′-diamidino-2-phenylindole (DAPI) (D9542, Sigma). Slides were washed with PBS and mounted with ProLong Gold Antifade (Thermo Fisher Scientific, P36930). Images were captured using an Olympus BX53 microscope.

TABLE 1 Modified SHIRPA assessment.

1. Neuropsychiatric state	Spontaneous activity, transfer arousal, touch escape, positional passivity, biting, fear, irritability, and aggression
2. Motor behavior	Body position, tremor, locomotor activity, pelvic elevation, tail elevation, gait, trunk curl, and limb grasping
3. Autonomic function	Respiration rate, piloerection, and heart rate
4. Muscle tone and strength	Grip strength, body tone, limb tone, and abdominal tone
5. Reflex and sensory functions	Visual placement, pinna reflex, toe pinch, righting reflex, palpebral reflex, whisker response, reach touch, and ear twitch

2.8 Antibody and SARS-CoV-2 spike (S) protein ELISA

As described previously (24, 31), ELISA plates were coated with 100 ng/well recombinant SARS-CoV-2 RBD protein (RayBiotech) overnight at 4°C. The plates were washed twice with PBS containing 0.05% Tween-20 (PBS-T) and then blocked with 8% FBS for 1.5 h. Sera were diluted 1:100 in blocking buffer and were added for 1 h at 37°C. Plates were washed five times with PBS-T. Goat anti-mouse IgG (Sigma) coupled to horseradish peroxidase (HRP) or alkaline phosphatase was added at a 1:2,000 dilution for 1 h at 37°C. This was followed by adding TMB (3,3,5,5′-tetramethylbenzidine) peroxidase substrate (Thermo Fisher Scientific) for approximately 10 min. The reactions were stopped with 1 M sulfuric acid, and the intensity was read at an absorbance of 450 nm. For SARS-CoV-2 S protein ELISA, plates were coated with 50 ng/well (5 µg/mL in coating buffer) of diluted capture antibody (Thermo Fisher Scientific, USA) overnight at 4°C. The plates were washed twice with PBS-Tween (PBS-T) and then blocked with 8% FBS for 1.5 h. Standard (recombinant SARS-CoV-2 S protein, Sino Biological, USA) was diluted in blocking buffer, and the brain suspension was added for 1 h at 37°C. Plates were washed five times with PBS-T. SARS-CoV-2 S1 protein primary antibody (Thermo Fisher Scientific) at 1 µg/mL was added for 1 h at 37°C. Plates were again washed five times with PBS-T. Goat anti-rabbit IgG (Thermo Fisher Scientific) coupled to horseradish peroxidase (HRP) was added at a 1:2,000 dilution for 1 h at 37°C, followed by adding TMB (Thermo Fisher Scientific) for 30 min. The reactions were stopped by 1 M sulfuric acid, and the intensity was read at an absorbance of 450 nm.

2.9 IFN-γ ELISPOT

As described earlier (24), Millipore ELISPOT plates (Millipore Ltd.) were coated with anti-mouse IFN-γ capture Ab at 1:100 dilution (Cellular Technology Ltd) at 4°C overnight. Splenocytes were stimulated with SARS-CoV-2 S peptide pools (2 µg/mL, Miltenyi Biotec) for 24 h at 37°C. Cells were stimulated with anti-CD3 (1 µg/mL, e-Biosciences) or medium alone, as controls. This was followed by incubation with biotin-conjugated anti-IFN-γ at 1:100 dilution (Cellular Technology Ltd.) for 2 h at room temperature, followed by incubation with alkaline phosphatase-conjugated streptavidin for 30 min. The plates were washed and scanned using an ImmunoSpot 6.0 analyzer and analyzed by ImmunoSpot software to determine the spot-forming cells (SFCs) per 10^6 splenocytes.

2.10 Intracellular cytokine staining

Splenocytes were incubated with SARS-CoV-2 S peptide pools (1 µg/mL, Miltenyi Biotec) for 6 h in the presence of GolgiPlug (BD Bioscience). Cells were harvested and stained with antibodies for CD3, CD4, or CD8, fixed in 2% paraformaldehyde, and permeabilized with 0.5% saponin before adding anti-IFN-γ (Thermo Fisher Scientific) (24, 31). Samples were acquired by a C6 Flow Cytometer instrument. Dead cells were excluded based on forward and side light scatter. Data were analyzed with a CFlow Plus Flow Cytometer (BD Biosciences).

2.11 Fluorescent focus reduction neutralization test

Neutralization titers of mice sera were measured by a fluorescent focus reduction neutralization test (FFRNT) using the mNG reporter SARS-CoV-2 as previously reported with some modifications (24, 34). Briefly, Vero E6 cells (2.5×10^4) were seeded in each well of a black µCLEAR flat-bottom 96-well plate (Greiner Bio-one™). The cells were incubated overnight at 37°C with 5% CO_2. On the following day, each serum was twofold serially diluted in the culture medium with the first dilution of 1:20. Each serum was tested in duplicate. The diluted serum was incubated with 100–150 fluorescent focus units (FFU) of mNG Delta and BA.2 SARS-CoV-2 at 37°C for 1 h (final dilution range of 1:20 to 1:20,480), respectively. After that, the serum–virus mixtures were inoculated onto the pre-seeded Vero E6 cell monolayer in 96-well plates. After 1 h of infection, the inoculum was removed and 100 µL of overlay medium (DMEM supplemented with 0.8% methylcellulose, 2% FBS, and 1% P/S) was added to each well. After incubating the plates at 37°C for 16 h, raw images of mNG fluorescent foci were acquired using CytationTM 7 (BioTek) armed with 2.5× FL Zeiss objective with a wide field of view and processed using the software settings [GFP (469,525) threshold 4,000, object selection size 50–1,000 µm]. The foci in each well were counted and normalized to the non-serum-treated controls (set as 100%) to calculate the relative infectivity. The neutralizing titer 50 (NT_{50}) was calculated manually as the highest dilution of the serum sample that prevents at least 50% fluorescence foci formation in infected cells. A titer is calculated for each of the two replicates of a sample and the geometric mean titer (GMT) of the two is reported as the final sample titer.

2.12 Statistical analysis

Survival curve comparison was performed using GraphPad Prism software 9.4.1, which uses the log-rank test. Values for viral load, cytokine production, antibody titers, and T-cell response experiments were compared using Prism software statistical analysis and were presented as means ± SEM. *P* values of these experiments were calculated with a non-paired Student's ***t***-test. Parameters of behavior changes at month 1 and results of parallel rod test were compared using Student's ***t***-test. For the categorical, longitudinal measures of each parameter in modified SHIRPA testing, we considered a score of 2 as normal activity and the other scores (0, 1, and 3) as abnormal activity. For selected parameters, changes were presented over time in a stacked bar chart with Sankey-style overlays using SAS version 9.4 (SAS Inc., Cary, NC). All tests were two-sided with a significance level of 0.05.

3 Results

3.1 SARS-CoV-2 variant replicated in brain and lung tissues and induced inflammatory responses in both tissues during the acute infection in K18-hACE2 mice

Several reports suggest that the frequencies of PASC symptoms increased with SARS-CoV-2 variants, in particular, with the pre-Omicron variant compared to the original prototype virus infection (3, 35–37). Thus, to investigate the mechanisms of SARS-CoV-2-induced PASC, we infected K18-hACE2 mice with the Delta variant. Viral load analysis, survival/weight changes monitoring, immunological and histopathology studies, and behavior assessment were performed at both acute infection and at 1 to 4 months post-acute COVID infection (Figure 1A). Initially, 6-to 8-week-old K18-hACE2 mice were intranasally (i.n.) inoculated with a sublethal dose of SARS-CoV-2 Delta variant strain and monitored daily for morbidity and mortality. Infected mice exhibited weight loss starting on day 6 pi and succumbed to infection as early as day 7. Approximately 22% of mice infected with the Delta variant survived the 4-week pi interval (Figures 1B, C). In the brain, viral RNA but not infectious virus was detected at day 3. Viral loads increased significantly and reached to the peak at day 6. Viral RNA levels were continuously detectable at days 7, 8, and 9 pi (Figures 1D, E, Supplementary Figure 1A). As in the most severe clinical cases, the prognosis can be worsened by the hyperproduction of proinflammatory cytokines (38, 39); we next measured expression levels of proinflammatory cytokines and chemokines. At days 3 and 6, proinflammatory cytokines, including IL-1β, IL-6, and TNF-α, and chemokines, such as CCL2, CCL5, CXCL10, and CCL11, were induced in the brains of infected mice (Figures 1F, G). Histopathology analysis was also performed to confirm these findings and revealed viral encephalitis with perivascular infiltrations and microglial activation in the cortex of infected mice, but not in mock-infected mice, which together suggest neuroinflammation induction in the Delta variant-infected mice (Figure 1H). As lung is the primary site of SARS-CoV-2 infection, we next measured viral loads in the lung and noted infection was high at day 3 but diminished at day 6 (Figures 2A, B). Viral RNA remained detectable in the lungs at days 7, 8, and 9 pi (Supplementary Figure 1B). Proinflammatory cytokines and chemokines, including IL-1β, IL-6, CCL2, CCL5, CXCL10, and CCL11, were triggered in the lungs of infected mice compared to the mock group (Figures 2C, D). Lung pathology study showed mononuclear cell infiltration in peribronchiolar and perivascular areas as well as in the alveolar septa of the infected mice, but not in the mock group (Figure 2E). Overall, these results suggest that the SARS-CoV-2 Delta variant replicated in lung and brain tissues and triggered inflammation during the acute infection phase in K18-hACE2 mice.

3.2 SARS-CoV-2 Delta variant-infected mice displayed neurological behavior changes months post-acute infection

Based on National Institute for Health and Care Excellence guidelines, PASC is displayed at 4 weeks or more after the start of acute COVID-19 infection [3]. Here, we did not detect infectious virus (data not shown) or viral RNAs (Supplementary Figures 1A, B) at 1 and 4 months pi in the brain and lung tissues. No significant levels of viral S1 protein were detected in the brain tissues beyond 1 month, which together indicate viral clearance at the post-acute phase (Supplementary Figure 1C). To further understand the effects of SARS-CoV-2 infection in PASC pathogenesis, viral loads in several other periphery tissues, including liver, kidney, and blood, were also measured during acute and post-acute infection. No detectable viral RNA was found in these tissues except at day 6 in the kidneys (Supplementary Figures 1D–F). Histopathological analysis revealed no changes in the various regions of brain in the Delta variant-infected mice at 1 or 4 months pi compared to the mock group (Supplementary Figure 2A). In the lung, there were notably increased levels of CCL7 and CXCL-10 at 1 month, and increased levels of IL-6, CCL5, CXCL10, and CCL11 at 4 months in the infected mice compared to the mock group (Supplementary Figure 2B), though no changes in the levels of IL-1β, TNF-α, and CCL2 were observed (data not shown). Thus, viral infection was cleared in the periphery and CNS tissues post-acute infection, and this was accompanied by mild and minimal local inflammatory responses in the lung and brain tissues, respectively.

To assess the infection impact on animals, behavior tests were performed in the surviving mice at 1 month pi using a modified Smith-Kline Beecham, Harwell, Imperial College, Royal London Hospital, phenotype assessment (SHIRPA) protocol (32, 33). Mock-infected mice were used as controls. The assay involves a battery of semi-quantitative tests for general health and sensory function, baseline behaviors, and neurological reflexes. The individual parameters assessed by SHIRPA were grouped into five functional categories (Table 1): (1) Motor behavior test includes body position, tremor, locomotor activity, pelvic elevation, tail elevation, gait, trunk curl, and limb grasping; (2) Autonomic function test includes respiration rate, palpebral closure, and piloerection; (3) Muscle tone and strength includes grip strength, body tone, and limb tone; (4) Neuropsychiatric state includes spontaneous activity, transfer arousal, touch escape, positional passivity, biting, fear, irritability, and aggression; and (5) Reflex and sensory functions include parameters such as visual placement, toe pinch, and righting reflex (40). The SHIRPA assay results showed that mice surviving acute infection with SARS-CoV-2 Delta variant displayed abnormalities mainly in neuropsychiatric state, motor behavior, autonomic function, and reflex and sensory function, compared to the mock group (Figure 3A). Weight loss was not noted in the surviving mice at 1 month pi, nor was it detected during the rest of

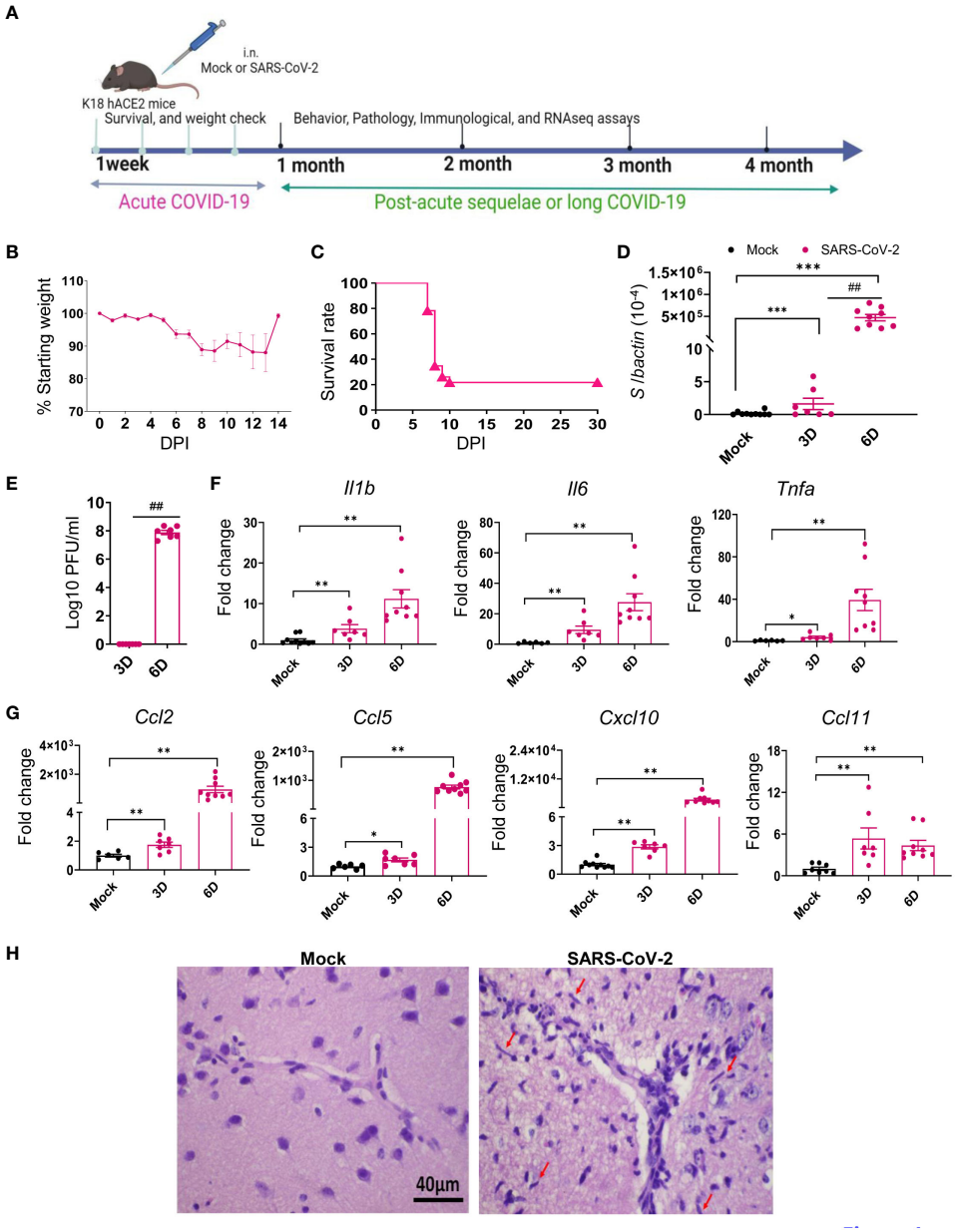

FIGURE 1
SARS-CoV-2 Delta variant replicated and induced inflammatory responses in brain tissues during acute infection in K18-hACE2 mice. Six- to eight-week-old K18-hACE2 mice were i.n. infected with a sublethal dose of SARS-CoV-2 Delta variant strain (or mock infected) and monitored daily for morbidity and mortality. **(A)** Study design. **(B)** Mouse weight loss. Weight loss is indicated by percentage using the weight on the day of infection as 100%. n = 23. **(C)** Survival rate. **(D, E)** SARS-CoV-2 viral loads in brain were measured by Q-PCR **(D)** and plaque assay **(E)** at indicated days **(D)** pi. **(F, G)** Cytokine **(F)** and chemokine **(G)** expression levels in the brains were measured by Q-PCR. Data are presented as the fold increase compared to mock-infected mice (means ± SEM). n = 7 to 10. **(H)** Histopathology of brains of Delta variant-infected mice revealed viral encephalitis with perivascular infiltrations and microglial activation (arrows) in the cortex, but not in mock-infected mice ***$p < 0.001$, **$p < 0.01$, or *$p < 0.05$ compared to mock. $^{\#\#}p < 0.01$ compared to 3D.

the 4-month pi interval (Figure 3B). Furthermore, the Delta variant-infected mice showed higher number of foot slips compared to the mock group at 3 months pi in a parallel rod test, which indicates ataxia in the infected mice (41) (Figure 3C).

To further assess the impact of SARS-CoV-2 infection on behavioral changes of surviving mice, SHIRPA analysis was performed longitudinally over the 4-month pi period. It was noted that in mice surviving Delta variant infection, the abnormal rates for the parameters such as body position, grip strength, touch escape, and reach touch remained unchanged during the 4-month period (Figure 4A). However, abnormal levels for gait, whisker response, ear twitch, and palpebral reflex decreased over time (Figure 4B). In contrast, abnormalities for parameters including spontaneous activity, tail position, and tremor increased over the 4-month period (Figure 4C). Overall, the neuropsychiatric state and motor behavior of Delta variant-infected mice remained impaired

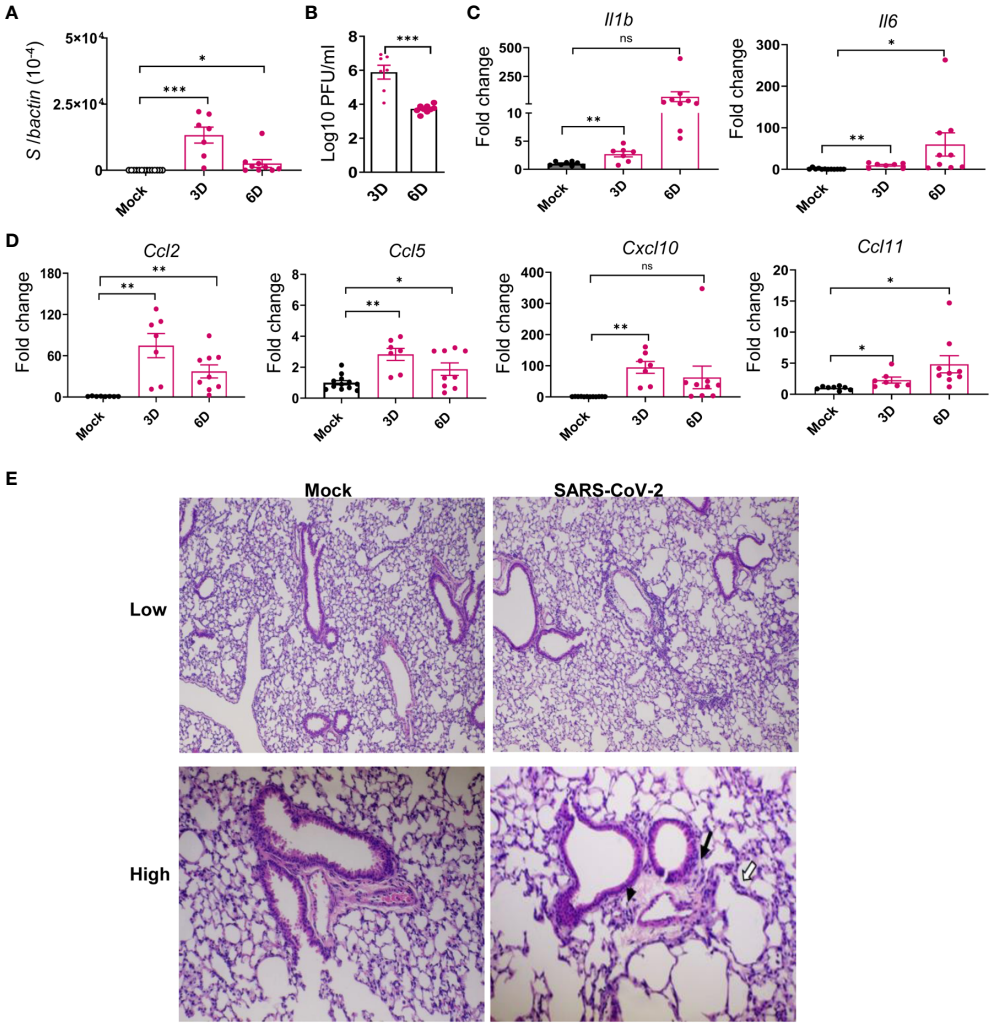

FIGURE 2
SARS-CoV-2 Delta variant replicated in the lung tissues and induced inflammatory responses in K18-hACE2 mice during acute infection. Six- to eight-week-old K18-hACE2 mice were i.n. infected with a sublethal dose of SARS-CoV-2 Delta variant strain or mock infected. Viral loads in lung tissues were measured by Q-PCR **(A)** and plaque assays **(B)** at indicated days **(D)** pi. **(C, D)** Cytokine **(C)** and chemokine **(D)** expression levels in the lung were measured by Q-PCR. Data are presented as the fold increase compared to mock-infected mice (means ± SEM). *n* = 7 to 9. **(E)** Histopathology of Delta variant-infected (right panels) or mock-infected (left panels) lungs. Low-power views of representative areas (top panels) from each group show moderate inflammation in the infected mice. At higher-power views (low panels), mononuclear cell infiltrations are observed in peribronchiolar (black arrow) and perivascular (arrowhead) areas as well as in the alveolar septa (white arrows). Bar = 200 µm for top panels; Bar = 80 µm for low panels. ns, not statistically significant ***$p < 0.001$, **$p < 0.01$, or *$p < 0.05$ compared to mock.

or even deteriorated months pi, whereas reflex and sensory functions appeared to recover over time.

Transcriptome analysis revealed persistent activation of immune pathways, and cognitive and neuronal dysfunction in the CNS months post-acute infection, though minimal microglia activation was observed. Potent Th1-prone cellular and antibody responses persisted in the periphery during post-acute infection.

To identify the host factors contributing to SARS-CoV-2 variant-induced PASC, we determined gene expression alterations by comparing the transcriptomes of mock and SARS-CoV-2 Delta variant-infected mouse brains using bulk RNA-seq. Transcriptome analysis identified 3,481 and 18 differentially expressed genes (FDR < 0.1) in 1-month and 4-month post-SARS-CoV-2 variant-infected mouse brains, respectively, compared to mock. We then performed GSEA to identify the biological processes that play a role in host response against SARS-CoV-2 infection. GSEA of RNA-seq data identified pathways related to immune signaling, such as the "complement activation pathway" and "phagocytosis recognition" (FDR < 0.01) as the top enriched pathways (Figure 5A, Supplementary Figure 3A, Supplementary Table S2). Interestingly, we also observed enrichment of the same immune signaling-related pathways "complement activation pathway," "phagocytosis recognition," and "humoral immune response mediated by circulating immunoglobulin" (FDR < 0.01) among the top five enriched pathways at 4-month pi (Figure 5B, Supplementary Figure 3B, Supplementary Table S2). Notably, the normalized enrichment scores (NESs) were higher for these pathways compared to the dataset at 1-month pi, an indication of persistence of immune activation in the CNS caused by infiltering immune cells and factors following the initial viral infection. Next,

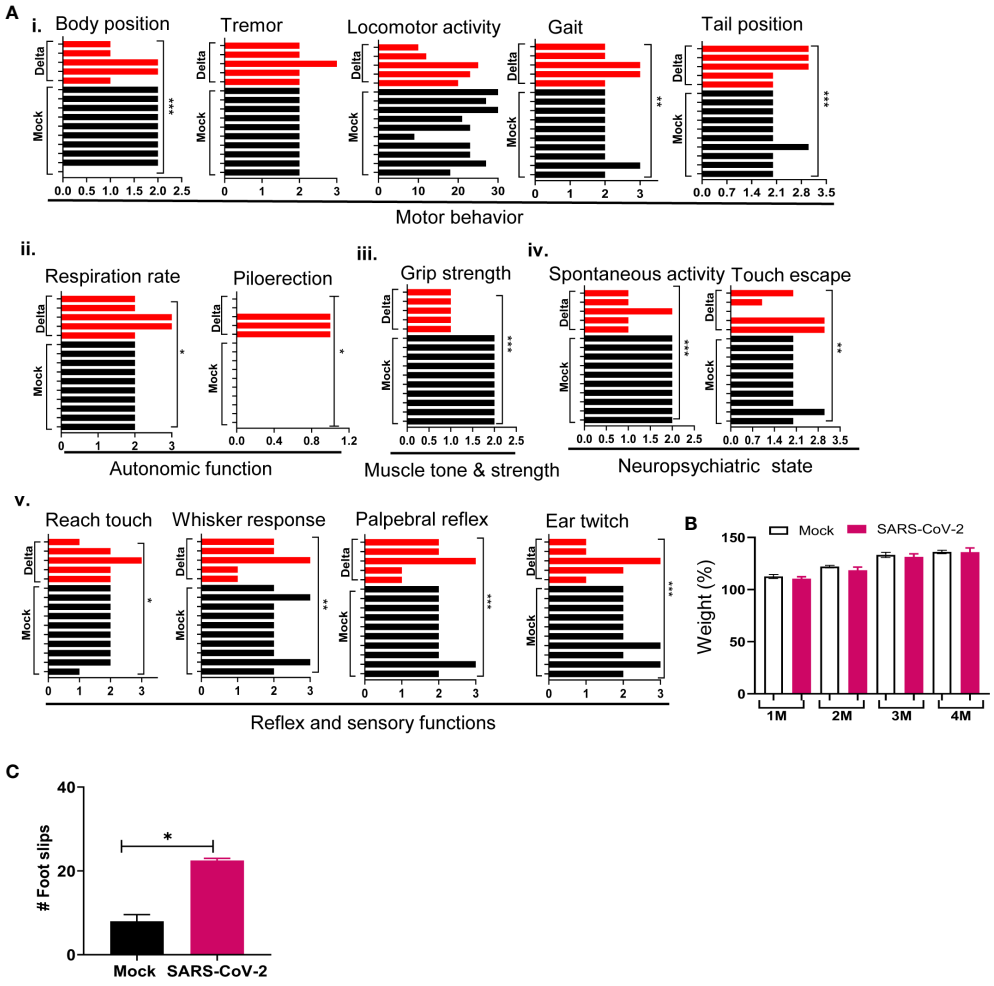

FIGURE 3
SARS-CoV-2 Delta variant induces behaviors changes post-acute infection. K18-hACE2 mice were infected with a sublethal dose of SARS-CoV-2 Delta variant, or PBS (mock). At 1 month pi or after, surviving mice (n = 10 for mock and n = 5 for SARS-CoV-2 Delta variant-infected mice) were assessed for behavior changes via SHIRPA **(A)**, weight changes **(B)**, and a parallel rod floor test **(C)**. **(A)** At 1 month pi, SARS-CoV-2 variant-infected mice showed impaired performance in the SHIRPA assessment. (i) Motor behavior; (ii) autonomic function; (iii) muscle tone and strength; (iv) neuropsychiatric state; and (v) Reflex and sensory functions. The y-axis represents individual mouse for the mock (n = 10) and Delta variant-infected groups (n = 5). **(B)** Weight changes during the 4-month pi interval presented as percentage using the weight on the day of infection as 100%. **(C)** Parallel rod floor test. At 3 months pi, surviving mice were placed in the center of the cage coated with horizontal rods for 2 min. Foot/paw slips were counted **(C)**. ***p < 0.001, **p < 0.01, or *p < 0.05 compared to the mock group.

we utilized Cytoscape Enrichment Map and AutoAnnotate tools to identify biological networks that are associated with the enriched gene sets and found immune response cluster in both 1-month and 4-month pi datasets. In addition, other enriched gene sets were annotated as "SARS-CoV-2 translation," "electron transport process," and "ribosomal small subunit," indicating that overall nervous system homeostasis is perturbed (Figures 5C, D). As microglia activation was often associated with cognitive changes and SARS-CoV-2 infection preferentially targets astrocytes (42–44), immunofluorescence staining was next performed to detect activation of microglia and astrocytes at both acute infection (day 6) and post-acute infection (1-month and 4-month pi). Microglia activation with increased cell processes was noted at day 6; however, minimal to mild activation of these cells were found at 1-month and 4- month pi (Figure 6A). Minimal astrocyte activation was detected at 1 month and 4 months pi (data not shown). These data suggest that infiltrating immune cells, not the residential immune cells, are involved in the immune pathway activation in the CNS at the post-acute stage. Q-PCR analysis was next utilized to determine eight differentially expressed genes identified by transcriptomic analysis. Reduced levels of expression of *Ddit4*, *Slc38a2*, *Tmem267m*, *Lrrc8c*, and *setd7* genes were noted at 4 months pi, which indicate ataxia telangiectasia neurodegenerative disease, impairment of memory, synaptic plasticity, motor, and cognitive abilities, neuronal dysfunction and degeneration, and cerebral ischemic stroke, respectively (45–48) (Figure 6B).

We next assessed systemic immune responses in surviving mice post-acute infection. At 1 month pi, splenic T cells, including both

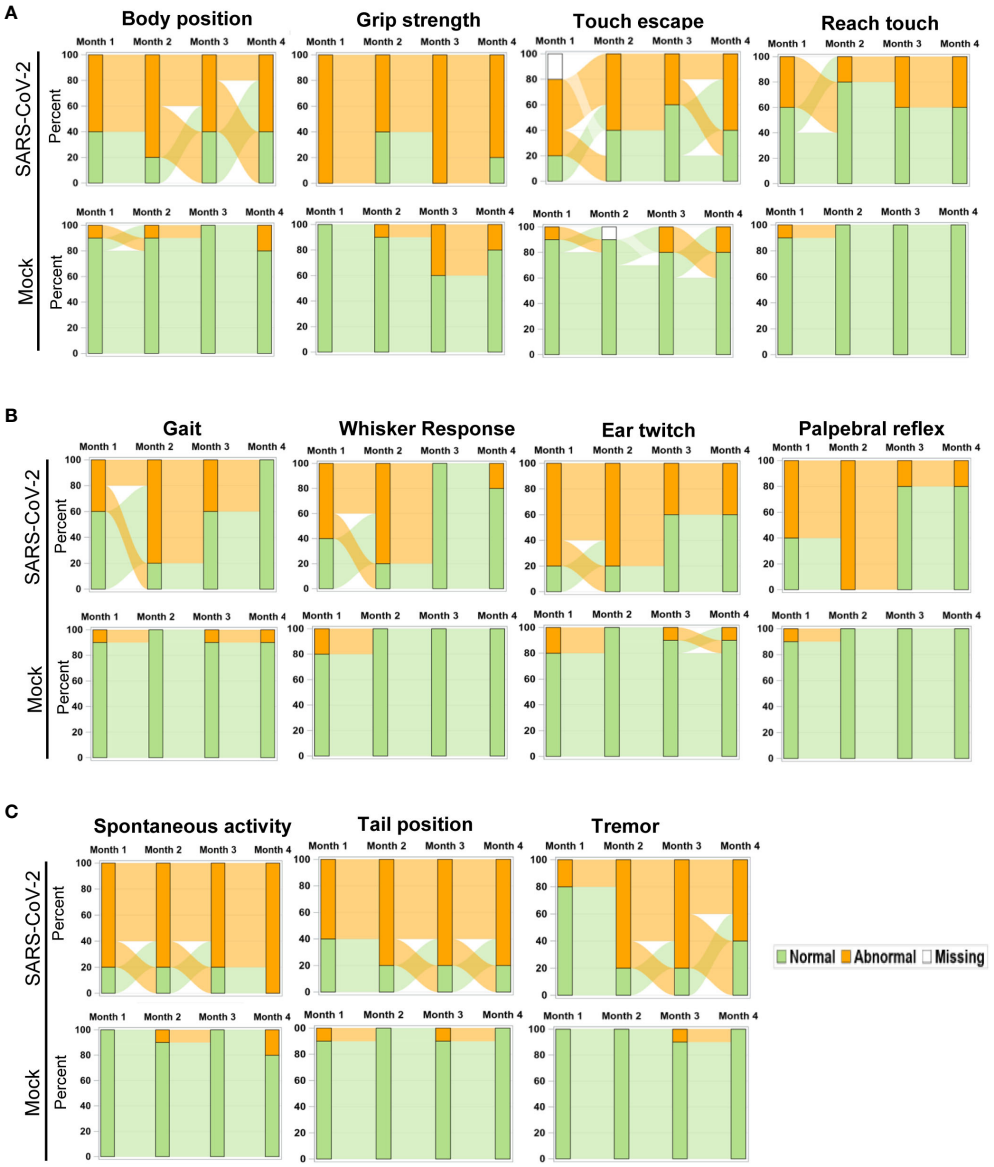

FIGURE 4
Longitudinal analysis of behavior changes 4 months post-acute infection in mice. Sankey bar charts were used to present results of SHIPA analysis. Functional status was defined as abnormal vs. normal and used to form the groups in the stacked bars at each time. Longitudinal stacked bar chart with Sankey-style overlays visualizes how the mice transit between abnormal and normal status over time. The *y*-axis is the percent in each group and the *x*-axis is the time for the measures collected. The link (bend) between the bars shows the transitions between two states over time. **(A)** Parameter abnormality patterns not changed within the 4-month pi period. **(B, C)** Parameter abnormality rates decreased **(B)** or increased **(C)** within the 4-month pi period.

$CD4^+$ and $CD8^+$ T-cell subsets, produced robust T helper (Th)-1 prone immune responses upon *in vitro* re-stimulation with S peptide pools compared to the mock group (Figures 7A–C). Furthermore, surviving mice also maintained high sera titers of RBD-binding IgG and neutralizing antibodies against Delta variant during the 4-month post-acute infection. Notably, similar levels of neutralization activity against Omicron strain were detected in the Delta variant-infected mice compared to mice surviving Omicron strain 4 months pi (Figures 7D, E). Overall, these results suggest that mice surviving SARS-CoV-2 Delta variant infection developed long-lasting Th1 and antibody responses in the periphery post-acute infection.

4 Discussion

The high risk of PASC is known to be associated with people with prior COVID-19 infection. The frequencies of PASC symptoms were reported to increase with SARS-CoV-2 variants, in particular, with those infected with the pre-Omicron variant compared to the original prototype virus infection (3, 35–37). Here, we infected K18 hACE2 mice with the SARS-CoV-2 Delta variant to recapitulate PASC in patients with COVID-19. We reported that the SARS-CoV-2 Delta variant replicated productively in lung and brain and triggered robust local inflammatory responses at acute infection in K18-hACE2 mice. Weight loss, neuroinflammation,

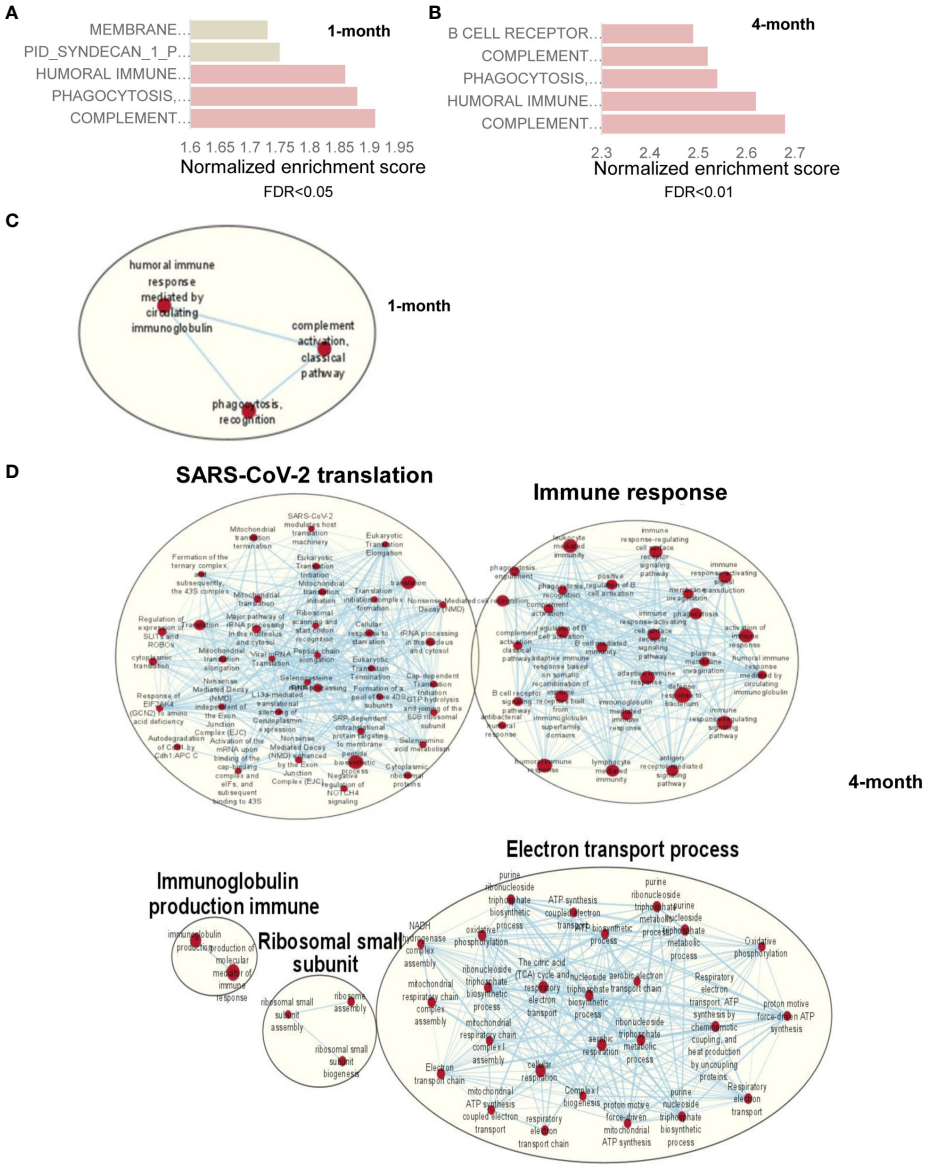

FIGURE 5
RNA-seq analysis of SARS-CoV-2- infected mouse brain shows genes with upregulation of immune signaling and enrichment of immune signaling-related pathways. **(A)** Pathway enrichment analysis of differential expressed genes using GSEA shows the top 5 upregulated pathways in SARS-CoV-2-infected mouse brain at 1 month **(A)** and 4 months **(B)** compared to mock-treated brain. Gray bar represents FDR >0.05 while orange bars represent FDR <0.05. **(C, D)** Cytoscape enrichment map (FDR *Q* value < 0.01) of GSEA pathways enriched in upregulated genes in SARS-CoV-2-infected mouse brain tissue at 1 month **(C)** and 4 months **(D)** pi compared to mock-treated sample. Clusters of nodes were labeled using the Auto Annotate feature of the Cytoscape application. Red nodes represent upregulated gene set enrichment and their node size represents the gene set size. The thickness of the line connecting the nodes represents the degree of overlap between two gene sets.

and mortalities were observed during acute infection. Surviving mice showed viral clearance with no additional weight loss, and minimal neuroinflammation. However, persistent neuropsychiatric state and motor-associated behavior changes were observed in surviving mice for months post-acute infection.

Our longitudinal behavior studies indicate development of ataxia and cognitive dysfunction in SARS-CoV-2 variant-infected mice post-acute infection. The behavior studies also suggest that the neuropsychiatric state and motor behavior of surviving mice remain impaired or deteriorated for months pi, whereas reflex and sensory functions appear to recover over time. These findings align with a recent 2-year retrospective cohort study that reported an increased risk of psychotic disorder, cognitive deficit, dementia, and epilepsy or seizures persisted in long-COVID patients (49). Furthermore, the downregulation of expression levels of *Ddit4*, *Slc38a2*, *Tmem267m*, *Lrrc8c*, and *setd7* genes in the brain at 4 months pi is associated with ataxia, impairment of memory, synaptic plasticity, motor, and cognitive abilities, neuronal dysfunction and degeneration, and cerebral ischemic stroke (45–48). RNA-seq analysis of brain samples showed activation of several immune pathways including "complement activation pathway," "phagocytosis recognition," and "humoral immune response mediated by circulating

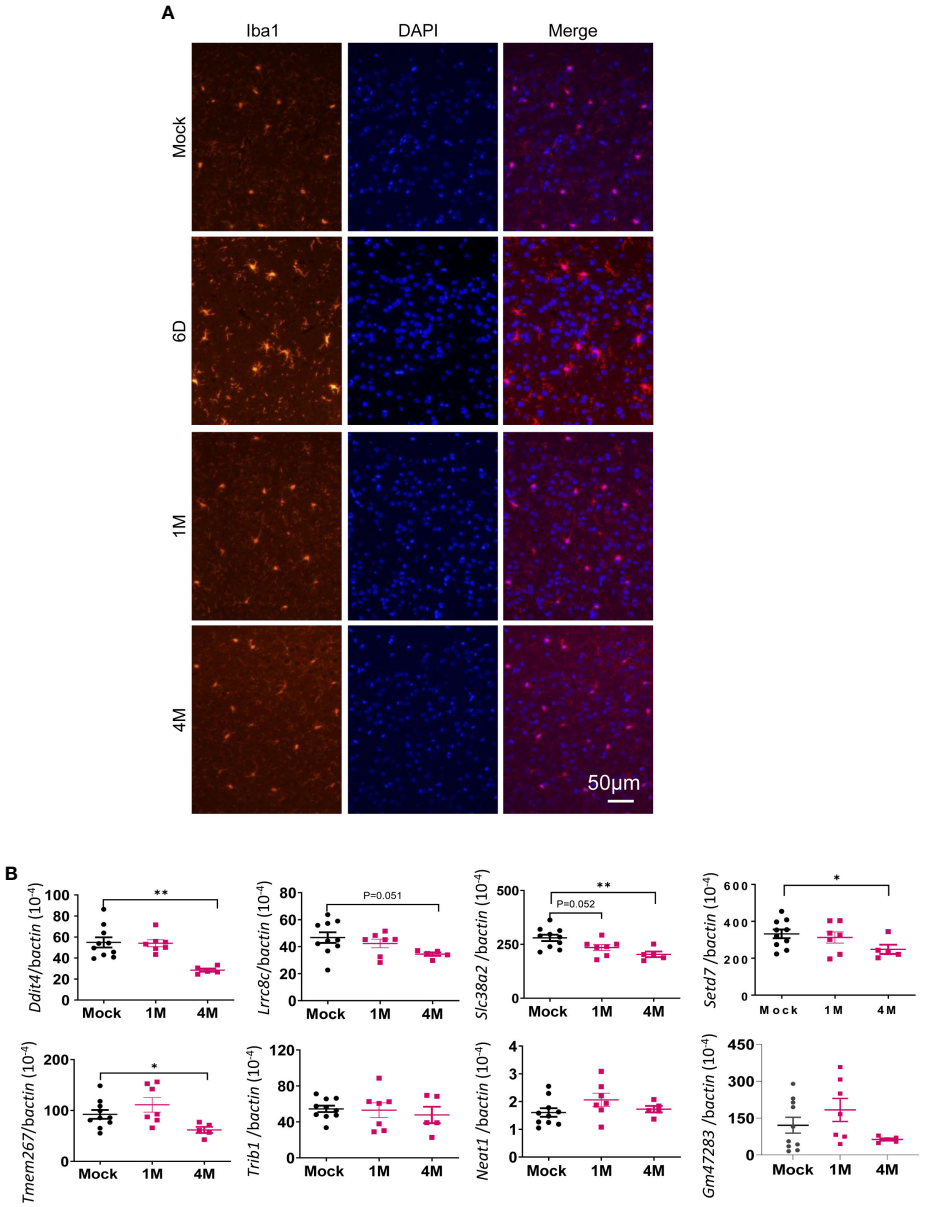

FIGURE 6
Microglia were activated during acute SARS-CoV-2 infection in the brain but were minimally activated post-acute infection. **(A)** Immunofluorescence images of brain tissues stained with DAPI (blue) and anti-Iba1 (red) at both acute infection (day 6, D6) and post-acute infection [1 month (M) and 4M pi]. **(B)** Q-PCR analysis of the levels of eight differentially expressed genes identified by transcriptomic analysis in the brain samples of mock- or SARS-CoV-2-infected samples at 1M and 4M. n = 5 to 10. $^{**}p < 0.01$ or $^{*}p < 0.05$, compared to the mock group.

immunoglobulin" at 1 month and 4 months pi. The transcriptome results support neurological behavior changes observed in the surviving mice. In addition, these findings suggest that infiltrating immune cells and circulating immune factors contribute to CNS disorder. For example, the complement-dependent engulfment of synapses may lead to the cognitive dysfunction. The spike protein and its fragment was reportedly to be able to cross the blood–brain barrier and enter the CNS (50), and this is directly involved in COVID-19-induced cognitive dysfunction (51) via complement-dependent engulfment of synapses in mice (52). In this study, no detectable levels of S1 in the CNS were found during the post-acute phase. We also noted neuroinflammation and microglia reactivity during acute infection, but minimal to mild microglia activation was found months post-acute infection. It is likely that PASC results from viral infection and/or viral fragment entry into the CNS and their associated neuroinflammation and neuronal injury during the acute infection stage. Interestingly, we also noted that surviving mice maintained potent protective systemic Th-1 prone and humoral immune responses post-acute infection. Combined with transcriptome results, these findings further suggest that systemic immune factors contribute to the development of PASC.

As reported earlier (24, 53), the K18-hACE2 mouse strain was generated by inserting multiple copies of the K18-hACE2 transgene on mouse chromosome 2. The K18-hACE2 transgene includes the

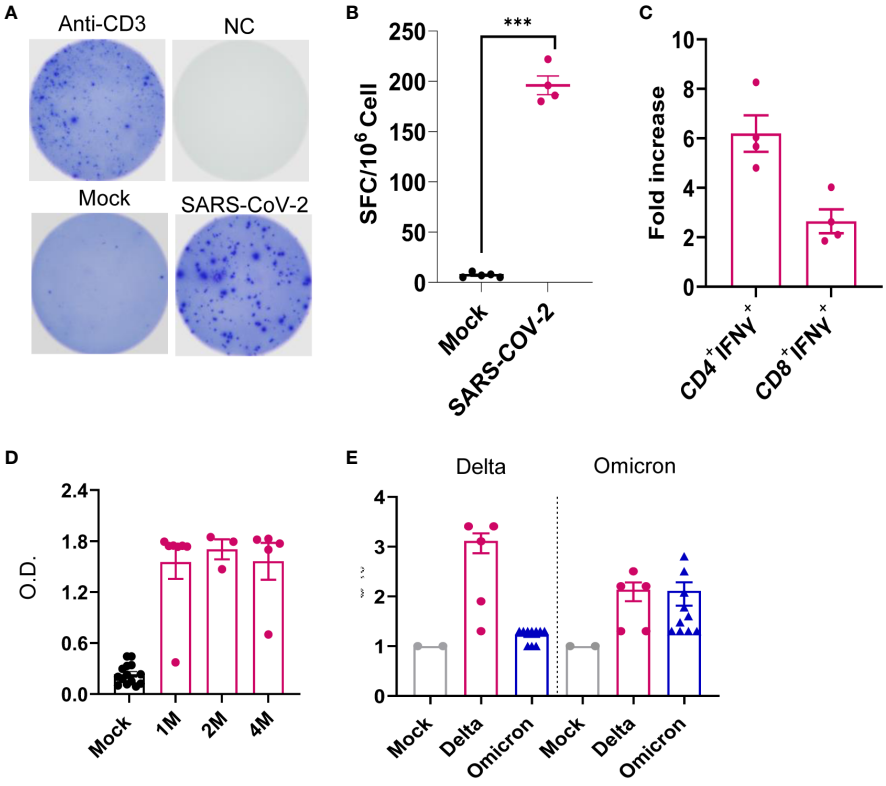

FIGURE 7
SARS-CoV-2 Delta variant induced persistent systemic cellular and humoral immune responses post-acute infection. K18-hACE2 mice were infected with a sublethal dose of SARS-CoV-2 Delta variant. **(A–C)** At 1 month pi, splenocytes were collected from surviving mice and mock group to measure T-cell responses. **(A, B)** ELISPOT quantification of splenic T-cell responses. Splenocytes were stimulated with SARS-CoV-2 S peptides, anti-CD3, or blank (negative control, NC) for 24 h. **(A)** Images of wells of T-cell culture. **(B)** Spot-forming cells (SFCs) were measured by IFN-γ ELISPOT. Data are shown as the number of SFCs per 10^6 splenocytes. n = 5. **(C)** Splenocytes were cultured *ex vivo* with S peptide pools for 6 h, and stained for IFN-γ, CD3, CD4, or CD8. Fold increase of IFN-γ^+ $CD4^+$ and $CD8^+$ T-cell expansion compared to the mock group is shown. **(D)** Sera of SARS-CoV-2 RBD-binding IgG titers at 1 month (M), 2M and 4M pi. O.D. values were measured by ELISA. n = 5 and 10 for Delta variant-infected and mock, respectively. **(E)** At 4M pi, sera neutralizing activity against SARS-CoV-2 Delta variant or Omicron B.A.2 variant was measured by plaque reduction neutralization test (PRNT). mNG-NT_{50} titers are shown, n = 2, 5, and 10 for mock-, Delta variant- or Omicron-infected group, respectively. ***p < 0.001, compared to the mock group.

K18 promoter, the first intron (with a mutation in the 3' splice acceptor site to reduce exon skipping) from the human keratin 18 (*KRT18*) gene, a translational enhancer sequence from alfalfa mosaic virus, *hACE2*) coding sequence, exons 6–7, and the poly (A) signal of the human *K18* gene. The K18 promoter confers efficient transgene expression in airway epithelial cells and epithelia of internal organs, including the liver, kidney, and gastrointestinal tract. Because of its high expression of hACE2 in the lung and kidney tissues, we noted Delta variant-induced productive viral replication in these tissues during acute infection. Viral loads in liver were nevertheless barely detectable at both acute and chronic stages. Liver injury has been reported to develop as a post-COVID sequela (54, 55). The primary focus of this study is SARS-CoV-2 variant infection in the CNS tissues and its contribution to PASC pathogenesis. Future investigation will also focus on the impacts of SARS-CoV-2 infection on liver, kidney, and other peripheral tissues and understand the association with PASC.

Several rodent models have been used to study long-COVID (56–58). In line with a recent report in a golden hamster model of long-COVID, we found detectable infectious virus in the CNS during the acute infection phase, which is correlated with behavioral changes at 1 month after viral clearance (57). Induction of CCL11 expression in the periphery tissues and CNS during acute COVID-19 infection was reported in a mild-respiratory COVID model in immunocompetent mice via delivery of an AAV vector to express human ACE2 to the trachea and lungs (58). Furthermore, results from the transcriptome analysis align with the impaired neurogenesis findings reported in both studies. Nevertheless, we did not note microglia reactivity, neuroinflammation, and induction of proinflammatory cytokines in the CNS post-acute phase as reported in these studies. Both prior studies are limited to a shorter time post-acute infection (4 to 7 weeks pi) and the use of wild-type prototype virus. Another study also reported increased reactive astrocytes and microglia, hyperphosphorylated TDP-43, and tau, and a decrease in synaptic protein synaptophysin-1 and defective neuronal integrity in A/J mice 12 months post-infection. Although the study recapitulates long-term sequelae of COVID-19, a mouse hepatitis virus 1 (MHV-1) was used in this study (59, 60).

In summary, our results suggest that infection in K18-hACE2 mice recapitulates the persistent clinical symptoms reported in long-COVID patients. Our immunological and transcriptomic

analysis provides new insights into the pathogenesis of the disease. Given that the formation and consolidation of learning and memory occurs primarily within the hippocampus region of the brain and alteration of hippocampal cells in clinical and preclinical model post SARS-CoV-2 infection along with its connection with the olfactory dysfunction worsen cognition, additional studies in K18-hACE2 mice for the evaluation of structure and function of hippocampus are needed (61, 62). Moreover, development of dysautonomia, a nervous system disorder that disrupts autonomic body processes, was reported to be the most recurrent type of neurological disorder post-COVID and has been linked to the neuro-psychological sequelae of long-COVID, for instance, cognitive impairment (63). Future investigation of autonomic neuropathy in the preclinical model is likely to provide novel insights into SARS-CoV-2-induced PASC. Overall, the K18-hACE model of long-COVID may be useful to evaluate efficacy for the future development of novel SARS-CoV-2 vaccines or therapeutics.

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: GSE260625 (GEO).

Ethics statement

The animal study was approved by Institutional Animal Care and Use Committee, University of Texas Medical Branch, Galveston, TX, USA. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AS: Conceptualization, Data curation, Formal analysis, Methodology, Visualization, Writing – review & editing, Investigation, Validation. AA: Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Writing – review & editing, Validation. A: Data curation, Formal analysis, Investigation, Writing – review & editing, Methodology, Software. B-HP: Formal analysis, Investigation, Methodology, Writing – review & editing. XY: Data curation, Formal analysis, Methodology, Software, Writing – review & editing. JZ: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. VK: Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – review & editing. PK: Conceptualization, Funding acquisition, Resources, Writing – review & editing. WJ: Data curation, Methodology, Software, Writing – review & editing. P-YS: Conceptualization, Methodology, Writing – review & editing. PS: Data curation, Formal analysis, Investigation, Methodology, Software, Writing – review & editing. IC: Investigation, Methodology, Software, Writing – review & editing, Conceptualization, Resources. TW: Methodology, Resources, Writing – review & editing, Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Supervision, Visualization, Writing – original draft.

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Conflict of interest

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

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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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.2024.1384516/full#supplementary-material.

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Neurological symphony: post-acute COVID-19 syndrome, an innovative pathophysiological exploration from neuraltherapeutic medicine

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The SARS-CoV-2 pandemic has affected 771 million people and caused 6.9 million confirmed deaths as of November 2023. Beyond the adversity, a crucial and less-explored chapter unfolds: adaptive sequelae. These have altered social, mental, and emotional conditions, leaving an imprint on biological systems. While some cases fully resolve the pathological process post-acute infection, others persist with symptoms, posing a challenge that underscores the need to comprehend pathophysiology from innovative perspectives. The article delves into "Long COVID" or Post-Acute COVID-19 Syndrome (PACS), where symptoms persist for ≥4 weeks irrespective of initial severity. Risk factors include a history of severe illness, in-hospital management, and intensive care. This article also explores theories, derived from various experimental models, that have demonstrated the involvement of the nervous system in coordination with the psychoneuroimmunoendocrine axes in the expression of inflammation. It is posited that PACS involves processes of peripheral and central sensitization (corticalization), facilitating dishomeostasis and the chronicity of the inflammatory process. In this context, various therapeutic strategies grounded in modulating the inflammatory reflex are reviewed, primarily through the infiltration of local anesthetics via linear and non-linear approaches. Neural therapeutic use is considered to stimulate the regulatory inflammatory circuits coordinated by the neuroimmune-endocrine system.

KEYWORDS

neural therapy, local anesthesia, inflammation, neuromodulation, post-acute COVID-19 syndrome

Introduction

The SARS-CoV-2 pandemic (Ritchie et al., 2022) has affected 771 million people and caused 6.9 million confirmed deaths as of November 2023 (WHO, 2022). Beyond the adversity, a crucial and less-explored chapter emerges: its adaptive aftermath. These have altered social, mental, and emotional conditions, leaving an imprint on biological systems. Yet, the fact that some cases completely resolve the pathological process while others persist with symptoms after acute infection remains an enigma. This phenomenon poses a challenge

that underscores the need to comprehend the pathophysiology from innovative perspectives, providing tools to address the post-pandemic landscape.

Persistent symptoms following acute infection, occurring between 4 to 12 weeks, are termed Long COVID or post-acute COVID sequelae. When these symptoms persist for ≥4 weeks, it is referred to as Post-Acute COVID-19 Syndrome (PACS) (Nalbandian et al., 2021; Shah et al., 2021; Greenhalgh et al., 2022; Li et al., 2023). These symptoms do not directly correlate with the severity of the acute infection, but there are risk factors such as a history of severe illness from SARS-CoV-2, in-hospital management, and intensive care. It is noteworthy that PACS can manifest in individuals with asymptomatic disease or without prior confirmed infection (Nalbandian et al., 2021). Despite the high effectiveness of vaccination in preventing severe acute illness from SARS-CoV-2, some authors have reported an increased risk of PACS if infection occurs within 14 days post-vaccination (Al-Aly et al., 2022) and on the other hand (Saheb Sharif-Askari et al., 2024) in a cohort study in 28,375 non-hospitalized adult patients diagnosed with mild to moderate COVID 19 in Dubai, emphasize the potential benefits of pre-COVID vaccination and timely treatment in the prevention of Long COVID.

To date, there is no diagnostic gold standard for Post-Acute COVID-19 Syndrome (PACS), and its symptoms are highly varied; moreover, they may be associated with other health issues (Nalbandian et al., 2021), complicating the diagnostic process. The multiorgan sequelae of PACS exhibit a broad spectrum of clinical manifestations (see Table 1), with the most common being fatigue (80%), weakness after physical exertion (73.3%), and cognitive impairment (58.4%), among others (Davis et al., 2021). Additionally, the pathophysiological mechanisms and effective therapeutic options have yet to be clearly defined (Nalbandian et al., 2021).

In a systematic review, 73% of individuals exhibited at least one persistent symptom 6 months after SARS-CoV-2 infection (Nasserie et al., 2021). In an observational study involving 3,762 patients from 56 countries, 62% presented with at least one persistent symptom at the 6-month mark (Davis et al., 2021). Due to the variability in clinical presentations and the diagnostic complexity of Post-Acute COVID-19 Syndrome (PACS), the prevalence remains uncertain, ranging between 5 and 80% (National Institutes of Health, 2021). The heterogeneity across studies underscores the necessity for more standardized diagnostic designs and prolonged follow-up.

This article reviews the pathophysiology of Post-Acute COVID-19 Syndrome (PACS), focusing on mechanisms of the nonspecific response to disease, particularly inflammation. It explores the nonspecific response to threats (see Figure 1) through Nervism theory, highlighting neurogenic dystrophy as a fundamental component of all diseases according to A.D. Speransky and an indispensable precursor for the development of pathologies according to G.N. Kryzhanovskysirve. Tracey's inflammatory reflex, Chiu's neurogenic inflammation, and Klein's neuroimmune axis are analyzed, comparing them with the central and peripheral sensitization model. The integration of these processes into the psychoneuroimmunoendocrine system is described. Additionally, other specific responses to SARS-CoV-2 related to symptom persistence are briefly addressed. Finally, it emphasizes how Neuraltherapeutic Medicine, using local anesthetics (AL), can modulate the nonspecific response and neurogenic inflammation.

TABLE 1 Persistent COVID-19 Symptoms, indicating damage caused by specific and non-specific mechanisms following SARS-CoV-2 infection.

General
Fatigue
Post-exertional discomfort
Fever
Deterioration of quality of life
Cardio-respiratory
Shortness of breath or trouble breathing
Cough
Chest pain
Palpitations
Postural orthostatic tachycardia syndrome
Neurological
Myalgia encephalomyelitis and chronic fatigue syndrome
Difficulty thinking or concentrating
Headache
Vision changes
Sleep disorders
Orthostatic hypotension
"Pins and needles" paresthesias
Smell or taste disorder
Depression or anxiety
Kidney
Decreased glomerular filtration rate
Gastrointestinal
Diarrhea
Constipation
Stomachache
Heartburn
Elevation of aminotransferases
Pancreatitis
Exocrine and endocrine pancreatic dysfunction
Osteo-articular
Myalgias-arthralgias
Muscular weakness
Sarcopenia
Blood vessels
Thrombogenesis
Dermatological
Rash
Alopecia
Urticaria
Reproductive system
Changes in menstrual cycles
Decreased libido
Difficulty ejaculating

Modified reference: NIH COVID-19 treatment guideline (NIH, April 21, 2021) (Nalbandian et al., 2021; Li et al., 2023).

Nervism: a physiological doctrine centered around the nervous system

Throughout 170 years of history, the so-called Russian nervism school has left an indelible mark on the study of the nervous system (NS). Initiated by the synthetic physiology of

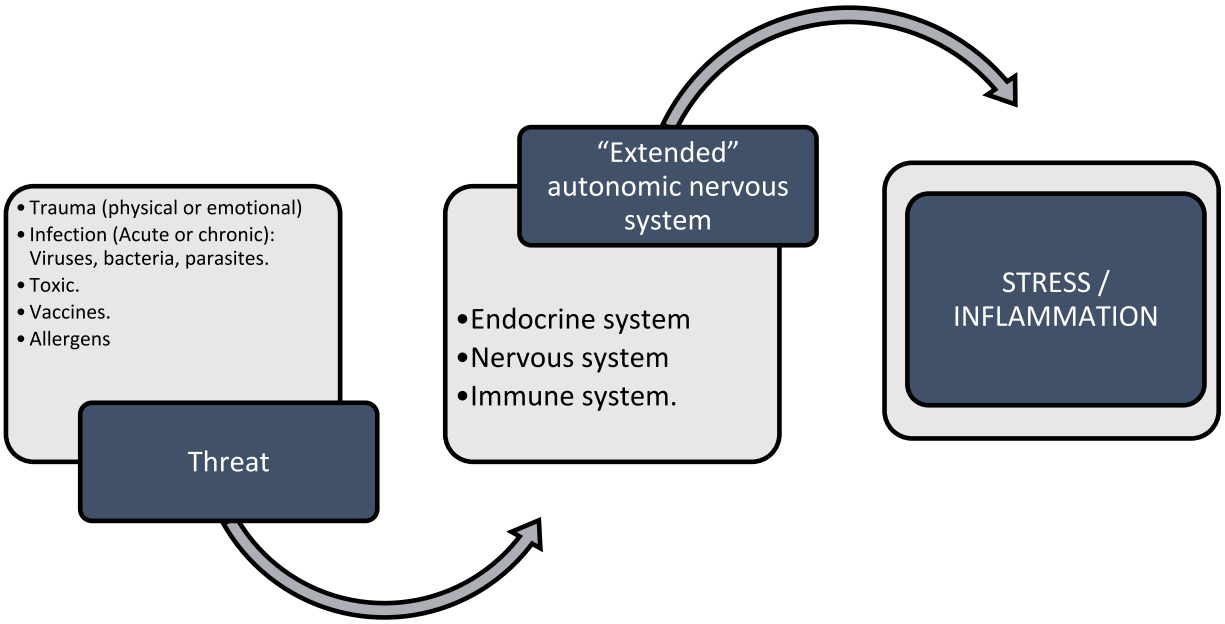

FIGURE 1
Nonspecific response to threatening stimuli such as trauma, microorganisms, toxins, vaccines, allergens that stimulate the extended autonomic nervous system or neuroimmunoendocrine system; and it responds as a unit through different cellular pathways and chemical communication with stress and inflammation.

Pavlov Smith G. P. (2000) and developed by Speransky (Speransky, 1954), Orbeli (1938), and Bykov (Bykov et al., 1958), this school has solidified the differential approach. Its concept of the living system as a biologically indivisible functional unit underscores the NS as the supreme director of all physiological and pathological processes (Sechenova et al., 1993; Ariza Tarazona et al., 2020).

Speransky (1954) developed a theory aiming to generate a profound understanding, based on experimental foundations, of organisms' responses to external irritating stimuli. This theory is grounded in reflex mechanisms of the nervous system, proposing a novel mechanism for the genesis of diseases overall. According to this theory, injurious stimuli have the potential to induce extreme irritation that affects tissue resilience—a phenomenon common to all pathophysiological processes known as the nonspecific response to damage, conceptualized by the theory of neurogenic dystrophies (Sechenova et al., 1993). This process, termed neurogenic tissue dystrophy, is caused by inadequate nervous influx to the involved tissue, disrupting the physiological cellular response and increasing tissue fragility, predisposing it to diseases (Akimov and Kositsyn, 2005).

On the other hand, Bykov and Anichkov suggested the occurrence of a reflex dystrophy associated with irritation of reflexogenic zones (Забродин, 1999), possibly linked to associated neuroanatomical circuits.

The influence of the nervous system (NS) on tissue homeostasis, along with its interaction with various physiological and pathophysiological phenomena, has been substantiated by other researchers. Its impact extends to cell division and differentiation (Bustamante et al., 2023), the modulation of hormonal or pharmacological responses (Sechenova et al., 1993; Akimov and Kositsyn, 2005), as well as changes in tissue ultrastructure and cytochemical profile (Tweedle et al., 1975; Kositsyn, 1978). Furthermore, regulatory effects on gene expression in tumors and their surrounding microenvironment have been observed (Cole et al., 2015). Its documented influence spans cellular metabolic processes (Pavlov and Tracey, 2012), thermogenesis, modulation of immunity, acute and chronic inflammation, and tissue repair (Tracey, 2002; Pavlov and Tracey, 2012; Klein Wolterink et al., 2022).

These findings support that the nervous system (NS) maintains the structural stability, functions, energy, and plastic processes of cells, tissues, organs, and the organism as a whole (Забродин, 1999).

Secondary reflex responses to irritative processes coordinated by the NS involve nonlinear physiological mechanisms that can be summarized into three main types (Speransky, 1954; Engel et al., 2022):

1 *Direct local irritation:* is generated by the direct irritation of the tissue and its corresponding nociceptors.
2 *Segmental metameric irritation:* is grounded in the embryogenesis of various tissues. This is primarily interconnected through the nervous system (NS), establishing segmental circuits that regulate function and communication between diverse structures. Reflex irritation from these circuits can trigger responses that impact innervation and, consequently, the function of related anatomical structures.
3 *Meta-segmental irritation:* is a reflex response that extends beyond the segment and lacks a local or embryogenic basis. It is currently referred to as "neuromodulatory trigger points" according to Engel et al. (Engel et al., 2022). Other authors have described it as a "neural interference field," especially in the context of the Neural Therapy school according to Huneke (Dosch and Dosch, 2007). Although its pathophysiological mechanism is not fully understood, it pertains to cortical coupling phenomena described by temporal associations of the nervous system, such as Pavlov's conditioned reflex (Bykov

et al., 1958), as well as polysegmental neuroanatomical connections (Engel et al., 2022).

These connections can influence irritation and reflex responses in distant anatomical sites, emphasizing the complexity of interactions within the nervous system.

Inflammatory reflex, neuroimmune circuit, and neurogenic inflammation

Bustamante et al. (Bustamante et al., 2023), Tracey (Tracey, 2002), Klein et al. (Klein Wolterink et al., 2022), and Chiu et al. (Chiu et al., 2012) have proposed that the nervous system (NS) and the immune system interact, forming the neuroimmune circuit to regulate homeostasis through the inflammatory reflex, also known as the neural phase of inflammation (see Figure 2). This process modulates the innate and adaptive immune response within seconds, acting as an autonomic reflex. The afferent pathway detects immune products secondary to tissue injury or infection through nerve terminals (Tracey, 2002), while the efferent pathway regulates the phenotypic expression of immune cells and the release of cytokines. The parasympathetic pathway is associated with anti-inflammation, whereas the sympathetic pathway is pro-inflammatory (Tracey, 2002; Chiu et al., 2012; Davidson et al., 2014; Cook et al., 2018; Klein Wolterink et al., 2022); this interaction becomes even more complex with a system of cells and chemical mediators such as neurotransmitters, neuropeptides, and cytokines interacting with each other (Goldstein, 2020).

Autonomic dysfunction of this inflammatory reflex, secondary to nociceptor sensitization, leaves the NS in a hyper-vigilant state (Cook et al., 2018), also known as "hyperinflammatory reflex" according to Engel et al. (Engel et al., 2022). This state triggers an axonal reflex that can induce neurogenic inflammation, leading to an increase in oxidative stress and its adverse effects. These alterations affect the biophysical properties of neuronal membrane potential, contributing to the development of intractable pain (Cook et al., 2018). In animal models, enteric inflammation affects sympathetic and sensory innervation, resulting in hypersensitivity associated with inflammatory bowel disease (Meade and Garvey, 2022).

Phenomenon of central and peripheral sensitization and neurogenic inflammation

Peripheral sensitization is defined as an increase in the sensitivity of nociceptors at the peripheral nerve endings of the body, facilitating the onset of pain and other discomfort. This phenomenon leads to changes in membrane potential and electrochemical balance, resulting from alterations in the redox balance, primarily induced by an increased production of free radicals (Davidson et al., 2014). Meanwhile, central sensitization at the spinal and cerebral levels amplifies nociceptive signals from the nervous system. Both phenomena generate inflammatory responses that contribute to the injury and dysfunction of the affected tissues (Davidson et al., 2014).

From the extended autonomic nervous system to psychoneuroimmunoendocrinology

The role of the nervous system (NS) in the trophic processes of all biological systems in the human body is closely tied to the information systems that integrate the complexity of organisms. Once considered isolated feedback systems, Goldstein (Goldstein, 2020) now refers to it as the "extended" autonomic nervous system with neuroimmuneendocrine circuits that interact harmoniously to maintain homeostatic balance. The cellular and chemical communication phase of this neuroimmuneendocrine system (NIES) shares common receptors and ligands, constituting interdependent axes of bioregulation (González-Díaz et al., 2017; Goldstein, 2020).

In 1891, Smith G. P. (2000) initiated research on digestive processes in dogs in his own laboratory, work that would earn him the Nobel Prize in physiology. His findings demonstrated that psychic variables influence physiological reactions, marking the beginning of his explorations into classical conditioning (Bykov et al., 1958). Subsequently, in 1975, Ader and Cohen (González-Díaz et al., 2017) described the integration of the psyche into the NIES, considering the psyche as a key determinant of biological response. This integration gives rise to a unique system called the psychoneuroimmunoendocrine system (see Figure 3), responsible for adaptive responses to various influences, both external and internal.

Selye (1990) defines stress as a nonspecific response to a demand imposed on the body Selye H. (1946). When the compensatory mechanism fails, regardless of its origin, it exhibits fundamental characteristics of "dishomeostasis," according to Goldstein, in the context of critical illness and chronic disease. These aspects support that dysfunction of the nervous system, known as dysautonomia, along with inflammation and dishomeostasis, are central mechanisms underlying the development and perpetuation of multiorgan failure (Toner et al., 2013; Zalewski et al., 2018).

Pathophysiology of long COVID (post-acute sequelae of SARS-CoV-2, SPAC)

Integrating the explored concepts (synthetic physiology, neurogenic dystrophy, inflammatory reflex, neuroimmune circuit, central and peripheral sensitization, and neurogenic inflammation as part of the nonspecific response) provides new elements for understanding the pathophysiological mechanisms triggered by SARS-CoV-2 injury that persists over time with Long COVID (Post-Acute Sequelae of SARS-CoV-2, SPAC).

Chronic inflammation is a common denominator in the pathophysiology of various chronic diseases such as diabetes, cardiovascular, respiratory, mental, epilepsy, obesity, and autoimmune diseases, among others (Tracey, 2002). The neuropathology of PACS is characterized by involvement of the nervous system (NS), dysautonomia, and subsequent neurogenic inflammation (Li et al., 2023). This process is imperative in the development of PACS and contributes to the understanding of related chronic diseases.

Publications correlate Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) with PACS, manifesting overlapping

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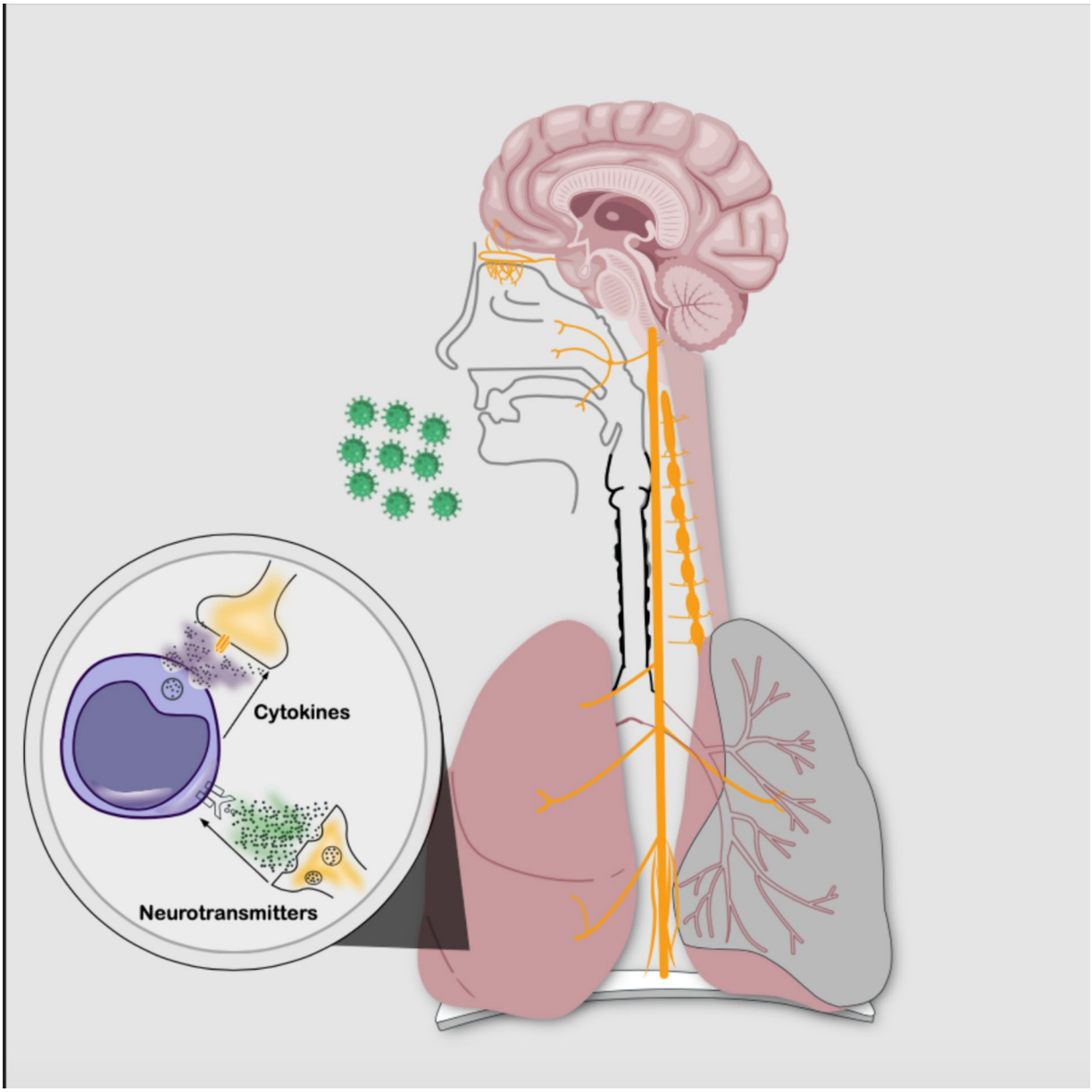

FIGURE 2
Neuroimmune circuit: bidirectional communication between the nervous system and the immune system through cellular phases and chemical signaling. The intricate interplay is established by neurotransmitters binding to immune cell membrane receptors and also by cytokines interacting with nerve cell membrane receptors. (Source: Own elaboration).

symptoms between both pathologies (Engel et al., 2022). In the early 20th century, Speransky (Speransky, 1954) proposed that a nonspecific stimulus associated with injury, either by its intensity and/or frequency, could trigger neurogenic dystrophy with disseminated encephalomyelitis, as demonstrated in animal experimental models.

Similarly, ME/CFS can be preceded by nonspecific triggering factors, such as viral, bacterial, parasitic (acute or chronic) infections, toxic exposures, vaccination, and trauma (physical or emotional) (DynaMed, n.d.). Other functional somatic syndromes without apparent cause, such as irritable bowel syndrome, fibromyalgia, temporomandibular disorders, vulvodynia, and interstitial cystitis, have been considered as dysautonomias (Meade and Garvey, 2022). Although the relationship is not linear, dysautonomic activation and hyperinflammatory reflex are common patterns in the expression of these syndromes.

VanElzakker hypothesizes that during acute SARS-CoV-2 infection, the terminal axons of the vagus nerve (VN) and other nerves in the respiratory epithelium become sensitized by immune factors. The reflex response involves various levels of the NS, such as the dorsal brainstem, the Nucleus Tractus Solitarius, and parabrachial regions with glial activation, manifesting systemic symptoms like fatigue, fever, myalgia, among others. These symptoms are common in the clinical expression of ME/CFS and PACS. As part of this reflex response, a "corticalization" pathway is observed, where peripheral sensitization spreads centrally, transmitting inflammatory signals

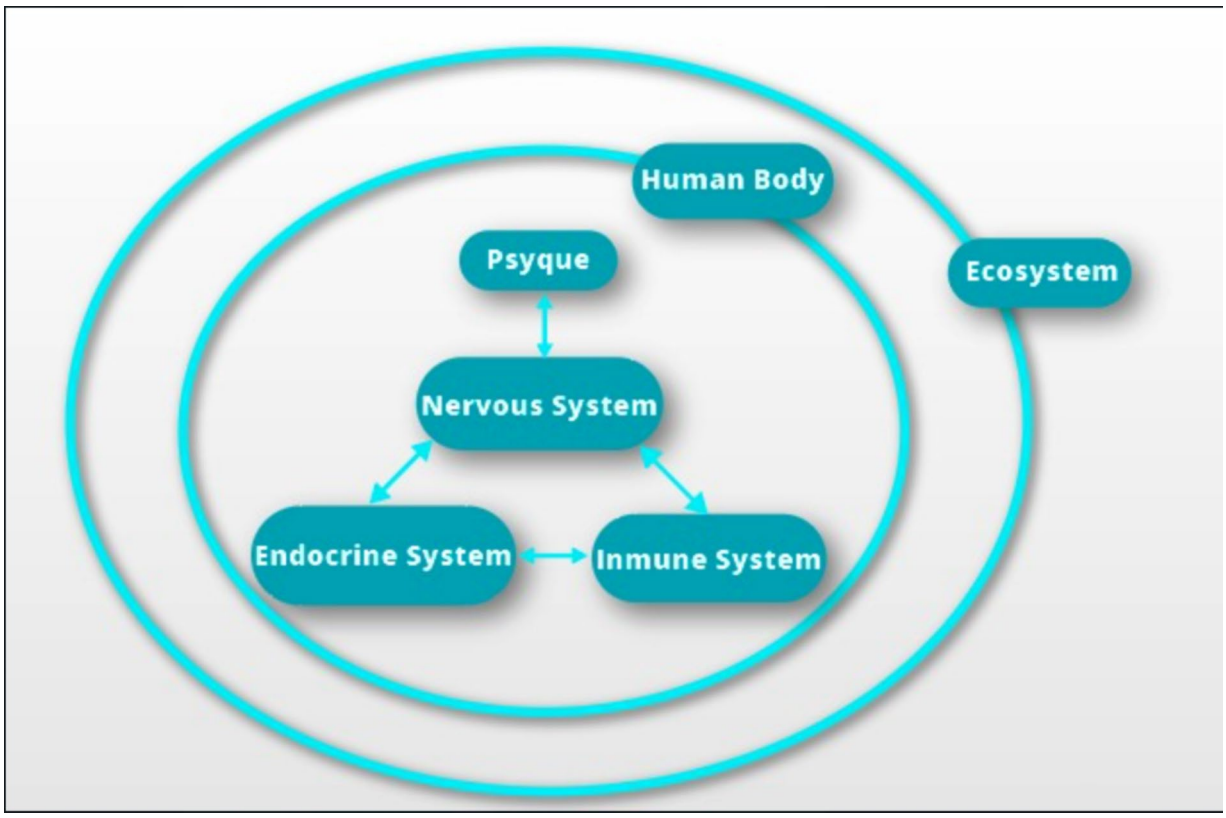

FIGURE 3
Information systems or psychoneuroimmunoendocrine axis. At the top within the human being, the psyche or consciousness harbors thoughts and emotions. The communication interface between the psyche and the physical body is carried out through the nervous system. The neuroimmune axis transmits chemical signals through cytokines and neurotransmitters. The neuroendocrine axis involves chemical transmitters, such as hormone-releasing factors and hormones. The immunoendocrine axis comprises chemical transmitters of hormones and cytokines. This dynamic adaptation of the unity of being takes place through feedback loops between the axes, adjusting to the internal circumstances of the human body and the external conditions of the ecosystem. Thus, the unity of being is configured, establishing a direct and continuous relationship with the environment. (Source: Own elaboration).

from VN terminals to the brainstem, limbic system, and neocortex (Figure 4) (VanElzakker, 2013).

The brainstem plays a fundamental role in the neural phase of the neuroimmune circuit and in the process of central sensitization, characterized by dysfunctional signaling of this component, which could be a key factor in PACS symptoms (Proal and VanElzakker, 2021). In autopsies of corpses affected by SARS-CoV-2, immune activation has been identified in the brainstem (Solomon, 2021). Furthermore, various studies have reported functional and structural abnormalities of the brainstem in ME/CFS (Shan et al., 2020), along with glial activation and cognitive impairment (Nakatomi et al., 2014). It is important to note that central sensitization of the brainstem can also be triggered by infections and inflammatory events "occurring outside the NS," such as nonspecific injurious stimuli (Proal and VanElzakker, 2021).

Within the NS, mast cells and microglia are activated in response to SARS-CoV-2 and other viruses. The inflammatory cascade is largely maintained by exposure to "multiple hits," which are diverse inflammatory events that collectively amplify their signaling (Proal and VanElzakker, 2021). Once activated, these immune cells retain a prepared functional state, leading to an even more robust response to subsequent challenges. This prepared state may also be crucial in symptoms such as sensory sensitivity in individuals who have survived acute neuroinflammatory events, such as encephalitis or concussion, or who may have low levels of persistent or latent neurotropic pathogenic microorganisms, including the herpes virus, among others (Proal and VanElzakker, 2021).

Any stimulus inducing the release of proinflammatory cytokines in a region of the body innervated by the VN can initiate or perpetuate this pathological response and associated chronic symptoms (Proal and VanElzakker, 2021).

Specific responses to SARS-CoV-2 infection in the pathophysiology of PACS

The specific responses to SARS-CoV-2 infection that contribute to the development of SPAC include: direct tissue damage in one or multiple organs, the persistence of SARS-CoV-2 reservoirs in certain tissues, reactivation of neurotrophic pathogens such as herpesviruses under conditions of immune dysregulation induced by SARS-CoV-2, interactions of SARS-CoV-2 with the host's microbiome/virome communities, and autoimmunity due to molecular mimicry between the pathogen and host proteins (Proal and VanElzakker, 2021).

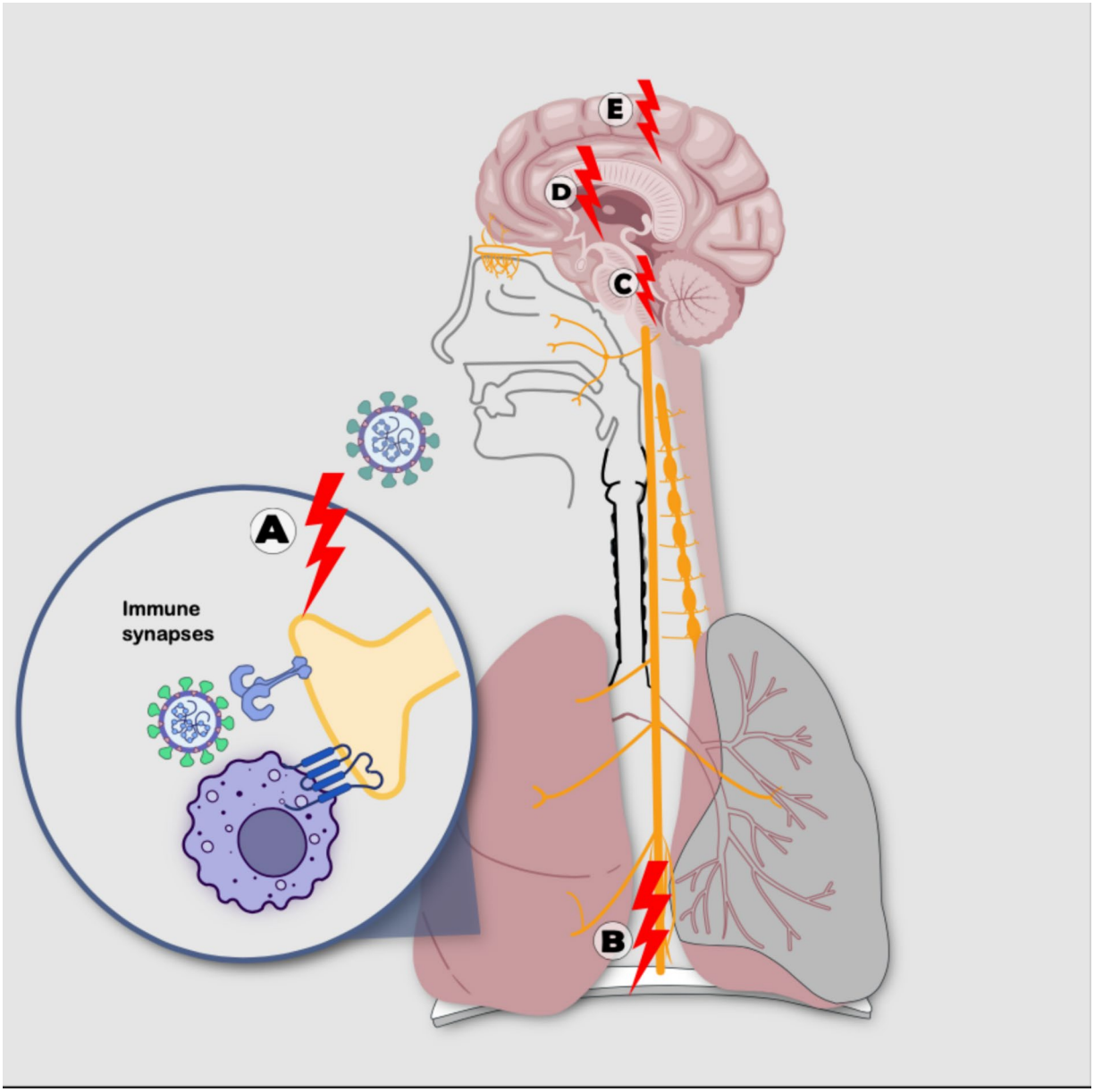

FIGURE 4
Sensitization processes: with nonlinear mechanisms following SARS-CoV-2 infection including: **(A)** sensitization-irritation of peripheral receptor through immunological synapse with immune cell or directly by the virus, **(B)** sensitization-irritation of the vagus nerve, **(C)** sensitization-irritation of the brainstem solitary tract nucleus and parabrachial regions, **(D)** sensitization-irritation of the limbic system, **(E)** sensitization-irritation of the cortex (Source: Own elaboration).

Direct tissue damage

SARS-CoV-2, similar to other virulent coronaviruses, initially enters through the respiratory epithelium using the angiotensin-converting enzyme 2 receptor. These receptors are expressed in cells of the respiratory system, brain endothelium, vascular smooth muscle, as well as gastrointestinal epithelial cells, pancreatic cells, and renal podocytes (Hamming et al., 2004). Although the mechanism of extrapulmonary spread is not yet clear, direct tissue damage from infection may be a primary mechanism contributing to long-term complications (Wiersinga et al., 2020).

Neuroinfection and subsequent neuroinflammation caused by SARS-CoV-2 have been documented in autopsy models, animal trials, and organoids. Infection can spread hematogenously through the blood–brain barrier or through the mechanism known as the "Trojan Horse," in which immune cells infected with intracellular pathogens are actively transported to the central nervous system. Additionally, retrograde transport of the virus through neuronal axons has been observed, originating from the olfactory, glossopharyngeal, or vagus nerves (Proal and VanElzakker, 2021). In some patients with neuroinflammation and conditions like ME/CFS, the possibility of viral or bacterial pathogens infecting the vagus nerve has been established (VanElzakker, 2013).

Persistent infection

In some cases, persistent symptoms may be related to prolonged infection with SARS-CoV-2, where the virus is not cleared for extended periods. The presence of viral reservoirs in tissues is evidenced by traces of positive PCR and amplified CD8 T-cell responses against SARS-CoV-2. Despite obtaining a negative PCR, the virus may persist in tissues, a phenomenon observed with other neurotropic viruses (Plebani, 2021; Proal and VanElzakker, 2021), thus perpetuating the low-grade chronic immune response and inflammation.

Autopsies have revealed persistent infection by SARS-CoV-2 in the human nervous system (Stein et al., 2022). Both SARS-CoV-2 and other neurotropic pathogens could be reactivated by acute SARS-CoV-2 infection. These pathogens rarely persist in the blood, typically being identified in tissues or nerves (Proal and VanElzakker, 2021).

Molecular mimicry and autoimmunity

Infection with SARS-CoV-2 can trigger "autoantibodies" due to similarities in sequence with proteins or metabolites derived from the virus and the host's own tissues. An important aspect in the study of PACS involves the analysis of the immune system and these autoantibodies as part of the underlying mechanisms (Proal and VanElzakker, 2021).

Genetic predisposition and immune response

Genetic variations in the immune response, coagulation, or expression of human endogenous retroviruses are associated with an increased risk of developing PACS. However, in a study involving twins, non-heritable factors were found to determine more than half of the variability in immune parameters. These findings highlight how at least one type of microbial exposure can significantly modulate the overall immune profile of healthy individuals (Brodin et al., 2015).

Microbiota/virome and neuroimmune dysregulation

The human body's microbiome consists of diverse and abundant microorganisms. During SARS-CoV-2 infection and PACS, changes in the composition of the intestinal microbiome have been identified (Liu et al., 2022). This dysbiosis of the microbiota/virome can have a profound impact on the host's genetics, immunity, metabolism, hormones, and nervous system (Ursell et al., 2012). During acute SARS-CoV-2 infection, some microorganisms undergo changes in their balance. This alteration may persist, allowing inactive pathogens to reactivate, colonize new sites, and trigger chronic symptoms (Proal and VanElzakker, 2021).

In the "multiple-hit model," one pathogen can support the virulence of the next infection (Proal and Marshall, 2018). In the acute progression of SARS-CoV-2 or PACS, persistent pathogens can be considered predisposing factors (Proal and VanElzakker, 2021), as they overload the neuroimmune circuit, predisposing it to a state of alertness or irritation (Pavlov and Tracey, 2012; Proal and VanElzakker, 2021).

Human bacteria have been shown to play a role in the production and/or consumption of various neurotransmitters such as norepinephrine, dopamine, serotonin, and gamma-aminobutyric acid (GABA) (Galland, 2014). Furthermore, proteins and metabolites derived from the microbiota/virome influence the activity of immune cells. Alterations in host signaling or the permeability of the gastrointestinal epithelial barrier, resulting from dysbiosis, could be contributors to the onset of PACS (Proal and VanElzakker, 2021).

Hydration, nutrition, and oxidative stress

Hydration and nutritional status are fundamental pillars of the body's capacity to respond to various stimuli. Dehydration and nutritional deficiencies are associated with alterations in the neuroimmunoendocrine response, stress, and aging, thus impacting general health status El-Sharkawy, et al., 2015; López Plaza, et al., 2017; Birnkrant, et.al., 2018; Moszak, et al,. 2020; Zhao, et al., 2020; Ciebiera, et al., 2021; Arab, et al., 2023; Schloss JV, 2023.

The evidence supporting the importance of stress-induced deficiencies in nutrients such as magnesium, zinc, iron, calcium, and niacin is strong (Ramsey et al., 2020). While it is crucial to acknowledge the impact of individual nutrients, it's essential to understand that the biological response to stress cannot be simplified to a single nutrient The synergistic interaction of macronutrients and micronutrients, encompassing high-quality sources of carbohydrates, fatty acids, and amino acids, along with vitamins, minerals, antioxidants, enzymes, coenzymes, and the contributions of phytochemicals, supports optimal biological function.

Maintaining adequate hydration and nutrition enhances resilience and improves the ability to adapt to both internal and external stressors. In the specific case of PACS, adequate hydration is an important factor in complete recovery (Barrea et al., 2022).

Some foods have shown notable effects on inflammatory pathways and have the potential to modulate inflammatory imbalances. Therefore, the careful selection of anti-inflammatory foods, while avoiding those with pro-inflammatory potential, is recommended as a fundamental strategy to alleviate diseases characterized by a significant inflammatory component in their pathophysiology. Moreover, a diet rich in anti-inflammatory nutrients, such as the Mediterranean diet, may prove beneficial in ameliorating sequelae secondary to COVID (Ricker and Haas, 2017; Ramsey et al., 2020).

Several clinical trials confirm the positive response of monotherapy with 22 different nutrients (cobalamin, calcium, zinc, thiamine, pyridoxine, asparagine, magnesium, niacinamide, riboflavin, oleic acid, glutamine, inositol, choline, selenium, vitamin D, iron, taurine, phosphorus, ascorbate, bioflavonoids, N-aceyl cysteine) on the probability of contracting COVID-19 and the severity of the disease (Ramsey et al., 2020). However, the response to monotherapy with these nutrients may be influenced by the nutritional exposome, biochemical individuality of each person, the concomitant deficiency of other nutrients and the special nutritional needs induced by the stress of dysfunction.

Supplementation with molecules like coenzyme Q10 and alpha-lipoic acid, targeting antioxidant cellular pathways, presents intriguing

alternatives explored in treating conditions with chronic inflammation, such as PACS (Tasneem et al., 2019; Akanchise and Angelova, 2023).

A wide variety of medicinal plants, such as *Camellia Sinensis, Tripterygium Wilfordii Hook F, and Zingiber officinale*, among others, display anti-inflammatory effects (Bustamante et al., 2023). Essential oils from species such as *Eucalyptus, Cinnamomum,* and *Juniperus* exhibit therapeutic potential in modulating immunity, reducing inflammation, and exerting antiviral effects. These plants and oils contain various phytochemicals, including phenolics, terpenoids, and alkaloids, which individually exhibit anti-inflammatory, immunomodulatory, and antiviral properties with curative potential for COVID-19 (Barletta et al., 2023).

The hydration and nutritional status significantly influence disease expression. While discussing the entire spectrum of therapeutic uses of diets, nutrients, phytochemicals, and essential oils is extensive, certain key areas and their direct impact on the neuroimmunoendocrine system and associated biological responses merit attention.

Neuraltherapeutic medicine

Neuraltherapeutic Medicine (NTM) (more commonly known as Neural Therapy) arises from the conjunction of understanding the synthetic physiology of Nervism with the discovery of the modulating effects of Local Anesthetics (LAs) on the nervous system, known as the neuraltherapeutic effect.

Researchers such as Speransky, Bykov, Orbeli, and Vischnevsky confirmed Pavlov's experimental findings and used infiltrations of LAs, known as "novocainic blocks," to address nervous system dysfunctions (Забродин, 1999). Rather than a simple transient anesthetic block, they described a lasting neuraltherapeutic effect that persists after the direct pharmacological effect, stimulating natural regulatory reflexes, such as anti-inflammation, that were somehow dysregulated. This approach allowed them, in a surgical context, to manage critical acute conditions such as septic or hypovolemic shock in war victims (Sechenova et al., 1993) and to modulate inflammation in both acute and chronic inflammatory and infectious pathologies, in both humans and animal models (Ariza Tarazona et al., 2020).

In Germany, a medical school named "Neural Therapy according to Huneke" was born, later enriched by scientific research from around the world. It presents itself as a therapeutic option to modulate the reflexes of the nervous system. In Colombia, a new school emerged in the 1970s, led by Julio Payán, which not only integrates Russian nervism and the foundations of the German school but also undergoes conceptual and scientific enrichment from the complexity sciences, leading to a change in its name to Neuraltherapeutic Medicine (Bustamante et al., 2023).

This school distinguishes itself by incorporating into its theoretical framework the concept of stimulating the psychoneuroimmunoendocrine system to modulate the nonspecific pathophysiological mechanism of neurogenic dystrophy and, consequently, inflammation. Additionally, it integrates reflex mechanisms of central (including the concept of corticalization) and peripheral sensitization in the process of neurogenic inflammation. These concepts manifest coherently within the semiological approach to patients and in diagnostic and therapeutic orientations, which focus depending on the level of irritation (local, segmental, or meta-segmental) (Ariza Tarazona et al., 2020).

Neuraltherapeutic Medicine, through stimuli applied to anatomical structures, usually through the use of Local Anesthetics (LAs) such as low-concentration procaine (between 0.5 and 1%), modulates the regulatory and plastic functions of the nervous system. Such application of procaine is usually very well tolerated, with minimal effects in patients such as transient dizziness and metallic taste (Dosch and Dosch, 2007). There is evidence of the neuroimmunomodulatory action of LAs, more extensively studied in inflammatory conditions and pain (Akimov and Kositsyn, 2005; Engel et al., 2022; Bustamante et al., 2023; Vinyes et al., 2023). Furthermore, other pathways of action on the information system that have not been completely elucidated have been indicated, such as influence through the microtubules of the living matrix (Cruz, 2011; Liu and Duricka, 2022; Bustamante et al., 2023).

Several authors have explored the therapeutic effect of Local Anesthetics (LA) on the Post-Acute Sequelae of COVID-19 (PACS), albeit diverging from the conceptual framework of Neuraltherapeutic Medicine. For instance, Liu reported symptom resolution in two PASC patients through the use of LA in two consecutive procedures near the stellate ganglion. Galvin conducted a retrospective review of medical records involving 195 PACS patients, noting statistically significant improvements in most symptoms following stellate ganglion block (Galvin et al., 2023). Typically, 5 mL of local anesthetic is used for this procedure (Restrepo-Garcés et al., 2012). Both authors describe the therapeutic effect as sympathetic blockade and acknowledge a lack of understanding of mechanisms beyond the anesthetic (Liu and Duricka, 2022).

Stellate ganglion block (SGB) can lead to various complications, including Horner's syndrome and potential impacts on nearby nerve structures like the recurrent laryngeal nerve, resulting in dysphonia, dysphagia, and dyspnea. Major risks involve inadvertent vascular injection causing seizures and cardiovascular toxicity, and neuroaxial block, often requiring immediate support. Other potential complications include esophageal perforation and thyroid puncture, which may lead to neck hematomas (Restrepo-Garcés et al., 2012).

Vinyes et al. (Vinyes et al., 2022), specifically, detailed a successful Neuraltherapeutic Medicine approach in a PACS patient, involving three procedures over 8 weeks with a 16-week follow-up. While Liu and Galvin follow a linear thinking approach with a standardized, non-individualized procedure, Vinyes considers a nonlinear approach, investigating the patient's past irritations throughout their life history, thus defining therapeutic orientations under this rationale.

The experience with neural therapeutic approaches, documented across centers in Switzerland (Mermod et al., 2008), Germany (Joos et al., 2011), Spain (Roca et al., 2010), and Colombia (Sarmiento Rodríguez, 2014), among other countries, has yielded promising outcomes in managing diverse chronic conditions. Presently, 14 scientific associations are affiliated with the International Federation of Medical Associations of Neural Therapy (IFMANT) (https://www.ifmant.at/es/), n.d. underscoring the increasing global recognition and adoption of this therapeutic modality.

Other neuroimmune modulation strategies

Few therapeutic advances address the modulation of the neuroimmune circuit. Some studies emphasize the role of the vagus

nerve (VN), a key representative of the parasympathetic nervous system, and the stimulation of the anti-inflammatory cholinergic efferent arm through implantable and external electronic devices, but these are costly and have limited access (Johnson and Wilson, 2018). Other researchers have explored the possibility of preventing neurogenic dystrophy by using adrenergic blockers during injury (Akimov and Kositsyn, 2005), but these remain limited.

Neurofeedback and biophotomodulation are potential non-invasive therapeutic tools in modulating inflammation of neurogenic origin, although there are no specific studies on their impact on the inflammatory reflex, these techniques have shown improvement in diseases with an inflammatory component and oxidative stress of the nervous system. Such as Alzheimer's and Parkinson's (Berman and Nichols, 2019).

Physical exercise in inflammatory disorders

The proper prescription of exercise holds potential benefits for inflammatory disorders and is essential in managing PACS (Metsios et al., 2020; Chuang et al., 2024).

Integral synthesis

Technological advancement has provided tools to delve deeper into the understanding of the nervous system. Although this system exhibits phenomena that are not fully explainable, the growing effort to integrate other branches of knowledge, such as quantum physics, into living systems (Tuszynski, 2020), reflects broad and ongoing scientific development.

Beyond reductionism, multiple interrelated non-linear connections are glimpsed in biological circuits following SARS-CoV-2 infection, prolonging symptoms. The properties of complex systems are not fully understood by analyzing isolated parts. Studying the organism as a whole, similar to the nervous system, represents a modernization of the biomedical paradigm.

Conclusion

In the pathophysiology of PACS, various hypotheses are proposed that involve common patterns of nonspecific response, such as inflammation and dysautonomia. These patterns may compromise the function of the neuroimmune-endocrine system, leaving it hypervigilant, primed, or hyperexcited, triggering neurogenic inflammation following neurogenic dystrophy. Although these patterns are also described in functional syndromes, from the perspective of nervism and Pavlovian synthetic physiology, they are involved in all pathophysiological processes.

Expanding the paradigm involves recognizing the crucial role of information systems in integrating the unity of organisms. Although the existential dilemmas of the psyche or soul and their physiological repercussions are not delved into, emerging approaches in medicine include innovative concepts of the neuroimmune-endocrine system in modulating all processes of the living body. It is worth highlighting the process of inflammation and tissue repair as a cornerstone of health and disease.

Neurogenic tissue dystrophy underlies the local, segmental, or distant clinical expression. This process resembles the model of central and peripheral sensitization. The described segmental irritation refers to the classic anatomical model of deep and superficial innervation, related in a transversal reflex arc from the somite to the medullary level. On the other hand, neuromodulatory trigger points correspond to polysegmental anatomical circuits, temporal associations, and other information interactions not yet described.

Neuraltherapeutic stimulation transcends the direct pharmacological response provided by the transient anesthesia of Local Anesthetics (LA). Potentially, this stimulation modulates nervous influx in tissues, simultaneously influencing physiological and pharmacological responses, especially in inflammatory processes. This capability involves modulating the non-specific response of the Neuroimmune-Endocrine System (SNIE), enhancing the resilience of the biological system independently of the specific mechanisms of the disease cause. This therapeutic modality could be potentially useful in pathologies with neurogenic inflammation, such as Post-COVID-19 Accumulative Syndrome (PACS).

More details about the Neural therapy should be given/discussed: the putative side effects of the blockade of the stellate ganglion, whether or not these procedures are routinely done in known Medical centers.

Examining points of convergence between the neuroscience paradigm and Neuraltherapeutic Medicine (NTM) allows for a more integrative synthesis, providing a fuller perspective on information systems and their interaction. Although much is yet to be understood, especially regarding the psyche, clinical practice offers a fertile ground for exploration and advancement in this field.

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CB: Writing – review & editing, Writing – original draft, Conceptualization. LBPB: Writing – review & editing. JCR: Writing – review & editing.

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Effects of COVID-19 virus-like particles on the behavioral and cognitive performance of human apolipoprotein E targeted replacement mice

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Introduction: The effects of viral infections might be apolipoprotein E (apoE) isoform-dependent. In humans, there are three major apoE isoforms, E2, E3, and E4. E4 is associated with the enhanced entry of several viruses into the brain and their disease progression. A concern of infection by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the development of post-acute COVID-19 syndrome, also known as long COVID. Genetic risk factors for developing long COVID were reported.

Methods: In this study, we used virus-like particles (VLPs) that include expression of the SARS-CoV-2 nucleocapsid (N), membrane (M), and envelope (E) structural proteins together with S. In the current study, we used human E2, E3, and E4 targeted replacement mice to assess whether these VLPs affect body weight, behavioral and cognitive performance, and circadian body temperatures. Using VLPs allow working outside an ABSL-3 facility.

Results: The effects of VLPs on some behavioral measures were apoE isoform-dependent, with the E2 mice being more affected than E3 or E4 mice. The overall decreased activity in the open field containing objects in week 2 indicate that VLPs can also reduce activity levels in an apoE isoform-independent fashion.

Discussion: The results of the current study indicate that even in the absence of viral replication, detrimental effects of VLPs on behavioral measures and circadian body temperatures are seen.

KEYWORDS

COVID-19, virus-like particles, apolipoprotein E, behavioral testing, cognitive testing

1 Introduction

The effects of viral infections might be apolipoprotein E (apoE) isoform-dependent. E4 is associated with enhanced entry of several viruses into the brain and their disease progression, including that of human immunodeficiency virus-1 (HIV) (1), herpes simplex virus-1, hepatitis C virus, hepatitis E virus, varicella zoster virus, Epstein–Barr virus, malaria, *Listeria monocytogenes* (LM), and *Klebsiella pneumoniae* (2). The impact of COVID-19 might also be apoE isoform-dependent. E4 modifies the associations of polymorphisms in angiotensin-converting enzyme (ACE), which plays a key role in COVID-19 (3), with neuropsychiatric syndromes in Alzheimer's disease (AD) (4). In addition, the *APOE* genotype is associated with survival in patients infected with COVID-19 (5); compared to E3 homozygous patients, E4 homozygous patients showed poorer survival. E2 homozygous patients showed a trend towards lower survival than E3 homozygous patients, but this did not reach significance, which might be related to the lower occurrence of E2 than E4 in the population. E4 is also associated with severe COVID-19 with more prevalent microhemorrhages in intensive care patients (6). Consistent with human studies, in human apoE mice, E2 and E4 mice showed worse survival than E3 mice following infection with COVID-19 (5).

Among the concerns of infection by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the development of post-acute COVID-19 syndrome, also known as long COVID. Many of the symptoms of long COVID, including fatigue, myalgia, learning and memory impairments, anxiety, and a post-traumatic stress disorder-like condition, are likely mediated through the central nervous system (CNS) (7) as well as PMC9537254. Pre-existing psychiatric conditions might increase risk to develop long COVID (8). Genetic risk factors for developing long COVID were reported (9). There might be an overlap between risk to develop long COVID and risk to develop age-related neurodegenerative conditions such as AD (10). apoE plays a role in cholesterol metabolism and neuronal repair after injury (11). Compared to apoE3 (E3), apoE4 (E4) is a risk factor for developing cardiovascular disease and AD (12–14), while apoE2 (E2) provides relative protections against developing AD (15). However, in adverse environments, E4 might provide relative protection (16).

COVID-19 is a betacoronavirus (17), which possesses a large (26–32 kb) positive-sense RNA genome that interacts with the nucleocapsid (N) protein to form the ribonucleoprotein core of the virion, encased in the viral envelope and shaped by the membrane (M) protein. The envelope (E) protein forms an ion channel that is required for the virulence of SARS- and MERS-CoVs and is also thought to close the virion during budding (18). The spike (S) protein is arranged in trimers on the virion surface and mediates viral entry into the host cell. The majority of the antibody response during coronavirus infection is directed against the N and S proteins, with virus neutralization and protection of the host being mediated primarily by anti-S antibodies (19). The N, M, and E proteins of SARS-CoV-2, or minimally N and M, are sufficient for efficient release of virus-like particles (VLPs) into the culture medium of transfected cells (20). The S protein, which can be incorporated into VLPs as well binds to humanACE2 on the host cell.

Dr. Sullivan developed targeted replacement (TR) E2 (21), E3 (22), and E4 (23) mice that express human apoE under control of the mouse apoE promoter on a C57BL/6J background. In the current study, we used these mice to assess whether VLPs affect body weight, behavioral and cognitive performance, and circadian body temperatures in E2, E3, and E4 TR mice. As markers of immune response were elevated in COVID-19-infected mice and exposed patients, we also assessed hippocampal mRNA levels of tumor necrosis factor (TNF)-α, interleukin (IL)-4, interferon (IFN)-γ, and C-C motif chemokine 11 (CCL11 or Eotaxin). Expression of TNF-α was reported to be induced in the brains of SARS-CoV-2-infected mice at 7 dpi, but not at 7 weeks, while CCL11 levels were induced long term (24). IL-4 levels are associated with COVID-19 severity (25). Low levels of IFN-γ were suggested as a risk factor for hospitalization following exposure to COVID-19 and IFN-γ was shown to be associated with recovery following COVID-19 exposure (26, 27).

2 Materials and methods

2.1 Mice

TR E2 (21), E3 (22), and E4 (23) mice [n = 51 (10.51 ± 0.12 months of age); E2: n =15 (10.65 ± 0.22 months of age; n = 6 males and n = 9 females); E3: n = 17 (10.47 ± 0.24 months of age; n = 8 males and n = 9 females); E4: n = 19 (10.43 ± 0.19 months of age; n = 10 males and n = 9 females)] expressing human apoE under control of the mouse apoE promoter on a C57BL/6J background were used in this study. Homozygous breeding of the mice was used to generate the experimental mice for this study. Throughout testing, all the mice were singly housed. Animals were maintained on a 12:00 h light/dark schedule (lights on at 06:00). Laboratory chow (PicoLab Rodent diet 20, # 5053; PMI Nutrition International, St. Louis, MO, USA) and water were provided *ad libitum*. Behavioral testing took place during the light cycle. All procedures complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with IACUC approval at Oregon Health & Sciences University. Experimenters were blinded to the genotype, sex, and treatment of the mice.

2.2 Implantation of temperature sensors

TS100 millimeter-scale (7.5 × 7.5 × 4.2 mm) CubiSensTM wireless sensors (CubeWorks, Ann Arbor, MI), packaged in bio-compatible epoxy and coated with parylene, were implanted in the abdomen for accurate, real-time temperature measurement. The TS100 is capable of transmitting up to 100 m in distance, lasts up to 2 years in sensing operation, and allows measuring circadian body temperature in individual mice. The sensors were sterilized using the Cidex solution (CubeWorks, Ann Arbor, MI). A heating pad and bead sterilizer were used for the surgeries.

For the surgery, the mice were anesthetized with isoflurane (4% for induction of the anesthesia and 1%–3% for maintenance of

the anesthesia). Lidocaine (7 mg/kg of 0.5%) was injected subcutaneously around the incision site, immediately prior to the aseptic preparation of the abdomen. To close the abdominal cavity, 4-0 undyed, unbraided, monofilament sutures were used. To close the skin, 9-mm AUTOCLIP stainless steel clips were used. For pain control, meloxicam (10 mg/kg) was administered orally prior to the induction of anesthesia and every 24 h for two additional days. The mice were treated and behaviorally tested starting 2 weeks after the surgeries. Body temperatures were acquired and analyzed for the first week of behavioral testing.

2.3 Generation of SARS-CoV-2 VLPs

The VLPs were generated to express the SARS-CoV-2 nucleocapsid (N), membrane (M), and envelope (E) structural proteins together with S, allowing us to perform SARS-CoV-2-related studies without replicating virus and outside a BSL-3 facility. Plasmid expression vectors encoding each of the SARS-CoV-2 N, M, E, and S proteins were constructed by standard cloning methods, using synthesized codon-optimized sequences. M, E, N, and S plasmids were transfected at a ratio of 5 µg:1 µg:5 µg:1 µg into suspension-grown Expi293F (25 mL at 3×10^6 cells/mL) cells using ExpiFectamine reagent (ThermoFisher). Cells were allowed to grow for 4–6 days post-transfection before harvesting. Cells were separated from culture supernatant by centrifugation at 2,000 rpm for 15 min, and culture supernatant was passed through a 0.45-µm filter. VLPs were further concentrated and purified by ultracentrifugation through a 20% sorbitol cushion at 30,000 rpm for 2 h. The pellet was resuspended in 1/100 original volume of phosphate-buffered saline (PBS). Viral proteins S and N were detected by Western blotting (Figures 1A, B), and the VLP structure was assessed by transmission electron microscopy of negatively stained samples (Figure 1C). Total protein content will be assessed by BCA assay.

2.4 Treatments

For two subsequent weeks, mice were injected daily (weekdays 1–5), each morning 1 h prior to the first behavioral test of that day, with virus-like particles (VLPs) (1 µg/mouse) or vehicle, intraperitoneally, in a volume of 100 µL. The dose was selected based on a preliminary study showing that that dose showed a robust threefold increase in plasma corticosterone levels 1 h following i.p. injection, while a lower dose of 0.3 µg did not.

2.5 Body weights

Body weights were taken prior to the surgery and at the time of the grip strength tests, on day 4 of weeks 1 and 2. The body weight

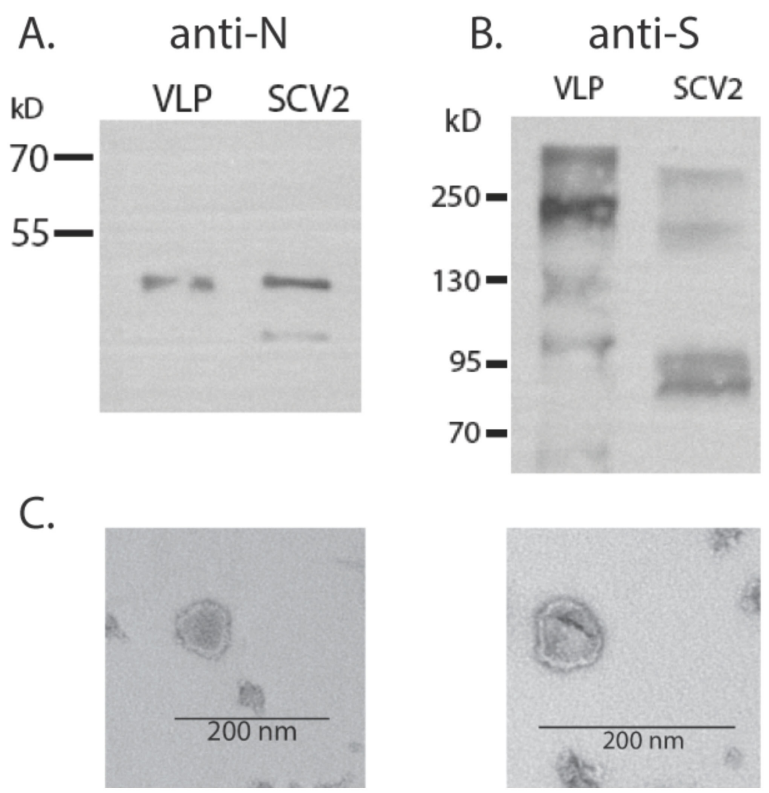

FIGURE 1
Production of SARS-CoV-2 VLPs. **(A)** Western blot detection of N protein in VLPs or SARS-CoV-2 virus (3×10^5 focus forming units). **(B)** Detection of S in VLP and virus. **(C)** Electron micrograph of VLP prep.

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ratio for both weeks was calculated as outcome measure and defined as: (body weight at the time of the grip strength test – body weight prior to the surgery)/(body weight prior to the surgery).

2.6 Behavioral testing

Mice were behaviorally tested as follows (Figure 2). In the morning of days 1 and 2 of both weeks, mice were tested for measures of activity, measures of anxiety, and spatial habituation in the open field. In the morning of days 3 and 4 of both weeks, the mice were tested for object recognition. In the morning of day 5 of both weeks, mice were tested for spontaneous alternation in the Y maze. In the afternoon of days 2 and 3 of both weeks, the mice were tested for sensorimotor function on the rotarod. In the afternoon of day 4 of both weeks, the mice were tested for grip strength. In the afternoon of day 5 of both weeks, the mice were tested for depressive-like behavior in the forced swim test. The behavioral tests were performed as described below.

2.7 Open field and novel object recognition

The mice were put in an open field enclosure (16 × 16 inches, Kinder Scientific, Poway, CA) for 10 min on two subsequent days. On day 3, the open field contained two identical objects for a 15-min trial. The next day, one object was replaced with a novel object for a 15-min trial. Between trial, the arenas and objects were cleaned with 0.5% acetic acid. Interaction within a 2-cm proximity with the object was coded as object exploration by hand scoring videos acquired with Noldus Ethovision software (version 17, Wageningen, The Netherlands). A discrimination index was defined as the time spent exploring the familiar object subtracted from the time exploring the novel object, and dividing the resulting number by the total time spent exploring both objects. A positive discrimination index indicates a preferential exploration of the novel object. A negative discrimination index indicated a preferential exploration of the familiar object. Different objects were used during the first and second week of testing.

The outcome measures in the open field analyzed were as follows (1): distance moved in the open field in the absence and presence of objects, an activity measure (2); the difference in the distance moved in the open field over days, habituation to the open field, a cognitive measure (3); time spent in the center of the open field, an anxiety measure; and (4) the discrimination index, a cognitive measure.

2.8 Grip strength

A Harvard Apparatus (Holliston, MA) grip strength meter for mice was positioned horizontally. The mice were allowed to grasp the metal grid and pulled backwards in the horizontal plane. The force applied to the grid was recorded as the peak tension. Three measurements were conducted at 1-min intervals (28). The peak grip strength for each mouse was recorded. In addition, we calculated the relative grip strength as the ratio of grip strength to body weight, as previously described (29). The outcome measures in the grip strength test were the peak grip strength and the ratio of grip strength to body weight.

2.9 Y maze

Activity levels and hippocampus-dependent spontaneous alternations were assessed in week 1 in the Y maze from Harvard Apparatus (Panlab, Holliston, MA, United States). This Y maze is smaller and distinct [raised sides and made of non-reflective opaque gray plastic (30 cm × 6 cm × 15 cm)] from the one used during the second week of testing. For the second week of testing, we used the Y-shaped maze from O' Hara & Co., Ltd. (Tokyo, Japan) that had raised sides (3.8 cm bottom width, 12.55 cm top width, and 12.55 cm height) with plastic, opaque gray arms (37.98 cm length). The maze was cleaned with 0.5% acetic acid between trials. Performance

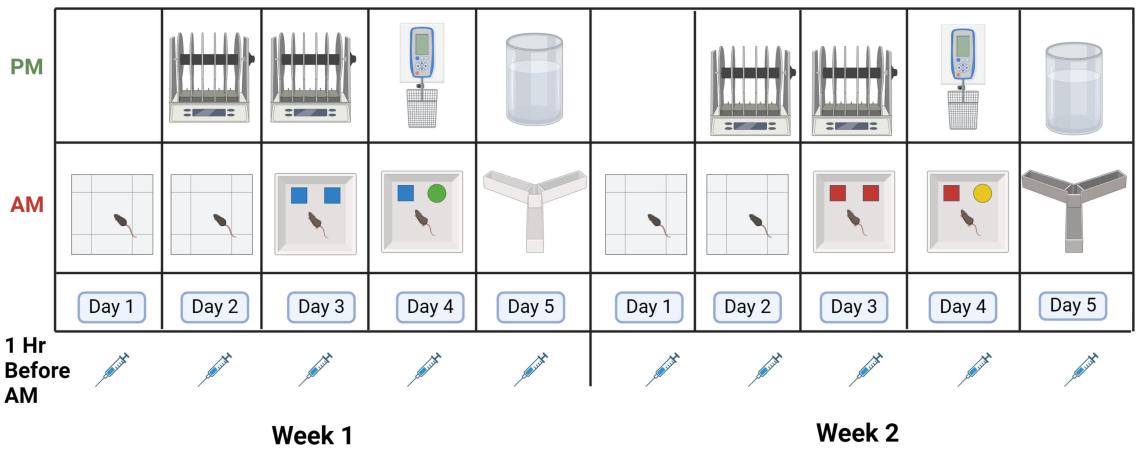

FIGURE 2
Schedule of behavioral testing. Mice were injected 5 days per week in weeks 1 and 2 with VLPs or vehicle. For details, see text.

was assessed during a 5-min trial. Performance was recorded using the Noldus Ethovision software and hand scoring was used to assess the number of arm entries and the percent spontaneous alternations. The outcome measures in the Y maze were total arm entries, an activity measure, and percent spontaneous alternations, a cognitive measure.

2.10 Rotarod

The rotarod test (rod diameter: 3 cm, elevated: 45 cm; Rotamex-5, Columbus Instruments, Columbus, OH, USA) was used to assess sensorimotor function. The rotation speed started at 5 rpm and accelerated 1.0 rpm every 3 s. Fall latency (s) was recorded. For both weeks of testing, mice received three subsequent trials on two subsequent days. The outcome measure in the rotarod test used was the mean fall latency of each mouse for each day.

2.11 Forced swim test

To assess depressive-like behavior, mice were placed for 6 min in a container with water (water height: 15 cm; container diameter: 16–20 cm; 25°C) not allowing the mouse's tail to touch the bottom. Immobility, defined as cessation of limb movements except for minor involuntary movements of the hind limbs or those movements necessary to stay afloat, was scored manually by an observer blinded to genotype and test history using a sampling technique every 5 s during the trial. The data are expressed as the percentage of immobility (number of immobility observations divided by the total number of observations) during the last 4 min (=48 observations) of the test, as previously described (30).

2.12 Hippocampal cytokine mRNA expression

Following the forced sim test, the mice were euthanized by cervical dislocation. The brain was quickly removed and the hippocampi were dissected in ice-cold PBS and stored at −80°C for analysis. RNA isolated from hippocampi was analyzed by qRT-PCR for expression of the inflammatory mediators TNF-α, IFN-γ, IL-4, and CCL11. RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Relative expression of cytokines was determined by qRT-PCR using gene-specific primer-probe sets (ThermoFisher) and normalized to β-actin mRNA expression using the ΔΔCt method (31).

2.13 Statistical analyses

All behavioral data are reported as mean ± standard error of the mean and were analyzed using SPSS v.22 (IBM, Armonk, NY, USA) or GraphPad v.8 (La Jolla, CA, USA) software. Genotype and treatment were included as factors in analysis of variance (ANOVA), and sex was used as a covariate. In case there were statistical genotype interactions, genotypes were analyzed separately, as indicated. There were no statistical treatment × sex interactions. When sex or an interaction with sex was not significant, we dropped sex as a covariate and reran the analysis. Repeated measures were used when appropriate. As the E2, E3, and E4 mice were of similar age, age was not included as part of the analysis. The 2 weeks of open field testing and the 2 weeks of testing behavioral performance in the open field containing objects were analyzed separately using day as the repeated measure. Because of the strong practice effects in the rotarod tests, we analyzed the four subsequent days of rotarod testing over the 2 weeks using a repeated measures ANOVA. For the other behavioral tests, week was used as the repeated measure in the ANOVA. For the circadian data, based on the pattern of the data, the light and dark periods for the 5 days were analyzed as separate analyses, with the mean body temperature in the light or dark period of each day as the repeated measure. Based on the three-way treatment × genotype × treatment interaction revealed, we next analyzed the VLP- and vehicle-treated genotype-matched group separately and finally performed an analysis of each light and dark period separately, with the hour as the repeated measure. Statistical significance was considered as $p < 0.05$. When sphericity was violated (Mauchly's test), Greenhouse–Geisser corrections were used. Mice were tested in separate cohorts, each containing mice of all experimental groups. All researchers were blinded to genotype and treatment, and the code was only broken after the data were analyzed.

To determine the relationships between behavioral performance measures on the different tests in individual mice, a principal components analysis (PCA) was performed. The behavioral measures used for this analysis are indicated in Table 1. The PCA was performed using SPSS software and using the varimax rotated matrix. Factors with eigenvalues > 1 were considered significant.

3 Results

3.1 Body weights/

For body weights, there was an effect of week [F (1,44) = 10.638, $p = 0.002$], with a lower body weight ratio in week 2 than in week 1, and there was a trend towards a week × genotype × treatment interaction [$F(2,44) = 2.810$, $p = 0.071$] (Figure 3). In E2 mice, there was a trend towards an effect of treatment [$F(1,13) = 3.976$, $p = 0.0676$], with a trend towards a lower body weight ratio in VLP- than vehicle-treated E2 mice. In E3 mice, there was only an effect of week [$F(1,15) = 8.858$, $p = 0.0094$], with a lower body weight ratio in week 2 than in week 1. Similarly, in E4 mice, there was only an effect of week [$F(1,17) = 12.85$, $p = 0.0023$], with a lower body weight ratio in week 2 than in week 1.

3.2 Open field and novel object recognition

When the activity levels in the open field during week 1 were analyzed, there was an effect of day, with lower activity levels on day 2

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TABLE 1 Behavioral measures used in the PCA.

Abbreviation	Behavioral Measure
TDOFD1w1	Total distance moved on the first day in the open field in week 1.
TDOFD2w1	Total distance moved on the second day in the open field in week 1.
TDOFD3w1	Total distance moved on the third day in the open field in week 1.
TDOFD4w1	Total distance moved on the fourth day in the open field in week 1.
TDOFD1w2	Total distance moved on the first day in the open field in week 2.
TDOFD2w2	Total distance moved on the second day in the open field in week 2.
TDOFD3w2	Total distance moved on the third day in the open field in week 2.
TDOFD4w2	Total distance moved on the fourth day in the open field in week 2.
CDOFD1w1	Distance moved in the center of the open field on the first day in the open field in week 1.
CDOFD2w1	Distance moved in the center of the open field on the second day in the open field in week 1.
CDOFD3w1	Distance moved in the center of the open field on the third day in the open field in week 1.
CDOFD4w1	Distance moved in the center of the open field on the fourth day in the open field in week 1.
CDOFD1w2	Distance moved in the center of the open field on the first day in the open field in week 2.
CDOFD2w2	Distance moved in the center of the open field on the second day in the open field in week 2.
CDOFD3w2	Distance moved in the center of the open field on the third day in the open field in week 2.
CDOFD4w2	Distance moved in the center of the open field on the fourth day in the open field in week 2.
DIw1	Discrimination Index in the object recognition test in week 1.
DIw2	Discrimination Index in the object recognition test in week 2.
Immobility FSTw1	Percent immobility in the Forced Swim Test in week 1.
Immobility FSTw2	Percent immobility in the Forced Swim Test in week 2.
Entries Ymazew1	Number of entries in the Y maze in week 1.
SpontAlternationw1	Spontaneous Alternations in the Y maze in week 1.
Entries Ymazew2	Number of entries in the Y maze in week 2.
SpontAlternationw2	Spontaneous Alternations in the Y maze in week 1.
GripStrengthw1	Grip strength in the grip strength test in week 1.
GripStrengthw2	Grip strength in the grip strength test in week 2.
RRD1w1	Fall latency on the first day of the rotarod test in week 1.
RRD2w1	Fall latency on the second day of the rotarod test in week 1.
RRD1w2	Fall latency on the first day of the rotarod test in week 2.
RRD2w2	Fall latency on the second day of the rotarod test in week 2.
BWRatiow1	Body weight ratio in week 1.
BWRatiow2	Body weight ratio in week 2.

(Continued)

TABLE 1 Continued

Hippocampal cytokine mRNA expression levels were analyzed in each genotype using *t*-tests.

than day 1 [$F(1,44) = 67.636$, $p < 0.001$] (Figure 4A). In addition, there was a trend towards an effect of treatment [$F(1,44) = 3.057$, $p = 0.087$]. When the activity level in the open field containing objects (days 3 and 4) during week 1 were analyzed, there was an effect of day [$F(1,44) = 41.925$, $p < 0.001$] (Figure 4A).

When the activity levels in the open field during week 2 were analyzed, there was an effect of treatment [$F(1,44) = 10.183$, $p = 0.003$] and a genotype × treatment interaction [$F(2,44) = 5.739$, $p = 0.006$] (Figure 4B). In E2 mice, there was an effect of day [$F(1,13) = 14.41$, $p = 0.0022$] and a trend towards an effect of treatment [$F(1,13) = 3.407$, $p = 0.0878$]. In E3 mice, there was only an effect of day [$F(1,15) = 25.7$, $p = 0.0001$]. Similarly, in E4 mice, there was only an effect of day [$F(1,17) = 36.75$, $p < 0.0001$]. When the activity level in the open field containing objects (days 3 and 4) during week 2 was analyzed, there was an effect of treatment [$F(1,44) = 4.588$, $p = 0.038$] (Figure 4B).

When time spent in the center of the open field during week 1 was analyzed, there was an effect of day [$F(1,44) = 29.993$, $p < 0.001$], with less time spent in the center of the open field on day 2 than day 1 (Figure 4C). When time spent in the center of the open field containing objects during week 1 was analyzed, there was an effect of sex [$F(1,43) = 4.463$, $p = 0.040$] and an effect of genotype

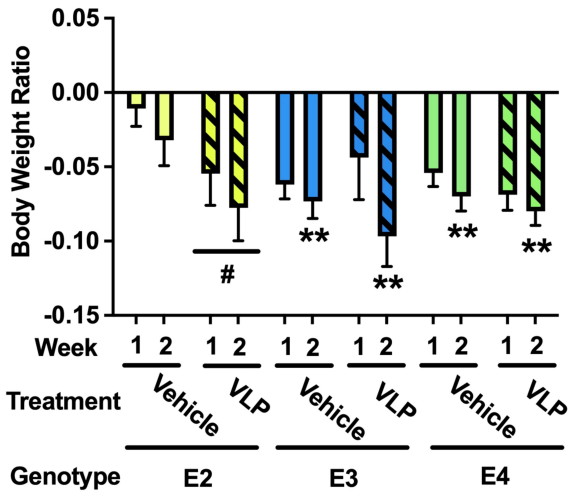

FIGURE 3
Body weight ratios in VLP- and vehicle-treated E2, E3, and E4 mice. In E2 mice, there was a trend towards an effect of treatment with a trend towards a lower body weight ratio in VLP- than saline-treated mice. $^{\#}p = 0.0676$. In E3 and E4 mice, there was only an effect of week. $^{**}p < 0.01$.

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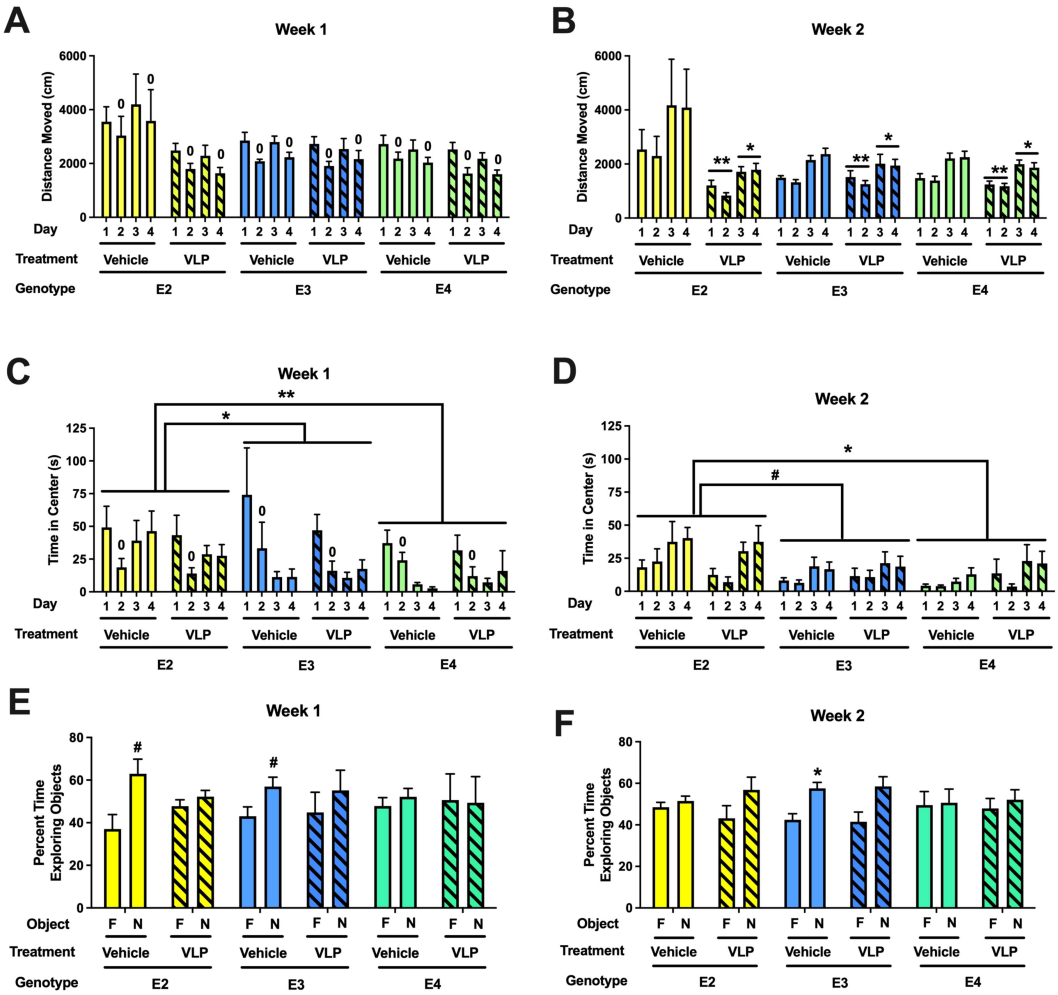

FIGURE 4
(A) Behavioral performance in the open field and novel object recognition of VLP- and vehicle-treated E2, E3, and E4 mice. Activity levels in the open field without (days 1 and 2) and with objects (days 3 and 4) during week 1. There was overall spatial habituation to the open field, with lower activity levels on day 2 than day 1. $^{0}p < 0.001$ versus day 1. **(B)** Activity levels in the open field without (days 1 and 2) and with objects (days 3 and 4) during week 2. VLP-treated mice moved less than vehicle-treated mice. $^{**}p = 0.003$ versus vehicle-treated mice on days 1 and 2, $^{*}p = 0.038$ versus vehicle-treated mice on days 3 and 4. **(C)** Time spent in the center of the open field without (days 1 and 2) and with objects (days 3 and 4) during week 1. Mice spent less time in the center of the open field on day 2 than day 1. $^{0}p < 0.001$. In addition, E2 mice spent more time in the center of the open field than E3 or E4 mice. $^{*}p = 0.0155$, $^{**}p = 0.0024$, Tukey's. **(D)** Time spent in the center of the open field without (days 1 and 2) and with objects (days 3 and 4) during week 2. E2 mice spent more time in the center of the open field than E4 mice and there was a trend towards E2 mice spending more time in the center of the open field than E3 mice. $^{*}p = 0.0333$, $^{\#}p = 0.056$, Tukey's. In the object recognition test, the time spent exploring the novel (N) and familiar **(F)** objects are analyzed for each group. **(E)** During week 1, vehicle-treated E2 ($^{\#}p = 0.0531$, paired *t*-test) and E3 mice ($\#p = 0.0783$, paired *t*-test) showed a trend towards exploring the novel object more than the familiar one. **(F)** During week 2, vehicle-treated E3 mice spent more time exploring the novel than the familiar object. $^{*}p = 0.0176$, paired *t*-test.

[$F(2,43) = 4.839$, $p = 0.013$], with E2 mice spending more time in the center than E3 ($p = 0.0155$, Tukey's) and E4 ($p = 0.0024$, Tukey's) mice (Figure 4C).

When time spent in the center of the open field during week 2 was analyzed, there was an effect of sex [$F(1,43) = 7.839$, $p = 0.008$] and a trend towards an effect of genotype [$F(2,43) = 2.708$, $p = 0.078$] (Figure 4D). When time spent in the center of the open field containing objects during week 2 was analyzed, there was a day × sex interaction [$F(1,43) = 4.637$, $p = 0.037$], an effect of genotype [$F(2,43) = 4.477$, $p = 0.017$], with E2 mice spending more time in the center than E4 mice ($p = 0.0333$, Tukey's) and a trend towards spending more time in the center than E3 mice ($p = 0.0928$, Tukey's), and a trend towards an effect of sex [$F(1,43) = 3.860$, $p = 0.056$].

Next, object recognition was assessed. During week 1, vehicle-treated E2 ($p = 0.0531$, paired t-test) and E3 mice ($p = 0.0783$, paired *t*-test) showed a trend towards exploring the novel object more than the familiar one (Figure 4E). In contrast, VLP-treated E2 and E3 mice and vehicle- and VLP-treated E4 mice did not. During week 2, only vehicle-treated E3 mice explored the novel object more than the familiar one ($p = 0.0176$, paired *t*-test) (Figure 4F).

3.3 Rotarod

When performance on the rotarod was analyzed, there was an effect of day [$F(1,44) = 62.701$, $p < 0.001$] with improved

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performance with training. There were no effects of genotype or treatment (Figure 5).

3.4 Grip strength

For grip strength, there was a week × sex [$F(1,44) = 5.25$, $p = 0.027$] and a week × genotype [$F(2,44) = 4.747$, $p = 0.014$] interaction. While there was no genotype or treatment effect in week 1 (Figure 6A) or week 2 (Figure 6B), the grip strength in week 2 was lower than that in week 1 in E2 ($t = 3.547$, $p = 0.0036$, paired t-test) and E3 ($t = 4.049$, $p = 0.0009$, paired t-test), but not E4 mice (Figure 6C).

3.5 Y maze

When spontaneous alternation was assessed in the Y maze, there was only an effect of week [$F(1,44) = 4.263$, $p = 0.045$], with lower spontaneous alternation in week 2 than in week 1 (Figures 6D, E). We recognize that this might be due to the larger Y maze used in week 2 than in week 1. When activity levels were analyzed in the Y maze, there was an effect of sex [$F(1,43) = 6.433$, $p = 0.015$] and a trend towards a week × genotype interaction [$F(2,43) = 3.024$, $p = 0.059$] (Figures 6F, G).

3.6 Forced swim test

When depressive-like behavior was tested in the forced swim test, there was an effect of week [$F(1,44) = 8.981$, $p = 0.004$], with more depressive-like behavior in week 2 than in week 1, but no effect of genotype or treatment (Figures 6H, I).

3.7 Circadian body temperatures of VLP- and vehicle-treated E2, E3, and E4 mice

The circadian body temperatures during the first week of behavioral testing are illustrated in Figure 7. Based on the pattern of the data observed, the light and dark periods were analyzed as separate analyses, with the mean body temperature in the light or dark period of each day as the repeated measure.

During the light periods, there was only an effect of day [$F(2.771,110.855) = 22.958$, $p < 0.001$, Greenhouse–Geisser correction]. However, during the dark periods, there was an effect of day [$F(2.495,99.780) = 22.958$, $p = 0.005$, Greenhouse–Geisser correction] and a day × genotype × treatment interaction [$F(4.989,99.780) = 2.518$, $p = 0.034$, Greenhouse–Geisser correction], with the E2 mice more affected by VLP treatment than E3 or E4 mice. In general, in E2 mice (Figure 7A), body temperatures were lower in VLP- than in vehicle-treated mice, with the most profound effect seen in the dark period on day 4. In contrast, in E3 (Figure 7B) and E4 (Figure 7C) mice, a more subtle higher body temperature in VLP- than in vehicle-treated mice was seen.

Based on this three-way interaction, we also analyzed each treatment group separately. In the vehicle treatment group, no significant effects or trends were seen. However, in the VLP treatment group, there was a trend towards an effect of day [$F(3,57) = 2.668$, $p = 0.056$] and a trend towards a day × genotype interaction [$F(6,57) = 2.159$, $p = 0.060$].

We also analyzed each light and dark period separately, using the hour as the repeated measure.

During the light period, on day 1, there was only an effect of hour [$F(5.238,183.317) = 22.958$, $p < 0.001$, Greenhouse–Geisser correction]. However, during the light period on day 2, there was an effect of hour [$F(6.385,197.920) = 22.958$, $p < 0.001$, Greenhouse–Geisser correction] and a hour × genotype interaction [$F(12.769,183.317) = 1.970$, $p = 0.026$, Greenhouse–Geisser correction]. During the light period on day 3, there was only an effect of hour [$F(3.501,108.519) = 8.609$, $p < 0.001$, Greenhouse–Geisser correction]. During the light period on day 4, there was an effect of hour [$F(4.920,142.685) = 13.965$, $p < 0.001$, Greenhouse–Geisser correction] and a trend towards an hour × genotype interaction [$F(9.840,142.685) = 1.832$, $p = 0.061$, Greenhouse–Geisser correction]. During the light period on day 5, there was an effect of hour [$F(2.072,64.239) = 1.832$, $p < 0.001$, Greenhouse–Geisser correction] and a trend towards an effect of genotype [$F(2,31) = 3.120$, $p = 0.058$].

During the dark period, on day 1, there was only an effect of hour [$F(6.971,223.069) = 5.847$, $p < 0.001$, Greenhouse–Geisser correction]. During the dark period, on day 2, there was only an

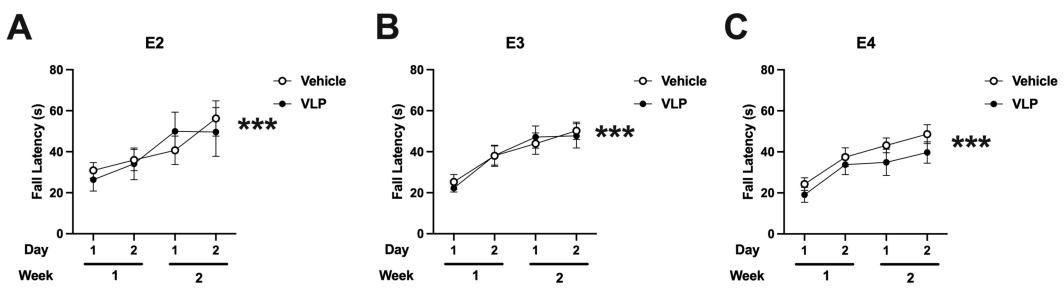

FIGURE 5
Rotarod performance of vehicle- and VLP-treated E2 **(A)**, E3 **(B)**, and E4 **(C)** mice. All groups improved their performance with training. Effect of day: ***$p < 0.001$.

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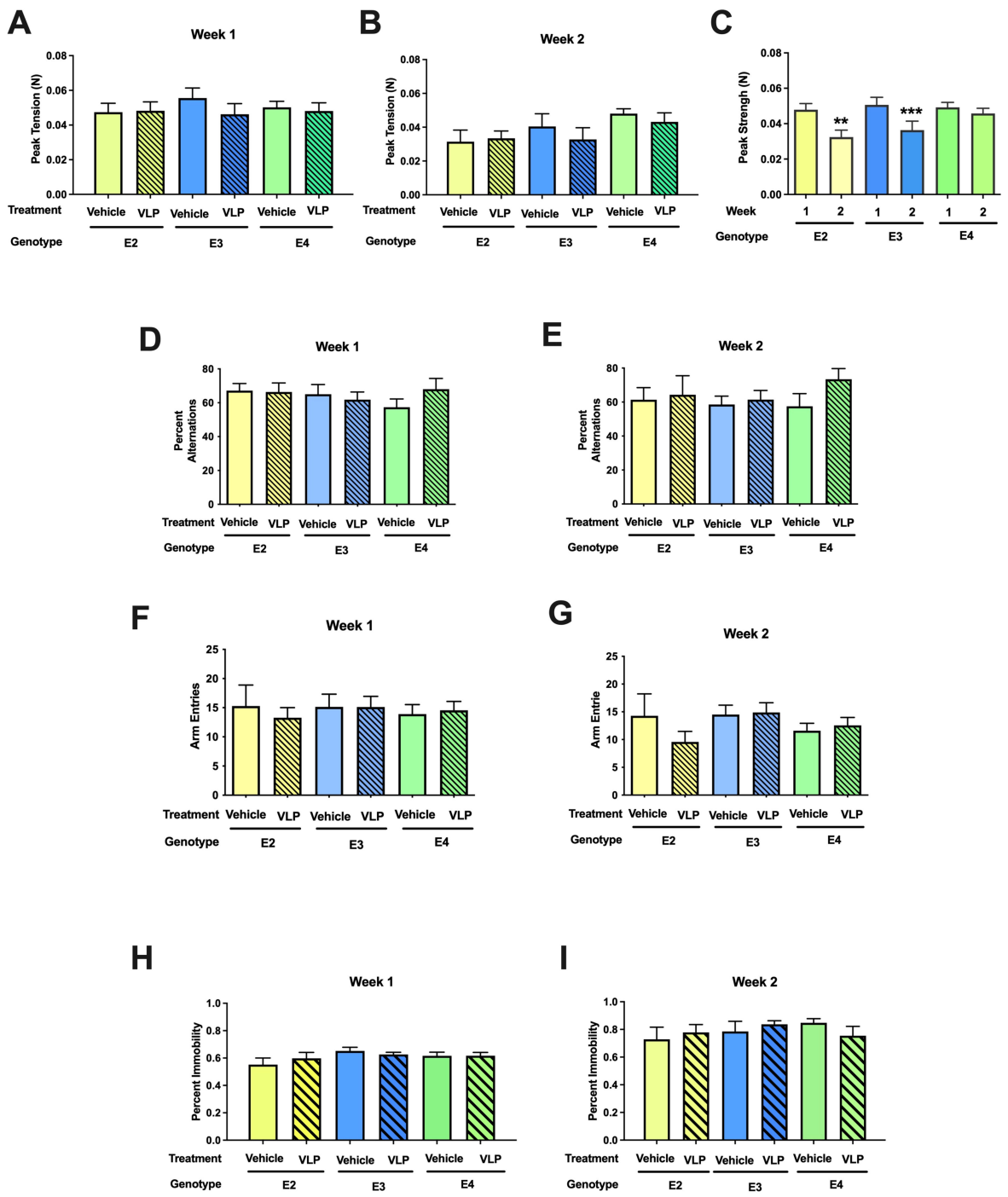

FIGURE 6
(A–C) Behavioral performance of VLP- and vehicle-treated E2, E3, and E4 mice in the grip strength test. There was a week × genotype [$F(2,44) = 4.747$, $p = 0.014$] interaction. In E2 and E3 mice, the grip strength in week 2 was lower than that in week 1 in E2. $^{**}p = 0.0036$, $^{***}p = 0.0009$, paired t-tests. This was not seen in E4 mice. **(D, E)** Spontaneous alternation of VLP- and vehicle-treated E2, E3, and E4 mice in the Y maze. **(F, G)** Arm entries of VLP- and vehicle-treated E2, E3, and E4 mice in the Y maze. **(H, I)** Depressive-like behavior of VLP- and vehicle-treated E2, E3, and E4 mice in the forced swim test.

effect of hour [$F(6.323,227.621) = 5.378$, $p < 0.001$, Greenhouse–Geisser correction]. During the dark period, on day 3, there was only an effect of hour [$F(7.293,269.855) = 9.508$, $p < 0.001$, Greenhouse–Geisser correction]. During the dark period, on day 4, there was an effect of hour [$F(5.745,172.353) = 7.596$, $p < 0.001$, Greenhouse–Geisser correction].

3.8 PCA

Nine factors were identified with eigenvalues < 1.0 and that explained a total of 81.3% of the variance among the behavioral measures (Table 2). Distance in the open field on 7 out of 8 days, distance moved in the center of the open field in 6 out of 8 days,

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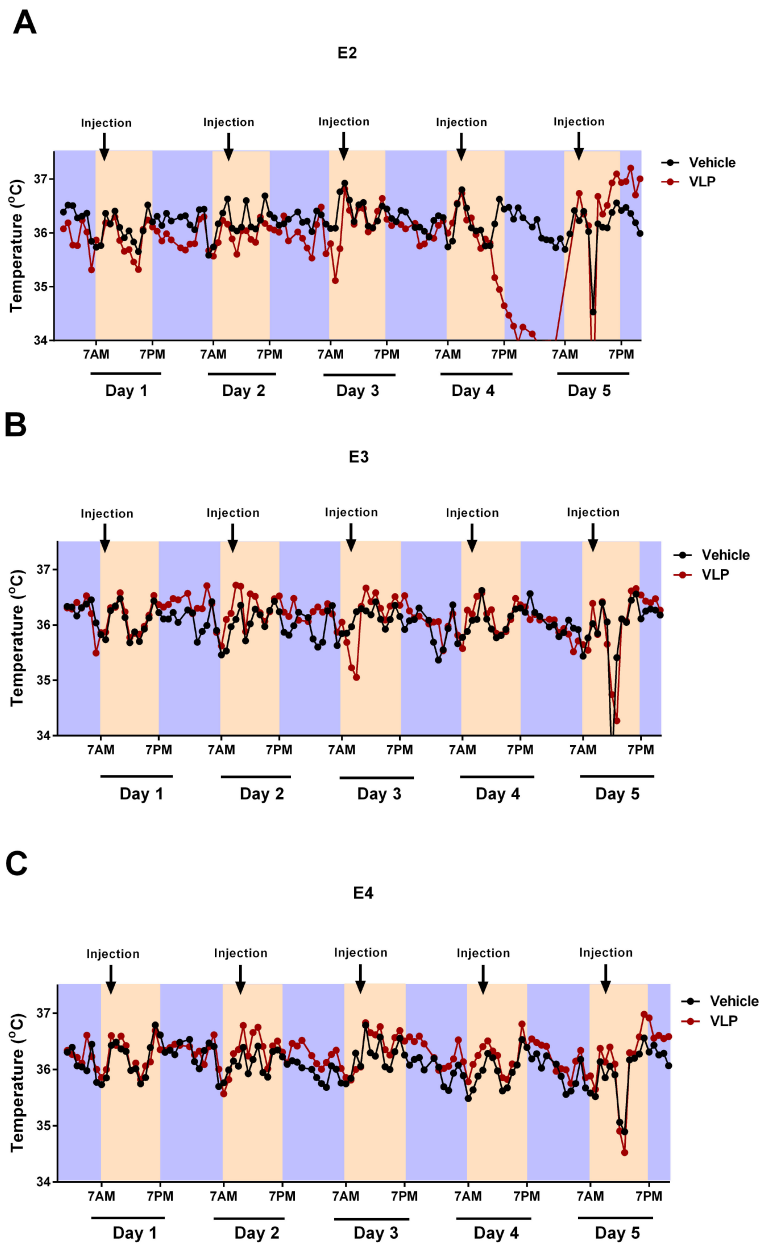

FIGURE 7
Circadian body temperatures in VLP- and vehicle-treated E2 **(A)**, E3 **(B)**, and E4 **(C)** mice. Black indicates the Vehicle groups and red the VLP groups. The dark periods are indicated in purple, the light periods in peach.

entries and spontaneous alternation in the Y maze in week 2, and the body weight ratio in week 2 all loaded on Factor 1, indicating a common underlying ability being assessed by all these behavioral measures. The directions of the component loadings in Factor 1 were such that increasing values of the factor indicate higher activity measures and increased cognitive performance in the Y maze and an increased body weight ratio in week 2.

Distance moved in the center of the open field in 6 out of 8 days, entries in Y maze in week 2, and fall latency on all 4 days in the rotarod test all loaded on Factor 2. The directions of the component loading in Factor 2 were such that reduced activity levels in the center of the open field and in week 2 and increased activity levels in the Y maze in week 2 indicate increased sensorimotor performance in the rotarod test.

Distance in the open field in 3 out of 4 days in the open field in week 2 and percent immobility in the forced swim test in week 2 loaded on Factor 3. The directions of the component loading in Factor 3 were such that decreased activity in the open field in week 2 indicates increased depressive-like behavior in the forced swim test in week 2.

Performance on all day of the rotarod test exclusively loaded on Factor 4.

Entries and spontaneous alternation in the Y maze in week 1 and grip strengths in weeks 1 and 2 loaded on Factor 5. The direction of the component loading was such that increased activity levels and increased cognitive performance in the Y maze in week 1 indicate increased grip strength in both weeks.

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TABLE 2 Component loadings of behavioral measures in the PCA[1].

	F1	F2	F3	F4	F5	F6	F7	F8	F9
TDOFD1w1	**0.732**								
TDOFD2w1	**0.799**								
TDOFD3w1	**0.825**								
TDOFD4w1	**0.772**								
TDOFD1w2	**0.844**								
TDOFD2w2	**0.526**		**−0.758**						
TDOFD3w2			**−0.841**						
TDOFD4w2	0.490		**−0.811**						
CDOFD1w1						**0.561**	0.451		
CDOFD2w1						0.480			
CDOFD3w1	**0.597**	−0.406							
CDOFD4w1	**0.685**	−0.470							
CDOFD1w2	**0.525**	**−0.601**							
CDOFD2w2	**0.514**	**−0.595**							
CDOFD3w2	0.434	**−0.733**							
CDOFD3w2	**0.620**	−0.481							
DIw1									
DIw2							**0.632**		**0.501**
Immobility FSTw1								**0.575**	0.414
Immobility FSTw2			**0.684**						
Entries Ymazew1					**0.593**				
SpontAlternationw1					**0.693**				
Entries Ymazew2	**0.516**	**0.509**							
SpontAlternationw2	0.476								
GripStrengthw1					0.470				0.426
GripStrengthw2					0.401			**−0.512**	
RRD1w1		0.474		**0.694**					
RRD2w1		0.481		**0.556**					
RRD1w2		0.463		**0.766**					
RRD2w2		**0.597**		**0.561**					
BWRatiow1						**0.547**	−0.471		
BWRatiow2	0.430					**0.566**	−0.401		
Eigenvalues	7.180	4.441	3.599	2.652	2.311	1.999	1.499	1.170	1.157
Percentage of variance explained	16.2	14.5	12.1	9.4	7.8	6.1	6.0	5.4	4.0

[1]Loadings higher than 0.5 are indicated in bold. F indicates the factor.

Activity levels in the center of the open field days 1 and 2 in week 1 and body weight ratios in both weeks loaded on Factor 6. The direction of the component loading was such that increased activity levels in the center of the open field in week 1 indicate increased body ratios in both weeks.

The activity levels in the center of the open field on day 1 of open field testing in week 1, the discrimination index in the object recognition test in week 2, and body weight ratios in both weeks loaded on Factor 7. The direction of the component loading was such that increased activity in the center of the open field in the first day of

open field testing in week 1 indicate better cognitive performance in the object recognition test but lower body weight ratios in both weeks.

Depressive-like behavior in the forced swim test in week 1 and grip strength in week 2 loaded on Factor 8. The direction of the component loading was such that increased depressive-like behavior in the forced swim test indicate reduced grip strength in week 2.

The discrimination index in the object recognition test in week 2, depressive-like behavior in the forced swim test in week 1, and grip strength in week 1 loaded on Factor 9. The direction of the component loadings was such that more depressive-like behavior in the forced swim test in week 1 and more grip strength in week 1 indicate better cognitive performance in the object recognition test in week 2.

3.9 Hippocampal cytokine expression of VLP- and vehicle-treated E2, E3, and E4 mice

Acute SARS-CoV-2 pathology is largely driven by inflammatory factors that mediate damage to the lungs. Notably, longer-term cognitive sequelae ("brain fog") are also associated with dysregulated cytokine expression in serum and CSF (24). We examined mRNA expression of several inflammatory cytokines in the hippocampi of VLP/saline-treated mice. Mice were euthanized after the last behavioral test (on the day of the last VLP injection). RNA isolated from hippocampi was analyzed by qRT-PCR for expression of the inflammatory mediators TNF-α, IFN-γ, and IL-4. We also analyzed expression of CCL11, which has been implicated in cognitive dysfunction post-COVID in experimentally infected mice and human patients (24) (Figure 8). Although no significant changes were observed for TNF-α, IFN-γ, and IL-4, CCL11 expression was elevated in VLP-treated animals of the E2 genotype. Thus, expression of TNF-α, which was reported to be induced in the brains of SARS-CoV-2-infected mice at 7 dpi, but not at 7 weeks (24), was not significantly changed in any genotype by VLP treatment.

4 Discussion

A summary of the effects of VLPs and trends towards effects of VLPs is indicated in Table 3. The genotype × treatment interaction

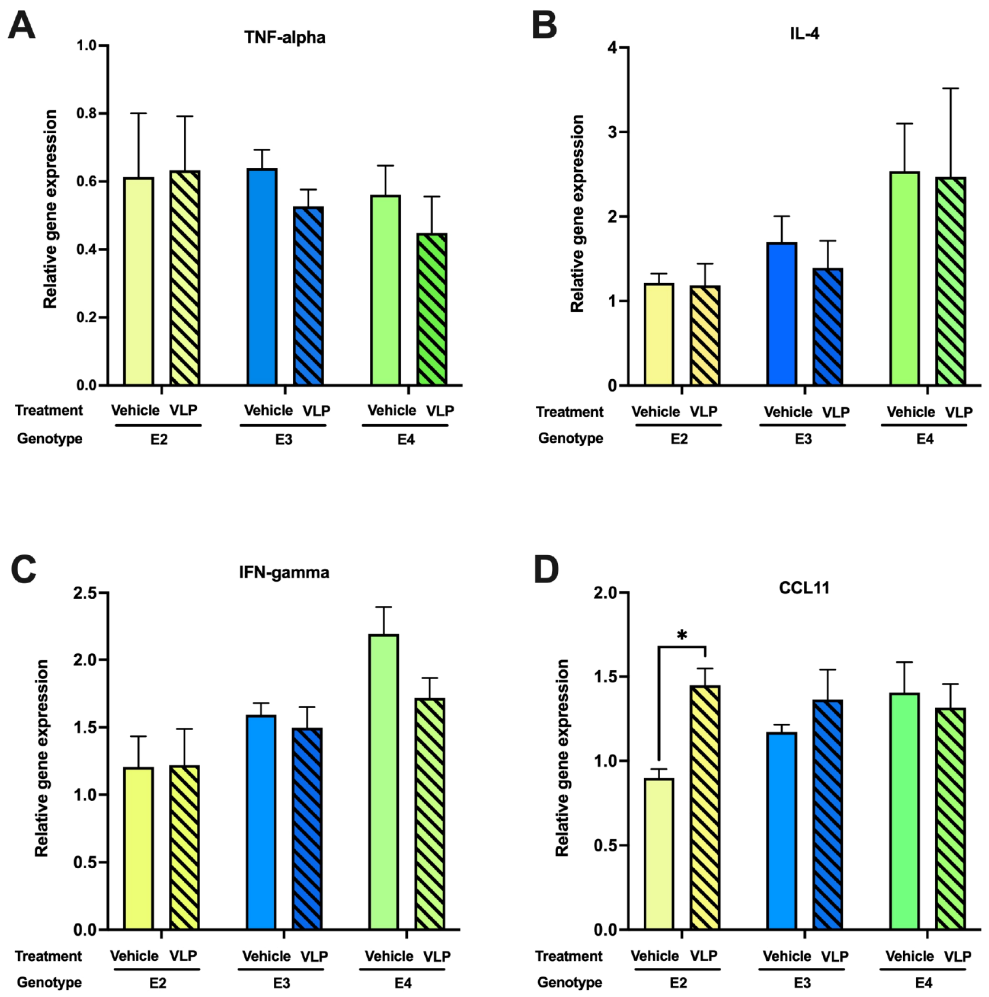

FIGURE 8
Hippocampal cytokine expression in VLP- and vehicle-treated mice. In E2 mice, **(A)** There were no genotype or treatment differences in TNF-alpha expression levels. **(B)** There were no genotype or treatment differences in IL-4 expression levels. **(C)** There were no genotype or treatment differences in IFN-gamma expression levels. **(D)** CCL11 mRNA expression was higher in VLP- than in vehicle-treated mice. *$p < 0.05$, *t*-test. $n = 3–5$/genotype/treatment.

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TABLE 3 Summary of effects and trends towards effects of VLPs[1].

Measure	E2	E3	E4	Overall
Activity levels in the open field without objects in week 2	Trend of a treatment effect: VLP < Vehicle			Genotype and genotype × treatment interaction
Activity levels in the open field with objects in week 2				Effect of treatment: VLP < Vehicle
Object recognition in week 2		Impaired object recognition in VLP-treated mice		
Circadian body temperatures during the dark period in week 1	VLP < Vehicle, most pronounced in the dark period of day 4	VLP > Vehicle	VLP > Vehicle	A day × genotype × treatment interaction: E2 mice more affected by VLPs than E3 or E4 mice

[1]The significant effects are indicated in orange.

seen for activity levels in the open field containing objects in week 2 and the day × genotype × treatment interaction observed for circadian body temperatures in week 1 indicate that for some behavioral measures, the effects of VLPs are apoE isoform-dependent, with the E2 mice being more affected than E3 or E4 mice. The overall decreased activity in the open field containing objects in week 2 indicates that VLPs can also reduce activity levels in an apoE isoform-independent fashion. The fact that effects of VLPs on activity levels in the open field in the absence and presence of objects were seen in week 2 but not in week 1 suggests that these effects might have been due to cumulative effects of VLPs, including administration to VLPs in week 1. The day × genotype × treatment interaction observed for circadian body temperature in week 1 is also consistent with the effects of cumulative exposure; the most pronounced effects of VLPs on body temperature in E2 mice were seen during the dark period on day 4, following the fourth administration of VLPs. The reduced body temperature in E2 mice is consistent with the reduced body temperature of K18-hACE2 transgenic mice that showed more severe disease, weight loss, decreased body temperature, and increased mortality following infection with a low dose of SARS-CoV-2 (32).

Compared to vehicle treatment, VLP treatment reduced activity levels in the open field containing objects in week 2. The directions of the component loadings in Factor 1 of the PCA were such that higher activity measures in both weeks indicate increased cognitive performance in the Y maze and an increased body weight ratio in week 2. The directions of the component loading in Factor 3 were such that decreased activity in the open field in week 2 indicate increased depressive-like behavior in the forced swim test in week 2. The direction of the component loading for Factor 5 was such that increased activity levels and increased cognitive performance in the Y maze in week 1 indicate increased grip strength in both weeks. The direction of the component loading for Factor 6 was such that increased activity levels in the center of the open field in week 1 indicate increased body ratios in both weeks. These data indicate that reduced activity levels might contribute to reduced cognitive performance, increased depressive-like behavior, and a reduced body weight ratio. This pattern is consistent with the beneficial effects of activity on cognitive performance (33) and reduced depression (34).

ApoE is involved in the pathogenesis and susceptibility to other infectious diseases, including herpes simplex virus-1, hepatitis C virus, hepatitis E virus, varicella zoster virus, Epstein–Barr virus, malaria, *L. monocytogenes* (LM), and *K. pneumoniae* (2). Compared to E3, E4 is proposed to be a risk factor for COVID-19 and other viruses. E4 modifies the associations of the angiotensin-converting enzyme (ACE) polymorphisms with neuropsychiatric syndromes in AD (4). E4 is also associated with enhanced entry of human immunodeficiency virus 1 (HIV-1) cell entry and HIV-1 disease progression (1). ApoE is an HIV-1-inducible inhibitor of viral production and infectivity in macrophages (2). Consistent with this pattern, the *APOE* genotype was associated with survival in patients infected with COVID-19 and part of the UK Biobank (5); E4 homozygote carriers showed poorer survival than E3 homozygote carriers. A trend towards lower survival of E2 homozygote carriers than E3 homozygote carriers was seen, but this did not reach significance. In a Finish Biobank study, E4 was associated with severe COVID-19 with more prevalent microhemorrhages in intensive care patients (6). It should be noted that in this study potential effects of E2 were not assessed.

Consistent with the human studies, in a mouse model of herpes simplex virus 1 (HSV-1), the cerebral load of latent HSV-1 genomic copies, which is associated with the reactivation risk (35), was 10-fold higher in E4 than E3 TR mice (36). In human apoE TR mice infected with mouse-adaptive COVID-19, both E2 and E4 mice showed a faster disease progression, increased viral loads and suppressive adaptive immune responses earlier after infection, and poorer survival than E3 mice. *In vitro*, viral infection was also higher in E2 and E4 than in E3 mice (5). In this study, E4 mice showed the most profound weight loss, with the E2 mice being less affected, as compared to E3 mice (5). In contrast to these viral studies, following VLP treatment, there was a trend towards a week × genotype × treatment interaction in E2, but not in E3 or E4 mice, and there was a trend towards an effect of treatment and a trend towards a lower body weight ratio in VLP- than in vehicle-treated E2 mice. The differences in pattern seen in relative susceptibility following mouse-adaptive viral COVID-19 infection versus following VLPs in E2 and E4 mice, compared to E3 mice, might be due to *APOE* genotype differences in viral replication. While it is an advantage to be able to perform COVID-19-related studies without replicating virus and outside a BSL-3 facility, we recognize that more severe effects and more profound apoE

isoform-dependent effects would likely be seen following mouse-adaptive COVID-19 viral inoculation.

In mice most affected by VLPs, E2 mice, hippocampal CCL11 levels were increased. Consistent with these data, long-term cognitive sequelae ("brain fog") are also associated with increased CCL11 levels in serum and CSF (24). Elevated CCL11 levels are seen in COVID-19 patients (37) and associated with aging and dementia, learning and memory impairments, and reduced neurogenesis (38). Interestingly, elevated CCL11 levels were also seen in a parabiosis study giving young blood to aged mice and *vice versa* and associated with learning and memory impairments when CCL11 was administered to younger mice (39).

Following exposure to COVID-19, men were more likely to be hospitalized, admitted to intensive care units, have a greater inflammatory cytokine production and antiviral antibody levels, and die (40, 41). Consistent with these human data, male Syrian hamsters showed more severe lung injury, a slower recovery, a greater percent body weight loss, and a reduced antibody response following inoculation with SARS-CoV-2/USA-WA1/2020, although viral titers in respiratory tissues and cytokine levels in pulmonary tissues were comparable in males and females (42). In addition, male K18-hACE2 transgenic mice showed more severe disease, weight loss, decreased body temperature, and increased mortality following infection with a low dose of SARS-CoV-2 (32). As no treatment × sex interactions were observed for any outcome measure, female and male data were not analyzed separately. However, a limitation of the current study is that we cannot exclude that increasing the sample of mice might have revealed a treatment × sex interaction. Alternatively, it is conceivable that viral replication is required to detect a treatment × sex interaction. Future efforts are warranted to consider using live-attenuated viral models in the absence and potentially in the presence of VLPs to compare their impacts on behavioral and cognitive performance.

A limitation of the current study design is that mice were behaviorally tested for 2 weeks. It is conceivable that in addition to the daily treatment injections, the behavioral testing itself might have contributed to fatigue in the mice. For example, activity levels in the open field and grip strength were lower in week 2 than in week 1. A study design with treatments for 2 weeks but only behavioral testing in the second week would allow addressing this.

Our VLPs include expression of the SARS-CoV-2 nucleocapsid (N), membrane (M), and envelope (E) structural proteins together with S. The results of the current study indicate that even in the absence of viral replication, detrimental effects on behavioral measures and circadian body temperatures are seen. Efforts are warranted to assess the pathways underlying these effects and to assess whether these effects are long-lasting and might model long COVID in humans. In addition to modeling COVID-19, this model is also relevant to assess the effects of using VLPs as immunization against COVID-19 on the brain. For example, it has recently been hypothesized that the inability to make long-lived plasma cells following COVID-19 vaccination might be related to the larger spacing of S1 molecules than required to bind and fully activate a single B-cell receptor, and there is therefore increased interest in using VLPs for vaccination against COVID-19 (43) (44).

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 Oregon Health & Science University IACUC. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AO'N: Formal analysis, Investigation, Visualization, Writing – review & editing. AP: Formal analysis, Investigation, Visualization, Writing – review & editing. ES: Formal analysis, Investigation, Visualization, Writing – review & editing. KN: Formal analysis, Investigation, Visualization, Writing – review & editing. MP: Formal analysis, Visualization, Writing – review & editing. MC: Formal analysis, Visualization, Writing – review & editing. PS: Formal analysis, Visualization, Writing – review & editing. EY: Formal analysis, Visualization, Writing – review & editing. HH: Investigation, Writing – review & editing. SB: Investigation, Writing – review & editing. AH: Conceptualization, Funding acquisition, Investigation, Resources, Writing – review & editing. JR: Conceptualization, Formal analysis, Funding acquisition, Investigation, Supervision, 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.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

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A blood-based mRNA signature distinguishes people with Long COVID from recovered individuals

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Introduction: Long COVID is a debilitating condition that lasts for more than three months post-infection by SARS–CoV–2. On average, one in ten individuals infected with SARS CoV- 2 develops Long COVID worldwide. A knowledge gap exists in our understanding of the mechanisms, genetic risk factors, and biomarkers that could be associated with Long COVID.

Methods: In this pilot study we used RNA-Seq to quantify the transcriptomes of peripheral blood mononuclear cells isolated from COVID-recovered individuals, seven with and seven without Long COVID symptoms (age- and sex-matched individuals), on average 6 months after infection.

Results: Seventy genes were identified as significantly up- or down-regulated in Long COVID samples, and the vast majority were downregulated. The most significantly up- or downregulated genes fell into two main categories, either associated with cell survival or with inflammation. This included genes such as *ICOS* (FDR p = 0.024) and *S1PR1* (FDR p = 0.019) that were both up-regulated, indicating that a pro-inflammatory state is sustained in Long COVID PBMCs compared with COVID recovered PBMCs. Functional enrichment analysis identified that immune-related functions were expectedly predominant among the up- or down-regulated genes. The most frequently downregulated genes in significantly altered functional categories were two leukocyte immunoglobulin like receptors *LILRB1* (FDR p = 0.005) and *LILRB2* (FDR p = 0.027). PCA analysis demonstrated that *LILRB1* and *LILRB2* expression discriminated all of the Long COVID samples from COVID recovered samples.

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Discussion: Downregulation of these inhibitory receptors similarly indicates a sustained pro-inflammatory state in Long COVID PBMCs. *LILRB1* and *LILRB2* should be validated as prospective biomarkers of Long COVID in larger cohorts, over time and against clinically overlapping conditions.

KEYWORDS

COVID-19, Long COVID, biomarker, transcriptomics, inflammation, LILRB1, LILRB2

1 Introduction

Millions of individuals are suffering from ongoing or new symptoms weeks, months or years after initial infection with SARS-CoV-2 (1). This condition is most often called Long COVID, also referred to as post-acute sequelae of SARS-CoV-2 infection (2). Many studies have now been conducted to measure the prevalence rates of Long COVID, varying widely from 9-83% of people who had been infected with SARS-CoV-2 (3–5). This large variation can be attributed to differences in clinical definition, methodology, vaccination status and the severity of acute infection. Conservative estimates suggest that one in ten people previously infected with SARS-CoV-2 have developed Long COVID (6). Long COVID has been defined by the World Health Organisation (WHO) as the occurrence of new or persistent symptoms three months after a SARS-CoV-2 infection, which persists for at least two months and cannot be explained by an alternative diagnosis (7). There have been over two-hundred symptoms reported which affect most body systems (8). The most common symptoms reported are fatigue (prevalence rate of 21.6%), respiratory problems (14.9%), cognitive impairment (10.1%) and joint/muscle pain (10.6%) (8). The underlying molecular mechanisms responsible for progression to chronicity following the initial infection are not known. Several theories focusing on immune dysfunction have been proposed, relating to viral persistence, reactivation of latent viruses, increased production of autoantibodies and persistent inflammation (6).

Despite the high occurrence rate of Long COVID there are no identified risk loci, diagnostic tests, treatments, or specific clinical biomarkers. This adds a layer of subjectivity and exclusionary process to Long COVID diagnosis that may delay or confound effective clinical management. It also poses a challenge in the recruitment of stringently diagnosed cohorts for research. Considering these issues, many studies have been undertaken which seek biomarkers of Long COVID. These efforts have mostly focused on attempting to identify immunological, neurological, vascular and cardiac signatures specific to the disease. Several studies have reported circulating inflammatory marker proteins to associate with Long COVID disease status, with this body of work strongly indicating a sustained pro-inflammatory state in at least a subset of affected individuals (9–16).

One study examined the immunological profile of Long COVID-affected individuals compared to COVID recovered individuals with varying severity of acute illness. Long COVID-affected individuals were characterized by increased IFN-γ and IL-2, indicative of a pro-inflammatory state and decreased CCL4 production. The authors propose that while the increased IFN-γ and IL-2 results in activation of effector T cells, the concomitantly reduced CCL4 leads to impaired recruitment of them to infected sites (17).

An Australian longitudinal study investigated the transcriptional and immunological blood profile of sixty-nine COVID recovered patients of varying severities, including twenty-one individuals referred to a Long COVID clinic (18). The study found that transcriptional changes which occurred during acute infection were still evident in recovered individuals for at least six months post-infection. This highlights the importance of including COVID recovered samples as controls in new studies. Crucially this study also reported that enriched immune signatures identified in whole blood transcriptomics withstood correction against the differential proportions of cell types in each PBMC sample, thereby demonstrating that transcriptomic assessment of heterogeneous PBMCs in COVID recovered samples is robust against individualized changes in cell type proportions and thus can be applied with confidence in future studies.

Measuring levels of specific mRNA transcripts from blood has high diagnostic potential since blood is readily accessible and the transcripts are measurable by existing Real-Time Quantitative Reverse Transcription PCR (qRT-PCR) infrastructure. This benefit would be accentuated if the number of transcripts required to indicate disease status is few. PBMCs are very easily isolated from whole blood and since, taken together, the prior studies strongly suggest persistent immunological disturbances (including robust changes to PBMCs), PBMC mRNA transcripts as potential discriminators of disease status are a promising avenue.

With this in mind, we undertook a small pilot study to test the effectiveness of using differences in PBMC gene expression to identify candidate biomarkers of Long COVID. We used RNA-Seq to sequence and quantify the transcriptomes of PBMCs isolated from seven Long COVID and seven COVID recovered age and sex matched individuals. The Long COVID participants had been infected with SARS-CoV-2 on average six months prior to sample collection. Functional enrichment analysis identified that immune-related

functions were unsurprisingly predominant among the up- and down-regulated genes. The differentially expressed genes supported a persistent or chronic inflammatory state in Long COVID. Using multivariate-based signature discovery, we identified a robust blood-based transcriptomic signature that effectively distinguished patients who have fully recovered from SARS-CoV-2 (COVID recovered) from those experiencing Long COVID using the reduced expression levels of only two genes (*LILRB1* and *LILRB2*). These results if validated in a larger cohort, over time and against similar conditions show promise for development into a simple blood based diagnostic tool for Long COVID.

2 Materials and methods

2.1 Recruitment, sampling and cohort characteristics

All participants were recruited in accordance with La Trobe University Human Ethics approvals HEC21207 and HEC21907. Seven Long COVID and seven COVID recovered participants were recruited for the study and all participants provided informed consent. Long COVID participants were defined according to the WHO description as exhibiting new or persistent symptoms three months following SARS-CoV-2 infection. Participants were asked to complete a symptom questionnaire and rate their illness severity using a five-point Likert scale. The cohort characteristics are shown in Table 1. COVID recovered participants were age- and sex-matched to the Long COVID cohort and on average all participants in this study were recruited approximately 6 months after an acute SARS-CoV2 infection. To exclude known, measurable alternative explanations for fatigue, blood samples collected from Long COVID participants were subject to pathology analysis conducted by Dorevitch Pathology, Melbourne, Australia. Blood samples for subsequent PBMC isolation were collected in lithium-heparin tubes.

2.2 PBMC isolation

PBMCs were isolated as previously published (19). Briefly, whole blood was separated by centrifugation in SepMate tubes (STEMCELL Technologies) at 1200×G for 10 minutes at RT. Resultant PBMCs were washed with RPMI 1640 to remove residual Ficoll. Aliquots were frozen in fetal bovine serum with 10% DMSO, gradually at -80°C.

2.3 RNA extraction and RNA-seq

RNA was extracted from PBMCs using the Monarch Total RNA Miniprep Kit (NEB, Ipswich, MA, USA). Approximately 5×10^6 PBMCs were harvested by centrifugation at 500 × g for 5 min and resuspended in 300 µL of RNA lysis buffer. The sample was transferred to a gDNA removal column fitted to a microcentrifuge tube and spun at maximum speed for 30 s to collect any contaminating genomic DNA. The RNA in the flow-through was precipitated by the addition of 300 µL ethanol (Chem Supply, Port Adelaide, SA, Australia) and mixed by repeated pipetting. The suspension was transferred to an RNA purification column fitted to a collection tube and again spun at max speed for 30 s and the flow-through discarded. The RNA was washed by the addition of 500 µL RNA wash buffer and centrifugation, as described previously, and the flow-through was discarded. The sample was DNase-treated by the addition of 5 µL DNase I and 75 µL of DNase I reaction buffer to the column matrix and incubated at RT for 15 min. A 500 µL volume of RNA priming buffer was added to the column, centrifuged for 30 s at max speed, and the flow-through was discarded. The RNA in the column was washed twice with 500 µL of RNA wash buffer, followed by centrifugation as described previously. RNA was eluted by the addition of 50 µL nuclease-free water and centrifugation at max speed for 30 s. RNA was then stored at -80°C before being sent to Novogene, Singapore on dry ice for mRNA sequencing and quantification using the Illumina NovaSeq 6000 platform and paired-end 150 bp reads.

2.4 Transcriptomic data analysis, enrichment analysis, and signature discovery

Transcriptomic profiling of PBMC samples from RNA-Seq Long COVID patients (n = 7) and COVID recovered individuals (n = 7) was performed by RNA-Seq (Illumina, paired end reads, 150bp). Quality control of the generated reads, mapping, and selection of genes with significant differential expression was carried out using CLC Genomics Workbench package 22 (QIAGEN) (20), HiSAT2 (21), and edgeR (22). Human genome 38 and its annotations (GRCh38) were downloaded from Ensembl genome browser (https://www.ensembl.org/index.html) and used for mapping and expression profiling. Mapping was carried out with the following parameters: mismatch cost of 2, insertion cost of 3, deletion cost of 3, minimum length fraction of 0.8, and minimum similarity fraction = 0.8. "Reads Per Kilobase of transcript per Million mapped reads" (RPKM) was used as the expression measurement. We used Generalized Linear Models (GLM) based on Negative Binomial distribution for differential expression analysis (22). The p-values were also corrected with false discovery rate (FDR) for multiple testing, and pFDR = 0.05 was used for selection of genes with statistically significant differential expression. The advantage of GLM is fitting the curve to expression values without the assumption that the error is normally distributed. Genes with an average RPKM lower than 4 in both cohorts were removed from the list of significant differentially expressed genes.

To find the key functions that the differentially expressed genes were involved in, enrichment analysis of Gene Ontology (GO) terms was analysed using the STRING web application tool (https://string-db.org/) (23). The significant molecular functions were selected based on pFDR = 0.05 and strength of the function, calculated by STRING (23).

Multivariate analysis, including principal component analysis (PCA) using correlation matrix and hierarchical clustering using average linkage method, was performed on expression values as previously described (24). PCA and clustering were utilized to

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TABLE 1 Participant cohort information.

Participant information	Long COVID	COVID recovered
Total Participants	7	7
Average age (years)	41	45
Age range (years)	23-53	23-63
Female (%)	85.7	71.4
Male (%)	14.3	28.6
Average time post-COVID (months)	6.1	5.5
Hospitalisation (number of participants)	1	n/a

evaluate the power of the developed transcriptomic signature as well as the functional pathways in distinguishing Long COVID from COVID recovered samples. Minitab Statistical Software 21 was employed for performing multivariate analysis.

3 Results

3.1 Long COVID participant symptoms

Seven Long COVID and seven COVID recovered participants were included in this study (Table 1). All participants had reported a SARS-CoV-2 infection on average six months prior to sample collection and pathology tests revealed no underlying conditions typically associated with fatigue. All pathology results were within the normal range. There were no significant difference in the mean age of each clinical group ($p = 0.58$, independent t-test) or gender, but both had a higher percentage of women and middle-aged participants. This is in line with the current literature that indicates a high prevalence in this age group and in women (25). Each of the seven Long COVID participants completed the symptom severity questionnaires which used a five-point Likert scale. Eighteen symptoms were included in the questionnaire and encompassed the main symptom clusters of neurocognitive, airway, cardiopulmonary, musculoskeletal and gastrointestinal issues. The most reported symptoms were fatigue, post-exertional malaise (PEM), muscle pain, concentration and memory issues, sore throat, headaches and temperature dysregulation. Fatigue was reported by each of the Long COVID participants and five out of the seven reported PEM. Fatigue and PEM were rated with the greatest overall severity of all symptoms (Figure 1).

3.2 Transcriptomic signature of long COVID

Total RNA was isolated from PBMCs of all participants and sent for RNA-Seq analysis (26). Transcriptomic analysis identified 5,144 transcripts, of which seventy were up- or down-regulated in Long COVID compared to COVID recovered controls (Figure 2). Most of the differentially expressed transcripts were downregulated (sixty-six down in total) and four were signficantly up-regulated in Long COVID compared to the COVID recovered controls (FDR p value < 0.05).

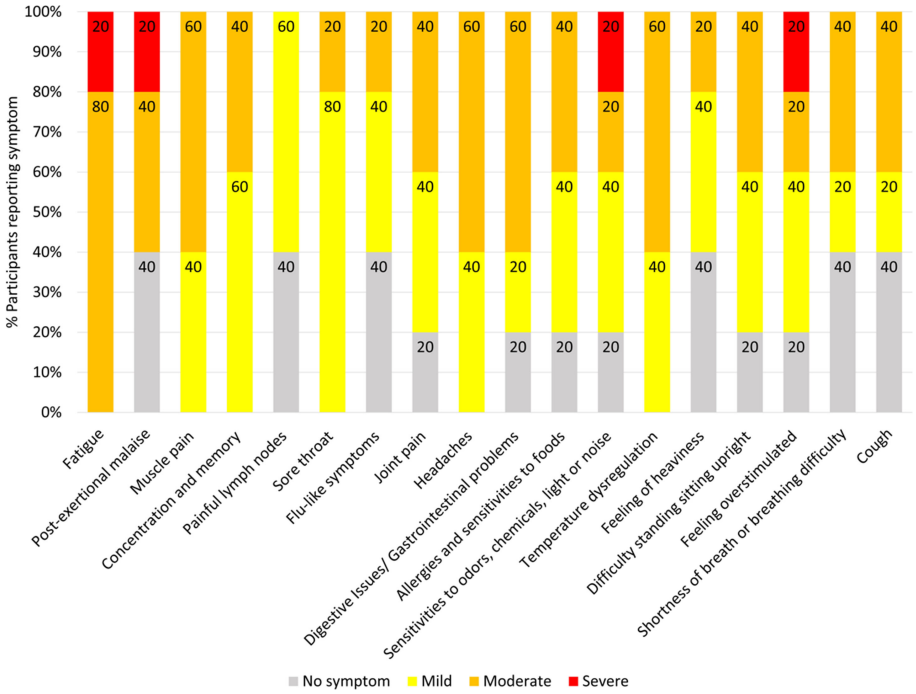

FIGURE 1
Symptoms affecting Long COVID participants and severity ratings. Seven of the participants with Long COVID completed questionnaires to list and rate the severity of their symptoms. Ratings of the symptoms were classified as 0 = no symptoms, 1-2 = mild, 3-4 = moderate, 5 = severe. Percentages are rounded to the nearest single decimal place.

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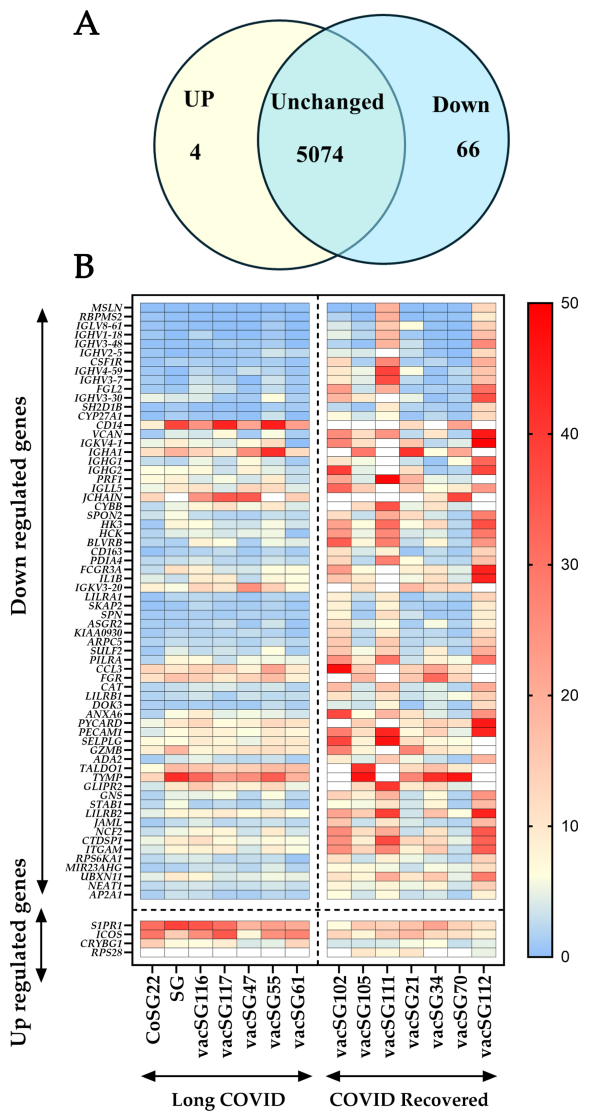

FIGURE 2
Long COVID versus COVID recovered PBMC transcriptomes. **(A)** Venn diagram depicting the number of gene transcripts significantly up- or down-regulated in PBMCs from people with Long COVID versus PBMCs from COVID recovered individuals. The p-values which determined significance were corrected with FDR for multiple testing, (pFDR < 0.05 was used as the threshold for significance). Genes with an average reads per kilobase per million mapped reads (RPKM) lower than four in both cohorts were removed from the list of significant differentially expressed genes. This was to ensure that any identified genes were present in sufficient amounts to be detected via clinically appropriate methods. **(B)** Heatmap representation of seventy differentially expressed genes between Long COVID and COVID recovered PBMC samples. Red indicates higher expression while blue indicates lower expression. Each column is a different sample and each row is a different gene.

Principal Component Analysis (PCA) of the seventy up- or down-regulated genes showed separation between Long COVID and COVID recovered samples (Figure 3) where only one COVID recovered sample was clustered in the Long COVID group. The first component was used to classify the samples and explained 81.7% of the variation in the expression data.

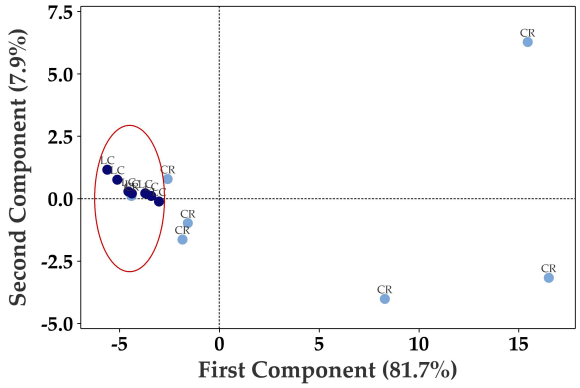

FIGURE 3
Principal component analysis (PCA) plot based on 70 differentially expressed genes in PBMCs efficiently distinguishes Long COVID (LC, red) PBMCs from most of the COVID recovered PBMCs (CR, blue) with one overlapping COVID recovered sample.

3.3 Dysregulation of immune related pathways

The list of genes with significant (pFDR < 0.05) differential expression between Long COVID and COVID recovered PBMC samples is presented in Table 2. Among the top ten downregulated genes in Long COVID as determined by fold change and FDR corrected p-value were genes associated with cell survival. This included Mesothelin (*MSLN*), expression of which was reduced more than sixty-fold. Mesothelin is a cell surface glycoprotein and is involved in cell signalling and adhesion. It is associated with various cancers and is thought to promote proliferation through activation of the NF-kB pathway. Downregulation of this gene has been connected with increased cell death via apoptosis (27). Another gene in the top ten which was associated with cell survival is colony stimulating factor receptor (CSF1R). This has been shown to be important for the survival, differentiation and proliferation of myeloid cells. The RNA binding protein, mRNA processing factor 2 (RBPMS2) was also highly downregulated and functions in post-transcriptional regulation of gene expression. Inactivation of this gene via methylation has also been associated with increased apoptosis (28).

Other genes among the top ten downregulated are associated with immune and inflammatory responses. This includes Fibrinogen-like protein 2 (FGL2), which is present in serum as a soluble protein and is a mediator of inflammation (29), and cytochrome b-245 beta chain (*CYBB*). *CYBB* encodes a subunit of the NADPH oxidase in phagocytes which is responsible for the microbicidal respiratory burst (30). *CYBB* is located in chromosome X and so may be related to the greater predisposition of females to Long COVID. Several Immunogloblin Light and Heavy Chain Variable (IGLV or IGHV) region genes were also present in the downregulated genes. All immunogloblin genes are located in chromosome fourteen, and as a group constituted the most frequently downregulated class of proteins in the Long COVID PBMCs. Seven out of the sixty-six downregulated genes belong to this class, namely *IGLV8-61*, *IGHV1-18*, *IGHV3-48*, *IGHV2-5*,

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TABLE 2 The blood-derived PBMC transcriptomic signature of Long COVID compared to COVID recovered individuals The top genes were selected by satisfying a FDR p-value < 0.05 threshold.

No	Name	Chromosome	Identifier	Fold change	FDR p-value	Long COVID
1	*MSLN*	16	ENSG00000102854	-60.251	0.005	DOWN
2	*RBPMS2*	15	ENSG00000166831	-17.944	0.005	DOWN
3	*IGLV8-61*	22	ENSG00000211638	-16.350	0.005	DOWN
4	*IGHV1-18*	14	ENSG00000211945	-9.798	0.047	DOWN
5	*IGHV3-48*	14	ENSG00000211964	-8.956	0.027	DOWN
6	*IGHV2-5*	14	ENSG00000211937	-8.055	0.031	DOWN
7	*CSF1R*	5	ENSG00000182578	-7.028	0.000	DOWN
8	*IGHV4-59*	14	ENSG00000224373	-6.375	0.009	DOWN
9	*IGHV3-7*	14	ENSG00000211938	-5.779	0.032	DOWN
10	*FGL2*	7	ENSG00000127951	-5.092	0.005	DOWN
11	*IGHV3-30*	14	ENSG00000270550	-4.992	0.023	DOWN
12	*SH2D1B*	1	ENSG00000198574	-4.900	0.011	DOWN
13	*CYP27A1*	2	ENSG00000135929	-4.607	0.032	DOWN
14	*CD14*	5	ENSG00000170458	-4.417	0.005	DOWN
15	*VCAN*	5	ENSG00000038427	-4.227	0.005	DOWN
16	*IGKV4-1*	2	ENSG00000211598	-3.987	0.038	DOWN
17	*IGHA1*	14	ENSG00000211895	-3.896	0.016	DOWN
18	*IGHG1*	14	ENSG00000211896	-3.868	0.024	DOWN
19	*IGHG2*	14	ENSG00000211893	-3.681	0.027	DOWN
20	*PRF1*	10	ENSG00000180644	-3.397	0.011	DOWN
21	*IGLL5*	22	ENSG00000254709	-3.336	0.017	DOWN
22	*JCHAIN*	4	ENSG00000132465	-3.321	0.034	DOWN
23	*CYBB*	X	ENSG00000165168	-3.262	0.015	DOWN
24	*SPON2*	4	ENSG00000159674	-3.189	0.006	DOWN
25	*HK3*	5	ENSG00000160883	-3.166	0.011	DOWN
26	*HCK*	20	ENSG00000101336	-3.104	0.027	DOWN
27	*BLVRB*	19	ENSG00000090013	-3.046	0.039	DOWN
28	*CD163*	12	ENSG00000177575	-3.040	0.027	DOWN
29	*PDIA4*	7	ENSG00000155660	-3.010	0.015	DOWN
30	*FCGR3A*	1	ENSG00000203747	-2.951	0.020	DOWN
31	*IL1B*	2	ENSG00000125538	-2.934	0.022	DOWN
32	*IGKV3-20*	2	ENSG00000239951	-2.899	0.038	DOWN
33	*LILRA1*	19	ENSG00000104974	-2.890	0.027	DOWN
34	*SKAP2*	7	ENSG00000005020	-2.867	0.046	DOWN
35	*SPN*	16	ENSG00000197471	-2.818	0.024	DOWN
36	*ASGR2*	17	ENSG00000161944	-2.724	0.027	DOWN
37	*KIAA0930*	22	ENSG00000100364	-2.687	0.020	DOWN
38	*ARPC5*	1	ENSG00000162704	-2.686	0.015	DOWN

(Continued)

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TABLE 2 Continued

No	Name	Chromosome	Identifier	Fold change	FDR p-value	Long COVID
39	*SULF2*	20	ENSG00000196562	-2.664	0.023	DOWN
40	*PILRA*	7	ENSG00000085514	-2.659	0.025	DOWN
41	*CCL3*	17	ENSG00000277632	-2.652	0.017	DOWN
42	*FGR*	1	ENSG00000000938	-2.632	0.005	DOWN
43	*CAT*	11	ENSG00000121691	-2.616	0.023	DOWN
44	*LILRB1*	19	ENSG00000104972	-2.540	0.005	DOWN
45	*DOK3*	5	ENSG00000146094	-2.526	0.045	DOWN
46	*ANXA6*	5	ENSG00000197043	-2.514	0.035	DOWN
47	*PYCARD*	16	ENSG00000103490	-2.502	0.017	DOWN
48	*PECAM1*	17	ENSG00000261371	-2.480	0.023	DOWN
49	*SELPLG*	12	ENSG00000110876	-2.479	0.031	DOWN
50	*GZMB*	14	ENSG00000100453	-2.459	0.048	DOWN
51	*ADA2*	22	ENSG00000093072	-2.457	0.027	DOWN
52	*TALDO1*	11	ENSG00000177156	-2.436	0.038	DOWN
53	*TYMP*	22	ENSG00000025708	-2.424	0.015	DOWN
54	*GLIPR2*	9	ENSG00000122694	-2.405	0.041	DOWN
55	*GNS*	12	ENSG00000135677	-2.398	0.038	DOWN
56	*STAB1*	3	ENSG00000010327	-2.380	0.023	DOWN
57	*LILRB2*	19	ENSG00000131042	-2.377	0.027	DOWN
58	*JAML*	11	ENSG00000160593	-2.376	0.032	DOWN
59	*NCF2*	1	ENSG00000116701	-2.356	0.047	DOWN
60	*CTDSP1*	2	ENSG00000144579	-2.286	0.018	DOWN
61	*ITGAM*	16	ENSG00000169896	-2.237	0.038	DOWN
62	*RPS6KA1*	1	ENSG00000117676	-2.212	0.027	DOWN
63	*MIR23AHG*	19	ENSG00000267519	-2.176	0.017	DOWN
64	*UBXN11*	1	ENSG00000158062	-2.071	0.023	DOWN
65	*NEAT1*	11	ENSG00000245532	-1.962	0.022	DOWN
66	*AP2A1*	19	ENSG00000196961	-1.951	0.043	DOWN
67	*S1PR1*	1	ENSG00000170989	2.134	0.019	UP
68	*ICOS*	2	ENSG00000163600	2.157	0.024	UP
69	*CRYBG1*	6	ENSG00000112297	2.232	0.027	UP
70	*RPS28*	19	ENSG00000233927	3.808	0.032	UP

Genes with low expression (RPKM < 4) were also filtered.

IGHV4-59, *IGHV3-7*, and *IGHV3-30*. It is important to note that IGLV8-61 is a secretory protein and is predicted to be active in the extracellular space. Integrated proteomics data analyses, based on the publicly available proteomics data in ProteomicsDB, PaxDb, and MOPED showed that at the protein level, IGLV8-61 is mainly present in urine and skin (https://www.genecards.org/cgi-bin/carddisp.pl?gene=IGLV8-61). Secretory proteins in urine and/or blood are attractive biomarker candidates.

Four genes were significantly up-regulated in the Long COVID PBMC samples and were also associated with survival or immune pathways. Two such genes, Sphingolipid phosphate receptor 1 (*S1PR1*) and inducible co-stimulator (*ICOS*) (Figure 4) are associated with inflammation and the differentiation of T cells into T helper cells which produce inflammatory cytokines. Another, the 40S small ribosomal protein 28 (RPS28) has been proposed to play a role in the presentation of Major Histocompatibility Complex (MHC) class I peptides. The

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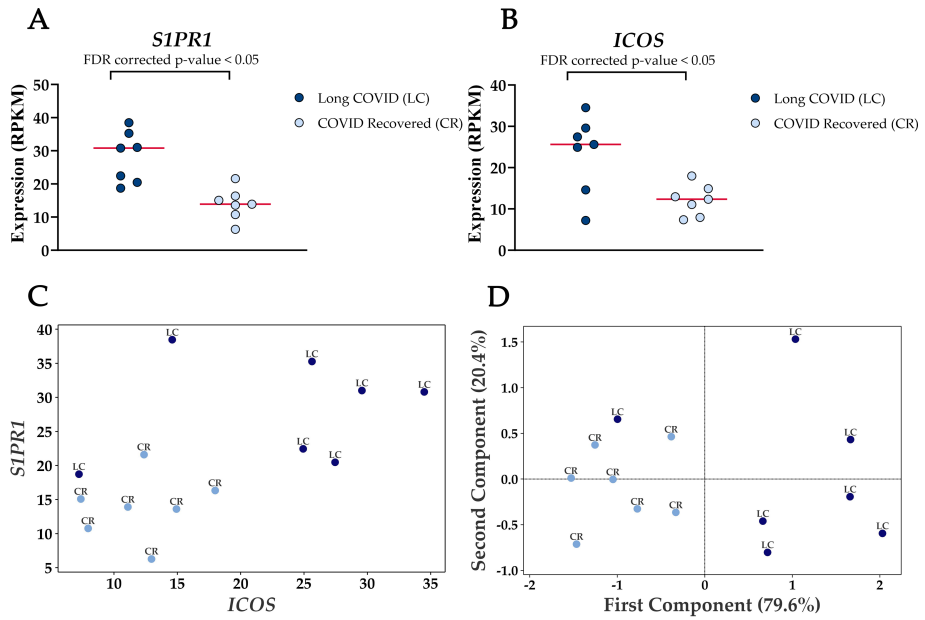

FIGURE 4
Elevated expression levels of *S1PR1* and *ICOS* in blood-derived PBMCs from Long COVID patients versus COVID recovered individuals. **(A)** Comparison of *S1PR1* expression between Long COVID and COVID recovered PBMCs. **(B)** Comparison of *ICOS* expression between Long COVID and COVID recovered PBMCs. **(C)** Scatter plot demonstrates the elevated expression of *S1PR1* and *ICOS* in Long COVID (LC) samples. **(D)** PCA analysis demonstrates that *S1PR1* and *ICOS* can discriminate all but one Long COVID (LC) sample from COVID recovered (CR), where the first component described 79.6% of variation in the expression data.

final up-regulated gene in Long COVID was crystallin beta-gamma domain containing 1 (CRYBG1) also known as Absent in Melanoma 1 (AIM1). This gene encodes an actin binding protein which acts as a tumour suppressor that is downregulated in various cancers (31).

Functional enrichment analysis was performed to further define the pathways that were differentially regulated in Long COVID PBMCs (Table 3). The "molecular function" Gene Ontology term was used to identify the molecular processes or activities enriched in the up- or down-regulated genes. Some of the molecular functions identified referred to very broad activities of signalling receptor binding, antigen binding and protein homodimerisation activity. However, the molecular functions with the highest strength were related to MHC class I molecules and immunoglobulin receptor activity. Immunoglobulin and MHC class I are essential for appropriate responses to infection and inflammation. Induction of the MHC class I pathway has been shown to be downregulated by SARS-CoV-2 infection (32) and the immunoglobulin receptor Fc receptor-like 2 has been identified as downregulated in several transcriptomic datasets from SARS-CoV-2 infected individuals (33).

The other molecular function which was enriched in the up- or down-regulated transcripts was glycosaminoglycan binding. Glycosaminoglycans (GAGs) are ubiquitously and abundantly expressed on the surface of cells or in the extracellular matrix and interact with many proteins including chemokines, cytokines and

TABLE 3 Enrichment analysis of genes that were up- or down-regulated in Long COVID compared to COVID recovered individuals in terms of molecular function.

"Molecular function" (STRING)	STRING pathway strength	pFDR	Proteins
inhibitory receptor binding to MHC class I	2.02	0.0099	LILRA1,LILRB1,LILRB2
Immunoglobulin receptor binding	1.82	0.0166	FGR,IGLL5,JCHAIN
MHC class I protein binding	1.74	0.0225	PILRA, LILRB1, LILRB2
Antigen binding	1.3	0.0374	LILRA1, IGLL5, JCHAIN, SPON2
Glycosaminoglycan binding	0.94	0.0414	GNS, VCAN, STAB1, ANXA6, SULF2, ADA2
Protein homodimerisation activity	0.77	0.0016	CAT, PYCARD, CSF1R, RBPMS2, LILRB1, JAML, GLIPR2, LILRB2, TYMP, ADA2, JCHAIN, PECAM1
Signalling receptor binding	0.63	0.00049	PILRA, SELPLG, PYCARD, FGL2, IL1B, S1PR1, LILRB1, JAML, AP2A1, FGR, HCK, LILRB2, TYMP, ADA2, IGLL5, JCHAIN, CCL3, ITGAM

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growth factors. Due to the diversity of these interactions, GAGs play important roles in countless biological functions including cell growth and proliferation, resistance to invasion by pathogens and migration of immune cells (34).

3.4 Reduced expression levels of LILRB1 and LILRB2 can effectively differentiate long COVID blood samples from those of recovered individuals

The most frequently occurring genes in the identified molecular functions were leukocyte immunoglobulin-like receptor subfamily B member 1 (*LILRB1*) and leukocyte immunoglobulin-like receptor subfamily B member 2 (*LILRB2*) (Table 3) and both genes were significantly downregulated in Long COVID (Figure 5). *LILRB1* and *LILRB2* are frequently expressed in immune cells and function largely to regulate antigen presenting cells such as macrophages, dendritic cells and B cells, thus are important in a variety of innate and adaptive immune responses (35, 36). Specifically, the gene products are involved in inhibitory responses which suppress downstream pathways to mediate immunosuppression. Downregulation of *LILRB1* and *LILRB2* as observed here in the Long COVID PBMCs is therefore indicative of a heightened immune response and inflammation.

PCA analysis demonstrates the power of *LILRB1* and *LILRB2* in completely discriminating Long COVID from COVID recovered PBMCs. The first component described 95.4% of variation in the expression data. The ability of only two genes to distinguish the two cohorts with no overlap is a promising avenue for future validation as a prospective biomarker.

4 Discussion

Utilizing PBMC transcriptomic profiling in a small cohort, this study has revealed information about the molecular mechanisms of Long COVID and has identified a prospective transcriptomic signature of the disease in PBMCs for subsequent validation.

Analysis of PBMC transcriptomes from Long COVID individuals compared to COVID recovered controls revealed that the vast majority of differentially expressed genes were downregulated in Long COVID. The most frequently downregulated family of genes in Long COVID PBMCs was Immunoglobulin Heavy Chain Variable Region (IGHV), constituting six of the top ten downregulated genes. This observation in Long COVID samples contrasts with the up-regulation of this family of genes reported in acute SARS-CoV-2 infection (33). An analysis of transcriptomic data from nine different studies that compared SARS-CoV-2 infected cohorts to either healthy controls or other respiratory diseases identified that the expression of IGHV genes was most consistently up-regulated specifically in severe SARS-CoV-2 infections (33). This is noteworthy since severe infections are the most associated with development of Long COVID. A shift from elevated IGHV expression during the acute phase to reduced IGHV expression in

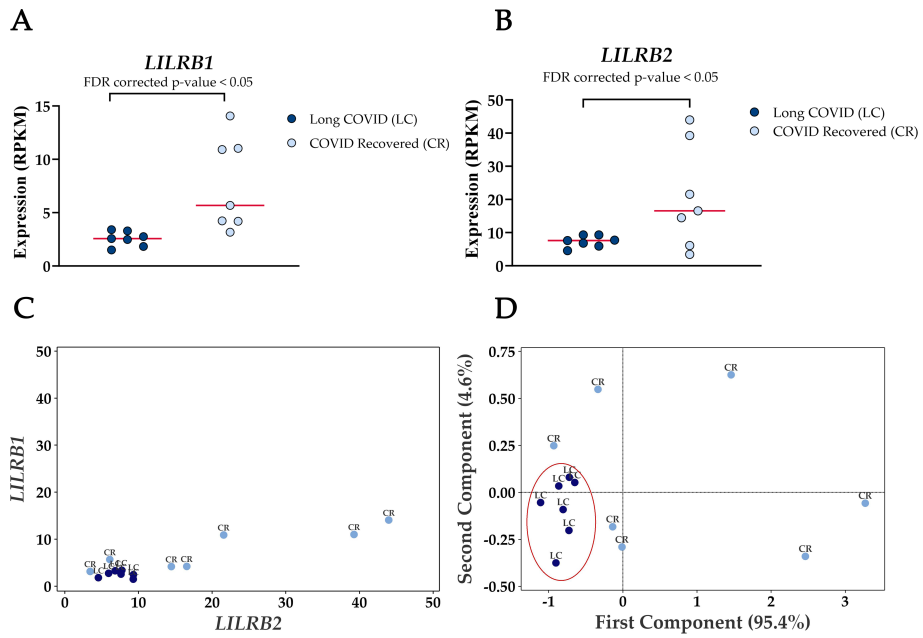

FIGURE 5
Reduced expression levels of *LILRB1* and *LILRB2* in blood-derived PBMCs effectively differentiate Long COVID patients from COVID recovered individuals. **(A)** Comparison of *LILRB1* expression between Long COVID and COVID recovered PBMCs. **(B)** Comparison of *LILRB2* expression between Long COVID and COVID recovered PBMCs. **(C)** Scatter plot demonstrates the reduced expression of *LILRB1* and *LILRB2* in Long COVID (LC) samples. **(D)** PCA analysis demonstrates the power of *LILRB1* and *LILRB2* to discriminate Long COVID (LC) samples from COVID recovered (CR) where the first component described 95.4% of variation in the expression data.

Long COVID suggests dysregulation, and perhaps overactivity of the regulatory pathways involved in bringing the immune response back down over time. Interestingly, all of these genes are located in chromosome fourteen which suggests a possible hotspot of shared regulatory control that should be investigated in future studies.

Induction of cell death pathways to decrease the numbers of activated immune cells is an important homeostatic mechanism which can backfire during severe viral infection. This depletion of lymphocytes is termed lymphopenia. The mechanisms of cell death underpinning lymphopenia are diverse. T cell lymphopenia is a feature of many respiratory viral infections and is common in SARS-CoV-2 infection, particularly severe patients (37). T cell lymphopenia has been reported to be more severe in COVID patients and takes longer to resolve than in other viral infections (38). In the current study we did observe a decrease in the expression of genes associated with cell survival. While not measured directly, we also observed that the Long COVID PBMC samples died more quickly than the COVID recovered controls once recovered from frozen storage. Together this is unsurprising given that lymphopenia has been reported to be common in Long COVID (39–41). What these observations do suggest is that the mechanism by which lymphopenia occurs in Long COVID may involve the downregulation of genes associated with cell survival that we observed.

In our data we observed a significant increase in expression of the inducible T cell co-stimulator *ICOS*. *ICOS* promotes all fundamental T cell responses to foreign antigen and is crucial in mediating inflammation (42, 43). Among many other functions, *ICOS* enhances differentiation of T cells into T helper cells which produce pro-inflammatory cytokines. Pro-inflammatory cytokines and persistent low-grade inflammation are ubiquitously reported in Long COVID, although elevated levels of pro-inflammatory cytokines have been reported in recovered individuals too (44). Our observation of elevated *ICOS* expression, on average six months after infection, confirms that a pro-inflammatory state exists in Long COVID PBMCs until at least six months and that *ICOS* levels do intuitively wane over time in COVID recovered individuals, as would also be expected of the levels of pro-inflammatory cytokines.

The lipid mediator sphingolipid phosphate (S1P) and the sphingolipid phosphate receptors (S1PR) play critical roles in immune responses. S1PR1 is highly expressed on immune cells and together with S1P has been implicated as a regulator of inflammatory diseases. The S1P/S1PR1 pathway is essential for the trafficking of immune cells and in the differentiation of T helper cells (45). *S1PR1* is up-regulated in several autoimmune diseases such as multiple sclerosis, systemic lupus erythematosus or rheumatoid arthritis and agents which inhibit S1PR1 are being investigated for their therapeutic potential (46). It is proposed that, in autoimmune conditions, increased S1PR1 inhibits the number and functions of regulatory T cells leading to increased production of inflammatory cytokines. Elevated *ICOS* expression is also associated with autoimmune diseases such as rheumatoid arthritis and lupus nephritis (47). Thus, the concomitant up-regulation of *ICOS* and *S1PR1* in our Long COVID PBMCs likely forms a regulatory axis underpinning persistent inflammation and could be related to an autoimmune component based on our knowledge of other diseases.

Regulation of an appropriate inflammatory response is largely dependent on the expression and activation of immunoregulatory receptors, of which the leukocyte immunoglobulin like receptors (LILRBs) are key players. These receptors regulate inflammation via the control of many cellular processes including cell survival, phagocytosis, cell migration, cytokine production and cell death (48). In our data we observed a large downregulation of two LILRBs, *LILRB1* and *LILRB2*, both of which inhibit inflammatory processes. Reduced expression or impaired function of LILRB1 and LILRB2 have been associated with inflammatory autoimmune conditions such as rheumatoid or psoriatic arthritis and systemic lupus erythematosus (49–52). This supports the potential role of reduced expression in promoting a sustained inflammatory state in Long COVID. Future studies characterizing *LILRB1* and *LILRB2* expression and function in SARS-CoV-2 infection and over time would be useful in determining how these receptors contribute to the severity of disease and susceptibility to development of Long COVID.

In this study we showed that the reduced expression of *LILRB1* and *LILRB2* alone discriminated a small pilot cohort of Long COVID PBMC samples from COVID recovered controls without overlap. This and the biological roles of these two genes being potentially related to the underlying pathology emphasize their potential as blood-based biomarkers of Long COVID. Given the small sample size of this pilot study this requires validation in larger cohorts, across different disease durations and in different disease groups.

Data availability statement

The data presented in the study are deposited in the Sequence Read Archive NCBI repository, accession number PRJNA1184005.

Ethics statement

The studies involving humans were approved by La Trobe University Human Ethics HEC21207 and HEC21907. 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

DM: Writing – original draft, Writing – review & editing. EE: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. MM: Data curation, Formal analysis, Methodology, Writing – review & editing. CA: Data curation, Formal analysis, Writing – review & editing. OS: Methodology, Writing – review & editing. PF: Conceptualization, Writing –

review & editing. SG: Resources, Writing – review & editing. SA: Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Supervision, 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.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

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Animal models of post-acute COVID-19 syndrome: a call for longitudinal animal studies

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Animal models are indispensable for unraveling the mechanisms underlying post-acute sequelae of COVID-19 (PASC). This review evaluates recent research on PASC-related perturbations in animal models, drawing comparisons with clinical findings. Despite the limited number of studies on post-COVID conditions, particularly those extending beyond three months, these studies provide valuable insights. Three hallmark features of PASC—lung fibrosis, hyperglycemia, and neurological sequelae—have been successfully replicated in animal models, paving the way for mechanistic discoveries and future medical interventions. Although most studies have reported post-COVID conditions within 14–60 days post-infection, they still offer critical reference for future long-term research. This review also explores potential mechanisms of persisting immune misfiring, a key factor in the chronicity of PASC symptoms. Moreover, challenges in modeling PASC are also discussed, including the limited genetic diversity in inbred animal strains and difficulties in accurately identifying PASC-affected individuals. To address these issues, we propose methodological improvements, such as comparing individual animal parameters with control averages and incorporating genetically diverse populations like collaborative cross models. These strategies will enhance the identification and characterization of PASC endotypes in animal studies. By integrating findings from animal models with clinical manifestations of PASC, future research can provide more valuable insights into its mechanisms and support the development of effective therapeutic strategies. Finally, we emphasize the urgent need for longitudinal studies in animal models to fully uncover the mechanisms driving PASC and guide interventions to mitigate its public health impact.

KEYWORDS

post-acute COVID-19 syndrome, animal models, long-term perturbations post coronavirus infection, immune tolerance, organ manifestations

1 Introduction

Microbial infections can induce long-term consequences beyond acute diseases or chronic infections. In particular, repeated viral exposure plays a key role in neurodegenerative diseases (1–3). COVID-19 patients not only suffered from acute-phase disease, but also experienced highly heterogeneous post-COVID conditions (4). Furthermore, SARS-CoV-2 infection has been associated with an increased risk of developing other non-infectious diseases such as diabetes (5), cardiovascular problems (6), autoimmune diseases (7), and neurological or psychiatric disorders (8). These findings reinforce this correlation. In the post-COVID era, PASC triggered by repeated infections from new variants of SARS-CoV-2, will continue to pose a significant threat to public health. Therefore, understanding the mechanisms behind long COVID and advancing therapeutic innovations remain crucial. However, currently, several limitations hinder the identification of a clear, reproducible, and generalizable long COVID/PASC signature, including sample accessibility, disease heterogeneity, inconsistent PASC definitions, variations in sample collection timing and methodologies, and uncontrollable factors such as infection severity, reinfection, co-infection, and subsequent infections. Therefore, the development and careful characterization of relevant animal models, which can can offer better control over certain factors, are crucial for revealing the underlying mechanisms of PASC.

Animal models are powerful tools for elucidating disease pathomechanisms. While post-infection perturbations and dyshomeostasis have been widely reported in COVID-19 models (9), research specifically focused on PASC is far less extensive. A key challenge is identifying PASC in animal models and appropriate control groups, often limiting comparisons to infected versus uninfected, vaccinated, or influenza-infected animals (10, 11). Ideally, more rigorous comparisons should be made between infected animals with and without PASC (12). Furthermore, the limited intraspecific diversity of most inbred animal strains may prevent them from fully exhibiting the heterogeneous symptoms observed in humans. Although animal models may not fully recapitulate the clinical manifestations of PASC, animal models offer several advantages for PASC studies, as long as findings from animal models are reproducible. 1. Systematic analysis can be conducted in animal models at serial time points, such as systematic histopathology analysis, multiple omics analysis, and viral detection. 2. Consistent pathogenic features in animal models can be profiled and reevaluated for further research. 3. Engineered animal models or experimental interventions can be applied for PASC research. Here, we systematically reviewed recently published research (including preprints) on PASC or coronavirus infection induced long-term perturbations, and present the similarities and differences by comparing these models with clinical findings (Table 1). Most importantly, these studies offer a valuable reference for future PASC research. Applying improved methodologies for identifying PASC in animals will offer more valuable insights though rigorous comparisons between infected animals with and without PASC.

2 Short and long term post-acute COVID-19 impact found in animal models

Due to the very few animal studies revealed that long-term sequelae of COVID-19 beyond three months, we employed a less stringent criteria for selecting studies investigating post-COVID conditions (9). According to the current consensus definition of PASC, only five animal studies qualify as PASC research. Three of these studies reported the fibrotic changes in rodent lungs (13–15), one reported a neurological sequelae in mice (16), and another reported hyperglycemia in a non-human primate model (17). Apart from the post-COVID studies, four studies revealed sequelae post mouse hepatic virus (MHV) infection were also included, including two studies on central nerve system impacts (18, 19) and two studies reflecting MHV-induced histopathological changes lasting for one year (20, 21). The MHV model represents a natural coronavirus infection process in mice. Although it may trigger different immune response compared with SARS-CoV-2, it may help to reveal some shared mechanisms of viral infection induced neurodegenerative diseases. Moreover, extensive longitudinal systematic histopathology examination is recommended in future PASC studies (20, 21).

While most included studies had durations of less than three months, many observed signs of exacerbation or permanent injury initiated during the acute phase of infection. These observations suggest that these sequelae may extend beyond the study durations, thus providing valuable references for future research. However, some short-term sequelae, such as olfactory dysfunction, testicular damage, and muscle atrophy (22–24), are likely to resolve within one or two regenerative cycles. Despite this tendency toward ceasing, it remains possible for individuals to experience long-term impacts, such as persistent muscle weakness or anosmia. Therefore, we compiled the main organ manifestations observed in these studies, along with their similarities to clinical findings and PASC sequelae at the study endpoints, in Table 1. Future research should prioritize monitoring these manifestations over extended timeframes to reveal the mechanisms underlying PASC. Findings from these animal models substantiate three pillars underlying the PASC (Figures 1A, B): 1. Damage caused during the acute infection phase that does not fully recover after viral resolution. 2. Subsequent and persistent damage caused by immune perturbations or other dyshomeostasis, with or without viral persistence. 3. Maladaptive tissue repair leading to conditions like interstitial pulmonary fibrosis and alveolar bronchiolization.

While most of the included post-COVID studies reported resolution of coronavirus infection within the acute phase, two PASC studies documented prolonged viral replication: one lasting 121 dpi (15) and the other persisting for 6 weeks post-infection (dpw) (17). However, recent research suggests that viral remnants, rather than actively replicating virus, may be responsible for the neurological sequelae of COVID-19 (25). Therefore, future PASC research employing small-micelle-mediated organ-efficient clearing and labeling techniques could be crucial in determining the duration of viral remnant persistence and their presence in relevant animal models.

TABLE 1 Organ manifestations.

Time scale	Manifestations	Tendency at study endpoint	Reference
Nervous system			
31 days	At 31 dpi, permanent injury to the lung and kidney was observed, characterized by peribronchiolar metaplasia and tubular atrophy, respectively. Transcriptional alterations in lungs, kidneys, heart and brain persisted up to 31 dpi. SARS-CoV-2 infection resulted in transient olfactory dysfunction in mice, resolving by 15 dpi. Similar to clinical findings, anosmia is normally resolved within 2-3 weeks (63). Notably, SARS-CoV-2 infection in mice induced persistent olfactory inflammation and reduced burying activity at 26 dpi, an assay commonly used to assess rodent repetitive and anxiety-like behaviors	Gene set enrichment analysis revealed an expansion of pathways involved in microtubular motor activity and axoneme assembly in the lungs, consistent with bronchiolization. Prolonged elevation of canonical interferon-stimulated genes and Cxcl10 was observed in the olfactory bulb and epithelium, with a gradual decline. However, Ccl5 expression kept increasing, as well as Iba1 levels, a biomarker for microglial and macrophage activation.	(11)
28 days	Neurological changes, such as reactive astrocytes and microglia, brain hypoxia, perivascular cuffs, degeneration of Purkinje cells, brain microhemorrhages with/without vascular injury, neuronal injury and apoptosis, were found in SARS-CoV-2 infected non-human primates, only with sparse viral infection in brain. Hypoxic-ischemic injury, reactive astrocytes and microglia observed align with autopsy findings in human brains (64).	While this study did not specifically compare early and late stages, neuronal apoptosis appeared to increase over time. Apoptosis was completely absent in primates that succumbed at acute phase, while exhibited more severe microhemorrhages.	(32)
14 days	Fibrotic scarring in lung and microglial activation and perivascular blood cuffing were also observed in brain at 14 dpi, even though no viral RNA and N protein were detected at this time point. SARS-CoV-2 infection through intranasal droplets leads to lethal outcomes and is not suitable for long-COVID model.	Lung pathology worsened, and brain Iba1 levels increased over time. Permanent damage caused during the acute phase may leave long lasting consequences.	(35)
21 days	Distinct infectivity and immune patterns were observed among variants. The expression of several cytokines remained elevated in the lungs, brain, and heart up to 21 dpi, varying depending on the viral strain. A resurgence in the numbers of B cells, dendritic cells, and macrophages at 14 dpi in bronchoalveolar lavage fluid was noted. A gradual accumulation of tau pathology was observed in cortex, hippocampus, striatum, and amygdala up to 21 dpi.	The expression of several cytokines (viral strain specific) and Tua pathology (AT8 staining) increased with time.	(34)
31 days	A type 1 IFN triggered neuropathic transcriptome alteration in dorsal root ganglia, coincided with SARS-CoV-2–specific mechanical hypersensitivity mechanical hypersensitivity, was found up to 31 dpi.	An expansion of differentially expressed genes was observed at 31 dpi compared to 4 dpi	(65)
4 months	Extensive longitudinal behavioral studies in mice surviving severe SARS-CoV-2 infection revealed a broad spectrum of neurological abnormalities in neuropsychiatric state, motor behavior, autonomic function, and reflex and sensory function. Minimal astrocyte activation and minimal to mild microglial activation were detected in brain, alongside with brain transcriptomic alteration in pathway related to complement activation, phagocytosis recognition, and humoral immune response mediated by circulating immunoglobulin up to 4 mpi. Persistent complement activation is revealed as a key feature of human long-COVID (30).	Abnormalities in spontaneous activity, tail position, and tremor increased, while gait, whisker response, ear twitch, and palpebral reflex decreased over the 4-month period. Mild inflammation persisted in the brain. The levels of IL-6, CCL5, CXCL10, and CCL11 in lungs remain elevated.	(16)
61 days	Persistent mild brain lesions with gliosis and hyperemic blood vessels and neuropsychiatric sequelae were observed up to 60 days after MHV infection. Extensive behavioral examination revealed female were more susceptible for post coronavirus infection syndrome, mirroring the sex difference observed in long-COVID (67). Persistent microglial activation and increased IL-6 levels were detected in both sexes. Female exhibited reduced marble burying activity at 34 dpi and significant cognitive dysfunctions at 60 dpi. The olfactory dysfunction in female was resolved by 38 dpi.	A resurgence of increased Iba1 levels was observed in the brain at 60 dpi. IL-6 levels remained elevated, particularly in males. Elevated levels of $S100B^{+}$ cells were detected in females	(19)
30 days	SARS-CoV-2 infection triggered monocyte infiltration and microglial activation in central nervous system. Myeloid cell-derived IL-1β impaired hippocampal neurogenesis, contributing to subsequent cognitive deficits. Adenoviral-vectored spike vaccination mitigated hippocampal degeneration.	T cells and myeloid cells remained elevated. Reduced double cortin-positive neuroblasts returned to bassline. Proliferating neuroblasts and synapses remain lower.	(10)
60 days	SARS-CoV-2 infection increases the Parkinson's disease susceptibility and cellular toxicity in a humanized Parkinson's disease model pretreated with human preformed fibrils by inducing	Astrocyte and microglial activation were gradually subsiding.	(68)

(Continued)

TABLE 1 Continued

Time scale	Manifestations	Tendency at study endpoint	Reference
Nervous system			
	a persisting astrocyte and microglial activation, even when the virus is undetectable.		
30 days	Expanded $CD8^+$ T cells found in the brain of aged mice after MHV infection caused neuronal cell death, neuronal regeneration in hippocampus, and subsequent spatial learning impairment, rather than microglial activation. These cytotoxic T cells induce neuronal apoptosis via IFNγ pathway, instead of antigen-specific killing. *in vitro*	Sustained microglial activation and elevated levels of IFN-γ and TNF-α were found in the central nervous systems of aged mice.	(18)
30/60 days	Microglial activation and lose of neuronal marker NeuN persisted in hippocampus of MA10 infection mice by 60 dpi. Perivascular lymphocyte cuffing was observed by 30 dpi.	Perivascular lymphocyte cuffing increased over time by 30 dpi.	(66, 69)
No infection	IgG from long COVID-19 patients can cause persistent sensory hypersensitivity or reduced locomotor activity in mice. These IgG can target on murine heart, skeletal muscles and spinal cord neurons.		(53)
No infection	Spike protein induced a long-term cognitive dysfunction via TLR4 signaling. Knockout of TLR4 and TLR4 blocking improves the synapse elimination and memory dysfunction.		(70)
Lung			
121 days	Aged hamsters suffered from persistent sub-pleural and interstitial pulmonary fibrosis, as well as alveolar bronchiolization, until 112 dpi after a mild physical exercise. A decrease of $CK8^+$ alveolar differentiation intermediate cells and a dominated $CK14^+$ airway basal cells population is correlated with bronchiolization.	$Azan^+$ area, an indicator of fibrosis gradually increased by 121 dpi.	(14)
30 days	Sustained lung inflammation, injury and airway wall thickening alongside with long-term neutrophil recruitment, fibrotic changes, and increased NET formation were observed in the lungs of mice for up to 30 dpi.	Net formation gradually increased from 5 dpi to 30 dpi.	(71)
35 days	IFNγ secreted by resident T cells recruited profibrotic macrophages to caused fibrosis in both human and mice model. Blocking IFNγ reduced lung inflammation and fibrosis.	Lung pathology and T cells peaked at 21 dpi and subsequently decline, while remained elevated by 35 dpi. IFNγ signaling waned by 35 dpi.	(12)
120 days	MA10 infection induced a heterogeneous and persistent pulmonary lesions in aged mice, mainly manifested as fibrosis and chronical inflammation in lungs. Similar severity in lungs were detected at acute phase between young and aged mice, while most young mice resolve these damage by 121 dpi.	Histopathological score and recruitment of macrophages and lymphocytes persisted up to 120 dpi.	(13)
120 days	SARS-CoV-2 persistently replicated in lungs of hamster from 42 dpi to 120 dpi, alongside with alveolar consolidation, chronic inflammation, alveolar-bronchiolization and fibrotic changes in lungs. Increased proliferation and differentiation of $CK14^+$ cells lead to alveolar bronchiolization	IL13, IL33, several gene involved with fibrosis remained elevated.	(15)
31 days	SARS-CoV-2 infection induced a long-term and sex-differential changes in lung proteome of hamsters with no remaining of virus.	Seven proteins remained elevated. Persistent upregulation of Muc5AC is found in other studies (13, 15).	(72)
14 days	A dysregulated alveolar regeneration is found in hamster model by 14 dpi even with no detectable viral antigen in hamster.		(73)
Heart			
24 weeks	Spike protein of SARS-CoV-2 induced a systematic suppression of mitochondrial genes and caused cardiac fibrosis and decreased ejection fraction in obese mice.		(74)
Heart			
4 weeks	Hamsters experienced a triphasic cardiac conduction system dysfunction, which peaked at 1-3 dpi, ceased by 7dpi and recurred	Cardiac conduction system dysfunction redeveloped at 28 dpi.	(75)

(Continued)

TABLE 1 Continued

Time scale	Manifestations	Tendency at study endpoint	Reference
Heart			
	and persisted up to 28 dpi. Persistent cardiac conduction system injury and dysfunction along with increased cardiac cytokines, interferon-stimulated gene expression, and macrophage remodeling in SARS-CoV-2 infected hamster model.		
Skeleton muscle			
60 days	SARS-CoV-2 infection left a long-lasting skeleton muscle atrophy and metabolisms suppression. Mitochondrial function is impaired by IFN-γ and TNF-α.	A significant decrease in myofiber cross-sectional area and an expansion of differentially expressed genes in muscles were detected by 60 dpi.	(24)
Intestine			
12 months	Nests of erythrocytosis, diffused inflammation and the infiltration of lymphocytes are found in mouse intestines 12 months after MHV-1 infection.		(21)
Testis			
4 weeks	Testicular morphological alterations, including interstitial edema, various tubular defects, and germ cell abnormalities, peaked during the acute infection phase (5 dpi). Most of these injuries resolved by 30 days post-infection, which mirrors the clinical findings (76). SARS-CoV-2 induces a transcriptomic changes in dysregulation of inflammatory, cell death, and steroidogenic pathways.	TNF and IL-6 levels in testis remained higher at 30 dpi, alongside with increased immune cells infiltration.	(23)
Gut-brain axis			
No infection	Mice that receives fecal transplantation from post-COVID patients exhibits poor cognitive performance.		(77)
Blood and liver			
18 weeks	SARS-CoV-2 infection induces a long-term elevation of blood glucose and a dysregulated blood chemokine signature in African green monkeys. No viral replication and inflammation are found in in the liver and pancreas, and an enhanced activity of gluconeogenic enzyme phosphoenolpyruvate carboxykinase is thought to cause hyperglycemia.		(17)
Skin			
12 months	The loss of hair follicles, damage to adipose tissues, and injury to epidermal layer are found in MHV infected mouse 12 months post infection.		(20)

3 Animal models of PASC

3.1 Lungs

Lung fibrosis, a consequence of maladaptive repair mechanisms following lung damage, is a key feature of PASC, which is significantly influenced by age (26). Two studies reported fibrosis and alveolar-bronchiolization in aged rodents over a three-month period, supporting this age-related predisposition (13, 14). Notably, in BALB/c mice, while similar mortality rates and subpleural opacities were observed in both young and aged animals, aged mice exhibited slower recovery and tendency toward fibrotic changes and chronic inflammation (13). Another study demonstrated that mild treadmill exercise at 21 dpi after the Delta variant infection re-induced lung dysfunction and fibrotic changes after apparent recovery (14). Moreover, long-term fibrosis can also be triggered by chronic viral infection in young hamsters (15). Notably, shared mechanisms underlying post-COVID sequelae in lungs between young and aged hamsters involve the $CK14^+$ cells' proliferation and differentiation into $SCGB1A^+$ club cells, leading to fibrosis and alveolar bronchiolization (14, 15). Mechanistically, dysregulated lung regeneration has been correlated with increased Notch3 and Hes1 protein expression (15). Given that Notch4 has been shown to hinder regulatory T-cell-mediated tissue repair and induce severe inflammation in an influenza model (27), these findings suggest that Notch signaling may disrupt normal tissue repair following viral infections.

Heterogeneity in damage resolution is observed both within the same strain and between different mouse strains, such as BALB/c and C57BL/6J (13), after MA10 infection. The resolution of fibrosis in some individuals highlights host-specific differences in damage repair. Recent research has identified IFNγ as a key factor in post-

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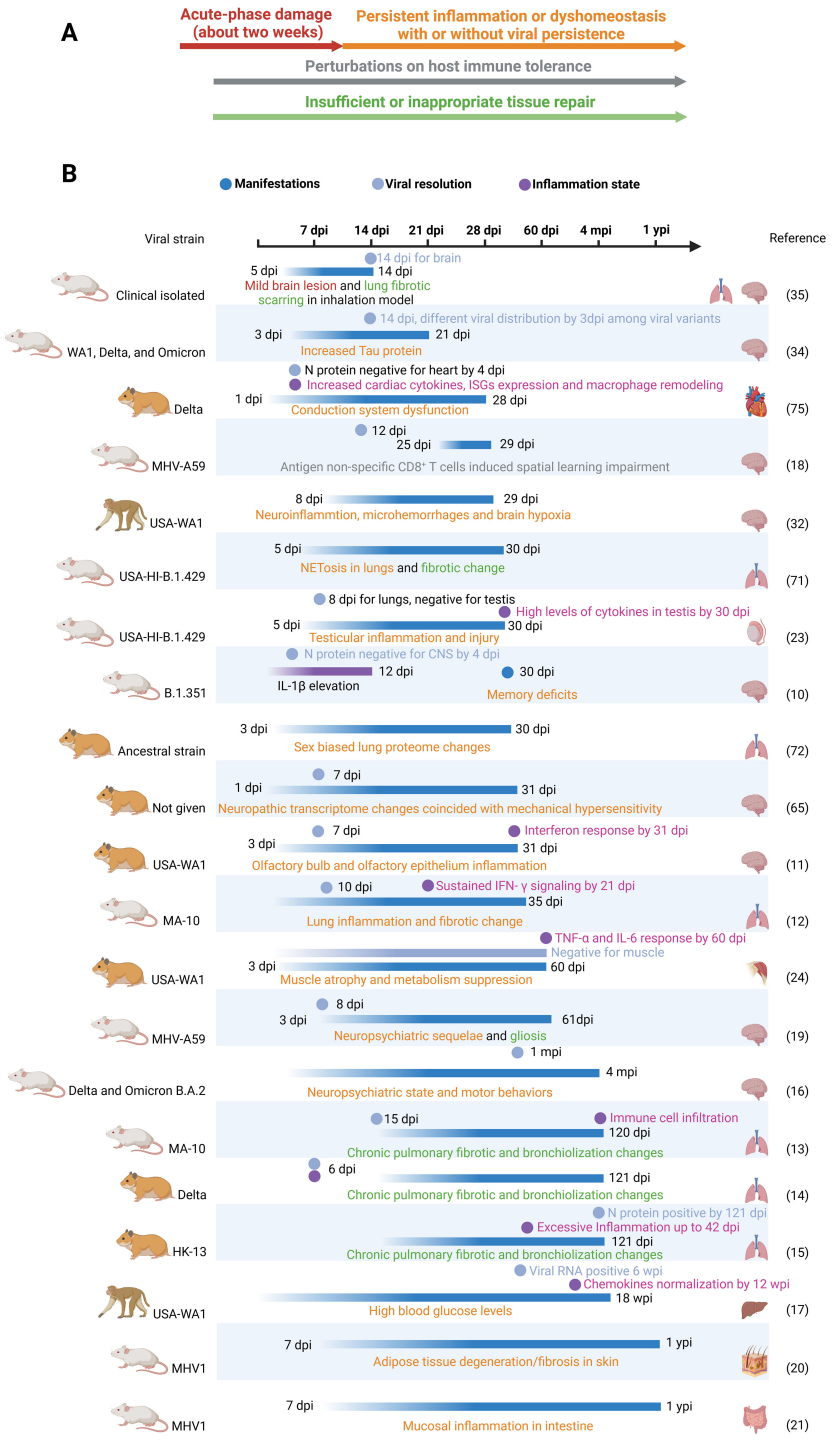

FIGURE 1
(A) Three pillars underlie long-COVID: acute phase damage, persistent inflammation or perturbations and maladaptive repair. Persistent inflammation can be caused by perturbations in immune tolerance (acquired immunity) and persisting activation of innate immunity by unknown reasons. In Figure **(A)**, four classes are represented by distinct colors. These colors correspond to the same classes depicted in **(B)**. **(B)** Timeframes for long-COVID animal models. In addition to only recording organ manifestations in long-COVID models, viral clearance and inflammation resolution can provide valuable insights into the underlying mechanisms. We put only one study with a duration of less than 2 weeks' here to address the importance of acute damage. Dpi, wpi, mpi and ypi represent days, weeks, months and years post infection respectively. .

COVID respiratory sequelae. By comparing single-cell RNA sequencing data from bronchoalveolar lavage fluid of convalescent donors with and without persistent respiratory complications, researchers found that IFNγ secreted by lung-resident T cells recruits pro-fibrotic monocyte-derived macrophages, contributing to fibrosis (12). Interestingly, blocking IFNγ alleviates fibrosis only in C57BL/6J mice and has no effect in BALB/c mice following MA10 infection (12). These findings

emphasize the distinct underlying mechanisms driving similar long-term pathological changes in these genetically different mouse strains.

3.2 Metabolism perturbations

A study reveals SARS-CoV-2 infection induces a lasting hyperglycemia for 18 weeks in African green monkeys without detectable virus or inflammation in the liver and pancreas, along with a dysregulated blood chemokine signature (14). Increased liver glycogen was found in this model, and an enhanced gluconeogenesis was thought to be a main cause of hyperglycemia. This research presents a lasting stress response in glycometabolism post-infection and highlights a link between immune response and metabolism. Apart from the previously reported pancreatitis from the same task group (28), this research provides insights into and may open a new avenue for immunometabolism perturbations after infection. Moreover, an increased risk of Type 2 diabetes is also found in children after COVID-19 (29). Future research on how an infection can induce a disorder in glycogen metabolism as well as insulin resistance is required.

3.3 Neurological sequelae

A four-month longitudinal study extensively monitoring mouse behavior after severe SARS-CoV-2 infection revealed a broad spectrum of neurological abnormalities in neuropsychiatric state, motor behavior, autonomic function, and reflex and sensory function (16). Minimal astrocyte activation and mild microglial activation persisted in the brain, accompanied by transcriptomic changes related to complement activation, phagocytosis, and humoral immune response and gene expression levels associated with ataxia telangiectasia, impaired cognitive function and memory recall, and neuronal dysfunction and degeneration. Notably, lingering complement activation is also revealed in PASC patients, which is correlated with the persistence of autoantibodies and antibodies against herpesviruses (30). This convergence of findings suggests a potential link between complement activation and neuropathological changes in both animal models and human PASC. Therefore, future research should focus on identifying the specific antibodies or other pathways triggering complement activation and mediating these neuropathological changes. The autoimmunity resulting from SARS-CoV-2 infection and its impact on immune tolerance will be discussed in the "Immune Tolerance" section.

Interestingly, although mild brain inflammation persisted throughout the study, some abnormalities in gait, whisker response, ear twitch, and palpebral reflex abnormalities, decreased over time. Others, including body position, grip strength, touch escape, and reach touch, remained relatively constant. Conversely, abnormalities in spontaneous activity, tail position, and tremor increased over the four-month period. These results suggest that the impact on the brain may stem from three distinct types of damage: permanent damage incurred during the acute phase, reversible damage incurred during the acute phase, and subsequent damage that gradually develops after the acute phase. The latter on aligns with the gradual exacerbation of cognitive deficits in some asymptomatic patients (31) highlighting the need for future PASC models induced by less severe infections.

4 Manifestations of short-term neuropathology changes

4.1 Persistent neuroinflammation

The most consistent finding across these studies is the persistence of neuroinflammation, primarily characterized by microglial activation (10, 11, 32), elevated levels of Iba1, elevated pro-inflammatory cytokines, such as IL-1β (10), IL-6 (16, 19), TNF-α (18), IFN-γ (18), and chemokines such as CCL5 and CXCL10 (11). While microglial activation is a normal response to infection or injury, its persistence can have detrimental effects on neuronal function and contribute to neurodegeneration. The prolonged elevation of these inflammatory mediators can disrupt neuronal signaling, impair synaptic plasticity, and promote neuronal damage or death. Notably, a research revealed gliosis in the mouse brain after MHV infection (19). Gliosis, as an end stage of microglial activation, is thought to be a main feature and driver for persistent depressive and cognitive symptoms in patients (33). In some cases, astrocyte activation (16, 32), tau pathology (34), and perivascular lymphocyte/blood cuffing (32, 35) can be salient in some animal models.

4.2 Neuronal damage and dysfunction

Several studies provide evidence of neuronal damage and dysfunction (32), including neuronal cell death (18, 32), impaired neurogenesis (36), and reduced synapses (10). These findings indicate that coronavirus infection can have a direct and lasting impact on neuronal function. Notably, one study revealed the neuronal apoptosis gradually increase over time in primates, and is absent in the acute phase (32), suggesting different damage in separate phases.

4.3 Age and sex bias changes

Aged animals often exhibit more severe and prolonged effects due to a heighten tone of basal inflammation (18), and female-specific neuropathological changes (19), which both mirroring the clinical findings. These findings highlight the importance of considering individual variability when studying the neurological impact of coronavirus infection.

5 Lasting immune misfiring caused by dysregulated immune tolerance

Perturbation of immune tolerance can lead to an autoimmune response. Immune tolerance serves as a supervisor in determining what our immune response should react to and what it should not react to. Perturbations of immune tolerance encompass two key concepts: failure to respond to a substance that should elicit an immune response, and responding to a substance that should be be indifferent to. During the pathogen infection, immune tolerance is expected to protect our normal tissue from immune misfiring and not to favor pathogen replication by tolerating pathogen. However, in the SARS-CoV-2 infection, it seem immune tolerance turn into malfunctioning mode to cause damage to innocent tissue, and lead to viral persistence. The process of PASC is a long-term immune misfiring of both innate and adaptive immune response triggered by infection.

5.1 Aspect from acquired immunity

For pathogens like viruses, the best way to evade our immunity is to mimic host molecular traits. Several studies reveal that SARS-CoV-2 can trigger self-reactive antibodies and T cells due to molecular mimicry between host proteins and virus (37, 38), which can trigger a post-infection malaise, such as multisystem inflammatory syndrome in children (39). Apart from the mimicry strategies, SARS-CoV-2 infection is known to manipulate (40) or relax (41–43) our immune tolerance and to trigger an autoimmunity (44, 45), resulting in both acute symptoms and post-COVID sequelae. For instance, autoantibodies in cerebrospinal fluid against brain antigens are found in COVID-19 patients with neurological symptoms (46). Moreover, disruption of peripheral tolerance in the pancreas caused by SARS-CoV-2 infection is thought to be a key driver of type 1 diabetes (5). Thus, within the large infected population, a subset of critical COVID-19 patients may have a previous immune tolerance dysregulation, resulting in the production of autoantibodies against immunomodulatory proteins (47) and interferons (48). This dysregulation can ensue from genetic deficiency in the NF-κB pathway (49) or age induced (50, 51) tolerance loss. Individuals in this group are more susceptible to SARS-CoV-2 infection and more likely to develop a lasting self-reactive acquired immune response, leading to immune misfiring (43). If these immune misfirings cannot be eliminated by central or peripheral tolerance, they may lead to long-term conditions.

Circulating autoantibodies have been linked to PASC, and their levels can predict PASC symptoms (46, 52). Recently, a study demonstrated that transferring IgGs from stratified PASC subgroups based on Glial Fibrillary Acidic Protein and type-I interferon expression can lead to sensory hypersensitivity or reduced locomotor activity in mouse models, depending on IgG cohorts (53). Even though the mechanisms by which these two cohorts of antibodies can trigger different symptoms in mice remain elusive, this research does necessitate the stratification of PASC patients and highlights the diverse mechanisms underlying PASC. Indeed, another research reveal similar finding that passive transfer of IgG from long-COVID patients with neurocognitive and neurological symptoms can cause increased sensitivity and pain (54). They further demonstrate these antibodies target mouse sciatic nerves, spinal cord, and meninges, which can lead to loss of balance and coordination in mice. Similar findings also reported in clinical that bispecific antibodies targeted on both spike protein and neural tissue were found in patients with neurological symptoms (55). In a mouse model, SARS-CoV-2 infection can also trigger anti-platelet factor-4 antibody production, causing coagulopathy (56). Apart from the interfering self-antigens' function, the activation of complement system is also a key driver of PASC (30). Further screening of human extracellular and secreted proteins will help to reveal what self-antigens are the antibodies from PASC patients react to (47), similar methods can be applied in animal models with PASC.

Similar to B cell tolerance, T cell dysfunction is also considered a significant driver of PASC. A recent study found that PASC patients can maintain robust SARS-CoV-2-specific T cells for two years compared to non-PASC controls (57). However, there are no report of immune misfiring caused by autoreactive cytotoxic $CD8^+$ T cells. Only one research revealed children with multisystem inflammatory syndrome had both anti-SNX8 autoantibodies and cross-reactive T cells engaged both the SNX8 and the SARS-CoV-2 nucleocapsid protein epitopes (39). Future screening of the T cells function may offer a clearer vision of the mechanisms of PASC (36).

5.2 Aspect from innate immunity

In a MHV post infection model, activated spike protein non-specific $CD8^+$ cells were increased in the brains of aged mice and correlated with neuronal death, further leading to cognitive decline (18). The authors further revealed these $CD8^+$ cells can induce primary neuronal cell death *in vitro*. Therefore, we may have underestimated the dysregulated T cell in PASC (58). The correlation of IFN-γ released by activated $CD8^+$ cells with PASC patients is also reported in clinical investigations (59, 60). Similarly, these $CD8^+$ were also found in an EBV infection-induced Alzheimer model (61). Moreover, expanded activated $CD8^+$ cells are found to be a key signature of Alzheimer's disease (61, 62). Thus, dysregulated T cells play a key role in dementia caused by different viral exposures. Furthermore, more research reflecting how these T cell responses leave a long-term impact on health is needed. Unlike the cytotoxic T cells, their functions relied on the target that react to. These by stander T cells in PASC can be evaluated by their populations. Therefore, future studies monitoring these by stander T cells in SARS-CoV-2 infection are required.

6 Challenge for PASC research in animal model and future prospect

Two main obstacles hinder the study of PASC in animal models: the limited heterogeneity of PASC symptoms due to the relatively low genetic diversity of inbred animals, and the difficulty

in accurately identifying PASC-affected animals. To address the first issue, using collaborative cross populations in future PASC studies could increase genetic diversity, facilitating the identification of genetic associations with PASC. However, the challenge of distinguishing PASC-affected animals remains. Simply comparing infected animals with uninfected controls or animals infected with other viruses may cover individuals with PASC and PASC phenotypes. For instance, female mice are predisposed to developing post-coronavirus syndrome. Therefore, to better discriminate PASC phenotypes, comparing individual animal parameters with the average of the control group is necessary (16). Furthermore, comparing each individual's pre-infection and post-infection parameters is also crucial (17). By combining these approaches with reference from human post-COVID sequelae, researchers can make more robust comparisons between animals with and without PASC, leading to valuable insights into PASC mechanisms and, ultimately, promoting the development of effective therapies.

7 Methods

1000 articles were scraped from Google scholar after searching for animal models of long-COVID and manually screened for eligibility.

Author contributions

JD: Writing – original draft, Conceptualization, Data curation, Supervision, Writing – review & editing, Funding acquisition, Resources, Visualization, Validation. FH: Data curation, Formal analysis, Software, Visualization, Writing – original draft, Writing – review & editing, Methodology. QC: Conceptualization, Data curation, Formal analysis, Software, Visualization, Writing – original draft, Writing – review & editing, Investigation. QL: Data curation, Formal analysis, Validation, Writing – review & editing, Conceptualization. LZ: Data curation, Formal analysis, Validation, Writing – review & editing, Investigation, Methodology. YD: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing, Writing – original draft.

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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.

Generative AI statement

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

Publisher's note

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