The SARS-CoV-2 S1 spike protein subunit potentiates the neuroinflammatory response to a subsequent immune challenge.

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1. ABSTRACT

By February 2021, the global death toll from SARS-CoV-2 reached approximately 2.5 million individuals. Evidence suggests that the S1 spike protein subunit of SARS-CoV-2 may initiate neuroinflammation through pattern recognition receptors (PRRs), contributing to post-acute sequelae of COVID-19 (PASC) manifestations, including cognitive and neuropsychiatric problems. However, it remains unclear whether persistent microglial activation beyond the acute phase modulates behavioral and neuroinflammatory responses, potentially priming microglia in a sustained proinflammatory state, introduced by the "two-hit" model. Therefore, we investigated whether the S1 spike protein would potentiate the neuroinflammatory response in the hypothalamus to a subsequent immune challenge of a bacterial mimetic. Adult male Sprague-Dawley rats received intra-cisterna magna (ICM) injections of vehicle or S1, followed by intraperitoneal (IP) injections of the bacterial mimetic, lipopolysaccharide (LPS), or vehicle 7 days later. Two days after LPS, neuroimmune gene expression was measured in the hypothalamus. Independent of LPS, S1 increased neuroimmune gene expression of MHCII, IL-6, and NLRP3 in the hypothalamus, indicative of heightened neuroinflammation from S1. S1 potentiated the effect of LPS on IL-1β and CD200R1. These findings underscore the potential of SARS-CoV-2 S1 spike protein subunits to exacerbate the neuroinflammatory process to subsequent immune challenges, potentially contributing to neurological manifestations of PASC.

2. INTRODUCTION

The emergence of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), and its subsequent global spread in 2019 led to the unprecedented coronavirus disease (COVID-19) pandemic, causing widespread illness, significant societal disruptions, and challenging public

healthcare systems worldwide (Zhou et al., 2020). It was estimated by the end of February 2021, approximately 2.5 million individuals died globally (Bouayed and Bohn, 2021). SARS-CoV-2 is transmitted via respiratory droplets, especially during sneezing, coughing, or even speaking by an infected individual. The range of clinical manifestations following a SARS-CoV-2 infection varies from asymptomatic cases to severe critical illnesses characterized by flu-like symptoms, ageusia, anosmia, malaise, dyspnea, hypoxemia, and respiratory failure (Dixon et al., 2020; Berlin et al., 2020; Gandhi et al., 2020). Other clinical complications following a SARS-CoV-2 infection can include dysfunction of the cardiorespiratory center located in the brain stem, neuroinflammation, and a spectrum of neurological, neuropsychiatric, and cognitive symptoms, including post-traumatic stress disorder (PTSD), depression, anxiety, and sleep disturbances, which are observed in approximately one-third of infected individuals (Han et al., 2021; Ahmed et al., 2022; Efstathiou et al., 2022; Schou et al., 2021). Particularly noteworthy, the enduring nature of this virus in brain-mediated complications and symptoms beyond the acute recovery phase of the infection can lead, in some cases, to the emergence of Post-Acute Sequelae of COVID-19 (PASC) (Ahmed et al., 2022; Proal and VanElzakker, 2021; Alcindor, 2023; Zhang et al., 2023). PASC, commonly known as “Long-COVID,” encompasses a range of physical, cognitive, and mental impairments that persist or resurface after the acute phase of COVID-19 has been resolved (Parotto et al., 2023). An estimated 10-35% of individuals who have recuperated from SARS-CoV-2 infection may encounter Long-COVID symptoms later in life (Huerne et al., 2023). However, this solely reflects individuals who have contracted the virus once and overlooks individuals who have experienced multiple SARS-CoV-2 infections. Research indicates that repeated SARS-CoV-2 infections are associated with an elevated risk of experiencing Long-COVID symptoms in the future.

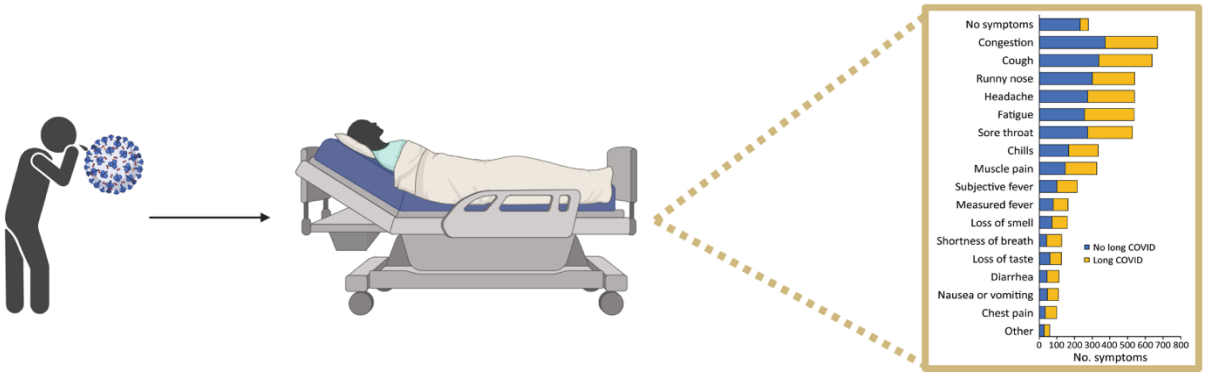


Fig. 1. **Diagram illustrating the pathological progression of SARS-CoV-2 infection leading to Post-Acute Sequelae of COVID-19 (PASC)/Long-COVID**. The diagram highlights the development of various chronic symptoms commonly observed in PASC/Long-COVID patients.

The mechanisms responsible for the brain-mediated impacts of SARS-CoV-2 remain unknown. Some recent work suggests that neuroinflammatory processes may play a role (Benameur et al., 2020; Eden et al., 2021; Farhadian et al., 2020; Nuovo et al., 2021; Song et al., 2020; Yang et al., 2021). SARS-CoV-2 might produce neuroinflammation in several ways. For example, direct entry of the SARS-CoV-2 virus into the brain is a possible mechanism, but there is limited evidence in support of this possibility (Mukerji and Solomon, 2021; Solomon et al., 2020). Alternatively, an emerging hypothesis suggests that the shedding of viral pathogen-associated molecular patterns (PAMPs) from SARS-CoV-2 may account for the range of symptoms observed following an infection (Liu et al., 2021; Proal and VanElzakker, 2021; Proal et al., 2023; Frank et al., 2022, 2023; Brusaferri et al., 2022). These mechanisms might produce proinflammatory cytokine signaling in the CNS through both direct infection of the CNS and/or indirect immune-to-brain signaling pathways (Han, et al., 2021; Boldrini et al., 2021; Kempuraj et al., 2020; Merad et al., 2020; Del Valle et al., 2020).

SARS-CoV-2 is a positive-sense, single-stranded, RNA, beta-coronavirus that is encapsulated within a virion capsid that consists of structural proteins, namely the spike (S), envelope (E), membrane (M), and nucleocapsid (N) (Goverdhan, 2024; Mariano et al., 2020). SARS-CoV-2 is littered with outward-facing spike proteins, made of 2 components: the S1 and the S2 protein subunits on the N and C terminal binding domains, respectively (Hoffmann et al., 2020). The S1 protein subunit aids in the entry of the virus into host cells, primarily through the highly specific binding to the Angiotensin-Converting-Enzyme 2 (ACE2) receptor (Frank et al., 2022; Beyerstedt et al., 2021). The ACE2 receptors exhibit significant expression in many areas of the body, including vital human organs: respiratory lungs epithelium, heart, kidneys, and intestines (Donoghue et al., 2000; Jackson et al., 2022). SARS-CoV-2 proteins, as evidenced by their detection in the circulation of COVID-19 patients long after the resolution of the infection, play a crucial role in the viral lifecycle (Swank et al., 2022). When the S1 subunit binds to the ACE2 receptor, it induces a conformational change in the spike PAMP protein on the SARS-CoV-2 virus, which leads to the shedding and release of the S1 subunit. This alteration exposes the S2 subunit, facilitating the fusion of the viral and host cell plasma membranes. The integration of these cells results in a shared cytoplasm, enabling the release of SARS-CoV-2 viral genetic material into the host cell. Consequently, this process grants access to and utilization of the host's cellular machinery for replication, transcription, and translation, thereby propagating the viral infection within the host (Jackson et al., 2022; Hoffman et al., 2020).

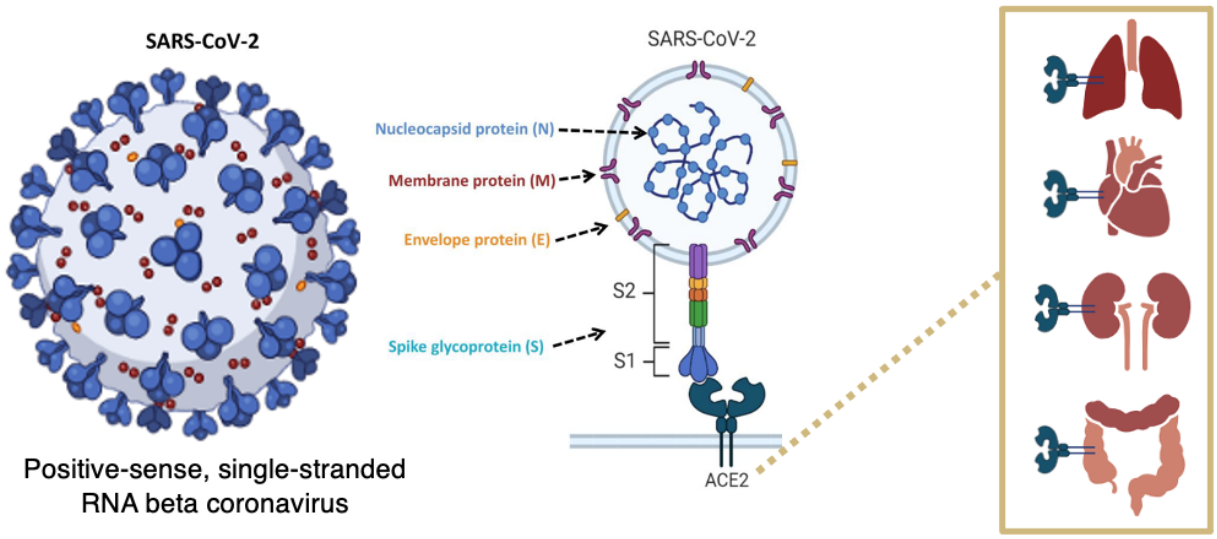


Fig. 2. Diagram representation of the SARS-CoV-2 virus structure and major protein components. The virus is enveloped and consists of the spike glycoproteins composed of two protein subunits: the N-terminal S1 and the C-terminal S2. The S1 subunit is responsible for binding to ACE2 receptors, which are expressed throughout the body, with high concentrations found in vital organs, such as the respiratory lung epithelium, heart, kidneys, and intestines.

Several studies have investigated the capacity of the SARS-CoV-2 S1 spike protein to act as a PAMP protein and mediate neuroinflammatory states by interacting with pattern recognition receptors (PRRs). PRRs are receptors expressed on innate immune cells, like macrophages, dendritic cells, and microglial cells, that recognize molecular motifs that are common to bacterial and viral pathogens, like the S1 protein. Specifically, there is evidence that the S1 protein binds to Toll-like receptor (TLR) 2 and 4 on the microglia, thereby inducing a neuroinflammatory state in the brain (Liu et al., 2022; Frank et al., 2022, 2023; Aboudounya and Heads, 2021). Proinflammatory responses mediated by the SARS-CoV-2 derived PAMPs binding to the TLRs are evident when microglial cells in the BV-2 cell line were exposed to S1 and produced high

levels of cytokines, chemokines, NFkB, NLRP3, and caspase-1 (Olajide et al., 2021a; Olajide et al., 2021b). These findings were corroborated by studies that blocked TLR4 receptor signaling, subsequently blocking the proinflammatory effects (Zhao et al., 2021). When the S1 subunit was injected intravenously, it was able to translocate across the blood-brain barrier (BBB) (Rhea et al., 2021). S1 increased the production of proinflammatory cytokines, like caspase 3, interleukin 6 (IL-6), and tumor necrosis factor (TNF); however, when the S2 subunit was injected intravenously, there was no detection of proinflammatory mediators in the brain (Nuovo et al., 2021). The occurrence of neuroinflammation has been correlated with prevalent cognitive and emotional disruptions in individuals experiencing PASC, which highlights how inflammatory processes within the central nervous system (CNS) can significantly contribute to cognitive and affective manifestations in individuals recovering from a SARS-CoV-2 infection (Stefano et al., 2021; Guo et al., 2023; Park et al., 2022). Collectively, these findings suggest that SARS-CoV-2-derived structural proteins, particularly the S1 spike protein subunit, may act as a PAMP agonist on PRRs to trigger the neuroinflammatory effect.

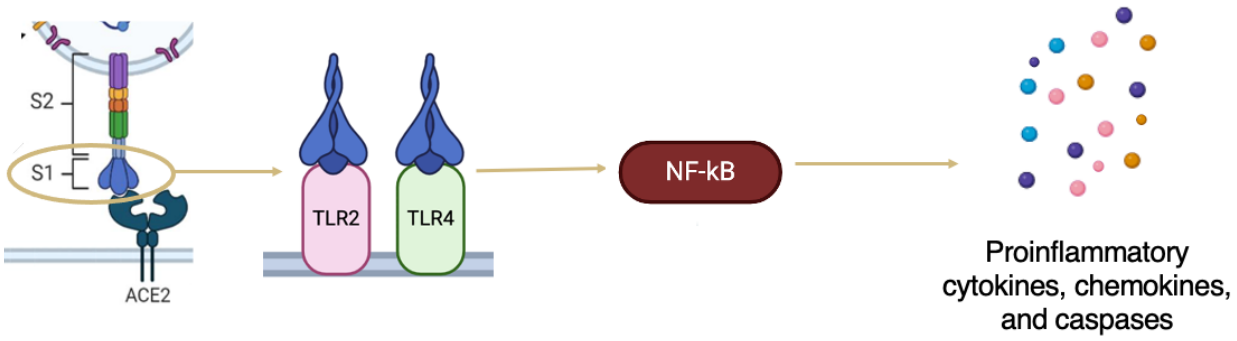


Fig. 3. Diagram of the proposed pathophysiological mechanism of S1 spike protein serving as a pathogen-associated molecular pattern (PAMP) and activating Toll-like receptors (TLR) expressed on immune cells within the body. The diagram illustrates the S1 spike

protein subunit dissociating from the S1-S2 dimerized protein complex after binding to ACE2 receptors. Once dissociated, the S1 acts as a PAMP and interacts with pattern recognition receptors (PRRs), specifically, TLR2 and TLR4. Activation of TLRs leads to the upregulation of NF-κB, a transcription factor that plays a pivotal role in the inflammatory response, resulting in increased production of proinflammatory cytokines, chemokines, and caspases, thereby contributing to systemic inflammation and associated pathologies.

Some patients report that after exposure to a mild bacterial or viral infection, their brain-mediated symptoms worsened or reappeared. (VanElzakker et al., 2023; Proal and VanElzakker, 2023; Moghimi et al., 2021). Lacking, however, are studies that explore the mechanisms behind the persistence or recurrence of brain-mediated symptoms following recovery from the acute phase of SARS-CoV-2 infection. We propose that the S1 spike protein subunit of SARS-CoV-2 primes microglia immune cells, placing the brain in a persistent proinflammatory state, such that microglia will respond excessively to subsequent challenges, often referred to as a "two-hit" exposure model. In this model, if the initial exposure primes the microglial cells within the brain, subsequent exposure to an immune challenge can lead to a potentiated neuroinflammatory response. This can exacerbate the production of neurotoxic agents, including proinflammatory cytokines, like IL-1β, TNF, and IL-6, as well as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Bouayed and Bohn, 2021; Dayarathna et al., 2020).

The present study is designed to test the hypothesis that S1 (first hit) in the brain results in sustained microglia priming, such that subsequent exposure to LPS (second hit) produces inappropriate inflammation. Results from this study will provide insight into the mechanisms

driving exaggerated neuroinflammation that may be responsible for prolonged and recurring brain-mediated symptoms in PASC patients.

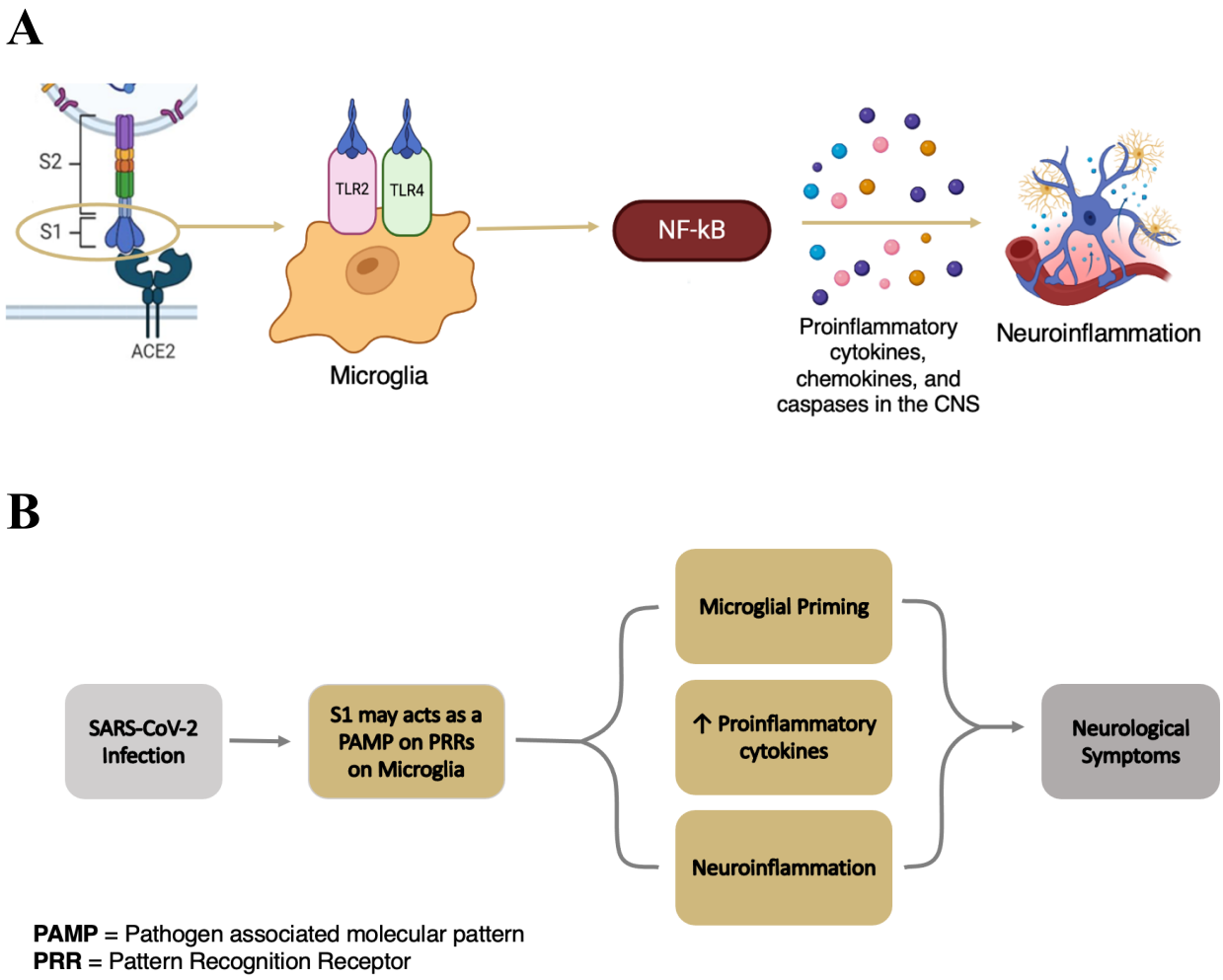


Fig. 4. (A) Diagram representation of S1 serving as a PAMP interacting with TLRs present on microglia within the central nervous system (CNS). This interaction stimulates the upregulation of NF-κB, a transcription factor, which subsequently leads to an increase in proinflammatory cytokines, contributing to neuroinflammation. **(B) A schematic of the proposed pathophysiological mechanism underlying the neurological, neuropsychiatric,**

and cognitive impairments observed in patients with post-acute sequelae of COVID-19 infection (PASC)/Long-COVID.

3. MATERIALS AND METHODS

3.1. Animals

Male Sprague-Dawley rats between the ages of post-natal day (PND) 60 and 90 (Envigo, Indianapolis, IN) were pair-housed in a 12-hour light-dark cycle, with lights on at 0700 h. The housing environment had food (standard laboratory rat chow) and water *ad libitum.* Following relocation into pair-housed cages, rats underwent a minimum acclimation period of one week before any experimentation was done. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Colorado Boulder in compliance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* as well as the ARRIVE guidelines for animal use.

3.2. Experimental Design

Animals were injected intra-cisterna magna (ICM) with a vehicle or the S1 subunit (first hit). Seven days after the ICM injection, animals were given an intraperitoneal (IP) injection of vehicle or LPS (second hit). Two days after LPS treatment brain tissue was collected.

3.3. SARS-CoV-2 S1 protein subunit (1st hit)

Recombinant SARS-CoV-2 S1 subunits were sourced from RayBiotech (cat#: 230–30161). The S1 proteins utilized in the study were derived from the HEK293 cells and lacked the LPS immunogen (Frank et al., 2023).

3.4. ICM

The rats were divided into two groups: the experimental S1 protein subunit (1 μg) group and the vehicle control group (2.5 μl, 0.2 μm filtered, sterile 1x PBX, pH 7.4). For direct exposure to the CNS, either the S1 or vehicle control was administered ICM (Frank et al., 2022; Hordeaux et al., 2018). This method of administration ensures that the material injected can reach forebrain regions and also eliminates the potential confounding variable of peripheral inflammation that could arise from other methods of administration, like intravenous (IV) injections (Frank et al., 2012; Nuovo et al., 2021). Previously, the 1 μg dosage of the S1 subunit used in this experiment was found to produce neuroinflammatory effects (Frank et al., 2022).

Animals were anesthetized with a pharmaceutical-grade isoflurane (2.5%). The ICM injection necessitated a concise anesthesia duration period of 2 to 3 minutes using isoflurane. Depth of anesthesia was assessed by observing the pedal response to a firm pinch of both the forelimb and hind limb paws; once the pedal response was absent, anesthesia was discontinued followed by the dorsal aspect above the cisterna magna being shaved and swabbed with 70% ethanol. Injection utilized a sterile 27-gauge needle connected to a sterile 25-uL Hamilton syringe via sterile PE50 tubing and then inserted carefully into the cisterna magna with the injection administered over a 30-second duration. The needle was left in place for an additional 30-second duration to allow for diffusion of the drug. All tubing and syringes underwent autoclave sterilization protocols. Following the injection, animals were placed in a heated recovery box and monitored at 5-minute intervals until they could maintain sternal recumbency. Recovery was deemed complete upon restoration of the righting reflex, typically occurring

within 5 minutes post-ICM injections. Once fully recovered, animals were returned to their respective home cages.

3.5. In vivo immune challenge (2nd hit)

Animals were injected IP with vehicle (0.9% saline) or LPS (10 μg/kg). LPS (E. coli serotype 0111:B4; Sigma, St. Louis, MO, L3012) was dissolved in pyrogen-free, sterile 0.9% saline, which serves as a vehicle. This dose is based on prior studies showing that stress potentiates the neuroinflammatory effects of LPS (Frank et al., 2012). 2 days after injection, all animals were anesthetized, saline perfused and brain tissue collected for mRNA analysis.

3.6. Tissue dissection of brain regions

Animals were euthanized in accordance with ethical protocols using a lethal dose of sodium pentobarbital. Subsequently, the rats underwent complete anesthetization followed by transcardial perfusion with ice-cold 0.9% saline for a duration of 3 minutes to separate the peripheral immune leukocytes from the CNS. Brains were rapidly extracted, and the hypothalamus was dissected (Frank et al., 2022). Tissue was flash-frozen in liquid nitrogen and stored in the freezer at a constant temperature of -80 ◦C to preserve the structural and cellular integrity of the tissue.

3.7. Real time RT-PCR measurement of gene expression

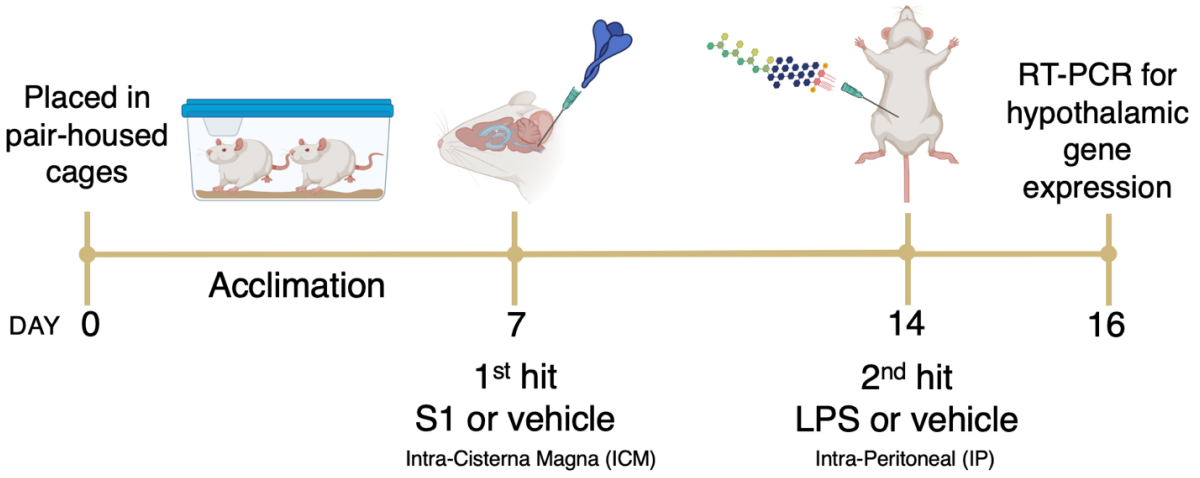
In this study, we assessed the immunophenotype and purity of microglial through real-time RT-PCR. Total RNA was extracted from the hypothalamic samples using TRI Reagent and a standard phenol:chloroform method. RNA concentration was measured using a ThermoFisher NanoDrop 2000 spectrophotometer, and cDNA synthesis was carried out with the SuperScript II

Reverse Transcriptase kit (ThermoFisher, cat#: 18054014). The PCR amplification protocol, detailed in Frank et al., 2006, was then followed. Genbank at the National Center for Biotechnology information provided the cDNA sequences, and primer sequences, obtained from Thermofisher, were designed using the Operon Oligo Analysis Tool, with specificity confirmed via the Basic Local Alignment Search Tool at NCBI (http://www.operon.com/tools/oligo-analysis-tool.aspx; https://www.ncbi.nlm.nih.gov/; Altschul et al., 1997). cDNA underwent PCR amplification using the Quantitect SYBR Green PCR Kit. Relative gene expression was determined using Actb as the housekeeping gene and the 2-ΔΔCT method (Livak and Schmittgen, 2001).

3.8. Statistical analysis

All the presented data is expressed as a mean value + standard error of the mean (SEM). Statistical analysis consisted of ANOVA for hypothalamic neuroinflammatory gene expression in a 2 (S1 protein vs. vehicle control) x 2 (vehicle vs LPS) factorial model. All statistical analyses were performed using Prism software, with the significance level set at $\alpha = 0.05$. The sample sizes are found in figure captions.

A



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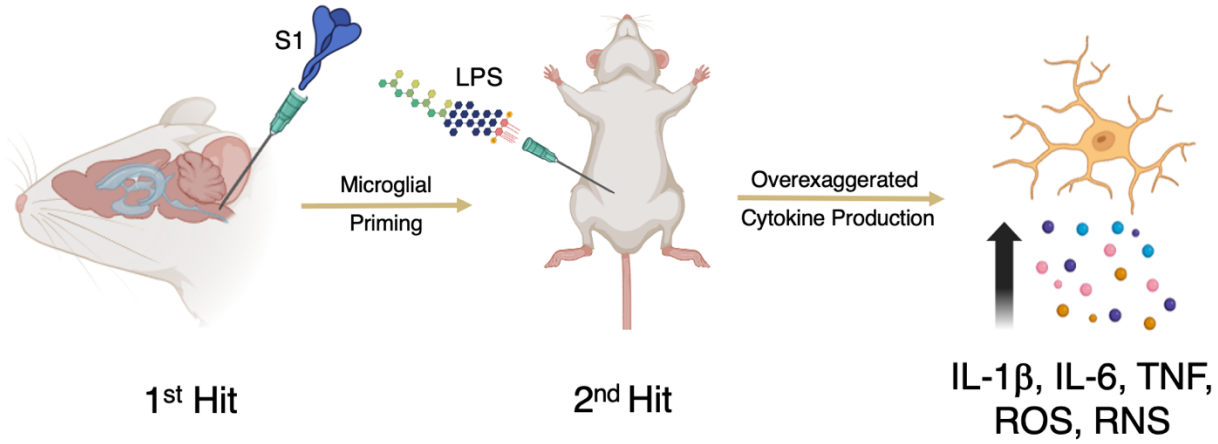

Fig. 5. (A) Experimental study design. Rats (*N* = 32*; n* = 8 per group*)* placed in pair-housed cages were immediately acclimated for at least 7 days. On day 7, rats received the 1st hit of S1 ICM. Day 14, rats received the 2nd hit of LPS IP. Day 16, rats were euthanized, and real-time RT-PCR analysis was conducted to assess the immunophenotype and purity of neuroinflammatory genes in microglia within the hypothalamus, following the “two-hit” exposure model. **(B) "Two-Hit" Exposure Model.** In this experimental paradigm, rats were

initially exposed to the 1st hit, S1 ICM, which serves as a primer for microglial activation. Subsequently, the 2nd hit of LPS IP, representing a subsequent immune challenge, was administered. The priming of microglia via S1 ICM amplifies the response to the 2nd hit of LPS IP, resulting in an exaggerated production of proinflammatory cytokines within the CNS, thereby leading to neuroinflammation.

4. RESULTS

We assessed the expression levels of various proinflammatory genes to examine the neuroinflammatory impacts of the "two-hit" exposure model of the S1 spike protein subunit followed by the LPS bacteria mimetic. Gene expression analysis encompassed markers related to microglial/brain macrophage activation (Major Histocompatibility Complex II, MHCII), microglial/brain macrophage checkpoint receptors, (Cluster of Differentiation 200 Receptor 1, CD200R1), an astrocyte activation indicator (Glial Fibrillary Acidic protein, GFAP), inflammasomes (NOD-like receptor protein 3, NLRP3), and proinflammatory cytokines (IL-1β, IL-6, and TNF).

4.1. Neuroinflammatory effects of S1 on mRNA gene expression

Relative mRNA gene expression levels were measured 2 days after LPS IP injection in the hypothalamus (Fig. 1-3). Independent of LPS, we found that S1 increased gene expression of NLRP3 (S1 main effect; df = 1, 28, F = 18.37, p = 0.0002), MHCII (S1 main effect; df = 1, 28, F = 15.36, p = 0.0005), and IL-6 (S1 main effect; df = 1, 28, F = 7.54, p = 0.01) compared to vehicle treatments. S1 by itself failed to significantly alter the expression of IL-1β, TNF, GFAP, and CD200R1.

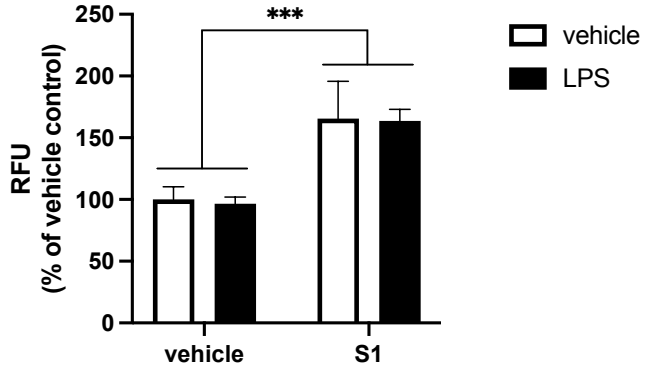

Fig. 6. Effect of LPS on hypothalamic MHCII 7d post-ICM injection of S1. Rats were injected ICM with vehicle (1x PBS) or S1 (1 ug). Seven days after ICM injection, vehicle (0.9% saline) or LPS (10 μg/kg) was injected IP. Two days after vehicle or LPS treatment, gene expression of MHCII was measured in the hypothalamus. RFU = relative fluorescent units. Data are presented as the mean+SEM. N = 8/group. S1 vs vehicle, ***p < 0.001.

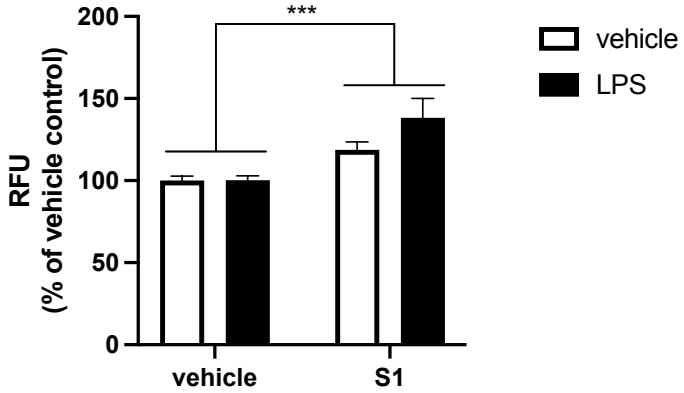

Fig. 7. Effect of LPS on hypothalamic NLRP3 7d post-ICM injection of S1. Rats were injected ICM with vehicle (1x PBS) or S1 (1 ug). Seven days after ICM injection, vehicle (0.9% saline) or LPS (10 μg/kg) was injected IP. Two days after vehicle or LPS treatment, gene expression of NLRP3 was measured in the hypothalamus. RFU = relative fluorescent units. Data are presented as the mean+SEM. N = 8/group. S1 vs vehicle, ***p < 0.001.

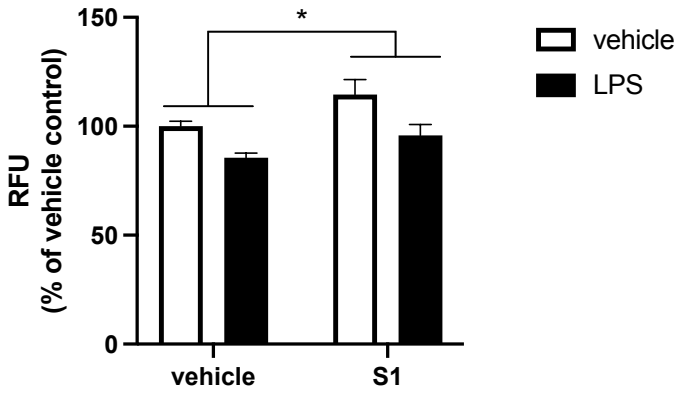

Fig. 8. Effect of LPS on hypothalamic IL-6 7d post-ICM injection of S1. Rats were injected ICM with vehicle (1x PBS) or S1 (1 ug). Seven days after ICM injection, vehicle (0.9% saline) or LPS (10 μg/kg) was injected IP. Two days after vehicle or LPS treatment, gene expression of IL-6 was measured in the hypothalamus. RFU = relative fluorescent units. Data are presented as the mean+SEM. N = 8/group. S1vs vehicle, *$p < 0.05$.

4.2. Neuroinflammatory effects of S1 potentiating LPS induced mRNA gene expression

Relative mRNA gene expression levels were measured 2 days after LPS IP injection in the hypothalamus (Fig. 4-5). Subsequent to LPS injections, we found that S1 potentiated the effect of LPS on CD200R1 (interaction effect; df = 1, 28, F = 12.04, p =0.0017) and IL-1β (interaction effect; df = 1, 26, F = 6.46, p = 0.017)..

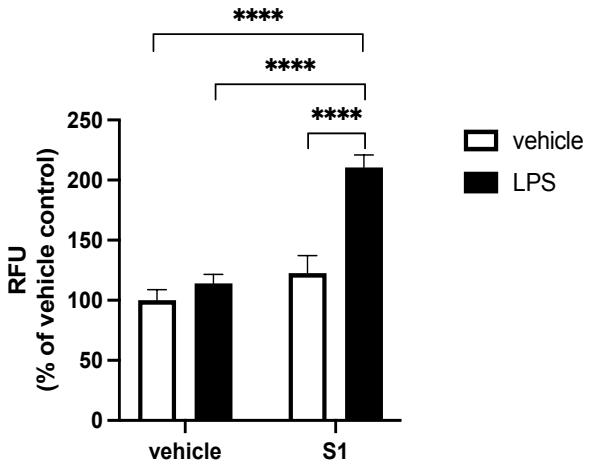

Fig. 9. Effect of LPS on hypothalamic CD200R1 7d post-ICM injection of S1. Rats were injected ICM with vehicle (1x PBS) or S1 (1 ug). Seven days after ICM injection, vehicle (0.9% saline) or LPS (10 μg/kg) was injected IP. Two days after vehicle or LPS treatment, gene expression of CD200R1 was measured in the hypothalamus. RFU = relative fluorescent units. Data are presented as the mean+SEM. N = 8/group. S1/LPS vs vehicle/vehicle, vehicle/LPS, and S1/vehicle, ****$p < 0.0001$.

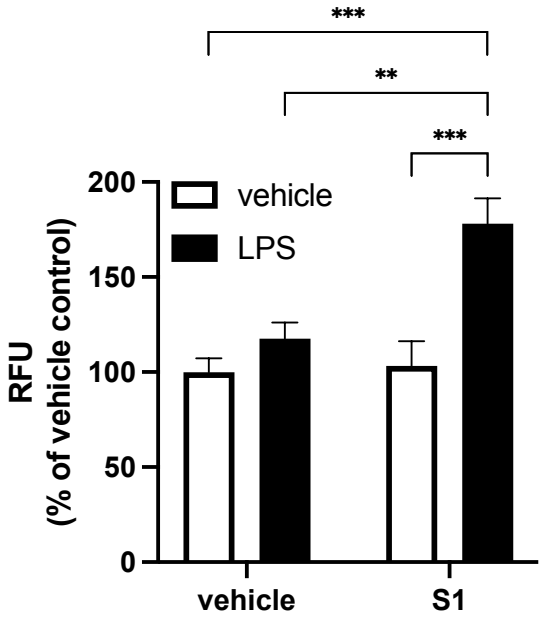

Fig. 10. Effect of LPS on hypothalamic IL-1β 7d post-ICM injection of S1. Rats were injected ICM with vehicle (1x PBS) or S1 (1 ug). Seven days after ICM injection, vehicle (0.9% saline) or LPS (10 μg/kg) was injected IP. Two days after vehicle or LPS treatment, gene expression of IL-1β was measured in the hypothalamus. RFU = relative fluorescent units. Data are presented as the mean+SEM. N = 8/group. S1/LPS vs vehicle/vehicle and S1/vehicle, ***p < 0.001. S1/LPS vs veh/LPS, **p < 0.01.

4.3. Null effects of S1 and LPS on mRNA gene expression

Relative mRNA gene expression levels were measured 2 days after LPS IP injection in the hypothalamus (Fig. 6-7). The main effects of both S1 or LPS as well as the interaction between both treatments did not significantly alter the expression levels of TNF and GFAP (Fig. 6-7).

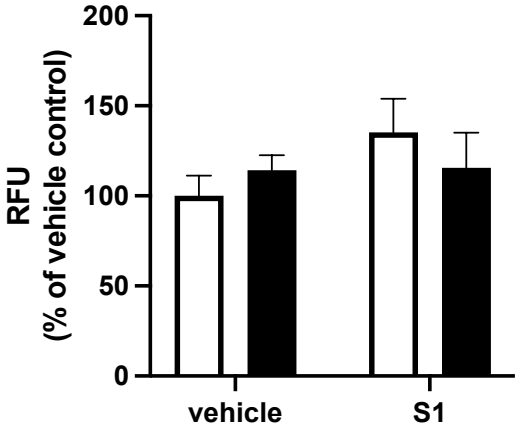

Fig. 11. Effect of LPS on hypothalamic TNF 7d post-ICM injection of S1. Rats were injected ICM with vehicle (1x PBS) or S1 (1 ug). Seven days after ICM injection, vehicle (0.9% saline) or LPS (10 μg/kg) was injected IP. Two days after vehicle or LPS treatment, gene expression of

TNF was measured in the hypothalamus. RFU = relative fluorescent units. Data are presented as the mean+SEM. N = 8/group.

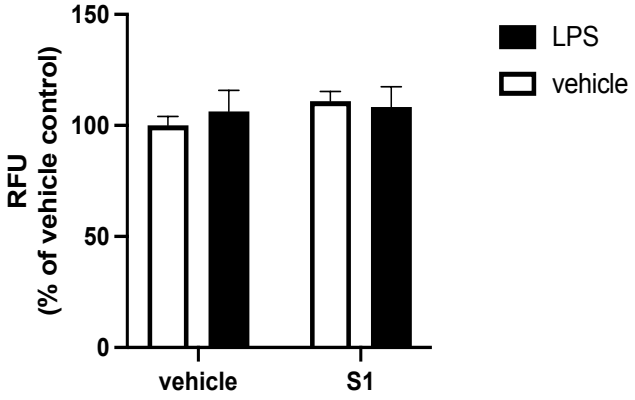

Fig. 12. Effect of LPS on hypothalamic GFAP 7d post-ICM injection of S1. Rats were injected ICM with vehicle (1x PBS) or S1 (1 ug). Seven days after ICM injection, vehicle (0.9% saline) or LPS (10 μg/kg) was injected IP. Two days after vehicle or LPS treatment, gene expression of GFAP was measured in the hypothalamus. RFU = relative fluorescent units. Data are presented as the mean+SEM. N = 8/group.

5. DISCUSSION

Collectively, current studies suggest that the SARS-CoV-2 S1 spike protein may trigger neuroinflammatory responses in the CNS by activating the PRRs of the innate immune system (Nuovo et al., 2021; Olajide et al., 2021a; Olajide et al., 2021b; Shirato and Kizaki, 2021). The S1 spike protein may potentially exacerbate microglial activation, a phenomenon termed microglial priming, when followed by a subsequent exposure to a mild bacterial mimetic, LPS. Such priming could lead to an inflammatory cascade, potentially contributing to the deterioration of neurological sequelae observed in patients suffering from post-acute sequelae of COVID-19 (PASC). Our research endeavored to investigate whether this "two-hit" model, involving initial

exposure to S1 followed by LPS, leads to heightened microglial activation, thus promoting pro-neuroinflammation and exacerbating neurological, neuropsychiatric, and cognitive manifestations observed in PASC/Long-COVID patients (VanElzakker et al., 2023; Proal and VanElzakker, 2023; Moghimi et al., 2021).

Our investigation revealed that S1, independent of LPS treatment, induced phenotypic changes and increased expression of microglial markers in the hypothalamus, like MHCII, NLRP3, and IL-6. These findings corroborate earlier research demonstrating that S1 induces upregulation of hypothalamic MHCII and NLRP3, which is indicative of microglial priming (Frank et al., 2022). It should be noted, however, that S1, independent of LPS treatment, also increased IL-6 proinflammatory expression, but its significance in microglial priming is still currently unknown. First, there was a significant augmentation in the transcription of the MHCII gene following S1 exposure. MHCII, a cell surface protein complex vital for immunological memory and antigen presentation, displayed elevated expression levels, consistent with the priming mechanism (Koss et al., 2019). The upregulation of MHCII on microglia indicates the priming of these cells for future encounters with bacterial or viral pathogens, leading to more antigen presentation to T-cells, thus allowing for the body to mediate a more efficient immune response. However, aberrant regulation of MHCII expression by microglia under specific neuroinflammatory conditions has been correlated with anomalous immune reactions, consequently leading to CNS tissue damage (Wong and Perlman, 2021). Likewise, S1 also upregulated NLRP3, a cytoplasmic protein integral to the inflammasome complex that plays a crucial role in the innate immune response. Upon activation, NLRP3 facilitates the maturation and release of proinflammatory cytokines, notably IL-1β, that are suggested to potentiate the

effect of S1 on LPS (He et al., 2019; Gustin et al., 2015). NLRP3 inflammasome activity is closely associated with a spectrum of neurological disorders, including Parkinson's disease, Alzheimer's disease, traumatic brain injury, multiple sclerosis, as well as neuropsychiatric conditions (Han et al., 2024; Feng et al., 2021; Liang et al., 2022; Jha et al., 2023; Liu et al., 2023; Cui et al., 2022). This suggests that NLRP3 activation leads to neuroinflammation, neuronal damage, and neuronal cell death, which mediates the pathogenesis and progression of these diseases.

Furthermore, S1 potentiated the effect of LPS exposure on both hypothalamic CD200R1 and IL-1β, indicative of microglial priming to a subsequent immune challenge. Upregulation of CD200R1, a cell surface receptor that is a distinguishing feature of microglia, contributes to the regulation of microglial activity (Zhao et al., 2020). CD200R1 contributes to microglia regulation and the upkeep of CNS homeostasis by implementing inhibitory signaling pathways within microglia, thus mitigating their activation and inflammatory responses, and averting excessive inflammation and tissue damage within the CNS. Fine-tuning microglia activation enhances their ability to modulate an effective inflammatory response to preserve CNS homeostasis; however, aberrant CD200R1 expression can lead to abnormal T-cell responsiveness and dendritic cell activity leading to autoimmune diseases, like Systemic Lupus Erythematosus (Li et al., 2012). Additionally, S1 potentiated the effect of LPS by increasing levels of IL-1β, which correlates with elevated pro-IL-1β encoding mRNA observed in a previous study examining microglial priming (Burm et al., 2015). As previously stated, NLRP3 mediates the maturation and release of IL-1β; therefore, with the observed significant increase in NLRP3 mRNA, it was expected that IL-1β mRNA levels would also rise accordingly, consistent with the

results obtained. The increase in CD200R1 and NLRP3-induced IL-1β mRNA levels, reinforces the concept of microglial priming, suggesting an augmentation in the responsiveness and vigor of the immune response to subsequent pathogenic challenges. This priming effect is of particular significance as it can exacerbate existing neurodegenerative conditions such as Parkinson's disease, Amyotrophic Lateral Sclerosis, Alzheimer's disease, and the general aging process (Nguyen et al., 2004; Field et al., 2010). The synergistic effects of S1 and LPS on CD200R1 and IL-1β show an interplay that leads to microglia potentiation and neuroinflammation to subsequent exposures, potentially exacerbating or contributing to the neurological outcomes associated with PASC.

In our study, we found that neither S1 nor LPS, when administered independently or in combination, led to significant changes in TNF and GFAP levels, suggesting minimal involvement of TNF and astrocytic reactivity, respectively, in microglial priming. TNF, a proinflammatory cytokine, is synthesized by various immune cells and holds significance in inflammation, immune regulation, neuroprotection, and cell fate determination; however, there were no discernable changes in TNF (Li et al., 2023). This discrepancy could be attributed to the fact that NLRP3 primarily mediates the production of IL-1β and may not be directly involved in the regulation of TNF. Consequently, the absence of TNF level alterations despite microglial priming could be elucidated by the specific involvement and presence of NLRP3 in IL-1β synthesis. The stable levels of GFAP expression imply minimal involvement of astrocytic reactivity in microglial priming. GFAP, a primary structural protein found in astrocytes, plays a vital role in maintaining the structural integrity and supportive functions of these glial cells in the CNS (Zheng et al., 2024). The absence of significant changes in GFAP suggests that astrocytes

may not substantially contribute to the inflammatory response or augment microglial priming induced by LPS exposure. While prior research has shown an increase in GFAP expression in response to LPS, serving as a potential indirect indicator of microglial priming due to the established astrocyte-microglia crosstalk, the constant GFAP levels in this context suggest that microglial priming induced by LPS might occur independently of astrocytic involvement (Tang et al., 2021; Olude et al., 2022). TNF and GFAP levels were notably subdued, likely due to the transient effectiveness of LPS, which has a short half-life and is rapidly cleared from the body. This characteristic, combined with the short half-life of proinflammatory cytokine mRNA transcripts, may explain the lack of significant effects on TNF and GFAP despite other indications of the microglial priming mechanism (Dinarello et al., 2017). Our findings underscore the importance of the temporal kinetics of LPS-induced inflammation; waiting for 2 days post-LPS injection to assess TNF, GFAP, and other proinflammatory cytokine mRNA transcript levels might allow sufficient time for LPS clearance and degradation of mRNA transcripts, thereby contributing to the observed minimal changes.

This study acknowledges limitations worth considering. Firstly, the study exclusively examined the proinflammatory response in male rats, thereby restricting our insight into potential sex-specific differences in the inflammatory response and the post-puberty susceptibility to neurological conditions observed in PASC patients (Loram et al., 2012). Another limitation stems from the rapid temporal kinetics and short half-life of LPS and proinflammatory cytokine mRNA transcripts, potentially overlooking the direct effects of LPS on important microglial markers, such as TNF and GFAP, within the two-day observation window following the LPS exposure. Future studies should analyze gene expression of these proinflammatory cytokines at

multiple time points within hours of LPS treatment to accurately capture immune activation dynamics.

6. CONCLUSION

Exposing adult male Sprague-Dawley rats to both the S1 spike protein of SARS-CoV-2 and the LPS bacterial mimetic in the "two-hit" exposure model was aimed to elucidate mechanisms underlying persistent neuroinflammation via microglial priming. We found that S1, independently, increased the expression of MHCII and NLRP3, which are markers of neuroinflammatory genes involved in microglial priming. In addition, we found that S1 potentiated the neuroinflammatory effects of LPS on both IL-1β and CD200R1, which is indicative of microglial priming. Finally, we found that S1 and LPS, independently as well as in combination, did not affect TNF and GFAP levels. Taken together, these findings suggest that prior exposure to the SARS-CoV-2 S1 subunit is sufficient to prime the neuroinflammatory response, potentiating microglia to subsequent immune changes.

The concept of microglia priming highlights how alterations in the microglial microenvironment and the release of specific proinflammatory cytokines can increase microglial sensitivity to subsequent proinflammatory pathogens, thereby augmenting neuroinflammation within the CNS. Our study adds valuable insight into the mechanisms underlying how the SARS-CoV-2 S1 spike protein subunit primes neuroinflammatory responses to subsequent immune challenges. Furthermore, these findings suggest that protracted neuroinflammatory priming following a SARS-CoV-2 infection might be a potential neuroimmune mechanism, which might

drive the neuroinflammatory processes underlying the cognitive, neurological, and neuropsychiatric manifestations observed in PASC/Long-COVID patients.

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