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Recombinant SARS-CoV-2 Spike Protein and Its Receptor Binding Domain Stimulate Release of Different Pro-Inflammatory Mediators via Activation of Distinct Receptors on Human Microglia Cells

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Abstract

SARS-CoV-2 infects cells via its spike (S) protein binding to its surface receptor angiotensin converting enzyme 2 (ACE2) on target cells and results in acute symptoms involving especially the lungs known as COVID-19. However, increasing evidence indicates that SARS-CoV-2 infection produces neuroinflammation associated with neurological, neuropsychiatric, and cognitive symptoms persists well past the resolution of the infection, known as post-COVID-19 sequalae or long-COVID. The neuroimmune mechanism(s) involved in long-COVID have not been adequately characterized. In this study, we show that recombinant SARS-CoV-2 full-length S protein stimulates release of pro-inflammatory IL-1b, CXCL8, IL-6, and MMP-9 from cultured human microglia via TLR4 receptor activation. Instead, recombinant receptor-binding domain (RBD) stimulates release of TNF-α, IL-18, and S100B via ACE2 signaling. These results provide evidence that SARS-CoV-2 spike protein contributes to neuroinflammation through different mechanisms that may be involved in CNS pathologies associated with long-COVID.

Keywords ACE2 · Brain · Corona virus · Cytokines · Inflammation · Microglia · Toll-like receptors · Spike protein

Abbreviations

ACE2	Angiotensin-converting enzyme-2
DAMPS	Damage-associated molecular patterns
MMP9	Matrix metalloproteinase 9
RBD	Receptor-binding domain
S	Spike protein
TLR	Toll-like receptor

Introduction

COVID-19 develops following infection with SARS-CoV-2 after binding to the surface receptor, angiotensin converting enzyme 2 (ACE2) via the receptor-binding domains (RBD) of its corona spike (S) protein [1–3]. COVID-19 is associated with a complex immune response that involves the release of a "storm" [4–6] of pro-inflammatory cytokines [4, 5, 7–13], especially IL-1β [14, 15] and IL-6 [16–19].

About 20% of patients infected with SARS-CoV-2 develop a post-acute syndrome [20] within months after the infection, named "Long-COVID" [20–27]. This condition is characterized by persistent fatigue, neurological [28–36], neurodegenerative [31, 37, 38], psychiatric [39–45], and cognitive [40–51] symptoms, especially brain fog [20, 22, 23, 39, 52–56]. In one study, most hospitalized patients had neurological symptoms that lasted at least 6 months [57].

Post-mortem analysis of brains obtained from deceased patients with COVID-19 infection showed extensive microglial activation and neuroinflammation associated with brain pathology [58–61]. SARS-CoV-2 neurotropism may trigger or exacerbate neuropsychiatric disorders [48], since microglia-induced neuroinflammation is a risk factor for the development of major depressive disorder [62, 63]. Increasing evidence indicates the involvement of neuro-inflammation [64–66] that may damage brain blood vessels [67, 68] and brain cells [64, 69, 70] possibly via activation of microglia [71, 72]. As such, long-COVID could be considered a state of "brain autoimmunity" [73].

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Microglia are resident macrophage-like cells of the central nervous system (CNS) that perform important functions [74–78] including COVID-19 [79, 80]. Microglia have also been implicated in neuroinflammatory [78, 81, 82] and neurodegenerative [74, 83, 84] diseases. Microglia express toll-like receptors (TLRs) [85] that are activated by damage associated molecular patterns (DAMPs) and one study demonstrated that SARS-CoV-2 S protein could trigger a pro-inflammatory response in cultured microglia cells [86].

Here, we show that SARS-CoV-2 spike protein contributes to neuro-inflammation through different mechanisms that may be involved in CNS pathologies associated with long-COVID.

Materials and Methods

Human Microglia Cell Culture

The immortalized human microglia-SV40 cell line derived from primary human microglia was purchased from Applied Biological Materials Inc. (ABM Inc.; Richmond, BC, Canada) and cultured in Prigrow III medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in type I collagen-coated T25-flasks (ABM Inc.). Microglia-SV40 maintain their phenotype and proliferation rates for over 10 passages, during which all experiments were performed using multiple microglia thaws and sub-cultured cells. Experiments were carried out in type I collagen-coated plates (BD PureCoat™ ECM Mimetic Cultureware Collagen I peptide plates, Becton Dickinson, Bedford, MA). Cell viability was determined by trypan blue (0.4%) exclusion.

Microglia Treatments

Human microglia were stimulated with 1–10 ng/mL of recombinant full-length SARS-CoV-2 S (Abcam, Waltham, MA and/or GeneTex, Irvine, CA), 1–10 ng/mL of RBD (Abcam, Waltham, MA and/or GeneTex, Irvine, CA), and/or pretreated with 2 µg/mL of anti-TLR2 Ab (InvivoGen, San Diego, CA), 2 µg/mL of anti-TLR4 Ab (InvivoGen, San Diego, CA), or 2 µg/mL of anti-ACE2 Ab (InvivoGen, San Diego, CA). Lipopolysaccharides (LPS) (10 ng/mL) and neurotensin (NT) (10 nM) were used as positive controls.

Proinflammatory Mediator Release

Microglia (0.5×10^5 cells/well) were seeded in 12-well, type I collagen, or poly-L-lysine–coated plates (Becton Dickinson, Bedford, MA) for 24 h before stimulation with full-length SARS-CoV-2 S (1–10 ng/mL) or RBD (1–10 ng/mL) was carried out. For selected experiments, microglia were pretreated with anti-TLR2 Ab or anti-TLR4 Ab or anti-ACE2 Ab for 24 h before stimulation with full-length SARS-CoV-2 S or RBD. After 24 h, supernatant fluids were collected and concentrations of IL-1β, CXCL8, IL-6, TNF-α, MMP9, S100B, and IL-18 were measured using commercially available ELISA DuoSet kits (DY201, DY208, DY206-05, DY210-05, DY911-05, DY1820-05, and DY318-05 respectively) from R&D Systems (Minneapolis, MN) according to the manufacturer's instructions. Control cells were treated with equal volume of culture medium in all experiments. The detection limits of IL-1β were 3.91–250 pg/mL, CXCL8 were 31.3–2000 pg/mL, IL-6 were 9.38–600 pg/mL, TNF-α were 15.6–1000 pg/mL, MMP9 were 31.3–2000 pg/mL, S100B were 46.9–3000 pg/mL, and IL-18 were 11.7–750 pg/mL.

Statistical Analysis

All in vitro conditions were performed in triplicate, and all experiments were repeated at least three times ($n = 3$). Results are presented as mean ± standard error of the mean (SEM). Differences between two groups were assessed using the Student's *t*-test. Comparisons among at least three groups were tested by one-way analysis of variance (ANOVA), and then post hoc comparisons to determine significant differences between several experimental groups and the control group and between two groups were performed using Dunnett's test and Bonferroni test, respectively. Differences with *P*-values less than 0.05 were considered statistically significant. All analyses were performed using Graph Pad Prism 5.

Results

SARS-CoV-2 Spike Protein Stimulates Secretion of Pro-Inflammatory Mediators from Human Microglia in a Dose-Dependent Manner

We analyzed the effect of full-length S protein on secretion of pro-inflammatory mediators, including IL-1β and CXCL8, in human cultured microglia. Upon stimulation with different concentrations (1, 5, or 10 ng/mL) of recombinant SARS-CoV-2 full-length S protein for 24 h, pro-inflammatory protein levels in the cell culture supernatants were significantly elevated, compared with those in controls in a dose-dependent manner (Fig. 1A, B). The data were confirmed by two different sources (Abcam and GeneTex) of recombinant SARS-CoV-2 full length S (Supplemental Fig. 1).

SARS-CoV-2 Spike Protein and RBD Stimulate Secretion of Different Pro-Inflammatory Mediators from Human Microglia

We examined the effects of recombinant SARS-CoV-2 full-length S and RBD on secretion of the pro-inflammatory mediators IL-1β, CXCL8, IL-6, TNF-α, IL-18, MMP9, and S100B from human cultured microglia. Stimulation with full-length S, but not RBD for 24 h, stimulated significant release of IL-1β,

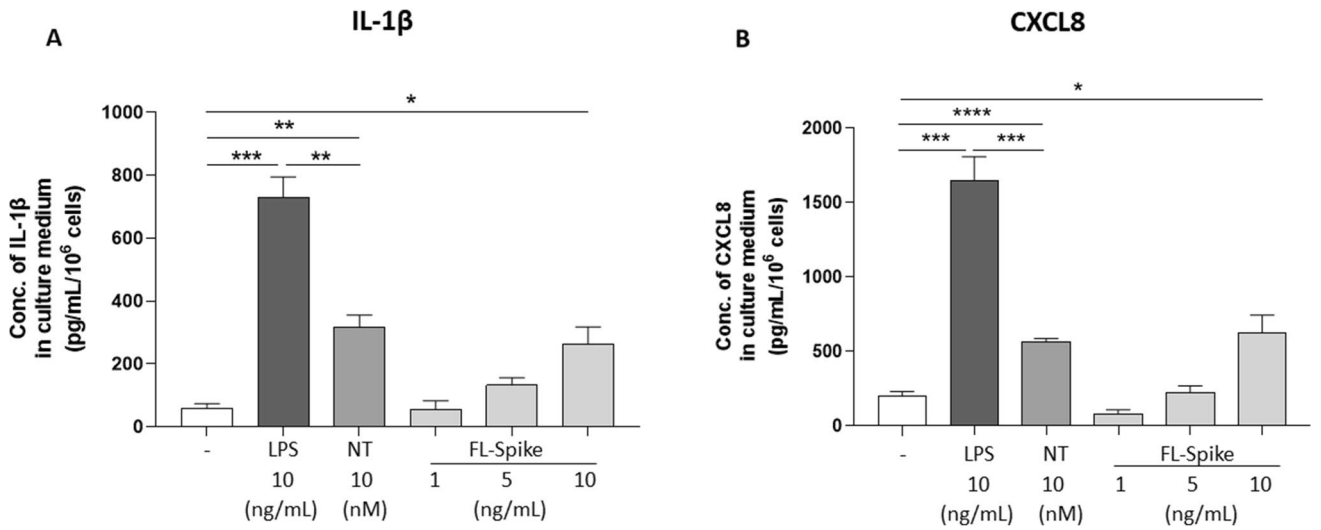


Fig. 1 SARS-CoV-2 spike protein stimulates secretion of proinflammatory mediators from human microglia in a dose-dependent manner. SV40 microglia ($1.0\times10.^5$ cells) were stimulated with recombinant SARS-CoV-2 full-length S protein (1–10 ng/mL) for 24 h. Secretion of IL-1β (**A**) and CXCL8 (**B**) was determined by specific ELISAs. LPS (10 ng/mL) and NT (10 nM) were used as "positive" controls. All conditions were performed in triplicate for each dataset and repeated 3 times ($n=3$). Significance of comparisons is denoted by $P<0.05$

CXCL8, IL-6 and MMP9 compared to controls (Fig. 2A–D). Interestingly, both full-length S and RBD were able to stimulate secretion of TNF-α and S100B levels (Fig. 2E and F). However, stimulation with RBD alone for 24 h was able to stimulate significant secretion of IL-18 compared to controls (Fig. 2G). The data were confirmed by two different sources (Abcam and GeneTex) of SARS-CoV-2 S and RBD (Supplemental Fig. 2).

SARS-CoV-2 Spike Protein Stimulates IL-1β, CXCL8, IL-6, and MMP9 Secretion from Human Microglia via TLR4

To examine whether the recombinant SARS-CoV-2 full-length S-induced pro-inflammatory responses in human microglia are mediated by TLR2 or TLR4 signaling, we pre-incubated human microglia with an anti-TLR2 Ab, anti-TLR4 Ab, and anti-ACE2 Ab. Stimulation of human microglia by full-length S (10 ng/mL) resulted in significant secretion of IL-1β, CXCL8, IL-6, and MMP9 for 24 h, which was completely suppressed by pretreatment with 2 μg/mL of anti-TLR4 Ab (Fig. 3A–D). However, pretreatment with anti-TLR2 Ab or anti-ACE2 Ab did not reduce proinflammatory mediator release (Fig. 3A, B).

SARS-CoV-2 Spike and RBD Stimulates TNF-α and S100B Secretion from Human Microglia via TLR2, TLR4, and ACE2

As TNF-α and S100B secretion from human microglia are stimulated by both full-length S and RBD, we further examined whether TLR2, TLR4, or ACE2 signaling mediate the pro-inflammatory responses. Human microglia stimulated by full-length S protein (10 ng/mL) secreted TNF-α for 24 h, which was completely suppressed by pretreatment with anti-TLR2 Ab or anti-TLR4 Ab (2 μg/mL), while S100B levels were suppressed by pretreatment with 2 μg/mL anti-TLR4 Ab only (Fig. 4A, B). RBD stimulated TNF-α and S100B secretion, which was completely suppressed by pretreatment with 2 μg/mL of anti-ACE2 Ab (Fig. 4A, B).

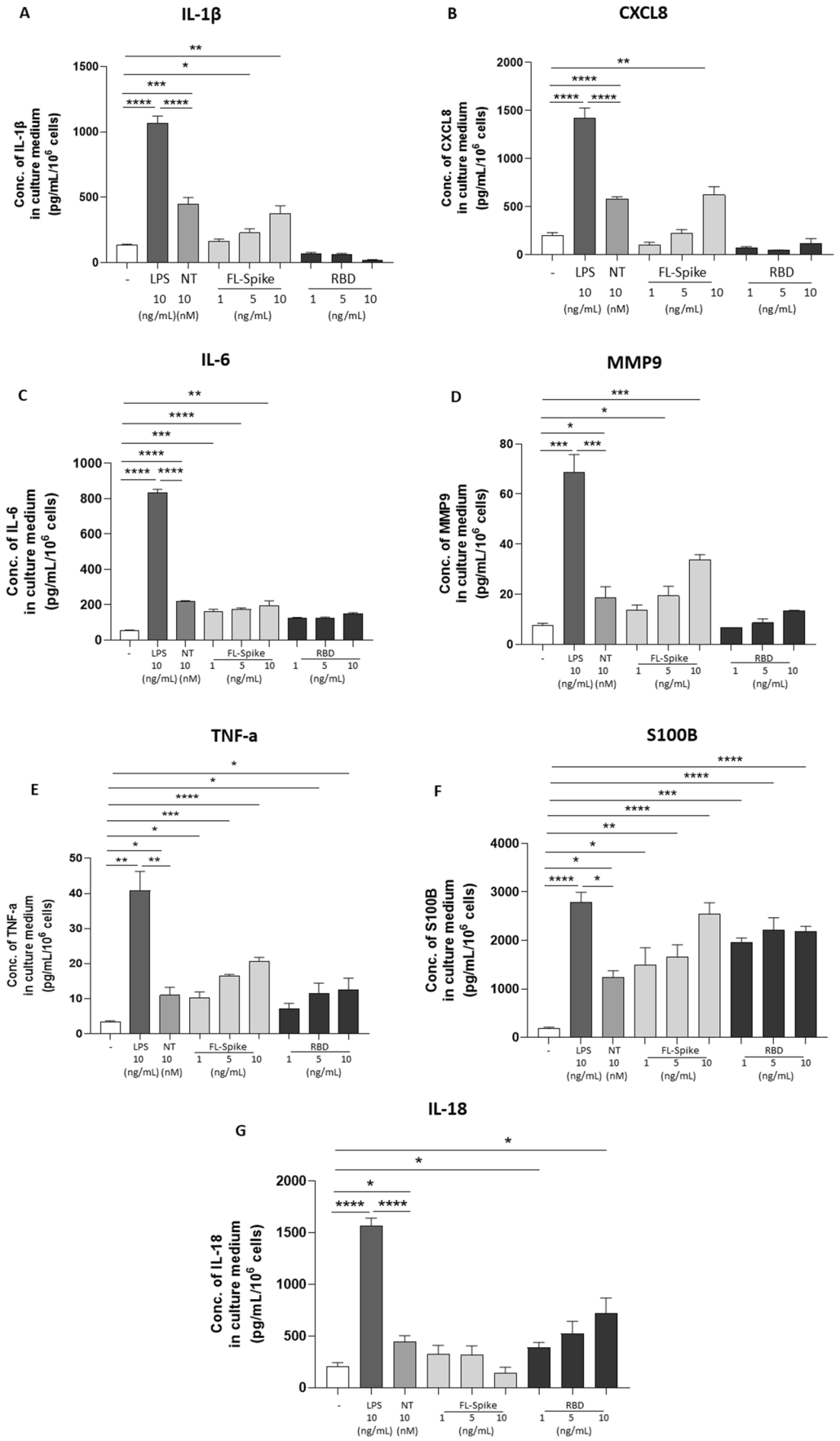
RBD Stimulates IL-18 Secretion from Human Microglia via ACE2

RBD (10 ng/mL, for 24 h) stimulated IL-18 secretion from human microglia which was completely suppressed by pretreatment with 2 μg/mL of anti-ACE2 Ab (Fig. 5).

Discussion

The SARS-CoV-2 S protein attaches to the surface receptor ACE2 via the S1 subunit containing the RBD, while the S2 subunit containing a transmembrane anchor that is needed to fuse the viral envelope with the host's cell surface membrane [1].

Our findings show that the recombinant whole length S protein and the RBD can stimulate human microglia to secrete distinct pro-inflammatory mediators via activation of different receptors. Our data further indicate that whole length S protein stimulates secretion of IL-1β and CXCL8 not via activation of ACE2, but rather activation of TLR-4. In contrast, RBD stimulates release of IL-18, TNF-α, and S100B via ACE2. SARS-CoV-2 has been reported to


A
IL-1β
Conc. of IL-1β in culture medium (pg/mL/10^6 cells)
B
CXCL8
Conc. of CXCL8 in culture medium (pg/mL/10^6 cells)
C
IL-6
Conc. of IL-6 in culture medium (pg/mL/10^6 cells)
D
MMP9
Conc. of MMP9 in culture medium (pg/mL/10^6 cells)
E
TNF-a
Conc. of TNF-a in culture medium (pg/mL/10^6 cells)
F
S100B
Conc. of S100B in culture medium (pg/mL/10^6 cells)
G
IL-18
Conc. of IL-18 in culture medium (pg/mL/10^6 cells)
-
LPS 10 (ng/mL)
NT 10 (nM)
FL-Spike 1 5 10 (ng/mL)
RBD 1 5 10 (ng/mL)

◂**Fig. 2** SARS-CoV-2 S and RBD stimulate a differential secretion of pro-inflammatory mediators from human microglia. SV40 microglia ($1.0\times10.^5$ cells) were stimulated with recombinant SARS-CoV-2 full-length S protein (1–10 ng/mL) and RBD (1–10 ng/mL) for 24 h. Secretion of IL-1β (**A**), CXCL8 (**B**), IL-6 (**C**), MMP9 (**D**), TNF-α (**E**), S100B (**F**), and IL-18 (**G**) was determined by specific ELISAs. LPS (10 ng/mL) and NT (10 nM) were used as "positive" controls. All conditions were performed in triplicate for each dataset and repeated 3 times ($n=3$). Significance of comparisons is denoted by $P<0.05$

activate TLRs [87, 88] leading to release of immune molecules that could contribute to neurologic symptoms [89]. In addition, increased levels of pro-inflammatory cytokines, especially IL-6 [90, 91], have also been detected in the CSF of COVID-19 patients [90]. SARS-CoV-2 spike protein has been reported to stimulate BV-2 microglia leading to release of IL-1β, IL-6, and TNF and increase expression of TLR4 [86]. In fact, TLR4 has been considered a therapeutic target

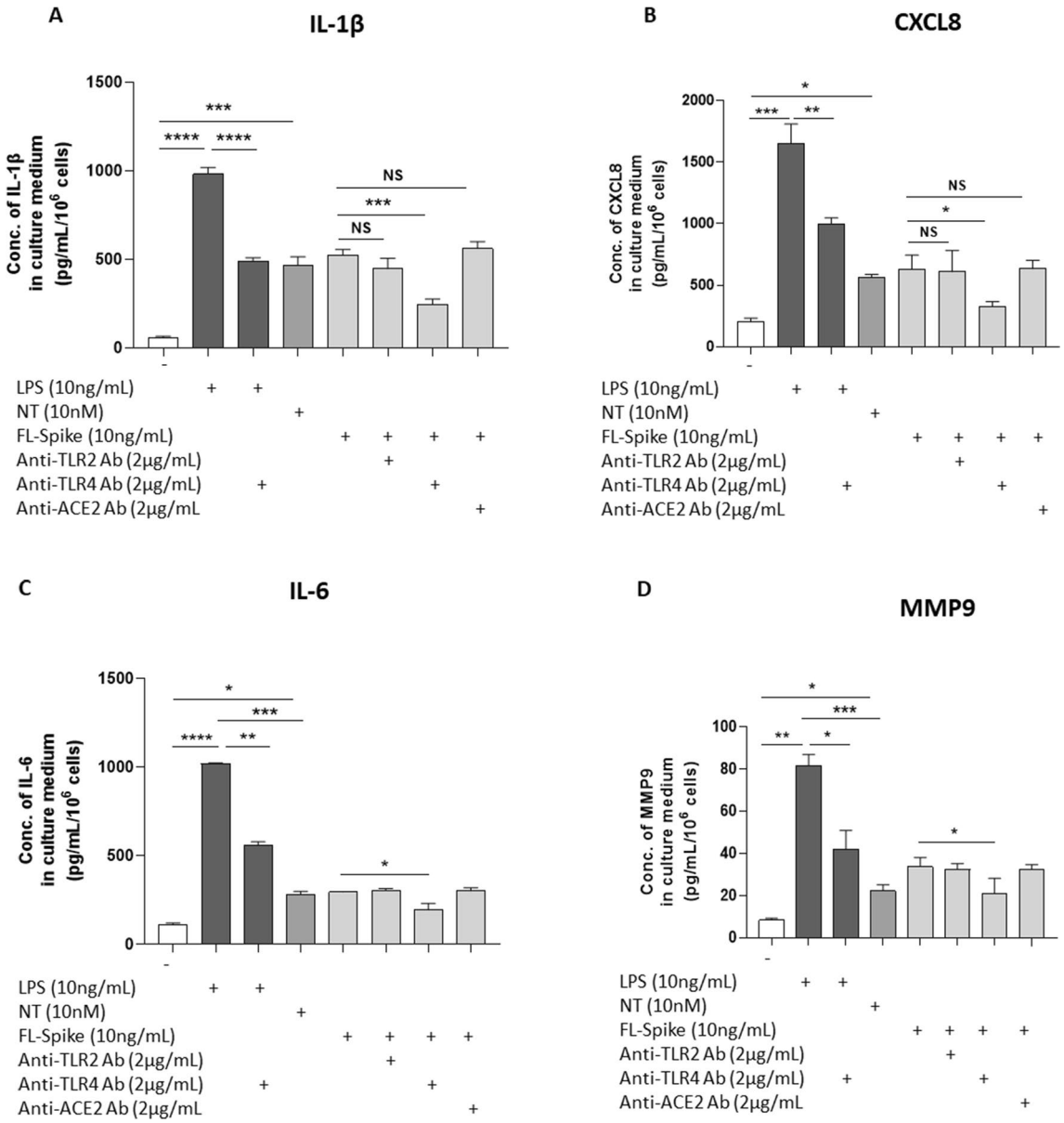


Fig. 3 Full-length SARS-CoV-2 S stimulates IL-1β, CXCL8, IL-6, and MMP9 secretion from human microglia via TLR4 signaling. SV40 microglia ($1.0\times10.^5$ cells) were pretreated with anti-TLR2 Ab (2 μg/mL), anti-TLR4 Ab (2 μg/mL), or anti-ACE2 Ab (2 μg/mL) for 24 h and then stimulated with recombinant SARS-CoV-2 full-length S (1–10 ng/mL) or RBD (1–10 ng/mL) for 24 h. Secretion of IL-1β (**A**), CXCL8 (**B**), IL-6 (**C**), and MMP9 (**D**) was determined by specific ELISAs. All conditions were performed in triplicate for each dataset and repeated 3 times ($n=3$). Significance of comparisons is denoted by $P<0.05$

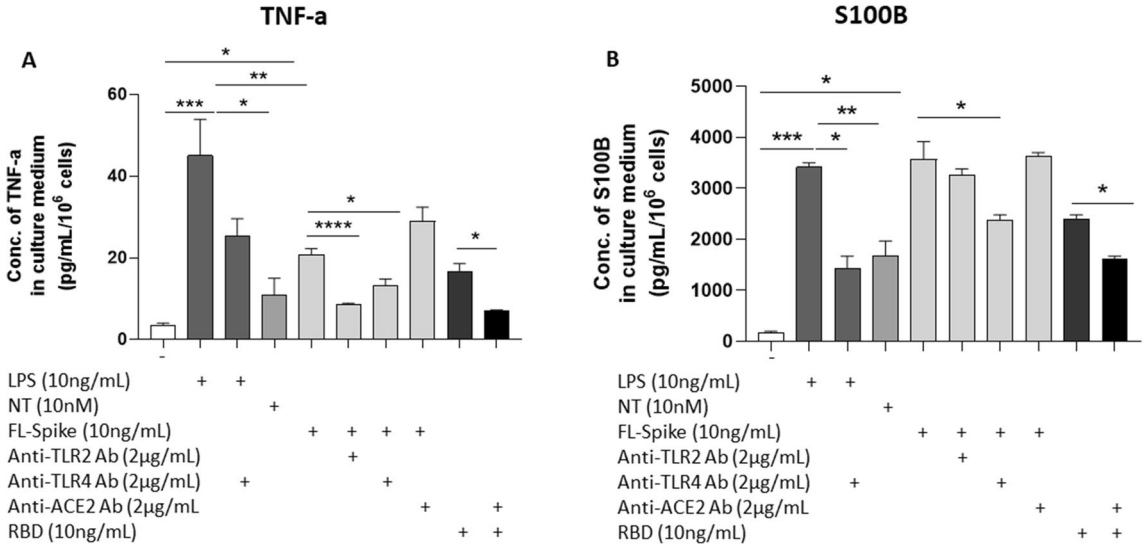


Fig. 4 SARS-CoV-2 S stimulates TNF-α and S100B secretion from human microglia via TLR2, TLR4, and ACE2. SV40 microglia ($1.0 \times 10.^5$ cells) were pretreated with anti-TLR2 Ab (2 µg/mL), anti-TLR4 Ab (2 µg/mL), or anti-ACE2 Ab (2 µg/mL) for 24 h and then stimulated with recombinant SARS-CoV-2 full-length S (1–10 ng/mL) and RBD (1–10 ng/mL) for 24 h. Secretion of TNF-α (**A**) and S100B (**B**) was determined by specific ELISAs. All conditions were performed in triplicate for each dataset and repeated 3 times ($n = 3$). Significance of comparisons is denoted by $P < 0.05$

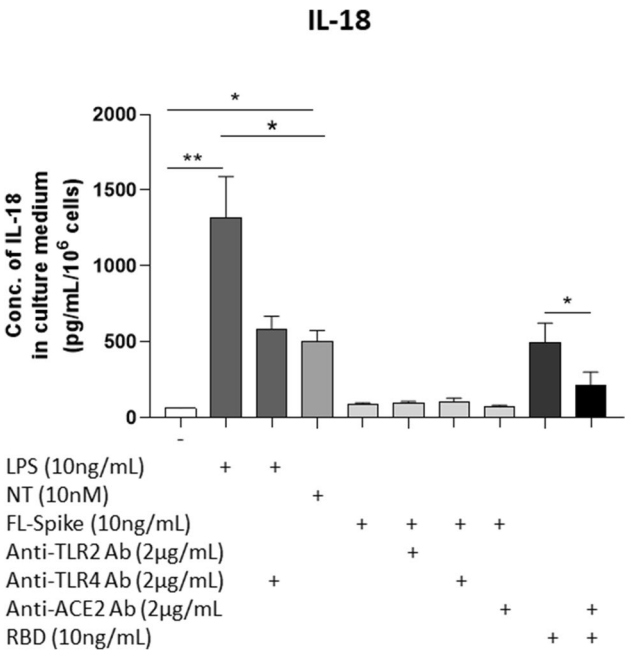


Fig. 5 RBD stimulates IL-18 secretion from human microglia via ACE2 signaling. SV40 microglia ($1.0 \times 10.^5$ cells) were pretreated with anti-TLR2 Ab (2 µg/mL), anti-TLR4 Ab (2 µg/mL), or anti-ACE2 Ab (2 µg/mL) for 24 h and then stimulated with recombinant SARS-CoV-2 full-length S (1–10 ng/mL) and RBD (1–10 ng/mL) for 24 h. Secretion of IL-18 was determined by specific ELISA. All conditions were performed in triplicate for each dataset and repeated 3 times ($n = 3$). Significance of comparisons is denoted by $P < 0.05$

for neurological complications associated with SARS-CoV-2 infection [92]. Moreover, activation of TLR4 increased expression of ACE2 [93] further enhancing viral infectivity in an autocrine loop. Another paper reported that infection of HMC3 microglia also led to the release of IL-1β, IL-6, and TNF-α [94].

Our results showing the release of IL-18 and S100B are novel. IL-18 is longer acting than other proinflammatory cytokines [95] and its expression was increased in the amygdala of children with ASD [96–98]. The mechanism of action of IL-18 is different than that of IL-1β because unlike the former, IL-18, the latter activates MAP kinases and not NF-kB [99].

S100B has been implicated in neurologic diseases [100–102]. In particular, elevated levels of S100B were associated with mild traumatic brain injury (TBI) [103], Parkinson's disease (PD) [104], ASD [105], and COVID-19 [106, 107]. In addition, S100B was reported to promote microglia polarization towards a pro-inflammatory (M1) phenotype [108, 109] suggesting autocrine effects.

Our data support the notion that the neurologic effects of COVID-19 [110–113] may be due to SARS-CoV-2 activating microglia that have been implicated in mental health disorders [114–117]. How SARS-CoV-2 enters the CNS is still unclear [118, 119]. One possibility is that the virus crosses the blood–brain barrier (BBB) [66, 120–122] or enters the brain via the olfactory nerve tract [123–125].

Our study has some limitations. We used SV40 immortalized microglia cells that are already fixed in M1 pro-inflammatory phase. Future studies will validate these results using iPSC-derived and primary microglia. Moreover, gene expression of the receptors and mediators should also be studied.

In conclusion, the SARS-CoV-2 spike protein can stimulate secretion of different pro-inflammatory molecules via activation of distinct receptors on cultured human microglia leading to neuro-inflammation [64–66] that could damage brain cells [64, 69, 70]. Preventing or minimizing the detrimental effects of the spike protein could lead to novel targeted treatment approaches [126, 127].

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12035-023-03493-7.

Author Contribution All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Eirini (Irene) Tsilioni. The first draft of the manuscript was written by Eirini (Irene) Tsilioni and Theoharis C. Theoharides. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data Availability The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

Declarations

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Research Involving Human Participants and/or Animals Not applicable.

Informed Consent Not applicable.

Competing Interests The authors declare no competing interests.

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