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SARS-CoV-2 spike glycoprotein S1 induces neuroinflammation in BV-2 microglia

Olumayokun A Olajide[+]*, Victoria U Iwuanyanwu, Oyinkansola D Adegbola

Department of Pharmacy, School of Applied Sciences, University of Huddersfield, Queensgate, Huddersfield, HD1 3DH, United Kingdom

[+] ORCID ID: 0000-0002-9254-8334

***Address for correspondence:**

Dr Olumayokun A Olajide
Department of Pharmacy, University of Huddersfield
Queensgate, Huddersfield, HD1 3DH, United Kingdom
Email: o.a.olajide@hud.ac.uk

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Abstract

The emergence of SARS-CoV-2 has resulted in a global pandemic. In addition to respiratory complications as a result of SARS-CoV-2 illness, accumulating evidence suggests that neurological and neuropsychiatric symptoms are associated with the disease caused by the virus. In this study, we investigated the effects of the SARS-CoV-2 spike glycoprotein S1 stimulation on neuroinflammation in BV-2 microglia. Analyses of culture supernatants revealed an increase in the production of TNFα, IL-6, IL-1β and iNOS/NO. SARS-CoV-2 spike glycoprotein S1 increased protein expressions of phospho-p65 and phospho-IκB, as well as enhancing DNA binding and transcriptional activity of NF-κB. Pro-inflammatory effects of the glycoprotein effects were reduced in the presence of BAY11-7082 (1 μM). The presence of SARS-CoV-2 spike glycoprotein S1 in BV-2 microglia increased the protein expression of NLRP3, as well as caspase-1 activity. However, pre-treatment with CRID3 (1 μM) or BAY11-7082 (1 μM) resulted in the inhibition of NLRP3 inflammasome/caspase-1. It was also observed that CRID3 attenuated SARS-CoV-2 spike glycoprotein S1-induced increase in IL-1β production. Increased protein expression of p38 MAPK was observed in BV-2 microglia stimulated with the spike glycoprotein S1, and was reduced in the presence of SKF 86002. These results have provided the first evidence demonstrating SARS-CoV-2 spike S1 glycoprotein-induced neuroinflammation in BV-2 microglia. We propose that promotion of neuroinflammation by this glycoprotein is mediated through activation of NF-κB, NLRP3 inflammasome and p38 MAPK. These results are significant because of their relevance to our understanding of neurological and neuropsychiatric symptoms observed in patients infected with SARS-CoV-2.

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Keywords

SARS-CoV-2; Spike glycoprotein S1; Neuroinflammation; Microglia; NF-κB

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Introduction

The emergence of the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) virus in 2019 has resulted in a global pandemic affecting most countries and territories. To date, there are 79,001,270 confirmed cases and 1,736,019 confirmed deaths, and high numbers of new cases are occurring daily [1]. The most common complications of illness due to SARS-CoV-2 infection are mainly related to respiratory symptoms [2-4]. SARS-CoV-2 infection causes significant damage to the alveolus, interstitial and intra-alveolar oedema, infiltration of inflammatory cells (mainly macrophages) [5].

In addition to respiratory complications of SARS-CoV-2 illness, accumulating evidence show that neurological and neuropsychiatric symptoms are associated with COVID-19, the disease caused by the virus [6]. These neurological and neuropsychiatric symptoms have been described in a review published by Iadecola et al. [7]. Furthermore, recent studies have suggested that SARS-CoV-2 can produce ACE2-dependent infection of neurons, resulting in neuronal damage [8]. Another study suggested that S1 promotes loss of barrier integrity and pro-inflammatory response in a 3D model of the human blood-brain barrier [9]. Other studies have proposed mechanisms of SARS-CoV-2-mediated dysfunction of the CNS [10, 11]. Further understanding of the cellular mechanisms involved in neurological and neuropsychiatric consequences of SARS-CoV-2 infection is critical for identifying pharmacological strategies for prevention and treatment.

Microglia are brain-resident macrophages that regulate brain development, maintenance of neuronal networks, and injury repair [12]. In the resting state, microglia survey their surrounding microenvironment through the projection and retraction of their highly motile processes [13]. However, in the presence of injury, or pathology within the CNS there is activation of microglia [14]. In neuroinflammation, polarised M1 microglia produce proinflammatory cytokines, neurotoxic molecules, such as tumour necrosis factor (TNF)-α, IL-6, IL-1β, nitric oxide (NO), reactive oxygen species (ROS), which contribute to neuronal dysfunction, while polarised M2 microglia secrete anti-inflammatory mediators such as IL-10 and transforming growth factor (TGF-β), which are involved in restoring homeostasis [15-18].

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Microglia-mediated neuroinflammation plays significant roles in many neurological and neuropsychiatry diseases, including neurodegenerative [19-23] and neuropsychiatric disorders [24-27].

In view of accumulating evidence linking SARS-CoV-2 infection with neurological and neuropsychiatric symptoms, this study was aimed at determining whether the SARS-CoV-2 spike S1 glycoprotein activates BV-2 microglia to cause elevated release of pro-inflammatory mediators.

Materials and methods

Materials

Recombinant human coronavirus SARS-CoV-2 spike glycoprotein S1 (Accession MN908947) was purchased from Abcam. The protein was suspended in sterile water for functional studies. The following drugs were used: BAY11-7082 (Sigma), CRID3 sodium salt (Tocris) and SKF 86002 dihydrochloride (Tocris).

Cell culture

BV-2 mouse microglia cell line (ICLCATL03001) was purchased from Interlab Cell Line Collection (Banca Biologica e Cell Factory, Italy) and cultured in RPMI medium supplemented with 10% foetal bovine serum.

Determination of cytokine production

Cultured BV-2 microglia were seeded out in 24-well plate at 5×10^4 cells/mL and treated with S1 glycoprotein (10, 50 and 100 ng/mL) for 24 h. Thereafter, medium was collected and centrifuged to obtain culture supernatants. Levels of TNFα in the supernatants were determined using mouse instant ELISA™ kit (Thermo Scientific). Concentrations of TNFα in supernatants were calculated from a mouse TNFα standard curve, and the assay range was 31.3-2,000 pg/mL. Levels of IL-6 in supernatants were determined using IL-6 mouse ELISA kit (Thermo Scientific). The range for IL-6 detection was 4-500 pg/mL. Similarly, levels of IL-1β were evaluated using IL-1β mouse ELISA kit (Thermo Scientific), with a range of detection of 7.8-500 pg/mL.

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Measurement of nitric oxide (NO) production

The Griess reagent (Promega) was used to determine the levels of levels of nitric NO) in supernatants of cultured BV-2 microglia treated with S1 glycoprotein (10, 50 and 100 ng/mL) for 24 h. Following treatment with S1 glycoprotein, the Griess assay was carried out by adding 50 μL of sulphanilamide to culture supernatants (50 μL), followed by incubation in the dark at room temperature for 10 min. of Thereafter 50 μL of N-1-naphthyl ethylenediamine dihydrochloride (NED) was added and the mixture incubated for a further 10 min. Absorbance was measured at 540nm using a Tecan Infinite M microplate reader. Nitrite concentrations were determined from a nitrite standard reference curve.

In cell-western/cytoblot

The in-cell western is a proven method for the rapid quantification of proteins in cells [28, 29]. BV-2 microglia were seeded into a 96-well plate at 5×10^4 cells/mL. The cells were treated at 70% confluence. At the end of each experiment, cells were fixed with 8% paraformaldehyde solution (100 μL) for 15 min., followed by washing with PBS. The cells were then incubated with primary antibodies overnight at 4°C. The following antibodies were used: rabbit anti-iNOS (Abcam), rabbit anti-phospho-p65 (Cell Signalling Technology), rabbit anti-phospho-IκBα (Santa Cruz Biotechnology), rabbit anti-NLRP3 (Abcam), and rabbit anti-phospho-p38 (Cell Signalling Technology) antibodies. Thereafter, cells were washed with PBS and incubated with anti-rabbit HRP secondary antibody for 2 h at room temperature. Then, 100 μL HRP substrate was added to each well and absorbance measured at 450nm with a Tecan Infinite M microplate reader. Readings were normalised with Janus Green normalisation stain (Abcam).

NF-κB luciferase reporter gene assay

BV2 cells were seeded in 24-well plate at a density of 4×10^4 cells/mL. At 60% confluence, RPMI medium was replaced with Opti-MEM, with a further incubation for 2 h at 37°C. Transfection of BV-2 cells was performed by preparing a Glia-Mag transfection reagent (OZ Biosciences) and Cignal NF-κB luciferase reporter (Qiagen) complex at a ratio 3:1 in 50 μL Opti-MEM. The complex was added to BV-2 cells, and the plate placed on a magnetic plate (OZ Biosciences) and incubated at 37°C for 30 min, followed by a further magnetic plate-free incubation for 2 h.

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Thereafter, medium was changed to serum-free RPMI and cells treated with S1 glycoprotein (10, 50 and 100 ng/mL) for 6 h. This was followed by a Dual-Glo® reporter assay (Promega). Firefly and renilla luminescence were measured using a FLUOstar OPTIMA microplate reader (BMG Labtech).

NF-κB transcription factor binding assay

The NF-κB p65 transcription factor assay is a non-radioactive ELISA-based assay for evaluating DNA binding activity of NF-κB in nuclear extracts. BV-2 microglia were seeded in a 6-well plate at a density of 4×10^4 cells/mL. The cells were then incubated with 10, 50 and 100 ng/mL of S1 glycoprotein for 60 min. At the end of the incubation, nuclear extracts were prepared from the cells and subjected to NF-κB transcription factor binding assay according to the instructions of the manufacturer (Abcam).

Caspase-Glo®1 inflammasome assay

The caspase-Glo®1 inflammasome assay (Promega) was used to measure the activity of caspase-1 directly in live cells or culture supernatants [30]. BV-2 microglia were seeded out in 24-well plate at a density of 4×10^4 cells/mL and stimulated with S1 glycoprotein (10, 50 and 10 ng/mL) for 24 h. After incubation, cell culture supernatants were collected and mixed with equal volume of Caspase-Glo® 1 reagent or Caspase-Glo® 1 reagent + YVAD-CHO (1 μM) in a 96-well plate. The contents of the wells were mixed using a plate shaker at 400rpm for 30 seconds. The plate was then incubated at room temperature for 60 min, followed by luminescent measurement of caspase-1 activity with a FLUOstar OPTIM reader (BMG LABTECH).

Effects of BAY11-7082, CRID3 and SKF 86002 on S1-induced neuroinflammation

The effects of the NF-κB inhibitor (BAY11-7082), NLRP3 inhibitor (CRID3) and p38 MAPK inhibitor (SKF 86002) on the release of TNFα, IL-6 and IL-1β production was investigated by pre-treating BV-2 microglia with BAY11-7082 (1 μM), CRID3 (1 μM), and SKF 86002 (1 μM) 60 min prior to stimulation with S1 glycoprotein (100 ng/mL) for a further 24 h. Culture supernatants were collected and analysed for levels of TNFα, IL-6 and IL-1β using mouse ELISA kits (Thermo Scientific).

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Statistical analyses

Data are expressed as mean ± SEM for at least three independent experiments (n=3) and analysed using one-way analysis of variance (ANOVA) with post hoc Tukey's test (for multiple comparisons). Statistical analysis were conducted using the GraphPad Prism software (version 9).

Results

Stimulation of BV-2 microglia with spike glycoprotein S1 resulted in elevated release of TNFα, IL-6 and IL-1β

In initial experiments to investigate the effects of S1 on the release of pro-inflammatory mediators in BV-2 microglia, 10, 50 and 100 ng/mL of the recombinant glycoprotein was incubated with the cells for 24 h. At 10 ng/mL of the glycoprotein, there was an insignificant ($p<0.05$) elevation in the secretion of TNFα. However, on increasing its concentration to 50 ng/mL, a significant ($p<0.05$) increase in the production of TNFα was observed (Figure 1A). On increasing the concentration of S1 to 100 ng/mL, there was a ~15-fold increase in the levels of TNFα in culture supernatants (Figure 1A).

Release of IL-6 was not significantly elevated in BV-2 microglia treated with 10 and 50 ng/mL of S1, in comparison with untreated (control) cells. Incubating the cells with 100 ng/mL of S1 resulted in ~10.2-fold and significant ($p<0.001$) increase in the secretion of IL-6 into culture medium (Figure 1B). Similar observations were made in experiments to determine the effects of S1 on IL-1β production; at 10 and 50 ng/mL of S1, there was no significant effect on the release of IL-1β. A ~5.5-fold elevation of IL-1β levels was however observed with 100 ng/mL of S1 (Figure 1C).

Spike glycoprotein S1 induced an increase in NO production and iNOS protein expression

Analyses of culture supernatants obtained from BV-2 microglia incubated with S1 (10 ng/mL) showed insignificant ($p<0.05$) increase in the levels of the inflammatory mediator, when compared with untreated (control) cells. We further showed that incubation with 50 and 100 ng/mL of the glycoprotein caused ~5.1 and 8.7 increase in NO production, respectively ($p<0.01$) (Figure 2A).

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We used cytoblot analyses to further demonstrate that incubation with S1 (50 and 100 ng/mL) resulted in significant ($p<0.05$) increase in protein levels of iNOS (Figure 2B).

Spike glycoprotein S1 activated NF-κB in BV-2 microglia

Following our observation that SARS-CoV-2 spike glycoprotein S1 induced an increase in the production of pro-inflammatory mediators at 100 ng/mL, we were next interested in evaluating the role of NF-κB in its activity at this concentration. Firstly, we investigated the effect of SARS-CoV-2 spike glycoprotein S1 on DNA binding activity of NF-κB, and observed that following treatment of BV-2 microglia with S1 (100 ng/mL) for 60 min, there was a significant ($p<0.001$) increase in the binding of NF-κB in nuclear extracts to consensus-binding sites. Interestingly, pre-treatment of BV-2 cells with the NF-κB inhibitor, BAY11-7082 (1 μM) prior to stimulation with S1 produced an inhibition in DNA binding (Figure 3A).

We also used the luciferase reporter gene assay to evaluate the effects of S1 on transcriptional activity of NF-κB. Incubation of S1 (100 ng/mL) with BV-2 microglia that were transfected with an NF-κB reporter for 60 min resulted in a significant ($p<0.01$) increase in luciferase activity, in comparison with untreated transfected cells. We further showed that when the cells were pre-treated with BAY11-7082 (1 μM), S1-induced increase in NF-κB luciferase activity was markedly reduced (Figure 3B).

The effects of S1 on nuclear events of NF-κB signalling prompted us to evaluate modulation of upstream targets in the pathway. Results of cytoblot analyses in Figures 3C and 3D show that S1 increased protein levels of both phospho-p65 and phospho-IκBα following incubation with BV-2 microglia for 15 min ($p<0.01$). Results also show that protein levels of phospho-p65 and phospho-IκBα were reduced in the presence of BAY11-7082 (1 μM).

Pre-treatment with BAY11-7082 reduced S1-induced production of pro-inflammatory mediators

In order to further confirm the roles of NF-κB in SARS-CoV-2 spike glycoprotein S1-induced neuroinflammation, BV-2 microglia were pre-treated with BAY11-7082 prior to stimulation with the glycoprotein for 24 h and levels of pro-inflammatory cytokines

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(TNFα, IL-6 and IL-1β) were measured in culture supernatants (Figures 4A, 4B and 4C). In the presence of BAY11-7082 (μM), S1-induced increases in the production of TNFα, IL-6 and IL-1β in BV-2 microglia were reduced by ~59.6%, ~40.6% and ~32.8%, respectively.

Results in Figures 5A and 5B also show that S1-induced increase in NO production and iNOS protein expression were significantly ($p<0.01$) reduced when the cells were treated with BAY11-7082 (1 μM) prior to S1 stimulation.

Spike glycoprotein S1 triggers activation of NLRP3 inflammasome/caspase-1 in BV-2 microglia

The NLRP3 inflammasome is known to contribute to the secretion of IL-1β during inflammation. Encouraged by the results of experiments showing an increase in the production of this cytokine following BV-2 stimulation with S1, we next investigated it's effect on protein levels of NLRP3 in the presence and absence of known NLRP3 inhibitors, CRID3 and BAY11-7082. Cytoblot analyses revealed that stimulation of BV-2 microglia with S1 (100 ng/mL) for 6 h resulted in ~11-fold increase in levels of NLRP3 protein (Figure 6A). It was also observed that S1-induced increase in NLRP3 protein levels were significantly reduced ($p<0.05$) in the presence of CRID3 (1 μM) and BAY11-7082 (1 μM) (Figure 6A).

We next determined measured caspase-1 activity in culture media of BV-2 cells that were stimulated with spike glycoprotein S1 for 6 h. Results in Figure 6B show a ~4.4-fold increase in caspase-1 activity as a result of S1 stimulation. Furthermore, both CRID3 (1 μM) and BAY11-7082 (1 μM) produced significant ($p<0.001$) inhibition in S1-induced increased caspase-1 activity (Figure 6B).

CRID3 prevented S1-induced increase in IL-1β production

Experiments to evaluate the effects of CRID3 on S1-induced increased secretion of TNFα, IL-6 and IL-1β in BV-2 microglia revealed that pre-treatment with CRID3 (1 μM) was ineffective in reducing TNFα (Figure 7A) and IL-6 (Figure 7B) production. However, in the presence of CRID3 S1-induced increased IL-1β production was significantly reduced ($p<0.001$) (Figure 7C). S1-induced NO production was not reduced by pre-treatment with CRID3 (Figure 7D).

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Effect of spike glycoprotein S1 on p38 MAPK activation

Incubation of spike glycoprotein S1 (100 ng/mL) with BV-2 microglia for 60 min resulted in a significant ($p<0.01$) and ~11.3-fold increase in protein levels of phospho-p38; an outcome that was prevented by pre-treating the cells with SKF86002 (1 μM) for 60 min prior to S1 stimulation (Figure 8).

S1-induced increase in pro-inflammatory cytokine production was reduced by SKF86002

Based on the results showing that S1 caused an increase in protein levels of phospho-p38 and its inhibition by SKF86002, we were next interested to determine the effects of this inhibitor on cytokine production in S1-stimulated BV-2 microglia. In the presence of SKF86002 (1 μM), TNFα production was reduced by ~47.9% compared with S1 alone (Figure 9A). Similarly, S1-induced increased production of IL-6 was significantly ($p<0.05$) reduced in the presence of SKF86002 (1 μM) (Figure 9B). However, there was insignificant reduction in the production of IL-1b by SKF86002 (Figure 9C).

Discussion

In SARS-CoV-2 infection, viral attachment, fusion and entry into the host's cells are facilitated by the spike glycoproteins which protrude from the surface of mature virions, by binding to the host ACE2 protein [31, 32]. Studies have shown that in addition to facilitating its fusion to the cell membrane, the location of the S glycoprotein on SARS-CoV-2 also makes it a direct target for host immune responses [31].

We report for the first time that a recombinant SARS-CoV-2 spike glycoprotein sub-unit S1 activated BV-2 microglia to induce significantly increased release of TNFα, IL-6 and IL-1β. This is a significant outcome based on the role of microglia activation in neurological and neuropsychiatric conditions, and findings linking SARS-CoV-2 infection with these conditions. It is highly likely that S1-induced production of high concentrations of pro-inflammatory cytokines in the microglia results in neuronal damage and/or dysfunction in some of these CNS conditions. In Parkinson's disease for example, significant increase in the expression of IL-1β was shown in the substantia nigra and frontal cortex, compared to controls [33]. Pro-inflammatory

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cytokine (IL-1β, TNF-α, IL-6) release has also been linked to depression-like behaviours and cognitive defects in mice [34]. Activation of neuroinflammatory processes by the spike S1 glycoprotein was further confirmed by results showing increased iNOS-mediated production of NO by the protein in microglia. Spike S1 glycoprotein-induced elevated NO production in the microglia is a significant finding in our understanding of the mechanisms involved in SARS-CoV-2-mediated neurological and neuropsychiatric symptoms. Elevated iNOS/NO has been strongly linked to a wide range of CNS disorders including Alzheimer's disease, Parkinson's disease, multiple sclerosis, epilepsy, and migraine [35]. These results reflect the outcome of a study which reports that the SARS-CoV-2 spike glycoproteins trigger a pro-inflammatory response on brain endothelial cells, thus contributing to an altered state of BBB function [6]. Our results showing SARS-CoV-2 spike S1 glycoprotein-induced neuroinflammation in the microglia suggests that this mechanism may be contributing to some of the neurological and neuropsychiatric symptoms in SARS-CoV-2 infected patients.

In neuroinflammation, the NF-κB transcription factor regulates the production of multiple pro-inflammatory genes, including the pro-inflammatory cytokines such as TNFα, IL-6 and IL-1β, as well as iNOS. We further showed that activation of microglial NF-κB signalling mediates the production of pro-inflammatory mediators in the microglia by SARS-CoV-2 spike S1 glycoprotein through its ability to promote cytoplasmic phosphorylation of the p65 sub-unit and IκBα, as well as DNA binding of p65 sub-unit and NF-κB transcriptional activity. Interestingly, these effects were blocked by the potent NF-κB inhibitor, BAY11-7082. The involvement of NF-κB in S1 glycoprotein-induced neuroinflammation was further confirmed by results showing the effectiveness of BAY11-7082 in blocking S1 glycoprotein-induced production of TNFα, IL-6 and IL-1β, and iNOS/NO in BV-2 microglia. In a study reported by Patra et al., SARS-CoV-2 spike glycoprotein was shown to promote IL-6 signalling through NF-κB in epithelial cells to initiate coordination of a hyper-inflammatory response [36]. To our knowledge, this is the evidence supporting the role of NF-κB in S1 glycoprotein-induced microglia activation.

There have been reports in the scientific literature suggesting that the NLRP3 inflammasome may be contributing to the release of cytokines such as IL-1β during

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SARS-CoV-2-induced hyper-inflammation [37-39]. In the microglia, activation of NLRP3 by extracellular ATP, certain bacterial toxins, crystalline and particulate matters results in caspase-1 activation which then cleaves the precursors of IL-1β and IL-18 to generate active IL-1β and IL-18. These mechanisms results in the neuroinflammation and pyroptosis [40-42]. In this study, we showed that in addition to increasing IL-1β production, SARS-CoV-2 spike S1 glycoprotein activated NLRP3 inflammasome and increased caspase-1 activity in BV-2 microglia. These effects were shown to be attenuated by CRID3 and BAY11-7082, which are known inhibitors of NLRP3. Furthermore, pre-treatment of BV-2 microglia with CRID3 prevented SARS-CoV-2 spike S1 glycoprotein-induced IL-1β-production, but not TNFα, IL-6 or NO, thereby confirming a role for NLRP3 in neuroinflammation induced by this glycoprotein in BV-2 microglia.

It is not clear from this study if the activation of NLRP3/caspase-1 by SARS-CoV-2 spike S1 glycoprotein was due to a direct effect on NLRP3 or an indirect action as a result of activating NF-κB. It has been suggested that activation of NF-κB could trigger NLRP3 through caspase-1, with subsequent release of mature IL-1β [43]. The roles of NLRP3 inflammasome activation in SARS-CoV-2 spike S1 glycoprotein-induced neuroinflammation requires further investigation.

SARS-CoV-2 spike S1 glycoprotein induced activation of p38 MAPK through an increase in protein levels of phospho-p38 in BV-2 microglia. This effect was blocked in the presence of SKF 86002, a p38 inhibitor. In addition, SKF 86002 reduced SARS-CoV-2 spike S1 glycoprotein-induced increased production of TNFα and IL-6, which further shows a modulation of p38 MAPK signalling by the glycoprotein. While studies have shown that there is a cross-talk between NF-κB and p38 MAPK activation which may account for these results, further studies will establish the exact molecular target of SARS-CoV-2 spike S1 glycoprotein effect on p38 activation pathway in the microglia.

The results obtained from these experiments have provided the first evidence demonstrating activation of BV-2 microglia by SARS-CoV-2 spike S1 glycoprotein, resulting in the production pro-inflammatory mediators TNFα, IL-6, IL-1β and NO. We propose that induction of neuroinflammation by this glycoprotein in the microglia is mediated through activation of NF-κB, through a potential cross-talk with NLRP3

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inflammasome and p38 MAPK. These results are significant because of their relevance to our understanding of neurological and neuropsychiatric symproms observed in patients infected with SARS-CoV-2.The possible involvement of microglia ACE2 and the toll-like receptors (TLRs) in the effects of the glycoprotein have not been evaluated in this study and will be a focus of follow-up investigations.

Author contributions

Olumayokun A Olajide: Conceptualisation, Methodology, Investigation, Writing - Original Draft, Writing- Review & Editing, Project administration. **Victoria U Iwuanyanwu**: Investigation. **Oyinkansola D Adegbola**: Investigation.

Declarations: Conflicts of Interest

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

Data availability statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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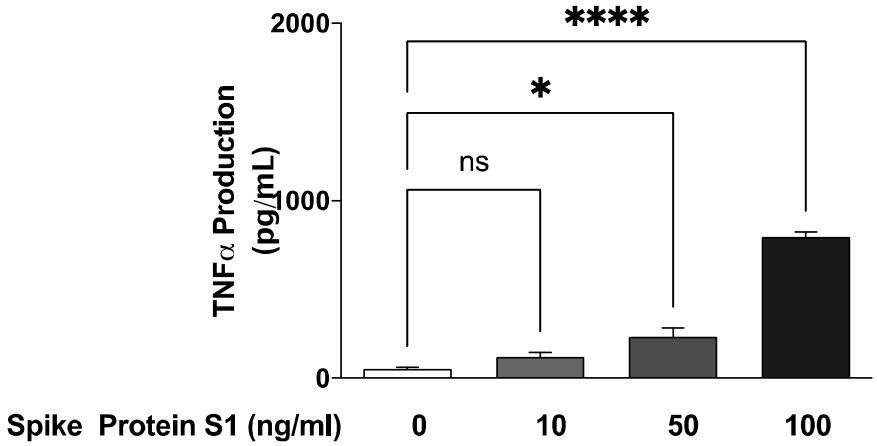
Figure 1A



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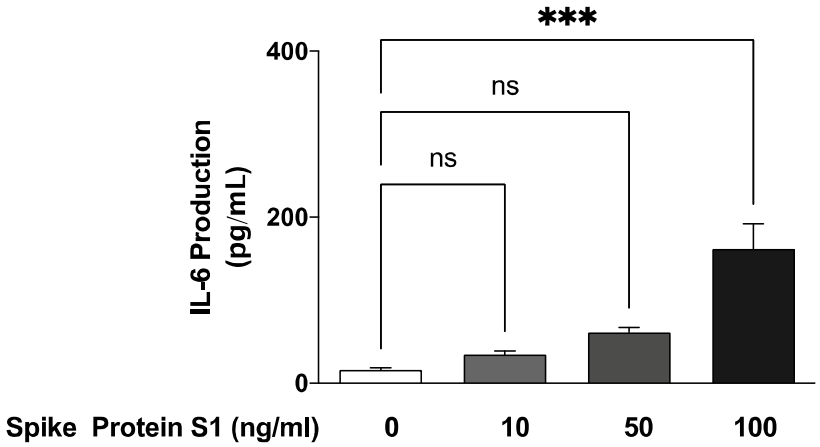

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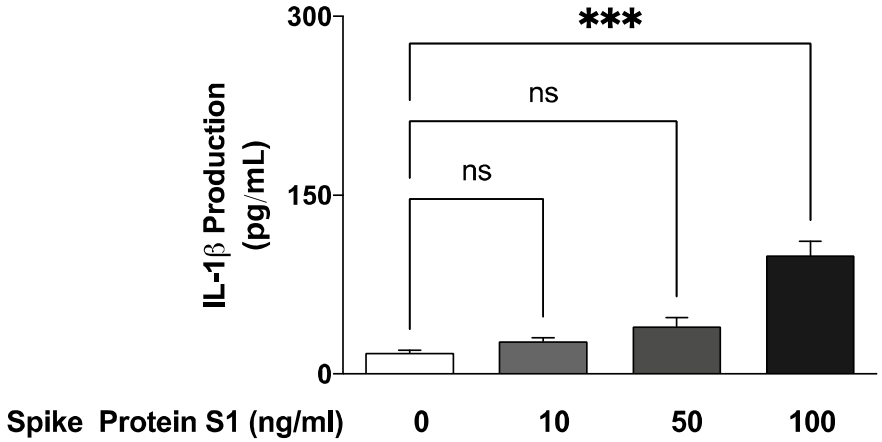

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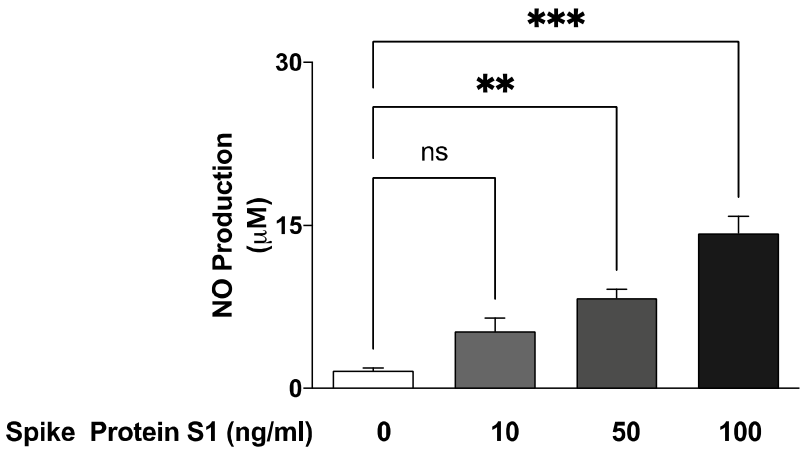

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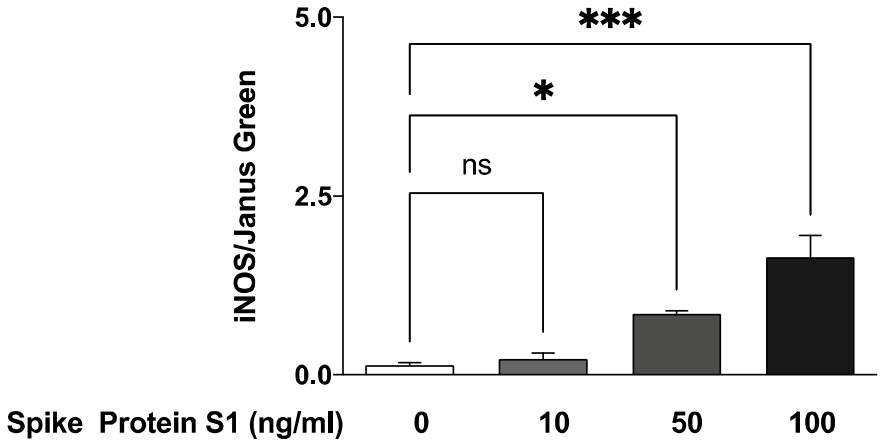

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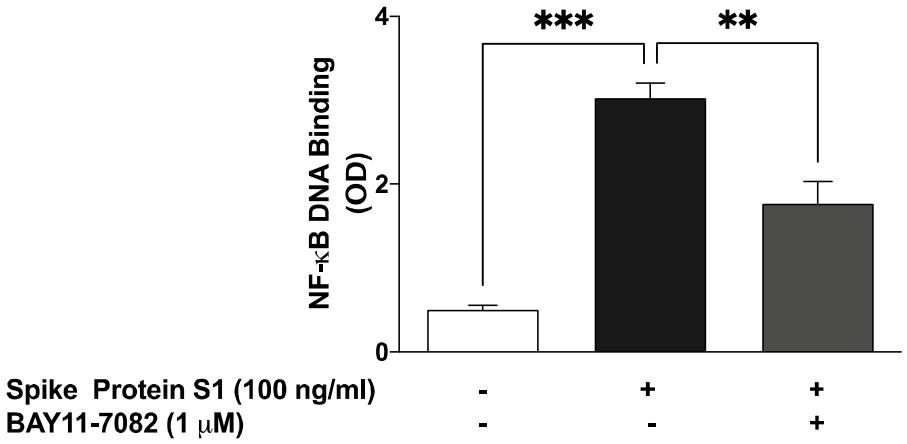
Figure 3A



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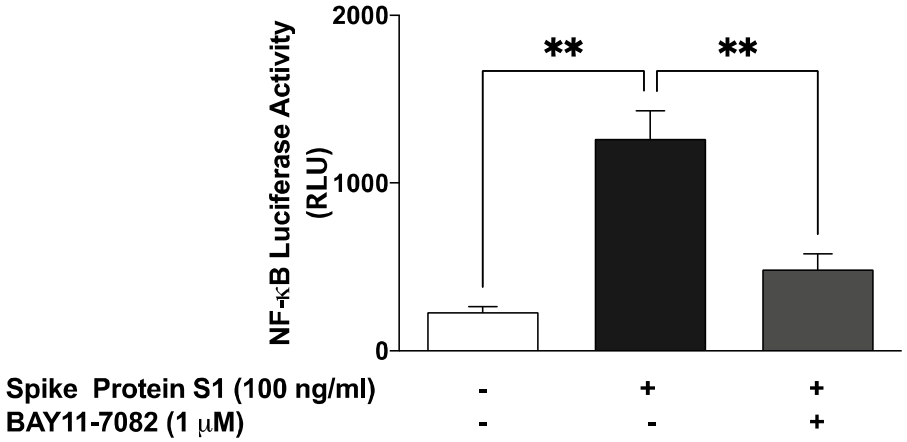

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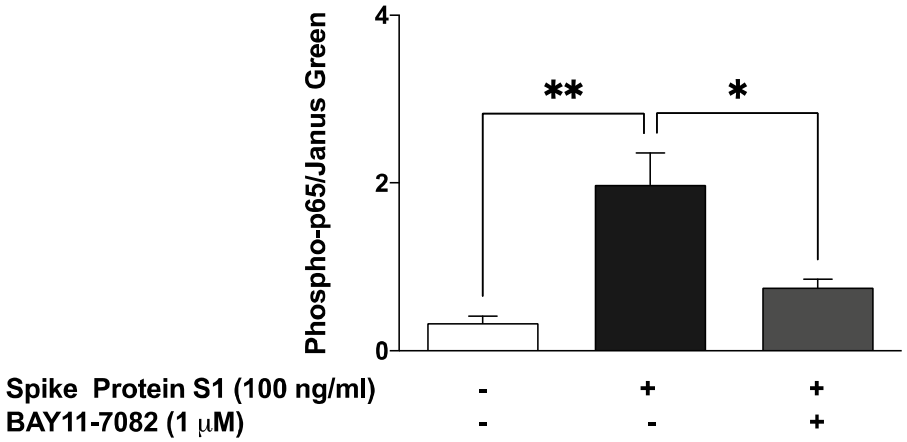

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Figure 3D

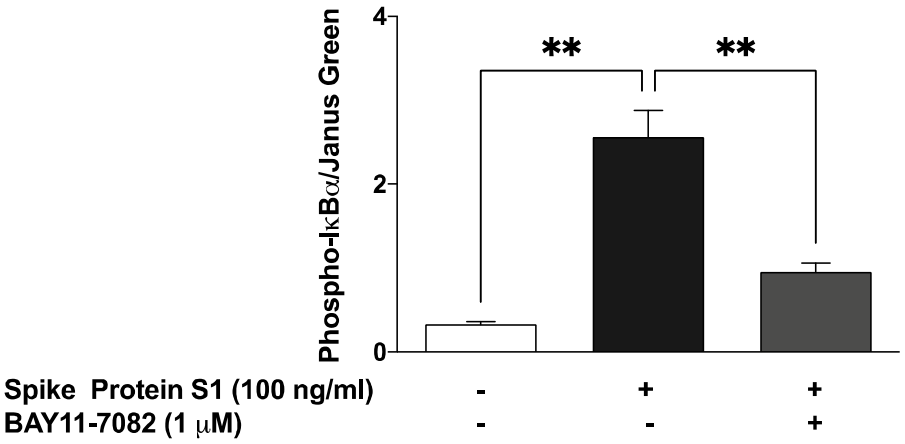

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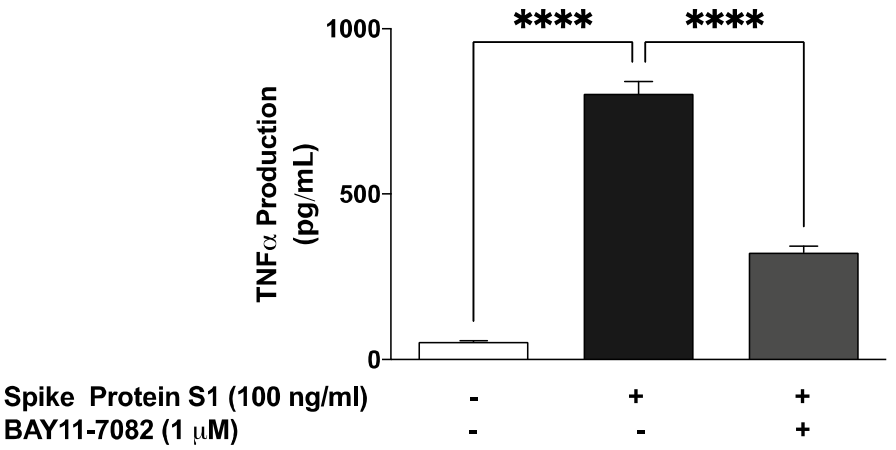
Figure 4A



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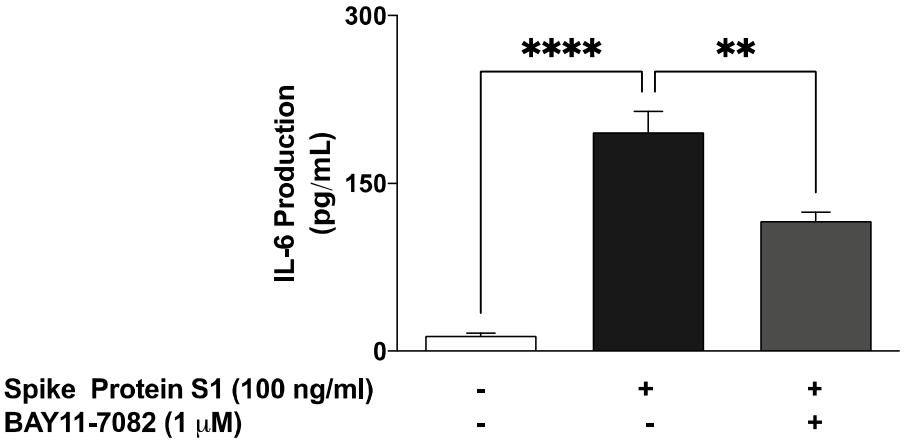

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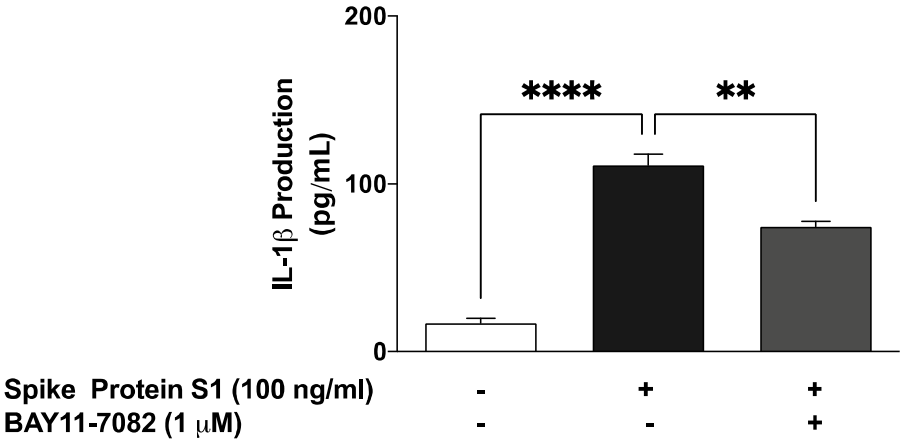

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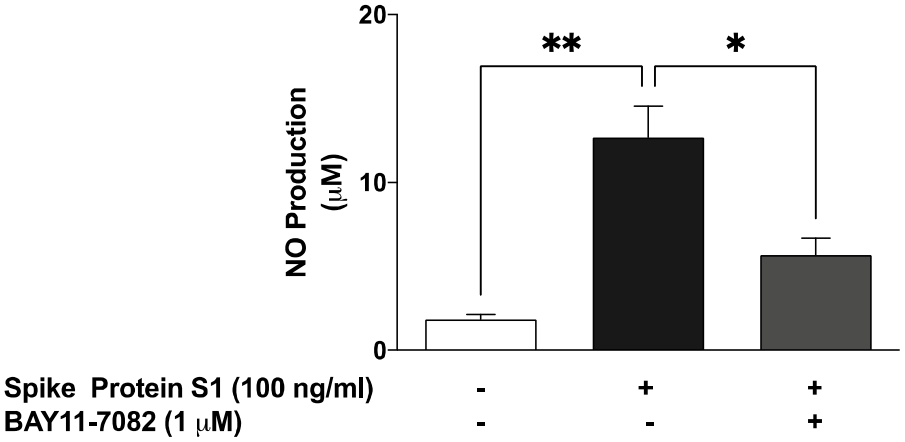
Figure 5A



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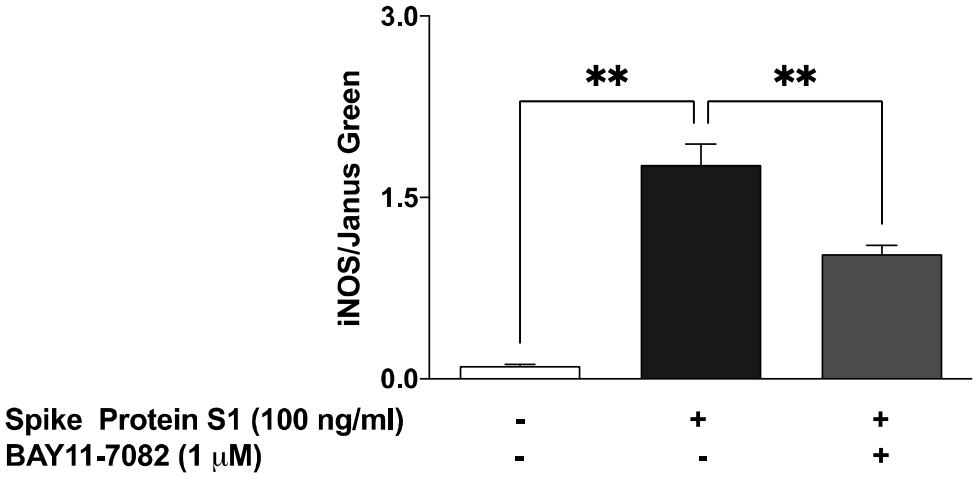

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Figure 6A

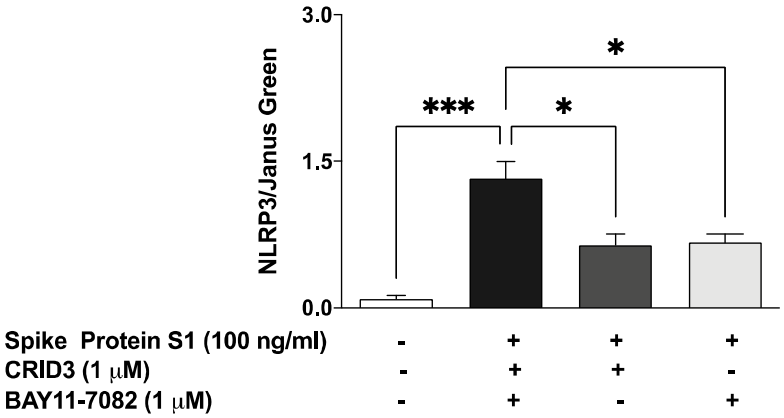

Figure 6B

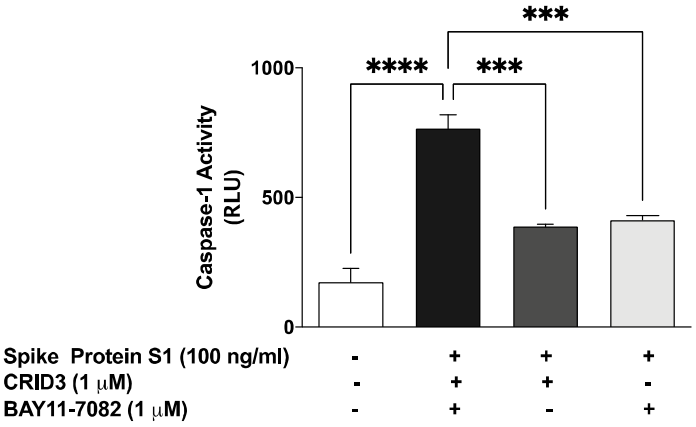

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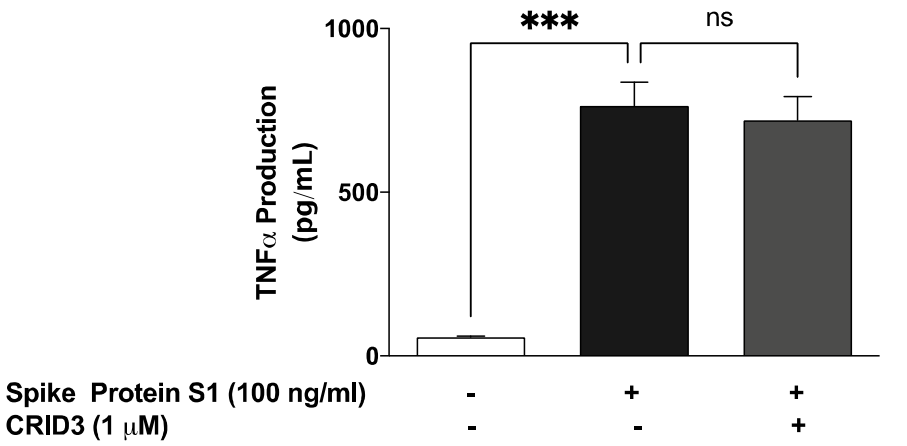
Figure 7A



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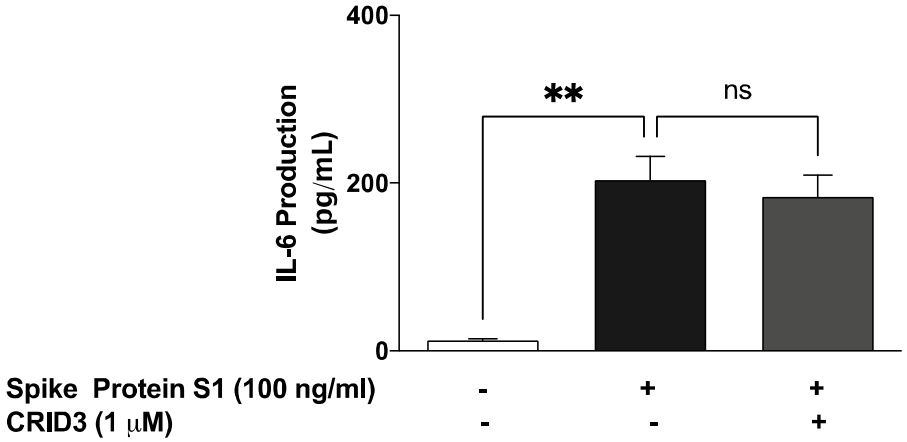

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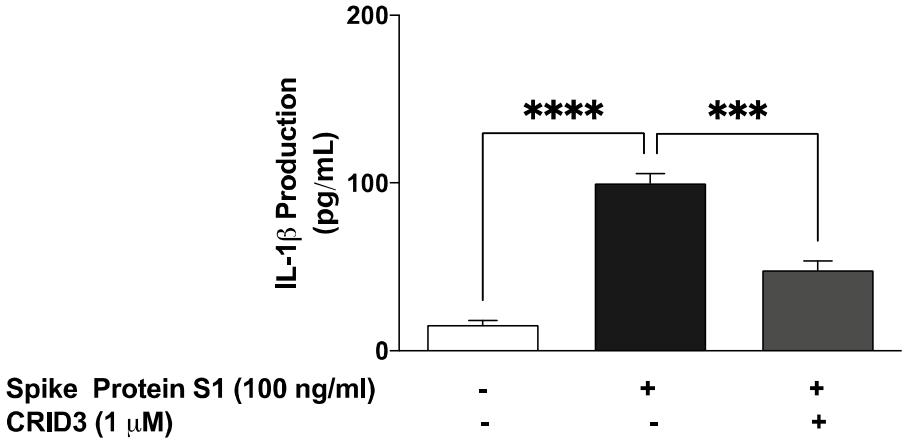

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Figure 7D

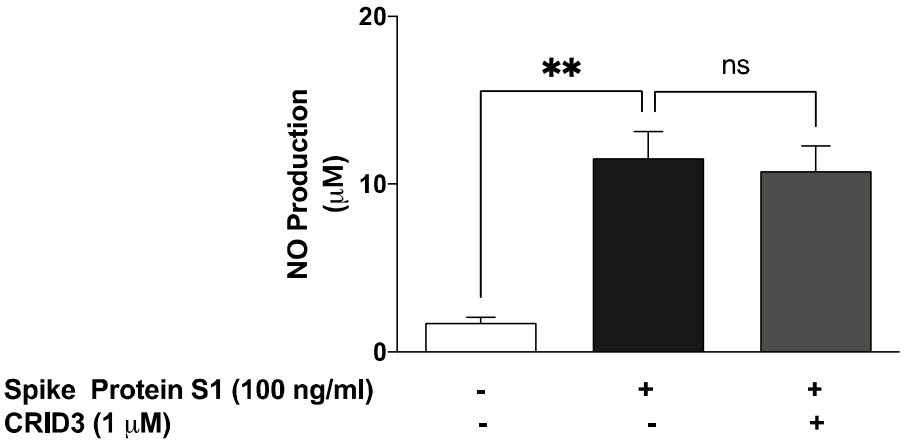

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Figure 8

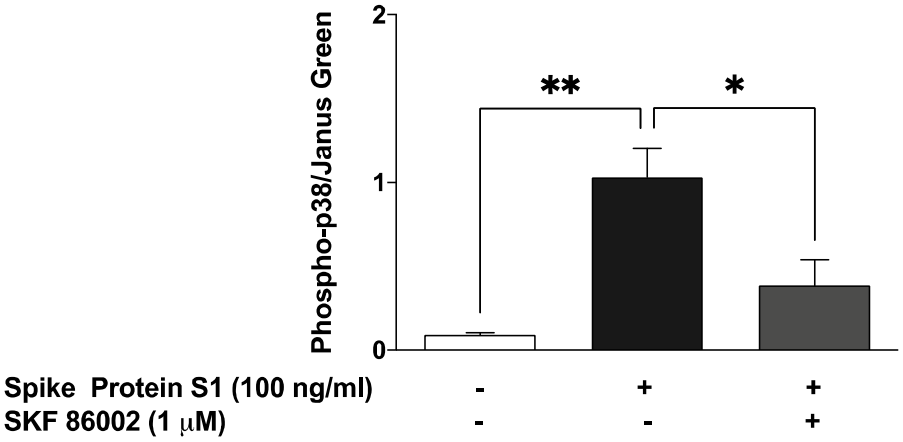

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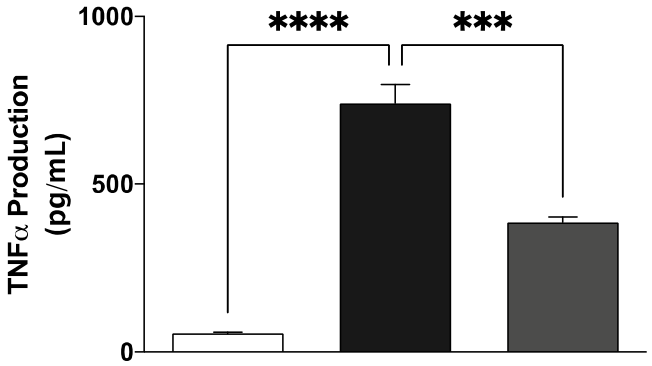
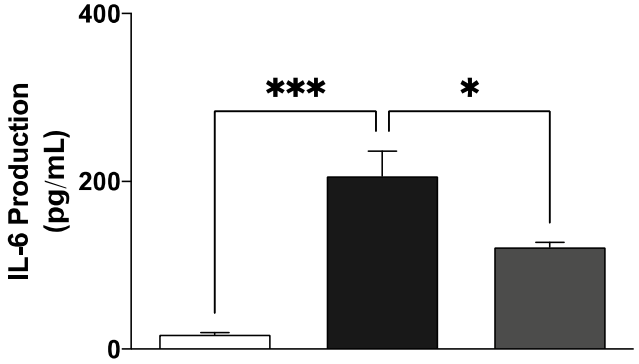
Figure 9A



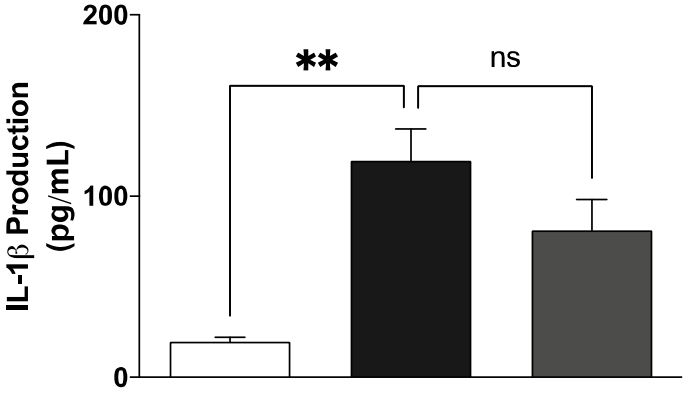
Spike Protein S1 (100 ng/ml)	-	+	+
SKF 86002 (1 μM)	-	-	+

Figure 9B



Spike Protein S1 (100 ng/ml)	-	+	+
SKF 86002 (1 μM)	-	-	+

Figure 9C



Spike Protein S1 (100 ng/ml)	-	+	+
SKF 86002 (1 μM)	-	-	+

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Figure Legends

Figure 1

SARS-CoV-2 spike S1 glycoprotein increased levels of TNFα (A), IL-6 (B) and IL-1β (C) in BV-2 microglia after a 24-h incubation. All values are expressed as mean□±□SEM for three independent experiments. Data were analysed using One-way ANOVA followed by a post hoc Tukey's multiple comparison test. *p□<□0.05, ***p□<□0.001, ****p<0.0001, ns (not significant at p<0.05), compared with untreated control.

Figure 2

SARS-CoV-2 spike S1 glycoprotein increased levels of NO (A) and iNOS protein (B) in BV-2 microglia after a 24-h incubation. NO levels in culture supernatants were measured using the Griess assay, while in-cell western (cytoblot) assay was used to detect iNOS protein expression. All values are expressed as mean□±□SEM for three independent experiments. Data were analysed using One-way ANOVA followed by a post hoc Tukey's multiple comparison test. *p<0.05, **p□<□0.01, ***p□<□0.001, ns (not significant at p<0.05), compared with untreated control.

Figure 3

SARS-CoV-2 spike S1 glycoprotein activated NF-κB signalling in BV-2 microglia. Nuclear extracts were collected from cells stimulated with SARS-CoV-2 spike S1 glycoprotein (100 ng/mL) in the absence or presence of BAY11-7082 (1 μM) for 60 min and subjected to DNA binding assays (A). Results of luciferase reporter gene assay showing increased NF-κB transcriptional activity by SARS-CoV-2 spike S1 glycoprotein and its inhibition by BAY11-7082 (1 μM) (B). In-cell western (cytoblot) analyses showing increased protein expressions of phospho-p65 sub-unit (C) and phospho-IκBα (D) following stimulation with SARS-CoV-2 spike S1 glycoprotein (100 ng/mL) for 15 min and inhibition by BAY11-7082 (1 μM). Data were analysed using One-way ANOVA followed by a post hoc Tukey's multiple comparison test. *p<0.05, **p□<□0.01, ***p□<□0.001, ns (not significant at p<0.05), compared with untreated control, or SARS-CoV-2 spike S1 glycoprotein stimulation alone.

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Figure 4

Pre-treatment with BAY11-7082 (1 μM) resulted in inhibition of SARS-CoV-2 spike S1 glycoprotein-induced increased production of TNFα (A), IL-6 (B) and IL-1β (C) in BV-2 microglia. Culture supernatants were analysed using ELISA following stimulation for 24 h. Data were analysed using One-way ANOVA followed by a post hoc Tukey's multiple comparison test. **p□<□0.01, ****p<0.0001, compared with untreated control, or SARS-CoV-2 spike S1 glycoprotein stimulation alone.

Figure 5

Pre-treatment with BAY11-7082 (1 μM) resulted in inhibition of SARS-CoV-2 spike S1 glycoprotein-induced increased production of NO (A), iNOS protein (B) in BV-2 microglia, following stimulation for 24 h. Culture supernatants were analysed using Griess assay, in-cell western (cytoblot) was used for iNOS detection. Data were analysed using One-way ANOVA followed by a post hoc Tukey's multiple comparison test. *p<0.05, **p□<□0.01, ***p<0.001, ****p<0.0001, compared with untreated control, or SARS-CoV-2 spike S1 glycoprotein stimulation alone.

Figure 6

(A) Stimulation of BV-2 microglia with SARS-CoV-2 spike S1 glycoprotein (100 ng/mL) increased protein expression of NLRP3 inflammasome, which was inhibited in the presence of CRID3 (1 μM) and BAY11-7082 (1 μM).

(B) Increased caspase-1 activity by SARS-CoV-2 spike S1 glycoprotein (100 ng/mL) BV-2 microglia was reduced in the presence of CRID3 (1 μM) and BAY11-7082 (1 μM).

Data were analysed using One-way ANOVA followed by a post hoc Tukey's multiple comparison test. *p<0.05, **p□<□0.01, ***p<0.001, ****p<0.0001, compared with untreated control, or SARS-CoV-2 spike S1 glycoprotein stimulation alone.

Figure 7

Pre-treatment with CRID3 (1 μM) did not prevent SARS-CoV-2 spike S1 glycoprotein-induced increased production of TNFα (A), IL-6 (B), NO (D) while IL-1β production was reduced (C) in BV-2 microglia. Culture supernatants were analysed using ELISA following stimulation for 24 h. Data were analysed using One-way ANOVA followed by a post hoc Tukey's multiple comparison test. **p□<□0.01,

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***p<0.001, ****p<0.0001, ns (not significant at p<0.05), compared with untreated control, or SARS-CoV-2 spike S1 glycoprotein stimulation alone.

Figure 8

Stimulation of BV-2 microglia with SARS-CoV-2 spike S1 glycoprotein (100 ng/mL) increased protein expression of phosphor-p38 MAPK, which was inhibited in the presence of SKF 86002 (1 µM). Data were analysed using One-way ANOVA followed by a post hoc Tukey's multiple comparison test. *p<0.05, **p<0.01, compared with untreated control, or SARS-CoV-2 spike S1 glycoprotein stimulation alone.

Figure 9

Pre-treatment with SKF 86002 (1 µM) resulted in inhibition of SARS-CoV-2 spike S1 glycoprotein-induced increased production of TNFα (A) and IL-6 (B) in BV-2 microglia. Culture supernatants were analysed using ELISA following stimulation for 24 h. Data were analysed using One-way ANOVA followed by a post hoc Tukey's multiple comparison test. **p<0.01, ***p<0.001, ****p<0.0001, ns (not significant at p<0.05), compared with untreated control, or SARS-CoV-2 spike S1 glycoprotein stimulation alone.