

## **Exaggerated cytokine production in human peripheral blood mononuclear cells by recombinant SARS-CoV-2 spike glycoprotein S1 and its inhibition by dexamethasone**

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## Abstract

An understanding of the pathological inflammatory mechanisms involved in SARS-CoV-2 virus infection is necessary in order to discover new molecular pharmacological targets for SARS-CoV-2 spike glycoprotein. In this study, the effects of a recombinant SARS-CoV-2 spike glycoprotein S1 was investigated in human peripheral blood mononuclear cells (PBMCs). Stimulation with spike glycoprotein S1 (100 ng/mL) resulted in significant elevation in the production of TNF $\alpha$ , IL-6, IL-1 $\beta$  and IL-8. However, pre-treatment with dexamethasone (100 nM) caused a significant reduction in the release of these cytokines. Further experiments revealed that S1 stimulation of PBMCs increased phosphorylation of NF- $\kappa$ B p65 and I $\kappa$ B $\alpha$ , while increasing I $\kappa$ B $\alpha$  degradation. DNA binding of NF- $\kappa$ B p65 was also significantly increased following stimulation with S1. Treatment of PBMCs with dexamethasone (100 nM) or BAY11-7082 (1  $\mu$ M) resulted in inhibition of S1-induced NF- $\kappa$ B activation. Activation of p38 MAPK by S1 was blocked in the presence of dexamethasone and SKF 86002. CRID3, but not dexamethasone pre-treatment produced significant inhibition of S1-induced activation of NLRP3/caspase-1. Further experiments revealed that S1-induced increase in the production of TNF $\alpha$ , IL-6, IL-1 $\beta$  and IL-8 was reduced in the presence of BAY11-7082 and SKF 86002, while CRID3 pre-treatment resulted in the reduction of IL-1 $\beta$  production. These results suggest that SARS-CoV-2 spike glycoprotein S1 stimulate PBMCs to release pro-inflammatory cytokines through mechanisms involving activation of NF- $\kappa$ B, p38 MAPK and NLRP3 inflammasome. It is proposed that clinical benefits of dexamethasone in COVID-19 is possibly due to its anti-inflammatory activity in reducing SARS-CoV-2 cytokine storm.

## **Keywords**

SARS-CoV-2 cytokine storm; SARS-CoV-2 spike glycoprotein S1; Inflammation;  
NF- $\kappa$ B, PBMCs

# 1. Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in China in December 2019. Since the first report of its emergence, there has been a global spread of the infection accompanied by widespread appearance of coronavirus disease 2019 (COVID-19) [1, 2]. As of 2<sup>nd</sup> February 2021, there were 102,817,575 confirmed cases and 2,227,420 confirmed deaths globally, and high numbers of new cases are occurring daily [3].

Symptoms of illness caused by SARS-CoV-2 infection at onset of illness are fever, cough, myalgia or fatigue, headache, dyspnoea and pneumonia, while the major complications identified were acute respiratory distress syndrome, cardiac injury and secondary infection [4]. Among these symptoms and complications, end organ damage and acute respiratory distress syndrome (ARDS) have been proposed to be the leading causes of death in critically ill patients [5]. This is not surprising as studies have suggested that excessive release of inflammatory mediators results in cytokine storm, which has been implicated in both ARDS and multi-organ failure [6-8].

Clinical evidence has proposed that the cytokine storm involves a vicious cycle of inflammatory responses which are characterised by the excessive release of pro-inflammatory cytokines including interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-12 (IL-12), interferon gamma (IFN $\gamma$ ), tumour necrosis factor (TNF $\alpha$ ), all of which target lung tissues [6, 9, 10]. During the pulmonary phase of SARS-CoV-2 infection, the virus infects the upper and lower respiratory tracts using angiotensin-converting enzyme 2 (ACE2) as the receptor for host entry [8]. However, in the pro-inflammatory phase, SARS-CoV-2 activates the host's immune system to trigger both adaptive and innate immune responses [11-13]. The resulting macrophage-mediated hyperactive immune response and stimulation of inflammatory cytokines results in acute lung injury, ARDS, systemic inflammatory response syndrome (SIRS), shock and multiorgan dysfunction [14, 15].

Viral attachment, fusion and entry into the host's cells during SARS-CoV-2 infection are known to be facilitated by the spike glycoproteins which protrude from the surface of mature virions, which bind to the host ACE2 protein [16, 17]. Studies have further 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 [16]. Of the two sub-units of the S glycoprotein, S1 and S2 the receptor-binding domain (RBD) of S1 is the main sub-unit of the spike protein for ACE2 binding. It is therefore hypothesised that the SARS-CoV-2 glycoprotein S1 triggers exaggerated response in immune cells to induce cytokine storm.

It has been widely suggested that macrophages/monocytes are immune cells which play significant roles in SARS-CoV-2-mediated cytokine storm. However, there is limited experimental data highlighting the mechanisms involved in the inflammatory responses by these cells during SARS-CoV-2 cytokine storm. Furthermore, there is a need to develop cellular pharmacological models for investigating anti-inflammatory drugs and novel compounds as adjuncts to treat immune cell-mediated cytokine storm in SARS-CoV-2 infection.

In this study, we have evaluated the effects of stimulating human peripheral blood mononuclear cells (PBMCs) with a recombinant human SARS-CoV-2 spike glycoprotein S1. We have further evaluated the effects of the anti-inflammatory drug, dexamethasone on SARS-CoV-2 spike glycoprotein S1-induced inflammation in PBMCs.

## **2. Materials and methods**

### **2.1 Materials**

Recombinant human coronavirus SARS-CoV-2 spike glycoprotein S1 (ab273068; Lots GR3356031-1 and 3353172-2; Accession [MN908947](#)) was purchased from Abcam. The protein was reconstituted in sterile water for functional studies. The following drugs were used: BAY11-7082 (Sigma), CRID3 sodium salt (Tocris), SKF 86002 dihydrochloride (Tocris) and dexamethasone (Sigma).

### **2.2 Cell culture**

Human peripheral blood mononuclear cells (hPBMCs) (Lonza Biosciences; Catalogue #: 4W-270; Batch: 3038013) were isolated from peripheral blood by apheresis and density gradient separation. Frozen cells were thawed, and transferred to a sterile centrifuge tube. Thereafter, warmed RPMI medium was added to the cells slowly, allowing gentle mixing. The cell suspension was then centrifuged at 400 x g for 10 min. After centrifuging, the supernatant was discarded

and fresh warmed RPMI was added to the pellet. This was followed by another centrifugation at 400 x g for 10 min. Supernatant was removed and cells were suspended in RPMI, counted and allowed to rest overnight.

## **2.3 Production of pro-inflammatory cytokines**

Human PBMCs were seeded out in 24-well plate at  $5 \times 10^4$  cells/mL and treated with S1 (10, 50 and 100 ng/mL) for 24 h. Thereafter, medium was collected and centrifuged to obtain culture supernatants. Experiments were also carried out in cells pre-treated with dexamethasone (1, 10 and 100 ng/ml) for 1 h prior to stimulation with spike glycoprotein S1 (100 ng/ml) for a further 24 h. Levels of  $\text{TNF}\alpha$  in the supernatants were determined using human ELISA™ kit (Abcam). Concentrations of  $\text{TNF}\alpha$  in supernatants were calculated from a mouse  $\text{TNF}\alpha$  standard curve, and the assay range was 15.63-1000 pg/mL. Levels of IL-6 in supernatants were determined using human IL-6 ELISA kit (Abcam). The range for IL-6 detection was 7.8-500 pg/mL. Similarly, levels of IL-1 $\beta$  were evaluated using human IL-1 $\beta$  ELISA kit (Abcam), with a range of detection of 14.06-900 pg/mL, while IL-8 production was evaluated using human IL-8 ELISA kit (Thermo Scientific), with assay range of 2-250 pg/mL.

## **2.4 In cell western (cytoblot) analyses**

The in cell western is a proven method for the rapid quantification of proteins in cells [18, 19]. PBMCs were seeded into a black 96-well plate at  $5 \times 10^4$  cells/mL. At 70% confluence, cells were stimulated with spike glycoprotein S1 (100 ng/ml) for different periods. At the end of each experiment, cells were fixed with 8% paraformaldehyde solution (100  $\mu$ L) 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-phospho-p65 (Cell Signalling Technology), rabbit anti-phospho-I $\kappa$ B $\alpha$  (Santa Cruz Biotechnology), rabbit total I $\kappa$ B $\alpha$  (Santa Cruz Biotechnology), rabbit anti-phospho-p38 (Cell Signalling Technology) and rabbit anti-NLRP3 (Abcam) antibodies. Thereafter, cells were washed with PBS and incubated with anti-rabbit HRP secondary antibody for 2 h at room temperature. Then, 100  $\mu$ L 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).

## **2.5 NF- $\kappa$ B p65 transcription factor binding assay**

The NF- $\kappa$ B p65 transcription factor assay is a non-radioactive ELISA-based assay for evaluating DNA binding activity of NF- $\kappa$ B in nuclear extracts. PBMCs were seeded in a 6-well plate at a density of  $4 \times 10^4$  cells/mL. The cells were then incubated with 100 ng/mL of spike glycoprotein S1 protein with or without dexamethasone (100 nM) or BAY11-7082 (1  $\mu$ M) for 60 min. At the end of the incubation, nuclear extracts were prepared from the cells and subjected to NF- $\kappa$ B transcription factor binding assay according to the instructions of the manufacturer (Abcam).

## **2.6 Caspase-Glo<sup>®</sup>1 inflammasome assay**

The caspase-Glo<sup>®</sup>1 inflammasome assay (Promega) was used to measure the activity of caspase-1 directly in live cells or culture supernatants. PBMCs were seeded out in 24-well plate at a density of  $4 \times 10^4$  cells/mL and pre-treated with dexamethasone (100 nM) or CRID3 (1  $\mu$ M) for 60 min prior to stimulation with spike glycoprotein S1 (100 ng/mL) for 6 h. After stimulation, cell culture supernatants were collected and mixed with equal volume of Caspase-Glo<sup>®</sup> 1 reagent or Caspase-Glo<sup>®</sup> 1 Reagent + YVAD-CHO (1  $\mu$ M) in a 96-well plate. The contents of the wells were mixed using a plate shaker at 400 rpm 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).

## **2.7 Human NLRP3 ELISA**

PBMCs were seeded out into a 6-well plate and allowed to settle overnight. Thereafter, cells were stimulated with spike protein S1 (100 ng/mL) in the presence or absence of dexamethasone (100 nM) or CRID3 (1  $\mu$ M) for 6 h. Cell lysates were prepared by centrifugation at 2000 x g for 7 min at 4°C. The supernatant was discarded and the cells washed in ice-cold PBS by centrifuging at 2000 x g for 7 min at 4°C. Thereafter, ice-cold lysis buffer was added to the cell pellet, followed by sonication for 30 min, and centrifugation at 16000 x g for 20 min at 4°C. Supernatants were collected and analysed for levels of NLRP3 protein using human NLRP3 ELISA kit (Abcam), according to the manufacturer's instructions.

## **2.8 Effects of NF- $\kappa$ B, p38, and NLRP3 inhibitors on cytokine production**

PBMCs were seeded out in 24-well plate at  $5 \times 10^4$  cells/mL and treated with BAY-11-7082 (1  $\mu$ M), SKF 86002 (1  $\mu$ M), or CRDI3 (1  $\mu$ M). One hour later, cells were stimulated with S1 protein (100 ng/mL) for a further 24 h. Culture media were collected and supernatants analysed for levels of TNF $\alpha$ , IL-6, IL-1 $\beta$  and IL-8 as described above.

## **2.9 Co-culture of human A549 epithelial cells and PBMCs**

Human A549 lung epithelial cells were co-cultured with PBMCs using the transwell system (0.4  $\mu$ m porous membrane; Corning). A549 cells were cultured in inserts that constituted the upper chamber until 70% confluent. Thereafter, they were incubated with S1 protein (100 ng/mL) with or without dexamethasone (100 nM) pre-treatment. One hour after the initiation of stimulation with S1 protein, the inserts were placed on overnight-rested PBMCs in the lower chamber for a further 24 h. At the end of the experiment, supernatants were collected from the PBMC layer and analysed for levels of TNF $\alpha$ , IL-6, IL-1 $\beta$  and IL-8 using human ELISA kits as described above.

## **2.10 Statistical analysis**

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

# **3. Results**

## **3.1 Stimulation of PBMCs with spike protein S1 resulted in increased production of TNF $\alpha$ , IL-6, IL-1 $\beta$ and IL-8**

Following incubation of spike protein S1 (10 ng/mL) with PBMCs for 24 h, analyses of cell supernatants showed no significant ( $p < 0.05$ ) increase in the release of TNF $\alpha$ . On increasing the concentration of the spike protein to 50 and 100 ng/mL, there was ~10 and ~24-fold increase in TNF $\alpha$  secretion, respectively (Figure 1A). Similarly, analyses of supernatants for levels of IL-6 (Figure 1B), IL-1 $\beta$  (Figure 1C) and IL-8 (Figure 1D) revealed that incubation with 10 ng/mL of spike protein S1 did not induce significant elevation in the production of the cytokines, while significant ( $p < 0.05$ )



increases were demonstrated in cells incubated with 50 and 100 ng/mL of the protein.

### **3.2 Increased production of pro-inflammatory mediators by S1 is reduced by dexamethasone**

We next evaluated effects of dexamethasone (1, 10 and 100 nM) on excessive production of pro-inflammatory cytokines in PBMCs stimulated with spike protein S1 (100 ng/mL) for 24 h. Results in Figure 2A show that pre-treatment with 1 nM of dexamethasone did not prevent S1-induced production of TNF $\alpha$ . On increasing the concentration of dexamethasone to 10 nM, there was a weak but insignificant ( $p < 0.05$ ) reduction in TNF $\alpha$  production. On pre-treating the cells with 100 nM of dexamethasone, a significant ( $p < 0.05$ ) reduction in S1-induced increased production of TNF $\alpha$  was observed.

Figure 2B shows that anti-inflammatory effect of dexamethasone on S1-induced exaggerated production of IL-6 in PBMCs was significantly reduced ( $p < 0.05$ ) by pre-treatment with 100 nM, but not the lower concentrations (10 and 50 nM) of the drug. Furthermore, analyses of samples obtained from PBMCs pre-treated with dexamethasone (1 nM) revealed no reduction in the production of IL-1 $\beta$ . However, pre-treatment with 10 and 100 nM of the drug resulted in significant ( $p < 0.001$ ) reduction in IL-1 $\beta$  production (Figure 2C).

Similarly, stimulation of PBMCs with S1 (100 ng/mL) for 24 h resulted in ~14-fold increase in the production of IL-8. This increase was not reduced by 1 and 10 nM of dexamethasone, while increasing drug concentration to 100 nM resulted in ~59% production of IL-8, when compared to S1 stimulation alone (100%) (Figure 2D).

### **3.3 Effects of spike protein S1 on NF- $\kappa$ B activation in PBMCs**

Based on results showing that spike protein S1 stimulates PBMCs to induce increased production of pro-inflammatory cytokines, and the inhibition of this action by dexamethasone, we investigated the roles of NF- $\kappa$ B activation in these actions. Firstly, we used in cell western assays to evaluate the effects of S1 stimulation on protein expression of phospho-p65, phospho-I $\kappa$ B $\alpha$  and total I $\kappa$ B $\alpha$  in the presence and absence of dexamethasone and BAY-11-7082. Results in Figure 3A show that following stimulation of PBMCs with S1 (100 ng/mL) for 15 min, there was ~12.7-fold increase ( $p < 0.001$ ) in protein expression of phospho-p65. Pre-treatment of PBMCs

with dexamethasone (100 nM) and BAY-11-7082 (1  $\mu$ M) for 60 min prior to stimulation with S1 resulted in significant ( $p<0.05$ ) inhibition of p65 phosphorylation. Similarly, significant ( $p<0.001$ ) spike protein S1-induced increase in phospho-I $\kappa$ B $\alpha$  and decrease in total I $\kappa$ B $\alpha$  protein levels were prevented by pre-treatment with dexamethasone (100 nM) and BAY-11-7082 (1  $\mu$ M) (Figures 3B and 3C).

Based on our results showing that spike protein S1 activates the processes resulting in translocation of NF- $\kappa$ B to the nucleus, we next asked whether the protein had any effect on DNA binding by NF- $\kappa$ B. Figure 3D illustrates an increase in DNA binding of NF- $\kappa$ B following stimulation of PBMCs with S1 (100 ng/mL) for 60 min, when compared with unstimulated cells. On the other hand, incubating the cells with either dexamethasone (100 nM) or BAY11-7082 (1  $\mu$ M) for 60 min prior to stimulation with S1 resulted in significant ( $p<0.001$ ) inhibition in DNA binding by NF- $\kappa$ B. These results indicate that dexamethasone and BAY11-7082 inhibited DNA binding by ~46% and ~60%, respectively in comparison with S1 stimulation alone.

### **3.4 Activation of p38 MAPK by spike glycoprotein S1**

In cell western blot analyses revealed that stimulation of PBMCs with S1 (100 ng/mL) for 60 min resulted in a significant ( $p<0.0001$ ) increase in phospho-p38 protein, when compared with unstimulated cells. In comparison with S1 stimulation alone (100% expression), pre-treatment with dexamethasone (100 nM) resulted in ~61.9% expression of phospho-p38 protein, while expression in cells pre-treated with SKF 86002 (1  $\mu$ M) was ~27.2% (Figure 4).

### **3.5 NLRP3 inflammasome/caspase-1 was activated by S1**

We previously showed that S1 induced an increase in IL-1 $\beta$  production in PBMCs. We next asked whether activation of NLRP3 inflammasome/caspase-1 pathway contributed to this increase. Results of ELISA and in-cell western assay in Figures 5A and 5B show that following stimulation with S1 (100 ng/ml) for 6 h, there was a significant ( $p<0.01$ ) increase in protein levels of NLRP3, in comparison with untreated control cells. It was further shown that S1-induced elevation of NLRP3 was significantly reduced in the presence of CRID3 (1  $\mu$ M), while pre-treatment with dexamethasone (100 nM) produced a slight and insignificant ( $p<0.05$ ) reduction in S1-induced increase in NLRP3 protein. Similarly, caspase-1 activity was increased in comparison with untreated PBMCs following stimulation with spike glycoprotein S1

(100 ng/ml). On pre-treating cells with CRID3 prior to S1 stimulation, reduction in caspase-1 activity was observed, while dexamethasone pre-treatment did not have a significant effect on S1-induced increase in caspase-1 activity (Figure 5C).

### **3.6 Effects of BAY11-7082, SKF 86002, SP600125 and CRID3 on SARS-CoV-2 spike protein S1-induced increased production of inflammatory cytokines**

Having demonstrated that spike glycoprotein S1 activates NF- $\kappa$ B, p38, and NLRP3 in PBMCs, we were then interested in establishing the possible roles of these targets in S1-induced exaggerated production of pro-inflammatory cytokines. Results in Figure 6 show that in the presence of BAY11-7082 (1  $\mu$ M), there were significant reductions in elevated secretion of TNF $\alpha$ , IL-6, IL-1 $\beta$  and IL-8 produced when PBMCs were stimulated with spike glycoprotein S1 (100 ng/mL) for 24 h. Similar reduction in S1-induced increased production of the cytokines were observed when cells were pre-treated with SKF 86002 (1  $\mu$ M) prior to S1 stimulation. However, CRID3 (1  $\mu$ M) produced significant ( $p < 0.01$ ) reduction in IL-1 $\beta$  production (Figure 6C), while having no effect on the release of TNF $\alpha$ , IL-6, and IL-8 (Figures 6A, 6B, and 6D).

## **4. Discussion**

The cytokine storm is now established to be a major contributor to fatalities of SARS-CoV-2 infection. Consequently, an understanding of the pathological inflammatory mechanisms involved in SARS-CoV-2 virus infection is necessary in order to discover new molecular pharmacological targets. This study demonstrated that stimulation of human PBMCs with a recombinant spike glycoprotein S1 for 24 hours resulted in significant release of pro-inflammatory cytokines TNF $\alpha$ , IL-6, IL-1 $\beta$  and IL-8. These results appear to explain the increased serum levels of inflammatory cytokines that have been widely reported in patients with severe COVID-19. It is therefore proposed that SARS-CoV-2 infection results in spike protein-mediated activation of monocytes, macrophages and dendritic cells, resulting in positive feedback involving dysregulated production of cytokines.

Several clinical studies have reported that hyper-inflammation, accompanied by increased serum levels of pro-inflammatory cytokines and chemokines are associated with disease severity and death in COVID-19 [14, 20-22]. In fact, post-mortem analyses have revealed that high levels of pro-inflammatory cytokines are

associated with cellular infiltration of organs such as the lungs, heart, and kidney [14, 23-24]. Furthermore, in a study reported by Han et al., cytokine storm characterised by increased serum levels of TNF $\alpha$  and IL-6 were observed and suggested to be predictive of disease severity [25]. Similarly, a retrospective observational study in hospitalised patients diagnosed with COVID-19 showed that serum levels of IL-6 greater than 30 pg/mL was a predictor of invasive mechanical ventilation requirement [26]. It is noteworthy that a similar pattern of cytokine storm was observed in preceding outbreaks such as MERS-CoV and SARS-CoV [27-30]. Pharmacological modulation of cytokine hypersecretion in coronavirus infections warrants further investigation due to fatalities involving multi-organ damage.

Dexamethasone is a corticosteroid employed in a wide range of conditions due to its anti-inflammatory and immunosuppressant activities. However, emerging evidence suggests that dexamethasone may provide some benefits in the treatment of COVID-19. In a controlled, open-label trial conducted by the RECOVERY group, dexamethasone treatment resulted in lower 28-day mortality among COVID-19 patients who were receiving either invasive mechanical ventilation or oxygen alone [31]. Results of the CoDEX clinical trial also show that in COVID-19 with moderate or severe ARDS, the use of intravenous dexamethasone plus standard care resulted in significant improvement in clinical outcome, in comparison with standard care alone [32].

We therefore hypothesised that the benefits of dexamethasone in treating patients with COVID-19 may be due in part to its anti-inflammatory effect through reduction in exaggerated cytokine production at the cellular level. To prove this hypothesis, we showed that treatment with dexamethasone prevented increased production of TNF $\alpha$ , IL-6, IL-1 $\beta$  and IL-8 in PBMCs stimulated with recombinant SARS-CoV-2 spike glycoprotein S1. It appears dexamethasone blocks cellular pathways that are responsible for exaggerated production of cytokines in monocytes, macrophages and lymphocytes that are recruited following infection by SARS-CoV-2. This anti-inflammatory activity may have contributed to the overall benefits of dexamethasone in both the RECOVERY and CoDEX trials.

Dexamethasone exerts anti-inflammatory partly through targeting the activation of the NF- $\kappa$ B transcription factor. This drug has been shown in many studies to block NF- $\kappa$ B activity in epithelial cells [33], as well as macrophages and monocytes

[34-36]. In this study, dexamethasone inhibited cytoplasmic activation and DNA binding of NF- $\kappa$ B in PBMCs stimulated with SARS-CoV-2 spike glycoprotein S1 suggesting a role for the transcription factor in the observed effects of dexamethasone on inflammatory cytokines. Our results showing a role for NF- $\kappa$ B in a cellular model of SARS-CoV-2-induced inflammation correlate with results of a recent study showing that SARS-CoV-2 infection of human ACE2-transgenic mice resulted in NF- $\kappa$ B-dependent lung inflammation [37]. Results demonstrating inhibition of SARS-CoV-2 spike glycoprotein S1-induced production of pro-inflammatory cytokines by BAY11-7082 further confirmed the direct involvement of NF- $\kappa$ B in their release during SARS-CoV-2 cytokine storm, and warrants further investigation.

Mitogen-activated protein kinases (MAPKs) are protein kinases that regulate various cellular proliferation, differentiation, apoptosis, survival, inflammation, and innate immunity [38, 39]. Specifically, p38 MAPK is activated by bacterial lipopolysaccharide and by pro-inflammatory cytokines, and plays a major role in regulating the production of pro-inflammatory cytokines in inflammation [38-42]. Results showing increased release of pro-inflammatory cytokines by SARS-CoV-2 spike glycoprotein S1 prompted investigations which revealed an activation of p38 MAPK by the protein in PBMCs. It was further established that S1-induced increased cytokine production was inhibited by the p38 MAPK inhibitor, SKF86002. These observations suggest that activation of p38 MAPK is a critical contributor to hypercytokinemia in COVID-19 patients. These findings appear to be consistent with the outcome of recent experiments which showed that infection of the African green monkey kidney epithelial (Vero E6) cells with the SARS-CoV-2 virus resulted in the activation of p38 MAPK [43]. In another study reported by Bouhaddou et al., SARS-CoV-2 activation of p38 MAPK was demonstrated in ACE2-expressing A549 cells, while SARS-CoV-2-induced increase in the production of inflammatory cytokines was inhibited by another p38 inhibitor, SB203580 [44].

While this study confirmed involvement of both NF- $\kappa$ B and p38 MAPK activation in SARS-CoV-2 spike glycoprotein S1-induced exaggerated release of inflammatory cytokines in PBMCs, it is not clear if these were as a result of activation of a critical convergence upstream process in the release of pro-inflammatory cytokines. The SARS-CoV-2 spike glycoprotein S1 is known to interact with the angiotensin-

converting enzyme 2 (ACE2) receptors to gain access to host cells, and has been suggested to induce immune responses. Interestingly, flow cytometry measurements of the human peripheral blood-derived immune cells revealed little or no expression of ACE2, while high expressions were reported in human tissue macrophages, such as alveolar macrophages, liver Kupffer cells, and brain microglia [45]. Other studies have reported expression of ACE2 in alveolar macrophages and human monocyte THP-1 cells [46]. Studies to further determine the roles of ACE2 in spike glycoprotein S1-induced inflammation are therefore needed. The unresolved roles of ACE2 in this respect will stimulate interest in the potential roles of toll-like receptors (TLRs) in the induction of exaggerated cytokine release by SARS-CoV-2 spike glycoprotein S1. Results of observational studies by Sohn et al. [47] revealed that TLR4 and its inflammatory signalling molecules were upregulated in PBMCs from COVID-19 patients, compared with healthy controls. These results, coupled with our data suggest that the SARS-CoV-2 spike glycoprotein S1 appear to be activating NF- $\kappa$ B and p38 MAPK signalling through activation of TLR4.

Our investigations further revealed that the spike protein S1 increased NLRP3 protein expression as well as caspase-1 activity in PBMCs, which may be contributing to the release of IL-1 $\beta$  by these cells. Interestingly, the activation of NLRP3/caspase-1 by S1 was not markedly dexamethasone, showing that the effects of the drug may be more pronounced on NF- $\kappa$ B signalling in these cells. Targeting of the NLRP3 inflammasome activation pathway in macrophages has been suggested as one of the mechanisms involved in SARS-CoV-2 cytokine storm [15, 48]. With data from studies suggesting that the spike protein S1 could be activating TLR4 to cause macrophage-mediated cytokine storm, coupled with our data showing activation of NLRP3 inflammasome by the protein, further investigations need to explore potential roles of TLRs in S1-induced activation of NLRP3 inflammasome, with subsequent increase in the release of pro-inflammatory cytokines.

The studies reported here have shown that the SARS-CoV-2 spike glycoprotein S1 induced exaggerated inflammation in PBMCs through mechanisms involving activation of NF- $\kappa$ B transcription factor, p38 MAPK and the NLRP3 inflammasome. It is proposed that the clinical benefits of dexamethasone in COVID-19 is possibly due to its anti-inflammatory activity in reducing SARS-CoV-2 cytokine storm and subsequent multi-organ failure. It is further proposed that S1-induced production of



cytokines in human peripheral blood mononuclear cells is therefore a potential cellular model to investigate anti-inflammatory compounds for reducing cytokine storm in SARS-CoV-2 infection. Further studies will focus on the possible interactions between the spike protein S1 and toll-like receptors in the activation of innate immunity in macrophages.

### **Conflict of interest**

There are no known conflicts of interest associated with this publication and there has been no financial support for this work that could have influenced its outcome.

### **CRediT author statement**

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## Figure Legends

**Figure 1:** Effects of spike glycoprotein S1 (10, 50 and 100 ng/mL) stimulation on TNF $\alpha$  (A), IL-6 (B), IL-1 $\beta$  (C) and IL-8 (D) production in human PBMCs. Culture supernatants were collected following stimulation for 24 h, and analysed using human ELISA kits for TNF $\alpha$ , IL-6, IL-1 $\beta$  and IL-8. Values are mean  $\pm$  SEM for at least 3 independent experiments (ns: not significant; \* $p$ <0.05; \*\*\* $p$ <0.001; \*\*\*\* $p$ <0.0001, compared with unstimulated control; one-way ANOVA with post-hoc Tukey test).

**Figure 2:** Effects of pre-treatment with dexamethasone (1, 10 and 100 nM) on increased TNF $\alpha$  (A), IL-6 (B), IL-1 $\beta$  (C) and IL-8 (D) production induced by spike glycoprotein S1 (100 ng/mL) in human PBMCs. Culture supernatants were collected following stimulation for 24 h, and analysed using human ELISA kits for TNF $\alpha$ , IL-6, IL-1 $\beta$  and IL-8. Values are mean  $\pm$  SEM for at least 3 independent experiments (ns: not significant; \* $p$ <0.05; \*\* $p$ <0.01; \*\*\* $p$ <0.001; \*\*\*\* $p$ <0.0001, compared with unstimulated control or spike glycoprotein S1 stimulation; one-way ANOVA with post-hoc Tukey test).

**Figure 3:** Spike glycoprotein S1 (100 ng/mL) stimulation of PBMCs activated NF- $\kappa$ B signalling, and was inhibited by dexamethasone (100 nM). In-cell western analyses of PBMCs revealed increased levels of phospho-p65 protein (A), phospho-I $\kappa$ B $\alpha$  (B), and a reduction in total I $\kappa$ B $\alpha$  protein (C) following stimulation for 15 min.

Dexamethasone pre-treatment prevented DNA binding of NF- $\kappa$ B following stimulation with Spike glycoprotein S1 for 60 min (D). Values are mean  $\pm$  SEM for at least 3 independent experiments (\* $p$ <0.05; \*\* $p$ <0.01; \*\*\* $p$ <0.001; \*\*\*\* $p$ <0.0001, compared with unstimulated control or spike glycoprotein S1 stimulation; one-way ANOVA with post-hoc Tukey test).

**Figure 4:** Stimulation of human PBMCs with spike glycoprotein S1 (100 ng/mL) for 60 min activated p38 MAPK, and was inhibited by treatment with dexamethasone (100 nM) or SKF 86002 (1  $\mu$ M). Values are mean  $\pm$  SEM for at least 3 independent experiments (\*\* $p$ <0.01; \*\*\*\* $p$ <0.0001, compared with unstimulated control or spike glycoprotein S1 stimulation; one-way ANOVA with post-hoc Tukey test).

**Figure 5:** Increase in protein expression of NLRP3 inflammasome following stimulation of human PBMCs with spike glycoprotein S1 (100 ng/mL) for 6 h, as

determined using human ELISA for NLRP3 (A) and in-cell western (B). Effects of stimulation with spike glycoprotein S1 on caspase-1 activity (C). Values are mean  $\pm$  SEM for at least 3 independent experiments (ns: not significant; \* $p < 0.05$ ; \*\* $p < 0.01$ , compared with unstimulated control or spike glycoprotein S1 stimulation; one-way ANOVA with post-hoc Tukey test).

**Figure 6:** Effects of pre-treatment with BAY11-7082 (1  $\mu$ M), SKF 86002 (1  $\mu$ M) and CRID3 (1  $\mu$ M) on TNF $\alpha$  (A), IL-6 (B), IL-1 $\beta$  (C) and IL-8 (D) production in human PBMCs. Culture supernatants were collected following stimulation for 24 h, and analysed using human ELISA kits for TNF $\alpha$ , IL-6, IL-1 $\beta$  and IL-8. Values are mean  $\pm$  SEM for at least 3 independent experiments (ns: not significant; ###  $p < 0.001$  versus unstimulated control; ####  $p < 0.0001$  versus unstimulated control; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ , compared with spike glycoprotein S1 stimulation; one-way ANOVA with post-hoc Tukey test).

Figure 1

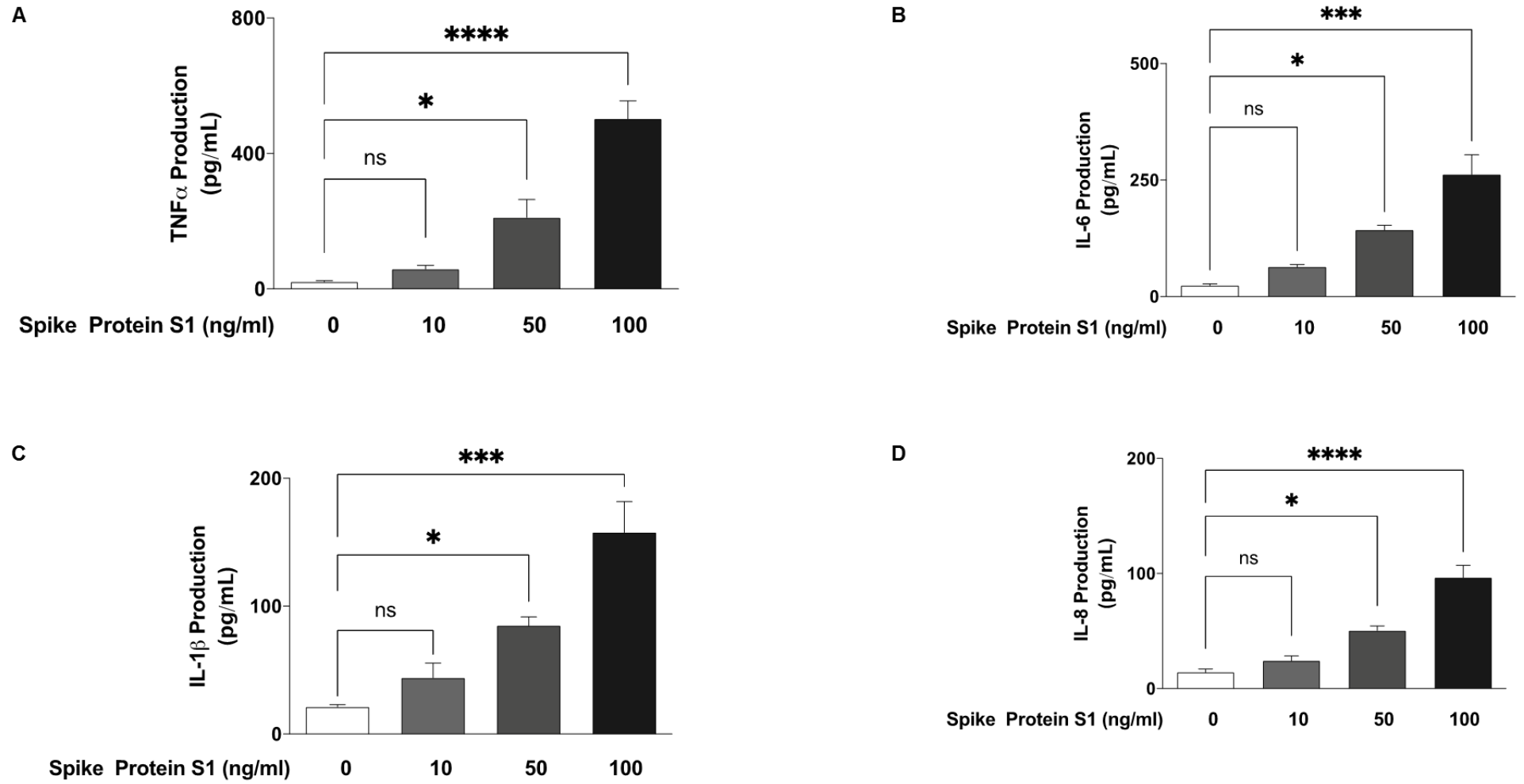


Figure 2

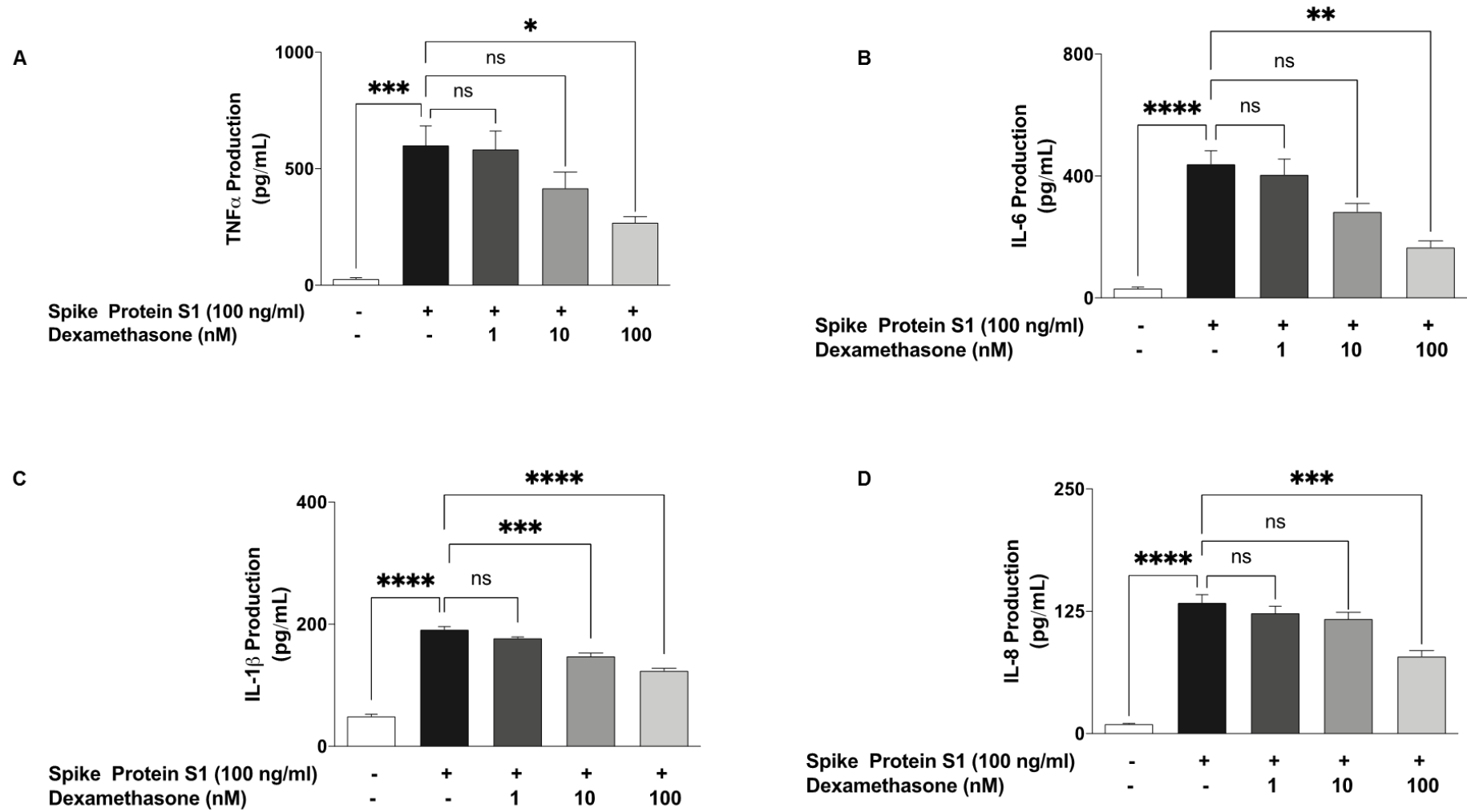




Figure 3

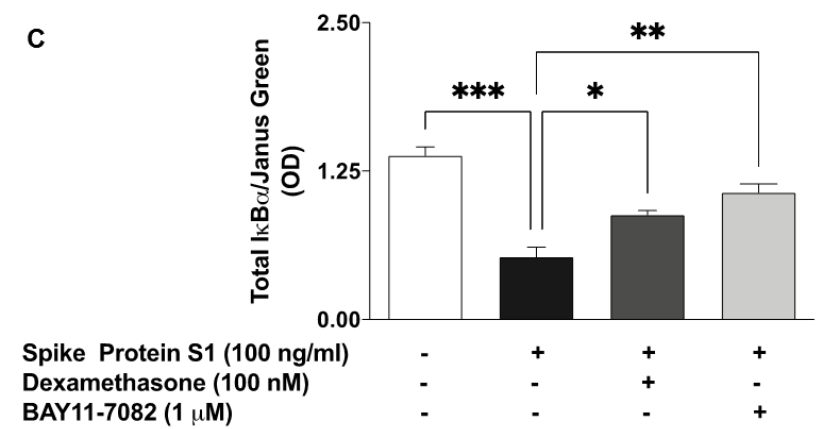
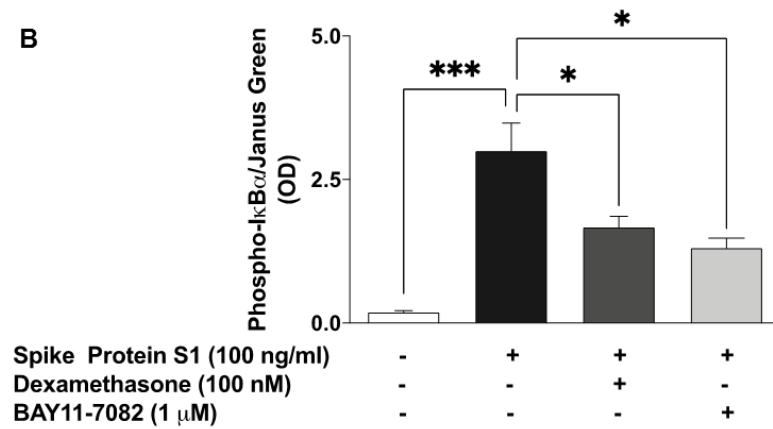
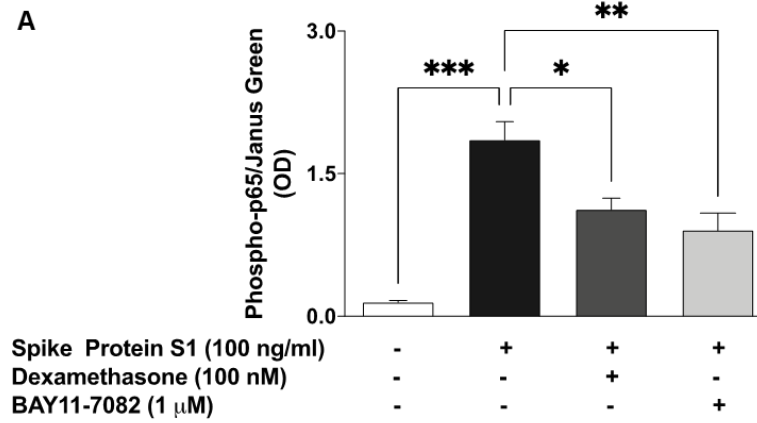


Figure 3

D

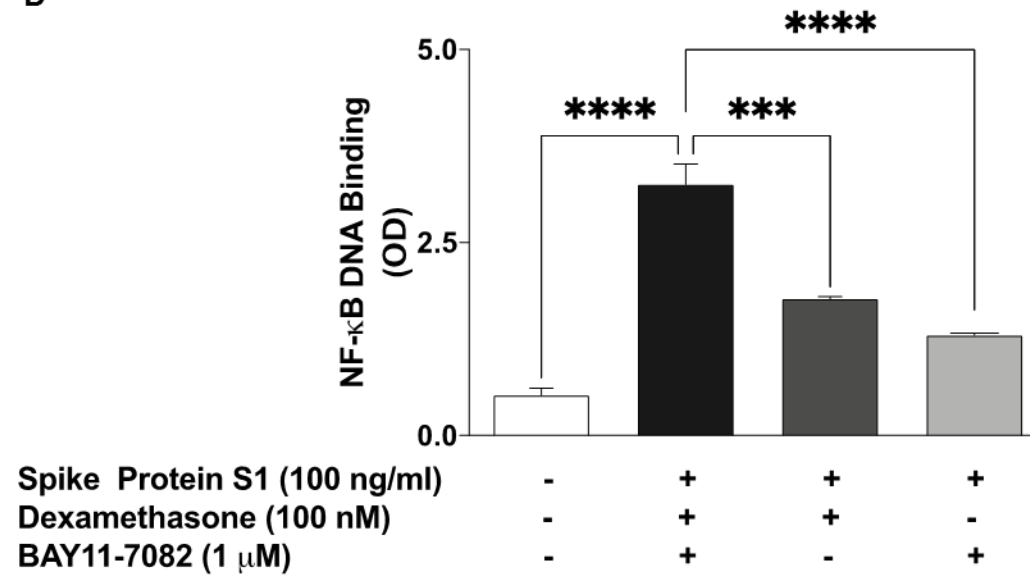


Figure 4

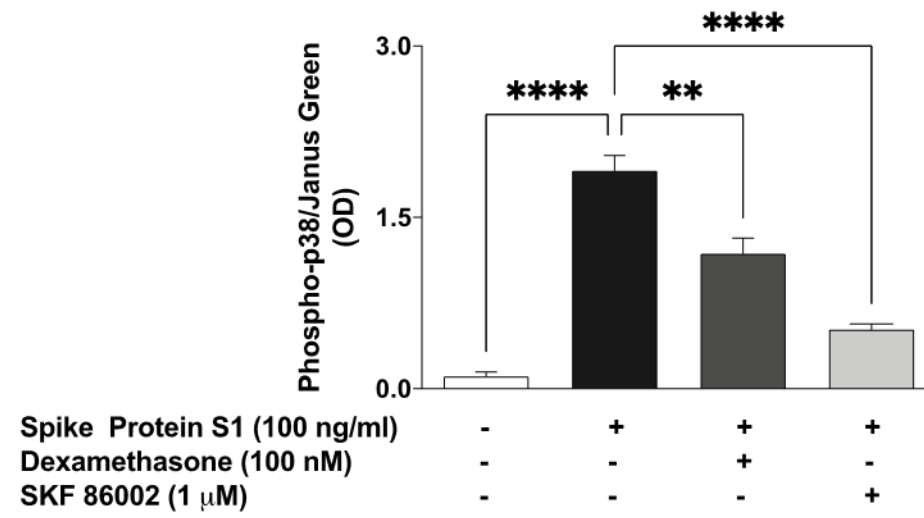


Figure 5

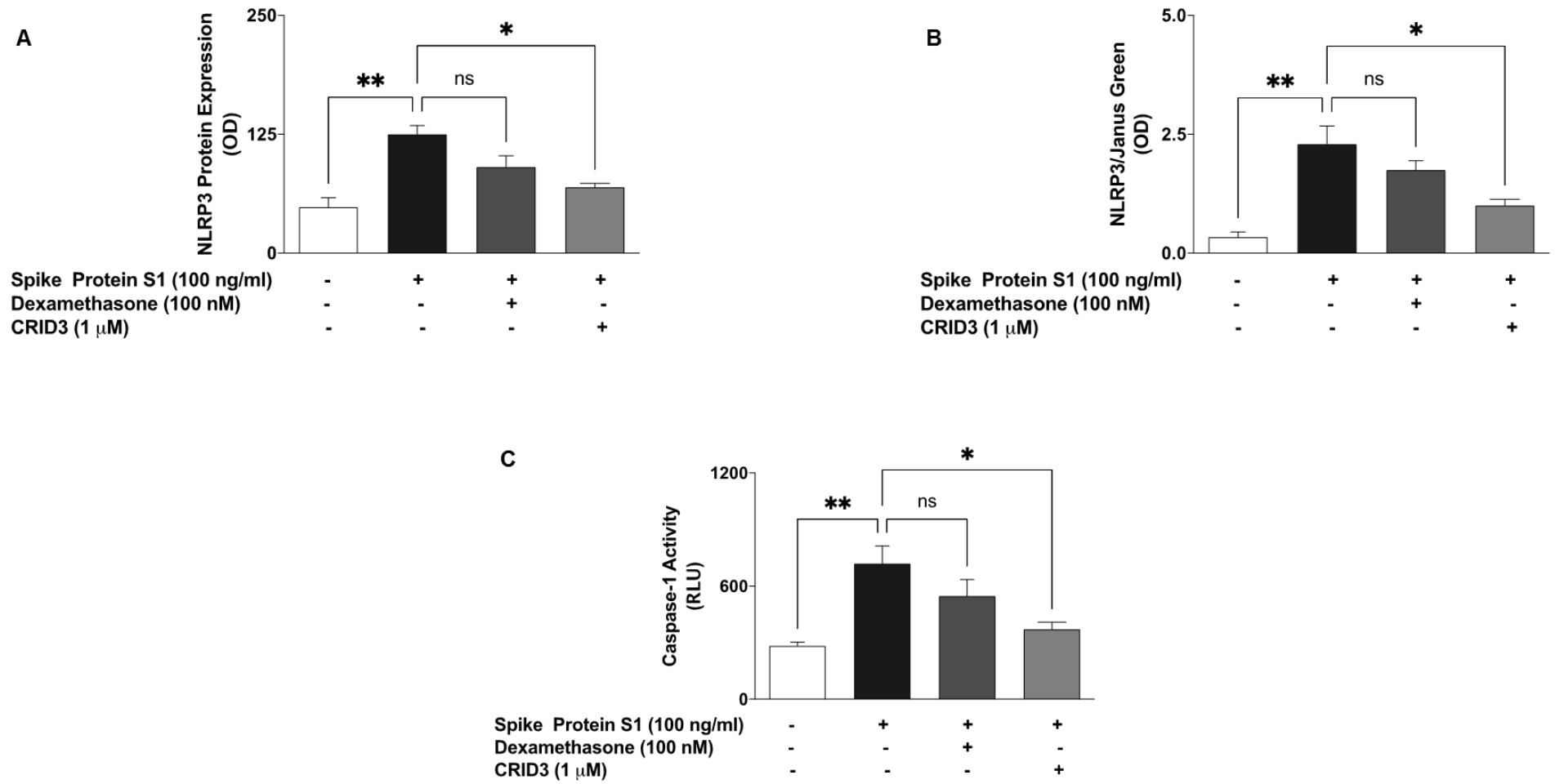
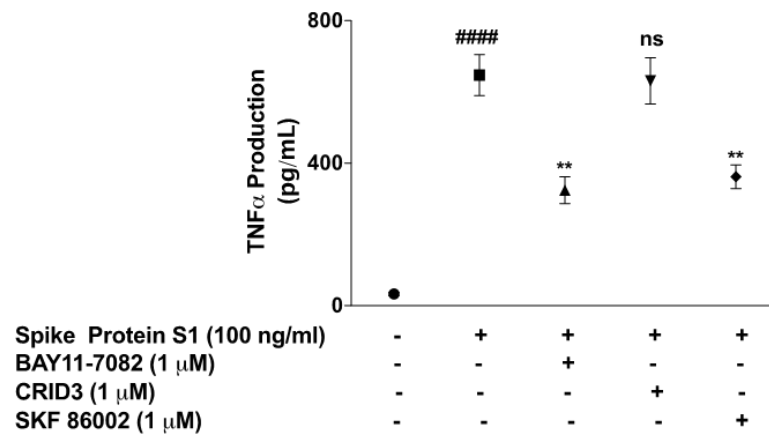
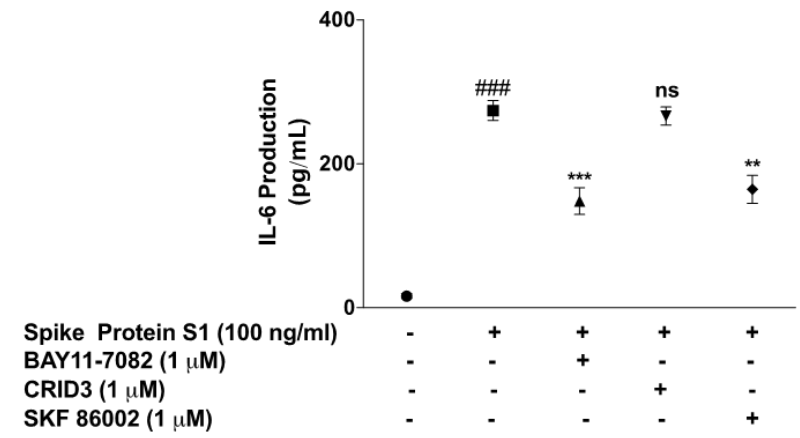


Figure 6

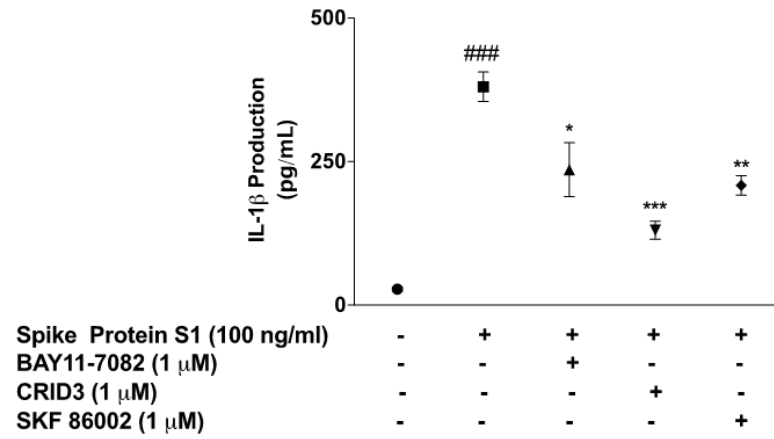
A



B



C



D

