Evidence for investigating GSK-3 inhibitors as potential therapeutics for severe COVID-19

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ABSTRACT

A key component of severe COVID-19 is a "cytokine storm" i.e., the excessive expression of unneeded cytokines. Previous studies suggest that SARS-CoV-2 proteins can induce macrophages to secrete pro-inflammatory cytokines; a process that may involve Toll-like receptors (TLRs). Glycogen synthase kinase-3 (GSK-3) has been implicated in TLR signal transduction and a selective GSK-3 inhibitor, termed COB-187, dramatically attenuates cytokine expression induced by the TLR ligand lipopolysaccharide (LPS). In the present study, we provide evidence that the SARS-CoV-2 spike protein (S) and the S2 subunit (S2) induce production of CXCL10 (a chemokine elevated in severe COVID-19) by a human macrophage cell line. Further, we report that two clinically relevant GSK-3 inhibitors and COB-187 attenuate S and S2 protein-induced CXCL10 production. Combined, our observations provide impetus for investigating GSK-3 inhibitors as potential therapeutics for severe COVID-19.

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1. Introduction

One of the key features of severe COVID-19 is a "cytokine storm" driven by a dysregulated innate immune response [1]. In general, innate immune cells (e.g., macrophages) sense pathogen associated molecular patterns (PAMPs) via pathogen recognition receptors (PRRs) [e.g., Toll-like receptors (TLRs)] [2]. Ligated PRRs trigger signaling pathways that activate transcription factors leading to the release of cytokines and chemokines (a subset of cytokines) [2]. The SARS-CoV-2 virus has four structural proteins namely, spike (S) which has two main subunits S1 and S2, envelope (E), membrane (M), and nucleocapsid (N) [3]. Multiple studies have probed SARS-CoV-2 protein induction of cytokine expression.

Khan et al. [4] provided evidence that S, but not M, E nor N protein induces cytokine (including chemokine) expression by THP-1 macrophages and human peripheral blood mononuclear cells (PBMCs) and that this induction occurs via TLR2 and a NF-kB,

MyD88-dependent pathway. Zheng et al. [5] reported that the E protein, but not S, induces the production of proinflammatory cytokines by mouse bone marrow-derived macrophages and human PBMCs. Karwaciak et al. [6] found that 3 day incubation of human monocytes and macrophages with the N or S protein induced IL-6 expression. Shirato et al. [7] and Chiok et al. [8] reported that S1 stimulates production of TNF-α by THP-1 macrophages and Chiok et al. [8] further reported S1 induction of CXCL10 (a.k.a. IP-10). Zhao et al. [9] found that only the trimeric form of the S protein, but not RBD nor the N-terminal domain of S, induce IL-1β expression by THP-1 cells and, through additional studies, concluded that the S protein activates TLR4. Finally, Pantazi et al. [10], using differentiated THP-1 cells, provided evidence that the S protein upregulates the expression of IL-6, MIP1a and TNF-α.

Thus, while the details of the above studies differ, and in certain cases are in conflict, an emerging hypothesis is that the SARS-CoV-2 spike protein itself can induce cytokine production. This potential mechanism, along with the effect of SARS-CoV-2 genomic material on cytokine production, are potential therapeutic targets for attenuating COVID-19 cytokine storms [11]. Central to induced cytokine expression is intracellular signaling involving a cascade of

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kinase reactions. Thus, altering these reactions could be a means to diminish the cytokine storm. Of particular interest is the multi-tasking serine/threonine kinase termed glycogen synthase kinase-3 (GSK-3) [12].

GSK-3, of which there are two isoforms namely GSK-3α and GSK-3β [13], was first identified in rabbit skeletal muscle [14] and has been hypothesized to play a key role in LPS (a component of gram-negative bacteria)-induced cytokine expression via the TLR signaling pathway [15]. Our lab and others have found evidence that GSK-3 inhibitors may attenuate the cytokine storm induced by LPS [16,17]. For example, we recently reported on the identification of a highly specific and potent inhibitor of GSK-3 [18], termed COB-187, that significantly abated the cytokine storm expressed by THP-1 macrophages in response to LPS [17]. Importantly, multiple PRR signal transduction pathways, while distinct in certain ways, clearly overlap in others. To the later point, both the TLR2 and TLR4 signal transduction pathways utilize the MyD88-dependent signaling pathway [19] and TLR3, thought to be mostly a "viral" PRR, and TLR4, thought to be mostly a "bacterial" PRR, both utilize the TRIF-dependent pathway [20]. This overlap, the above discussion, and the fact that CXCL10 has been reported to be elevated in severe COVID-19 [21], led us to investigate the propositions that (i) the SARS-CoV-2 spike protein induces CXCL10 expression and (ii) GSK-3 inhibitors attenuate this induction.

2. Materials and methods

2.1. Cell culture

The THP-1 human monocyte cell line was purchased from American Type Culture Collection (ATTC, Manassas, VA) and cultured according to the manufacturer's protocol. In brief, THP-1 cells were cultured in RPMI-1640 medium (ATCC, Manassas, VA) supplemented with 10% FBS (ATCC, Manassas, VA), 0.05 mM of 2-mercaptoethanol (Millipore, Billerica, MA), and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO), at 37 °C and 5.0% CO_2. To differentiate THP-1 monocytes to a macrophage phenotype, THP-1 cells were treated with 50 ng/mL of phorbol 12-myristate 13-acetate (PMA) (5244001 MG, MilliporeSigma) in growth media for 48 h. Subsequently, the adherent differentiated cells were washed with growth media and incubated for 24 h with growth media in the absence of PMA at 37 °C and 5.0% CO_2 [18]. We refer to the differentiated THP-1 cells as THP-1 macrophages throughout the paper.

2.2. Treatment of THP-1 macrophages with SARS-CoV-2 proteins and LPS

We obtained the SARS-CoV-2 proteins from ACROBiosystems (Delaware Technology Park). ACROBiosystems altered the amino acid sequence of the recombinant S and S2 proteins in the middle of our study. Specifically, proline substitutions [F817P, A892P, A899P, A942P, K986P, V987P] were introduced for both proteins. Note that both versions of the S protein had alanine substitutions [R683A and R685A]. We refer to the constructs with the proline substitutions as mutated S and S2; unless otherwise noted, the "wild type" version of these proteins was used. The mutated and wild type S have different catalog numbers while the mutated and wild type S2 have the same catalog number but different lot numbers. Currently only the mutated version of S and S2 are listed in the catalog and the wild type can be obtained via special request.

THP-1 macrophages were treated with growth media containing one of the following proteins: SARS-CoV-2 full length S protein (S) (cat# SPN-C52H8), mutated S (cat# SPN-C52H9), the S1 subunit (S1) (cat# S1NC52H3), the S2 subunit (S2) (cat# S2N—C52H5, lot#

3621-206GF1-SG and lot#3621-2042F1-X5), mutated S2 (cat# S2N—C52H5, lot# P3840b-2087F1-T7), nucleocapsid (N) (cat#-NUN-C5227) all from ACROBiosystems, or a non-SARS-CoV-2 protein GFP (cat# 23030040; RayBiotech). All proteins were expressed in human embryonic kidney 293 (HEK293) cells and contained a poly-His Tag. The concentration of spike protein in a given sample reported in the figures was calculated using the molecular weight of the monomer for the spike protein. Communication with the manufacturer indicted that the S proteins exist predominantly as a trimer and the S2 and S1 as a monomer. To estimate the concentration of trimeric S and mutated S used, divide the values reported in the figures for these two proteins by 3.

LPS was used as a positive control inducer of CXCL10 and was either from Millipore Sigma (cat# LPS25; used in Fig. 1A), or from InvivoGen (cat# tlrl-3pelps, Ultrapure LPS; used in all other figures). Polymyxin B (PB) was from Sigma-Aldrich (cat# 92283-10 ML) and LPS-RS, which is a potent and selective antagonist of TLR4 [22], was from Invivogen (cat# tlrl-prslps). To determine the effect of GSK-3 inhibitors on S and S2 induction of CXCL10, THP-1 macrophages were treated with S or S2 proteins in the presence of 25 or 12.5 μM of GSK-3 inhibitors [9-ING-41 (cat# AOB33534, AOBIOUS INC) [23], Lithium carbonate (cat# 413261000, Thermo Scientific Chemicals) [24], LY2090314 (cat# SML1438, MilliporeSigma) [25], Tideglusib (cat# SML0339, MilliporeSigma) [26], AZD-1080 (cat# HY-13862, MedChemExpress) [27], or COB-187 [17,18,28] generated by S. Bergmeier at Ohio University], or dexamethasone (cat# 0219004090, MP Biomedicals, Inc) which is currently used in the treatment of SARS-CoV-2 patients [29]. Negative controls were THP-1 macrophages treated with fresh media in the presence of 1% DMSO (i.e., carrier control for the GSK-3 inhibitors).

Subsequent to the treatments described in the above paragraphs, the THP-1 macrophages were incubated for 6 h under standard cell culture conditions. After the incubation, the supernatants were harvested and the level of CXCL10 in the supernatants determined via ELISA as described below.

2.3. ELISA quantification of CXCL10 protein in culture supernatants

The human CXCL10/IP-10 ELISA kit (cat# KAC2361, R&D Systems) was used to quantify the presence of CXCL10 protein in culture supernatants. The assay was performed according to the manufacturer's protocol with the exception that the supernatants were diluted 1:2 rather than 1:10 with Standard Diluent Buffer. A standard curve was generated using CXCL10 provided by the supplier. This curve was used to estimate the level of CXCL10 present in supernatants from the S and S2 treated THP-1 macrophages. To maximize utilization of resources, this analysis was only done for a small set of experiments.

2.4. LAL assay

Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo-Fisher Scientific, A39553, Waltham, MA) was used to indirectly estimate the level of endotoxin in the spike protein samples. This assay was performed as per the manufacturer's protocol. Briefly, the samples were incubated with the Amebocyte Lysate reagent that interacts with endotoxin, if present, and initiates a cascade of reactions which ultimately leads to activation of a pro-clotting enzyme. The activated enzyme facilitates the release of p-nitro-aniline (pNA) from a chromogenic substrate which produces a yellow color. The solutions were read at 405 nm using a Synergy HT Multi-Mode Microplate Reader (BioTek; Winoooski, VT).
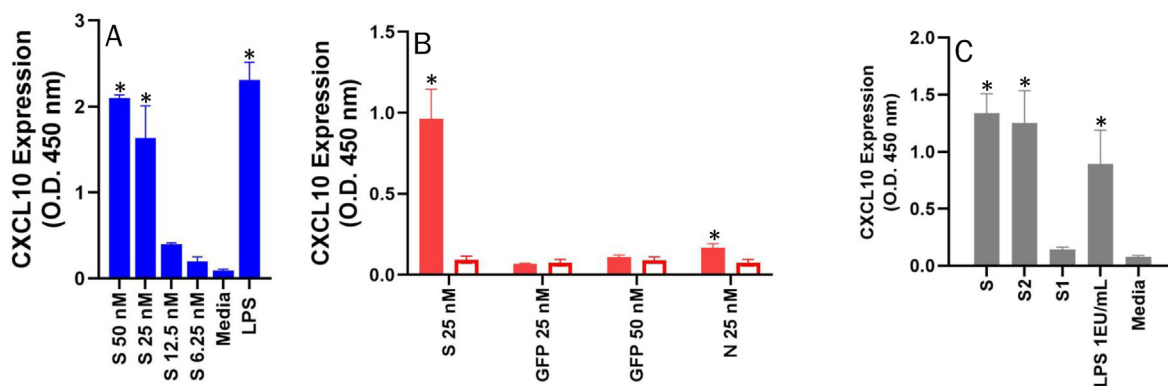
Fig. 1. S and S2 induce CXCL10 expression by THP-1 macrophages. **(A)** THP-1 macrophages were treated with various concentrations of S, media alone, or LPS (10 ng/mL) for 6 h and the level of CXCL10 in the supernatants subsequently determined. Error bars: SD generated from a single experiment performed in duplicate; *$p < 0.05$ determined by Dunnett's comparing treatments to media. **(B)** THP-1 macrophages were treated with S, GFP, N (filled bars), or media alone (open bars) for 6 h and the level of CXCL10 in the supernatants subsequently determined. Error bars: SEM ($n \geq 4$); *$p < 0.05$ determined by a two-tailed *t*-test between treatment and its media control. **(C)** THP-1 macrophages were treated with S, S2, S1, 1 EU/mL ultrapure LPS, or media alone for 6 h and the level of CXCL10 in the supernatants subsequently determined. Error bars: SEM ($n \geq 4$). *$p < 0.05$ as determined by Dunnett's comparing treatments to media. Protein concentration, 25 nM.

2.5. EndoLISA assay

EndoLISA (BioVendor, LLC, 609033) was used to directly measure the level of endotoxin in the spike protein preparations. The assay was performed according to the manufacturer's protocol. In brief, samples were added to the wells of an EndoLISA microtiter plate pre-coated with a capture construct that has high selectivity and affinity for the conserved core region of LPS i.e., lipid A. Subsequently, the wells were washed and then exposed to a solution containing the zymogen form of factor C which detects the endotoxin, if present, and generates a fluorescent signal through conversion of a substrate [30]. The solutions were read at 380 nm and 485 nm using the Synergy HT Multi-Mode Microplate Reader.

2.6. Statistics

All statistical analyses were performed using the GraphPad Prism 8.3.2 (GraphPad Software Inc., San Diego, CA). Specific analyses are listed in the figure captions.

3. Results and discussion

3.1. SARS-CoV-2 S, S2, but not N nor S1, induce robust CXCL10 expression by THP-1 macrophages at the conditions tested

Treatment of THP-1 macrophages with S for 6 h induced CXCL10 expression in a dose dependent manner with 25 nM of S inducing significant and relevant (compared to LPS) levels of CXCL10 (Fig. 1A). In contrast, an irrelevant protein, GFP, did not induce CXCL10 expression while N induced quite limited, albeit statistically significant, CXCL10 expression (Fig. 1B). S2, but not S1, as well as LPS (positive control) induced significant CXCL10 at the conditions tested (Fig. 1C).

3.2. SARS-CoV-2 S and S2 induction of CXCL10 expression is not due to contaminating endotoxin in the protein preparations

While the above observations suggest that S and S2 can induce robust CXCL10 expression, commercially available protein preparations can be contaminated with endotoxin which may confound
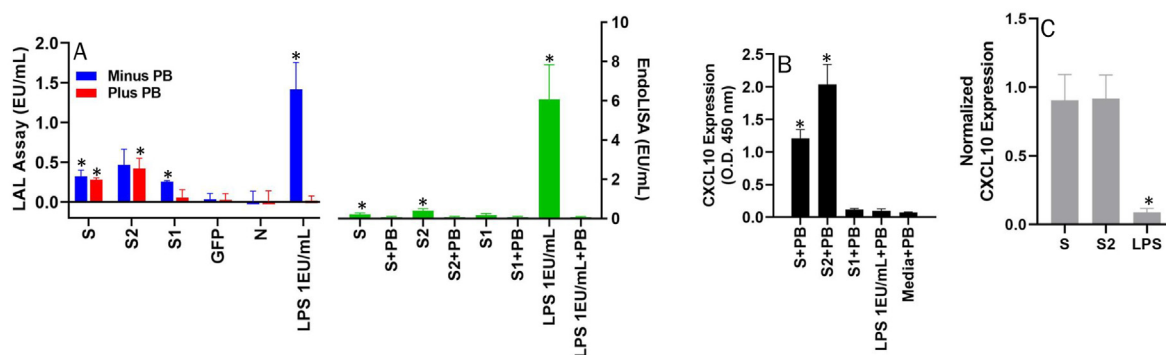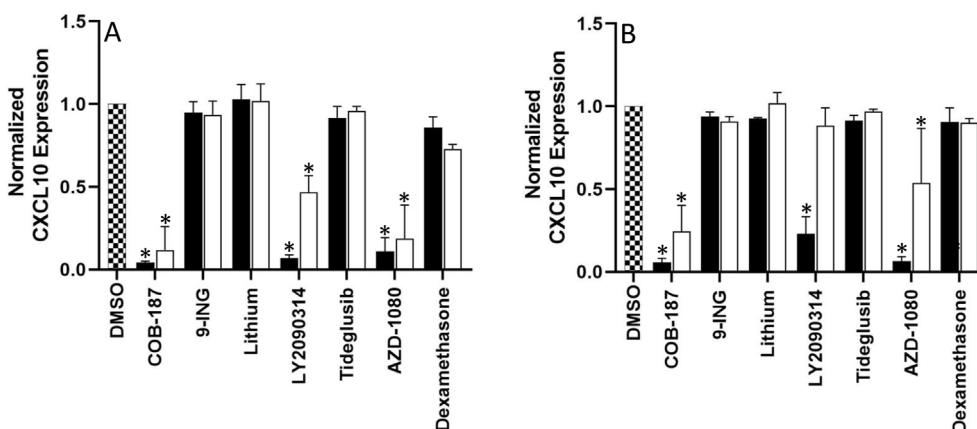

Fig. 2. LAL and EndoLISA analysis of protein preparations, and the effect of PB and a TLR4 antagonist on S and S2 induction of CXCL10. **(A)** Protein samples and ultrapure LPS (nominal 1 EU/ml), in the absence (left bar in each pair) or presence (right bar in each pair) of 30 μg/mL PB, were evaluated in an LAL assay (left panel) or an EndoLISA (right panel). Protein concentration, 25 nM. Error bars: SEM ($n \geq 2$). *$p < 0.05$ indicates significantly greater than zero based on a one-tailed, one-variable *t*-test. [Note: the LAL LPS signal (1.4 ± 0.7SD) was similar to the nominal value of 1 EU/ml (determined from company supplied LAL data), while the EndoELISA signal was much higher (6.0 ± 3.5SD) suggesting the EndoLISA is more sensitive.]. **(B)** THP-1 macrophages were treated with S, S2, S1, 1 EU/mL ultrapure LPS, or media alone for 6 h in the presence of 30 μg/ml PB and the level of CXCL10 in the supernatants subsequently determined. Error bars: SEM ($n \geq 4$). *$p < 0.05$ as determined by a Dunnett's comparing treatments to media + PB. Protein concentration, 25 nM. **(C)** THP-1 macrophages were treated with S, S2, or 1 EU/mL ultrapure LPS in the absence or presence of 100 ng/mL LPS-RS, an antagonist of TLR4. 30 μg/ml PB was also present in the S and S2 samples. The level of CXCL10 in the supernatants was determined subsequent to a 6 h incubation. The level of CXCL10 observed in the presence of LPS-RS was divided by the level of CXCL10 observed in the absence of LPS-RS. The average from the results of 4 experiments is shown. Error bars: SD ($n = 4$). *$p < 0.05$ determined by a one-tailed *t*-test comparing the observed ratio to unity. S, S2 concentration, 25 nM.
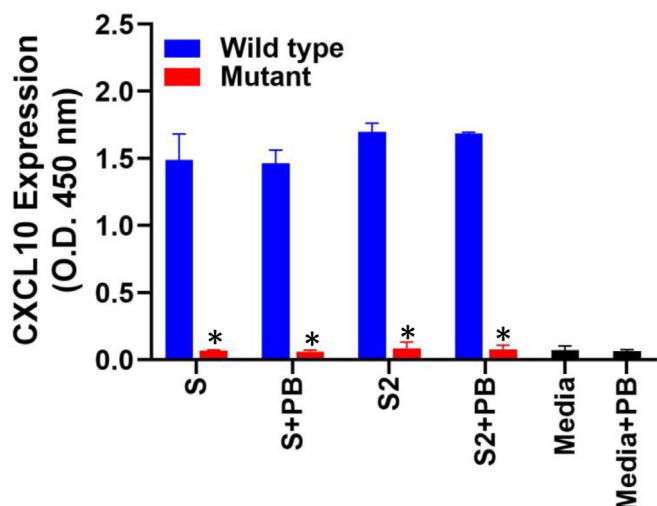
Fig. 3. Mutated S and S2 do not induce CXCL10 expression. THP-1 macrophages were treated with wild type (left bar in each pair) or mutant (right bar in each pair) S or S2 proteins, or media alone, in the absence or presence of 30 μg/mL PB for 6 h and the level of CXCL10 in the supernatants subsequently determined. Error bars: SEM (n = 2). *p < 0.05 as determined by a two-tailed *t*-test comparing wild type to mutant for each condition. Mutant S and S2 are described in the methods section.

data interpretation [31]. Thus, we assayed for the presence of endotoxin using the indirect LAL assay (Fig. 2A left panel), which measures sample-induced coagulation, and the EndoLISA which is a direct measure of endotoxin (Fig. 2A, right panel). The LAL results were conflicted regarding the presence of endotoxin in the samples. While both the S and S2 preparations appeared to give a modest signal, the signal was not reduced by polymyxin B (PB) which can neutralize endotoxin present in protein preparations [32] (Fig. 2A left panel). To the latter point, PB did indeed abolish the LPS signal in both the assays (Fig. 2A left and right panels). The EndoLISA appeared to detect a low level of endotoxin in the S and S2 protein preparations (~4% and ~7%, respectively, of the nominal 1 EU/ml LPS signal) which were reduced to background levels by PB (Fig. 2A, right panel).

Treatment of THP-1 macrophages with LPS in the presence of PB abolished LPS-induced CXCL10 expression by THP-1 macrophages

(Fig. 2B). In sharp contrast, treatment of THP-1 macrophages with S or S2 in the presence of PB yielded robust CXCL10 expression (Fig. 2B). S1, which gave a signal in the LAL assay, did not induce significant CXCL10 expression in the absence or presence of PB (Figs. 1C and 2B). Several reports demonstrate that LPS utilizes TLR4 to induce cytokine expression [33]. LPS-RS is a potent and selective antagonist of TLR4 [22]. Thus, to further probe the possibility of contaminating endotoxin underlying S and S2 induction of CXCL10 expression, and to begin to gain insight into the mechanism of induction, we treated THP-1 macrophages with S, S2 or 1 EU/ml LPS in the presence of 100 ng/mL of LPS-RS. We observed that LPS-RS had no significant effect on S and S2 induction of CXCL10 (Fig. 2C). In clear contrast, LPS-RS significantly diminished LPS-induced CXCL10 expression (Fig. 2C). Combined, the data presented in Fig. 2 are consistent with the interpretation that it is the S and S2 proteins, and not contaminating endotoxin, that induces CXCL10 expression.

3.3. A mutated form of S and S2 do not induce CXCL10 expression

During the course of this study, the supplier of S and S2 altered the amino acid sequence of the S and S2 recombinant proteins. Specifically, they modified F817P, A892P, A899P, A942P, K986P, V987P on both proteins. Surprisingly, the modified versions of S and S2 did not induce CXCL10 (Fig. 3). Data provided by the supplier indicated that both the "wild-type" and proline mutated S protein exist predominantly in the trimeric state while both versions of the S2 proteins are in the monomeric state. This observation would appear to eliminate the possibility of a change in the trimeric/monomeric state of the proteins as the cause of the observed differences in the activity of the "wild-type" versus the mutated proteins.

3.4. Two clinically relevant GSK-3 inhibitors and COB-187 attenuate S and S2 induction of CXCL10 expression by THP-1 macrophages

We next explored the effect of GSK-3 inhibitors on S and S2 induction of CXCL10 using COB-187 and more clinically advanced GSK-3 inhibitors, specifically: lithium (a weak GSK-3 inhibitor used clinically [24]); Tideglusib [26] and 9-ING-41 [23] (currently in clinical trials); and AZD-1080 [27] and LY2090314 [25] (previously in clinical trials). We observed that 25 and 12.5 μM of COB-187, LY2090314, and AZD-1080, significantly reduced S-induced

Fig. 4. Effect of GSK-3 inhibitors on S and S2-induced CXCL10 expression. THP-1 macrophages were treated with S (**A**), or S2 (**B**) in the presence of 25 μM (black bars) or 12.5 μM (white bars) GSK 3 inhibitors, or 1% DMSO (carrier control). The y-axis represents S or S2-induced CXCL10 expression in the presence of the listed inhibitor normalized to S or S2-induced CXCL10 expression observed in the presence of 1% DMSO. Normalized DMSO bar, whose value is unity due to being normalized to itself, is shown for comparison. Error bars: SD; (n ≥ 2). *p < 0.05 as determined by a Dunnett's test comparing S or S2-induced CXCL10 expression in the presence of a given GSK-3 inhibitor to S or S2-induced CXCL10 expression in the presence of 1% DMSO control. (Note that the raw data, not the normalized data, was used in the statistical analysis.) Experiments were done in the presence of 30 μg/ml PB and utilized 25 nM of S or S2.

CXCL10 expression (Fig. 4A). Lithium, Tideglusib, and 9-ING-41 appeared to have little, if any effect. Dexamethasone, currently used to treat severe COVID-19 in the later stages of the disease [29], did not significantly inhibit S-induction of CXCL10 (Fig. 4A). Similar results were observed with S2-induction of CXCL10 (Fig. 4B) with the exception that 12.5 μM LY2090314 had little, if any, effect. While we did not observe inhibition by Lithium, 9-ING-41 and Tideglusib, we only tested a limited number of conditions. It is entirely possible that inhibition could be observed under a different set of conditions [e.g., lower concentration of S or S2 protein (the level of CXCL10 induced by 25 nM of the spike proteins was ~500—1000 pg/ml), pre-incubation with the inhibitor prior to treatment with spike protein, higher concentration of inhibitor]. This caveat might be particularly important for lithium since it is known to be a weak GSK-3 inhibitor [34].

In summary, in line with other recent studies [4,10] we have found evidence that SARS-CoV-2 S and S2 proteins induce cytokine expression, in particular the chemokine CXCL10. Under the conditions tested, we found that two clinically relevant GSK-3 inhibitors and COB-187, a novel GSK-3 inhibitor, attenuate S and S2 induction of CXCL10. These results provide impetus for investigating GSK-3 inhibitors as potential therapeutics for COVID-19.

Declaration of competing interest

Ohio University owns patents related to COB-187. SCB, KDM, and DJG are inventors on the patents.

Acknowledgments

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