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Communication

The SARS-CoV-2 S1 Spike Protein Promotes MAPK and NF-kB Activation and Inflammatory Cytokine Production In Human Lung and Intestinal Epithelial Cells

Christopher B. Forsyth[1], Lijuan Zhang[2], Abhinav Bhushan[3], Barbara Swanson[4], João I. Mamede[5], Maliha Shaikh[2], Li Zhang[5], and Ali Keshavarzian[1*]

Affiliations:

1. Department of Internal Medicine, Section of Gastroenterology; Rush Center for Integrated Microbiome and Chronobiology Research; Department of Anatomy and Cell Biology; and Rush University Graduate College, Rush University Medical Center, Chicago, IL 60612 USA; ali_keshavarziasn@rush.edu; christopher_forsyth@rush.edu

2. Rush Center for Integrated Microbiome and Chronobiology Research, Rush University Medical Center, Chicago, IL 60612 USA maliha_shaikh@rush.edu; lijuan_zhang@rush.edu

3. Department of Biomedical Engineering, Illinois Institute of Technology, Chicago, IL 60616 USA abhushan@iit.edu

4. Department of Adult Health & Gerontological Nursing, Rush University Medical Center, Chicago, IL 60612 Barbara_A Swanson@rush.edu

5. Department of Microbial Pathogens and Immunity, Rush University Medical Center, Chicago, IL 60612 joao_mamede@rush.edu li_zhang@rush.edu

* Correspondence: ali_keshavarzian@rush.edu

Abstract: The Coronavirus disease 2019 (COVID-19) pandemic began in Jan. 2020 in Wuhan, China with a new coronavirus designated SARS-CoV-2. The principle cause of death from COVID-19 disease quickly emerged as Acute Respiratory Distress Syndrome (ARDS). A key ARDS pathogenic mechanism is the "*Cytokine Storm*". This is a dramatic increase in the blood of inflammatory cytokines. In the last 2 years of the pandemic new pathology has emerged in COVID-19 survivors in which a variety of long-term symptoms emerge. This condition is called "*Long COVID*". The spike protein on the surface of the virus (target for the new mRNA/DNA vaccines) is composed of joined S1-S2 subunits. Upon S1 binding to the human ACE2 receptor on cells, the S1 subunit is cleaved and the S2 subunit mediates entry of the virus. The S1 protein is then released into the blood, which might be one of the pivotal triggers for initiation and/or perpetuation of the cytokine storm. In this study, we tested the hypothesis that the spike S1 protein may activate inflammatory signaling and cytokine production independent of the virus. Our data support a potential role for spike S1 activation of inflammatory signaling and cytokine production in human lung and intestinal epithelial cells in culture. These data support a potential role for the SARS-CoV-2 spike S1 protein in COVID-19 pathogenesis.

Keywords: COVID-19, ARDS, Cytokine Storm, Spike S1 protein, SARS-CoV-2, Long COVID, ACE2, A549 cells, Caco-2 cells

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1. Introduction

In Dec. 2019-January 2020 a pneumonia illness originating in Wuhan, China designated as *coronavirus disease 2019* (COVID-19) was shown to be caused by a novel RNA coronavirus designated as *severe acute respiratory syndrome coronavirus 2* (SARS-CoV-2)[1-3]. The WHO declared COVID-19 as an International Health Emergency on Jan. 30th 2020[4,5]. This SARS-CoV-2 coronavirus represents one of seven *coronaviridae* now identified to cause disease in humans [1,6,7]. The current COVID-19 pandemic has spread globally with current world estimates of World: 529 mil.+ cases and 6.3 mil.+ deaths; USA 85 mil.+ cases and 1+ mil. deaths (5/22) [8]. Coronaviruses are typically respiratory viruses and SARS-CoV-1 (SARS), MERS-CoV and SARS-CoV-2 have as their major cause of death the Acute Respiratory Distress Syndrome (ARDS). ARDS is a severe and often fatal form of acute lung injury (ALI) that occurs in critically ill patients [9,10]. A key to severe COVID-19 disease is the so-called *Cytokine Storm* [11,12]. This is a dramatic increase in the blood of inflammatory cytokines/chemokines (especially the signature IL-1β, IL-6, IL-8 and TNF-α) and other inflammatory factors that predicts COVID-19 severity and survival [11]. COVID-19 disease has a unique cytokine profile compared to other respiratory viruses [13]. However, the pathogenesis of COVID-19 disease has become more mysterious and complex as the pandemic evolves. A large body of data now supports diverse COVID-19 pathology including gastrointestinal, cardiovascular, and neurological disease in addition to respiratory disease [14,15]. These diverse pathologies apply not only to acute COVID-19 disease but also to a condition now known as '*Long COVID*' (also called post-acute sequelae of COVID-19, PASC; or 'Long haulers') that persists in the absence of a positive test for the virus beyond six months after infection [16,17].

One emerging candidate for COVID-19 ARDS/cytokine storm pathology as well as *Long COVID* is the S1 spike region of the SARS-CoV-2 spike protein [18-20]. In SARS-CoV-2 infection, the 'spike' (S) protein on the surface of each coronavirus binds to the ACE2 receptor on human cells to gain entry by a cleavage/endocytosis process [21-23]. The spike proteins on the virus surface give the virus the "corona" appearance. SARS-CoV-2 spike protein is a class I viral fusion protein [19]. The complete S spike protein is coded for in the 3 major vaccines developed against SARS-CoV-2 by Pfizer-Biontech and Moderna (S mRNA), and Johnson & Johnson/Janssen (S DNA) [24]. These vaccines work by instructing human cells to make the S protein (complete S1-S2) to stimulate a protective SARS-CoV-2 immune response. SARS-CoV-2 mutations in variants of concern (VOC) occur mainly in the spike (S) protein, which is also the target of most natural protective antibodies [25]. Each surface spike receptor is actually a trimer composed of 3 identical spike protein monomers tightly associated with each other and each spike monomer contains 2 distinct joined subunits: S1 and S2 [24,26]. The S1 outer subunit makes first contact with ACE2 and S1 contains the 'receptor-binding-domain' (RBD) region that provides the binding site and specificity for ACE2 [27,28]. Importantly for pathogenesis and our study, after the S1 subunit/RBD binding to ACE2, the region between S1-S2 is cleaved to release S1, and S2 mediates viral fusion to the cell membrane and viral entry [23]. This S1-S2 cleavage site is not present in the SARS virus spike protein and correlates with COVID-19 pathogenesis [29-31]. Thus the S1 spike subunit is released with each infection and S1 protein is also shed spontaneously [32]. One study estimates 300/1 fold greater numbers of free S1 spike in the blood during infection vs. virions in COVID-19 disease [33]. Previously this S spike protein in COVID-19 was assumed to not have independent biological activity and thus was the target protein produced by the mRNA and DNA COVID-19 vaccines [24]. However, recent data shows high S1 spike blood levels correlate with COVID-19 poor prognosis, ICU admission, and death [34,35]. S1 spike is the target of most neutralizing antibodies after infection with 34 mutations in the recent original Omicron variant [25]. Thus, S1 Spike protein might be one of the pivotal triggers for initiation and/or perpetuation of the cytokine storm. The primary goal of our study is to test this hypothesis.

As the COVID-19 pandemic has evolved, the effects of the SARS-CoV-2 virus in humans appear more complex than simple respiratory symptoms and ARDS [36,37]. The additional symptoms can occur more than 6 months later in patients who test negative for the virus and involve virtually every organ system including respiratory, cardiovascular, gastrointestinal, and neurological dysfunction [14,37]. This condition is now being called "*Long COVID Syndrome*" (or

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simply *Long COVID*) and the causes are unknown [16,38]. The spike S1 protein is also recently implicated in a pediatric condition called *Hyper Inflammatory Syndrome* after mRNA vaccination in children [39,40]. Since patients with severe COVID-19 also have significant GI symptoms, S1 spike protein might also trigger cytokine production by the intestinal epithelial cells. In this study, we also test this hypothesis. Several recent studies suggest that the S1 spike protein may be one driving agent of '*Long COVID*' such as long term lung, gastrointestinal and brain related symptoms [18,20,36]. Spike S1 can cross the BB barrier and is found in neurons [41-43]. In further support of this model, blood levels of the S1 spike protein were found in 64% of COVID-19 positive patients and spike S1 blood levels correlated with disease severity and ICU admission [34,35]. The S1 protein has been shown to alter barrier function in lung endothelial, BBB and cardiovascular endothelial cell models [44,45]. Direct injection of S1 into rat brain activated multiple inflammatory pathways including IL-1β, IL-6 and TNF-α showing S1 may act like a PAMP (pathogen-associated molecular protein) similar to LPS [42].

Accordingly, we hypothesized that S1 spike protein alone might exhibit proinflammatory activity and sought to test that hypothesis. In our study we used an in vitro model to treat human A549 Type 2 lung epithelial cells (stably overexpressing ACE2 to ensure robust ACE2 expression) as well as Caco-2 human intestinal epithelial cells (which express high levels of ACE2). Both cell types have been shown to exhibit expression of ACE2 and have been used in several studies of SARS-CoV-2 infection of human cells [46]. We sought to determine if S1 spike protein alone could activate MAPK (ERK1/2) or NF-kB (p-65) signaling and/or inflammatory cytokine/chemokine production as a potential mechanism of inflammation-mediated COVID-19 pathology.

2. Materials and Methods

Reagents and antibodies. SARS-CoV-2 Spike recombinant protein S1 subunit #230-01101-100, mw 75kD (Val16 - Gln690), RayBiotech (Peachtree Corners, GA); Chemical inhibitor for MEK1/2 and thus ERK1/2 #S1177 PD98059 Selleck Chemicals, LL (Houston TX). Antibodies: from Cell Signaling Technology (Danvers, MA) the following: #7074 HRP-linked goat anti-rabbit IgG; #4370 phospho-p44/42 MAPK (ERK1/2) Thr202/Tyr204 rabbit mAb; #9102 total p44/42 MAPK (ERK1/2) rabbit poly; #3033 phospho-NF-kB p-p65 Ser536 rabbit mAb; #8242 total NF-kB p65 rabbit mAb; #A2066 Anti-β-actin human rabbit poly from Millipore-Sigma (Burlington, MA); #NBP2-67692 Anti-ACE2 human/mouse, rabbit monoclonal, Novus Biologicals (Centennial CO);

Cell culture. A549 Human lung Type 2 alveolar epithelial cancer cells, ATCC#CCL-185 and Caco-2 human colon epithelial carcinoma ATCC #HTB-37 were purchased from ATCC (Bar Harbor, ME). Cells were cultured in DMEM (Fisher Scientific, Waltham, MA) supplemented with 10% FBS (Gibco, Waltham, MA), 1 mM sodium pyruvate (Life Technologies, Waltham, MA), 2 mM L-glutamine (Fisher Scientific), 10 mM HEPES (Fisher Scientific),100 U ml of penicillin and 100 µg ml of streptomycin (Fisher Scientific). For S1 peptide stimulation experiments, S1 peptide was dissolved in complete media and cells were grown to 80% confluence in 12-well plates (Costar #3513; Corning, NY) with 1ml media for all experiments. Our group has extensive experience with A549-ACE2 overexpressing cells and Caco-2 cells [47,48].

A549 stable ACE2 overexpressing cells (designated A549+ cells in this paper). To produce A549 cells overexpressing ACE2 (A549+), the following protocol we previously published to express ACE2 in HeLa cells was used: A549 cells were transfected with psPAX2, pCMV-VSVG, and pRRL.sin.cPPT.SFFV/Ace2.IRES-puro.WPRE (a gift from Caroline Goujon; Addgene plasmid # 145839; RRID:Addgene145839)(Addgene, Watertown, MA). A549 cells were then transduced with the lentiviral particles and puromycin (InvivoGen, San Diego, CA) selected as in Mamede et al.[49]. ACE2 overexpression was validated by western blotting as described [47].

Western blotting analysis. Cells at 80% confluence in 12 well plates were lysed with Tris-triton buffer (Bioworld, Fisher, Pittsburg, PA) with phosphatase/protease inhibitor cocktail (Sigma, St. Louis, MO) For western blots, total protein was determined (Bio-Rad, Hercules, CA), and samples were prepared with Laemmli sample buffer with 2-ME (Bio-Rad) as

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described [50]. Thirty micrograms of protein/lane was loaded into a 4%/10% stacking acrylamide Tris gel and electrophoresed at 100 V for 2 h. Protein was then transferred to a nitrocellulose membrane (GE Healthcare Limited, Buckinghamshire, UK) for 1.5 h at 130 V. Nonspecific binding was blocked by incubation of the membrane with 5% milk TBST for 1 h. Membranes were then incubated overnight at 4°C with antibodies for p-ERK 1/2, or p-p65 NF-kB, or hACE2, or h-actin in TBST and 5% nonfat dry milk. Membranes were subsequently washed with TBST for 1 h and incubated with the appropriate horseradish peroxidase (HRP)-conjugated anti-secondary antibody (#7074 above). For p-ERK 1/2, or p-p65, or ACE2 or β-actin membranes were subsequently washed with TBST for 1 h. Chemiluminescent substrate (ECL, GE Healthcare) was applied to the membrane for protein visualization using autoradiography film (HyBlot CL, Denville Scientific, Metuchen, NJ). Optical density was determined via densitometric analysis with Image J Software (NIH, Bethesda, MD) [50]. After stripping the p-ERK1/2 and NF-kB p-p65 blots the blots were assessed for total ERK 1/2 or total NF-kB p65. The ERK1/2 and NF-kB p65 blots were treated with anti-total ERK1/2 or total NF-kB p65 in TBST for 1h and developed as described above. Data was normalized to actin for each lane for densitometry comparisons [50].

Spike S1 subunit stimulation, Cytokine and inhibitors analysis. For data in Figures 2-4, cell media cytokine analysis (IL-8 is a chemokine cytokine) was carried out in our lab as we have recently described using the Meso Scale Diagnostics platform. (Meso Scale Diagnostics LLC, (MSD), Rockville, MD)[51]. For this analysis we utilized a V-Plex 96 well plate listed under 'COVID-19 resources' that analyzes 10 inflammatory cytokines identified in the COVID-19 cytokine storm in each well at picogram/ml sensitivity. Specifically, the Meso Scale V-Plex Proinflammatory Panel 1 Human Kit # K15049D (10 cytokine targets: IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNF-α). A549+ and Caco-2 cells in 1 ml/well complete media at 80% confluence in 12-well plates were stimulated with the indicated doses of SARS-CoV-2 recombinant Spike protein S1 subunit (dissolved in complete media). Final S1 concentrations utilized in Figures 2-3 initial experiments were 10ng/ml (0.13nM), 25ng/ml(0.325nM), and 50ng/ml(0.65nM) per well in complete media and in triplicate experiments for both 6h and 24h. Data from Figures 2-3 supported the use of 50ng/ml and 24h as our optimal conditions to move forward with inhibitor experiments data in Figures 4-6. For inhibitor studies MEK1/2 inhibitor PD98059 (30μM) was used to pretreat the cells 1h before the S1 protein was added and inhibitor remained. At the end of each time period 25μl of media from each well was combined with 25μl MSD test solution and processed in the MSD instrument according to manufacturer's instructions. Cell lysates were processed for western blotting and phosphoprotein analysis as described (Methods).

Statistical analysis. All statistical analysis was carried out using 1-way ANOVA with GraphPad Prism software with $p<.05$ significance level (San Diego, CA).

3. Results

Expression of SARS-CoV-2 receptor ACE2 by the A549, A549+, and Caco-2 cell lines in this study.

As shown in Figure 1a lanes 1-4 (brackets) western blotting data, the native ATCC cell line human lung Type 2 alveolar A549 cells express very low levels of ACE2 protein. We have previously established an ATCC HeLa cell line stably overexpressing ACE2 [47] and used the same methods to establish an A549 cell line stably expressing greater ACE2 levels for this study because of the low levels of ACE2 in native ATCC A549 cells. This strategy has been used by others [46]. We designate these cells as A549+(Methods). As shown in Figure 1a lanes 5-8 these A549+ cells robustly express ACE2 receptor protein and were used for all of the A549+/S1 experiments in this study. Shown in Figure 1b (lanes 1-8) is robust ACE2 protein expression western blotting data for the ATCC Caco-2 cells used in this study (Figure 1b. lane 9 is A549+ cells for comparison). These ACE2 expression data are consistent with those from previous COVID-19 studies using these cell lines.[52] Figure 1c compares for the average ACE2 protein relative density values from Figures 1a and 1b (normalized to actin) compared to native ATCC A549 cells in Figure 1a (lanes 1-4), ACE2 in A549+ (Figure 1a lanes 5-8, and Figure 1b lane 9) and Caco-2 cells in Figure 1b lanes 1-8. Summarized in Fig. 1c, The actual Image J density

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values for relative ACE2 protein expression vs. actin are for A549: mean 0.004207; A549+ mean 0.3384 and for Caco-2 cells mean 0.8054 (Figure 1c****p<.0001signif.).

Clearly the Caco-2 cells are expressing about twice the amount of ACE2 receptor protein as the ACE2+ cells and, as others have shown, native A549 cells or primary Type II alveolar cells (not shown) do express ACE2 but not at high levels although they robustly support SARS and SARS-CoV-2 infection as do Caco-2 cells [46]. However, based on these data we went forward to only compare A549+ cells with Caco-2 cells for stimulation with the S1 spike subunit protein.

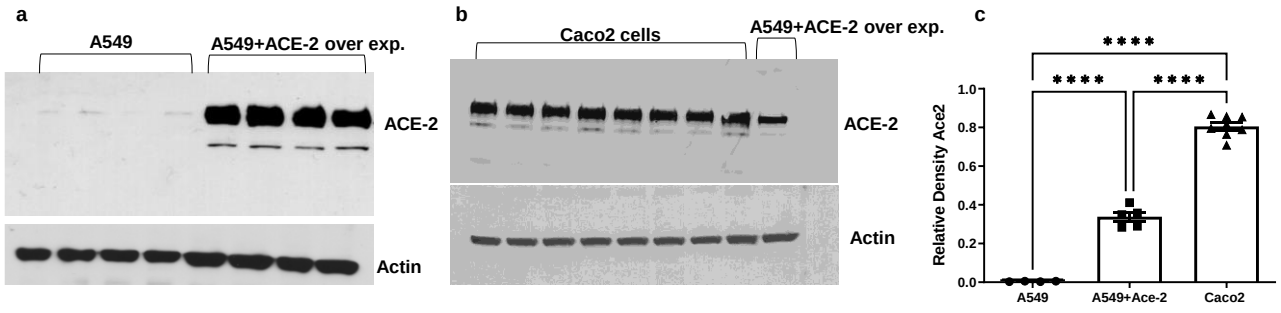

Figure 1. (a-c). ACE2 receptor protein expression in A549, A549+, and Caco-2 cells in this study.

The three cell lines utilized in this study were evaluated for ACE2 receptor protein expression using western blotting (Methods): A549 and A549+ human Type 2 lung alveolar cells and Caco-2 human intestinal epithelial cells were all grown to 80% confluence in 12 well plates, each lane (30ug/lane) in the figures is from a separate well. Cells were lysed and analyzed with Ab to human ACE2 (Methods). **1a.** Lanes 1-4 native ATCC A549 cells; lanes 5-7 are from A549+ ACE2 stable overexpressing cells. **1b.** Lanes 1-8 are lysates from 8 wells containing the human Caco-2 intestinal epithelial cells; lane 9 is data from a single well of A549+ cells for comparison on the same blot. **1c.** Shows relative ACE2 expression density comparison (each point is from a well) for the 3 cell types. The actual Image J mean density values for relative ACE2 protein expression vs. actin are for A549: mean 0.004207; A549+ mean 0.3384 and for Caco-2 cells mean 0.8054. ****p<.0001 Significance 1-way ANOVA (GraphPad, all Figures).

The S1 Spike protein subunit of SARS-CoV-2 stimulates inflammatory cytokine production in A549+ and Caco-2 cells.

We first sought to determine if the S1 Spike protein stimulated cytokine production by A549+ and Caco-2 cells and determine an optimal dose and time for S1 spike protein concentration for treatment of A549+ and Caco-2 cells. Data for A549+ cells are shown in Figures 2a-2h and Caco-2 cells in Figures 3a-3f. A549+ and Caco-2 cells were grown in triplicate wells in 12 well plates to 80% confluence for treatment with initial doses of S1 peptide ranging from 10ng/ml, 25ng/ml, and 50ng/ml for both 6h or 24h. Supernatants from these studies were then analyzed for key inflammatory cytokines that have been associated with the cytokine storm in COVID-19 (IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNF-α)[11,53,54] using the ultrasensitive Meso Scale platform (Methods). Not all cytokines resulted in measurable levels and those cytokine data have been omitted. Means from triplicate wells were analyzed for significance (Methods). Because of numerous studies using media alone as a control group in S1 protein experiments we chose this as a control as well for our studies [55-57]. As shown in Figures 2g and 2h the only significantly increased cytokines in A549+ cells were IL-8 after 24h with 50ng/ml S1 (Figure 2g, p<.001***) and TNF-α resulting in significantly increased TNF-α (Figure 2h) after 24h treatment with 50ng/ml S1 protein (p<.001***) For the treatment of Caco-2 cells with S1 in Figure 3 the data reveals significantly increased IL-6 with 24h and 50ng/ml of S1 treatment (Figure. 3c; p<.001***) as well as significantly increased IL-8 in Figure 3f with 50ng/ml of S1 at both the 6h (p<.0001****) and 24h (p<.001***) time points (Figure 3f).

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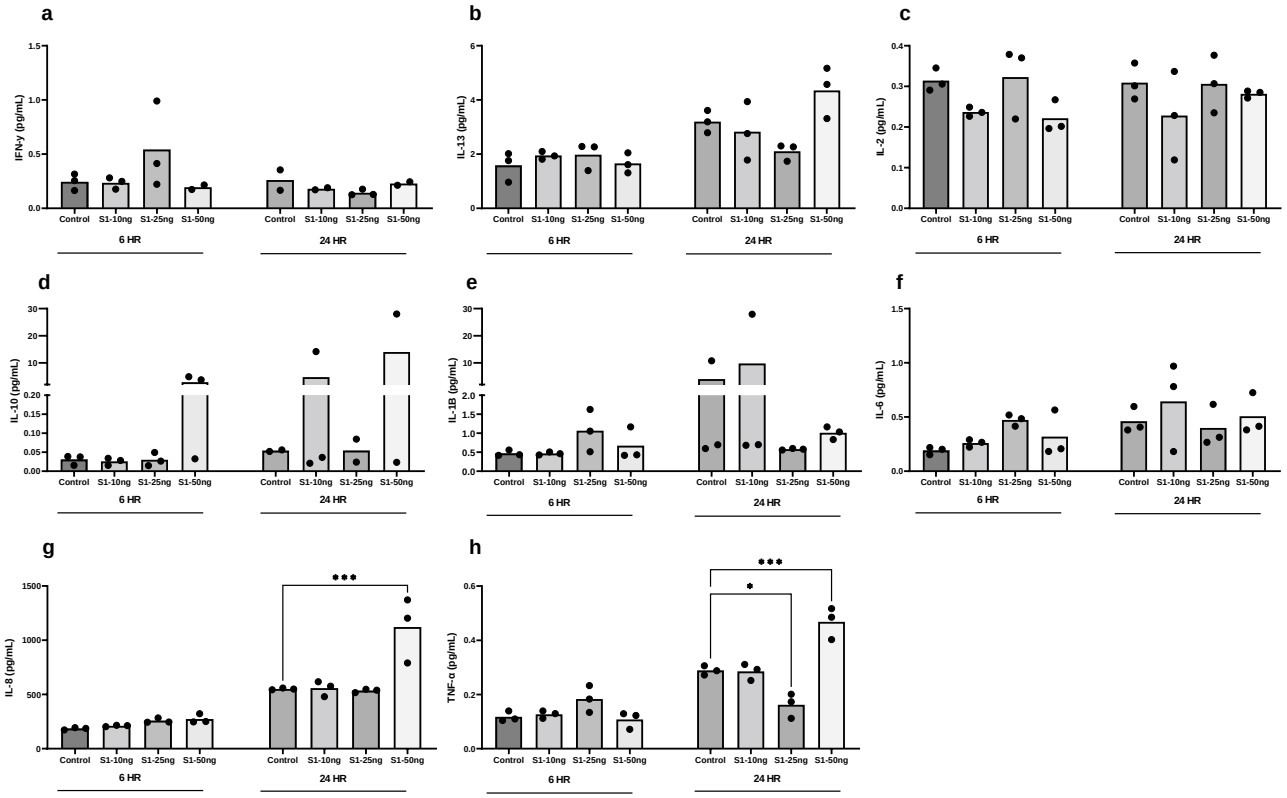

Figure 2 (a-h). Supernatant cytokines for A549+ cells with S1 spike stimulation.

A549+ cells in triplicate 12-well plates at 80% confluence in 1ml media were stimulated with media control or 3 doses of the SARS-CoV-2 spike S1 protein at final concentrations of 10ng/ml (0.13nM), 25ng/ml (0.325 nM), or 50ng/ml (0.65 nM) for either 6h (labeled left) or 24h (right). Supernatant cytokines were measured using the Meso Scale platform (Methods). Some wells had no reading for some cytokines and so had less than 3 data points. *p< 0.5; ***p< .001 significance vs. media control for either 6h or 24h.

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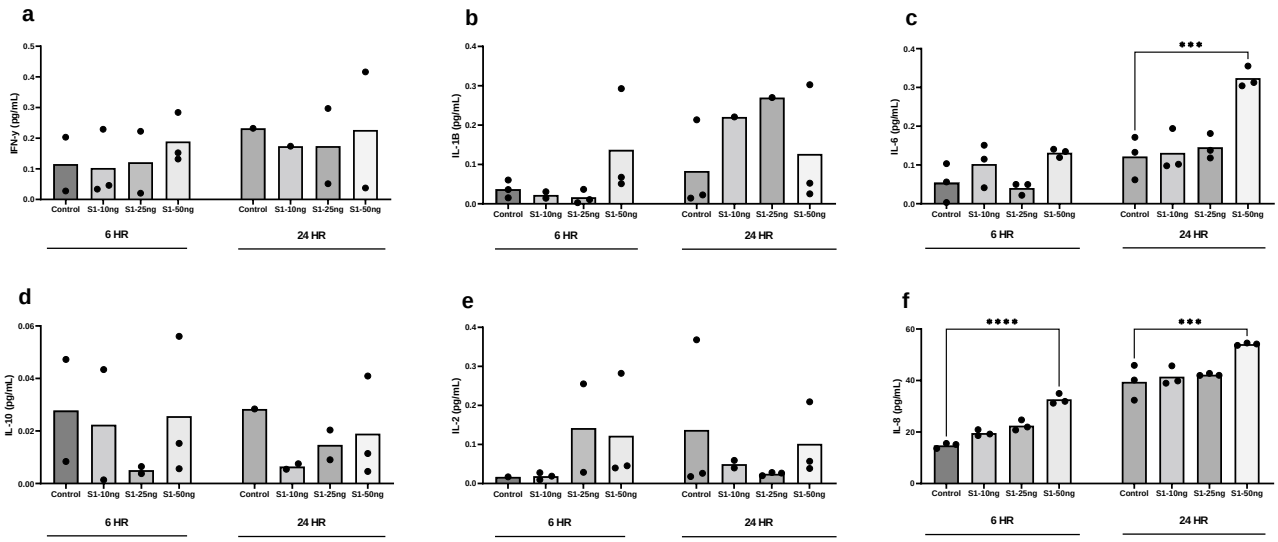

Figure 3 (a-f). Supernatant cytokines for Caco-2 cells with S1 spike stimulation.

Caco-2 cells in triplicate 12-well plates at 80% confluence in 1ml media were stimulated with media control or 3 doses of the SARS-CoV-2 spike S1 protein at final concentrations of 10ng/ml (0.13nM), 25ng/ml (0.325 nM), or 50ng/ml (0.65 nM) for either 6h (labeled left) or 24h (right). Supernatant cytokines were measured using the Meso Scale platform (Methods). Some wells had no reading for some cytokines and so had less than 3 data points. ***p< .001, ****p<.0001 significance vs. media control for either 6h or 24h.

With the overall results from these experiments we chose to continue some experiments only in A549+ cells using only a S1 spike protein concentration of 50ng/ml for 24h in addition to much greater numbers of wells for each test. In some wells (designated "Inhibitor") 30μM MEK1/2 (ERK 1/2) inhibitor PD98059 was added to separate wells 1h before the S1 spike protein was added and remained for 24h. Figures 4a-4J represent data for cytokines that were measureable with our Meso Scale panel (those omitted had no measureable cytokines). Importantly, in Figure 4b we found 50ng/ml S1 spike protein for 24h stimulated a significant increase in IL-1β (p<.05*) that was also significantly inhibited by MAPK inhibitor (p<.05*) Several of the cytokines shown exhibited measureable levels of cytokines but no significant difference between Control and S1 peptide treated A549+ cells, although several cytokines exhibited inhibition by MAPK inhibitor including: Figure 4d IL-12p70, Figure 4e IL-13, Figure 4i IL-8, and Figure 4j TNF-α. Other A549+ cells cytokines measured but not significantly greater than control or affected by S1 or MAPK inhibitor were Figure 4a IFNy, Figure 4c IL-10, Figure 4f IL-2, Figure 4g IL-4, and Figure 4h IL-6.

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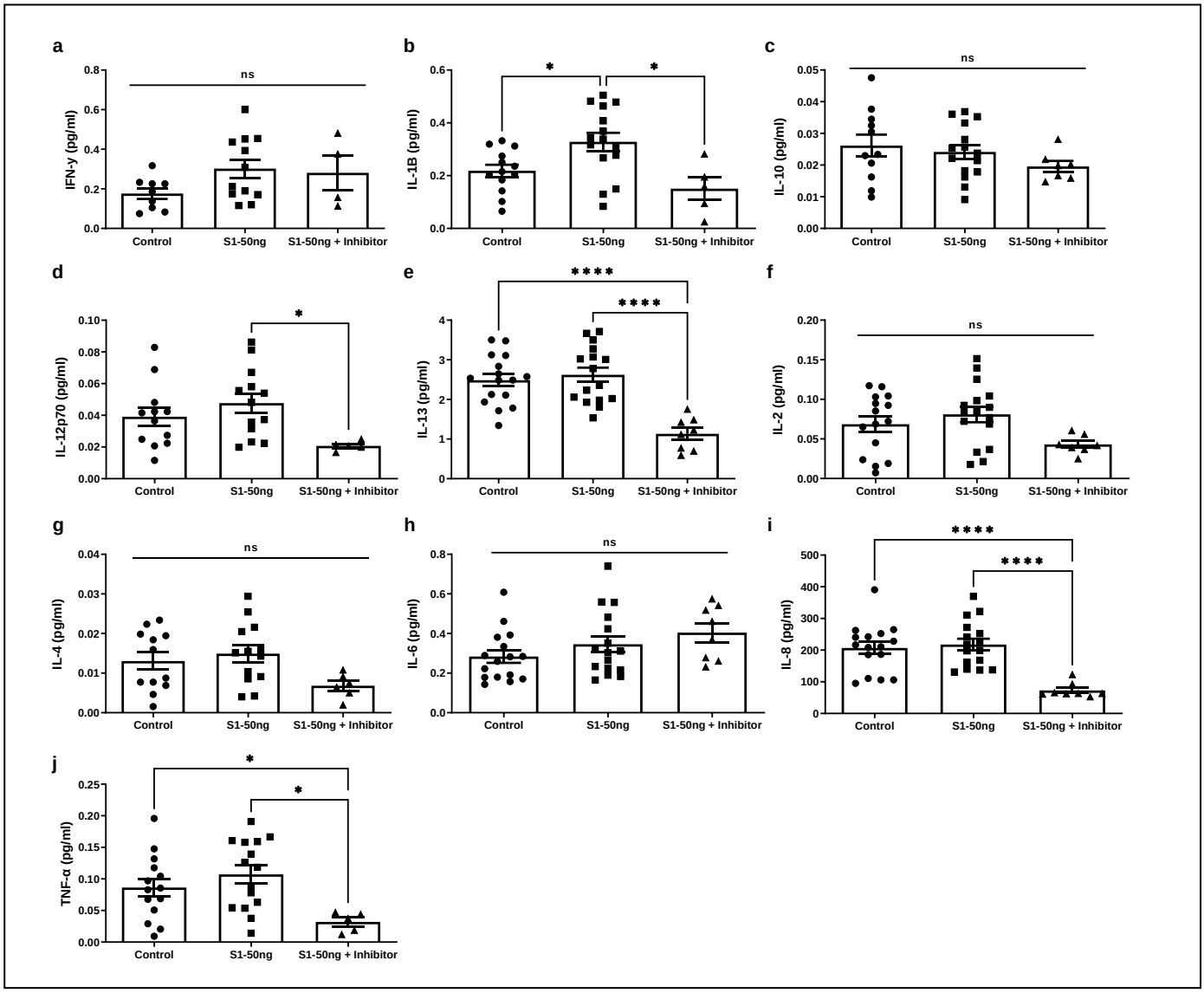

Western blotting data supports S1 spike protein signaling activation of MAPK p-ERK1/2 and NF-kB p-p65 in A549+ cells but not Caco-2 cells.

We next sought to use another avenue to explore possible SARS-CoV-2 S1 protein stimulation of signaling in our A549+ lung and intestinal Caco-2 cells both expressing robust levels of the SARS-CoV-2 ACE2 receptor protein. Figures 5 and 6 show western blotting data for phospho ERK1/2 MAPK ('activated') as well as phospho p65 NF-kB ('activated') after

Figure 4 (a-j). Supernatant cytokines for A549+ cells stimulated with 50ng/ml S1 spike for 24h with/without MAPK inhibitor. A549+ cells in 12-well plates at 80% confluence in 1ml media were stimulated for 24h with media control or 50ng/ml (0.65 nM) of the SARS-CoV-2 spike S1 protein (middle column) or 50ng/ml S1 plus 30μM PD98059 MAPK/ERK1/2 inhibitor for 24h (right). Inhibitor was added 1h before S1 stimulation and not removed. Supernatant cytokines were measured using the Meso Scale platform (Methods). *p< 0.5, ****p<. 0001 significance vs. media control (left) or S1 protein stimulation alone (middle). NS= not significant at p<.05.

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treatment of A549+ and Caco-2 cells with the S1 Spike protein for 24h (50ng/ml) +/- the MEK/ERK 1/2 inhibitor PD98059 (30μM). As in Figures 4a and 4b above, the PD98059 MAPK inhibitor was added 1h before the S1 spike protein and

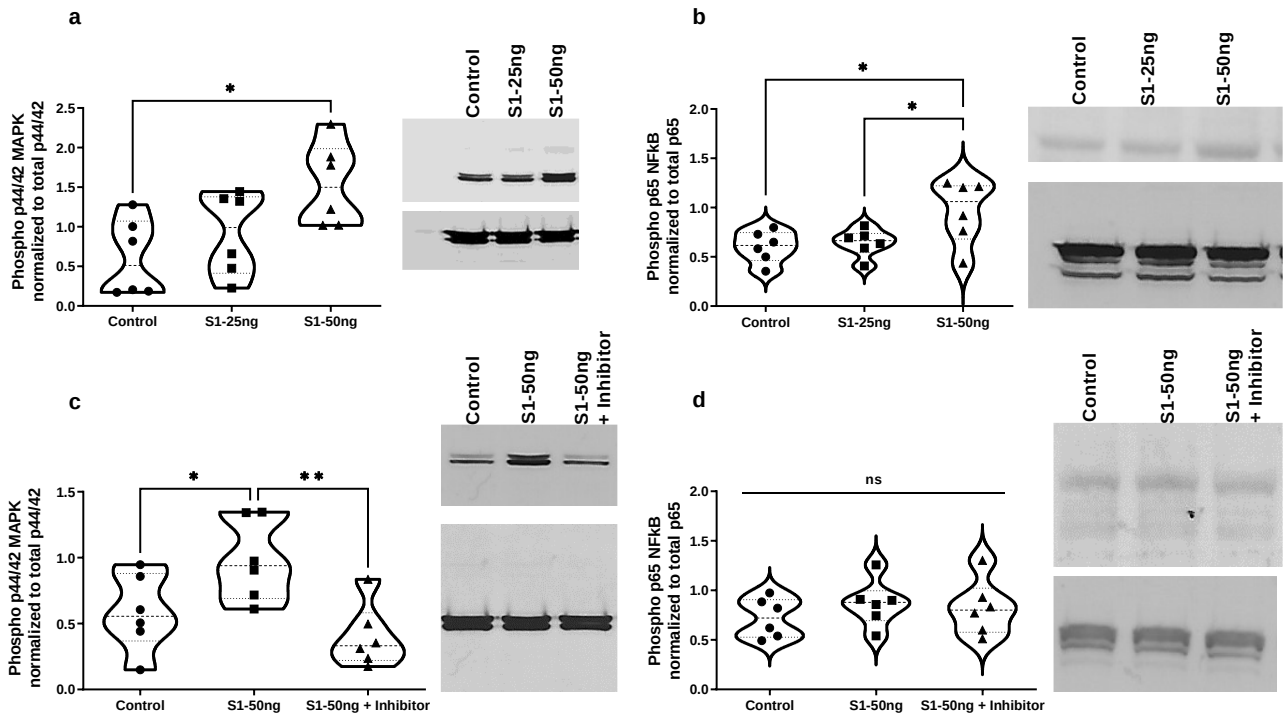

Figure 5. A549+ cells 24h p-ERK 1/2 and NF-kB p-p65 stimulation with S1 Spike 25ng/ml and 50ng/ml and MAPK inhibitor. A549+ cells were stimulated for 24h with either media control (left) or 25ng/ml S1 spike or 50ng/ml S1 spike. Cell lysates were analyzed for: **a.** MAPK p-ERK1/2 (activated) and total ERK1/2 with representative blots at right, or: **b.** Analyzed for NF-kB p-p65 (activated) and total NF-kB with data for each well graphed and representative blots at right. **c.** A549+ cells were treated with only media or 50ng/ml S1 spike or 50ng/ml S1 spike plus 30 μM MAPK inhibitor PD98059 for 1 hour before S1 stimulation and inhibitor was not removed for the 24h. Cell lysates were analyzed as above for p-ERK1/2 vs. total ERK1/2 or: **d.** NF-kB p-p65 vs. total NF-kB p65. *p<.05, **p<.01 significance vs. control or stimulated without inhibitor. NS= not significant at p<.05.

remained for the 24h duration. Both Figure 5 and Figure 6 also contain a representative western blot of either p-ERK1/2

MAPK and total ERK1/2 or p-p65 NF-kB and total p-65 NF-kB proteins +/- the MAPK inhibitor. Figure 5a shows clear dose response inflammation signaling effect data for A549+ cell stimulation with 50ng/ml S1 spike protein (but not 25ng/ml S1) promoting significant activation of phospho-ERK1/2 MAPK proteins (Figure 5a). In Fig. 5b, importantly we show significant S1 spike protein activation of A549+ p-p65 NF-kB with 50ng/ml but not 25ng/ml (Figure 5b) for n=6 wells in each case. In addition, the MEK-MAPK inhibitor significantly blocked activation of ERK1/2 (a target of MEK1/2) with 50ng/ml stimulation in Figure 5c. NF-kB is perhaps the major signaling pathway in the COVID-19 inflammatory response [58]. However, the MEK-MAPK inhibitor did not block A549+ cell activation of p-p65 NF-kB in Figure 5d. However, NF-kB activation was low in these experiments and will need to be re-examined. One preprint suggested MAPK inhibition could block S1 activation of NF-kB [59]. Figure 6 data from Caco-2 cells treatment with 50ng/ml spike S1 +/- MAPK inhibitor despite also n=6 wells in each case tells a different story. The Caco-2 cell data in Figure 6a show a trend in S1 spike protein dose response activation of p-ERK 1/2 by 50ng/ml as well as for NF-kB p-p65 activation in

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Figure 6b but not a significant effect for either case. Surprisingly, Figure 6c shows the ERK 1/2 MAPK inhibitor had no effects on even the slight increase in p-ERK 1/2 or on p-p65 NF-kB levels in S1 stimulated Caco-2 cells.

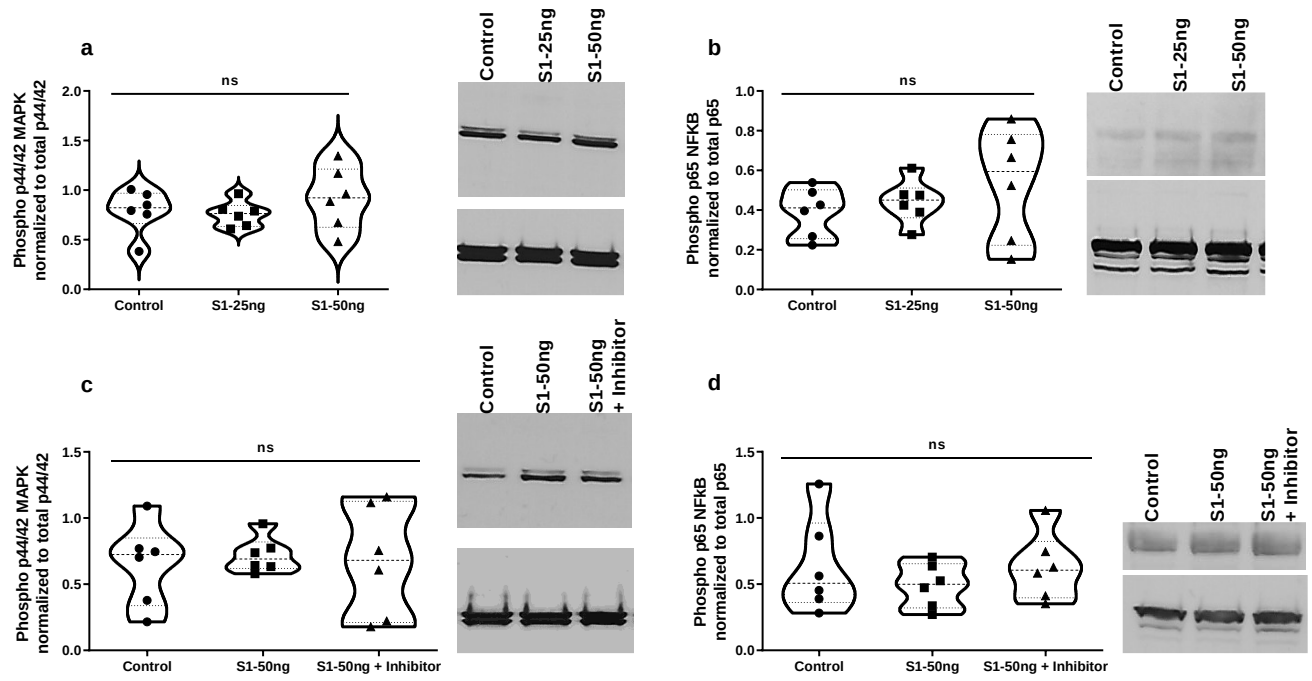

Figure 6. Caco-2 cells 24h p-ERK 1/2 and NF-kB p-p65 stimulation with S1 Spike 25ng/ml and 50ng/ml and MAPK inhibitor.

Caco-2 cells were stimulated for 24h with either media control (left) or 25ng/ml S1 spike or 50ng/ml S1 spike. Cell lysates were analyzed for: **a.** MAPK p-ERK1/2 (activated) and total ERK1/2 and representative blots at right, or: **b.** Analyzed for NF-kB p-p65 (activated) and total NF-kB with data for each well graphed and representative blots at right. Trends in increased p-ERK1/2 and p-p65 are seen but no significance. **c.** Caco-2 cells were treated with only media or 50ng/ml S1 spike or 50ng/ml S1 spike plus 30 uM MAPK inhibitor PD98059 for 1 hour before S1 stimulation and inhibitor was not removed for the 24h. Representative blots at right. Cell lysates were analyzed as above for p-ERK1/2 vs. total ERK1/2 or: **d.** NF-kB p-p65 vs. total NF-kB p65. Representative blots at right. No effect of the MAPK inhibitor was observed for p-ERK1/2 or p-p65. NS= not significant at p<.05.

In summary these data support several points. First, the S1 spike protein of SARS-CoV-2 does appear to demonstrate signaling properties in our A549+ and Caco-2 cells. Importantly, the only significant production of cytokines collectively by both cell types were the four cytokines cited as the principle drivers of the cytokine storm in COVID-19: IL-1β, IL-6, IL-8, and TNF-α [11]. However S1 spike does not appear to have the same signaling effects on Caco-2 cells compared to A549+ cells. Second, A549+ cells responded to S1 stimulation by significant production of IL-8, TNF-α, and IL-1β. We also found in A549+ cells S1 spike protein mediated activation of ERK1/2 MAPK (blocked by MEK/MAPK inhibitor) as well as significant activation of p-p65 NF-kB (not blocked by MAPK inhibitor). Third, S1 Spike protein treatment of Caco-2 cells resulted in significant stimulation of IL-8 and IL-6 but only trends in p-ERK 1/2 activation and NF-kB p-p65 activation. No effects of the MAPK inhibitor were found for S1 stimulation of p-ERK1/2 or NF-kB p-p65 in Caco-2 cells. Therefore, it appears that while both ACE2 expressing cells responded to S1 spike protein stimulation to produce

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inflammatory cytokines characteristic of COVID-19, the intracellular signaling mechanisms appear to be different for A549+ versus Caco-2 cells.

4. Discussion

In this study, we sought to investigate the hypothesis that the COVID-19 virus SARS-CoV-2 S1 spike protein could independently promote cell signaling and inflammatory cytokine production as a potential mechanism for disease in COVID-19. We used two cellular in vitro models that have both been used extensively in studies of infection with intact SARS and SARS-CoV-2 viruses [46,60,61]. The parent ATCC A549 human lung Type 2 epithelial cell line has relatively low expression of ACE2 and other investigators have chosen to overexpress ACE2 to ensure a robust signal as we have in this study [46]. Caco-2 cells express high levels of ACE2 protein and were used to isolate the first COVID-19 virus in Wuhan and to affirm ACE2 as the principle receptor for SARS-CoV-2 in early 2020 [46,62]. Both cell types are relevant because COVID-19 patients (both acute and *Long COVID*) exhibit both respiratory and gastrointestinal symptoms and pathology [63,64]. The levels of ACE2 protein in these two cell lines is demonstrated by western blot in Figure 1.

A growing number of studies have also shown that treatment of cells with the S1 spike protein results in cytokine production and changes in cell signaling [19,20,36]. Characteristic of these studies is variability of the results between studies but overall these studies support our data in A549+ cells. We chose to use the recombinant Raybiotech S1 protein which is the most widely used S1 spike in these experiments [44,57,65] as well as the most widely tested cell line in COVID-19 studies, human lung Type II alveolar A549 cells with ACE2 overexpressed in these cells (ACE2+) and also the human Caco-2 intestinal epithelial cells [46,61]. Caco-2 cells also express ACE2 at high levels and are widely used for SARS and SARS-CoV-2 infection studies as well as other respiratory viruses [46,62]. We found only one study in which Caco-2 were stimulated with the whole S1-S2 spike protein (discussed below).

In our first set of experiments, we sought to develop what we believe is a physiologically relevant SARS-CoV-2 spike protein S1 stimulation dose response. Beginning with only 10ng, 25ng, and 50ng/ml doses we stimulated A549+ and Caco-2 cells with S1 spike for 6h or 24h time periods as seen in Figure 2 and Figure 3. We then measured cytokines released into culture supernatants using the sensitive MesoScale V-Plex Proinflammatory Panel 1 (Methods) for 10 inflammatory cytokines these include: IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF-α. Our data in Figure 2 show significant production of IL-8 and TNF-α at 24h with 50ng/ml stimulation of ACE2+ cells while Caco-2 cells showed significant production of IL-6 and IL-8 for 50ng/ml stimulation at 24h in Figure 3. Later experiments in Figure 4b add IL-1β as an increased cytokine in S1 stimulation of A549+ cells. These data are remarkable for several reasons. First, the collective profile of cytokines produced matches the core cytokines of the COVID-19 cytokine storm: IL-1β, IL-6, IL-8, and TNF-α[11]. Also we are using a concentration of spike S1 protein significantly less than the concentrations in the μg/ml range in several other S1 spike protein studies [45,65]. Perhaps most important is that we know of no other studies demonstrating inflammatory cytokine production in vitro using S1 spike and Caco-2 intestinal epithelial cells. This is a novel and significant effect for S1 spike protein. To our knowledge no in vitro studies have examined effects of the S1 spike protein on Caco-2 inflammatory signaling or cytokine production. However Caco-2 have a history of robust expression of ACE2 and infection by SARS[66] as well as SARS-CoV-2 [46,61]. Only one study we found showed Caco-2 cell expression of inflammatory cytokines IL-β, TNF-α, IL-6 and IL-18 after 24h stimulation with the entire (S1-S2) spike protein [67]. Significant to our data supporting an alternative pathway to MAPK in Caco-2, these cytokines increase was dependent on PPARy signaling [67]. Two recent studies noted that while Caco-2 cells permit robust infection by the COVID-19 virus the cells exhibit no cell death or other pathology [68,69]. Thus Caco-2 response mechanisms to SARS-CoV-2 virus may be quite different than A549+ [68]. As noted above, COVID-19 disease often exhibits severe GI symptoms and diarrhea and intestinal discomfort was added to the list of COVID-19 symptoms early and is now included in symptoms of *Long COVID*[17]. Finally, these data are important in supporting the growing body of data that shows that the spike S1 protein alone has proinflammatory biological activity.

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Our MAPK and cytokine data are supported by other recent studies that report stimulation of MAPK cell signaling pathways as well as inflammatory cytokines by S1 spike protein alone in several cell types and two in vivo mice models. In A549 cells in vitro studies (using a media control as in our studies), S1 spike was found to stimulate NF-kB as well as TNF-α, IL-1β, and IL-6 cytokines. Blocking NF-kB prevented the cytokine increase [56,70]. The entire S1-S2 spike protein was recently also shown to stimulate MAPK, ERK1/2 and NF-kB p65 activation as well as cytokines IL-6 and IL-8 in A549 cells that could be blocked with MAPK and NF-kB inhibitors [71]. Studies in human primary lung bronchial and alveolar mucosal models showed S1 protein stimulated RNA expression in over 100 genes and increased cytokines including the cytokine storm members IL-1β, TNF-α, IL-8 and IL-6 as well as IFNγ, IL-2, IL-4, IL-10, and IL-13 [72]. S1 treatment of BV-2 microglia with 10ng and 50ng/ml as we used increased NLRP3 inflammasome activation and production of the signature IL-1β, TNF-α, and IL-6 as we found (Figures 2-4)[73].

A recent *Science* paper looked at S1 spike protein treatment of older senescent endothelial cells and found S1 spike stimulation of the entire senescent SASP cytokine profile including IL-1α/β, TNF-α, IL-6, IL-8 (as we found) and many other cytokine storm members [74,75]. Significant for potential mechanisms is a recent study using the same recombinant S1 Raybiotech protein that we utilized in this study [65]. They found S1 spike promotes senescence in human endothelial cells and in lungs of hACE2 mice after tracheal gavage and with it the SASP (senescence-associated secretory phenotype) cytokine profile[76] that contains the signature cytokine storm members including IFNγ, TNF-α, IL-6 and IL-1β both in mice lung cells/BALF and systemically in serum [11,65]. Brain injection of the S1 protein promotes neuroinflammation in mice including IL-1β, TNF-α, and IL-6 [42]. Stimulation of human PBMC with S1 also results in monocyte activation (via ACE2 or TLR4) and production of cytokine storm cytokines including TNF-α, IL-6, IL-1β and IL-8 [77]. The S1 protein also stimulates leakiness of 2D and 3D models of human endothelial cell BBB barrier in an in vitro model, a key element in COVID-19 lung damage [44]. These support earlier studies with S1 that showed S1 disrupts and crosses the blood brain barrier in mice [43]. Finally in a recent outstanding paper, tail vein injection of S1 spike in mice resulted in the S1 subunit localized to the endothelia of blood vessels in the mice brain and showed co-staining with caspase-3, ACE2, IL-6, TNF-α, and C5b-9 [78]. In that study, this pattern of S1 spike co-localization with inflammatory cytokines and ACE2 was also found in the brains of 13/13 deceased COVID-19 patients [78].

In our next series of experiments in Figure 4, we focused on cell signaling by stimulating A549+ cells with our optimized 50ng/ml S1 spike protein for 24h and measured supernatant cytokine production with and without the MAPK inhibitor. In this series, some S1 spike protein treated cells we pre-incubated with MEK1/2 (MAPK, ERK1/2) inhibitor PD98059 for 1h before 24h treatment (and it remained for 24h). These data show significant stimulation of IL-1β vs. control (Figure 4b) that was inhibited by the MAPK inhibitor. Stimulation of other cytokines were inhibited by MAPK inhibitor but not compared to controls included IL12p70, IL-13, IL-8 and TNF-α. Other cytokines not inhibited by the MAPK inhibitor were INFy, IL-10, IL-2, IL-4 and IL-6. The key data here is that in human lung epithelial cells, S1 spike stimulated MAPK-mediated IL-1β, an established driver of COVID-19 inflammation which was inhibited by the MAPK inhibitor. Taken together with Figures 2 and 3, our data support S1 spike protein stimulation in A549+/Caco-2 of four inflammatory cytokines thought to be possibly the most key in the cytokine storm: IL-1β, IL-8, Il-6, and TNF-α[11].

Finally we wished to compare S1 spike protein activation of cell signaling ERK1/2 MAPK and NF-kB p65 using the 25ng/ml or 50ng/ml S1 concentrations and a 24h period. As seen in Figures 5a and 5b we see a critical dose dependent stimulation by S1 protein of both p-ERK1/2 MAPK and NF-kB p-p65 in A549+ lung Type II alveolar cells. The MAPK inhibitor blocked the increase in ERK1/2 phosphorylation (activation)(Figure 5c) but not NF-kB (p-p65) activation (Figure 5d) as in some studies [59]. In the Caco-2 cells in Figures 6a and 6b we see a trend but no significance in p-ERK1/2 and NF-kB p-p65 activation by S1 with a slight but not significant inhibition of the MAPK p-ERK1/2 or NF-kB p-p65 pathways in Figures 6c and 6d respectively.

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Our data are supported by other S1 spike protein studies in A549 and other cells as well as in vivo studies in mice. In human platelets, S1 spike induced MAPK activation of ERK1/2, p38, and JNK that was blocked by the same MAPK inhibitor (PD98059) in our study[79]. Our data for this S1 spike protein proinflammatory signaling are supported by studies in A549 as well as other models. Several studies in human primary lung vascular cells using the same S1 spike (Raybiotech) and concentrations in our studies also show that S1 spike mediates activation of MAPK, ERK1/2. MAPK inhibition prevents cytokine production after S1 stimulation. (RBD or S2 peptides alone had no effect)[19,57]. Importantly to COVID-19 pathogenesis, S1 spike also activates human platelets via ACE2 to result in thrombus formation (a hallmark of COVID-19 pathogenesis)[17] as well as TNF-α, IL-8, and IL-1β production by humanized (hACE2) platelets in mice[80]. This pathway of S1 mediated MAPK, ERK1/2 signaling in platelets is blocked by the PD98059 MAPK inhibitor we used in our study. Interestingly, whole spike protein (S1-S2) from SARS-CoV-1 virus also activates ERK1/2 MAPK in human A549 cells[81]. A study in *Science* also showed activation of MAPK by S1 in human pulmonary and endothelial cells [28]. Significant to this mechanism and other S1 MAPK mediated inflammatory signaling, a very recent study shows that a MAPK inhibitor blocks inflammatory cytokines in a COVID-19 humanized mouse model. They found that SARS-CoV-2 virus transiently activates Raf/MEK/ERK signaling in the very early COVID-19 infection phase and that MAPK ERK1/2 knockdown limits virus replication in cell culture models and prevents inflammatory cytokines production in A549 cells.(IL-6, TNF-α). They propose MAPK inhibitors as a possible early treatment for COVID-19 [61].

Limitations of this study

There are limitations of our study that should be noted. This study was a funded NSF RAPID exploratory concept study and future studies will include a broader range of time course data. Interestingly, other studies with the Raybiotech S1 spike used in our study found only transient activation of MEK (which activates ERK1/2 in our study) after 10 minutes in human lung vascular endothelial and smooth muscle cells. In our discussion we also point the great variability in the published data which is typical of the COVID-19 mysteries we have all come to know. Our future studies will include a greater number of human cell types, a larger dose response range and extended time course data. However, despite these limitations our study does support the proof of concept for SARS-CoV-2 spike S1 protein stimulating proinflammatory cytokine production in human lung epithelial A549+ and human Caco-2 intestinal epithelial cells and ERK1/2 and NF-kB signaling in A549+ cells.

5. Conclusion

In conclusion, our study using the SARS-CoV-2 S1 spike protein stimulation of human A549+ lung epithelial and Caco-2 intestinal epithelial cells support several significant results. Our data show spike S1 activation of ERK 1/2 MAPK as well as NF-kB p-65 in A549+ cells. We also found S1 spike stimulated production of IL-1β, TNF-α, and IL-8 in A549+ human lung as well as IL-6 and IL-8 in Caco-2 human intestinal cells. These are all core elements of the cytokine storm in COVID-19 infection [11]. We also show that MAPK/ERK1/2 inhibition blocks S1 stimulation of IL-1β in A549+ cells. We also demonstrate for the first time that S1 spike also stimulates Caco-2 human intestinal epithelial cells cytokine production (IL-6 and IL-8). But our MAPK inhibitor had no effect on S1 cytokine stimulation in Caco-2. These blunted effects for S1 stimulation have also been seen in Caco-2 infection with SARS-CoV-2 virus in which Caco-2 cells show no pathogenic effects in contrast to A549 and other cells [68]. These differences will need to be further investigated. Together these data support a possible independent role for the S1 spike protein in COVID-19 inflammation pathogenesis and possibly MAPK inhibitors as potential therapies for COVID-19 or *Long COVID* as some have suggested [18,20,36]. Also, as others have pointed out these biological effects of S1 protein could have implications for side effects of the COVID-19 mRNA and DNA vaccines that promote the body to synthesize the S1-S2 spike protein[19].

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Supplementary Materials: We have no supplemental material at this time. We have saved images of whole western blots if needed.

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