ORIGINAL ARTICLE

# Inhibition of IRAK4 dysregulates SARS-CoV-2 spike protein-induced macrophage inflammatory and glycolytic reprogramming

Sadiq Umar[1,2] · Karol Palasiewicz[1,2] · Anja Meyer[1,2] · Prabhakaran Kumar[3] · Bellur S. Prabhakar[3] · Michael V. Volin[4] · Rani Rahat[2] · Mina Al-Awqati[1,2] · Huan J. Chang[1,2] · Ryan K. Zomorrodi[2] · Jalees Rehman[5,6] · Shiva Shahrara[1,2]

## Abstract

Escalated innate immunity plays a critical role in SARS-CoV-2 pathology; however, the molecular mechanism is incompletely understood. Thus, we aim to characterize the molecular mechanism by which SARS-CoV-2 Spike protein advances human macrophage (MΘ) inflammatory and glycolytic phenotypes and uncover novel therapeutic strategies. We found that human MΘs exposed to Spike protein activate IRAK4 phosphorylation. Blockade of IRAK4 in Spike protein-stimulated MΘs nullifies signaling of IRAK4, AKT, and baseline p38 without affecting ERK and NF-κB activation. Intriguingly, IRAK4 inhibitor (IRAK4i) rescues the SARS-CoV-2-induced cytotoxic effect in ACE2$^+$HEK 293 cells. Moreover, the inflammatory reprogramming of MΘs by Spike protein was blunted by IRAK4i through IRF5 and IRF7, along with the reduction of monokines, IL-6, IL-8, TNFα, and CCL2. Notably, in Spike protein-stimulated MΘs, suppression of the inflammatory markers by IRAK4i was coupled with the rebalancing of oxidative phosphorylation over metabolic activity. This metabolic adaptation promoted by IRAK4i in Spike protein-activated MΘs was shown to be in part through constraining PFKBF3, HIF1α, cMYC, LDHA, lactate expression, and reversal of citrate and succinate buildup. IRAK4 knockdown could comparably impair Spike protein-enhanced inflammatory and metabolic imprints in human MΘs as those treated with ACE2, TLR2, and TLR7 siRNA. Extending these results, in murine models, where human SARS-CoV-2 Spike protein was not recognized by mouse ACE2, TLRs were responsible for the inflammatory and glycolytic responses instigated by Spike protein and were dysregulated by IRAK4i therapy. In conclusion, IRAK4i may be a promising strategy for severe COVID-19 patients by counter-regulating ACE2 and TLR-mediated MΘ hyperactivation.

✉ Shiva Shahrara
shahrara@uic.edu

1 Jesse Brown VA Medical Center, Chicago, IL, USA

2 Department of Medicine, Division of Rheumatology, University of Illinois at Chicago, 840 S Wood Street, CSB suite 1114, Chicago, IL 60612, USA

3 Department of Microbiology and Immunology, University of Illinois at Chicago, Chicago, IL, USA

4 Department of Microbiology and Immunology, Midwestern University, Downers Grove, IL, USA

5 Department of Pharmacology and Regenerative Medicine, The University of Illinois at Chicago, Chicago, IL, USA

6 Department of Medicine, Division of Cardiology, University of Illinois at Chicago, Chicago, IL, USA

**Graphical abstract**
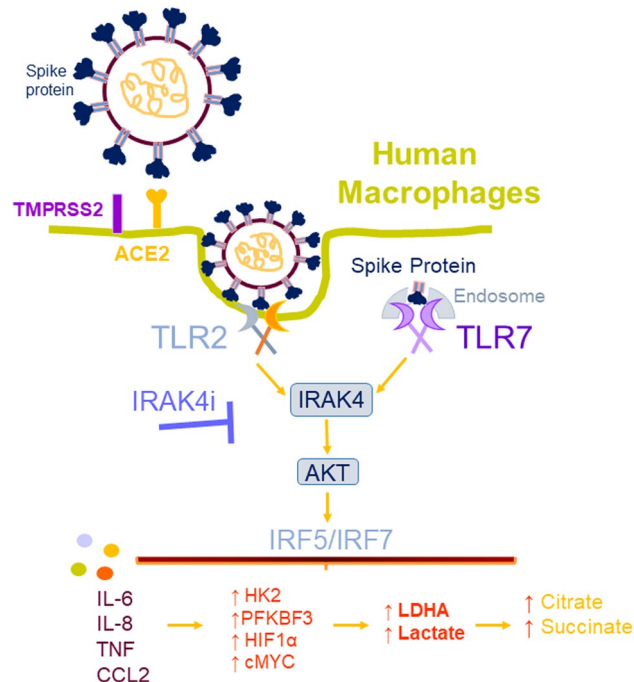


IRAK4i therapy counteracts MΘ inflammatory and glycolytic reprogramming triggered by Spike protein. This study illustrates that SARS-CoV-2 Spike protein activates IRAK4 signaling via ACE2 as well as TLR2 and TLR7 sensing in human MΘs. Remarkably, IRAK4i treatment can dysregulate both ACE-dependent and independent (via TLR sensing) SARS-CoV-2 Spike protein-activated inflammatory and metabolic imprints.

## Introduction

The emergence of the 2019 strain of coronavirus has resulted in profound morbidity and mortality worldwide. Severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) is a novel enveloped RNA virus that is associated with circulating hypercytokinemia and lymphopenia, in addition to substantial mononuclear cell infiltration in the lungs, heart, spleen, and lymph nodes in patients with severe disease [1–3]. These manifestations are observed as a result of excessive innate response and restrained adaptive immune defense leading to tissue damage in SARS-CoV-2-infected patients.

Several studies have characterized the peripheral blood transcriptome profile of COVID-19 patients at different disease stages compared to healthy donors [4, 5]. It was shown that the number of the classical $CD14^+$ $IL-1\beta^+$ monocytes, as well as IFN-activated monocytes, were expanded in COVID-19 patients relative to controls [6]. The classical $CD14^+$ monocytes displayed amplified levels of IL-1β, JUN, FOS, JUNB, KLF6, CCL4 and CXCR4 in SARS-CoV-2-infected patients compared to the control group [7]. An independent study substantiated that the classical monocytes in COVID-19 peripheral blood mononuclear cells (PBMCs) were the main source of inflammatory monokines including IL-1β, IL-18, CCL2, CXCL8, and TNFα [4]. These authors also observed that the classical monocyte markers (*S100A8, S100A9, and S100A12*) dominated the nonclassical counterparts (*C1AQ, C1BQ, LSTB1*) in critical compared to mild/moderate COVID-19 patients [4]. In contrast, IFNγ, CXCL8, and CXCL9 had comparable expression levels in moderate and critical COVID-19 patients [4]. Others have shown that myeloid cell reprogramming by SARS-CoV-2 infection is partially due to hypermetabolic activity [8, 9].

The receptor-binding domain (RBD) of Spike protein binds to ACE2 to facilitate SARS-CoV-2 entry [10–13]. Recent findings demonstrated that coronavirus envelope protein can directly interact with TLR2 [14]. Others have shown that the Spike protein but not the envelope protein is responsible for sensing and activation of TLR2 in human and murine MΘs [15]. Conversely, the S1 subunit of Spike protein was shown to be responsible for advancing inflammation

in connection with TLR4 but not TLR2 [16]. These studies suggest that TLRs may be accountable for SARS-CoV-2 pathology via different structural proteins. However, the question remains whether the SARS-CoV-2 virus can utilize multiple strategies to enter cells and trigger the host cell's immune response.

Interestingly, the expression of TLR1, TLR4, TLR5, TLR8, and TLR9 was associated with the severity of COVID-19 in patients with severe and critical disease relative to controls [5, 14]. Further, TLR7 transcription levels were elevated in patients with moderate COVID-19, while MyD88 and TLR2 transcriptomes were potentiated in all analyzed patients compared to healthy controls [14]. Consistently, activation of NLRP3-inflammasome and escalation of IL-1β secretion were aligned with COVID-19 severity [17, 18]. TLR and IL-1β bind to MyD88 and recruit Interleukin-1 receptor-activated kinase 4/2 into the Toll–IL-1 receptor (TIR) domain. Moreover, IRAK4 is recruited with other partners including IRAK1, TRAF6, and IRF5 to form the myddosome complex which can be triggered by TLRs [19]. Consequently, human and murine cells with IRAK4 deficiency are nonresponsive to TLR or IL-1β signaling [20, 21].

In this study, we sought to characterize the mechanism of Spike protein-instigated pathology and strategies that may subside its function. We exhibit that in human MΘs, Spike protein phosphorylates IRAK4 and AKT which is counteracted by IRAK4i. Remarkably, IRAK4i disrupts Spike protein-induced pathogenesis by restoring oxidative phosphorylation via suppression of citrate and succinate buildup as well as downregulating the expression of inflammatory monokines and glycolytic intermediates. Similar to IRAK4 siRNA, the inflammatory and metabolic reprogramming exacerbated by Spike protein in human MΘs were reversed by ACE2, TLR2, and TLR7 knockdown. Our findings indicate that in human MΘs, IRAK4 inhibition impedes Spike protein signaling through ACE2 in addition to its potentiated TLR sensing.

## Results

### IRAK4i impairs spike protein-induced AKT signaling in myeloid cells and nullifies SARS-CoV-2 infection

Myeloid cells are the first line of defense in SARS-CoV-2 infection, we therefore, characterized the signaling pathways that were activated by Spike protein in these cells. In these studies, the dose of Spike protein was titrated based on its EC50 value (29.1 nM) in monocyte-differentiated MΘs by TNFα quantification (Suppl. 6A) and earlier studies [22]. We found that human myeloid cells exposed to Spike protein lead to activation of IRAK4 and AKT phosphorylation, while p38, ERK, and NF-κB signaling

were not implicated in this process (Fig. 1A, Suppl. 2D, Suppl. 6). We further authenticated that similar to R837, Spike protein expands IRAK4 phosphorylation by twofold, whereas the pan IRAK4 levels were unaffected in human myeloid cells (Suppl. 6B). Interestingly, IRAK4i represses Spike protein-enhanced AKT and IRAK4 signaling as well as baseline p38 phosphorylation in human myeloid cells (Fig. 1B).

Next, the efficacy of IRAK4i therapy was evaluated on SARS-CoV-2-mediated infection and cytopathic effect (CPE) in hACE2$^+$ HEK 293 T cells. At the dose of 1 μM, IRAK4i did not display any cytotoxic effect in hACE2$^+$ HEK 293 T cells. However, SARS-CoV-2 infection in hACE2$^+$HEK 293 T cells was constrained by 85% through IRAK4i therapy (Fig. 1C, D). Interestingly, we found that while IRAK4i interferes with cell death mediated by SARS-CoV-2 infection in hACE2$^+$ HEK 293 T cells, this compound alone is inconsequential on ACE2 internalization in the absence of Spike protein (Fig. 1E, F). Collectively, in human MΘs, IRAK4i interferes with Spike protein downstream signaling including IRAK4 and AKT activation.

### IRAK4i dysregulates spike protein-enhanced IRF5/IRF7 transcription and inflammatory response in human MΘs

Several other groups have exhibited that MΘ inflammatory reprogramming is accompanied by specific IRF transcriptional regulation [22, 23]. In this study, we examined the inflammatory imprint of Spike protein in human monocyte-derived MΘs and delineated the mechanism by which IRAK4i intercepts this phenomenon. Interestingly, Spike protein induction of IRF5 and IRF7 was 80–70% nullified by IRAK4i, this was followed by a 50% reduction of IRF1/8/9 in human monocyte-derived MΘs (Fig. 2A). Transcription of IL-1β (130x), IL-6 (60x), IL-8 (34x), and CCL2 (9x), but not CCL5, was highly potentiated by Spike protein and was ablated by IRAK4i therapy (70–95%) in human monocyte-derived MΘs (Fig. 2B). Likewise, type I (α, β) IFN and II (γ) responses amplified by Spike protein were diminished by IRAK4i (Fig. 2C, D), Suppl. 3A). Extending these findings, secretion of IL-8 was upregulated by Spike protein exposure trailed by CCL2, TNFα, and IL-6 in human monocyte-derived MΘs (Fig. 2E–H). Nevertheless, Spike protein-induced IL-6 production levels were more potently reduced (90%) by IRAK4i relative to other monokines (40–50%). Taken together, IRAK4i reverses Spike protein-mediated inflammatory phenotype especially by disrupting IL-6 expression via IRF5 and IRF7 suppression in human monocyte-derived MΘs.
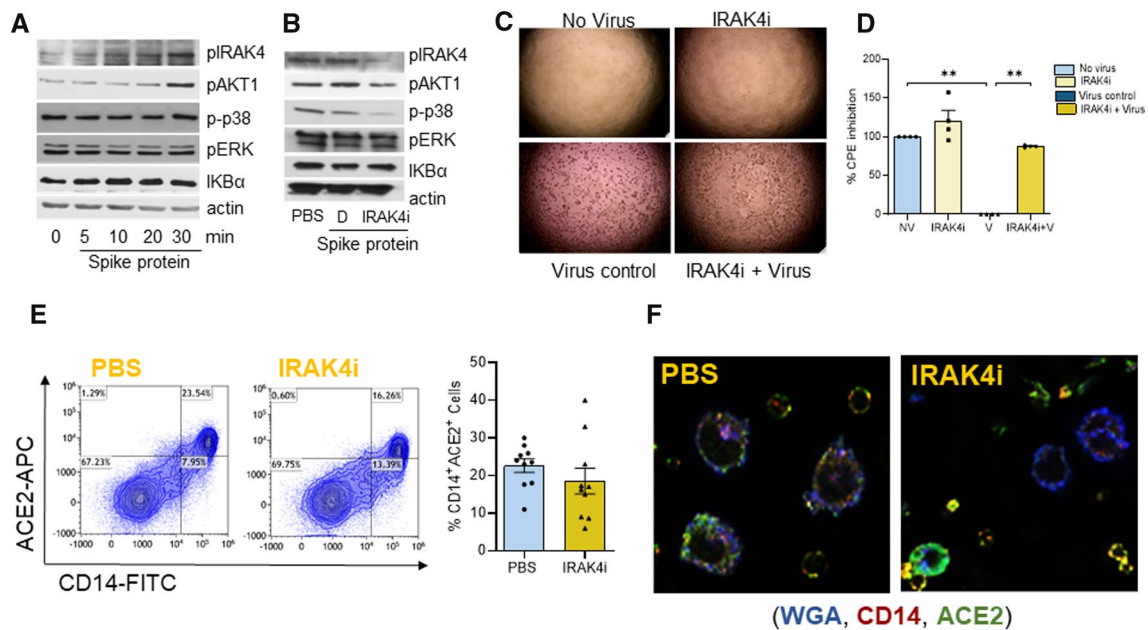
Fig. 1 IRAK4 signaling is triggered by SARS-CoV-2 Spike protein in human myeloid cells and inhibition of IRAK4 prevents SARS-CoV-2-mediated viral infection in hACE2 + 293 T cells. Human myeloid cells were untreated or stimulated with Spike Protein (30 nM; ExonBio) for 0–30 min (**A**). In a different set of studies, human myeloid cells were either untreated or activated with Spike protein (Exonbio; 30 nM) in the presence of DMSO (D) or IRAK4i (1 μM, PF06650833, Sigma #PZ0327) for 30 min (**B**). Lysates from both studies were examined for IRAK4, AKT1, p38, ERK phosphorylation, or IKBα degradation (1:1000, Cell Signaling) and actin (1:3000, Santa Cruz), $n = 3$. hACE2 + HEK 293 T cells were either uninfected or infected with SARS-CoV-2, (Isolate USA-WA1/2020, BEI) in the presence of IRAK4i (1 μM, PF06650833). Cytopathic effect (CPE) was imaged (**C**) at 48 h post-infection and quantified using ATPLite luminescence assay (**D**), $n = 3$. **E** Negatively selected human monocytes were either untreated (PBS) or treated with IRAK4i (1 μM, PF06650833) for 18 h before staining with FITC conjugated CD14 (Biolegend) and APC labeled ACE2 (R&D Systems) staining for 1 h, $n = 10$. **F** Human monocyte-differentiated macrophages seeded on a coverslip were either untreated (PBS) or treated with IRAK4i (1 μM) for 18 h. Thereafter, cells were fixed in 10% formalin and stained with WGA (5 μg/ml, Biotium), ACE2 (1:200 dilution; Cell Signaling), CD14 (1:100 dilution; Proteintech), and a representative image is shown, $n = 3$. The data are shown as mean ± SEM, *$p < 0.05$, **$p < 0.01$. Significant differences were determined by one-way ANOVA followed by Tukey's multiple comparisons or unpaired student's *T*-test

## IRAK4i transposes spike protein-induced metabolic reconfiguration in Mϴs

Next, experiments were performed to determine the impact of Spike protein on Mϴ immunometabolism and IRAK4i ability to counteract its function. Surprisingly, while glycolytic activity measured by extracellular acidification rate (ECAR) was amplified in Mϴs and the oxygen consumption rate (OCR) was reduced in response to Spike protein, only downregulation of OCR was rescued by IRAK4i (Fig. 3A–E). Nevertheless, expression of the glycolysis master regulators, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFKFB)3, hexokinase (HK)2, HIF1α, cMYC, LDHA, and lactate was amplified by Spike protein in human Mϴs and abrogated via IRAK4i, while pyruvate levels were unchanged (Fig. 3B–D), Suppl. 3D). Spike protein-triggered Mϴ glycolysis was also accompanied by transcriptional downregulation of non-oxidative PPP intermediates, CARKL which was restored by IRAK4i to the baseline level (Suppl. 3F). Remarkably, Spike protein inhibitory effect on oxidative phosphorylation was linked to citrate and succinate buildup in human monocyte-derived Mϴs which was counteracted by IRAK4i (Fig. 3F, G) In short, IRAK4i shifts the Spike protein-induced glycolytic Mϴs to oxidative cells by impairing the upregulation of metabolic intermediates and accumulation of citrate or succinate.

## Spike protein-elevated inflammatory and metabolic imprints are abrogated by TLR2, TLR7, and ACE2 knockdown in Mϴs

Since IRAK4 signaling is required for innate immune responses escalated by cell surface or endosomal TLRs, we asked if TLR2 or TLR7 signaling is involved in Spike protein-instigated Mϴ pathology. We found that Spike protein-expanded IL-6, TNFα, IL-8, or IL-1β transcription and/or production were nullified by inhibitors or knockdown of TLR2 or TLR7 in Mϴs relative to counterpart controls (Figs. 4A–C, 5A–C, Suppl. 4–5). In line with these observations, inhibition or knockdown of TLR2 or TLR7 blunted
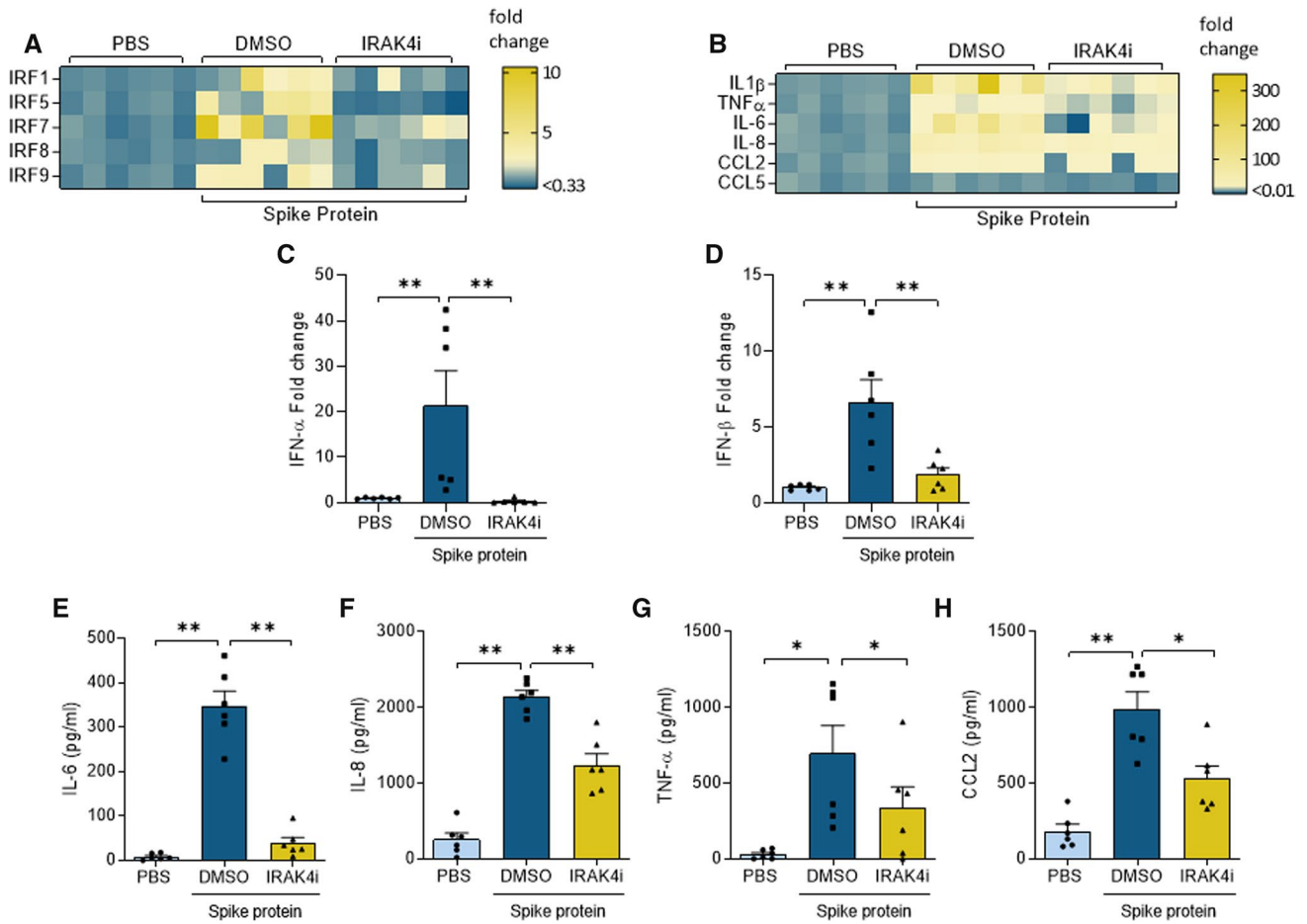
**Fig. 2** IRAK4i therapy disrupts SARS-CoV-2 Spike protein-mediated inflammatory imprints in human MΘs. Human monocytes-differentiated MΘs were pretreated with DMSO (D) or IRAK4i (1 μM, PF06650833) before treating the cells with PBS or Spike protein (30 nM) for 8 h. Cells were utilized to determine the transcription of IRFs (**A**) or monokines including IL-1β, TNFα, IL-6, IL-8, CCL2, CCL5 (**B**), IFNα, and IFNβ (**C–D**) were quantified by real-time RT-PCR, n = 6. Concurrently, IL-6, IL-8, TNFα, and CCL2 production (**E–H**) were measured in the conditioned media by ELISA, $n = 6$. The data are shown as mean ± SEM, *$p < 0.05$, **$p < 0.01$. Significant differences were determined by one-way ANOVA followed by Tukey's or Šídák's multiple comparison test

Spike protein transcriptional upregulation of metabolic markers, HIF1α, cMYC, LDHA in MΘs compared to the control group (Figs. 4D–F, 5D–F). To further substantiate the specificity of TLR2i and TLR7i compounds, their impact was examined on LPS-elevated inflammatory and metabolic phenotypes in human monocyte-differentiated MΘs. LPS signals through TLR2/TLR4 pathways, hence blockade of TLR2 impairs LPS-induced TNFα and lactate secretion, while dysregulation of TLR7 was ineffective on these manifestations (Suppl. 6G-H).

Subsequently, experiments were performed to substantiate the specificity of IRAK4i and to compare its action with ACE2 ablation in Spike protein-activated MΘs. Similar to the blockade of TLR2 and TLR7, the knockdown of IRAK4 and ACE2 could effectively restrain Spike protein-potentiated inflammatory monokines and glycolytic metabolites, HIF1α, cMYC, LDHA in MΘs (Figs. 4D–F, 5D–F). In short,

our results suggest that dysregulation of ACE2 or TLR2 and TLR7 function can intercept Spike protein-induced pathology in MΘs. Hence, in cells that express ACE2, IRAK4 inhibition blocks Spike protein-induced inflammatory profile through 2 distinct pathways that are dependent on ACE or TLR-mediated function. These findings are in line with IRAK4i therapy rescuing ACE2^{+} HEK 293 T cells from SARS-CoV-2-induced cell death.

## IRAK4i reverses spike protein-induced inflammatory and metabolic landscape in vivo

SARS-CoV-2 does not cause infection in the upper and lower airways as the human and mouse ACE2 are not homologous [24], and the murine ACE2 is not recognized by the human SARS-CoV-2 Spike protein [25]. Hence, we asked if the inflammatory response can be locally triggered
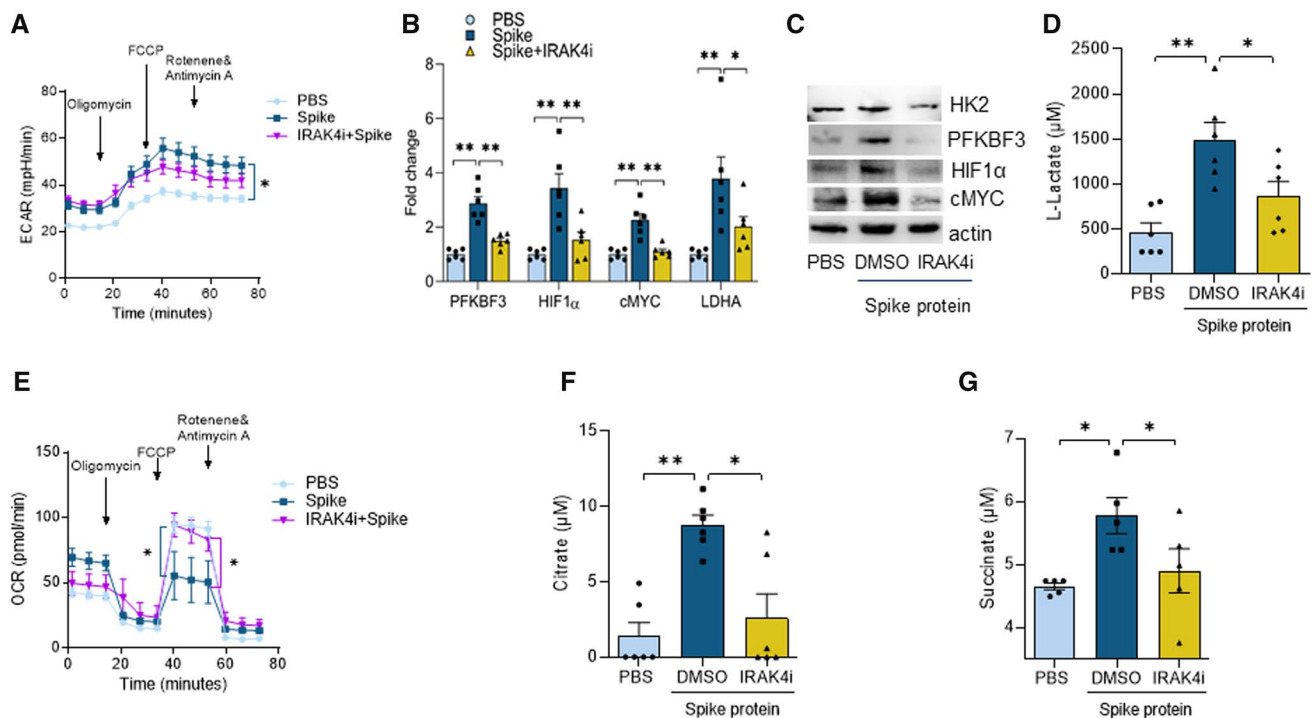
**Fig. 3** IRAK4i counter-regulates glycolysis potentiated by SARS-CoV-2 Spike protein in MΘs and rebalances oxidative phosphorylation. Glycolytic capacity (ECAR) and oxygen consumption (OCR) were tested in RAW 264.7 cells ($5 \times 10^3$ cells/well) treated with PBS, Spike Protein (300 nM; ExonBio) with or without IRAK4i (10 μM) using the XF Cell Mito Stress Test kit (103,015–100; Agilent Technologies) as per manufacturer's instructions. Cells were pre-conditioned with the stimuli in 0% FBS/DMEM for 24 h before ECAR (**A**) and OCR (**E**) evaluation, $n = 5$. Human monocytes-differentiated MΘs were pretreated with DMSO (**D**) or IRAK4i (1 μM,

PF06650833) before treating the cells with PBS or Spike protein (30 nM) for 8 h. Cells were utilized to determine the transcription of glycolytic intermediates by real-time RT-PCR (**B**) and the protein expression by Western blot analysis (**C**) and conditioned media was employed to quantify levels of Lactate (**D**), Citrate (**F**), and Succinate (**G**) by colorimetric assay, $n = 5-6$. The data are shown as mean ± SEM, $*p < 0.05$, $**p < 0.01$. Significant differences were determined by one-way ANOVA followed by Šídák's multiple comparison test

by Spike protein in the peritoneal cavity and whether this function can be negated by IRAK4i or TLR blockade. Interestingly, we found that in IRAK4i treated mice and TLR7-/-animals, IL-1β transcription and secretion as well as IL-6, CCL2, and LDHA mRNA levels were compromised in response to systemic injection of Spike protein (Fig. 6A–D, F). Yet, treatment with IRAK4i showed a greater potency by suppressing several Spike protein-activated monokines that were unchanged by TLR7 deficiency including CCL5, TNFα, and MIP2 (Fig. 6E, G–H). In conclusion, in the absence of ACE2 ligation, Spike protein's modest inflammatory phenotype can be effectively ablated by IRAK4i in part through TLRs.

## Discussion

The current study uncovers that IRAK4 dysregulation obstructs the SARS-CoV-2-induced cytotoxic effect in ACE2+ HEK 293 *T* cells. Although IRAK4i therapy alone

does not interfere with ACE2 internalization, it can effectively transpose Spike protein-driven inflammatory and metabolic reprogramming in human MΘs. IRAK4i intercepts the metabolic activity potentiated by Spike protein via HIF1α, cMYC, LDHA, and lactate downregulation. While Spike protein-mediated suppression of oxidative phosphorylation is counter-regulated by IRAK4i through constraining citrate and succinate buildup in MΘs. In murine models, where human SARS-CoV-2 Spike protein is unable to internalize mouse ACE2, its inflammatory and metabolic imprints are deregulated by IRAK4i therapy in part through TLR sensing. Our results indicate that blockade of IRAK4 signaling interferes with Spike protein-driven pathology through ACE2 dependent and independent strategies that include TLR deactivation (Graphical abstract).

A growing body of evidence has established that SARS-CoV-2's, Envelope, and Spike protein advance inflammation in murine myeloid cells through NF-κB activation [14–16]. Others have elucidated that SARS-CoV-2's Envelope or

**Fig. 4** Spike protein-induced inflammatory and metabolic responses are dysregulated by TLR2 and TLR7 inhibitors. Human monocytes, differentiated into MΘs were either untreated (PBS) or treated with Spike protein (30 nM; ExonBio) for 8 h in the presence of DMSO, TLR2i (oxPAPC, InvivoGen, 30 μg/ml), or TLR7i (A151 InvivoGen, 1 μM). Conditioned media was utilized for quantifying monokines such as IL-6, TNFα, and CCL2 (**A–C**) protein levels by ELISA as well as employing the harvested cells to evaluate glycolytic metabolite including HIF1α, cMYC, and LDHA (**D–F**) transcriptional regulation by real-time RT-PCR, $n = 3$. The data are shown as mean ± SEM, *$p < 0.05$, **$p < 0.01$. Significant differences were determined by one-way ANOVA followed by Bonferroni's or Šídák's multiple comparison test
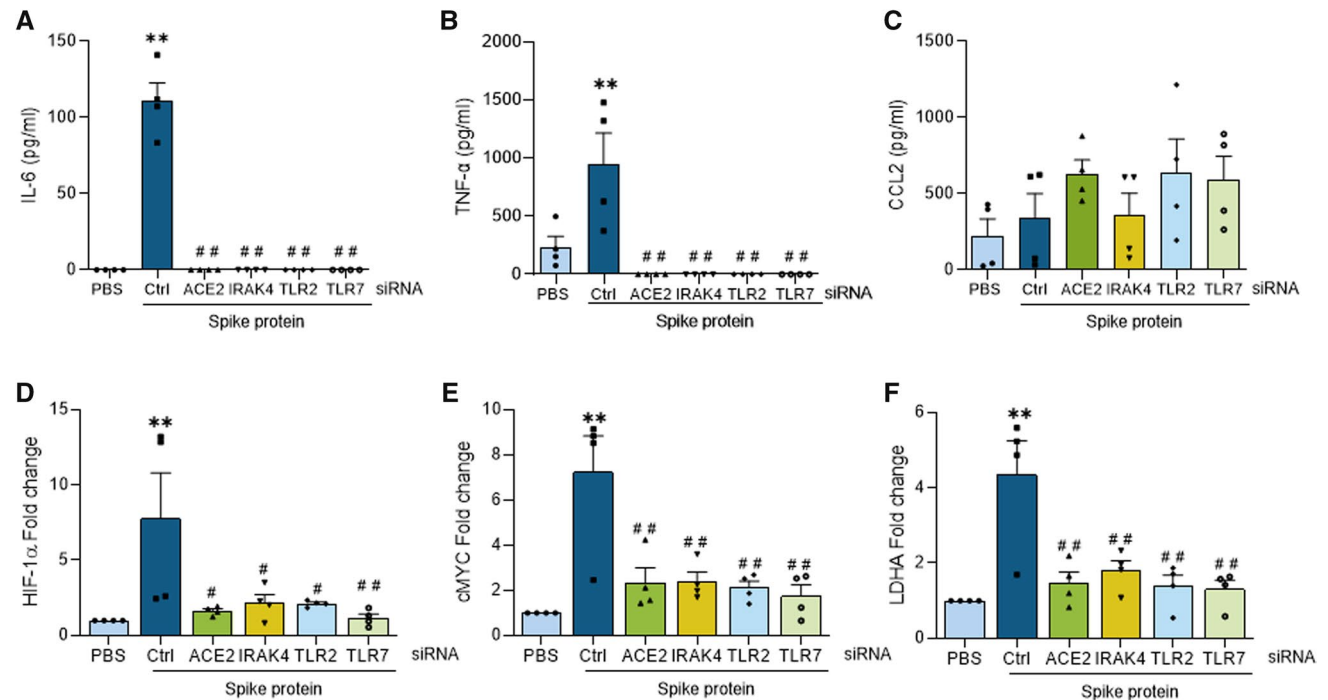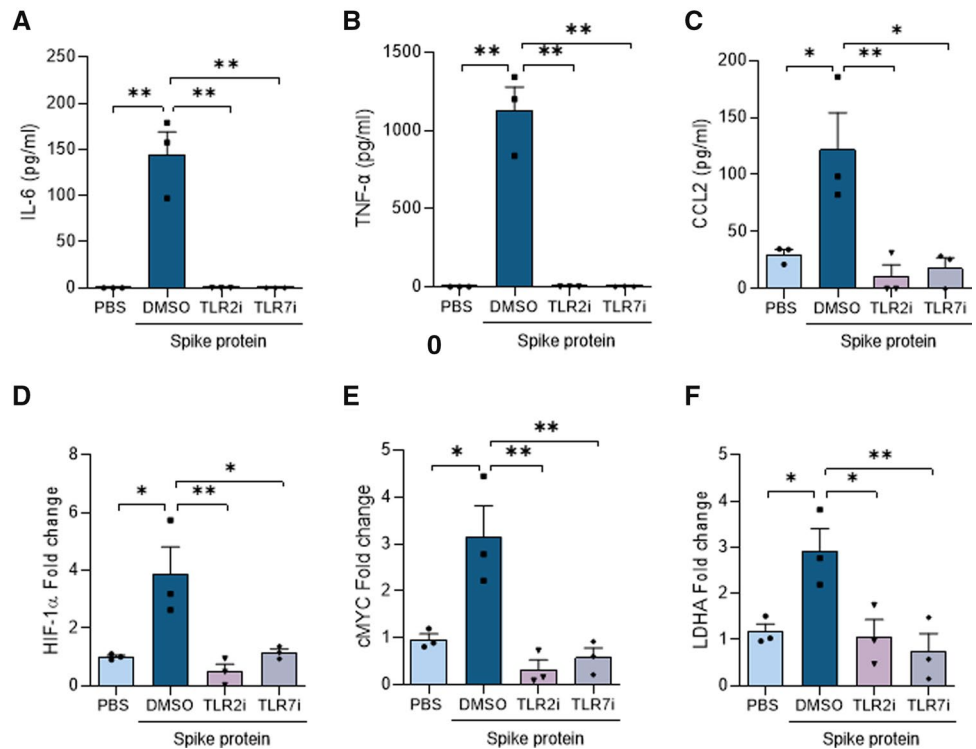


**Fig. 5** Spike protein-potentiated inflammatory and metabolic markers were impaired by ACE2, IRAK4, TLR2, and TLR7 knockdown in MΘs. Human monocyte-differentiated MΘs were transfected with ACE2, IRAK4, TLR2, and TLR7 specific and nonspecific control siRNA (Santa Cruz Biotechnologies) at a final concentration of 100 nM using Lipofectamine 3000 (Thermo Fisher) following the manufacturer's instruction. The transfected cells were utilized following 48 h of transfection and were either untreated or stimulated with Spike protein (30 nM) for 8 h. Conditioned media was utilized for quantifying monokines such as IL-6, TNFα, and CCL2 (**A–C**) secretion by ELISA as well as employing harvested cells to characterize glycolytic metabolite mRNA expression including HIF1α, cMYC, and LDHA (**D–F**) by real-time RT-PCR, $n = 3$. The data are shown as mean ± SEM, *$p < 0.05$ compared to PBS, **$p < 0.01$ compared to PBS, #$p < 0.05$ relative to Spike protein, and ##$p < 0.01$ relative to Spike protein. Significant differences were determined by one-way ANOVA followed by Šídák's multiple comparison test
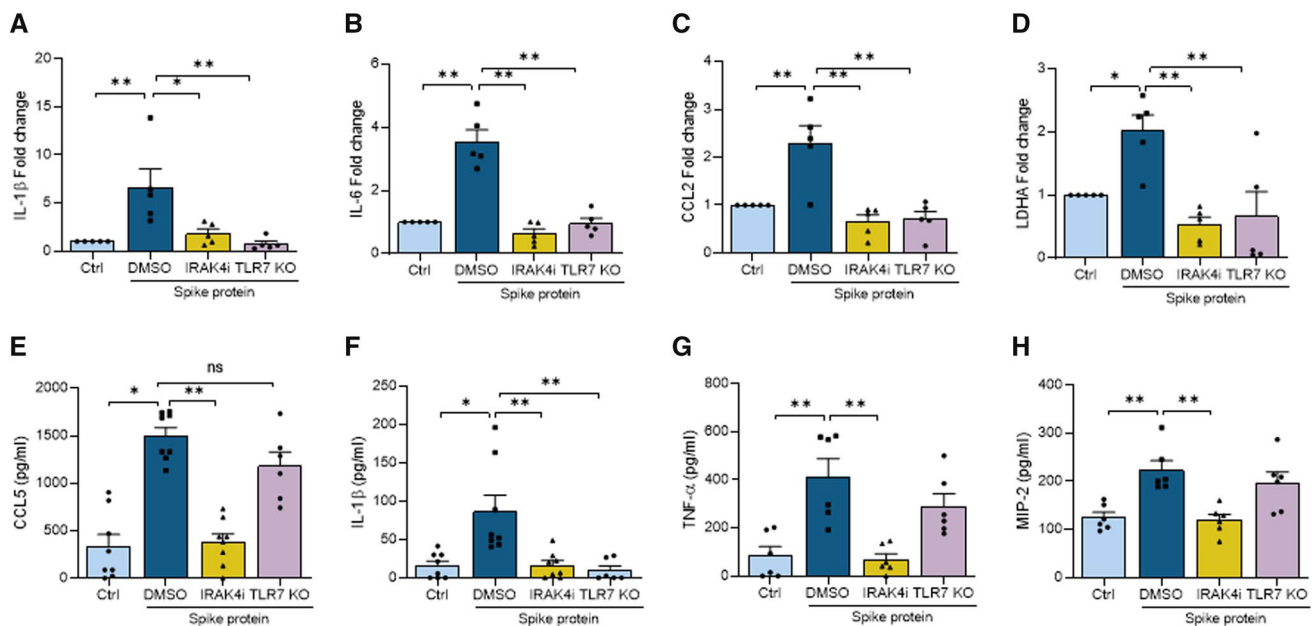
**Fig. 6** IRAK4i therapy reverses Spike protein-induced inflammatory and metabolic landscape in vivo. Wild-type C57BL6 and/or TLR7-/- mice were pretreated with IRAK4i (10 mg/kg BW only in WT) or untreated for 18 h before receiving Spike protein (2 μg, Exon-Bio) i.p. injection for 24 h. The peritoneal gavage and the exudate cells were harvested. Cells were utilized to quantify the expression of IL-1β, IL-6, CCL2, LDHA (**A–D**) $n = 5$, by real-time RT-PCR, and peritoneal gavage was employed to determine protein levels of CCL5, IL-1β, TNFα, and MIP2 (**E–H**), $n = 6$–8. The data are shown as mean ± SEM, *$p < 0.05$, **$p < 0.01$. Significant differences were determined by one-way ANOVA followed by Šídák's multiple comparison test

Spike protein S1 subunit is involved in ERK and JNK signaling in murine MΘs [14, 16]. Although we were unable to show ERK involvement, a strong and time-dependent IRAK4, and AKT signaling was exhibited in human MΘs exposed to Spike protein, which was impaired by IRAK4 inhibition. Notably, NF-κB activation was transient and sporadic in Spike protein-stimulated human MΘs and was unaffected by IRAK4i.

ACE2 is the main port of SARS-CoV-2 Spike protein entry into the host cells [10–13]. The contact between ACE2 and Spike protein is facilitated by its RBD; hence, viral entry can be prevented by therapeutics that disengage this interaction [10, 26, 27]. More recent studies have identified alternative receptors that allow SARS-CoV-2 to access the host cells. Lectins, CD147 glycoprotein, and neuropilin (NRP1) were identified as the additional port of entry, however, only NRP1 was shown to bind to Spike protein S1 [13]. Despite IRAK4i's ability to reverse cell death in ACE2+HEK 293 *T* cells infected with SARS-CoV-2, this compound alone was inconsequential on ACE2 expression and internalization in MΘs. These findings imply that in ACE2+HEK 293 *T* cells and human ACE2+ MΘs, IRAK4i disrupts IRAK4 signaling potentiated by SARS-CoV-2 Spike protein interaction with ACE2.

Earlier studies have revealed that transcriptomes of innate immunity and TLR signaling are closely connected to disease severity in COVID-19 PBMCs [5]. While MYD88 and TLR2 expression were exponentially elevated in patients with moderate to critical disease, TLR7 levels were upregulated in those with moderate disease compared to healthy controls [14]. Since TLR expression was distinctly upregulated in COVID-19 patients we asked whether blockade of a common signaling molecule, IRAK4, may effectively mitigate disease pathology expanded by Spike protein.

The SARS-CoV-2-induced inflammatory phenotype has been associated with the massive influx of monocytes that exacerbate lung injury and promote acute respiratory distress syndrome (ARDS) [28]. Moreover, monocytes recruited to the inflamed tissues are reconfigured into inflammatory cells. We found that in human MΘs, amplification of IRF5 and IRF7 was instrumental in Spike protein-induced inflammatory landscape and was potently negated by IRAK4i. Extending these observations, IRF7 transcription was increased in COVID-19 patients with moderate disease compared to healthy individuals [5]. Similarly, transcriptional upregulation of IRF5 and IRF7 observed in TLR7-activated RA MΘs was markedly reversed by IRAK4i [29]. A recent review has postulated that SARS-CoV-2 infection advances MΘ metabolic remodeling in part through IRF5 activation [19]. Hence, IRF5 may be a critical link between the so-called "metabolic inflammation" and the amplified monokine profile in COVID-19 that can be disrupted by IRAK4i.

Additionally, IRAK4i counteracted Spike protein MΘ inflammatory reprogramming that was accompanied by the expansion of IL-6, IL-8, TNFα, and CCL2 at transcriptional and translational levels. Corroborating these findings, exposure of human PBMCs to Envelope protein contributed to an increase in IL-6 and TNFα transcription [14], which was shown to be TLR2 dependent and TLR7 independent using deficient mice compared to control animals. On the contrary, our data indicate that akin to IRAK4 blockade, inhibition, and knockdown of TLR2 or TLR7 abrogate Spike protein-induced MΘ inflammatory and metabolic rewiring.

Enriched levels of lactate dehydrogenase (LDHA) and lactate are linked to disease severity and hypermetabolic activity in COVID-19 patients [5]. Glucose can dose-dependently enhance viral load, ACE2, and IL-1β expression in SARS-CoV-2-infected monocytes, thus upregulated blood glucose levels are a major risk factor for these patients [8]. Spike protein misregulates ECAR over OCR by increasing glycolytic intermediates including, HK2, PFKBF3, HIF1α, cMYC, LDHA, and lactate in circulating monocyte-differentiated MΘs. Notably, IRAK4i can more effectively reverse Spike protein-induced glycolytic reprogramming in MΘs compared to Tofacitinib in part by impairing HIF1α transcription [22]. Nonetheless, both IRAK4i and Tofacitinib therapies rebalance OCR through a different mechanism of action [22]. IRAK4i advances mitochondrial oxidative phosphorylation by repressing citrate and succinate buildup, whereas Tofacitinib restores PPARγ transcription. We also showed that knockdown of ACE2, TLR2, or TLR7 counteracts Spike protein-potentiated glycolytic reprogramming in MΘs by downregulating transcription of cMYC, LDHA, and HIF1α. In line with our data, inhibition of glucose uptake or HIF1α activity dysregulated SARS-CoV-2 replication and monocyte inflammatory response [8]. It was also observed that SARS-CoV-2-induced mitochondrial ROS and HIF1α stability were responsible for IL-1β transcription and inflammatory response in monocytes [8].

Human ACE2 transgenic mice are required for SARS-CoV-2 infection in the lungs [24]. Yet, a modest local inflammation can be triggered by Spike protein in the absence of human ACE2 expression in vivo, suggesting that alternative strategies may function in parallel to ACE2. To delineate the influence of IRAK4 and TLR7 signaling on Spike protein-modulated inflammatory phenotype in vivo, wild type, or TLR7-/- mice were systemically injected with Spike protein in the presence or absence of IRAK4i. Interestingly, Spike protein administration activated peritoneal IL-1β secretion that was impeded by IRAK4i and TLR7 deficiency, while CCL5, TNFα, and MIP2 production were unaffected by TLR7-/- mice. In contrast, the modest inflammatory and glycolytic imprints accentuated by local Spike protein injection were strongly intercepted by IRAK4i treatment. Distinct from

the in vivo findings, IL-1β production was unaltered in Spike protein stimulated monocyte-differentiated MΘs, perhaps due to low pro-IL-1β or caspase1 expression. However, downregulation of IL-1β production by IRAK4i in response to Spike protein in vivo administration may indicate that both TLR and IL-1βR function can be nullified by this compound. A recent elegant study exhibits that in humanized K18-hACE-2 mice, treatment with IL-1 receptor antagonist rescues SARS-CoV-2-mediated lung infection [30], substantiating the significance of IL-1β in COVID-19 pathogenesis.

Supporting IRAK4i efficiency in counter-regulation of Spike protein-induced macrophage activation syndrome, others have shown that TLR2 sensing is involved in SARS-CoV-2 amplified infection [14, 15]. Intriguingly, the significance of IRAK4 signaling in SARS-CoV-2 infection was shown in an independent study where IRAK4 deficiency leads to resistance to COVID-19 through impaired IFNα2, CXCL10/IP-10, and IL-6 secretion from blood myeloid cells [31]. Supporting this notion, IRAK4 was phosphorylated following SARS-CoV-2 infection in Vero E6 cells [32] which was shown to be abrogated by Capmatinib an IRAK4 inhibitor [33]. More importantly, IRAK4i (PF-06650833) is currently in phase 2 clinical trial for hospitalized patients with COVID-19 pneumonia that suffer from exuberant inflammation.

Spike-human ACE2 interaction is considered the canonical pathway of SARS-CoV-2 entry into cells and initiation of signals, which mirrors our human data. However, our animal data most likely represents an ACE2 independent mechanism as the mice do not express human ACE2 and thus have a low affinity for the Spike protein. As recent studies have shown, treatment with ACE2 inhibitor (MLN-4760) did not abrogate the inflammatory phenotype triggered by Spike protein in human myeloid cells [15, 34]. In agreement with this notion, others have validated that TLR7-MyD88 signaling is critical for SARS-CoV-2-induced IFN secretion, while TLR2 is responsible for the IL-6 response in dendritic cells [35].

The current study uncovers that inhibition of IRAK4 signaling can reverse Spike protein-induction of inflammatory and metabolic profiles in human MΘs that express ACE2 as well as in vivo models where ACE2 is absent, highlighting its potential use for treating patients with severe COVID-19.

# Materials and methods

## Human myeloid cells

Studies were approved by the University of Illinois at Chicago (UIC) Institutional Ethics Review Board and all donors

gave informed written consent. Human mononuclear cells were isolated by Histopaque gradient centrifugation and monocytes were isolated from human peripheral blood (PB) using a negative selection kit according to the manufacturer's instruction (StemCell Technology) [36–43].

## Western blot analysis

Human myeloid cells were untreated or stimulated with Spike Protein (30 nM; ExonBio) for 0–40 min before cells were harvested. In a different set of studies, human myeloid cells were either untreated or activated with Spike protein (Exonbio; 30 nM) in the presence of DMSO (D) or IRAK4i (1 μM, PF06650833, Sigma #PZ0327) [23] for 30 min. Lysates from both studies were examined for IRAK4, AKT1, p38, ERK phosphorylation, or IKBα degradation (1:1000, Cell Signaling) and actin equal loading (1:3000, Santa Cruz). For glycolytic metabolites, human myeloid cells were untreated or stimulated with Spike protein (30 nM) with DMSO or IRAK4i (1 μM, PF06650833) for 8 h. Lysates were probed for HK2, PFKBF3, (1:1000, Cell Signaling), HIF1α (1:1000, Santa Cruz), cMYC (1:1000, Novus Biologicals), and actin (1:3000, Santa Cruz). Raw Western blots and their quantification are shown in Suppl-2.

## Flow cytometry

Negatively selected human monocytes were either untreated (PBS) or treated with IRAK4i (1 μM, PF06650833, Sigma #PZ0327) [23] for 18 h before staining with FITC conjugated CD14 (Biolegend) and APC labeled ACE2 (R&D Systems) staining for 1 h. Zombie Violet PB450 was used to exclude the dead cells. The gating strategy is shown in Suppl-1.

## Immunofluorescence visualization

Human monocyte-differentiated macrophages seeded on a coverslip were either untreated (0 h) or treated with IRAK4i (1 μM) for 18 h. Thereafter, cells were fixed in 10% formalin and stained with WGA (5 μg/ml, Biotium), ACE2 (1:200 dilution; Cell Signaling), CD14 (1:100 dilution; Proteintech), and a representative image is shown.

## CPE assay using SARS-CoV-2 infection assay in hACE2-293 T cells

hACE2-293 T cells were either uninfected or infected with SARS-CoV-2, (Isolate USA-WA1/2020, BEI) in the presence or absence of IRAK4i (1 μM, PF06650833, Sigma #PZ0327) [23] and the assay was performed in triplicates. Cytopathic effect (CPE) was imaged at 48 h post-infection

and %CPE was quantified using ATPLite luminescence screening. For quantification purposes, the medium was removed and following the addition of Cell Titer Glo assay reagent (Promega Inc.) for 15 min, luminescence was measured [44]. The virus-induced cytotoxicity % was calculated as follows: $100 \times [1–(X–MIN)/(MAX–MIN)]$. $X$ = Mean RLU of triplicates; Min = Mean RLU of infected control wells with the virus; Max = Mean RLU of uninfected wells without virus. These experiments were performed in collaboration with Drs. Kumar and Prabhakar in a BCL3 certified facility. Dr. Prabhakar's laboratory was responsible for the virus propagation experiments which were performed under BSL3 containment with the approval of the University of Illinois at Chicago Institutional Biosafety Committee.

## Use of inhibitors and/or knockdown cells

Human monocytes were differentiated into MΘs for 3 days in 10% RPMI. On day 4, MΘs were either untreated (PBS) or treated with Spike protein (30 nM; ExonBio) for 8 h in the presence of DMSO, TLR2i (oxPAPC, InvivoGen, 30 μg/ml) [14, 45] or TLR7i (A151 InvivoGen, 1 μM) [23, 46] and IRAK4i (1 μM, PF06650833, Sigma #PZ0327)[23]. Cells were harvested in Trizol for mRNA quantification and conditioned media was collected for ELISA. Human monocyte-differentiated MΘs were transfected with ACE2, IRAK4, TLR2, and TLR7 specific and nonspecific control siRNA (Santa Cruz Biotechnologies) at a final concentration of 100 nM using Lipofectamine 3000 (Thermo Fisher) complying with the manufacturer's instruction. The transfected cells were utilized following 48 h of transfection and were either untreated or stimulated with Spike protein (30 nM) for 8 h.

## Seahorse assay

Glycolytic capacity (ECAR) and oxygen consumption (OCR) were tested in RAW 264.7 cells ($5 \times 10^3$ cells/well) treated with PBS, Spike Protein (300 nM; ExonBio) with or without IRAK4i (10 μM) using the XF Cell Mito Stress Test kit (103,015–100; Agilent Technologies) as per manufacturer's instructions. Cells were pre-conditioned with the stimuli in 0% FBS/DMEM for 24 h before ECAR and OCR evaluation [22, 47].

## Spike protein immune response in vivo

Next, we examined if the inflammatory response can be activated by local Spike protein administration and whether this function can be negated by IRAK4i or TLR blockade. For this purpose, wild-type C57BL6 and/or TLR7-/- mice

were pretreated and/or untreated with IRAK4i (10 mg/kg BW only in WT) for 18 h before receiving Spike protein (2 μg, ExonBio) i.p. injection for 24 h. Thereafter, peritoneal gavage and peritoneal exudate cells were harvested.

## Statistical analysis

For comparison among multiple groups, one-way ANOVA followed by Tukey's, Bonferroni's, or Šídák's multiple comparison test was employed, using Graph Pad Prism8 software. The data were also analyzed using a two-tailed Student's T test for paired or unpaired comparisons between two groups. Values of $p < 0.05$ were considered significant.

**Author contributions** All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Shahrara had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study conception and design. SU and SS. Acquisition of data. SU, KP, AM, PK, MVV, RKZ, and SS. Analysis and interpretation of data. SU, KP, AM, PK, BP, MVV, RR, MA, HJC, RKZ, JR, and SS. Providing crucial reagents. MA and HJC.

**Data availability** All findings are exhibited in the paper and the material and data are available for transparency.

**Code availability** Not applicable.

## Declarations

**Conflict of interest** The authors have declared that no commercial or financial conflict of interest exists.

**Ethical approval** All peripheral blood (PB) was collected in accordance with our protocol approved by the University of Illinois at Chicago Institutional Ethics Review Board. Furthermore, all animal studies were approved by the University of Illinois at Chicago Animal Care and Use Committee following the legal requirements and guidelines of the state of Illinois in the USA and NIH. Dr. Prabhakar's laboratory was responsible for the virus propagation experiments which were performed under BSL3 containment with approval of the University of Illinois at Chicago Institutional Biosafety Committee.

**Consent to publication** All authors have been involved in writing the manuscript and consented to publication.

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