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LETTER TO THE EDITOR

SARS-CoV-2 spike protein interacts with and activates TLR41

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Dear Editor,

Accumulating clinical data suggest the main causes of death by COVID-19 include respiratory failure and the onset of sepsis.[1] Importantly, sepsis has been observed in nearly all deceased patients.[2–5] It remains elusive how SARS-CoV-2 infection results in viral sepsis in humans. Toll-like receptor 4 (TLR4) mediates anti-gram-negative bacterial immune responses by recognizing lipopolysaccharide (LPS) from bacteria.[6] We recently found that SARS-CoV-2 infection provoked an anti-bacterial like response at the very early stage of infection via TLR4. However, the identity of the original trigger initiating these abnormal immune responses during SARS-CoV-2 infection is unknown.

Previous in silico studies predicted cell surface TLRs, especially TLR4, are most likely to be involved in recognizing molecular patterns, probably spike protein, from SARS-CoV-2 to induce inflammatory responses.[7,8] Consistently, we found that the induction of IL1B by SARS-CoV-2 was completely blocked by TLR4-specific inhibitor Resatorvid (Fig. 1a). Combined with our recent data that TLR4 signaling was activated by SARS-CoV-2, we hypothesized that spike protein could activate TLR4 pathway. A recent study has reported that trimeric SARS-CoV-2 spike proteins are high quality antigens.[9] To this end, we purified the trimeric spike protein (1–1208 aa) (Fig. 1b; Supplementary information, Fig. S1a), as this form of spike protein presents on the surface of viral particle, which most likely interacts with the proteins on the cell surface. Results of the surface plasmon resonance (SPR) assay showed that SARS-CoV-2 spike trimer directly bound to TLR4 with an affinity of ~300 nM (Fig. 1b), comparable to many virus-receptor interactions.

We then treated THP-1 cells, a cell line of human monocytes, with purified spike protein. IL1B was robustly induced by spike protein in a dose-dependent manner (Fig. 1c), which was comparable to LPS (Supplementary information, Fig. S1b). IL6 was also induced by spike protein (Supplementary information, Fig. S1c). As IL1B induction was much more robust than that of IL6, we chose IL1B production as a marker of immune activation. Moreover, the pseudovirus expressing spike protein can also induce IL1B production (Fig. 1d). Neutrophils also express TLR4 on their cell surface and play an important role in the development of sepsis. We utilized all-trans retinoic acid (ATRA) to treat HL-60 cell (a promyelocytic leukemia cell line), which directed those cells to differentiate into neutrophils. Spike proteins significantly induced IL1B production in HL-60 cells after ATRA treatment (Fig. 1e; Supplementary information, Fig. S1d).

We treated THP-1 cells with the N-terminal domain (NTD) or the receptor-binding domain (RBD) of spike protein. Only the trimeric protein could induce IL1B and IL6 (Fig. 1f; Supplementary information, Fig. S1e). To examine if this activation was mediated by TLR4, we treated cells with Resatorvid. Resatorvid greatly blocked induction of IL1B by spike protein and LPS (Fig. 1g). Moreover, spike protein was also able to induce IL1B production in murine macrophage cell line (Raw 264.7) in a TLR4- and MyD88-dependent manner (Fig. 1h). Consistently, spike protein induced production of IL1B in the primary bone marrow-derived macrophages (BMDM) and peritoneal macrophages from wild-type but not TLR4-deficient mice (Fig. 1i). The NF-κB inhibitor (JSH-23) was able to suppress IL1B induced by spike protein (Supplementary information, Fig. S1f), suggesting that NF-κB was involved in this immune response. Trypsin digestion almost completely abolished the activation of IL1B by spike protein ruling out the possibility that the protein was contaminated by LPS (Fig. 1j; Supplementary information, Fig. S1g). Collectively, SARS-CoV-2 spike protein is capable of interacting with and activating TLR4.

To determine if other coronaviruses could activate TLR4 signaling, we treated THP-1 cells with murine coronavirus MHV-A59. As expected, MHV-A59 significantly induced IL1B (Fig. 1k), which was blocked by Resatorvid (Supplementary information, Fig. S1h). Theoretically, there is no MHV-A59 receptor (murine *Ceacam1*) expression in THP-1 cells, so MHV-A59 was not able to infect and enter this type of human monocytes. To confirm this, we washed those cells following the treatment with virus. After washing with PBS, the viral load was significantly decreased, so was the induction of IL1B (Fig. 1l; Supplementary information, Fig. S1i). These data suggested that MHV-A59 could trigger TLR4 signaling probably via spike–TLR4 interaction. Moreover, we treated macrophages with the spike protein trimer of SARS-CoV or infected these cells with human coronavirus 229E (HCoV-229E; Fig. 1m, n). Both treatments can induce production of IL1B, which was suppressed by Resatorvid. Together, different coronaviruses were able to activate TLR4 via their spike proteins.

MD2 and CD14 are the coreceptors of TLR4 for sensing LPS. We suppressed MD2 with its specific inhibitor (T5342126) and blocked CD14 with anti-CD14 antibody (Supplementary information, Fig. S1j, k).[10,11] Both treatments significantly suppressed IL1B induction by spike protein suggesting that MD2 and CD14 were involved in TLR4 activation. TRIF is another adaptor protein downstream of TLR4. We treated macrophages from TRIF-deficient mice with spike protein. Consistent with reported studies,[12,13] TRIF was dispensable for transcription activation of IL1B by LPS (Supplementary information, Fig. S1l). Deficiency of TRIF also did not affect induction of IL1B by spike protein (Supplementary information, Fig. S1l). However, TRIF was required for IFNB induction by LPS or spike protein (Supplementary information, Fig. S1m). To address if other TLRs are activated by spike protein, we treated macrophages from TLR2- or TLR3-deficient mice with SARS-CoV and SARS-CoV-2 spike protein. Deficiency of TLR2 or TLR3 did not affect induction of IL1B by spike protein (Fig. 1o). In addition to IL1B, the transcriptomic analysis showed that spike protein of SARS-CoV-2 was able to induce a number of immune-related genes, including interleukins, chemokines and IFN-stimulated genes (ISGs) (Fig. 1p, q; Supplementary information, Fig. S1s).

Spike protein also interacts with host proteins ACE2 and TMPRSS2. We treated macrophages from ACE2-deficient or human ACE2-transgenic mice with spike protein. Deficiency of ACE2 or overexpression of human ACE2 did not affect the induction of IL1B (Supplementary information, Fig. S1n, o). Treatment with ACE2 inhibitor (MLN-4760) or soluble ACE2 was not able to inhibit the induction of IL1B by LPS or spike protein (Supplementary information, Fig. S1p, q). Moreover, TMPRSS2-specific inhibitor (Bromhexine hydrochloride) did not alter the induction of IL1B by

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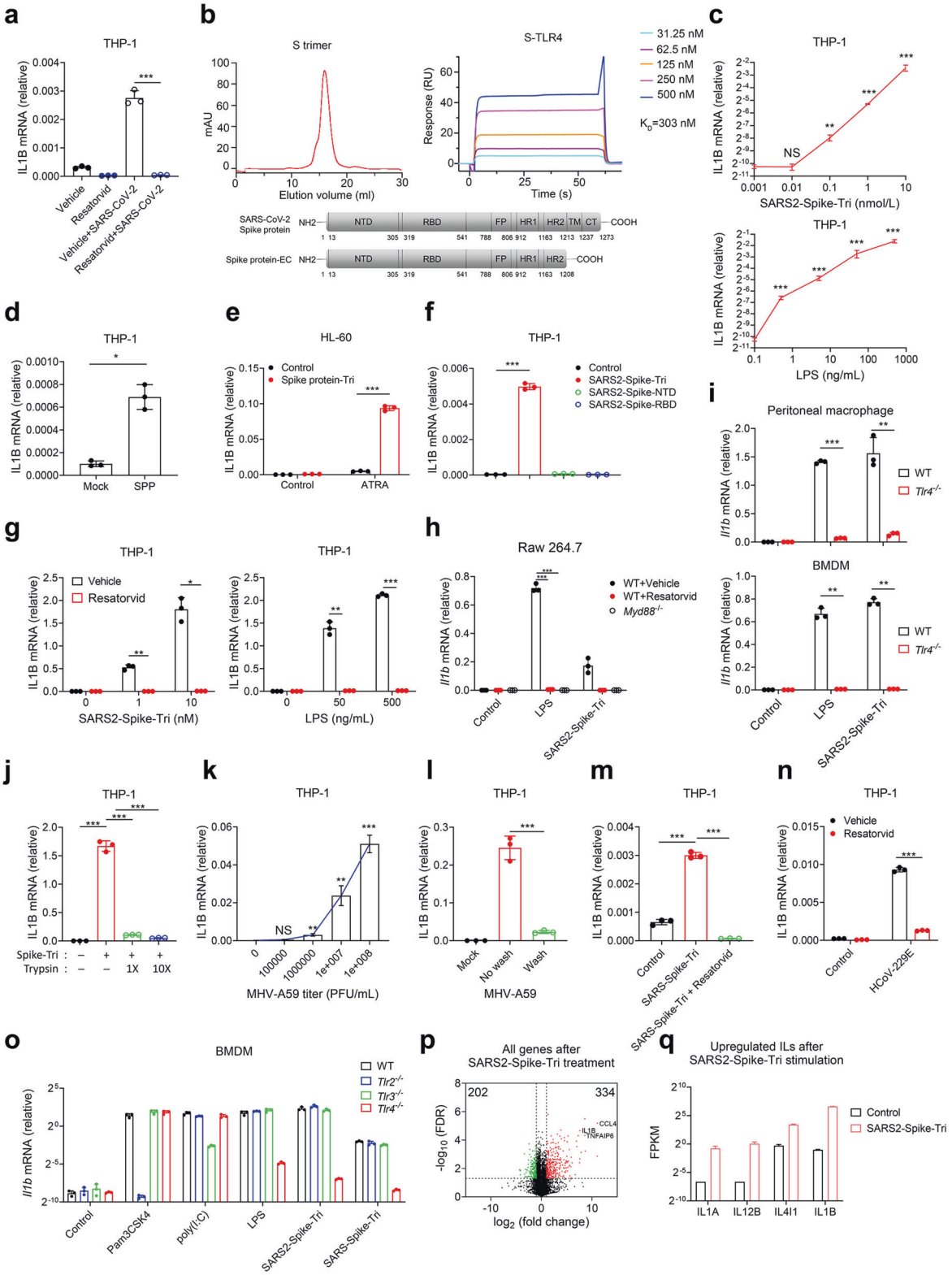

spike protein (Supplementary information, Fig. S1r). Thus, activation of TLR4 by spike protein was not regulated by ACE2 and TMPRSS2 or virus entry.

The induction of IL1B by trimeric spike proteins from SARS-CoV-2 or SARS-CoV was comparable to LPS treatment. Moreover, IL1B was induced by SARS-CoV-2, HCoV-229E and MHV-A59 via TLR4. These observations indicated at least some coronaviruses might share common/conserved abilities to interact with and activate TLR4 or TLR4-related signalings. Multiple sequence alignments of spike proteins from these coronaviruses reveal the higher conservation for S2, rather than RBD or NTD (Supplementary information, Fig. S1t), but this does not indicate TLR4 binding to

Fig. 1 Spike protein of SARS-CoV-2 directly interacts with TLR4 and activates related immune responses. a qRT-PCR analysis for the expression of IL1B in the THP-1 cells infected with 10^7 PFU/mL SARS-CoV-2 for 2 h, with or without 100 μM Resatorvid treatment. **b** Schematic representation of full-length spike protein and ectodomain (EC)-spike protein of SARS-CoV-2 (bottom), and affinity analysis of the binding of TLR4 to SARS-CoV-2 spike trimer (top). Purified SARS-CoV-2 spike trimer (SARS2-Spike-Tri, hereafter) was immobilized onto a CM5 sensor chip surface and tested for real-time association and dissociation of TLR4. **c** qRT-PCR analysis for the expression of IL1B in THP-1 cells treated with control, 0.01 nM, 0.1 nM, 1 nM, 10 nM SARS2-Spike-Tri (top) or control, 0.5 ng/mL, 5 ng/mL, 50 ng/mL, 500 ng/mL LPS (bottom) for 12 h. **d** qRT-PCR analysis for the expression of IL1B in THP-1 cells treated with control and Spike protein-pseudotyped (SPP) lentivirus for 12 h. **e** qRT-PCR analysis for the expression of IL1B in the control group and ATRA-differentiated HL-60 cells treated with 1 nM SARS2-Spike-Tri for 12 h. **f** qRT-PCR analysis for the expression of IL1B in THP-1 cells treated with 10 nM Trimer Ectodomain (Tri), 10 nM N-terminal domain (NTD), 10 nM Receptor binding domain (RBD) of SARS-CoV-2 spike protein and control for 12 h. **g** qRT-PCR analysis for the expression of IL1B in THP-1 cells treated with control, 1 nM SARS2-Spike-Tri, and 10 nM SARS2-Spike-Tri (left) or control, 50 ng/mL LPS, and 500 ng/mL LPS (right) for 2 h with or without 100 μM Resatorvid treatment. **h** qRT-PCR analysis for the expression of *Il1b* in WT and Myd88$^{-/-}$ Raw 264.7 cells treated with control, 500 ng/mL LPS, 10 nM SARS2-Spike-Tri for 6 h with or without 1 μM Resatorvid treatment. **i** qRT-PCR analysis for the expression of *Il1b* in WT and *Tlr4*$^{-/-}$ mouse peritoneal macrophage (top) or BMM (bottom) treated with control, 500 ng/mL LPS, 10 nM SARS2-Spike-Tri for 6 h. **j** qRT-PCR analysis for the expression of IL1B in THP-1 cells treated with control, 10 nM SARS2-Spike-Tri, 10 nM SARS2-Spike-Tri + 0.025% Trypsin (1×), 10 nM SARS2-Spike-Tri + 0.25% Trypsin (10×) for 2 h. **k** qRT-PCR analysis for the expression of IL1B in THP-1 cells treated with control, 1×10^5 PFU/mL, 1×10^6 PFU/mL, 1×10^7 PFU/mL, 1×10^8 PFU/mL MHV-A59 for 12 h. **l** qRT-PCR analysis for the expression of IL1B in THP-1 cells treated with 1×10^7 PFU/mL MHV-A59 for 0 and 12 h with or without washing by PBS. **m** qRT-PCR analysis for the expression of IL1B in THP-1 cells treated with control, 10 nM SARS-Spike-Tri, 10 nM SARS-Spike-Tri + 100 μM Resatorvid for 2 h. SARS-Spike-Tri is short for the spike protein trimer of SARS-CoV. **n** qRT-PCR analysis for the expression of IL1B in THP-1 cells infected with 1×10^7 PFU/mL HCoV-229E for 2 h, with or without 100 μM Resatorvid treatment. **o** qRT-PCR analysis for the expression of *Il1b* in BMDMs of WT, *Tlr2*$^{-/-}$, *Tlr3*$^{-/-}$ and *Tlr4*$^{-/-}$ mice. BMDMs were treated with control, 0.5 μg/mL Pam3CSK4, 20 μg/mL poly(I:C), 50 ng/mL LPS, 10 nM SARS2-Spike-Tri or 10 nM SARS-Spike-Tri for 2 h. **p** Volcano plots depicting the transcriptomes in THP-1 cells with or without 10 nM SARS2-Spike-Tri treatment for 2 h. All genes were shown with a false discovery rate (FDR) and fold change (FC) (red dots, FC > 1 and FDR < 0.05; green dots, FC < −1 and FDR < 0.05; black dots, −1 < FC < 1 or FDR > 0.05). **q** RNA-seq analysis for the upregulated interleukins (ILs) in THP-1 cells after 10 nM SARS2-Spike-Tri treatment for 2 h. The expression levels of each gene were showed by the fragments per kilobase of exons per million fragments mapped (FPKM). NS, non-significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

S2. Structural analysis by molecular docking suggests that TLR4 more likely targets a conformational concave constructed by RBD and NTD (Supplementary information, Fig. S1u). Therefore, the detailed molecular basis of the interaction between spike trimer with TLR4 and the activation of TLR4 or TLR4-related signaling by coronaviruses still remains open questions for future studies.

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AUTHOR CONTRIBUTIONS

F.Y., X.W., L.Z. and Y.Z. conceived the study and analyzed the data. Y.Z., M.K. and Z.J. performed most experiments and analyzed the data. X.G. performed RNA sequencing analysis. Y.H. performed SARS-CoV-2 infection experiments. J.L., H.Y. and J.K. provided support on literature search. F.Y. wrote the manuscript. F.Y. and X.W. revised the manuscript.

ADDITIONAL INFORMATION

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