Supplementary Information for

SARS-CoV-2 spike protein interacts with and activates TLR4

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Materials and Methods

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Fuping You (fupingyou@hsc.pku.edu.cn).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

The datasets generated during this study are available at Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/). The accession number for the original RNA-Seq data reported in this paper is GEO: GSE167480.

METHOD DETAILS

Facility and ethics statements

All experiments with live SARS-CoV-2 viruses were carried out in the enhanced biosafety level 3 (P3+) facilities in the Sinovac Biotech Ltd approved by the National Health Commission of the People's Republic of China. All animals care and use were in accordance with the Guide for the Care and Use of Laboratory Animals of the Chinese Association for Laboratory Animal Science. All procedures of animal handling were approved by the Animal Care Committee of Peking University Health Science Center.

Cell culture

293T cells, Vero cells, Huh7, Raw 274.7 cells, 17CL-1 cells, THP-1 cells and HL-60 cells were maintained in our lab. 17CL-1 cells, Huh7, 293T cells and Vero cells were cultured in DMEM medium (Gibco) supplemented with 10% FBS (PAN), 100 U/mL Penicillin-Streptomycin. Raw 274.7 cells, THP-1 cells and HL-60 cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% FBS, 100 U/mL Penicillin-Streptomycin. Cells were negative for mycoplasma. Isolation of BMDMs (bone-marrow derived macrophages) and peritoneal macrophages was performed as

described[1].

To isolate thioglycolate (TG)-elicited mouse primary peritoneal macrophages, cells were obtained from lavage of the peritoneal cavity with RPMI 1640 medium and were centrifuged and resuspended and cultured in indicated culture medium. For BMDMs, bone marrow cells were isolated from femurs and tibiae of 8-12 weeks old mice. Cells were cultured with 20 ng/mL recombinant murine GM-CSF (315-03; Peprotech) in a 10-cm dish for 7 d before experiments. Mature macrophages were harvested by incubating with PBS-EDTA for 10 min and cultured on 12-well plates for further experiments.

Animal experiments

All animals were kept and bred in specific pathogen-free conditions. Male Wild-type (WT) mice were purchased from Department of Laboratory Animal Science of Peking University Health Science Center, Beijing. Male $TLR4^{-/-}$ mice on a C57BL/10 background were a gift from Pro. Zhihua Liu (Tsinghua University). Male $TLR2^{-/-}$ mice on a C57BL/10 background were a gift from Pro. Liyun Shi (Nanjing University of Chinese Medicine).

Viruses

The SARS-CoV-2 which has been used in this paper is named strain HB-01. The complete genome for this SARS-CoV-2 had been put in to GISAID (BetaCoV/Wuhan/IVDC-HB-01/2020|EPI_ISL_402119).

MHV-A59 (mouse hepatitis virus A-59) has been described previously[2]. MHV-A59 was propagated in 17CL-1 cells followed by 3 cycles of freezing and thawing. The large debris was spun down and the supernatants were ultrafiltered and concentrated by 100 kDa cutoff ultrafiltration device (Millipore). The concentrated supernatants were used as viral stocks. The titer of the viruses was determined by plaque assay in 17CL-1 cells. HCoV-229E (human coronavirus 229E) was a gift from Prof. Yanjun Zhang in Zhejiang Provincial Center for Disease Control and Prevention. HCoV-229E was propagated in Huh7 cells followed by 3 cycles of freezing and thawing. The large debris was spun down and the supernatants were ultrafiltered and concentrated by 100 kDa cutoff ultrafiltration device (Millipore). The concentrated supernatants were used as viral

stocks. The titer of the viruses was determined by qRT-PCR.

Expression constructs

The plasmids used for protein expression were constructed by insertion of the coding sequences for SARS-CoV-2 spike trimer (residues 1–1208, GenBank:MN908947.3), RBD (residues 319-541) and NTD (residues 1-307), respectively into the mammalian expression vector pCAGGS with a C-terminal twin Strep tag to facilitate protein purification. The spike protein gene was constructed with proline substitutions at residues 986 and 987, a "GSAS" instead of "RRAR" at the furin cleavage site according to Jason S. McLellan's research[3].

Protein expression and purification

Expi293F cells (Thermo Fisher) were transiently transfected with the spike protein or RBD or NTD expression plasmids using polyethylenimine. To purify the spike trimer protein or RBD or NTD, filtered cell supernatants were loaded onto a Strep-tactin resin column (IBA). The column was then washed with 5 column volumes of Buffer W (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA). The spike trimer or RBD or NTD was eluted by Buffer W containing 50 mM biotin. Elution fractions were analyzed by SDS-PAGE. The protein was subjected to additional purification by gel filtration chromatography using a Superose 6 10/300 column (GE Healthcare) in 20 mM Tris, 200 mM NaCl, pH 8.0.

Silver staining

Silver staining is used to evaluate the purity of SARS-CoV-2 spike trimer using electrophoretic separation on polyacrylamide gels. The processes of silver staining include protein fixation, then sensitization and rinses, then silver impregnation with a silver nitrate solution and finally image development. After the desired image level obtained, development was stopped by dipping the gel in a stop solution containing 5% acetic acid to reach a pH of 7.0.

Surface plasmon resonance (SPR)

For the binding affinity assay, purified SARS-CoV-2 spike trimer was immobilized onto a CM5 sensor chip surface by using the NHS/EDC method to a level of ~ 700 response units (RUs) using Bia-core 8000 (GE Healthcare) and a PBS running buffer

(supplemented with 0.05% Tween-20) was prepared for assay. Serial dilutions of purified TLR4 were injected. The sample flew over the chip at a rate of 20 μL/min for 30 s, then the dissociation of the sample was done at the same rate for another 30 s. The responses of the sample to the spike trimer were recorded at room temperature and the data was analyzed by Bia-core 8000 Evaluation Software (GE Healthcare).

Generation of pseudotyped lentivirus

pMD2.G-SARS-CoV-2-Spike was a gift from Pro. Demin Zhou. 293T cells were grown in DMEM containing 10% FBS and co-transfected by pCDH-eGFP (6000 ng), psPAX2 (2000 ng) and pMD2.G-SARS-CoV-2-Spike (2000 ng) or pMD2.G-VSVG (2000 ng). The supernatant with produced virus (Spike protein-pseudotyped (SPP) or (VSV-G lentivirus) was harvested 48-hours post transfection, clarified by centrifuging at 8000 g for 10 min at 4ºC. The virus was collected by an ultracentrifugation run at 50000 g for 2 hours (hrs) using Beckman SW41 rotor. The viral pellets were resuspended in FBS free RPMI 1640 medium and stored at -80°C before use. The viral particle number was determined using a real time RT-PCR assay to quantify the RNA copies of eGFP.

HL-60 cells differentiation

HL-60 cells were cultured in RPMI 1640 medium (Gibco) with 10% FBS in 5% CO2 humidified air at 37ºC. Cells were passaged every 3 days and only cells passaged no more than 15 times were used for all experiments. Differentiation of HL-60 cells into granulocyte-like cells was performed as described[4]. The cells were incubated for 5 days with ATRA (1 μmol L^{-1}). After 5 days of differentiation, cells were collected into a 15 mL tube and precipitated naturally for 2 hours. Cell pellet was resuspended by RPMI 1640 medium with 10% FBS. Differentiated cells were counted before experiment.

Quantitative RT-PCR (qRT-PCR) analysis

Total RNA was isolated from the tissues by TRNzol reagent (DP424, Beijing TIANGEN Biotech, China). Then, cDNA was prepared using HiScript III 1st Strand cDNA Synthesis Kit (R312-02, Nanjing Vazyme Biotech, China). qRT-PCR was performed using the Applied Biosystems 7500 Real-Time PCR Systems (Thermo Fisher Scientific, USA) with SYBR qPCR Master mix (Q331-02, Nanjing Vazyme Biotech, China). The data of qRT-PCR were analyzed by the Livak method ($2^{-\Delta\Delta Ct}$).

Ribosomal protein L19 (RPL19) was used as a reference gene for mouse cell line, and GAPDH for human cell line.

RNA sequencing (RNA-seq)

Whole RNA of tissues with specific treatment were purified using TRNzol reagent. The transcriptome library for sequencing was generated using VAHTSTM mRNA-seq v2 Library Prep Kit for Illumina® (Vazyme Biotech Co.,Ltd, Nanjing, China) following the manufacturer's recommendations. After clustering, the libraries were sequenced on Illumina Hiseq X Ten platform using (2 × 150 bp) paired-end module. The raw data were transformed into raw reads by base calling using CASAVA.

QUANTIFICATION AND STATISTICAL ANALYSIS

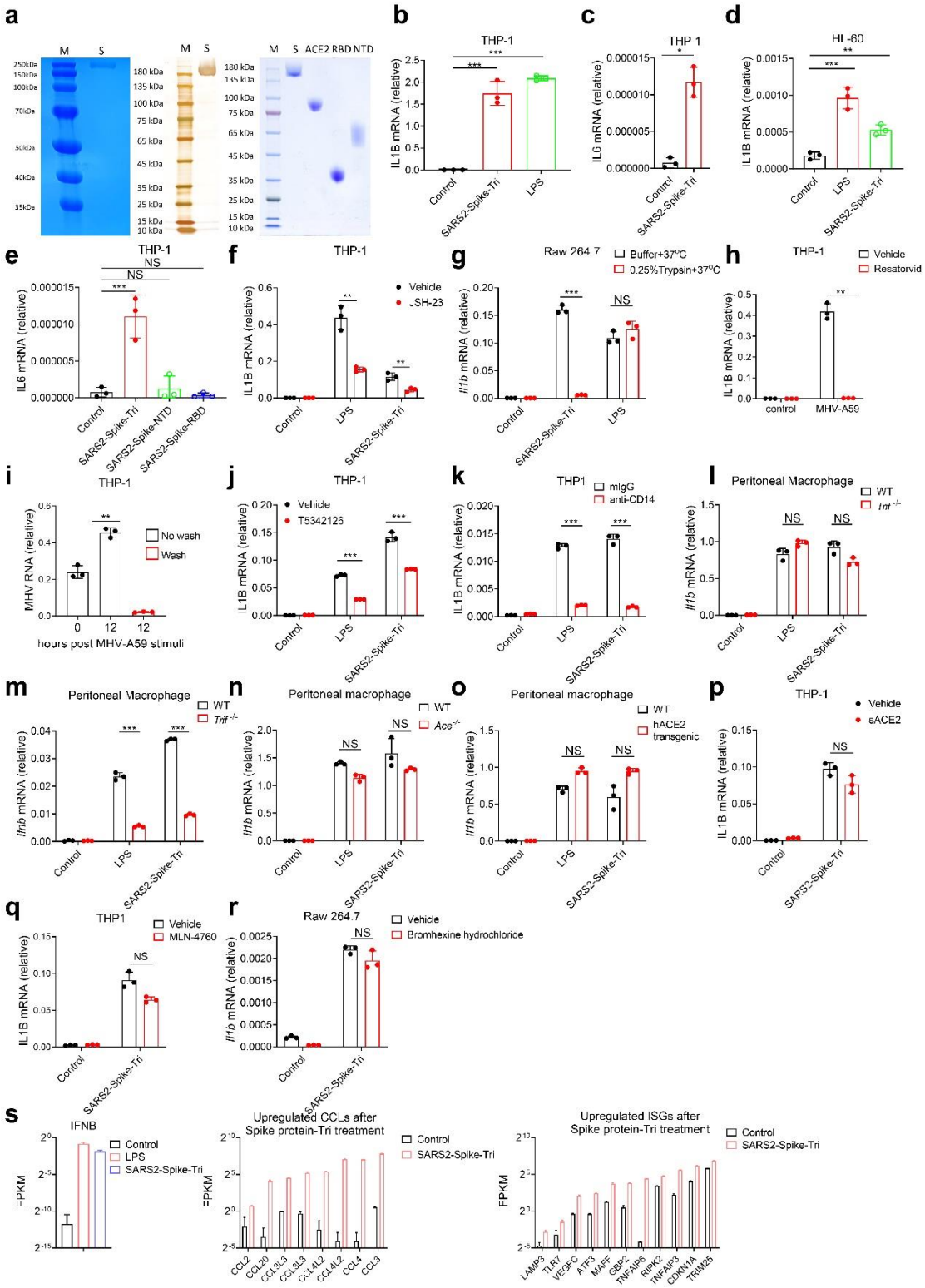
RNA-seq analysis

The FastQC and Trim Galore were used for raw data quality control, then the R package Rsubread was used for mapping and counting the reads. The count matrix was normalized by FPKM. The differentially expressed genes were identified by the GFOLD, a Linux software.

Statistical analysis

All analyses were repeated at least three times, and a representative experimental result was presented. Data were analyzed using GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA). Continuous variables with normal distribution are expressed as the mean ± standard deviation (SD). Comparisons between groups were all verified for normal distribution by D'Agostino-Pearson omnibus test. Student's t-test (for pairwise comparisons) and one-way ANOVA (for comparisons among three or more groups) were used. The post hoc test with Bonferroni correction was performed for multiple comparisons following ANOVA. Asterisks denote statistical significance (*$P < 0.05$; **$P < 0.01$; *** $P < 0.001$). Data are shown as means ± SD ($n \geq 3$).

Supplementary information, Fig. S1



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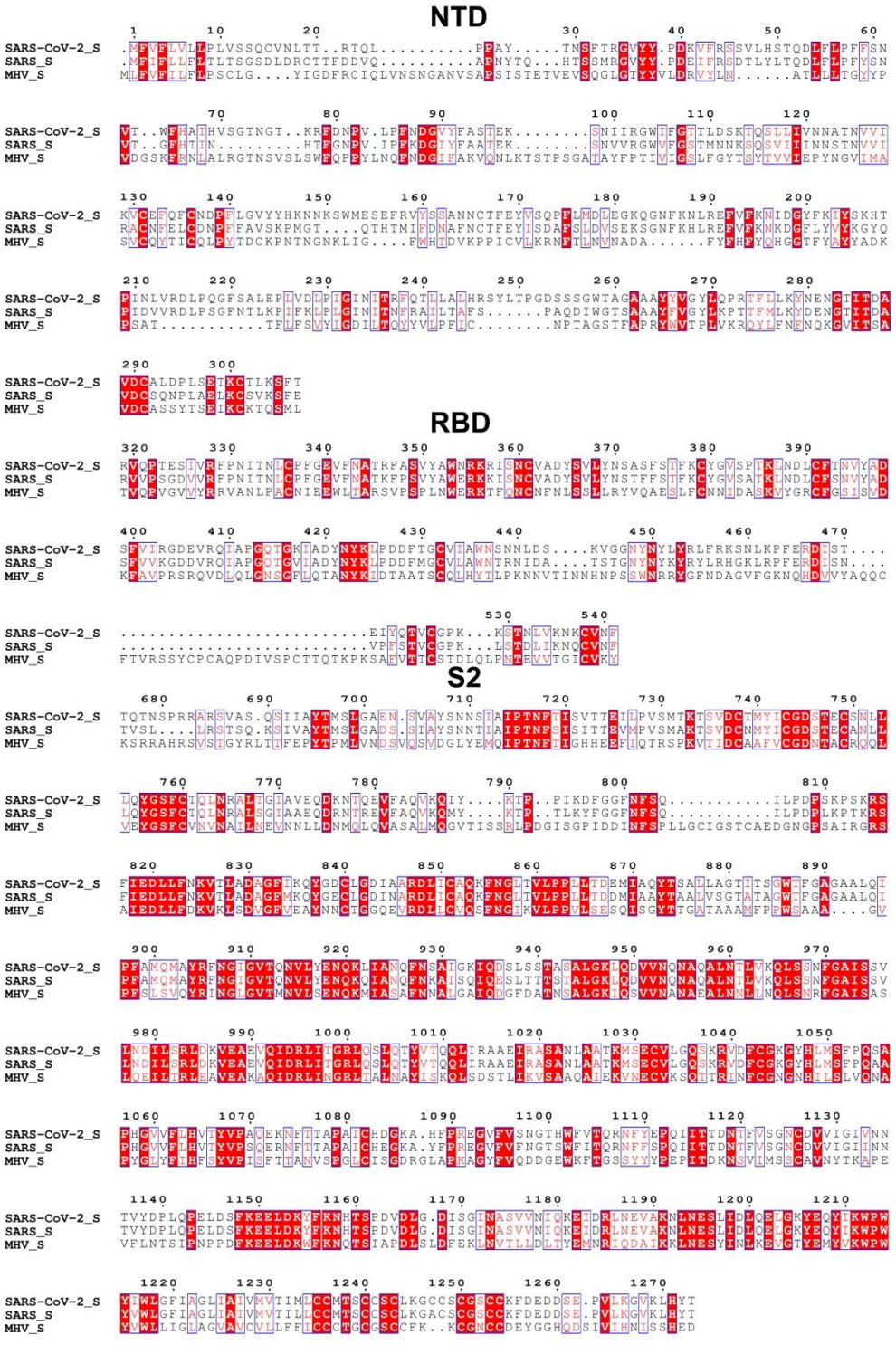

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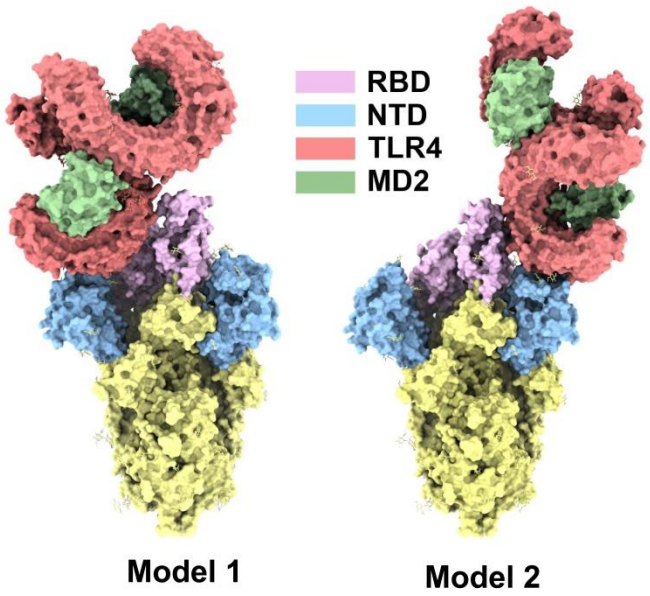

a SDS-PAGE results analysis for the eukaryotic expressed SARS-CoV-2 spike trimer (left). Silver-staining analysis for the eukaryotic expressed SARS-CoV-2 spike trimer (middle). Note：The lower two bands are contaminations from the protein marker. SDS-PAGE results analysis for the eukaryotic expressed SARS-CoV spike trimer, soluble hACE2, SARS-CoV-2 spike protein Receptor binding domain (RBD) and N-terminal domain (NTD) (left).

b qRT-PCR analysis for the expression of IL1B in the THP-1 cells treated with control, 500 ng/mL LPS, and 10 nM SARS2-Spike-Tri for 2 hours.

c qRT-PCR analysis for the expression of IL6 in the THP-1 cells treated with or without 10 nM SARS2-Spike-Tri for 2 hours.

d qRT-PCR analysis for the expression of IL1B in the HL-60 cells treated with control, 500 ng/mL LPS, and 10 nM SARS2-Spike-Tri for 12 hours.

e qRT-PCR analysis for the expression of IL6 in the 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 hours.

f qRT-PCR analysis for the expression of IL1B in the THP-1 cells treated with control, 500 ng/mL LPS, and 10 nM SARS2-Spike-Tri for 2 hours with or without 50 μM JSH-23 treatment.

g qRT-PCR analysis for the expression of *Il1b* in the Raw 264.7 cells treated with

control, 500 ng/mL LPS, and 10 nM SARS2-Spike-Tri for 2 hours. LPS and SARS2-Spike-Tri were preincubated with or without 0.25% trypsin in 37℃ for 2 hours.

h qRT-PCR analysis for the expression of IL1B in the THP-1 cells treated with 10^7 PFU/mL MHV-A59 for 2 hours with or without 100 μM Resatorvid treatment.

i qRT-PCR analysis for the MHV-A59 titer in the THP-1 cells treated with $1x10^7$ PFU/mL MHV-A59 for 0 and 12 hours with or without washing by PBS.

j qRT-PCR analysis for the expression of IL1B in the THP-1 cells treated with control, 500 ng/mL LPS, and 10 nM SARS2-Spike-Tri for 2 hours with or without 50 μM T5342126 treatment.

k qRT-PCR analysis for the expression of IL1B in the THP-1 cells treated with control, 500 ng/mL LPS, and 10 nM SARS2-Spike-Tri for 2 hours. THP-1 cells were pretreated with 5 μg/mL normal IgG or CD14 antibody.

l qRT-PCR analysis for the expression of *Il1b* in the WT and *Trif*$^{-/-}$ mice peritoneal macrophage treated with control, 500 ng/mL LPS, 10 nM SARS2-Spike-Tri for 2 hours.

m qRT-PCR analysis for the expression of *Ifnb* in the WT and *Trif*$^{-/-}$ mice peritoneal macrophage treated with control, 500 ng/mL LPS, 10 nM SARS2-Spike-Tri for 2 hours.

n qRT-PCR analysis for the expression of *Il1b* in the WT and *Ace2*$^{-/-}$ mice peritoneal macrophage treated with control, 500 ng/mL LPS, 10 nM SARS2-Spike-Tri for 2 hours.

o qRT-PCR analysis for the expression of *Il1b* in the WT and hACE2 transgenic mice peritoneal macrophage treated with control, 500 ng/mL LPS, 10 nM SARS2-Spike-Tri for 2 hours.

p qRT-PCR analysis for the expression of IL1B in the THP-1 cells treated with or without 10 nM SARS2-Spike-Tri for 2 hours with or without 1 μM soluble hACE2 protein.

q qRT-PCR analysis for the expression of IL1B in the THP-1 cells treated with control, 1 nM SARS2-Spike-Tri for 12 hours with or without 10 μM MLN-4760 treatment.

r qRT-PCR analysis for the expression of *Il1b* in the Raw 264.7 cells treated with or without 10 nM SARS2-Spike-Tri for 2 hours with or without 20 μM Bromhexine hydrochloride.

s Analysis for IFNB expression after 10 nM SARS2-Spike-Tri or 50 ng/mL LPS treatment for 2 hours (left). Analysis for upregulated chemokine CCLs (middle) and ISGs (right) after 10 nM SARS2-Spike-Tri treatment for 2 hours (left). The expression levels of each gene were showed by the fragments per kilobase of exons per million fragments mapped (FPKM).

t ESPript representation of sequence alignments of spike proteins of SARS-CoV-2, SARS-CoV and MHV. Conserved analysis of NTD, RBD and S2 are presented at the top, middle and bottom, respectively.

u Two proposed binding models of SARS-CoV-2 spike trimer to TLR4 dimer. Surface representations of SARS-CoV-2 spike trimer[5] in complex with TLR4 dimer as well as MD2[6]. TLR4, MD2 and NTD, RBD and other regions of SARS-CoV-2 trimer are colored in red, green, sky-blue and violet, respectively. Glycans are shown as sticks. TLR4 likely binds to a conformational concave constructed by RBD and NTD from two neighboring monomer spike molecules (model 1) or one same momomer spike (model 2).

(NS=non-significant, *P < 0.05; **P < 0.01; ***P < 0.001)

References

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