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Supplemental information

ORF8 contributes to cytokine storm during SARS-CoV-2 infection by activating IL-17 pathway

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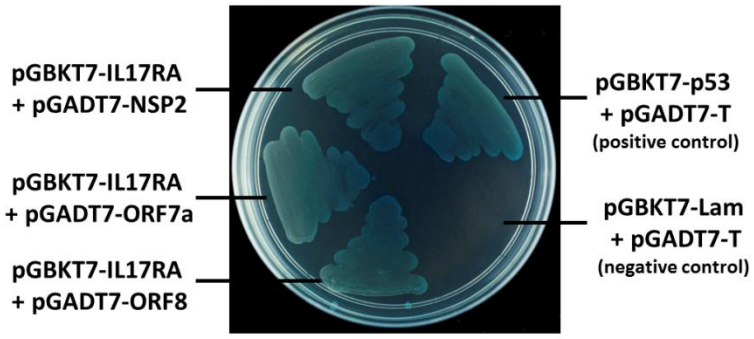

Figure S1 **NSP2, ORF7a and ORF8 are the potential candidates that interacts with IL17RA. Related to Figure 1.**

Positive clones obtained by yeast two-hybrid screening. pGBKT7-p53+pGADT7-T: positive control; pGBKT7-Lam+pGADT7-T: negative control.

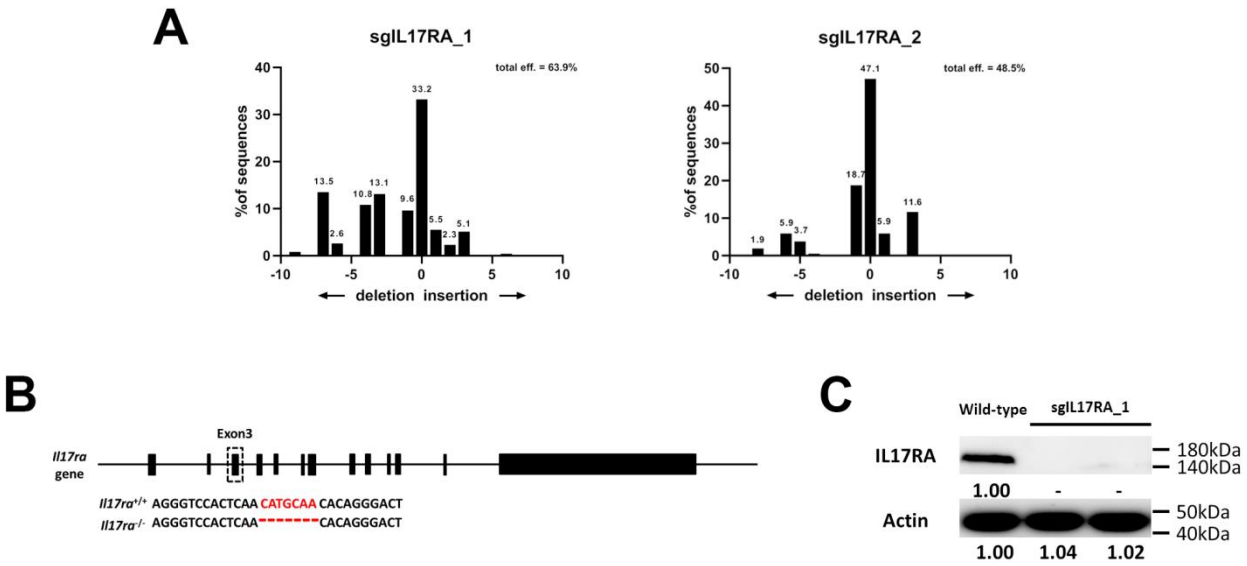

Figure S2 **Validation of *Il17ra*-deficient RAW264.7 cells. Related to Figure 1.**

(A) TIDE analysis of *Il17ra*$^{-/-}$ RAW264.7 cell pools produced by sgRNA1 and sgRNA2. (B) Schematic illustration of the target region of *Il17ra*$^{-/-}$ RAW264.7 cell clone. (C) IL17RA expression in *Il17ra*$^{-/-}$ RAW264.7 cell clone was analyzed by Western Blotting. The clone was derived from the cell pool produced by sgRNA1. Data are representative of three independent experiments (C).

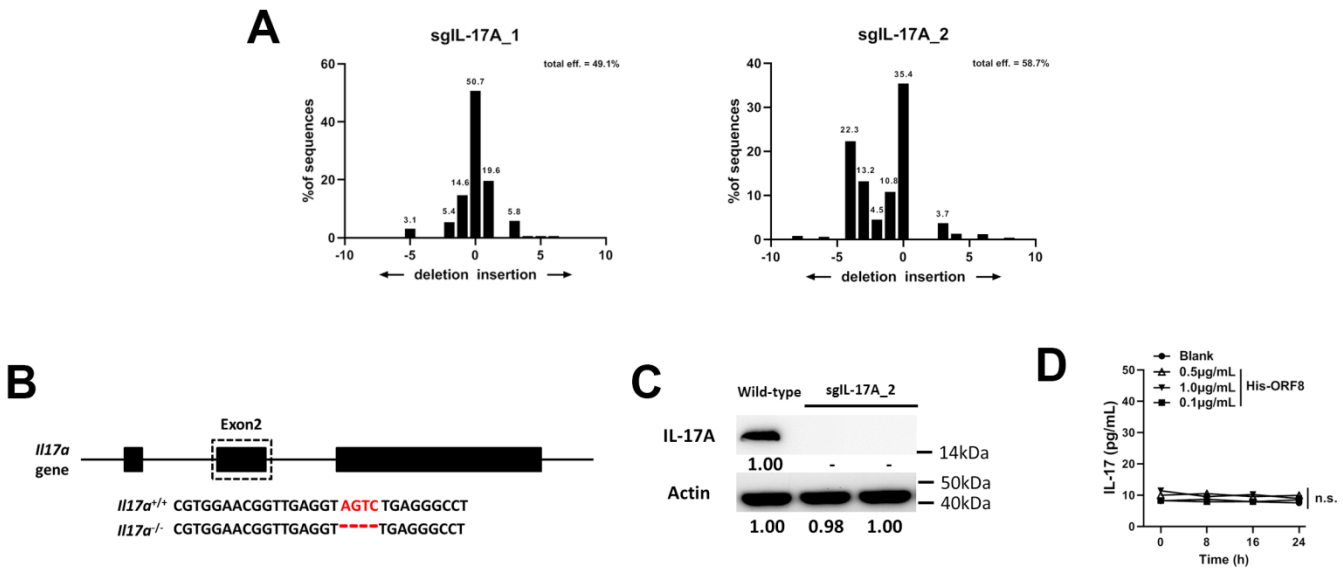

Figure S3 **Validation of *Il17a*-deficient RAW264.7 cells. Related to Figure 1.**

(A) TIDE analysis of *Il17a*$^{-/-}$ RAW264.7 cell pools produced by sgRNA1 and sgRNA2. (B) Schematic illustration of the target region of *Il17a*$^{-/-}$ RAW264.7 cell clone. (C) IL-17A expression in *Il17a*$^{-/-}$ RAW264.7 cell clone was analyzed by Western Blotting. The clone was derived from the cell pool produced by sgRNA2. (D) His-ORF8 was added to culture media of *Il17a*$^{-/-}$ RAW264.7 cells, and IL-17 expression was analyzed by ELISA. Data are representative of three independent experiments (C) or three independent experiments with n = 3 technical replicates (D) (shown as mean ± s.e.m. in D). Data are analyzed by two-tailed Student *t* test (D). Abbreviations: n.s., not significant.

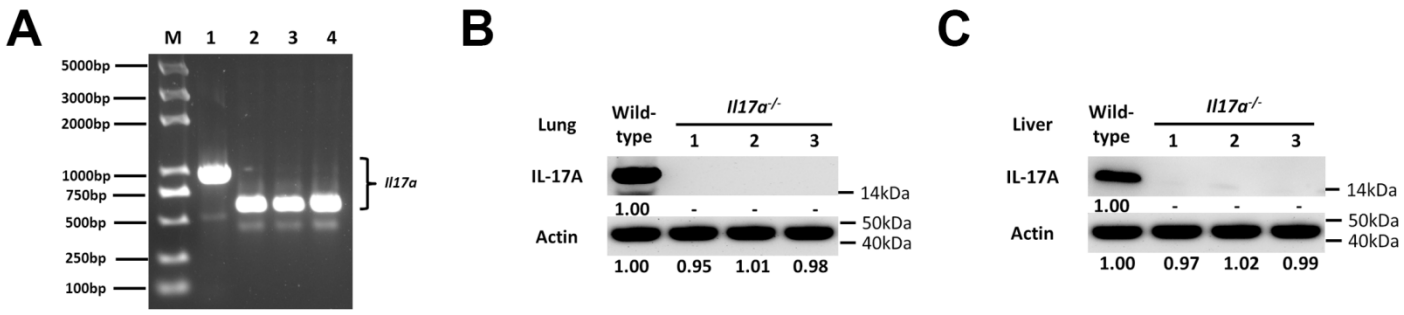

Figure S4 **Validation of *Il17a*-deficient mice. Related to Figure 2.**

(A) IL-17A expression in *Il17a*-deficient mice was analyzed by gel electrophoresis. M: marker; 1: wild-type mice; 2-4: *Il17a*-deficient mice. (B-C) IL-17A expression in lungs (B) and livers (C) of *Il17a*-deficient mice. Data are representative of three independent experiments.

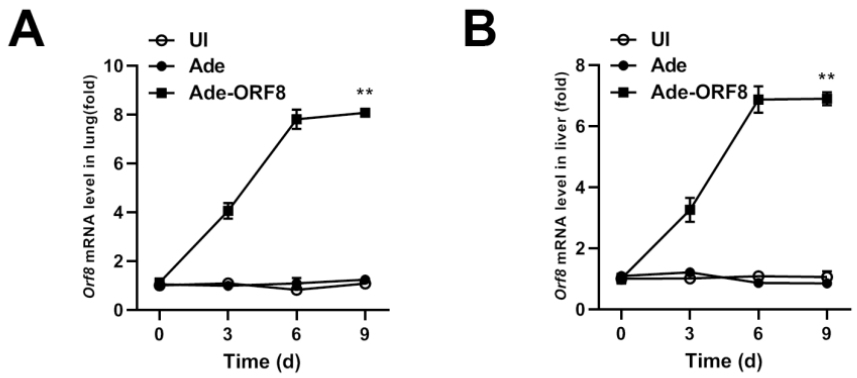

Figure S5 **Adenovirus-mediated ORF8 expression in mice. Related to Figure 2.**

(A-B) ORF8 expression in lungs (A) and livers (B) of *Il17a*-deficient mice after injection of Ade-ORF8. UI: uninfection. Data are representative of three independent experiments with n = 3 technical replicates (shown as mean ± s.e.m.). Data are analyzed by two-tailed Student *t* test. **, $p < 0.01$.

Transparent Methods

Ethic statement

This study was carried out in accordance with the Guidelines for the Care and Use of Animals of Chongqing University. All animal experimental procedures were approved by the Animal Ethics Committees of the School of Life Sciences, Chongqing University.

Mice

Six- to eight-week-old wild-type C57BL/6 mice and *Il17a*-deficient mice (C57BL/6N-Il17a^{em1cyagen}) were purchased from Cyagen Biosciences (Guangzhou, China). Mice used in each experiment were half male and half female, and age- and sex- matched in experimental group and control group. All animal study protocols were reviewed and approved by Chongqing University School of Life Sciences review boards for animal studies. The upstream and downstream primers were designed on exon 1 and exon 3 of mouse *Il17a* (NM_010552) (F-GCAAACATGAGTCCAGG, R-TGGTTTTCACCCCATTC). Three knockout mice were randomly selected to extract genomic DNA, and PCR was used to detect the length of the knockout fragment (~212bp). Meanwhile, lung and liver were taken to detect IL17RA expression by Western Blotting.

Plasmids construction

Full-length coding sequence (CDS) of SARS-CoV-2 NSP2, ORF7a and ORF8 (NCBI

Accession number: NC_045512.2) were synthesized by Beijing Genomics Institute (BGI, Beijing, China). NSP2, ORF7a, or ORF8 CDS was inserted into pCMV-HA (for eukaryotic expression) or pET-28a (+) (for protein production and purification), respectively. For GST pulldown assay, ORF8 CDS was inserted into pGEX-4T-1. Full-length CDS of IL17RA (NCBI Accession number: NM_008359.2) was inserted into pCMV-Myc. Primers are as follows: F-AATTGTCGACTATGGCGATTCGG, R-ATAAGCGGCCGCCCAAATGTCTGAT. The pNL3.2.NF-κB-RE plasmid used in the measurement of NF-κB activity was purchased from Promega (Madison, WI, USA).

Yeast two-hybrid screening

Yeast two-hybrid screening was performed using the Matchmaker Gold Yeast Two-Hybrid System (Takara, Dalian, China). Briefly, a SARS-CoV-2 protein expressing library was constructed by using the Make Your Own "Mate & Plate" Library System (Takara) strictly according to the manufacturer's instructions. Then the library was cloned to a yeast Gal4 activation domain (AD) vector pGADT7, and transformed into yeast strain Y187 to serve as "prey"; IL17RA cDNA was cloned to a Gal4 binding domain (BD) vector pGBKT7, and transformed into yeast strain Y2HGold to serve as "bait". Prey and bait were combined together to screen for positive interactions. Colonies grown on the synthetic defined (SD) plate lacking adenine, histidine, leucine, and tryptophan (SD/-Ade-His-Leu-Trp) were picked for Sanger sequencing (Supplemental File Sets).

Protein production and purification

Production and purification of ORF8, NSP2, or ORF7a protein were performed as follows(Walls et al., 2020): pET-28a(+)-ORF8, pET-28a(+)-NSP2, or pET-28a(+)-ORF7a construct was transformed into *E. coli* BL21 (DE3) and cultured in LB media at 37 °C until OD600 reached 0.6. The recombinant expression of His-tagged protein was induced by adding isopropyl β-D-thiogalactoside (IPTG) with a final concentration of 125 μM and stimulating for 16 h at 12 °C. Cells were harvested by centrifugation at 4 °C, and lysed by freezing/thawing method. Purification of the supernatants containing His-tagged protein was performed by Ni-affinity chromatography in an ÄKTA Primer FPLC system (GE Healthcare Life Sciences, Chicago, IL, USA) using the HisTrap FF columns (GE Healthcare Life Sciences) according to the manufacturer's instructions.

Cell culture and treatment

HEK293T cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The culture medium was composed of Dulbecco's Modified Eagle's Medium (DMEM, Gibco, San Jose, CA, USA) and 10% fetal bovine serum (Gibco). Plasmid DNA was transfected into indicated cells using Lipofectamine 3000 Transfection Reagent (Invitrogen, Life Technologies, CA, USA) according to the manufacturer's instructions. *Il17ra*$^{-/-}$ and *Il17a*$^{-/-}$ RAW264.7 were generated using CRISPR-Cas9. To be detailed, two sgRNAs were designed for each gene

(*Il17ra*-sgRNA1: TCCACTCAACATGCAACACA; *Il17ra*-sgRNA2: GGGGGTGGATTCATTCCACA; *Il17a*-sgRNA1: CTCAGCGTGTCCAAACACTG; *Il17a*-sgRNA2: GAACGGTTGAGGTAGTCTGA), and ligated into pSpCas9(BB)-2A-Puro (PX459) after being digested by Bbs I. The recombinant was then transfected into RAW264.7 (ATCC) using Lipo 3000 Transfection Reagent (Invitrogen). After 48 h, DMEM containing 3 μg/mL puromycin was used for screening for 7 days to obtain the cell pool. Half of the cells were taken for TIDE analysis (http://shinyapps.datacurators.nl/tide/), and the remaining cells were used for limiting dilution to obtain the cell clone. Genomic DNA was extracted and sequenced, and the indels were analyzed. For the obtained homozygous knockout monoclone, total cell protein was extracted and Western Blotting was used to detect expressions of IL17RA and IL-17A. *Il17ra*$^{+/+}$ PMs were isolated as follows(Kim et al., 2016) : mice were intraperitoneally injected with HBSS containing 2 mM EDTA and 2% FBS. After flushing the abdominal cavity, 5 ml of flushing solution was collected and centrifuged 10 min at 400×g, 4°C. Supernatant was discarded and cell pellet was resuspended in cold DMEM/F12. The cells were cultured at 37°C for 2 h and attached to the substrate. The nonadherent cells were removed by gently washing with warm PBS three times. The purified PMs were plated at a density of 1×10^6 cells/60 mm plastic dish. Afterwards, purified NSP2, ORF7a, or ORF8 was added to culture media of *Il17ra*$^{+/+}$ PMs for treatment. After 24 h, cells were harvested for immunoprecipitation. In addition, purified ORF8 was added to culture media of *Il17ra*$^{-/-}$ RAW264.7 or *Il17a*$^{-/-}$ RAW264.7 for treatment. After 24 h, cells were

harvested for immunoprecipitation.

GST pulldown assay

GST pulldown assay was performed using the GST Protein Interaction Pull-Down Kit (Thermo Fisher Scientific, San Jose, CA, USA) following the manufacturer's instructions. Briefly, the glutathione-S-transferase (GST)-tagged SARS-CoV-2 ORF8 fusion proteins were expressed in *Escherichia coli* (*E. coli*) and immobilized on the glutathione agarose resin, and then incubated with HEK293T cell lysates transfected with pCMV-Myc-IL17RA. After incubation at 4°C for at least 4 h (overnight if possible) with gentle rocking motion on a rotating platform, elution was collected for detection of protein interaction by Western Blotting.

Immunoblot and Immunoprecipitation

Immunoblot analysis was performed as follows(Fu et al., 2020) : total proteins were collected and separated by SDS-PAGE, and transferred to PVDF membrane. Blots were probed with 1/1000 anti-Actin (AF5001), 1/1000 anti-GST (AF2299) (Beyotime, Shanghai, China), 1/1000 anti-HA (SAB2702196), 1/1000 anti-Myc (SAB2702192) (Sigma-Aldrich, St. Louis, MO, USA), 1/200 anti-IL-17 (sc-374218) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1/200 anti-IL17RA (PA5-47199), 1/200 anti-ACT1 (14-4040-82) (Invitrogen), 1/200 anti-phospho-IκBα (Ser32/36) (9246), 1/500 anti-IκBα (9242) (Cell Signaling Technology, Inc., Danvers, MA, USA) antibodies. Co-immunoprecipitation was performed according to previous studies

(Lafont et al., 2018; Su et al., 2019). Briefly, cells were harvested and lysed with RIPA Lysis Buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) (P0013B, Beyotime) containing protease inhibitor cocktail. Cell lysate was centrifuged at 12, 000×g for 10 min. Part of the supernatant was taken to determine the total protein concentration and used as the input for immunoblotting, and the remaining supernatant was incubated with appropriate antibodies and Protein A/G beads (Thermo Fisher Scientific) overnight at 4°C. Precipitated protein complex was mixed with 5× SDS Loading Buffer and boiled at 98°C for 8 min, followed by immunoblotting with indicated antibodies.

NF-κB activity assay

Dual Luciferase Reporter (DLR) Assay System (Promega) was used to perform NF-κB activity assays as follows(Fu et al., 2020) : firefly luciferase plasmid pNL3.2.NF-κB-RE and Renilla luciferase plasmid pRL-SV40 (internal control) were co-transfected to cells. Twenty-four h after treatment or stimulation, cell lysates were harvested for DLR assays. Data were collected with a VICTOR X5 Multilabel Plate Reader (PerkinElmer, Waltham, MA, USA). The relative NF-κB activity was measured by Firefly luciferase luminescence divided by Renilla luciferase luminescence.

Exposure of mice to adenoviral vectors

The construction and characterization of recombinant adenovirus vector encoding

SARS-CoV-2 ORF8 (Ade-ORF8) were referred as follows(Huang et al., 2019): the CDS of ORF8 was cloned into pENTR™/D-TOPO vector (Thermo Fisher Scientific) followed by recombination into the pAd/CMV/V5-DEST (Thermo Fisher Scientific). The replication-deficient recombinant Ade-ORF8 adenovirus was produced in HEK293A cells. Mice were anesthetized, and then intratracheally instilled with Ade-ORF8 at 10^8 PFU/mouse diluted in 50 μL PBS.

IL17RA blocking

Anti-mouse IL17RA antibody (MAB4481) and mouse IgG1 isotype control (MAB002) were purchased from R&D Systems. For injections, antibody stocks were diluted in sterile PBS and each mouse received 200 μg per injection.

H&E staining

On the 9^{th} day after infection, the lung and liver of mice were fixed with 10% buffered formaldehyde for more than 24 h, embedded in paraffin, sectioned, and stained with H&E according to the standard procedure. Photographs were obtained by microscope (Carl Zeiss, Jena, Germany). A scoring system was set as follows (Kleiner et al., 2005; Matute-Bello et al., 2011) : five fields at 200× magnification were randomly selected for each slice. The lung scoring criteria are as follows: 0, pulmonary lobes lacked lesions; 1, multifocal lesions with mild lymphocyte and macrophage infiltration; 2, mild infiltration of peri-bronchial, peri-vascular and alveolar; 3, small range of blocked terminal bronchioles, fibroplasia or organization; 4, wide range of alveolar

necrosis and hyaline thrombus. The liver scoring criteria are as follows: 0, hepatic lobules lacked lesions; 1, scattered inflammation with ≤3 lesions in hepatic lobules; 2, 3-7 lesions in hepatic lobules, accounting for＜1/3 of the hepatic lobule; 3, scattered inflammation with＞7 lesions, accounting for 1/3-2/3 of the hepatic lobule; 4, inflammatory lesions spread throughout hepatic lobules, with large areas of hepatocyte necrosis.

Enzyme-linked immunosorbent assay (ELISA)

Mouse TNF-α, IL-1β, IL-6, IL-12 ELISA kits were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Cell culture supernatants were assayed according to the manufacturer's protocols. Mice were sacrificed and lungs/livers were quickly excised, rinsed of blood, and homogenized by adding 1 mL homogenization buffer (PBS containing 0.05% sodium azide, 0.5% Triton X-100, and a protease inhibitor cocktail, pH 7.2, 4˚C), and then sonicated for 10 minutes. Homogenates were centrifuged at 12,000×g for 10 minutes, and the supernatant was taken to determine the total protein concentration, followed by ELISA analysis. The concentration of each cytokine was calculated against a standard curve.

Statistical analysis

Two-tailed Student's *t* test was used to compare the means between two groups. A value of $P < 0.05$ was considered significant.

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