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# SARS-CoV-2 ORF3a positively regulates NF-κB activity by enhancing IKKβ-NEMO interaction

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ABSTRACT

Coronavirus disease 2019 (COVID-19) is a global pandemic caused by SARS-CoV-2 infection. Patients with severe COVID-19 exhibit robust induction of proinflammatory cytokines, which are closely associated with the development of acute respiratory distress syndrome. However, the underlying mechanisms of the NF-κB activation mediated by SARS-CoV-2 infection remain poorly understood. Here, we screened SARS-CoV-2 genes and found that ORF3a induces proinflammatory cytokines by activating the NF-κB pathway. Moreover, we found that ORF3a interacts with IKKβ and NEMO and enhances the interaction of IKKβ-NEMO, thereby positively regulating NF-κB activity. Together, these results suggest ORF3a may play pivotal roles in the pathogenesis of SARS-CoV-2 and provide novel insights into the interaction between host immune responses and SARS-CoV-2 infection.

## 1. Introduction

The coronavirus disease 2019 (COVID-19) outbreak is still a pandemic and continues to threaten public health and security. The clinical symptoms of COVID-19 vary widely from asymptomatic to mild or atypical pneumonia and acute respiratory distress syndrome (ARDS) (Fara et al., 2020). Studies evidenced that patients with severe and critical COVID-19 showed excessive NF-κB–driven inflammatory response (Hadjadj et al., 2020; Nilsson-Payant et al., 2021; Schultze and Aschenbrenner, 2021). In the serum of severe and critical COVID-19 patients, proinflammatory cytokines/chemokines, such as IL-1β, TNF-α, IL-6, and IP-10, are significantly increased (Blanco-Melo et al., 2020; Hadjadj et al., 2020; Karki et al., 2021; Kouhpayeh, 2022; Schultze and Aschenbrenner, 2021; Shen et al., 2020; Tang et al., 2020). Continuously high cytokine levels are closely associated with ARDS, multiple organ failure, and even a fatal outcome (Napoli et al., 2022; Sun et al., 2020; Vaninov, 2020; Yang et al., 2020).

SARS-CoV-2, the causative agent of COVID-19, is an enveloped β-coronavirus with a large single-stranded, positive-sense RNA genome. The genome encodes sixteen nonstructural proteins (NSP1–16), four structural proteins (S [spike], E [envelope], M [membrane], and N [nucleocapsid]), and nine predicted accessory proteins (ORF3a, ORF3b, ORF6, ORF7a, ORF7b, ORF8, ORF9b, ORF9c, and ORF10) (Fig. 1A) (Gordon et al., 2020). The nonstructural proteins contribute to mass viral replication, the structural proteins form new virions, and the accessory proteins play critical roles in viral pathogenesis (Peng et al., 2021; Rashid et al., 2022).

Upon SARS-CoV-2 infection, the host cells utilize germline-encoded pattern recognition receptors (PRRs) to recognize the virus-derived pathogen-associated molecular patterns (PAMPs) and initiate host antiviral immune response to produce type I/III interferons (IFNs) and proinflammatory cytokines by activating the interferon regulatory factors (IRFs) and NF-κB transcription factors (Diamond and Kanneganti, 2022). Like most viruses, SARS-CoV-2 has evolved multiple strategies to antagonize the IFN system. At least ten SARS-CoV-2 encode proteins, such as M, NSP1, and ORF9b, are known to counteract the production and signaling of type I/III IFNs (Rashid et al., 2022). On the contrary, SARS-CoV-2 infection selectively activates NF-κB, leading to abnormal proinflammatory cytokines production (Neufeldt et al., 2022; Nilsson--Payant et al., 2021). Additionally, silencing of NF-κB effectively blocks

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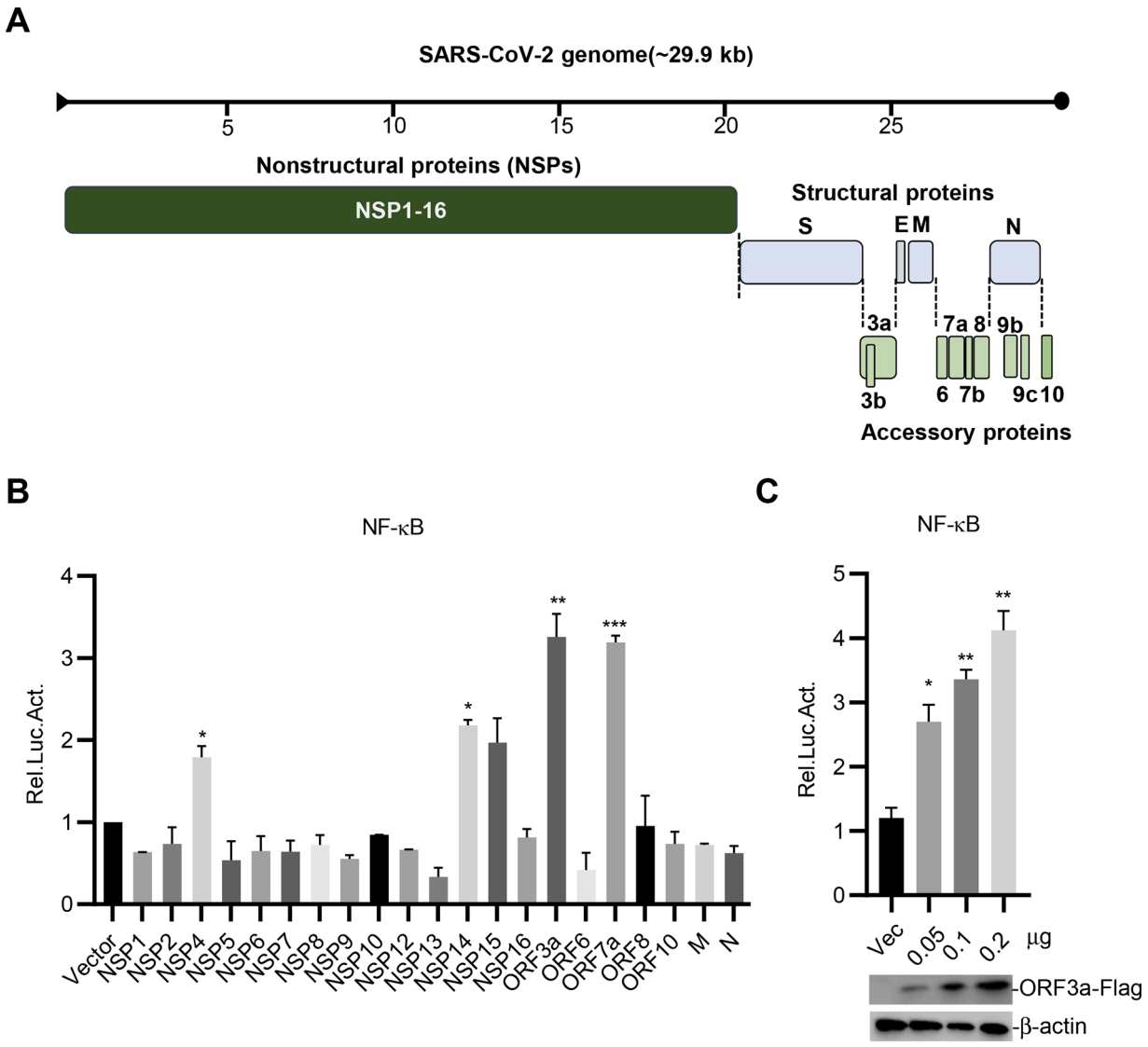


**Fig. 1.** Identification of SARS CoV-2 ORF3a as an activator of NF-κB luciferase reporter. (A) Schematic of SARS CoV-2 encoded proteins. Nonstructural proteins (NSPs), structural proteins, and predicted accessory proteins are shown. (B) HEK293T cells ($1 \times 10^5$) were cotransfected with NF-κB luciferase reporter, pRL-TK, along with empty vector or the viral expression plasmids (0.1 μg). Twenty-four hours after transfection, cells were lysed for luciferase assays. (C) HEK293T cells ($1 \times 10^5$) were cotransfected empty vectors or increased amounts of ORF3a expression plasmids with NF-κB luciferase reporter. Twenty-four hours post-transfection, cell lysates were divided into two aliquots; one aliquot was used for luciferase assays, and the other was used for Western blot analysis. Graphs show mean ± SD. $n = 3$. $^*P<0.05$, $^{**}P<0.01$ (Student's *t*-test).

SARS-CoV-2 replication (Nilsson-Payant et al., 2021). Thus, NF-κB signaling has been regarded as a potential and promising pharmacological target for COVID-19 treatment (Carcaterra and Caruso, 2021; Gudowska-Sawczuk and Mroczko, 2022; Kircheis et al., 2020; Moradian et al., 2020).

In mammals, the NF-κB family consists of five members named p65 (RelA), RelB, c-Rel, p105/p50 (NF-κB1), and p100/52 (NF-κB2) (Capece et al., 2022). There are two types of NF-κB pathways: the canonical and the noncanonical pathway. The canonical NF-κB pathway is rapidly and transiently activated by various signals, including PAMPs and proinflammatory cytokines, from a variety of receptors. The receptors recruit the adaptor proteins, including myeloid differentiation primary response gene 88 (MyD88), receptor-interacting protein (RIP1) and TNF receptor-associated factors (TRAFs), and TGF-beta-activated kinase 1 (TAK1) into a megacomplex, leading to the activation of the IκB kinase (IKK) complex which is composed of two catalytically active kinases (IKKα and IKKβ) and a regulatory scaffolding protein called NEMO (NF-κB essential modulator, also known as IKKγ). The active IKK complex phosphorylates the IκB protein and promotes its degradation by the proteasome, releasing NF-κB into the nucleus and inducing the production of inflammation (Hayden and Ghosh, 2008). More recently, several studies showed the association between SARS-CoV-2 proteins and the NF-κB pathway activation. S and E induce inflammatory cytokines and chemokines via TLR2-mediated activation of the NF-κB pathway (Khan et al., 2021; Planès et al., 2022). NSP14 activates NF-κB by binding to IMPDH2, a regulator of NF-κB signaling (Qin et al., 2022). Moreover, N, NSP6, and ORF7a target TAK1 and components of the IKK complex to facilitate NF-κB activation (Nishitsuji et al., 2022; Wu et al., 2021).

The involvement of ORF3 in inflammatory response has also been reported (Su et al., 2021; Xu et al., 2022). However, its underlying molecular mechanisms remain unknown. Here we show that SARS-CoV-2 ORF3a induces proinflammatory cytokines through NF-κB signaling pathway. Mechanistically, ORF3a interacts with IKKβ and NEMO, enhancing the interaction of IKKβ and NEMO. Our results reveal a novel mechanism by which SARS-CoV-2 induces abnormal proinflammatory response.

## 2. Materials and methods

### 2.1. Cells, virus, reagents, and antibodies

HEK293T cells (ATCC, CRL-3216™). A549 cells (SCSP-503) and THP-1 cells (TCHu 57) were purchased from the National Collection of Authenticated Cell Cultures (Shanghai, China). Sendai virus (SeV) was kindly provided by Prof. Yanyi Wang (Wuhan Institute of Virology, CAS) and propagated in SPF chicken embryonated eggs. Lipo293™ Transfection Reagent (Beyotime Biotechnology, C0521), Lipofectamine 2000 (Invitrogen, 11,668,019), dual-specific luciferase assay kits (Promega, E1980), human recombinant TNFα (R&D Systems, 210-TA-020/CF), BAY 11–7082 (MCE, HY-13,453), mouse anti-Flag (Sigma, F3165), rabbit anti-Flag (Proteintech, 20,543–1-AP), anti-β-actin (Cell Signaling Technology, # 3700S), anti-GAPDH (HuaBio, #R1210–1), anti-LMNB1 (Proteintech, 2987–1-AP), anti-HA (Origene, TA100012), anti-TNF-α (Proteintech, 60,291–1-Ig), anti-phospho-NF-κB p65(Cell Signaling Technology, #3033S), anti-phospho-IκBα (Cell Signaling Technology, #9246S), anti-phospho-IKKα/β (Cell Signaling Technology, # 2697S), anti-IKK-β (Proteintech, 15,649–1-AP) anti-IκBα (Santa Cruz, sc-1643), anti-p65 (Santa Cruz, sc-8008), donkey anti-mouse IgG-Cy3 (Absin, abs20015) and goat anti-Rabbit IgG-FITC (Absin, abs20004ss) were purchased from the indicated companies.

### 2.2. Plasmid construction

Mammalian expression plasmids for HA-tagged MyD88, TAK1, IKKα, IKKβ, NEMO, and Flag-NEMO were purchased from the Miaoling Plasmid Sharing Platform. IKKβ S177/181A (the kinase-dead mutant of IKKβ) (Mercurio et al., 1997) and dnIκBα (S32R/S36R) were generated from the HA-IKKβ plasmids and pCMV6-IκBα plasmids (Origene), respectively, by using site-directed mutagenesis. NF-κB and pRL-TK luciferase plasmids were reported previously (Nie et al., 2017). Nucleotides for twenty SARS-CoV-2 (Wuhan-Hu-1, NC_045512.2) genes were codon optimized and synthesized by Sangon Biotech and then cloned into pCMV14–3xFlag-C by standard molecular biology techniques.

### 2.3. Dual-luciferase reporter assays

HEK293T cells ($1 \times 10^5$) were seeded on 48-well plates and cotransfected with 5 ng NF-κB firefly luciferase reporter plasmid, 50 ng pRL-TK Renilla luciferase reporter plasmid and the indicated expression plasmids mixed with Lipo293™ Transfection Reagent according to the manufacturer's instructions. At 24 h post-transfection, cells were harvested, or treated with TNF-α (10 ng/ml) for 12 h or infected with SeV (100 HAU/ml) for 12 h, followed by an analysis of cell lysates for luciferase assays with a dual-specific luciferase assay kit (Promega). Firefly luciferase activities were normalized based on Renilla luciferase activities.

### 2.4. Construction of THP-1 cells stably expressing ORF3a

To establish a stable ORF3a-expressed THP-1 cell line, ORF3a was subcloned into a lentiviral transfer vector pCDH—CMV-MCS-EF1-copGFP-T2A-Puro (Miaoling Plasmid Sharing Platform). Subsequently, HEK293T cells were seeded in 10 cm dishes and transfected with pCDH—CMV-ORF3a-EF1-copGFP-T2A-Puro or empty vector, along with the packaging vectors pGag/Pol, pRev and pVSVG using Lipo293™ Transfection Reagent. The lentiviral supernatants were collected after 72 h and used to infect THP-1 cells in the presence of polybrene (8 μg/mL) (Absin, abs42025397). Polyclonal ORF3a stable expression THP-1 cell line was selected for 2–4 weeks with 1 μg/ml puromycin dihydrochloride (MCE, HY-B1743A). The stable THP-1 cell line with copGFP expression efficiency over 80% was maintained and passaged for the subsequent experiments.

### 2.5. Quantitative real-time PCR

HEK293T cells and HeLa cells were transfected with indicated plasmids using Lipo293™ or Lipo2000 Transfection Reagent, respectively. Twenty-four hours after transfection, cells were untreated or treated with TNF-α or SeV for 12 h. Total RNA was isolated for quantitative real-time PCR (qPCR) analysis to measure the mRNA levels of the indicated genes. The expression values of targeted genes were normalized to the values of *GAPDH*. Human gene-specific primer sequences were as follows: *TNF-α*: 5′-CCTCTCTCTAATCAGCCCTCTG-3′ (forward), 5′-GAGGACCTGGGAGTAGATGAG-3′ (reverse); *IL-1β*: 5′-AGCTACGAATCTCCGACCAC-3′ (forward), 5′-CGTTATCCCATGTGTCGAAGAA-3′ (reverse); *IL-6*: 5′-AAACAACCTGAACCTTCCAAAGA-3′ (forward), 5′-GCAAGTCTCCTCATTGAATCCA-3′ (reverse); *IL-8*: 5′-GGTGCAGTTTTGCCAAGGAG-3′ (forward), 5′-TTCCTTGGGGTCCAGACAGA-3′ (reverse); *CXCL1*: 5′-CTTCAGGAACAGCCACCAGT-3′ (forward), 5′-TCCTGCATCCCCCATAGTTA-3′ (reverse); *IFNB1*: 5′-TTGTTGAGAACCTCCTGGCT-3′ (forward), 5′-TGACTATGG TCCAGGCACAG-3′ (reverse); *ISG15*: 5′-AGGACAGGGTCCCCCTTGCC-3′ (forward), 5′- CCTCCAGCCCGCTCACTTGC-3′ (reverse); *ISG56*: 5′-TCATCAGGT CAAGGATAGTC-3′ (forward), 5′-CCACACTGTATTTGGTGTCTAGG-3′ (reverse); *ORF3a*: forward: 5′-ATAGCACCCAACTGAGCACC-3′, reverse: 5′- TTGAACGTGTTCCTCCGGTT −3′; *GAPDH*: 5′-AGGTCGGTGTGAACG GATTTG-3′ (forward); reverse:5′-TGTAGACCATGTAGTTGAGGTCA-3′ (reverse).

### 2.6. Subcellular fractionation

The experiments were performed with a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotechnology, P0027) following protocols recommended by the manufacturer. GAPDH and LMNB1 were used as cytoplasmic and nuclear markers to determine the quality of the fraction.

### 2.7. Coimmunoprecipitation and western blot analysis

HEK293T cells were cotransfected with the indicated expression plasmids. Twenty-four hours after transfection, cells were collected and lysed in l ml NP-40 lysis buffer (20 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 10 mg/ml aprotinin, 10 mg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Lysates were incubated with indicated antibodies and protein G-Sepharose beads (GE Healthcare) for 2–4 h. The Sepharose beads were washed five times with 1 ml NP-40 lysis buffer containing 0.5 M NaCl. For the western blot (WB) assay, the protein beads or whole cell lysates were subjected to SDS-PAGE and transferred to nitrocellulose (NC) membrane. After blocking with 5% nonfat dried milk, the membranes were incubated with indicated primary antibodies, followed by HRP-conjugated secondary antibodies. The secondary antibodies (goat anti-mouse IgG light chain (A25012), Abbkine) were used for the Co-IP assay to avoid the noise of heavy chains.

### 2.8. Immunofluorescence assay

A549 cells were transfected with the indicated plasmids for 24 h, then washed with PBS. The cells were fixed with 4% paraformaldehyde/PBS for 10 min and permeabilized with 0.1% Triton X-100/PBS for 10 min at room temperature. Next, the cells were blocked with 5% BSA and incubated with the indicated primary at 4 °C overnight and with secondary antibodies at room temperature for 1 h. The nuclei were stained with DAPI (Absin, abs47047616). The cells were observed by Olympus confocal microscope.

### 2.9. Statistical analysis

Data are represented as mean ± SD. Data were analyzed by Prism8 (GraphPad Software), and statistical significance was determined by

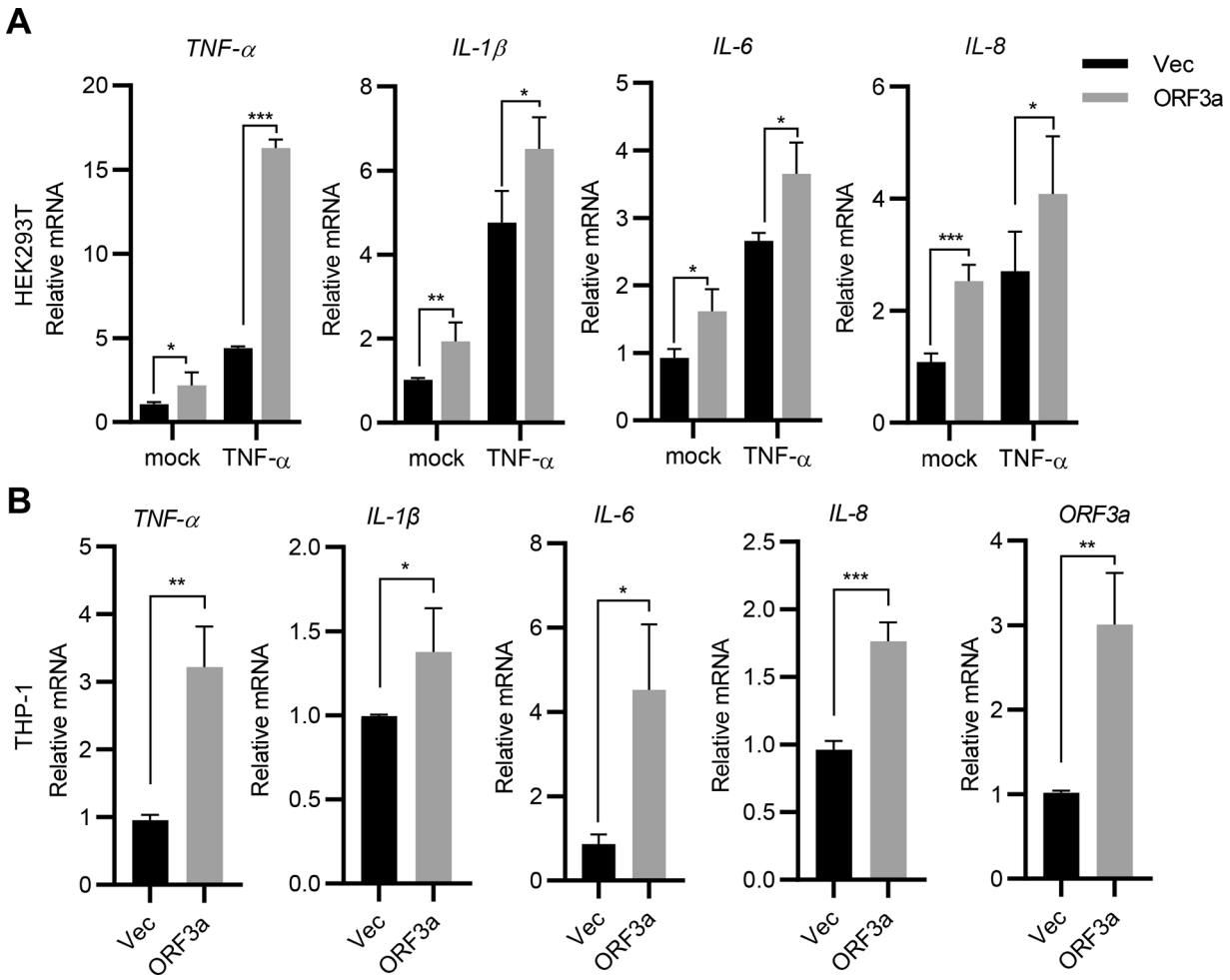


**Fig. 2.** SARS-CoV-2 ORF3a promotes cytokines production. (A) HEK293T cells ($4 \times 10^5$) were transfected with empty vector or ORF3a expression plasmids (2 μg). Twenty-four hours after transfection, cells were untreated or treated with TNF-α (10 ng/ml) for 12 h, and the mRNA levels were measured by qPCR. (B) The mRNA levels indicated genes in the stable THP-1 cells were analyzed by qPCR. Graphs show mean ± SD. $n = 3$. *$P$<0.05, **$P$<0.01, ***$P$<0.001 (Student's $t$-test).

two-tailed unpaired Student's $t$-test. *$P$<0.05 was considered statistically significant.

## 3. Results

### 3.1. Identification of SARS-CoV-2 ORF3a as an activator of NF-κB

We cloned 20 genes of SARS-CoV-2 to a C-terminal Flag tagged-mammalian expression plasmid. The expression of these viral proteins was confirmed by Western blotting (Figure S1A). The results showed that all proteins were expressed with the expected molecular sizes and expressed at various levels. To screen for SARS-CoV-2 proteins involved in modulating the activation of NF-κB signaling, HEK293T cells were cotransfected with individual SARS-CoV-2 gene and NF-κB luciferase reporter. We found that NSP4, NSP14, NSP15, ORF3a, and ORF7a significantly activated NF-kB reporter (Fig. 1B). The binding of TNF-α to its receptor TNFR induces the activation of the canonical NF-κB pathway (Taniguchi and Karin, 2018). We treated HEK293T cells with TNF-α and found that NSP15, ORF3a, and ORF7a markedly increased NF-κB activity (Figure S1B). Collectively, Only ORF3a and ORF7a significantly activated NF-κB signaling in both untreated and TNF-α-treated cells. The exact mechanisms of ORF7a in modulating NF-κB activity have been reported recently (Nishitsuji et al., 2022). Here, we focus on how ORF3a regulates NF-κB signaling. To further investigate the function of ORF3a in NF-κB activation, cells were transfected with gradient doses of ORF3a with NF-κB reporter plasmids. Results demonstrated that ORF3a enhanced NF-κB activity in a dose-dependent manner (Fig. 1C).

### 3.2. SARS-CoV-2 ORF3a induces proinflammatory cytokines

Activation of NF-κB is known to induce proinflammatory cytokines and chemokines. Since ORF3a enhances NF-κB activation, we further examined the production of NF-κB-mediated proinflammatory cytokines. In HEK293T cells transfected with ORF3a expression plasmids, the mRNA levels of *TNF-α, IL-1β, IL-6*, and *IL-8* were markedly upregulated with or without TNF-α stimuli (Fig. 2A). We also performed Western blotting to assess the protein levels of TNF-α. The result showed that treatment of TNF-α induced a significant increase in the production of TNF-α protein, whereas ORF3a enhanced this effect within 30 min (Figure S2A). To investigate whether ORF3a also regulates Sendai virus (SeV)-induced NF-κB activation, HEK293T cells expressing ORF3a were subjected to infection with SeV. We observed that overexpression of ORF3a significantly increased the expression of SeV-induced *TNF-α* and *CXCL1* gene (Figure S2B). We also attempt to determine the roles of ORF3a in innate immune evasion. Results showed that ORF3a did not decrease SeV-induced expression of *IFNB1, ISG15*, and *ISG56* (Figure S2C). Immune cells, including macrophages and monocytes, are cornerstone of the immune system during viral infection. We first established an ORF3a-overexpressing stable THP-1 cell line and confirmed the ORF3a mRNA levels by qPCR. Then, increased expression of *TNF-α, IL-1β, IL-6*, and *IL-8* were detected in ORF3a-expressed THP-1 cells (Fig. 2B).

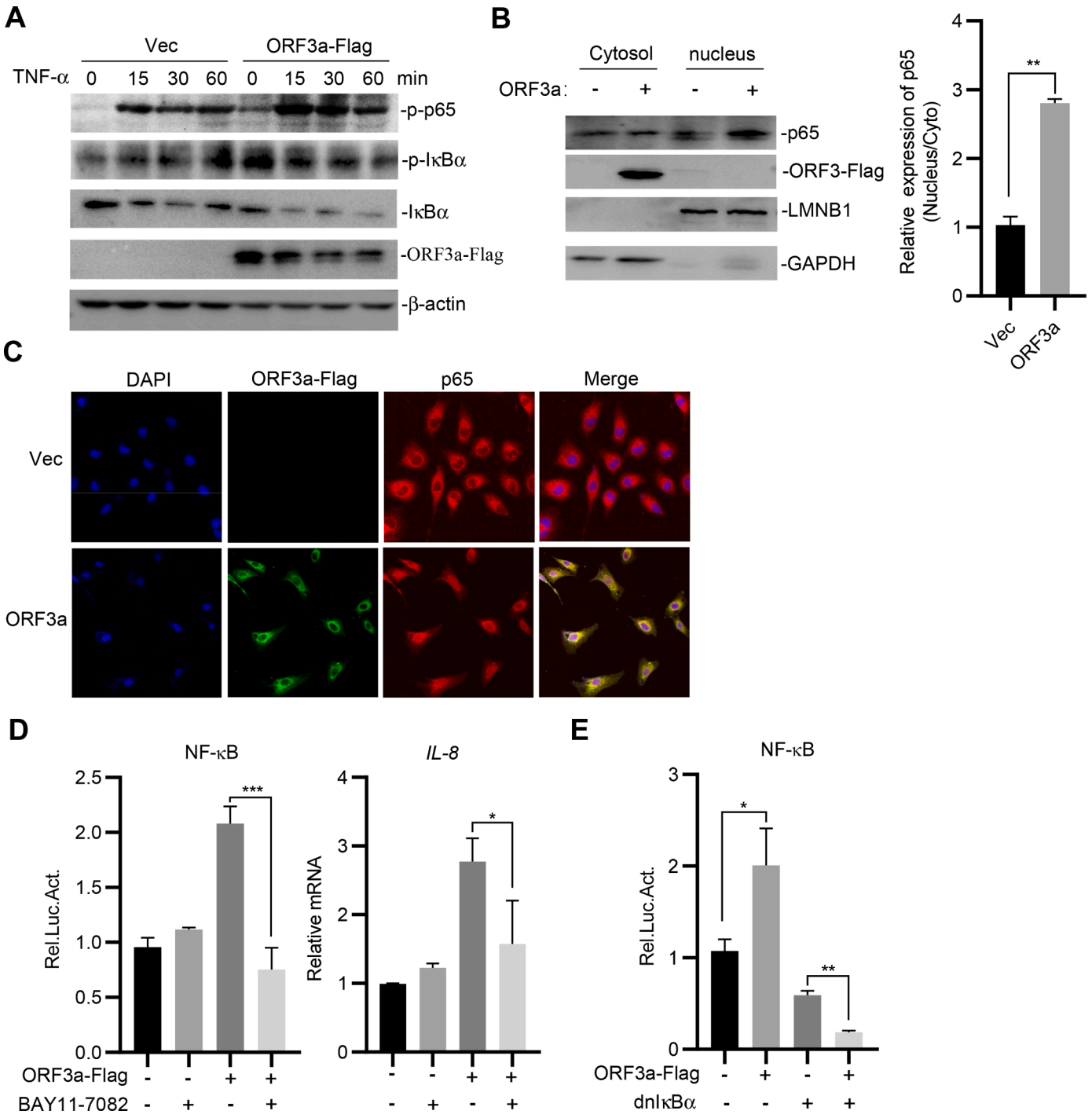


**Fig. 3.** SARS-CoV-2 ORF3a induces pro-inflammatory production via NF-κB. (A) HEK293T cells ($4 \times 10^5$) transfected with empty vector or ORF3a-Flag (2 μg) were treated with TNF-α (10 ng/ml) for the indicated times. Cell lysates were analyzed by Western blotting with the indicated antibodies. (B) HEK293T cells ($4 \times 10^5$) were transfected with empty vector or ORF3a-Flag (2 μg). Twenty-four hours after transfection, the cells were fractionated. The subcellular fractions, including cytosol and nucleus were equilibrated to equal volumes and analyzed by Western blotting with the indicated antibodies. (C) A549 cells ($2 \times 10^5$) were transfected with empty vector or ORF3a-Flag (1 μg) for 24 h, cells were fixed and stained with the anti-p65 antibody (red) and anti-Flag (green). Cellular nuclei were stained with DAPI. (D) In the left panel, HEK293T cells ($1 \times 10^5$) were cotransfected with empty vector or ORF3a-Flag (0.1 μg), NF-κB luciferase reporter, pRL-TK. In the right panel, HEK293T cells ($4 \times 10^5$) were cotransfected with empty vector or ORF3a-Flag (2 μg). At 24 h post-transfection, cells were treated or untreated with BAY 11–7082 (10 μM) for 6 h before luciferase assays or qPCR were performed. (E) HEK293T cells ($1 \times 10^5$) were cotransfected with empty vector or ORF3a-Flag (0.1 μg), along with NF-κB luciferase reporter and pRL-TK in the absence or presence of dnIκBα (0.05 μg). Graphs show mean ± SD. $n = 3$. *$P$<0.05, **$P$<0.01 (Student's $t$-test).

### *3.3. SARS-CoV-2 ORF3a activates NF-κB signaling in the upstream level of IκBα*

The expression of proinflammatory cytokines/chemokines such as TNF-α, IL-6, and IL-8 is dependent on the IKK complex-mediated degradation of IκBα. IKK also phosphorylates the NF-κB subunit p65, which is required for the nuclear translocation of NF-κB. Therefore, we investigated the regulatory effects of ORF3a on NF-κB signaling by

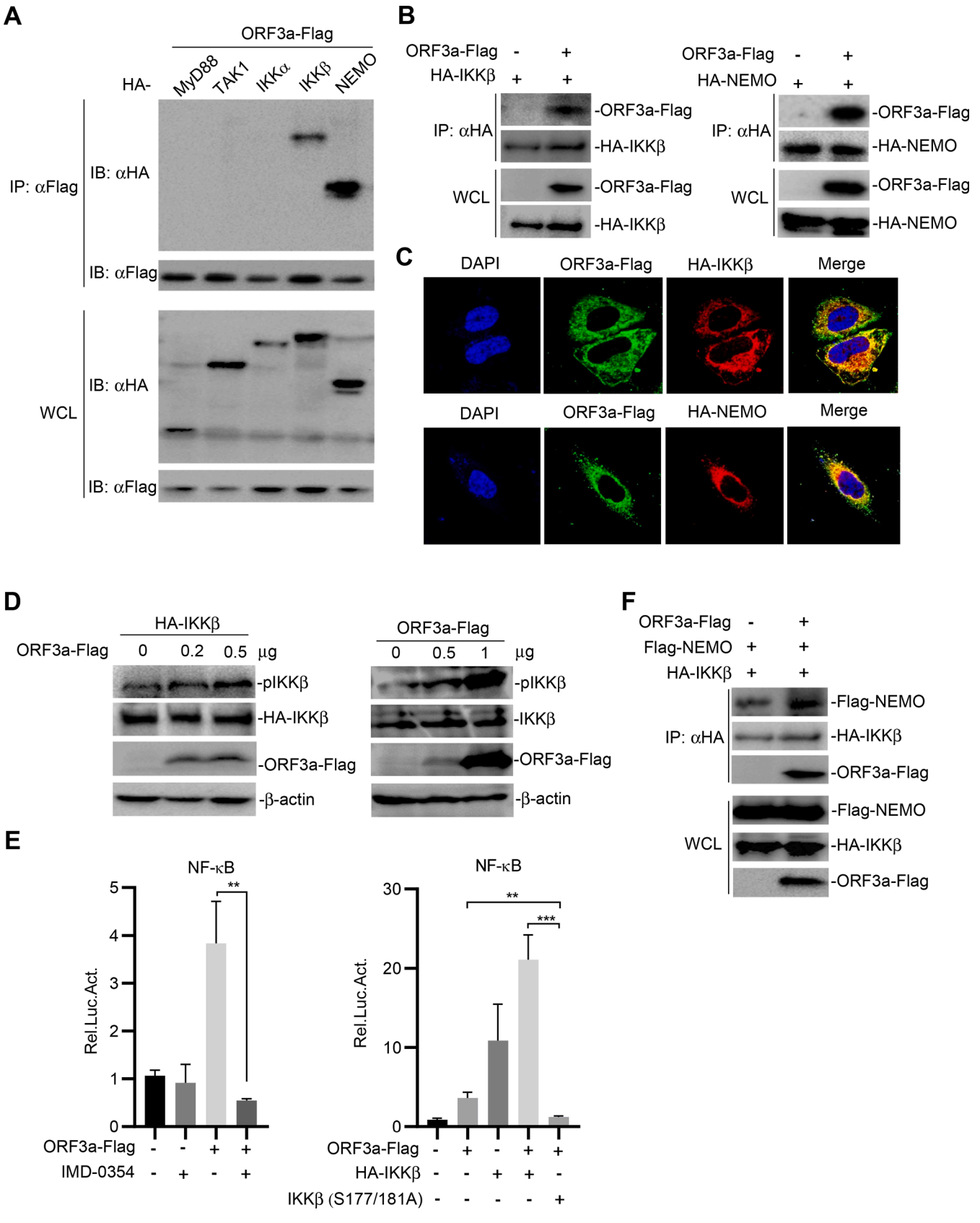


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**Fig. 4.** SARS-CoV-2 ORF3a interacts with IKKβ and NEMO. (A) HEK293T ($2 \times 10^6$) cells were cotransfected with ORF3a-Flag and HA-tagged MyD88, TAK1, IKKα IKKβ, and NEMO for 24 h. Coimmunoprecipitation and immunoblotting were performed with the indicated antibodies. (B) HEK293T ($2 \times 10^6$) cells were cotransfected with HA-IKKβ (left panel) or -NEMO (right panel) and ORF3a-Flag or empty vector for 24 h. Coimmunoprecipitation and immunoblotting were performed with the indicated antibodies. (C) A549 cells ($1 \times 10^5$) were cotransfected with ORF3a-Flag (0.5 μg) and HA-IKKβ (upper panels) or -NEMO (lower panels) (0.5 μg). At 24 h post-infection, cells were stained with the indicated antibodies and analyzed by confocal microscopy. (D) In the left panel, HEK293T ($4 \times 10^5$) cells were cotransfected with HA-IKKβ (0.5 μg) and increased amounts of ORF3a-Flag for 24 h. In the right panel, HEK293T cells were transfected with increased amounts of ORF3a-Flag for 24 h. Cell lysates were analyzed by Western blotting with the indicated antibodies. (E) In the left panel, HEK293T cells ($1 \times 10^5$) were cotransfected with empty vector or ORF3a-Flag (0.1 μg), NF-κB luciferase reporter. At 24 h post-transfection, cells were treated or untreated with IMD-0354 (10 μM) for 6 h before luciferase assays were performed. In the right panel, HEK293T cells ($1 \times 10^5$) were transfected with indicated plasmids for 24 h before luciferase assays were performed. (F) HEK293T ($2 \times 10^6$) cells were cotransfected with HA-IKKβ, Flag-NEMO, along with ORF3a-Flag or empty vector for 24 h. Coimmunoprecipitation and immunoblotting were performed with the indicated antibodies. Graphs show mean ± SD. $n = 3$. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ (Student's *t*-test).

assessing phosphorylation of the IκBα and p65, as well as nuclear translocation of p65. As shown in Fig. 3A, TNF-α-induced degradation of IκBα, and phosphorylation of p65 and IκBα were markedly increased in ORF3a expressed HEK293T cells. In subcellular fractionation experiments, ORF3a was distributed in the cytoplasm, and the amount of nucleus-p65 was significantly increased in HEK293T cells transfected with ORF3a compared to those transfected with empty vector (Fig. 3B). Immunofluorescence analysis also confirmed that ORF3a promoted the nuclear translocation of p65 (Fig. 3C).

In addition, we analyzed the effects of NF-κB inhibitor BAY 11–7082 on ORF3a-mediated NF-κB activation. BAY 11–7082 irreversibly inhibits TNF-α-induced phosphorylation and degradation of IκBα (Chen et al., 2014). In our results, incubation with BAY 11–7082 impaired ORF3a-mediated NF-κB luciferase activity and transcription of *IL-8* (Fig. 3D). To further confirm these results, we generated a dominant-negative IκBα that cannot be phosphorylated and degraded (Wang et al., 1996). Coexpression of dnIκBα significantly inhibited NF-κB activity mediated by ORF3a (Fig. 3E). These observations suggest that ORF3a functions upstream of IκBα in the canonical NF-κB pathway.

### 3.4. ORF3a targets IKKβ and NEMO to enhance the interaction of IKKβ-NEMO

To determine the potential regulatory targets of ORF3a, we performed coimmunoprecipitation (Co-IP) in HEK293T cells transfected ORF3a-Flag with HA-tagged MyD88, TAK1, IKKα, IKKβ, and NEMO, upstream components of NF-κB signaling using anti-Flag precipitating antibody. The results showed that ORF3a strongly interacted with IKKβ and NEMO but not MyD88, TAK1, and IKKα (Fig. 4A). Next, cells were cotransfected with HA-IKKβ or -NEMO and ORF3a-Flag or empty vector, and Co-IP was performed by anti-HA precipitating antibody. This effort further verified that ORF3a interacted with IKKβ and NEMO, and ORF3a did not affect their protein levels (Fig. 4B). Furthermore, immunofluorescence staining consistently revealed the co-localization of ORF3a with IKKβ, and ORF3a with NEMO (Fig. 4C).

In the IKK complex, the binding of NEMO to IKKβ leads to conformational changes of IKKβ and facilitates the phosphorylation of IKKβ, which is essential for the activation of canonical NF-κB (Ko et al., 2022; Schröfelbauer et al., 2012). We first investigated whether ORF3a enhanced the phosphorylation of IKKβ. As shown in Fig. 4D, phosphorylation of IKKβ was markedly increased by ORF3a. In addition, ORF3a induced NF-κB induction was reversed by IKKβ inhibitor IMD-0354 and IKKβ (S177/181A) (the kinase-dead mutant), indicating that IKKβ activity is required for ORF3a-mediated NF-κB activation (Fig. 4E). Since ORF3a has the ability to interact with IKKβ and NEMO, we then determined whether ORF3a enhanced the association of IKKβ with NEMO to active IKKβ and NF-κB. Cells were cotransfected IKKβ with NEMO, along with ORF3a or empty vector. Co-IP results indicated that the interaction of IKKβ-NEMO was significantly increased by ORF3a (Fig. 4F). These data suggest that SARS-CoV-2 ORF3a targets IKKβ and NEMO, and enhances the association of IKKβ with NEMO, thereby positively regulating NF-κB activity.

## 4. Discussion

In the late stage of severe COVID-19 patients, SARS-CoV-2 infection induces excessive immune response and cytokine storm (Tian et al., 2020). NF-κB activation is central to SARS-CoV-2 induced cytokine storm (Attiq et al., 2021; Millar et al., 2022). Anti-inflammation has been regarded as an essential strategy for treating severe COVID-19 patients. How SARS-CoV-2 infection activates NF-κB signaling is not well understood. In this study, our screening results showed that several SARS-CoV-2 encoded proteins promote NF-κB promoter activity, particularly ORF3a and ORF7a proteins. Our study further identified SARS-CoV-2 ORF3a as a positive regulator of NF-κB signaling by enhancing the association of IKKβ and NEMO.

Several lines of evidence suggest that the SARS-CoV-2 ORF3a positively regulates NF-κB activity. ORF3a significantly increased NF-κB activity in untreated and TNF-α- or SeV-treated cells, leading to the release of proinflammatory cytokines/chemokines. In addition, BAY 11–7082 (NF-κB inhibitor) and dnIκBα (dominant negative mutant of IκBα) effectively blocked ORF3a-mediated NF-κB activation, suggesting that ORF3a functions in the upstream levels of IκBα. Co-immunoprecipitation experiments indicated that ORF3a interacted with IKKβ and NEMO. Further studies suggested that ORF3a enhanced the association of IKKβ-NEMO. These results suggest that ORF3a positively regulates NF-κB activity by enhancing IKKβ-NEMO interaction.

With the discovery of the ORF3a protein in 2003, it has been considered to play vital roles in viral pathogenesis (Lu et al., 2010; Zhang et al., 2022a). The ORF3 protein from SARS-CoV, MERS-CoV and SARS-CoV-2 can induce apoptosis in cells (Freundt et al., 2010; Law et al., 2005; Ren et al., 2020; Zhou et al., 2022). SARS-CoV ORF3a interacts with Receptor Interacting Protein 3 (Rip3), causing necrotic cell death and lysosomal damage (Yue et al., 2018). In addition, the ORF3a protein of SARS-CoV activates NLRP3 inflammasome by targeting TRAF3 and ASC to promote TRAF3-mediated ubiquitination of ASC (Siu et al., 2019). The ability of SARS-CoV-2 ORF3a to induce proinflammatory response has also been descried. SARS-CoV-2 ORF3a causes RETREG1/FAM134B-related reticulophagy, triggering ER (endoplasmic reticulum) stress and inflammatory responses during SARS-CoV-2 infection (Zhang et al., 2022b). Moreover, SARS-CoV-2 ORF3a activates the NLRP3 inflammasome with both ASC-dependent and -independent manner (Xu et al., 2022).

Here, we demonstrated that SARS-CoV-2 ORF3a targets IKKβ and NEMO to induce proinflammatory response. Interestingly, recent studies uncovered that, besides NLRP3 priming, IKKβ plays important roles in NLRP3 activation via NF-κB independent way (Schmacke et al., 2022; Unterreiner et al., 2021). Based on these results, it is possible that ORF3a enhances association of IKKβ-NEMO and then increases the activity of IKKβ to activate NF-κB and also NLRP3 inflammasome, inducing robust proinflammatory cytokines. Our current findings may provide potential therapeutic targets for the treatment of COVID-19.

## Authors' contributions

YN, LMM and JHW conceived and designed the study. YN, LMM, QZL, DQD, RYH, and JZC carried out the experiments. YN, LMM, and

JHW analyzed and interpreted the data. YN, LMM and JHW wrote and revised the manuscript. All authors read and approved the final manuscript.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2023.199086.

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