

## ORIGINAL RESEARCH

## RAGE Is a Receptor for SARS-CoV-2 N Protein and Mediates N Protein–induced Acute Lung Injury

Jie Xia<sup>1\*</sup>, Jiangmei Wang<sup>1\*</sup>, Liyang Ying<sup>1</sup>, Ruoqiong Huang<sup>1</sup>, Kai Zhang<sup>2</sup>, Ruoyang Zhang<sup>1</sup>, Wenqi Tang<sup>1</sup>, Qi Xu<sup>3</sup>, Dengming Lai<sup>1</sup>, Yan Zhang<sup>1</sup>, Yaoqin Hu<sup>1</sup>, Xiaodie Zhang<sup>1</sup>, Ruoxi Zang<sup>1</sup>, Jiajie Fan<sup>1</sup>, Qiang Shu<sup>1‡</sup>, and Jianguo Xu<sup>1‡</sup>

<sup>1</sup>The Children's Hospital of Zhejiang University School of Medicine and National Clinical Research Center for Child Health, Hangzhou, China; <sup>2</sup>The First Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou, China; and <sup>3</sup>Hangzhou Medical College, Hangzhou, China

## Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) nucleocapsid protein (N-protein) increases early in body fluids during infection and has recently been identified as a direct inducer for lung injury. However, the signal mechanism of N-protein in the lung inflammatory response remains poorly understood. The goal of this study was to determine whether RAGE (receptor for advanced glycation endproducts) participated in N-protein–induced acute lung injury. The binding between N-protein and RAGE was examined via assays for protein–protein interaction. To determine the signaling mechanism *in vitro*, cells were treated with recombinant N-protein and assayed for the activation of the RAGE/MAPK (mitogen-activated protein kinase)/NF- $\kappa$ B pathway. RAGE deficiency mice and antagonist were used to study N-protein–induced acute lung injury *in vivo*. Binding between N-protein and RAGE was

confirmed via flow cytometry–based binding assay, surface plasmon resonance, and ELISA. Pull-down and coimmunoprecipitation assays revealed that N-protein bound RAGE via both N-terminal and C-terminal domains. *In vitro*, N-protein activated the RAGE-ERK1/2–NF- $\kappa$ B signaling pathway and induced a proinflammatory response. RAGE deficiency subdued N-protein–induced proinflammatory signaling and response. *In vivo*, RAGE was upregulated in the BAL and lung tissue after recombinant N-protein insult. RAGE deficiency and small molecule antagonist partially protected mice from N-protein–induced acute lung injury. Our study demonstrated that RAGE is a receptor for N-protein. RAGE is partially responsible for N-protein–induced acute lung injury and has the potential to become a therapeutic target for treating coronavirus disease.

**Keywords:** SARS-CoV-2; receptor for advanced glycation endproducts; nucleocapsid protein; receptor; acute lung injury

The coronavirus disease (COVID-19) pandemic, caused by infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has resulted in profound public health and socioeconomic consequences around the globe (1). Acute lung injury and acute respiratory distress syndrome (ARDS) are the most serious and

frequent complications of COVID-19. The mortality rate of COVID-19 ARDS is estimated to be ~45%, whereas the incidence of ARDS among COVID-19 nonsurvivors is ~90% (2). Both classic and COVID-19 ARDS are characterized by increased endothelial permeability, infiltration of inflammatory cells, and elevated

proinflammatory cytokines, such as IL-6, IL-8, and TNF- $\alpha$  (3).

SARS-CoV-2 is an enveloped, positive-sense, single-stranded RNA virus with a genome size of ~30,000 nucleotides. The virion envelope is anchored with three of the four structural proteins: the spike (S), the membrane (M), and the envelope (E)

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\*These authors contributed equally to this work.

‡Co-senior authors.

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Correspondence and requests for reprints should be addressed to Qiang Shu, M.D., Department of Thoracic and Cardiovascular Surgery, The Children's Hospital of Zhejiang University School of Medicine, 3333 Binsheng Road, Hangzhou, Zhejiang 310052, China. E-mail: shuqiang@zju.edu.cn.

This article has a related editorial.

This article has a data supplement, which is accessible from this issue's table of contents at [www.atsjournals.org](http://www.atsjournals.org).

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## Clinical Relevance

RAGE (receptor for advanced glycation endproducts) is a receptor for the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) N protein. RAGE is partially responsible for N protein-induced acute lung injury and has the potential to become a therapeutic target for treating coronavirus disease (COVID-19).

proteins (4). The fourth, the nucleocapsid protein (N-protein), is located inside of the viral envelope. It packages the RNA genome into a helical ribonucleoprotein complex enclosed within the capsid, which is essential for viral replication and infection (5). The N-protein is composed of two RNA-binding domains, the N-terminal domain (NTD) and the C-terminal domain (CTD), which are connected by a Ser/Arg-rich linker region (LR) (6). Several studies have documented that SARS-CoV-2 N-protein is directly involved in lung inflammation and acute lung injury. Gao and colleagues reported that N-protein aggregated LPS-induced lung injury via binding to MASP-2, leading to complement activation (7). Pan and colleagues found that N-protein boosted NLRP3 inflammasome activation to induce lung inflammation (8). Our results showed that recombinant N-protein induced acute lung injury in mice via NF- $\kappa$ B activation (9). However, the upstream mechanism of NF- $\kappa$ B activation is unknown.

One of the upstream mediators of NF- $\kappa$ B activation is RAGE (receptor for advanced glycation endproducts). RAGE is a transmembrane glycoprotein and belongs to the immunoglobulin superfamily. In addition to binding with advanced glycation endproducts, RAGE is also a receptor for many other ligands, such as LPS, the family of S100 proteins, and HMGB1 (high mobility group box 1) (10). Compared with other tissues, RAGE is most highly expressed in the lung, particularly in the alveolar type I cells, type II cells, and macrophages (11, 12). A potential role of RAGE in the pathogenesis of COVID-19 has been proposed by several groups (13). However, the role of RAGE in N-protein-induced acute lung injury has not been reported. In the present study, we aimed to examine whether RAGE is

a receptor for N-protein and determine the consequent impact on acute lung injury of N-protein-RAGE interaction.

## Methods

Detailed methods are provided in the data supplement.

### Plasmids and Fusion Proteins

N-protein NTD (1–174 AA), LR (175–246 AA), and CTD (247–419 AA) constructs were amplified from a full-length N-protein cDNA via PCR, confirmed via sequencing, and subcloned into the pcDNA3.1-GFP plasmid in frame for mammalian expression. The human RAGE cDNA construct was amplified from a full-length RAGE cDNA via PCR, confirmed via sequencing, and subcloned into the pcDNA3.1-Flag plasmid. The construct for full-length pET28a-His-N-protein for bacterial expression was generously provided by Dr. Feng Cong from Guangdong Laboratory Animals Monitoring Institute. Recombinant His-protein constructs for NTD, LR, and CTD were amplified from an N-protein cDNA via PCR, confirmed via sequencing, and inserted into the pET28a vector. Recombinant RAGE protein was purchased from SinoBiological.

### Preparation of Recombinant N-Protein

The proteins were purified from bacterial lysate via nickel affinity chromatography (BBI Life Sciences) and dialyzed to remove extra salt and imidazole. To remove the contaminated endotoxin, the recombinant proteins were passed through a column from ToxinEraser Endotoxin Removal Kit (GenScript). The endotoxin levels were  $<0.1$  EU/ $\mu$ g protein ( $<0.01$  ng/ $\mu$ g) as determined by the ToxinSensor Chromogenic LAL Endotoxin Assay Kit (GenScript). To neutralize the possible protein-bound LPS, recombinant proteins were pretreated with polymyxin B (250  $\mu$ g/ml, MilliporeSigma) for 1 hour at room temperature before all *in vitro* and *in vivo* experiments.

### Flow Cytometry-based Binding Assay

Cells were detached by cell scrapers and rinsed with flow cytometry buffer (PBS, 0.5% BSA) to block nonspecific binding. Cells were then incubated with His-tagged full-length N-protein (5  $\mu$ g/ml) at 37 °C for 1 hour. Cells were washed with flow cytometry buffer three times for

3  $\times$  5 minutes and incubated with an Alexa Fluor 488-conjugated anti-His antibody (Biolegend) diluted 1:100 for 30 minutes. Then, cells were rinsed, detected by a BD LSRFortessa flow cytometer, and analyzed using FlowJo V10 software.

### Surface Plasmon Resonance

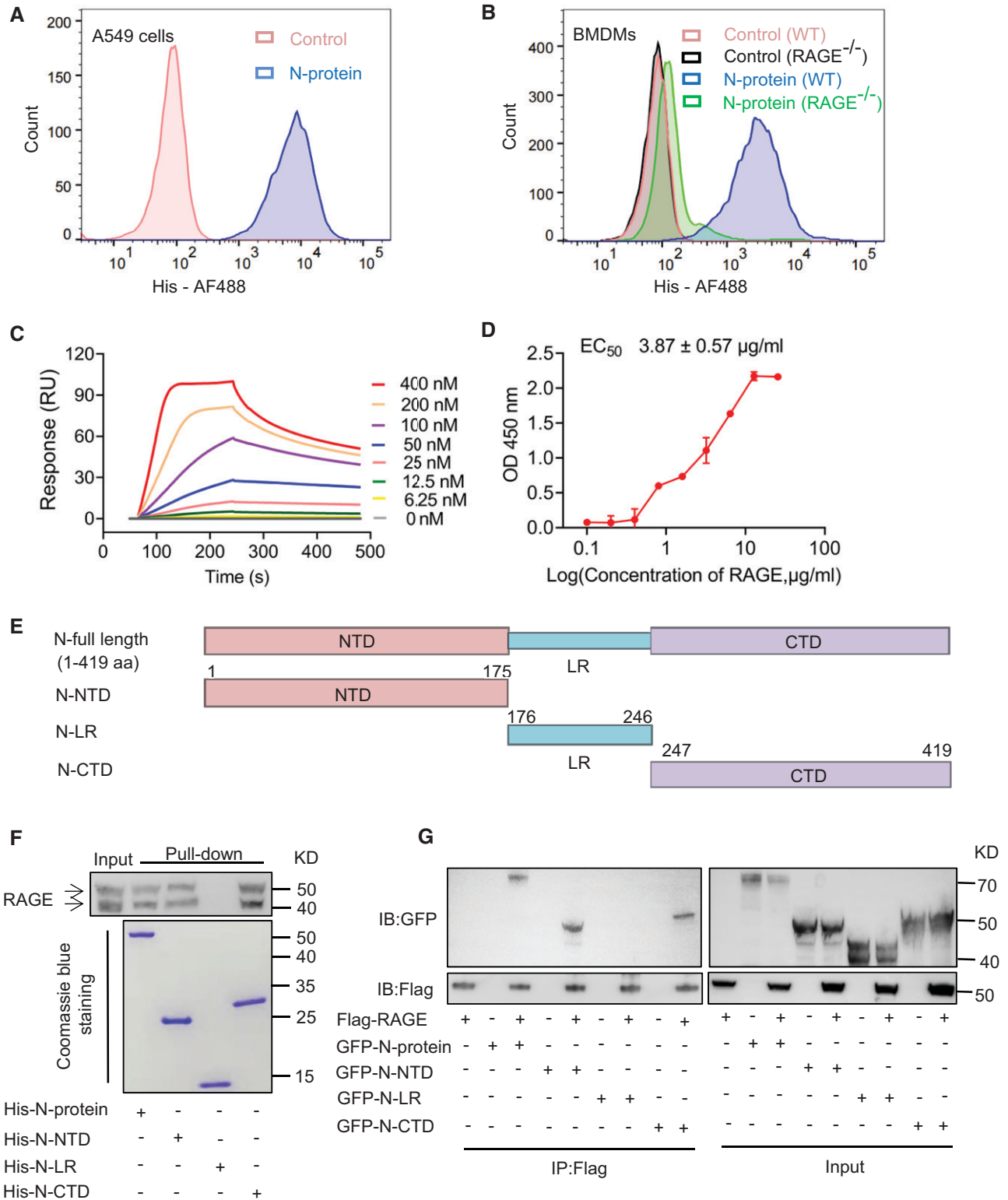
Surface plasmon resonance was performed using a Biacore 3000 system (Cytiva). N-protein was diluted in PBS to generate a series of concentrations (6.25–400 nM). After immobilizing RAGE at the 100 resonance unit on the surface of a CM5 sensor chip (Cytiva), N-protein samples were injected for 4 minutes across the sensor surface at a flow rate of 20  $\mu$ l/min. At the end of the sample injection, PBS was flowed over the sensor surface for 4 minutes to promote dissociation. The response was monitored as a function of time at room temperature. Sensorgrams were obtained by subtraction of the zero concentration curve. The binding data were fitted to a 1:1 binding model using BLAEvaluation software (Cytiva).

### N-Protein-induced Lung Injury Model

C57BL/6 mice were acquired from the Shanghai Laboratory Animal Center. RAGE knockout mice with C57BL/6 background were generated by the Nanjing Biomedical Research Institute of Nanjing University via CRISPR/Cas9 technology (Project number XM709828). Animal experimental protocols were approved by the Institutional Animal Care and Use Committee at Zhejiang University School of Medicine. Mice were anesthetized with an injection of phenobarbital (50 mg/kg) intraperitoneally. Acute lung injury was induced via intratracheal instillation of recombinant full-length N-protein (75  $\mu$ g per mouse in 50  $\mu$ l) as reported previously (9). To study the effect of RAGE inhibition on N-protein-induced lung injury, mice were subjected to intraperitoneal injection of RAGE antagonist RAP (50 or 100  $\mu$ g/mouse) 1 hour before N-protein administration. Based on our experience, the optimal time point for assessing N-protein-induced lung injury was 24 hours after insult. Serum, lung, and BAL samples were harvested at 24 hours from mouse groups (48 h for Figure E1 in the data supplement).

### Western Blot

Antibodies for phosphor-ERK1/2 and ERK1/2 were purchased from Abcam. Antibodies for phosphor-JNK1/2, JNK1/2,



**Figure 1.** N-protein bound to RAGE (receptor for advanced glycation endproducts). (A) Detached A549 cells were incubated with or without His-tagged full-length N-protein under unpermeabilized conditions for 1 hour and washed with PBS + 0.5% BSA three times. Binding of N-protein to A549 cells was measured by flow cytometry using an Alexa Fluor 488–conjugated anti-His antibody. (B) Detached bone marrow–derived macrophages (BMDMs) from RAGE<sup>-/-</sup> and wild-type (WT) C57BL/6 mice were incubated with or without His-tagged full-length N-protein under unpermeabilized conditions for 1 hour and washed with PBS + 0.5% BSA three times. Binding of N-protein to BMDMs was measured by flow cytometry using an Alexa Fluor 488–conjugated anti-His antibody. (C) Surface plasmon resonance sensorgrams show the binding of

phosphor-p38, p38, phosphor-NF- $\kappa$ B p65, and NF- $\kappa$ B p65 were purchased from Cell Signaling Technology. Equal amounts of cell lysates (30  $\mu$ g) were separated on 12% SDS-PAGE gels, transferred via polyvinylidene difluoride membranes, blocked in TRIS-buffered saline with Tween-20 containing 5% nonfat milk (blot buffer), and probed with the primary antibodies. After washing with blot buffer, membranes were incubated with specific secondary antibodies linked to horseradish peroxidase. Signal detection was conducted using an enzyme-linked chemiluminescence kit. Blot images were analyzed by ImageJ software.

### Statistical Methods

Data are presented as mean  $\pm$  SD. Data normality was examined using the D'Agostino-Pearson Omnibus normality test, the Shapiro-Wilk normality test, or the Kolmogorov-Smirnov test with Dallal-Wilkinson-Lilliefors-corrected *P* value. Student's *t* test, one-way ANOVA with Tukey's *post hoc* test, or two-way ANOVA with Tukey's *post hoc* test was conducted for parametric values. In data sets with nonparametric values (Figure 2F, and TNF- $\alpha$  of Figure 3B), the Kruskal-Wallis test followed by Dunn's *post hoc* test was performed for analysis. Statistical analysis was conducted using the GraphPad Prism 8.0.2. Results were deemed significant if *P* < 0.05.

## Results

### RAGE Is a Receptor for N-Protein

RAGE is constitutively expressed in A549 human alveolar epithelial cells (14). The first experiment was to test whether recombinant full-length N-protein could bind to the surface of A549 cells using a flow cytometry-based binding assay. Detached cells were incubated with or without His-

tagged N-proteins and then analyzed by flow cytometry after incubation with an anti-His antibody. The results suggested that N-protein bound with surface molecules of A549 cells (Figure 1A). To determine whether N-protein could specifically bind with RAGE on the cell surface, bone marrow-derived macrophages (BMDMs) were isolated from RAGE knockout mice with C57BL/6 background (RAGE<sup>-/-</sup>) and wild-type (WT) C57BL/6 mice. We observed that full-length N-protein bound BMDMs from WT mice, whereas the binding was substantially reduced in BMDMs from RAGE<sup>-/-</sup> mice (Figure 1B). Surface plasmon resonance analysis demonstrated that N-protein had a strong binding affinity with RAGE, with an equilibrium dissociation constant (*K<sub>d</sub>*) of 34.5  $\pm$  10.6 nM (Figure 1C). It has been documented that the binding between the receptor binding domain of the SARS-CoV-2 S-protein and ACE2 (angiotensin-converting enzyme 2) has a *K<sub>d</sub>* in the range of 6–133 nM (15). To demonstrate that RAGE possesses potent binding with N-protein, N-protein was coated on plates, and the binding of various concentrations of RAGE was assayed via ELISA. The half-maximal effective concentration (EC<sub>50</sub>) was 3.87  $\pm$  0.57  $\mu$ g/ml for N-protein (Figure 1D). Lopez and colleagues reported that ACE2 bound with the receptor binding domain of SARS-CoV2 S-protein with a half-maximal effective concentration value of 13.5  $\mu$ g/ml (16).

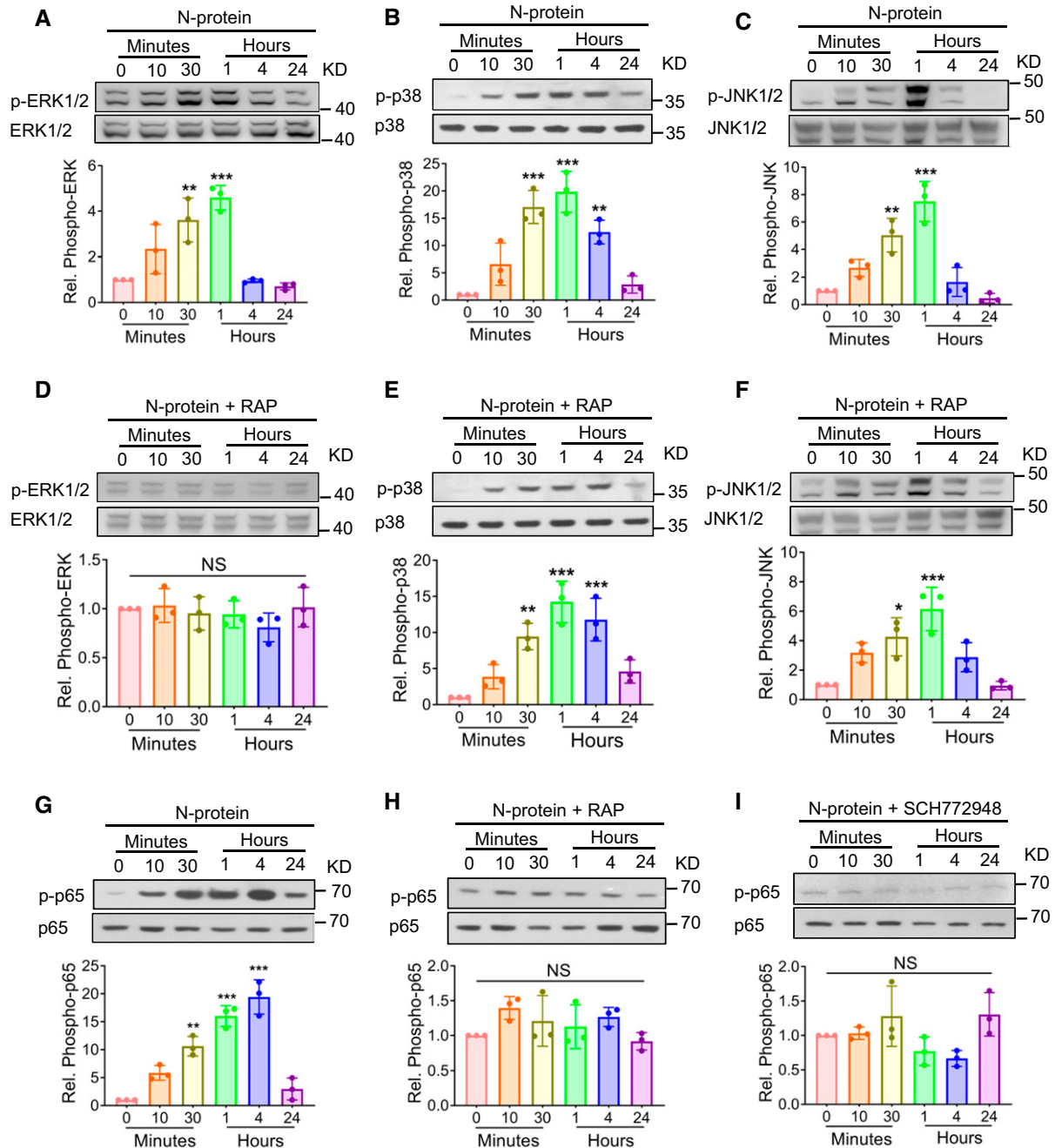
To provide more evidence for N-protein and RAGE association, His-tagged proteins expressing full-length, NTD (N-NTD), LR (N-LR), and CTD (N-CTD) (Figure 1E) were used in a pull-down assay of mouse lung lysate. Full-length N-protein, N-NTD, and N-CTD were able to pull down a considerable amount of RAGE, as detected by Western blot, whereas N-LR did not show binding activity (Figure 1F). To confirm the physical association of N-protein and RAGE

in a cellular context, constructs of GFP-N-protein and Flag-RAGE were expressed in HEK-293 cells either alone or in combination via transient transfection. When cell lysates were immunoprecipitated with an anti-FLAG antibody, the cotransfected full-length N-protein, N-NTD, and N-CTD, but not N-LR, were robustly coimmunoprecipitated (Figure 1G). These results indicate that N-protein has two binding sites for RAGE, one at the CTD and the other at the NTD. Similarly, it has been reported that HMGB1 has two RAGE-binding domains (17).

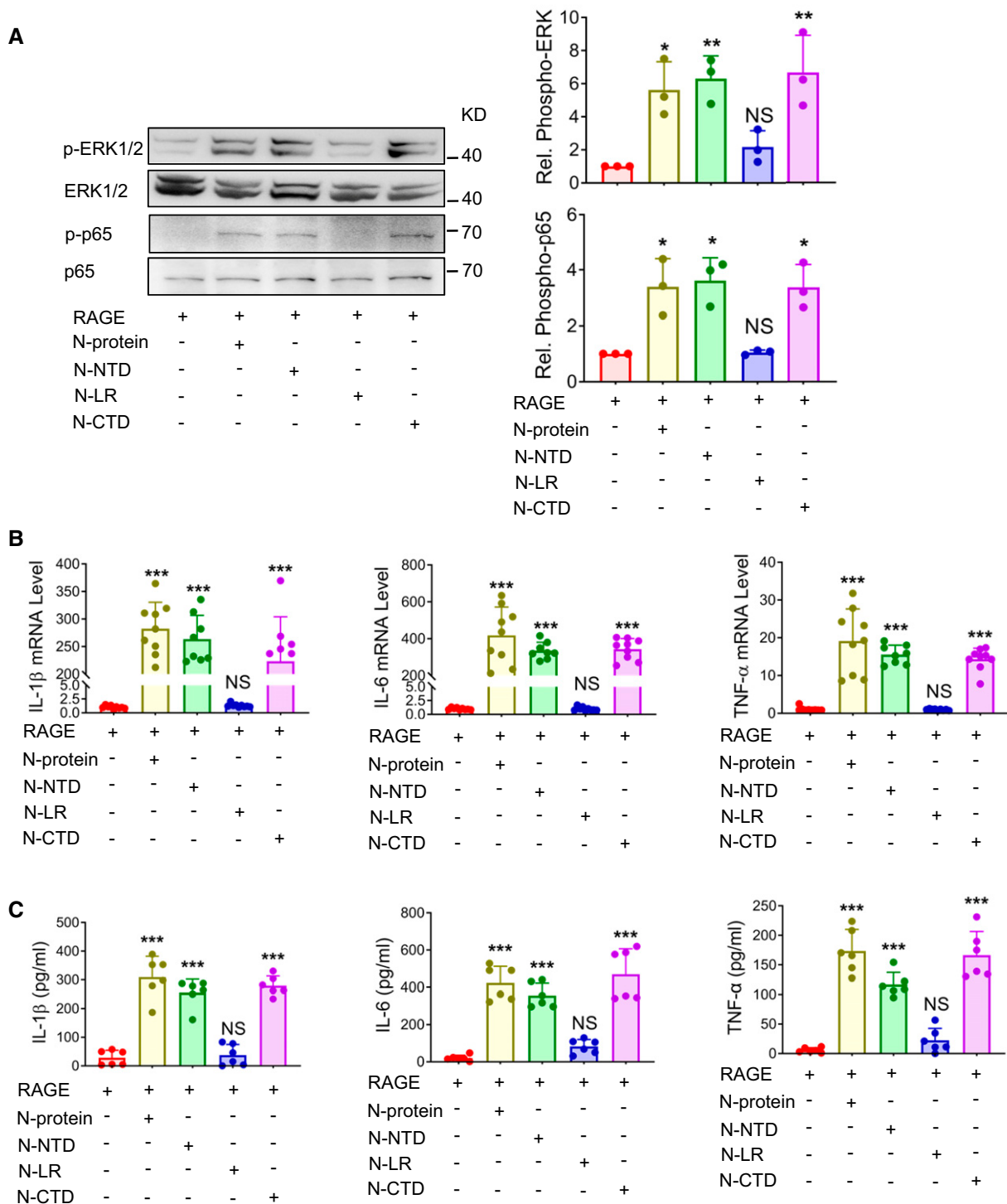
### N-Protein Activates the RAGE-ERK1/2-NF- $\kappa$ B Pathway

Previously, our group reported that recombinant N-protein induced lung injury and NF- $\kappa$ B activation, independent of endotoxin (9). To investigate the upstream signaling mechanism of NF- $\kappa$ B activation, RAW 264.7 macrophages were treated with recombinant full-length N-protein for a series of time points. Cell lysates were examined for the phosphorylation of MAPK (mitogen-activated protein kinase; ERK1/2, p38 MAPK, and JNK1/2) via Western blot analysis. The results showed that phosphorylation of ERK1/2, p38 MAPK, and JNK1/2 was elevated in a time-dependent manner, reaching a peak between 30 minutes and 1 hour (Figures 2A–2C). Pretreatment of cells with RAGE antagonist (RAP, 20  $\mu$ M) blocked N-protein-induced ERK1/2 activation, indicating that RAGE is upstream of ERK1/2 (Figure 2D). The phosphorylation of p38 MAPK and JNK1/2 evoked by N-protein was unaltered by RAGE antagonist, suggesting that the activation of p38 MAPK and JNK1/2 is independent of RAGE (Figures 2E and 2F). This phenomenon is similar to a previous report that S100B increased the phosphorylation of p38 MAPK and JNK1/2 independent of RAGE (18). Compared with the phosphorylation pattern of MAPK

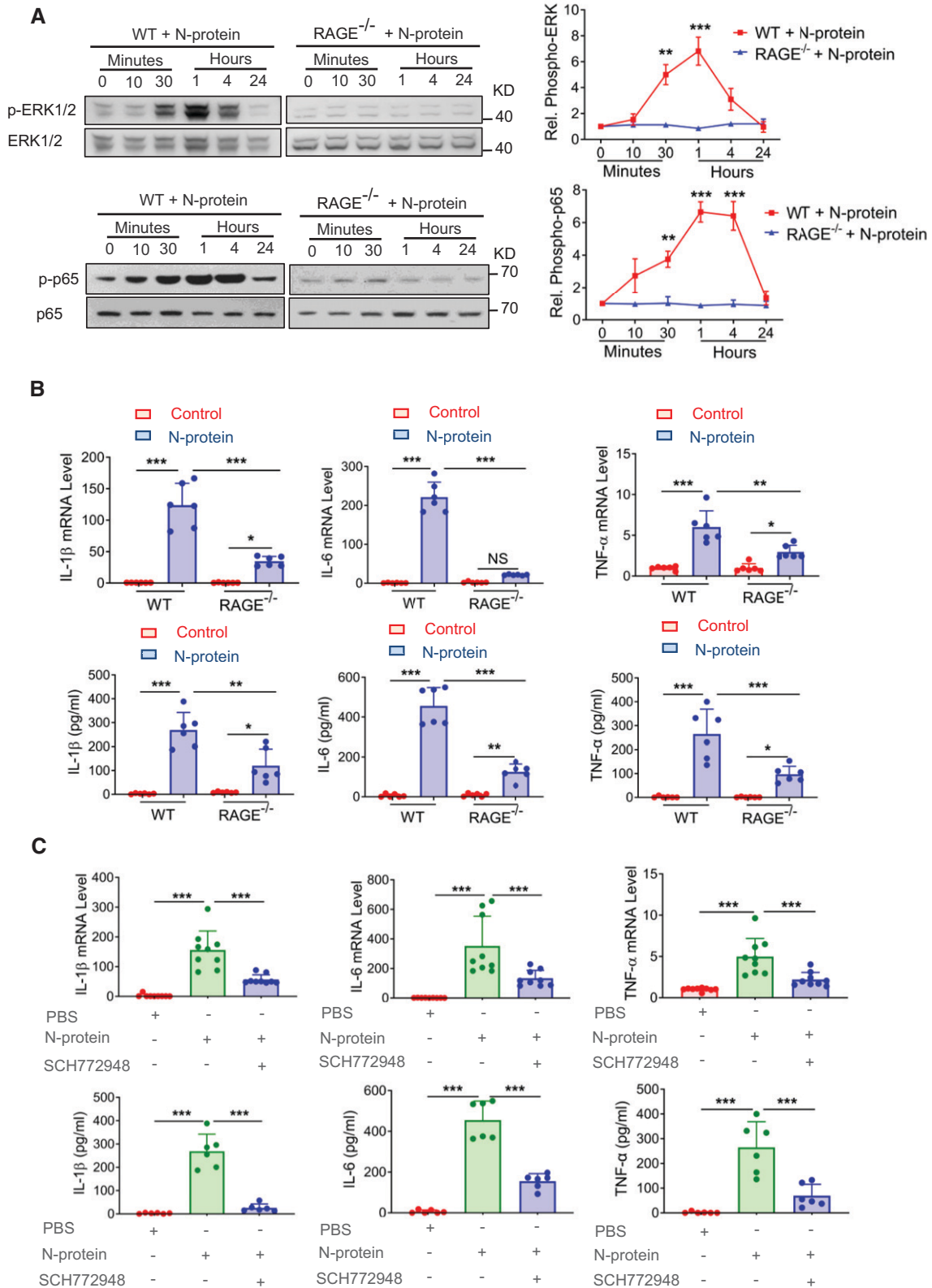
**Figure 1.** (Continued). immobilized RAGE to N-protein at concentrations of 400, 200, 100, 50, 25, 12.5, and 6.25 nM. The data were fitted to the one-to-one kinetic model. (D) Recombinant RAGE of different concentrations (0.1–25.6  $\mu$ g/ml) was applied to ELISA plates coated with N-protein. Bound RAGE was detected using a rabbit anti-RAGE antibody followed by a goat anti-rabbit secondary antibody conjugated to horseradish peroxidase. After adding substrate, OD was measured at 450 nm and normalized against the negative control. Data are presented as mean  $\pm$  SD. (E) A schematic diagram shows full-length and truncated N-proteins. (F) Lung lysates were prepared from C57BL/6 mice and pulled down with His-tagged full-length N-protein, N-N-terminal domain (N-NTD), N-C-terminal domain (N-CTD), or N-linker region (N-LR). The lysates and pull-down products were examined via Western blot using an anti-RAGE antibody. The two bands detected in the pull-down assay represent the RAGE protein before and after glycosylation (upper panel). Bait proteins were visualized with Coomassie staining (lower panel). (G) Cell lysates from HEK 293 cells expressing FLAG-RAGE and GFP-N-proteins of full-length, N-NTD, N-CTD, or N-LR were subjected to immunoprecipitation using an anti-FLAG antibody. The cell lysates and immunoprecipitation fractions were examined by Western blot using an anti-GFP or anti-Flag antibody. Data shown are representative of three independent experiments. EC<sub>50</sub> = half-maximal effective concentration; OD = optical density; RU = resonance unit.



**Figure 2.** N-protein prompted ERK-mediated NF- $\kappa$ B phosphorylation via RAGE. (A–C) RAW 264.7 cells were treated with recombinant full-length N-protein (5  $\mu$ g/ml) for the indicated times. Cell lysates were analyzed via Western blot using antibodies specific to phosphorylated (upper panels) or total (lower panels) ERK1/2 (A), p38 MAPK (mitogen-activated protein kinase) (B), or JNK1/2 (C). (D–F) RAW 264.7 cells were pretreated with RAGE antagonist (RAP, 20  $\mu$ M) for 1 hour and then incubated with recombinant full-length N-protein (5  $\mu$ g/ml) for the indicated times. Cell lysate was examined via Western blot using specific antibodies to detect phosphorylation of ERK1/2 (D), p38 MAPK (E), or JNK1/2 (F). (G and H) RAW 264.7 cells were pretreated without (G) or with (H) RAGE antagonist RAP (20  $\mu$ M) for 1 hour and then stimulated with recombinant full-length N-protein (5  $\mu$ g/ml) for the indicated times. Cell lysate was assessed via Western blot using antibodies specific to phospho-NF- $\kappa$ B p65 (upper panel) or total NF- $\kappa$ B p65 (lower panel). (I) RAW 264.7 cells were pretreated with ERK inhibitor (SCH772948, 500 nM) for 1 hour and then stimulated with recombinant full-length N-protein (5  $\mu$ g/ml) for the indicated times. Cell lysate was examined via Western blot using antibodies specific to phospho-NF- $\kappa$ B p65 (upper panel) or total NF- $\kappa$ B p65 (lower panel). N-protein for all groups was pretreated with polymyxin B (250  $\mu$ g/ml) for 1 hour at room temperature before all experiments to neutralize endotoxin. The band density of phosphorylation was quantified with ImageJ software, normalized to that of total protein, and expressed as relative fold change compared with time 0. Data are presented as mean  $\pm$  SD,  $n=3$ . \* $P<0.05$ , \*\* $P<0.01$ , and \*\*\* $P<0.001$  versus time 0. One-way ANOVA with Tukey's *post hoc* test (A–E, G–I) or Kruskal-Wallis test with Dunn's *post hoc* test (F) was used for the analysis.  $n$  = number of independent experiments; NS = not significant.



**Figure 3.** N-NTD and N-CTD induced proinflammatory signaling and immune response. (A) HEK293 cells were transiently transfected with RAGE for 24 hours and treated with recombinant N-proteins expressing full-length, N-NTD, N-CTD, or N-LR (5  $\mu$ g/ml) for 30 minutes. Cell lysates were analyzed via Western blot using antibodies specific to phosphorylated (upper panels) or total (lower panels) ERK1/2 or NF- $\kappa$ B p65. The levels of phosphorylation were quantified with ImageJ software ( $n=3$ ). (B and C) HEK293 cells were transiently transfected with RAGE for 24 hours and treated with recombinant proteins expressing full-length, NTD, CTD, or LR of N-protein (5  $\mu$ g/ml) for 24 hours. (B) The mRNA expression of proinflammatory cytokines IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in HEK293 cells was quantitated by qRT-PCR ( $n=8-9$ ). (C) Culture supernatants were analyzed by ELISA for IL-1 $\beta$ , IL-6, and TNF- $\alpha$  ( $n=6$ ). Data are presented as mean  $\pm$  SD. \* $P<0.05$ , \*\* $P<0.01$ , and \*\*\* $P<0.001$  versus no N-protein treatment. One-way ANOVA with Tukey's *post hoc* test (A, B, and C, except TNF- $\alpha$  in B) or Kruskal-Wallis test with Dunn's *post hoc* test (TNF- $\alpha$  in B) was used for the analysis.



induced by N-protein (Figures 2A–2C), NF- $\kappa$ B p65 phosphorylation lasted longer, reaching a peak between 1 and 4 hours (Figure 2G). Similar to that of ERK1/2, N-protein-induced NF- $\kappa$ B p65 phosphorylation was blocked by RAGE antagonist (Figure 2H). Furthermore, ERK1/2 inhibition (SCH772948, 500 nM) abolished the effect of N-protein on NF- $\kappa$ B activation, suggesting ERK1/2 is upstream of NF- $\kappa$ B (Figure 2I). These findings support that N-protein induces signaling through RAGE-dependent activation of the ERK1/2–NF- $\kappa$ B pathway.

### N-NTD and N-CTD Mimic Full-Length N-Protein in Signaling and Inflammatory Response

It has been reported that HEK 293 cells do not express RAGE or express low levels of RAGE (19). To examine whether N-NTD and N-CTD could promote RAGE-mediated signaling, HEK 293 cells were transfected with Flag-tagged RAGE and stimulated without or with recombinant proteins expressing full-length N-protein, N-NTD, N-CTD, or N-LR (5  $\mu$ g/ml) for 30 minutes. Without stimulation, the phosphorylation of ERK1/2 and NF- $\kappa$ B p65, as detected by Western blot, was almost undetectable. Treatment with full-length N-protein, N-NTD, and N-CTD, but not N-LR, elevated the phosphorylation of ERK1/2 and NF- $\kappa$ B p65 (Figure 3A). To study whether N-NTD and N-CTD could enhance the RAGE-mediated inflammatory response, transfected HEK293 cells were stimulated without or with recombinant proteins expressing full-length, N-NTD, N-CTD, or N-LR (5  $\mu$ g/ml) for 24 hours. Full-length N-protein, N-NTD, and N-CTD significantly elevated both mRNA expression (Figure 3B) and cellular release (Figure 3C) of proinflammatory cytokines IL-1 $\beta$ , IL-6, and TNF- $\alpha$ , as determined by qRT-PCR and ELISA, respectively.

### RAGE Deficiency Reduces N-Protein-induced ERK1/2–NF- $\kappa$ B Signaling and Proinflammatory Response

To further explore the role of RAGE in the N-protein-induced proinflammatory

pathway, BMDMs were isolated from RAGE<sup>-/-</sup> and WT C57BL/6 mice and treated with recombinant full-length N-protein for different time points. Compared with WT BMDMs, phosphorylation of ERK1/2 and NF- $\kappa$ B p65 was abolished in RAGE<sup>-/-</sup> BMDMs in response to N-protein (Figure 4A). Compared with WT, RAGE deficiency significantly decreased the effect of N-protein on mRNA expression and cellular release of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  at 24 hours (Figure 4B). However, RAGE deficiency did not totally wipe out the proinflammatory effect of N-protein. To further support the role of ERK1/2 in regulation of the N-protein-induced proinflammatory response, WT BMDMs were treated with ERK1/2 inhibitor (SCH772948, 500 nM) before N-protein insult. ERK1/2 inhibition significantly inhibited the mRNA expression and cellular release of proinflammatory cytokines IL-1 $\beta$ , IL-6, and TNF- $\alpha$  at 24 hours (Figure 4C). Taken together, these findings indicate that RAGE–ERK1/2–NF- $\kappa$ B pathway is essential in the N-protein-induced proinflammatory response.

### RAGE Deficiency and Inhibition Alleviate N-Protein-induced Acute Lung Injury

Previously, our group reported that recombinant N-protein-induced lung injury was dose dependent. Intratracheal administration of a low dose of N-protein (3  $\mu$ g/mouse) failed to trigger an acute lung injury at 24 hours, whereas a high dose (75  $\mu$ g/mouse) had a more pronounced effect than a medium dose (15  $\mu$ g/mouse) (9). To study whether N-protein induces RAGE expression *in vivo*, WT C57BL/6 mice were treated intratracheally with recombinant full-length N-protein (75  $\mu$ g/mouse) or PBS. At 24 hours, N-protein insult significantly increased RAGE levels in the BAL and serum relative to the control in the ELISA assay (Figure 5A). The treatment also elevated the total RAGE mRNA levels in BAL cells (Figure 5B). In addition, lungs with N-protein insult showed extensive RAGE reactivity in immunohistochemistry

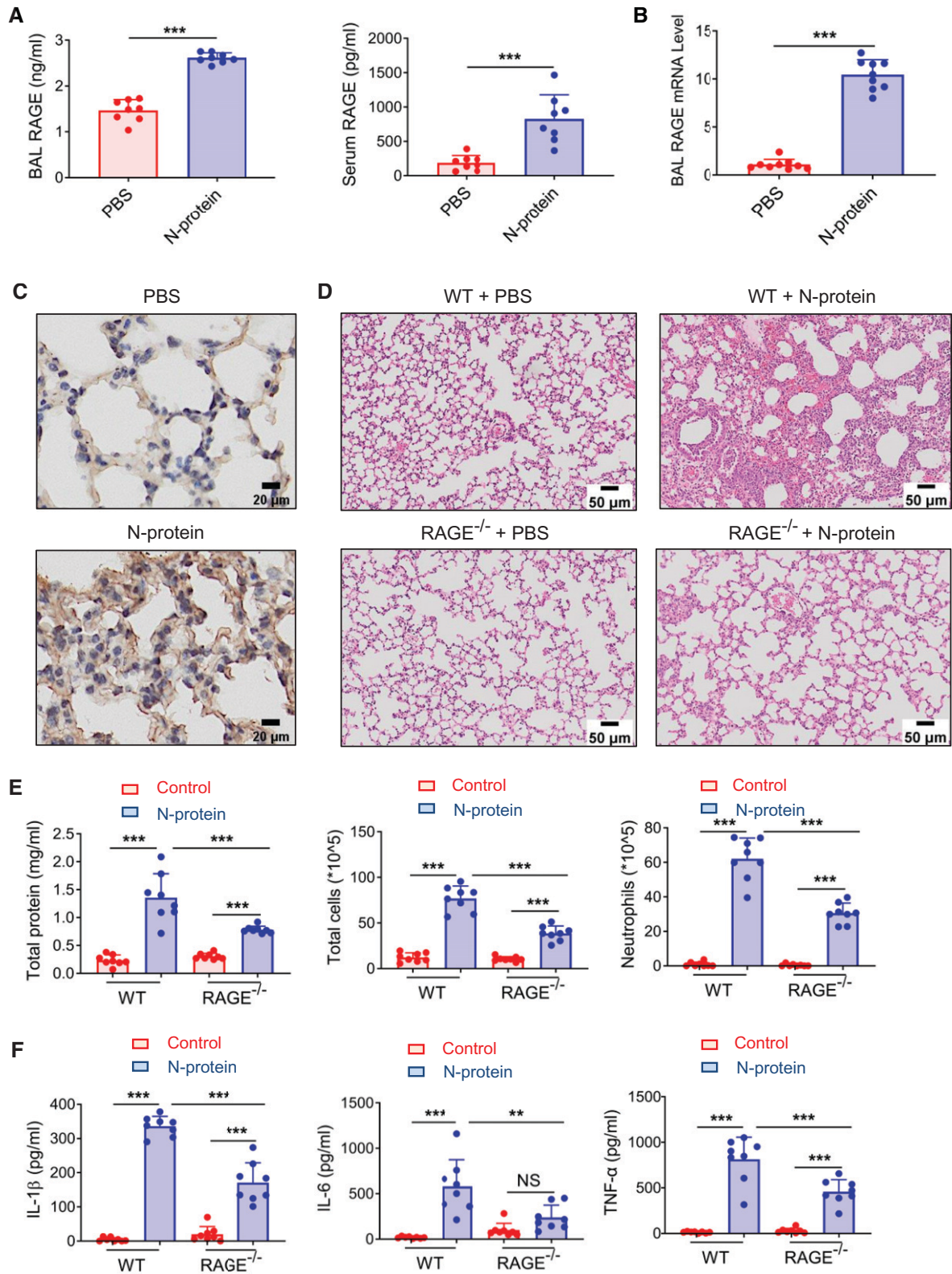
(Figure 5C), indicating that N-protein upregulates both membrane and soluble RAGE.

To examine the role of RAGE in N-protein-induced acute lung injury, RAGE<sup>-/-</sup> and WT C57BL/6 mice were treated intratracheally with recombinant full-length N-protein (75  $\mu$ g/mouse) or PBS. Lung samples and BAL were harvested at 24 hours. Treatment of N-protein elicited edema and higher cellularity in WT mice compared with control mice. However, inflammatory cell infiltration and septal thickening were remarkably less prominent in treated RAGE<sup>-/-</sup> mice compared with WT mice (Figure 5D). Total protein levels, the number of total cells, and the number of neutrophils in the BAL were significantly reduced (by 42.4%, 49.5%, and 50.8%, respectively) in treated RAGE<sup>-/-</sup> versus WT mice (Figure 5E). Proinflammatory cytokines, including IL-1 $\beta$ , IL-6, and TNF- $\alpha$ , in the BAL were substantially decreased (by 49.1%, 59.1%, and 43.7%, respectively) in treated RAGE<sup>-/-</sup> mice versus WT mice (Figure 5F). However, lung injury was not totally abolished in RAGE<sup>-/-</sup> mice compared with control mice (Figures 5E and 5F). Reduced lung injury in treated RAGE<sup>-/-</sup> versus WT mice was still observed at 48 hours after N-protein insult. However, increased protein leakage in BAL was no longer present in treated RAGE<sup>-/-</sup> mice versus their control mice (Figure E1). These results show that N-protein-induced acute lung injury is partially protected by RAGE deficiency. Using a lentivirus-mediated N-protein expression system, Pan and colleagues reported that N-protein mediated NLRP3 inflammasome activation and prompted hyperinflammation in the lung (8). Intraabdominal administration of the NLRP3 inhibitor MCC950 (10 mg/kg) alleviated lung injury induced by recombinant N-protein (Figure E2), indicating NLRP3 activation is also partially responsible for N-protein-induced acute lung injury.

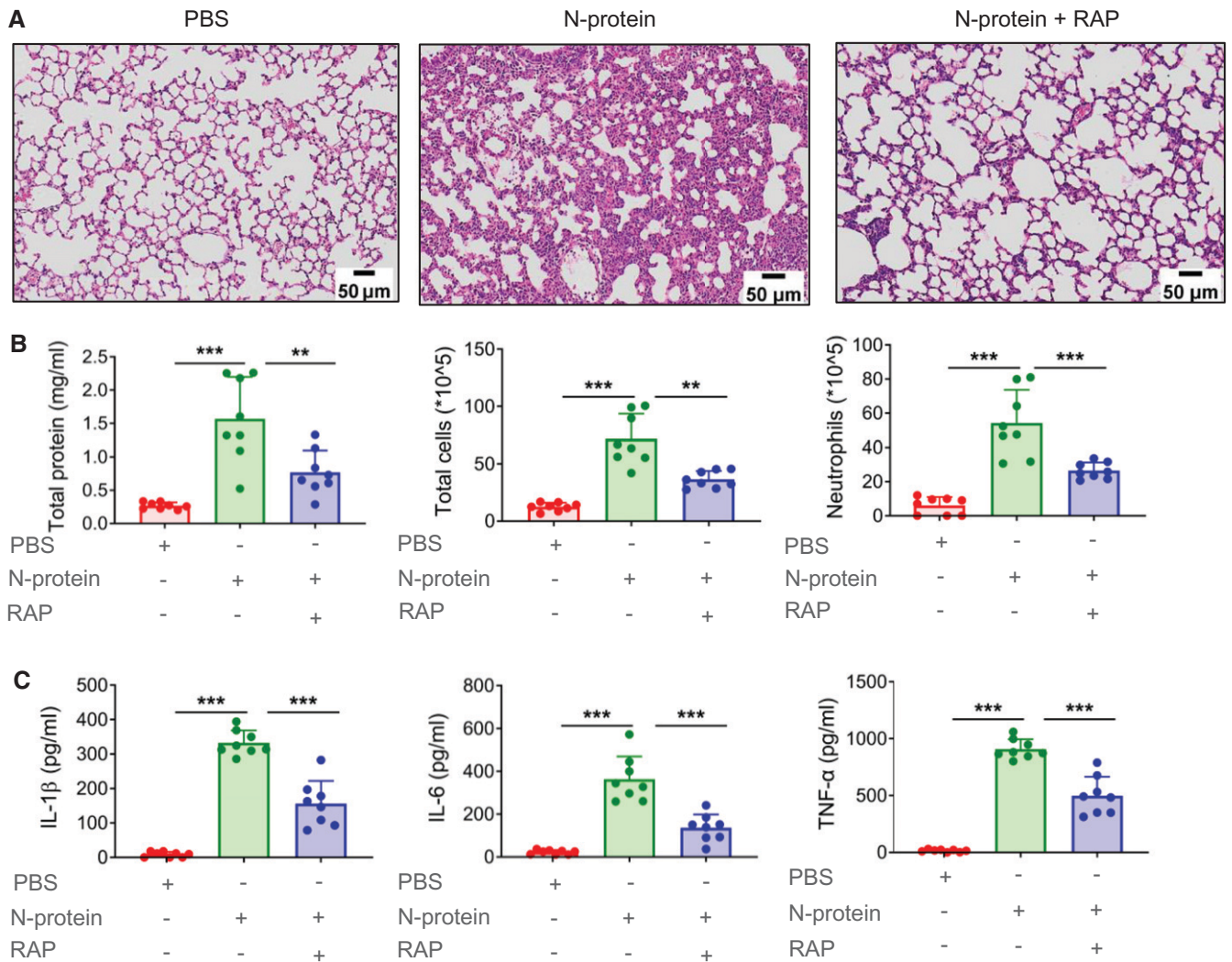
To determine the therapeutic potential of RAGE inhibition in N-protein-induced lung injury, C57BL/6 mice were treated with recombinant full-length N-protein

**Figure 4.** (Continued). expression of proinflammatory cytokines IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in BMDMs was quantitated by qRT-PCR (upper panel,  $n=6$ ). Culture supernatants were analyzed by ELISA for IL-1 $\beta$ , IL-6, and TNF- $\alpha$  (lower panel,  $n=6$ ). (C) BMDMs from WT mice were pretreated with ERK1/2 inhibitor (SCH772948, 500 nM) for 1 hour and then stimulated with recombinant N-protein (5  $\mu$ g/ml) for 24 hours. The mRNA expression of proinflammatory cytokines IL-1 $\beta$ , IL-6, and TNF- $\alpha$  was quantitated by qRT-PCR (upper panel,  $n=9$ ). Culture supernatants were analyzed by ELISA for IL-1 $\beta$ , IL-6, and TNF- $\alpha$  (lower panel,  $n=6$ ). Data are presented as mean  $\pm$  SD. \* $P<0.05$ , \*\* $P<0.01$ , and \*\*\* $P<0.001$ . One-way ANOVA with Tukey's *post hoc* test versus time 0 (A). Two-way ANOVA with Tukey's *post hoc* test (B). One-way ANOVA with Tukey's *post hoc* test (C).





**Figure 5.** RAGE mediated N-protein-induced acute lung injury. (A–C) WT C57BL/6 mice were administered intratracheally with His-tagged full-length N-protein (75  $\mu$ g/mouse in 50  $\mu$ l) or PBS. (A) BAL and serum samples were harvested at 24 hours. RAGE levels in the BAL and serum were determined by ELISA ( $n=8$ ). (B) Total RAGE mRNA expression in BAL cells was examined via qRT-PCR ( $n=9$ ). (C) RAGE expression in lung tissue was detected via immunohistochemistry ( $n=3$ ). Scale bars, 20  $\mu$ m. (D–F) RAGE<sup>-/-</sup> mice with C57BL/6 background and



**Figure 6.** RAGE antagonist reduced N-protein-induced acute lung injury. (A–C) WT C57BL/6 mice were treated with PBS, full-length N-protein (75  $\mu$ g/mouse), or N-protein + RAGE antagonist RAP (100  $\mu$ g/mouse, intraperitoneal injection 1 h before N-protein administration). Lung tissue and BAL samples were collected at 24 hours after N-protein insult. (A) Tissue sections were stained with hematoxylin and eosin ( $n=3$ ). Scale bars, 50  $\mu$ m. (B) Total protein, total cells, and neutrophils in the BAL were determined to evaluate lung injury ( $n=8$ ). (C) Levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in the BAL were analyzed via ELISA ( $n=8$ ). Data are presented as mean  $\pm$  SD. \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . One-way ANOVA with Tukey’s *post hoc* test (B and C).

intratracheally (75  $\mu$ g/mouse), N-protein + RAGE antagonist RAP (100  $\mu$ g/mouse intraperitoneally 1 h before N-protein insult), or PBS. RAGE inhibition alleviated the effect of N-protein on lung pathology (Figure 6A). It also significantly reduced total protein concentration (by 51.1%), total cell count (by 49.1%), and neutrophil infiltration (by 51.2%) in the

BAL (Figure 6B). Proinflammatory cytokines, including IL-1 $\beta$ , IL-6, and TNF- $\alpha$ , in the BAL were significantly decreased (by 53.1%, 62.6%, and 45.2%, respectively) by RAGE inhibition (Figure 6C). RAGE inhibition with a lower dose of RAP (50  $\mu$ g/mouse) had a similar effect as the 100- $\mu$ g dose in alleviating protein leakage in the BAL, while failing to reduce neutrophil

infiltration (Figure E3). Several studies have reported that RAGE ligands, such as S100A8 and S100A9, were upregulated in patients with COVID-19 (20). S100A8 and S100A9 were shown to exacerbate lung inflammation via neutrophil accumulation in tuberculosis (21). Our results revealed that N-protein had an effect similar to S100A8 and A9 in inducing acute lung

**Figure 5.** (Continued). WT C57BL/6 mice were treated with PBS or N-protein (75  $\mu$ g/mouse in 50  $\mu$ l) intratracheally. Lung tissue and BAL samples were collected at 24 hours after N-protein insult. (D) Tissue sections were stained with hematoxylin and eosin ( $n=3$ ). Scale bars, 50  $\mu$ m. (E) Total protein, total cells, and neutrophils in the BAL were assayed to evaluate lung injury ( $n=8$ ). (F) Levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in the BAL were determined via ELISA ( $n=8$ ). Data are presented as mean  $\pm$  SD. \*\* $P < 0.01$  and \*\*\* $P < 0.001$ . Student’s two-tailed *t* test (A and B). Two-way ANOVA with Tukey’s *post hoc* test (E and F).

injury (Figure E4). These findings suggest RAGE inhibition might be beneficial in alleviating lung injury from both N-protein and other RAGE agonists in COVID-19.

## Discussion

It has been well established that the association between SARS-CoV-2 S-protein and ACE2 mediates the viral entry to cells. To our knowledge, this is the first study to report the binding between N-protein and RAGE, linking another SARS-CoV-2 structural protein with a membrane receptor. This conclusion is substantiated by the following findings: 1) there was a direct high-affinity physical association between N-protein and RAGE (Figures 1A–1D); 2) N-protein bound RAGE via both the NTD and the CTD (Figures 1F and 1G); 3) N-protein activated the ERK1/2–NF- $\kappa$ B pathway through RAGE (Figure 2); 4) N-NTD and N-CTD mimicked full-length N-protein to induce an inflammatory response (Figure 3); 5) genetic deletion of RAGE significantly decreased N-protein–induced signaling and proinflammatory response *in vitro* (Figures 4A and 4B); 6) in mice, N-protein treatment elevated RAGE levels in the BAL and the lung (Figures 5A–5C); 7) RAGE deficiency protected against N-protein–induced acute lung injury in mice (Figures 5D–5F); and 8) inhibition of RAGE signaling via antagonist significantly alleviated N-protein–induced acute lung injury (Figure 6).

Previously, our group reported that N-protein induced acute lung injury in mice via activation of NF- $\kappa$ B (9). We speculated that a membrane receptor may mediate the binding with N-protein and activation of NF- $\kappa$ B. In the preliminary experiments using pull-down and coimmunoprecipitation assays, we found that N-protein was unable to physically associate with Toll-like receptor 4, the receptor for LPS (data not shown). Then, we turned our attention to exploring the binding between N-protein and RAGE. Our results demonstrated that the NTD and CTD of N-protein, but not the LR, bound with RAGE. Similarly, HMGB1 has two RAGE-binding domains, one in the box A domain (23–50 AA) (22) and the other in the box B domain (150–183 AA) (23). It has

been documented that both the NTD and the CTD of N-protein possess the ability of RNA binding (24). The presence of two domains with similar functions may help to reinforce the activation of the RAGE-mediated signaling pathway. However, we did not examine the ligand-binding domains for N-protein in RAGE. The mature human RAGE is a glycoprotein with 404 amino acids. It consists of an extracellular domain (1–342 AA), a single transmembrane domain (amino acids 343–363), and a cytoplasmic tail (amino acids 364–404). The extracellular part consists of one variable domain (V) and two constant domains (C1 and C2). The majority of binding studies showed that ligands bind with the V domain of RAGE; however, the C1 domain also participates in the recognition of ligands (25). The molecular mechanism underlying the binding between N-protein and RAGE warrants further investigation.

The association between RAGE and COVID-19 has been reported in the literature. Lim and colleagues showed that serum sRAGE is a biomarker for predicting disease severity of COVID-19 and the need for mechanical ventilation (26). mRNA and protein levels of RAGE ligands, such as S100A8, S100A9, S100A11, S100A12, and S100 P, were significantly higher in the lungs of COVID-19 fatal cases compared with healthy control subjects (27). Serum S100B levels were elevated in patients with COVID-19 and correlated with disease severity (28). In addition, serum levels of S100A8/A9 and HMGB1 at admission had prognostic value for ICU admission and hospital death in patients with COVID-19 (20). Furthermore, Jessop and colleagues found that RAGE antagonist improved the survival of SARS-CoV-2–infected mice and alleviated lung inflammation and perivascular pathology (29). The present study brings new light to the role of RAGE in COVID-19.

Using immunohistochemistry, Massoth and colleagues found that N-protein localized predominantly extracellularly and within the hyaline membrane in a large percentage of patients who died of COVID-19 (3). During SARS-CoV-2 infection, N-protein can be released into extracellular space and blood via several mechanisms. First, N-protein can be naturally secreted from SARS-CoV-2–infected cells.

When transfected alone in HEK 293 cells, N-protein and S-protein, but not E- and M-proteins, were readily detected in the cell lysate (30). Second, several studies showed that SARS-CoV-2 infection induced cell necrosis, leading to the passive release of virions, viral proteins, and cytoplasmic components into the extracellular space and blood (31). Third, one report showed that extracellular vesicles carrying S-protein were generated by host cells and budded from the plasma membrane, indicating that N-protein might also be released via the same machinery (32). Clinical studies showed that serum N-protein concentration was closely correlated with disease severity and had a peak value from 1 ng/ml to 2.7  $\mu$ g/ml in patients with COVID-19 (33, 34). The present study revealed that N-protein activated the membrane receptor RAGE on target cells and mediated the inflammatory response, providing the mechanism of N-protein in SARS-CoV-2–induced lung injury.

In the present study, inhibition or knockout of RAGE did not completely abolish the effects of N-protein on acute lung injury, which suggests that N-protein may have other binding partners. It has been reported that N-protein interacted directly with NLRP3 and promoted NLRP3 inflammasome activation to induce an inflammatory response in the lung (8). Here, we showed that N-protein–induced lung injury was partially blocked by the NLRP3 inhibitor MCC950. Another study showed that N-protein bound with Smad3 and enhanced TGF- $\beta$ /Smad3 signaling to induce acute kidney injury via the G1 cell cycle arrest mechanism (35). Moreover, the interaction between N-protein and  $\alpha$ -synuclein accelerated amyloid formation and cell death, which might explain the reported correlation between COVID-19 and Parkinsonism (36).

There are several other findings worth mentioning. In the SDS-PAGE analysis, we noticed that N-NTD migrated slightly more slowly than the predicted 20 kD, whereas the N-CTD (also expected 20 kD) moved even more slowly than NTD (Figure 1D). The same phenomenon was also seen in the report from Nakayama and colleagues (37). This finding suggests that N-NTD and N-CTD have differences in post-translation modification. Second, our study demonstrated that N-protein induced

activation of ERK1/2, p38 MAPK, and JNK1/2. However, the activation of p38 MAPK and JNK1/2 was not affected by RAGE inhibition, suggesting that this effect is independent of RAGE. Ishihara and colleagues found that RAGE bound specifically with ERK but not with JNK or p38 MAPK (38). Somensi and colleagues documented that heat shock protein 70 induced activation of ERK1/2, p38

MAPK, and JNK. However, only ERK1/2 activation was blocked by RAGE knockdown (39).

In conclusion, the present study reveals that RAGE is a cell surface receptor for N-protein. N-protein activates the RAGE-ERK1/2–NF- $\kappa$ B pathway and promotes the production of proinflammatory cytokines. N-protein-induced acute lung injury is partially mediated by RAGE. Therapeutic

interventions targeting RAGE may alleviate cytokine storm and ARDS in COVID-19. ■

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