ORIGINAL RESEARCH

MicroRNA-574-5p Attenuates Acute Respiratory Distress Syndrome by Targeting HMGB1

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Abstract

Acute respiratory distress syndrome (ARDS) is a critical condition with high mortality. HMGB1 (high-mobility group protein B1) is one of the key proinflammatory factors in the ARDS "inflammatory storm." According to previous studies, some microRNAs (miRNAs) play important roles in this process. We aimed to determine the contributing miRNAs targeting the expression and release of HMGB1. miRNA expression in the peripheral blood of patients with ARDS was measured by miRNA microarray. miRNAs targeting HMGB1 were screened and explored for further study. In LPS-induced cell and mouse ARDS models, we explored the effect of this miRNA on the expression and secretion of HMGB1 by Western blot, real-time qPCR, and ELISA. The effects of this miRNA on the NF-κB signaling pathway, proinflammatory cytokines, and NLRP3 (nod-like receptor protein 3) inflammasome were detected by Western blot and real-time qPCR. In ARDS models, microRNA-574-5p (miR-574-5p) expression could be induced by the TLR4/NF-κB pathway upon LPS stimulation. It could suppress the inflammatory response by targeting HMGB1. Enforcing the expression of miR-574-5p or HMGB1 siRNA silencing inhibits the activation of NF-κB signaling pathway and the NLRP3 inflammasome. Moreover, overexpression of HMGB1 reversed the antiinflammatory effect of miR-574-5p. In ARDS mice, overexpression of miR-574-5p suppresses alveolar leukocytes infiltration, interstitial edema, protein effusion, and inflammation. This study demonstrated that miR-574-5p provided negative feedback to LPS-induced inflammation and relieved ARDS. It may provide new therapeutic strategies for ARDS.

Keywords: miR-574-5p; HMGB1; NLRP3 inflammasome; NF-κB

Clinical Relevance

These findings provide insight into the role of microRNA-574-5p (miR-574-5p) in acute respiratory distress syndrome (ARDS) and may indicate a novel therapeutic approach to relieve ARDS.

Acute respiratory distress syndrome (ARDS) refers to a devastating medical condition with a high mortality rate of 20–50% worldwide (1). It is characterized by exaggerated pulmonary inflammation, which may be caused by sepsis, pneumonia, pancreatitis, drug toxicity, or multiple blood transfusions (2). Recently, patients infected with coronavirus disease (COVID-19) may develop ARDS, and the cytokine storm may associated with disease severity (3). The activation of lung microvascular cells is one of the most significant initiating events of ARDS (4). The pulmonary vascular endothelium has a vast surface area, and it is an ideal place to interact with bloodborne cells and vasoactive mediators to sense chemical, mechanical,

(Received in original form March 26, 2020; accepted in final form October 6, 2020)

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Supported by the National Natural Science Foundation of China (grant 81670073) and the Natural Science Foundation of Jiangsu Province (BK20161383).

Author Contributions: Conception, design, and experiments: B.H., W.Z., Y.R., and X.S. Analysis and interpretation: B.H., L.L., B.C., and X.S. Drafting the manuscript for important intellectual content: B.H. and X.S.

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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.

Am J Respir Cell Mol Biol Vol 64, Iss 2, pp 196–207, Feb 2021

Originally Published in Press as DOI: 10.1165/rcmb.2020-0112OC on November 17, 2020
Internet address: www.atsjournals.org

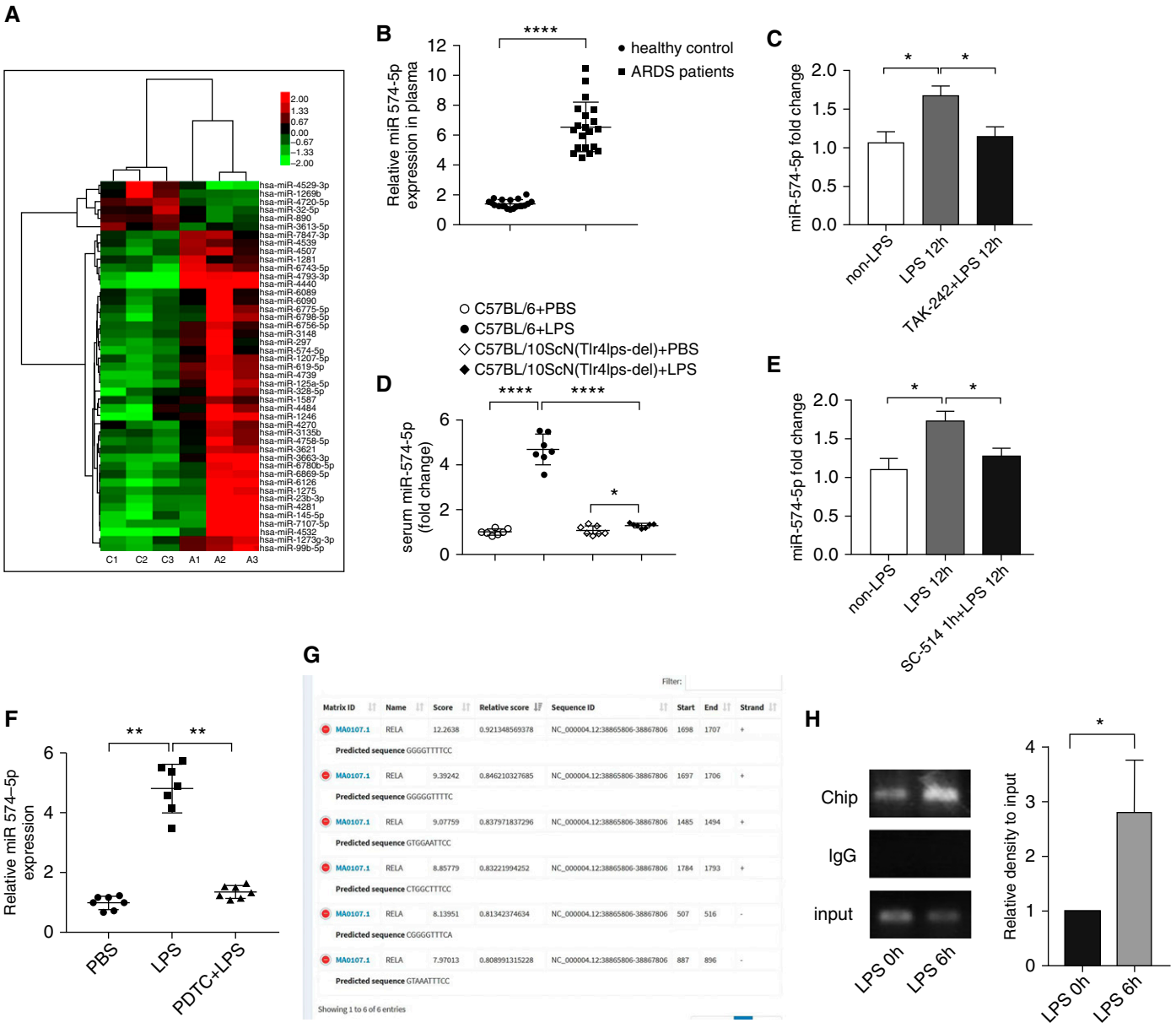

Figure 1. miR-574-5p expression was upregulated in acute respiratory distress syndrome (ARDS) and LPS-stimulated human pulmonary microvascular endothelial cells (HPMECs) and was induced by TLR4 via the NF-κB signaling pathway. (*A*) Microarray analysis of miRNA expression in healthy subjects (C1, C2, and C3) versus patients with ARDS (A1, A2, and A3) using a red, black, and green color scale (red indicates high expression, green indicates low expression, and black indicates unchanged expression). (*B*) Real-time qPCR analysis of miR-574-5p expression in the plasma of 20 healthy people and 20 patients with ARDS. (*C*) HPMECs were cultured for 45 minutes with medium only or LPS in the presence or absence of TAK-242. The expression of miR-574-5p was analyzed by real-time qPCR analysis ($n = 7$). (*D*) Wild-type and TLR4$^{-/-}$ mice were administered PBS or LPS and killed at 24 hours after infection. Serum was obtained, and the concentrations of miR-574-5p were measured ($n = 7$). (*E*) HPMECs were cultured for 1 hour with medium only or LPS in the presence or absence of SC-514. The expression of miR-574-5p was analyzed by real-time qPCR analysis ($n = 7$). (*F*) Wild-type and ammonium pyrrolidine dithiocarbamate (PDTC)-pretreated mice were administered LPS and killed at 24 hours after infection. Serum was obtained, and the concentrations of miR-574-5p were measured ($n = 7$). (*G*) The predicted potential binding sites between NF-κB p65 and the promoter of the miR-574-5p gene. (*H*) Binding of the NF-κB p65 subunit to the promoter of the miR-574-5p gene. IgG was used as a negative control in the chromatin IP assay ($n = 3$). The values presented are the mean ± SD. Comparisons were made by *t* test in *B*, *D*, and *H*. Comparisons were made by one-way ANOVA followed by Dunnett Multiple Comparison in *C*, *E*, and *F*. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{****}P < 0.0001$. Chip = chromatin IP; miR-574-5p = microRNA-574-5p; WT = wild-type.

and cellular stimuli to participate in inflammation (5). Lung endothelium disruption can progress to an activated proinflammatory phenotype (6). The pulmonary endothelium is a key regulator of innate cellular and cytokine responses (7, 8).

HMGB1 (high-mobility group box-1) is a nonhistone chromosomal protein that is expressed by almost all eukaryotic cells (9). It has been demonstrated to be secreted by activated immune cells and serves as a cytokine that mediates

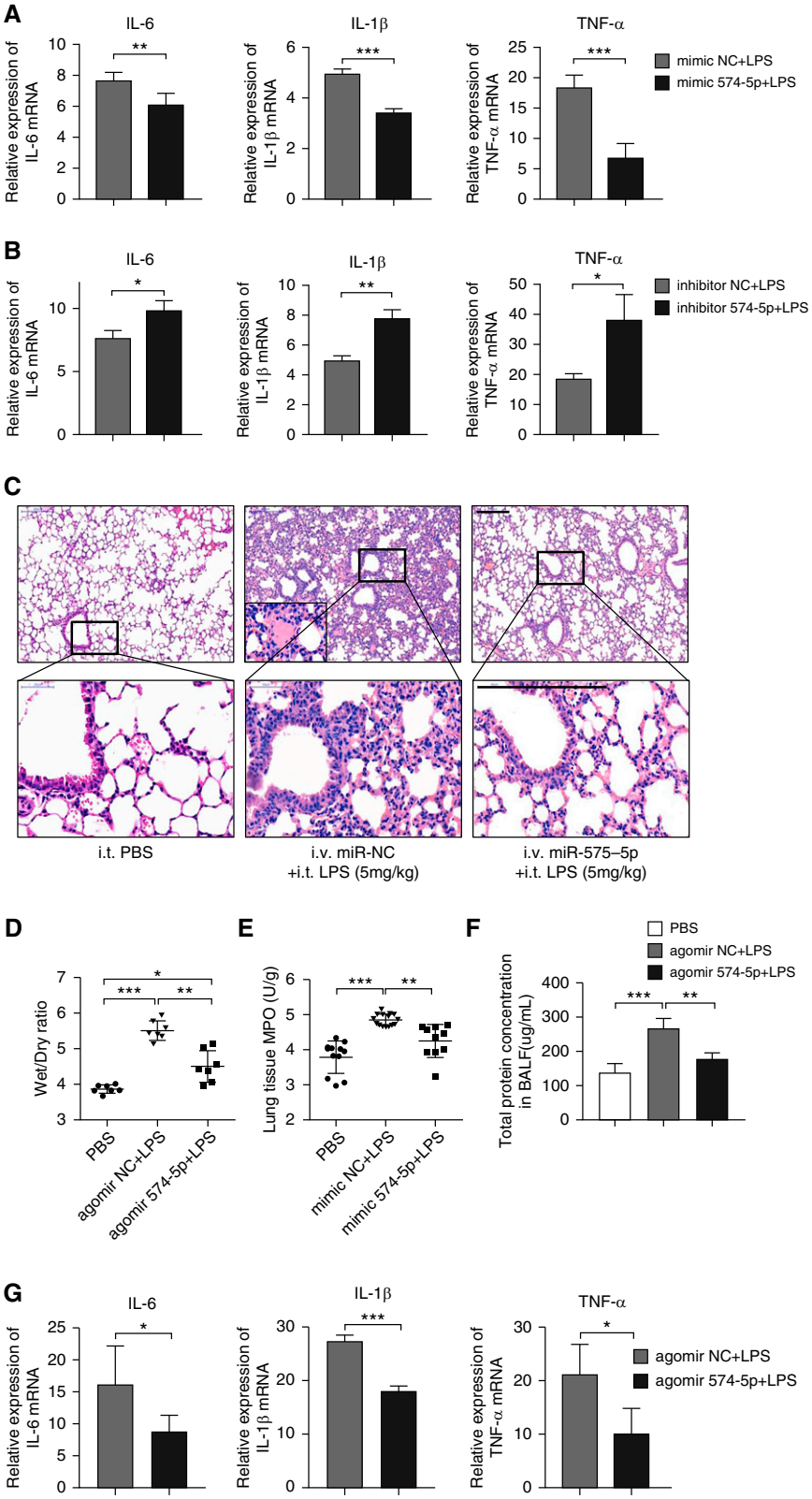

Figure 2. miR-574-5p inhibited LPS-induced ARDS *in vitro* and *in vivo*. (*A* and *B*) HPMECs were transfected with miR-574-5p mimics (*A*) or inhibitor (*B*) for 24 hours followed by stimulation with LPS for 6 hours. IL-6, IL-1β, and TNF-α mRNA concentrations were measured by real-time qPCR ($n = 7$). (*C*) Mice were intravenously injected with miR negative control agomir or miR-574-5p agomir; 24 hours later, the mice were intratracheally instilled with PBS or 5 mg/ml LPS. Twenty-four hours later, lung inflammation was assessed by morphologic analysis. The lungs were embedded in formalin, and sections were analyzed by

the response to inflammation, injury, and infection (10, 11). Under inflammation conditions, active HMGB1 is able to translocate from the nucleus to the cytoplasm for release into the extracellular milieu (12). HMGB1 has been identified as a late mediator of endotoxin lethality (13). Circulating concentrations of HMGB1 increase after the administration of endotoxin (13), and the administration of HMGB1 through trachea can directly induce ARDS (14). In endotoxin-induced ARDS, administration of anti-HMGB1 antibody before or after endotoxin exposure is able to alleviate ARDS (14).

MicroRNAs (miRNAs) are a class of noncoding RNAs that regulate gene expression. MiRNAs play an important role in the maintenance of immune homeostasis by binding to complementary target mRNAs, resulting in mRNA degradation or translational inhibition (15). Some miRNA mimics or molecules targeted at miRNAs (antimirs) have progressed to the clinical development pipeline for use as therapeutics (16). Antimirs targeted at miR-122 have reached phase II trials for the treatment of hepatitis. miRNAs, such as miR-146, miR-21, and miR-17, also play an important role in ARDS-associated inflammation (17–20). Sun and colleagues observed that overexpression of miR-181b inhibited the expression of importin-a3 and further reduced lung injury and mortality in ARDS mice (21). Rao and colleagues found that targeting SOCS1 (suppressor of cytokine signaling 1) in ARDS mice led to had diminished inflammatory cytokine production and inflammatory cell accumulation in miR-155$^{-/-}$ mice compared with wild-type mice (22). In this study, we aimed to determine which miRNA could ameliorate ARDS by targeting HMGB1.

Methods

An extended description of the Methods is provided in the data supplement.

Mice and Infection Experiment

C57BL/6 male mice and C57BL/10ScN (Tlr4lps-del) male mice (6–8 wk) were obtained from Nanjing Biomedical Research Institute of Nanjing University. All of the experimental procedures involving animals were approved by the Jinling Hospital Animal Care Committee and strictly followed the International Recommendations for the Use and Care of Animals (National Institutes of Health). All of the mice were housed in bright rooms at the appropriate temperature (20–25°C), and humidity (40–70%), with a 12 hour light and 12 hour dark cycle. The mice had *ad libitum* access to food and water. All of the mice were allowed to adapt to the experimental environment for at least 1 week before experimentation and were monitored once a day. Mice were anesthetized with sodium pentobarbital (60 mg/kg i.p.) and then intratracheally instilled with PBS or 5 mg/kg LPS. Twenty-four hours after the intratracheal injection, BAL fluid, blood, and lung tissues were harvested immediately after the animal was killed. Blood was centrifuged at 3,000 rpm for 10 minutes at 4°C to obtain the supernatant for further testing. BAL fluid and lung tissues were stored at −80°C for later tests.

Cell Culture and Treatment

Human pulmonary microvascular endothelial cells (HPMECs) were cultured in endothelial cell medium (ScienCell Research Laboratories) supplemented with 5% FBS (ScienCell Research Laboratories), 1% endothelial cell growth supplement (ScienCell Research Laboratories), and 1% penicillin/streptomycin solution (ScienCell Research Laboratories). The cells were incubated at 37°C with 5% CO_2. HPMECs were stimulated with 10 μg/ml LPS for 6 hours.

Reagents

LPS (serotype 055:B5) purified by phenol extraction from *Escherichia coli* was supplied by Sigma Chemicals (St. Louis). HPMECs were obtained from ScienCell Research Laboratories.

Statistical Analysis

Statistical analysis was performed using the SPSS 24 Package (SPSS Inc.). For comparative studies, Student's *t* test (unpaired) was used among two groups. The analysis was conducted using one-way ANOVA followed by Dunnett multiple comparison test in data with more than two groups. *P* values <0.05 were considered significant. These data are expressed as the mean and SD of the mean (mean ± SD). All of the graphs were generated with GraphPad Prism 7.0 (GraphPad Software Inc.).

Results

miR-574-5p Expression Is Upregulated in Patients with ARDS

We performed miRNA expression profiling on plasma samples from three cases with pneumonia-related ARDS and three healthy control subjects using Affymetrix microRNA 4.0 Array. We identified 45 miRNAs that were differentially expressed in plasma from patients with ARDS compared with control subjects. The miRNAs were analyzed using hierarchical clustering to reveal the miRNA expression patterns (Figure 1A).

From these 45 miRNAs, miRNAs with binding potential to HMGB1 were selected for further study. It was predicted on the TargetScan and RNA22 bioinformatics websites that 7 of the 45 microRNAs had potential binding sites for HMGB1. Among them, miR-574-5p and miR-890 were predicted to have potential binding sites for HMGB1 by both bioinformatics websites. RNA22 showed that miR-574-5p had four potential binding sites for HMGB1 mRNA 3′ untranslated region (UTR). Compared with that in healthy control subjects, miR-574-5p expression in the ARDS samples was increased 5.48-fold. This differentially expressed plasma miRNA was validated by real-time qPCR in 20 patients with ARDS and 20 healthy control subjects (Figure 1B).

Figure 2. (*Continued*). hematoxylin and eosin staining. Scale bars, 200 mm ($n = 7$). (*D* and *E*) Lungs were harvested to measure wet/dry (*D*) ratio and myeloperoxidase (*E*) activity ($n = 7$). (*F*) BAL fluid was collected, and the protein concentration was measured ($n = 7$). (*G*) Real-time qPCR analysis of the IL-6, IL-1β, and TNF-α mRNA concentrations in lung tissues ($n = 7$). The values presented are the mean ± SD. Comparisons were made by *t* test in *A*, *B*, and *G*. Comparisons were made by one-way ANOVA followed by Dunnett Multiple Comparison in *D–F*. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. MPO = myeloperoxidase; NC = negative control.

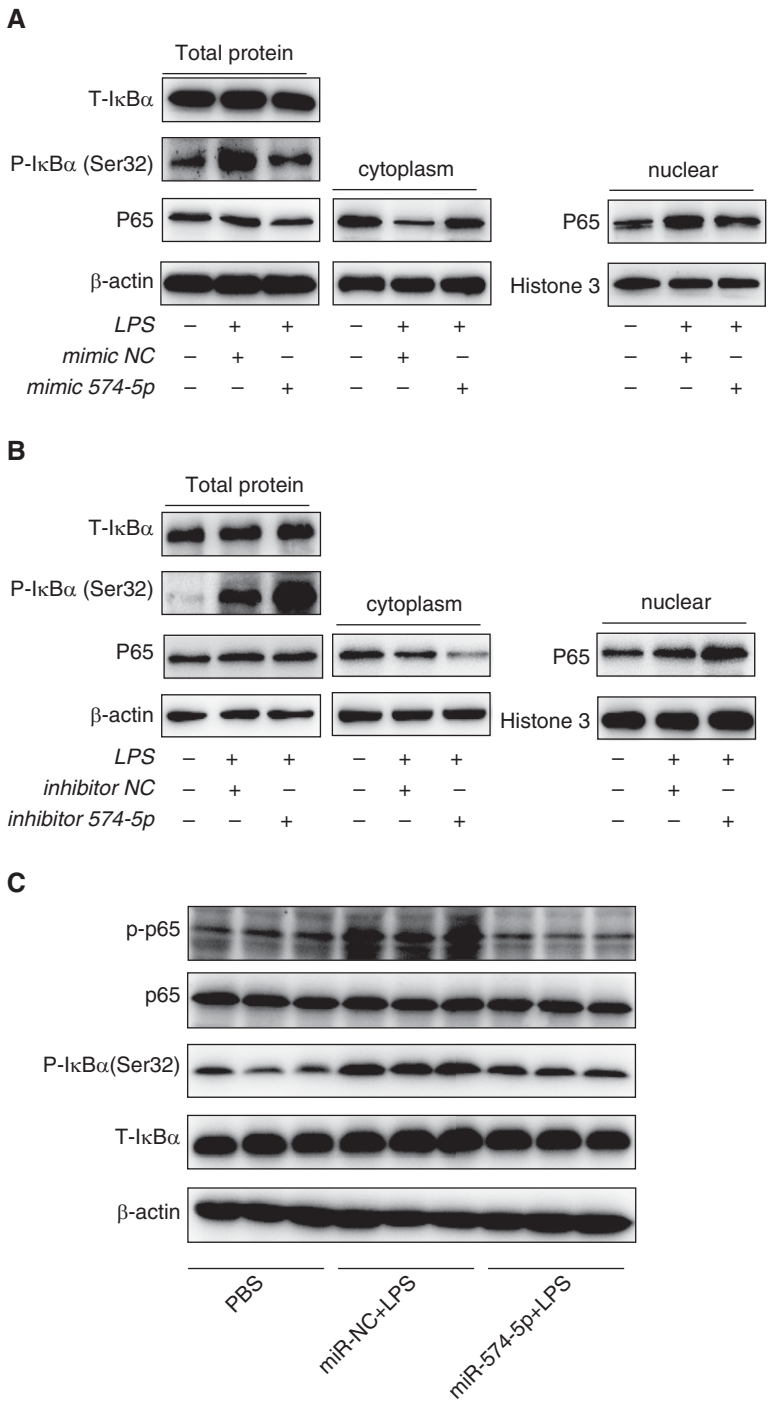

Figure 3. miR-574-5p suppressed the activation of the NF-κB signaling pathway *in vitro* and *in vivo*. (*A* and *B*) HPMECs were transfected with miR-574-5p mimics (*A*) or inhibitor (*B*) for 48 hours followed by stimulation with LPS for 6 hours and Western blot analysis of the total and phosphorylated IκBα in HPMECs and the translocation of NF-κB p65 in HPMECs. (*C*) Mice were intravenously injected with miR-NC agomir or miR-574-5p agomir, and 24 hours later, the mice were intratracheally instilled with PBS or 5 mg/ml LPS. Twenty-four hours later, the lungs were harvested for total and phosphorylated IκBα and NF-κB p65 analysis. All images are representatives of five independent experiments.

miR-574-5p Expression Is Upregulated upon LPS Stimulation *in Vitro* and *in Vivo*

We investigated whether miR-574-5p was differentially expressed in LPS-stimulated HPMECs versus unstimulated control cells and ARDS mice versus control mice. The results revealed that miR-574-5p was both upregulated in LPS-stimulated HPMECs and ARDS mice compared with their control counterparts (Figures 1C and 1D). These findings suggest that miR-574-5p is involved in ARDS.

miR-574-5p Is Induced by TLR4 Signaling

It has been demonstrated that TLR4 plays a prominent role in bacterium-induced inflammatory responses. Considering the regulatory mechanism of miR-574-5p, we hypothesized that TLR4 could induce the expression of miR-574-5p. HPMECs were pretreated with 100 nM TAK-242, a potent TLR4 signaling inhibitor (23), for 45 minutes followed by LPS stimuli, and miR-574-5p expression was detected by real-time qPCR analysis. We found that miR-574-5p induction by LPS challenge was significantly impaired in TLR4 signaling–inhibited HPMECs (Figure 1C), suggesting that LPS-induced miR-574-5p expression was dependent on the activation of TLR4 signaling. Then, we evaluated miR-574-5p concentrations in TLR4-knockout mice. Importantly, we observed that LPS-induced miR-574-5p expression was impaired in TLR4-deficient mice compared with wild-type C57BL/6J mice (Figure 1D), strongly indicating that TLR4 is involved in LPS-induced miR-574-5p expression.

NF-κB Activation Regulates miR-574-5p Expression in Response to LPS Stimuli

Recognition of microbial components by TLRs triggers a cascade of cellular signals that culminates in the activation of NF-κB, which leads to inflammatory gene expression and clearance of the infectious agent (24). NF-κB is an important transcription factor that regulates the expression of a variety of miRNAs (25, 26). To identify whether the TLR4-mediated increase in miR-574-5p is

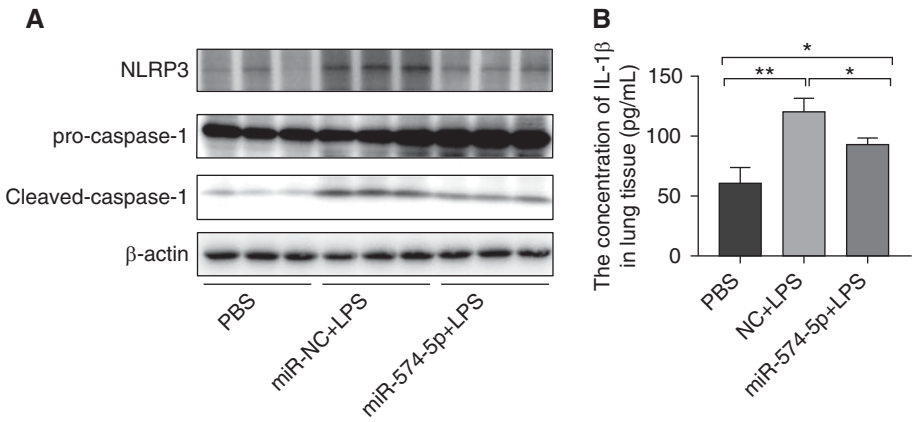

Figure 4. miR-574-5p suppressed the activation of the NLRP3 (nod-like receptor protein 3) inflammasome *in vitro* and *in vivo*. (*A*) Mice were intravenously injected with miR-NC agomir or miR-574-5p agomir, and 24 hours later, the mice were intratracheally instilled with PBS or 5 mg/ml LPS. Twenty-four hours later, the lungs were harvested, and NLRP3, pro–capsase-1, cleaved caspase-1 were analyzed by Western blot. (*B*) IL-1β was analyzed by ELISA. All images are representatives of five independent experiments. The values presented are the mean ± SD. Comparisons were made by one-way ANOVA followed by Dunnett Multiple Comparison. $^{*}P < 0.05$ and $^{**}P < 0.01$.

dependent on the activation of NF-κB pathways, we pretreated HPMECs with 100 μM SC-514, an NF-κB inhibitor (27), for 1 hour followed by LPS stimulation. We measured miR-574-5p expression and showed that inhibition of NF-κB reduced the induction of miR-574-5p in HPMECs (Figure 1E). Importantly, we observed that LPS-induced miR-574-5p expression was impaired in PDTC (ammonium pyrrolidine dithiocarbamate, a potent NF-κB signaling inhibitor [28])-pretreated mice (Figure 1F).

It is essential to clarify how NF-κB is associated with the increase in miR-574-5p in response to LPS stimulation. In humans, the miR-574 gene is believed to be located in the first intron of the gene encoding Noxp20 on human chromosome 4 (29). To address this issue, we first used JASPAR (http://jaspar.genereg.net/) to predict potential binding sites between NF-κB p65 and the promoter of Noxp20, and the results showed that there was at least one NF-κB binding site (Figure 1G). Importantly, the binding site was validated via chromatin IP analysis. As presented in Figure 1H, binding activity of NF-κB p65 was detected in the promoter region of miR-574-5p, and this interaction was enhanced by LPS stimulation.

miR-574-5p Suppresses LPS-induced Inflammatory Responses

To assess the function of elevated concentrations of miR-574-5p in endothelial cells, we overexpressed miR-574-5p via transfection of miR-574-5p mimic (Figure 2A). Overexpression of miR-574-5p in HPMECs inhibited LPS-induced IL-6, IL-1β, and TNF-α mRNA expression compared with the miRNA mimic negative control (Figure 2A), whereas miR-574-5p inhibitor increased the expression levels of these cytokines compared with the miRNA inhibitor negative control (Figure 2B). In addition, we explored the role of miR-574-5p in RAW 264.7. It showed that miR-574-5p also suppressed the transcription of inflammatory factors in RAW 264.7 (Figure E1 in the data supplement).

To dissect the physiological impact of altered miR-574-5p concentrations, we further investigated whether systemic administration of miR-574-5p could relieve LPS-induced ARDS *in vivo*. miR-574-5p agomir or miRNA agomir negative control was administered via tail vein injection in C57BL/6 mice 24 hours before PBS or LPS stimulation. As shown in Figure 2C, the lung morphology of mice in the ARDS group changed greatly, showing alveolar infiltration by leukocytes, alveolar wall thickening, patchy hemorrhage, and interstitial edema. However, these histological changes were ameliorated by miR-574-5p. The collected lung tissue was assayed for its wet:dry ratio and myeloperoxidase (MPO) activity, and it was found that the LPS-induced increase in wet:dry ratio and MPO activity were reversed by miR-574-5p (Figures 2D and 2E). The total protein concentration in BAL fluid was elevated significantly in ARDS mice, and this effect was ameliorated by miR-574-5p (Figure 2F). Similarly, administration of miR-574-5p agomir ameliorated the induction of cytokine mRNA expression in lung tissue (Figure 2G). These results showed that miR-574-5p could relieve LPS-induced inflammation and edema in the lung tissue.

miR-574-5p Inhibits the Activation of NF-κB Signaling

The expression of proinflammatory cytokine genes is usually mediated by TLR signaling in bacterial infection via phosphorylation of IBκα, followed by the release of the NF-κB p65 protein into the nucleus and the activation of NF-κB, which is a TLR-signaling downstream event (30). To determine whether miR-574-5p affects NF-κB activation, we next investigated the effect of miR-574-5p on the phosphorylation of IκBα and the NF-κB p65 subunit. Western blot results showed that the phosphorylation of IκBα and nuclear translocation of NF-κB p65 subunit in HPMECs transfected with miR-574-5p mimic was significantly decreased compared with that in the cells transfected with miRNA mimic negative control (Figure 3A), whereas miR-574-5p inhibitor showed the opposite trend (Figure 3B). Similarly, in ARDS mice, miR-574-5p administration ameliorated the concentration of phospho-IκBα and phospho-NF-κB p65 (Figure 3C). These data suggest that the inhibitory role of miR-574-5p in LPS-induced proinflammatory gene expression may be due to its effects on the TLR-signaling pathway via downregulation of NF-κB activation.

miR-574-5p Inhibits the Activation of the NLRP3 Inflammasome

Recent data have indicated that the NLRP3 (nod-like receptor protein 3) inflammasome is essential for the development of experimental acute lung injury (ALI) (31, 32). The products of inflammasome

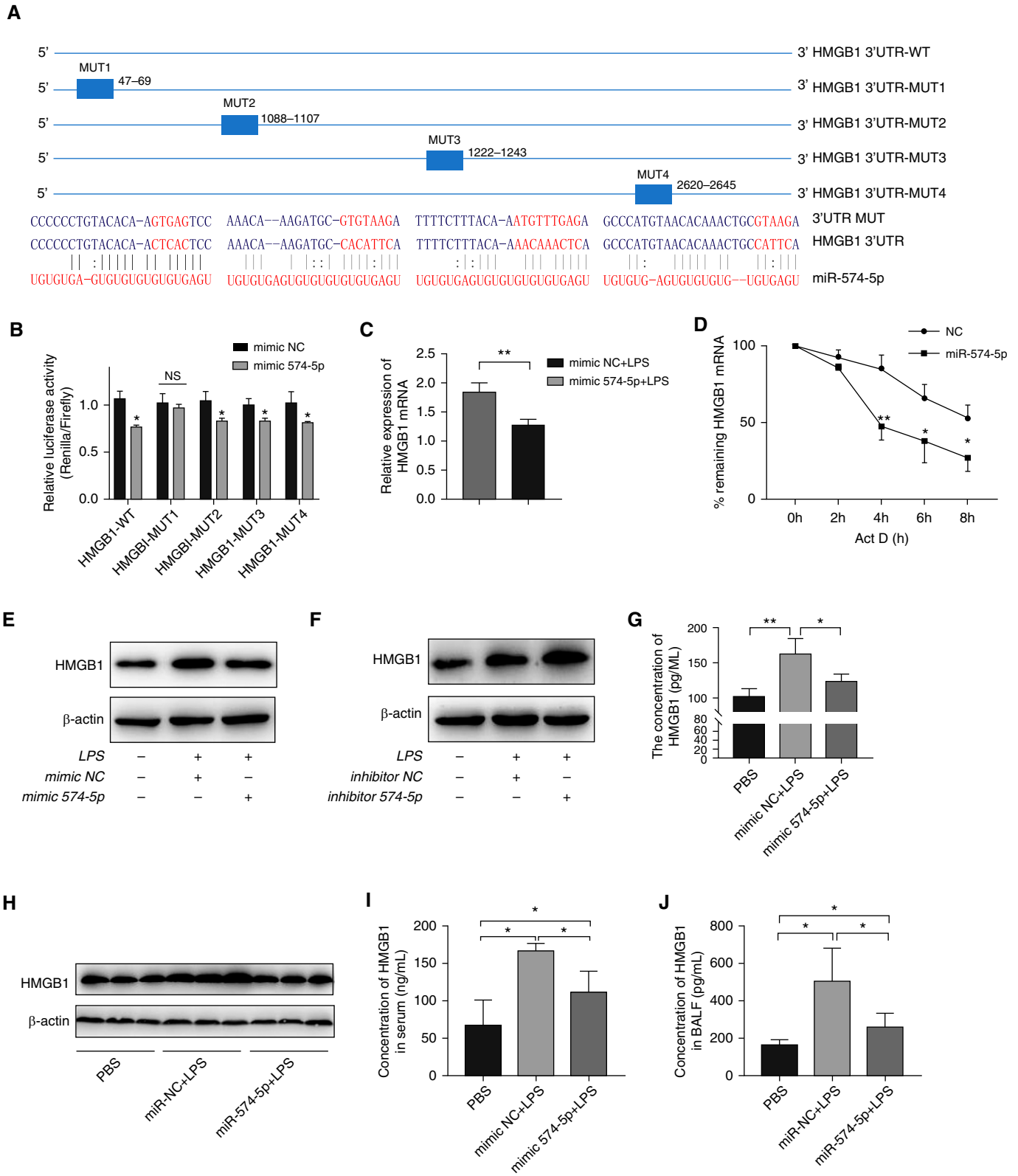

Figure 5. HMGB1 is the functional target of miR-574-5p. (*A*) The HMGB1 3′ untranslated region (UTR) contains four predicted miR-574-5p binding sites. The figure shows the predicted binding interactions between the HMGB1 3′ UTR (middle) and miR-574-5p (bottom). The sites of targeted mutagenesis (top) are also indicated. (*B*) Normalized luciferase activity of a reporter containing the WT or point-mutated (MUT1, MUT2, MUT3, and MUT4) 3′ UTR

activation (IL-1β and IL-18) are important for exacerbating ALI (33). To determine whether miR-574-5p could inhibit activation of the NLRP3 inflammasome, we detected the concentration of NLRP3, caspase-1, and mature IL-1β in lung homogenate. We detected high concentrations of NLRP3, caspase-1 p20 (an autoprocessed fragment of caspase-1), and IL-1β in the lung homogenate of ALI mice. However, the concentrations of NLRP3, caspase-1 p20, and IL-1β were decreased when miR-574-5p agomir was administered (Figures 4A and 4B). These results demonstrate that the administration of miR-574-5p could effectively attenuate the activation of the NLRP3 inflammasome.

HMGB1 Is the Functional Target of miR-574-5p

miRNA target prediction program (RNA22 and miRanda) suggested that HMGB1 might be a direct target of miR-574-5p and that the HMGB1 3′ UTR contains four predicted miR-574-5p binding sites (Figure 5A). Overexpression of miR-574-5p inhibited the activity of a luciferase reporter construct containing the HMGB1 3′ UTR. In contrast, the activity of luciferase constructs containing the site mutant 1 3′ UTR of HMGB1 was not inhibited, whereas the activity of luciferase constructs containing the site mutant 2–4 3′ UTR of HMGB1 was still inhibited by overexpressing miR-574-5p (Figure 5B). It was demonstrated that among the four predicted miR-574-5p binding sites, only one binding site (47–69) in the 3′ UTR of HMGB1 proved to be the unique binding site of miR-574-5p.

Next, the expression levels of HMGB1 were detected in LPS-stimulated HPMECs. As shown in Figure 5C, HMGB1 mRNA expression decreased in miR-574-5p–overexpressing cells. To clarify whether miR-574-5p affected the stability of HMGB1 mRNA, 24 hours after the transfection of HPMECs with miR-574-5p mimics or negative control mimics, we used actinomycin D to inhibit mRNA transcription. We collected cell samples at different time points and detected HMGB1 mRNA abundance by real-time qPCR. As shown in Figure 5D, miR-574-5p mimics indeed decreased HMGB1 mRNA stability in HPMECs. The HMGB1 protein concentrations decreased significantly in miR-574-5p–overexpressing HPMECs (Figure 5E), and miR-574-5p inhibitor showed the opposite trend (Figure 5F). In addition, we explored at the role of miR-574-5p in RAW 264.7. It showed that miR-574-5p also reduced the expression of HMGB1 in RAW 264.7 (Figure E1). Similarly, miR-574-5p mimics decreased the HMGB1 concentrations in the supernatant of HPMECs (Figure 5G). To test whether miR-574-5p suppresses endogenous HMGB1 expression *in vivo*, we next injected mice with miRNA agomir negative control or miR-574-5p agomir via intravenous injection. As shown in Figures 5H–5J, the enforced expression of miR-574-5p significantly decreased the amount of HMGB1 protein in lung tissue, serum, and BAL fluid. Thus, we demonstrated that HMGB1 may be a target of miR-574-5p.

miR-574-5p Suppresses Inflammatory Responses and NLRP3 Inflammasome Activation through HMGB1 Inhibition

To further determine whether the suppressive role of miR-574-5p on LPS-induced inflammatory responses is mediated through the inhibition of HMGB1, we subsequently analyzed the effect of HMGB1 on NF-κB activation, NLRP3 inflammasome activation, and cytokine expression. As shown in Figure 6A, HPMECs transfected with HMGB1 siRNA had reduced expression of IL-6, IL-1β, and TNF-α compared with cells transfected with negative control siRNA. The specific siRNA significantly reduced the protein concentrations of HMGB1. Importantly, transfection of HMGB1 siRNA into HPMECs resulted in decreased phosphorylation of NF-κB and IκBα protein as well as NLRP3 and cleaved caspase-1 protein concentrations compared with scrambled siRNA–transfected cells (Figure 6B).

Furthermore, we showed that the reduction in NLRP3 inflammasome activation and cytokines expression by the miR-574-5p mimics was abolished by HMGB1 overexpression after HPMECs were transfected with the pEGFP-N1-HMGB1 plasmid (Figures 6C, 6E). Similarly, silencing of HMGB1 abolished the effect of miR-574-5p inhibitor on NF-κB activation, inflammasome activation, and inflammatory gene expression (Figures 6D, 6F). Collectively, these data strongly indicate that the negative regulation of miR-574-5p on LPS-induced inflammatory responses is mediated through HMGB1.

Discussion

ARDS is characterized by an exaggerated inflammatory reaction. To this end, negative regulators are very important to avoid excessive inflammation or uncontrolled inflammatory responses. In recent years, a number of negative regulators of the inflammatory response have been reported (34). For instance, gram-negative bacterial infection can induce the expression of miR-302b in epithelial cells through TLR/NF-κB–dependent pathways. Then,

Figure 5. (*Continued*). reporter constructs of HMGB1 in 293T cells cotransfected with miR-NC mimics or miR-574-5p mimics ($n = 5$). (*C*) Twenty-four hours later, the cells were stimulated with LPS for 6 hours. Real-time qPCR analysis of HMGB1 mRNA concentrations in HPMECs transfected with miR-NC mimics or miR-574-5p mimics ($n = 5$). (*D*) Twenty-four hours after transfection of HPMECs with miR-NC mimics or miR-574-5p mimics, 5 μg/ml actinomycin D was added, the cell samples were collected at different time points, and HMGB1 mRNA abundance in HPMECs was detected by real-time qPCR ($n = 5$). (*E* and *F*) Western blot analysis of HMGB1 protein concentrations in HPMECs. HPMECs were transfected with miR-574-5p mimics or miR-574-5p inhibitors ($n = 5$). (*G*) HPMECs were transfected with miR-574-5p mimics for 48 hours followed by stimulation with LPS for 6 hours and ELISA analysis of HMGB1 protein concentrations in cell culture supernatants ($n = 5$). (*H*) Western blot analysis of HMGB1 protein in lung tissues ($n = 5$). (*I* and *J*) ELISA analysis of HMGB1 protein expression in serum (*I*) and BAL fluid (*J*) of ARDS mice ($n = 5$). The values presented are the mean ± SD. Comparisons were made by *t* test in *C* and *D*. Comparisons were made by one-way ANOVA followed by Dunnett Multiple Comparison in *G*–*J*. $^*P < 0.05$ and $^{**}P < 0.01$. NS = not significant.

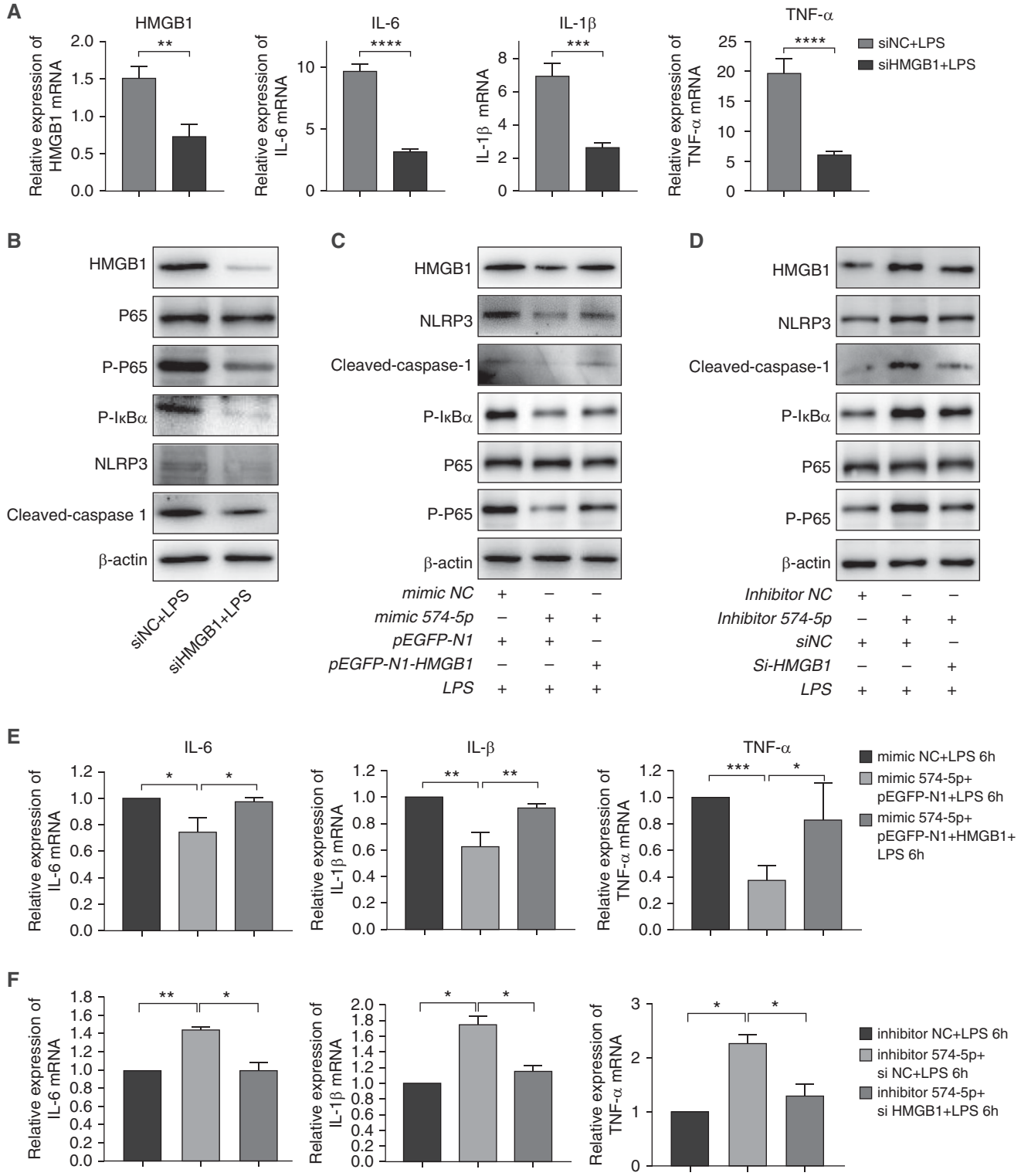

Figure 6. miR-574-5p regulates the inflammatory response through HMGB1. (*A*) Real-time qPCR analysis of HMGB1, IL-6, IL-1β, and TNF-α mRNA concentrations in HPMECs transfected with NC-siRNA or HMGB1-siRNA followed by LPS stimulation ($n = 5$). (*B*) Western blot analysis of HMGB1, total and phosphorylated NF-κB p65 and IκBα, NLRP3, and cleaved caspase-1 in HPMECs transfected with NC-siRNA or HMGB1-siRNA followed by LPS stimulation ($n = 3$). (*C*) HPMECs were cotransfected with miR-574-5p mimics/miR-NC mimics and pEGFP-N1-HMGB1/pEGFP-N1. After 48 hours, the cells were treated with LPS for 6 hours. The HMGB1, NLRP3, and cleaved caspase-1 protein concentrations were evaluated by Western blotting ($n = 3$).

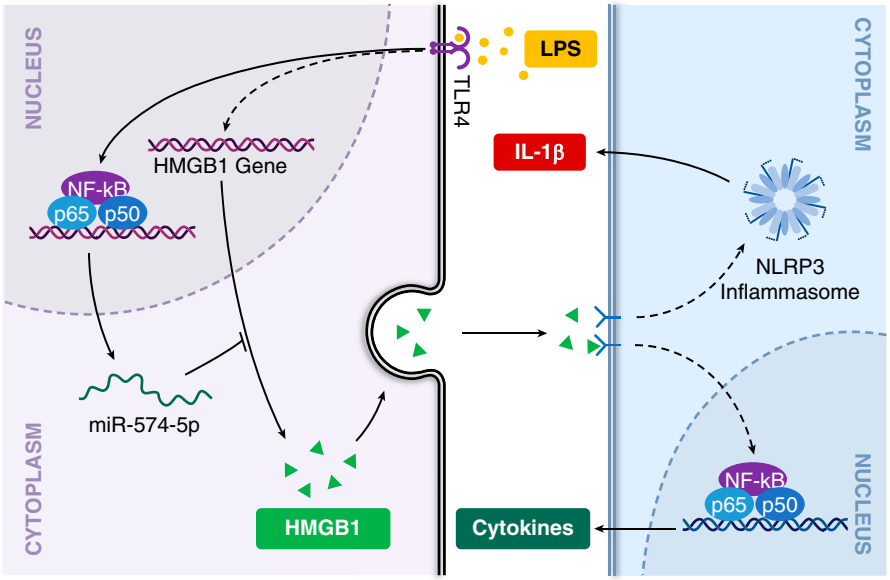

Figure 7. Schematic model of the critical role of miR-574-5p in ARDS pathogenesis. miR-574-5p is induced by TLR4 through the NF-κB signaling pathway upon LPS stimulation. It functions as a negative feedback regulator in NF-κB signaling and NLRP3 inflammation activation by targeting HMGB1.

miR-302b can negatively regulate bacteria-induced inflammation by targeting IRAK4. The cross-talk between the positive and negative regulation of the signaling pathways involved is complex; thus, detailed mechanisms for new positive or negative regulators need to be further studied.

In this article, we found a novel negative regulatory network for TLR4/NF-κB signaling at the miRNA level (Figure 7). We demonstrated that LPS stimulation could induce the upregulation of miR-574-5p in HPMECs and C57BL/6 mice through TLR4/NF-κB–dependent pathway. Then, we found that the overexpression of miR-574-5p inhibited the activation of an NF-κB–dependent pathway and negatively regulated the production of proinflammatory cytokine stimulated by LPS in HPMECs and in ARDS mice. Moreover, miR-574-5p could also inhibit the activation of the NLRP3 inflammasome. Furthermore, it is indicated that HMGB1 is a target of miR-574-5p and that their binding site was determined by dual-luciferase reporter assay. Similarly, the direct inhibition of HMGB1 had similar effects as the overexpression of miR-574-5p. Transfection of the pEGFP-N1-HMGB1 plasmid abrogated the beneficial effect of miR-574-5p mimics. These data further indicated that the negative regulation of miR-574-5p on LPS-induced inflammatory responses is mediated through HMGB1.

Therefore, we present a new feedback model in which LPS is first sensed by TLR4 and activates the NF-κB pathway, which initiates the inflammatory response. At the same time, the activation of NF-κB signaling can also upregulate the expression of miR-574-5p, which in turn inhibits the inflammatory response by targeting HMGB1, thus providing a balanced response.

It has been proven that HMGB1 and the NLRP3 inflammasome can activate each other in inflammatory cells (35–39) to increase inflammation. Thus, reduction in HMGB1 and/or NLRP3 can disrupt this positive feedback loop. In our study, we demonstrated that the downregulation of HMGB1 reduced the activation of the NLRP3 inflammasome as well as the production of inflammatory factors. Thus, as an upstream molecule of HMGB1, miR-574-5p plays a key role in ameliorating excessive inflammatory responses.

The expression of miR-574-5p changes in a variety of diseases (40–46). miR-574-5p is a candidate oncogene in various types of cancer (42, 47, 48). Conversely, other studies have found that miR-574-5p can also target a number of oncogenic proteins (40, 49). Serum miR-574-5p was found to be a prognostic predictor of patients with sepsis, and the expression levels of miR-574-5p were higher in survivors (50). However, there are very few studies about the biological function and molecular mechanism of miR-574-5p in patients with inflammatory disease and none in ARDS. Our study is the first to show the mechanism of how serum miR-574-5p concentrations are elevated and the biological function in ARDS.

This study has limitations. There were 45 miRNAs identified in the microRNA array that were differentially expressed in patients with pneumonia-related ARDS compared with healthy subjects, yet we restricted the focus of this work to miR-574-5p because we consider HMGB1 to play a key role in ARDS. However, we cannot rule out the possibility that other miRNAs also contribute to the beneficial effects in ARDS, and additional studies should be performed in the future.

In summary, we have determined that miR-574-5p regulates the expression of HMGB1, a key proinflammatory factor of ARDS that is involved in the excessive inflammatory responses both *in vitro* and *in vivo*. These studies support an idea that miR-574-5p may serve as an important negative regulator of inflammatory responses by controlling critical aspects of respiratory system homeostasis. Thus, these findings provide insight into the role of miR-574-5p in ARDS and may indicate a novel therapeutic approach to relieve ARDS. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Figure 6. (*Continued*). (*D*) HPMECs were cotransfected with miR-574-5p inhibitor/miR-NC inhibitor and si-HMGB1/si-NC. After 48 hours, the cells were treated with LPS for 6 hours. The HMGB1, NLRP3, and cleaved caspase-1 protein concentrations were evaluated by Western blotting ($n = 3$). (*E*) mRNA concentrations of IL-6, IL-1β, and TNF-α were measured by real-time qPCR in HPMECs treated as described in *C* ($n = 5$). (*F*) mRNA concentrations of IL-6, IL-1β, and TNF-α were measured by real-time qPCR in HPMECs treated as described in *D*. The values presented are the mean ± SD. Comparisons were made by *t* test in *A*. Comparisons were made by one-way ANOVA followed by Dunnett Multiple Comparison in *E* and *F*. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

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