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ORIGINAL RESEARCH

Haploinsufficiency of Cnot3 Aggravates Acid-Induced Acute Lung Injury Likely Through Transcriptional and Post-Transcriptional Upregulation of Pro-Inflammatory Genes

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Background: Acute lung injury (ALI) is caused by a variety of illnesses, including aspiration pneumonia and sepsis. The CCR4-NOT complex is a large multimeric protein complex that degrades mRNA through poly(A) tail shortening, whereas it also contributes to regulation of transcription and translation. Cnot3 is a scaffold component of the CCR4-NOT complex and is essential for the integrity of the complex; loss of Cnot3 leads to depletion of whole complex. While the significance of cytokine mRNA degradation in limiting inflammation has been established, the roles of CCR4-NOT complex-mediated in ALI remain elusive.

Methods: The effects of Cnot3 haploinsufficiency in the pathology and cytokine expression were analyzed in the mouse lungs of acid aspiration-induced acute lung injury. The decay rate and transcription activity of cytokine mRNAs under Cnot3 heterozygous deletion were analyzed in lipopolysaccharide (LPS) -stimulated mouse embryonic fibroblasts (MEFs).

Results: Tamoxifen-induced heterozygous deletion of Cnot3 in adult mice (Cnot3 Hetz) did not show body weight loss or any apparent abnormality. Under acid aspiration-induced acute lung injury, Cnot3 Hetz mice exhibited increased pulmonary edema, worse lung pathologies and more severe inflammation compared with wild type mice. mRNA expression of pro-inflammatory genes *Il1b* and *Nos2* were significantly upregulated in the lungs of Cnot3 Hetz mice. Consistently, mRNA expression of *Il1b* and *Nos2* was upregulated in LPS-stimulated Cnot3 Hetz MEFs. Mechanistically, while heterozygous depletion of Cnot3 stabilized both *Il1b* and *Nos2* mRNAs, the nascent pre-mRNA level of *Il1b* was upregulated in Cnot3 Hetz MEFs, implicating Cnot3-mediated transcriptional repression of *Il1b* expression in addition to destabilization of *Il1b* and *Nos2* mRNAs. PU.1 (Spi1) was identified as a causative transcription factor to promote *Il1b* expression under Cnot3 haploinsufficient conditions.

Conclusion: CNOT3 plays a protective role in ALI by suppressing expression of pro-inflammatory genes *Il1b* and *Nos2* through both post-transcriptional and transcriptional mechanisms, including mRNA stability control of Spi1.

Keywords: acute lung injury, ALI, ARDS, CNOT3, CCR4-NOT complex, deadenylation

Introduction

Acute respiratory distress syndrome (ARDS)/acute lung injury (ALI) is a life-threatening form of rapid progressive respiratory failure globally affecting approximately 3 million patients annually that accounts for 10% of intensive care unit admissions and is associated with 40% mortality in severe cases.[1,2] The ARDS is caused by a variety of pulmonary

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or non-pulmonary insults including aspiration pneumonia, sepsis, infection, trauma or major surgery, leading to acute onset of diffuse inflammatory lung injury which increases alveolar-capillary permeability causing the pathogenesis of pulmonary edema and loss of aerated lung tissue.[1,3] Despite the high mortality rate and extremely poor prognosis, treatment options for ARDS are limited as supportive care with fluid control and mechanical ventilation remains the mainstay of management.[4,5] There are no effective drugs for improving the clinical outcome of ARDS, and thus new mechanistic insights into the pathogenesis of ARDS/ALI are needed.

The CCR4-NOT (carbon catabolite repression 4–negative on TATA-less) complex is a large multimeric protein complex that is involved in multi-steps of mRNA regulations including transcription, translation and degradation. In mammals, the CCR4-NOT complex consists of two units, a catalytic unit containing two exonucleases [CNOT6 (or CNOT6L) and CNOT7 (or CNOT8)] and a non-catalytic unit including the subunits of CNOT1, CNOT2, CNOT3, CNOT9, CNOT10, and CNOT11.[6–9] While the catalytic unit is required for mRNA degradation via its deadenylase activity, the non-catalytic unit is involved in other RNA regulations, such as transcription and translation. CNOT3, a core scaffold subunit, is necessary to maintain the stability of CCR4–NOT complex. Thus, CNOT3 deficiency decreases the abundance of whole complex and impairs tissue homeostasis in vivo leading to embryonic lethality, osteoporosis, heart failure, liver damage, anemia and lipodystrophy.[10–14] A previous study reported that CNOT7(Caf1)-knockout mice worsened the symptoms of lipopolysaccharide(LPS)-induced ALI and that expression of IL-8 (CXCL1) and ICAM-1 was upregulated in Cnot7 knockout mice compared to wild type (WT) mice, suggesting the protective roles of CNOT7 deadenylase in ALI.[15] However, the involvement of CNOT3 in the pathogenesis of ALI remains elusive.

While transcriptional regulation for induction of inflammatory genes is critical for immune response to various threats such as infection or tissue damage, post-transcriptional regulation of inflammatory genes, such as degradation of cytokine mRNA, is important for cease inflammation. The conserved cis-element including AU-rich elements (AREs) and stem-loop (SL) structures are frequently found in the 3'UTR of mRNAs encoding inflammation-related genes and are involved in the regulation of mRNA stability.[16,17] The interaction of RNA binding proteins with cis-element, such as TTP or AUF binding to AREs or Roquin binding to SLs recruits the CCR4-NOT complex, thereby leading to degradation of mRNAs.[18–21] On the other hand, in our previous study, Cnot3 heterozygous knockout in mice downregulated histone modifications for active transcription (eg H3K9 acetylation) in the hearts, thereby exacerbating heart failure in response to pressure-overload stress.[10] In addition, we have also shown that the mRNAs encoding transcription factors and histone modification factors were co-immunoprecipitated with Cnot3 protein.[11] Thus, Cnot3 regulates transcription and epigenetics in maintaining cardiac functions. However, if any contribution of Cnot3 to transcription of inflammatory genes remains elusive. Thus, in case Cnot3 is involved in the pathogenesis of ALI, Cnot3 potentially plays roles in both transcriptional and post-transcriptional regulation of inflammatory gene expression, which needs to be explored.

In this study, to determine the roles of CNOT3 in ALI, we investigated whether Cnot3 haploinsufficiency in mice affects the severity of hydrochloric acid (HCl) aspiration-induced acute lung injury, which mimics the clinical situation of inhalation of acidic gastric contents by directly insulting lung epithelial cells and causes sterile inflammation.[22–24] To circumvent lethality in Cnot3 homozygous knockout mice or lean phenotypes in the mice with congenital Cnot3 heterozygous deletion,[10,25] we employed the strategy of tamoxifen-driven Cre-loxP-mediated heterozygous deletion of Cnot3 in adult mice (Cnot3 Hetz). We here show that Cnot3 Hetz mice aggravated acid-induced ALI accompanied with the upregulation of mRNAs encoding *Il1b* and *Nos2*. Heterozygous depletion of Cnot3 also led to stabilization and increased expression of *Il1b* and *Nos2* mRNAs in LPS-stimulated mouse embryonic fibroblasts (MEFs), whereas pre-mRNA expression of *Il1b* but not *Nos2* was upregulated in Cnot3 Hetz MEFs. Failed mRNA destabilization of the transcription factor *Spi1* was causative of increased *Il1b* expression in Cnot3 Hetz MEFs. Thus, CNOT3 represses expression of pro-inflammatory genes through both transcriptional and posttranscriptional regulation thereby exerting protective effects in ALI.

Materials and Methods

Heterozygous Knockout of Cnot3 in Mice

Cnot3 flox mice were generated by homologous recombination in ES cells, in which loxP sites flank exons 2 and 3 of the *Cnot3* gene.[11] The mice carrying heterozygous floxed allele of Cnot3 ($Cnot3^{+/flox}$) were crossed with CAG-MerCreMer

Tg mice (*CAG-cre/Esr1*5Amc*$^{Tg/+}$ mice).[26] *Cnot3*$^{+/flox}$*;CAG-MerCreMer*$^{+/Tg}$ mice and *Cnot3*$^{+/+}$;*CAG-MerCreMer*$^{+/Tg}$ mice served as WT mice are intraperitoneally administrated with 4-hydroxytamoxifen (4-OHT) (20 mg/kg per day; H6278, Sigma-Aldrich) for three times once two days. Mice were genotyped by PCR[11] and maintained at the animal facilities of Akita University and Kyushu University. All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals, Eighth Edition, updated by the US National Research Council Committee in 2011, and approvals of the experiments were granted by the ethics review board of Akita University and Kyushu University.

Acid-Induced Acute Lung Injury

For acid aspiration-induced acute lung injury, five-month-old mice at 18 days after 4-OHT treatment were anesthetized with anesthesia with cocktails of 0.2 mg/kg medetomidine, 6 mg/kg midazolam and 10 mg/kg butorphanol tartrate, and were intratracheally instilled with 0.02 M HCl (in 50 μL PBS). To evaluate lung pathologies, the mice were euthanized with overdose of anesthesia at 24 hours after acid instillation, and lungs were excised, as described previously.[27]

Lung Histology

Lung tissues were fixed with 4% formalin and embedded in paraffin. Five-μm-thick sections were prepared and stained with Hematoxylin & Eosin (H&E). For semi quantitative assessment of lung injury, the high-resolution images (x200 magnification) of the lung sections stained with H&E were taken with microscope (Nikon). Three randomly chosen fields of each section were scored for Lung injury score in a blinded fashion using a previously defined score consisting of alveolar congestion, hemorrhage, neutrophil infiltration, thickness of alveolar wall, and hyaline membrane formation, as follows: 0 = minimal (little) damage, 1 = mild damage, 2 = moderate damage, 3 = severe damage and 4 = maximal damage. The average Lung injury score of three fields of each section was used as the one individual Lung injury score.[28] For immunohistochemistry of Cnot3 in the lungs, sections were stained with anti-Cnot3 antibody[11] or Normal Mouse IgG (Merck, 12–371).

Cell Cultures

For induction of heterozygous deletion of Cnot3 in MEFs, MEFs were isolated from 13.5 days post-coitum embryos from the crossing of *Cnot3*$^{+/flox}$ mice with *CAG-MerCreMer*$^{+/Tg}$, and the genotypes were determined by PCR.[11] When plating, the MEFs of *Cnot3*$^{+/flox}$*;CAG-MerCreMer*$^{+/Tg}$ and *Cnot3*$^{+/+}$*;CAG-MerCreMer*$^{+/Tg}$ served as WT MEFs (passages 2 to 5) were treated with 1 μM 4-OHT. At 24 hours after plating, 100 ng/mL *Escherichia coli*-derived LPS were added. Twenty-four hours after LPS treatment, MEFs were subjected to RNA extraction or Actinomycin D chase. To examine whether Spi1 is involved in cytokine gene expression in response to LPS stimulation, MEFs were plated with siRNAs for control or two kinds of *Spi1* siRNAs. siRNA target sequences are listed in Supplementary Table 1. 4-OHT and LPS were added at 24 and 48 hours after cell seeding, respectively, and RNA was extracted at 24 hours after LPS treatment.

RNA Analyses

qRT-PCR analysis was conducted as previously described.[11] Briefly, total RNA was extracted from mice lungs or MEFs using TRIzol reagent (Invitrogen) and cDNA was synthesized using the PrimeScript RT reagent kit (RR037; TAKARA). Quantitative real-time PCR was run in 96 well plates using a SYBR Premix ExTaq II (RR820; TAKARA) according to the instructions of the manufacturer. Relative gene expression levels were quantified by using the Thermal Cycler Dice Real Time System II software (TAKARA). Sequences of the forward and reverse primers of the genes studied are shown in Supplementary Table 2. To assess mRNA stability, we treated MEFs with actinomycin D (2.5 μg/mL; Wako). Total RNA was extracted at the indicated time points after actinomycin D treatment and subjected to qRT-PCR.

Measurements of Transcriptional Activity of NF-κB

At 24 hours after plating of MEFs with 4-OHT, the MEFs were treated with LPS and nuclear protein was extracted using NE-PER Nuclear and Cytoplasmic Extraction Kit (78833, Thermo Fisher Scientific) at the indicated time points after LPS treatment. The concentration of nuclear protein was quantified using BCA Protein Assay Kits (23225, Thermo Fisher Scientific) and 3 μg of nuclear protein was applied to p65 Transcription Factor Assay Kit (Abcam, ab133112) to assess the transcriptional activity of NF-κB.

Western Blotting

Lung proteins were extracted with TNE lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP40, protease inhibitor (Complete Mini; Roche), 20 mM NaF, 2 mM Na_3VO_4) with Microsmash (MS-100R; TOMY), were sonicated and denatured with LDS sample buffer (Invitrogen) at 70 °C. Proteins were electrophoresed on NuPAGE bis-tris precast gels (Invitrogen) and transferred to nitrocellulose membranes (Invitrogen). Membranes were probed with anti-IL-1β antibody (CST, 12242, 1:1000 diluted) or anti-GAPDH antibody (Proteintech, 10494-1-AP, 1:3000 diluted). The blotting bands visualized with ECL reagent (Bio-Rad) using ChemiDoc Touch Imaging System (Bio-Rad).

Statistical Analyses

Data are presented as mean values ± SEM. Comparisons of parameters among groups were analyzed by one-way analysis of variance (ANOVA), followed by Sidak's multiple-comparisons test. When a comparison is done for groups with two factor levels, two-way ANOVA with Sidak's multiple-comparisons test were used. $P < 0.05$ was considered significant.

Results

Cnot3 Haploinsufficiency Worsens Acid-Induced ALI

To determine the roles of Cnot3 in ALI, we generated inducible Cnot3 heterozygous mice, because homozygous deletion of Cnot3 results in die in early embryos. We crossed the mice carrying heterozygous floxed allele of Cnot3 (*Cnot3*$^{+/flox}$) with CAG-MerCreMer Tg mice to generate *Cnot3*$^{+/flox}$*;CAG-MerCreMer*$^{+/Tg}$ mice and induce Cnot3 haploinsufficiency in the adult mice by tamoxifen treatment (Figure 1A). We confirmed that expression of *Cnot3* mRNA was decreased in the lungs of *Cnot3*$^{+/flox}$*;CAG-MerCreMer*$^{+/Tg}$ mice approximately to the half of that in Cnot3 WT mice (*Cnot3*$^{+/+}$*;CAG-MerCreMer*$^{+/Tg}$) after intraperitoneal injection of tamoxifen (Figure 1B). Hereafter, tamoxifen-treated *Cnot3*$^{+/flox}$*;CAG-MerCreMer*$^{+/Tg}$ mice are referred to as Cnot3 Hetz mice. Cnot3 Hetz mice were apparently healthy and had normal reproductive function.

Acid aspiration-induced ALI model mimics the aspiration pneumonia while is useful to evaluate the significance of genes/molecules in ALI pathologies, because the model exhibits severe lung pathologies in a reproducible manner.[27,29] Acid instillation induced modest but significant injury and lung edema in WT mice (Figure 1C). Lung edema was quantified by the ratio of wet weight to dry weight of the lungs (Wet/Dry ratio), lung weight to body weight (LW/BW) or lung weight to tibia length (LW/TL), and all the parameters of lung edema were upregulated in WT mice with acid instillation compared with sham-operated WT mice (Figure 1D–F). When Cnot3 Hetz mice were subjected to acid lung injury, the mice showed more severe lung injury with massive hemorrhage and edematous appearance than WT mice with acid injury (Figure 1C). The parameters of lung edema were significantly increased in Cnot3 Hetz mice compared with WT mice (Figure 1D–F). Consistently, histological analysis showed the pathologies of severe lung inflammation and damage in Cnot3 Hetz mice (Figure 1G and H; Table 1).

Cnot3 Haploinsufficiency Upregulates mRNA Expression of Il1b and Nos2 in the Lungs of Acid-Induced ALI

While mRNA expression of various pro-inflammatory cytokines was upregulated at 5 h after acid instillation in the lungs of WT mice (not shown), expression of all pro-inflammatory genes was downregulated to baseline at 24 h after acid aspiration (Figure 2A). By contrast, *Il1b* and *Nos2* mRNA levels were significantly up-regulated in Cnot3 Hetz mice with acid injury (Figure 2A). Furthermore, both precursor and mature form of IL-1β proteins were up-regulated in Cnot3 Hetz mice with acid injury compared to WT mice with acid injury (Figure 2B). A trend toward elevated, but not significant, expression of *Tnfa, Il6* and *Cxcl1* was also observed in Cnot3 Hetz mice.

CNOT3 Promotes mRNA Decay of Il1b and Nos2 Genes, and Represses Transcription of Il1b mRNA

Immunohistochemical analysis revealed that Cnot3 protein is primarily located in the interstitial area of alveoli, most likely in lung fibroblasts, suggesting that Cnot3 in the fibroblasts is involved in inflammatory responses in lungs (Supplementary Figure 1).

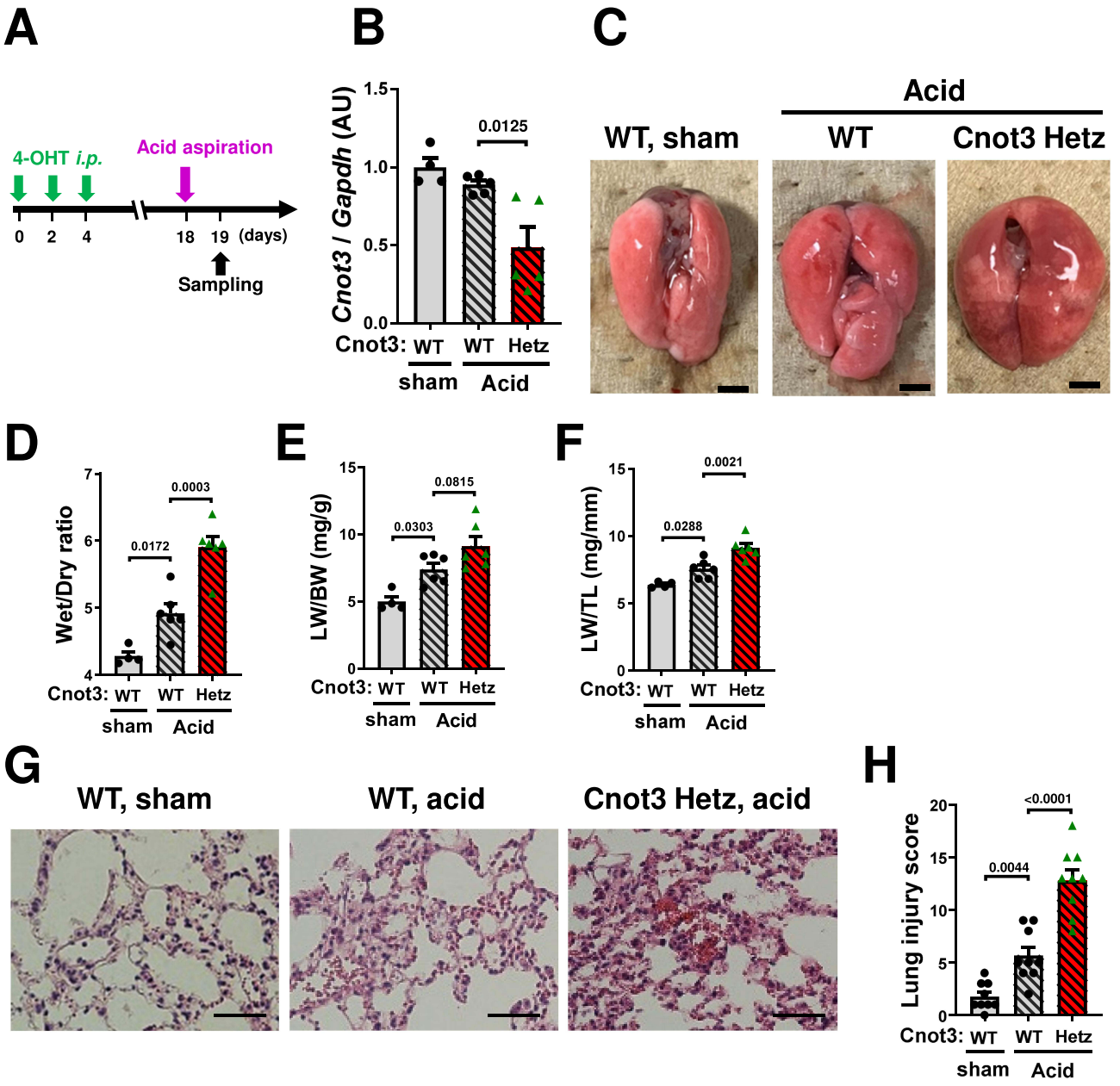


Figure 1 Cnot3 haploinsufficiency worsens acid-induced acute lung injury (ALI). (**A**) Experimental protocol; For induction of Cnot3 heterozygous deletion (Cnot3 Hetz), 5-month-old mice were intraperitoneally (*i.p.*) administrated with 4-hydroxytamoxifen (4-OHT) (20 mg/kg per day) once in two days for three times. Eighteen days after the first 4-OHT administration, the mice were intra-tracheally instilled with acid (0.02 M HCl, 50 μL per body). Lungs were harvested at 24 hours after acid instillation. (**B**) mRNA expression of *Cnot3* in mouse lungs. WT + sham (n = 4), WT + Acid (n = 5), Cnot3 Hetz + Acid (n = 5). (**C**) Representative photograph of mouse lungs. Bars indicate 2 mm. (**D**) Wet to dry weight ratio of lungs. (**E**) The ratio of lung weight (LW) to body weight (BW). (**F**) The ratio of lung weight (LW) to tibia length (TL). WT + sham (n = 4), WT + Acid (n = 6), Cnot3 Hetz + Acid (n = 6) (**D–F**). (**G**) Representative images of lung histopathology. Bars indicate 200 μm. (**H**) Lung injury score measurements. WT + sham (n = 9), WT + Acid (n = 9), Cnot3 Hetz + Acid (n = 9). All values are means ± SEM. One-way ANOVA with Sidak's multiple-comparisons test. Numbers above square brackets show significant *P*-values.

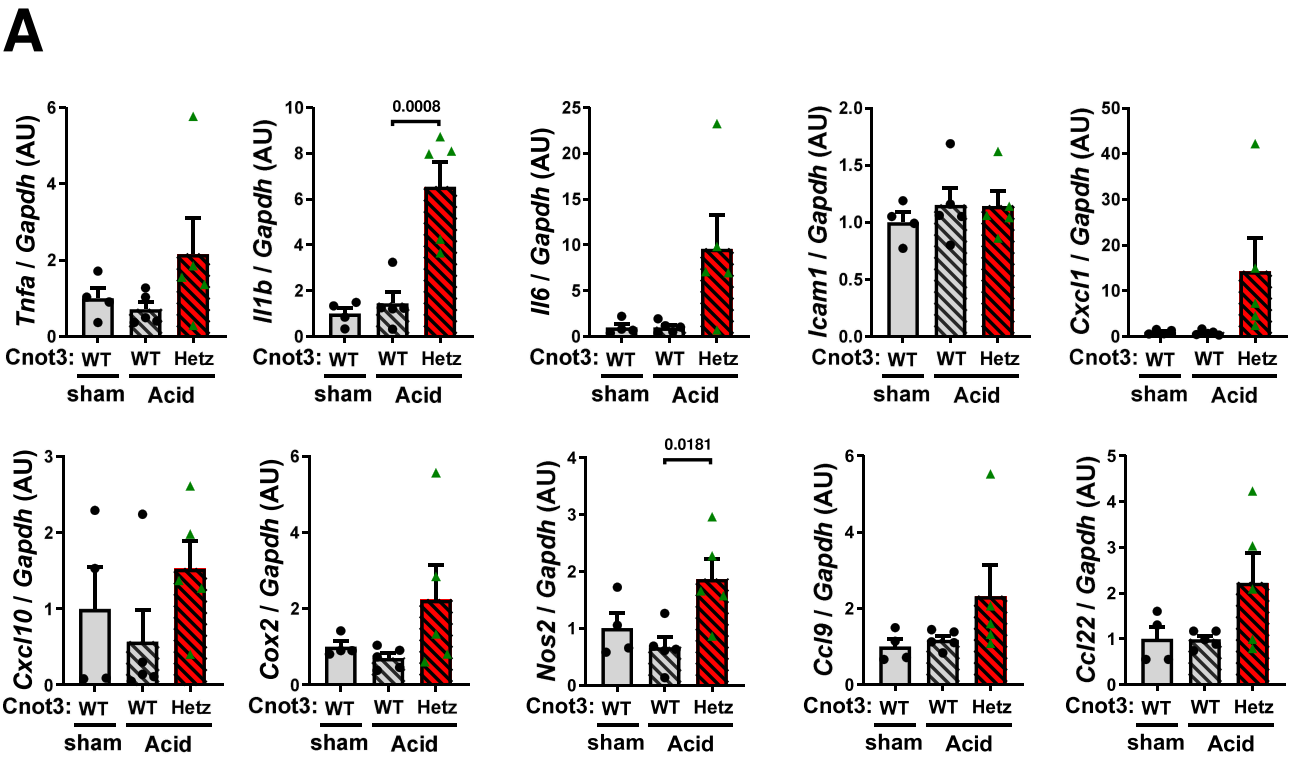
Thus, we next examined the effects of Cnot3 haploinsufficiency on *Il1b* and *Nos2* mRNA expression in the fibroblasts (mouse embryonic fibroblasts; MEFs). The MEFs of *Cnot3*$^{+/flox}$;*CAG-MerCreMer*$^{+/Tg}$ were isolated from embryos at 13.5 dpc and treated with tamoxifen to obtain Cnot3 Hetz MEFs. RT-qPCR analysis confirmed that Cnot3 mRNA levels were halved in Cnot3 Hetz MEFs compared to those in WT MEFs (Figure 3A). Cell growth and viability of Cnot3 Hetz MEFs were comparable to those of WT MEFs (not shown). Cnot3 Hetz MEFs showed the same levels of *Il1b* and *Nos2* mRNA expression as WT MEFs in vehicle-treated conditions (Figure 3B). On the other hand, when the MEFs were treated with LPS for 24 h, Cnot3 Hetz MEFs

Table 1 Lung Injury Score for Mice with Acid-Induced Lung Injury

Group	Alveolar Congestion	HemorrhAge	Neutrophil Infiltrates	Alveolar Wall Thickness	Hyaline Membrane Formation	Total Scores
Saline, WT	0.22±0.15	0.33±0.17	0.89±0.11	0.33±0.17	0.00±0.00	1.78±0.43
Acid, WT	0.56±0.29	0.56±0.29	1.89±0.11	1.33±0.24	1.33±0.17	5.67±0.82
Acid, Cnot3 Hetz	2.11±0.26**	2.11±1.17**	3.22±0.22**	2.56±0.29**	2.78±0.22**	12.78±1.04**

Notes: Data are shown as mean values ± SEM. n = 9 each for experimental groups. **$P < 0.01$, one-way ANOVA with Sidak's multiple comparisons test. Independent experiments were performed two times, and consistent results were obtained.

showed significantly higher expression of *Il1b* and *Nos2* (Figure 3B), recapitulating gene expression in the lungs of Cnot3 Hetz mice with acid injury. Since haploinsufficiency of Cnot3 potentially impairs the deadenylase activity of CCR4-NOT complex, we examined the stability of *Il1b* and *Nos2* mRNAs by analyzing mRNA decay under transcription-halted condition with



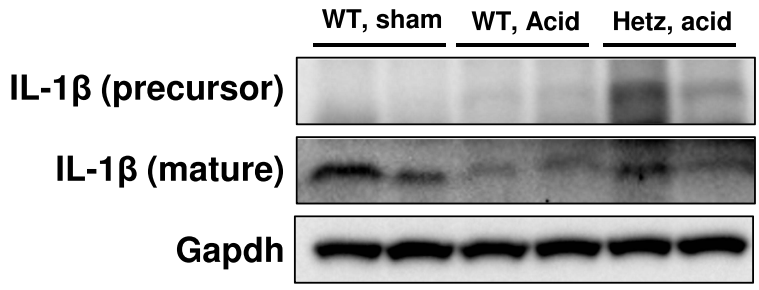


Figure 2 mRNA levels of *Il1b* and *Nos2* are upregulated in the lungs of Cnot3 Hetz mice with acid aspiration. (**A**) mRNA expression of *Tnfa, Il1b, Il6, Icam1, Cxcl1, Cxcl10, Cox2, Nos2, Ccl9, Ccl22* in acid-instilled mouse lungs. WT + sham (n = 4), WT + Acid (n = 5), Cnot3 Hetz + Acid (n = 5). All values are means ± SEM. One-way ANOVA with Sidak's multiple-comparisons test. Numbers above square brackets show significant *P*-values. (**B**) Protein expression of IL-1β in acid-instilled mouse lungs.

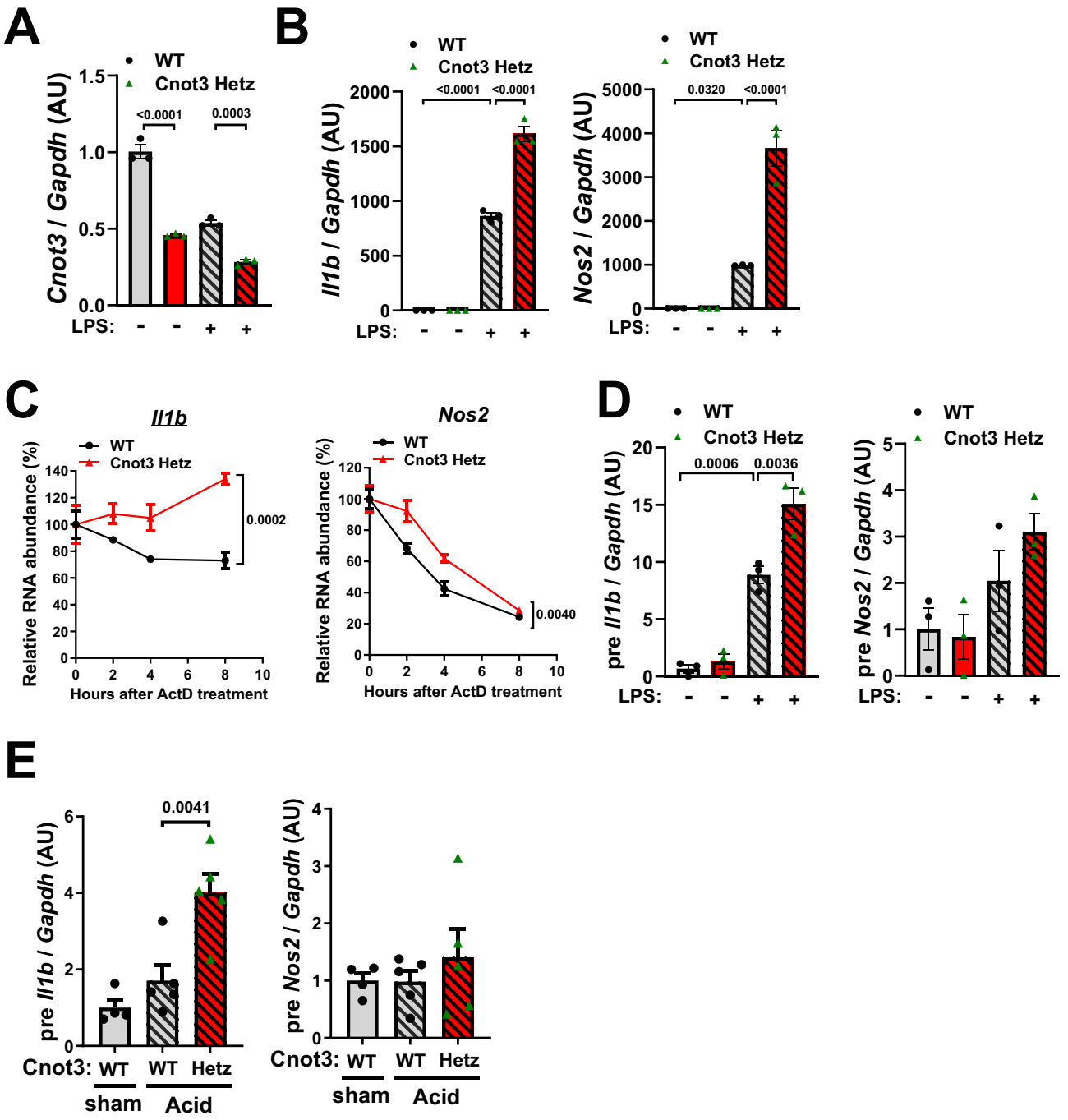


Figure 3 CNOT3 promotes degradation of *Il1b* and *Nos2* mRNAs, and represses transcription of *Il1b* in response to inflammation stimulus. (**A** and **B**) mRNA expression of *Cnot3* (**A**), *Il1b* and *Nos2* (**B**) in MEFs. MEFs were treated with 1 μM 4-OHT to induce the heterozygous deletion of Cnot3. Twenty-four hours after 4-OHT treatment, MEFs were treated with 100 ng/mL *Escherichia coli*-derived lipopolysaccharide (LPS). Cells were harvested at 24 hours after LPS treatment (n = 3). (**C**) mRNA stability of *Il1b* and *Nos2* mRNAs in MEFs. Cells were harvested at the indicated time points after actinomycin D (ActD) treatment (n = 3). (**D**) Expression of nascent pre-mRNA of *Il1b* and *Nos2* in MEFs (n = 3). (**E**) Nascent pre-mRNA expression of *Il1b* and *Nos2* in acid-instilled mouse lungs. WT + sham (n = 4), WT + Acid (n = 5), Cnot3 Hetz + Acid (n = 5). All values are means ± SEM. One-way ANOVA with Sidak's multiple-comparisons test (**A** and **B, D** and **E**). Two-way ANOVA with Sidak's multiple-comparisons test (**C**). Numbers above or next to square brackets show significant *P*-values.

Actinomycin D treatment. Both *Il1b* and *Nos2* mRNAs were stabilized in Cnot3 Hetz MEFs compared with WT MEFs, albeit the decay rate of *Il1b* mRNA in WT MEFs is so slow and thus the half-life is quite long. Thus, Cnot3 haploinsufficiency impairs the degradation of *Il1b* and *Nos2* mRNAs (Figure 3C). Due to the long half-life of *Il1b* mRNA, we reasoned that transcription might be also involved in regulation of *Il1b* mRNA expression. To address the effects of Cnot3 haploinsufficiency on transcription of *Il1b* and *Nos2* genes, we measured the nascent pre-mRNA levels. As such, expression of *Il1b* pre-mRNA was up-regulated in

Cnot3 Hetz MEFs compared with WT MEFs, whereas that of *Nos2* pre-mRNA was not affected (Figure 3D). Consistently, nascent pre-mRNA level of *Il1b* but not *Nos2* was significantly up-regulated in the lungs of Cnot3 Hetz mice with acid injury (Figure 3E). These results suggest that CNOT3 promotes degradation of *Nos2* mRNA and to a lesser extent *Il1b* mRNA, while it represses the transcription of *Il1b* in the lungs of acid-induced ALI.

Cnot3 Haploinsufficiency Promotes Il1b Transcription Due to Impaired mRNA Degradation of Spi1

To address transcriptional regulation of *Il1b* expression, we examined the effects of Cnot3 haploinsufficiency on transcriptional activity of NF-κB, an essential transcriptional regulator in the TLR4 signaling driven by LPS,[30,31] by measuring the binding activity of p65 subunit of NF-κB to oligo DNAs of NF-κB response element. However, the transcriptional activity of p65 in LPS-treated Cnot3 Hetz MEFs was comparable to that in LPS-treated WT MEFs (Figure 4A). Spi-1 Proto-Oncogene /PU.1 (*Spi1*) is a transcription factor that is involved in the differentiation of immune cells and transcriptional regulation of

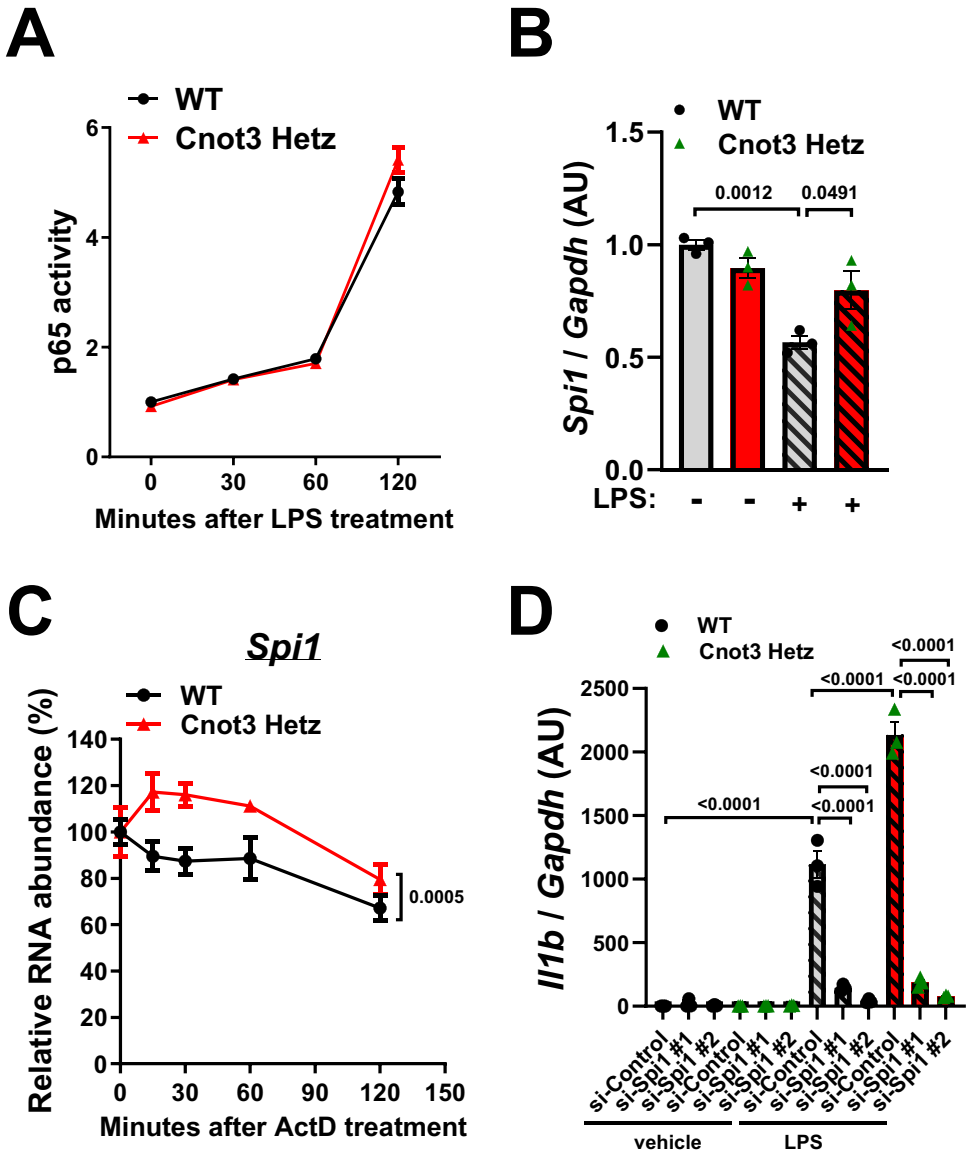


Figure 4 Cnot3 downregulates transcription of *Il1b* by promoting *Spi1* mRNA degradation. (**A**) Time course analyses of NF-κB transcriptional activity after LPS treatment in MEFs. Cells were harvested at the indicated time points after LPS treatment (n = 3). (**B**) mRNA expression of *Spi1* in MEFs (n = 3). (**C**) mRNA stability of *Spi1* in MEFs. Cells were harvested at the indicated time points after ActD treatment (n = 3). (**D**) mRNA expression of *Il1b* in MEFs (n = 3). All values are means ± SEM. One-way ANOVA with Sidak's multiple-comparisons test (**B** and **D**). Two-way ANOVA with Sidak's multiple-comparisons test (**C**). Numbers above or next to square brackets show significant *P*-values.

Il1b.[32,33] In response to LPS stimulation, *Spi1* mRNA level was downregulated in WT MEFs, whereas it was maintained and higher in Cnot3 Hetz MEFs than WT MEFs (Figure 4B). Furthermore, *Spi1* mRNA was stabilized in Cnot3 Hetz MEFs under Actinomycin D treatment compared with WT MEFs, suggesting that the up-regulation of *Spi1* in Cnot3 Hetz MEFs is likely due to impaired deadenylation of *Spi1* mRNA (Figure 4C). The upregulated mRNA expression of *Il1b* in Cnot3 Hetz MEFs was suppressed by knockdown of *Spi1* (Figure 4D). These results indicate that CNOT3 promotes destabilization of *Spi1* mRNA thereby suppressing the transcription of *Il1b* gene in response to inflammatory stimuli.

Discussion

In this study, we showed that tamoxifen-induced heterozygous deletion of Cnot3 in adult mice aggravates the pathologies of acid aspiration-induced lung injury compared with WT mice, and that Cnot3 haploinsufficiency upregulated *Il1b* and *Nos2* mRNAs in the lungs of acid aspirated. Mechanistically, Cnot3 haploinsufficiency leads stabilization of mRNAs encoding pro-inflammatory genes *Il1b* and *Nos2*, and a transcription factor *Spi1*, which in turn promotes transcription of *Il1b*, resulting in exacerbation of acute lung injury.

Previous reports showed that germline CNOT3 heterozygosity exhibits lean phenotypes due to the upregulation of mRNAs encoding energy metabolism in liver and adipose tissues,[25] while the tamoxifen-inducible heterozygous knock-out of Cnot3 in adult mice used in the present study showed no weight loss, suggesting that effects of Cnot3 haploinsufficiency in development can be ruled out in these mice. A previous study showed that knockout mice of CNOT7, a deadenylase of the CCR4-NOT complex, exacerbates LPS-induced acute lung injury due to the upregulation of ICAM-1 and IL8 (CXCL1).[15] The expression of both mRNAs in the lungs of Cnot3 Hetz mice after acid aspiration was not significantly changed. IL-1β has previously been shown to exacerbate lung edema by increasing lung vascular permeability via an αvβ5/6 integrin and TGF-β–dependent mechanisms.[34,35] NOS2 is involved in the regulation of pulmonary inflammation, and administration of 1400W, a specific NOS2 inhibitor, has been reported to attenuate bleomycin-induced lung inflammation.[36] Thus, Cnot3 plays a protective role in the lungs of ALI by repressing the expression of IL-1β and NOS2.

Previous studies had shown that tristetraprolin (TTP) interacts with AU-rich elements in the 3' UTR of mRNAs encoding *Il1b* and *Nos2*, and that loss of TTP increases the expression of *Il1b* and *Nos2* mRNAs.[37,38] Since TTP promotes mRNA destabilization by recruiting the mRNA to CCR4-NOT deadenylase complex through direct binding to the central domain of CNOT1,[39] a core scaffold protein of the complex, Cnot3 haploinsufficiency might have resulted in impaired deadenylation of TTP-interacting mRNAs. On the other hand, the post-transcriptional regulation for *Spi1* mRNA has been reported based on the discovery of reduced expression and destabilization of *Spi1* mRNA on differentiating murine erythroleukemia cells,[40] but the underlying mechanisms remain unclear. The present study suggests that Cnot3 contributes to the repression of *Spi1* expression via deadenylation, which is crucial for the dampening of *Il1b* expression. The fact that *Spi1* mRNA is silenced as a target of MicroRNA-155 in B cells[41] and that the CNOT9, a non-enzymatic protein of CCR4-NOT complex, directly binds to GW182/TNRC6 in the RISC complex harboring microRNA,[42] suggests that Cnot3 is required for deadenylation of *Spi1* mRNA recruited by microRNA-155 and RISC complex.

Recently, the integrated analyses of multiple RNA-seq datasets in blood samples and leukocytes derived from COVID-19 patients identified SPI1 as a central transcriptional factor associated with the up-regulated genes by SARS-CoV2 infection.[43] In addition, the functional roles of Spi1 in lung macrophages promoting pro-inflammatory cytokines in sepsis-related lung inflammation had been demonstrated by using bone marrow chimera mice with conditional Spi1 knockout.[44] Thus, Spi1 has a pivotal role in the pathogenesis of various inflammatory diseases in the lung. In the present study, we used MEFs to evaluate the general immune response under the Cnot3 haploinsufficiency, however, further addresses were needed to dissect the interaction of CNOT3 with SPI1 worsening pneumonia in the cell consisting of the lung including epithelial cells, hematopoietic cells, endothelial cells, and fibroblasts for the best understanding the mechanisms leading aggravation of ALI.

Conclusion

In summary, our findings of transcriptional- or posttranscriptional regulation mediated by Cnot3 for *Il1b, Nos2*, and *Spi1* gene expression may contribute to suppressing the exacerbation of ALI. Since there are no effective drugs for improving the clinical outcome of ALI, future work is needed to evaluate whether compounds modulating levels of CNOT3 protein or target mRNAs for deadenylation are beneficial for reducing the severity of ALI. Since siRNA knockdown of Spi1 markedly abolished IL-1β expression in LPS-stimulated cells, the compounds targeting SPI1 might be novel therapeutics for treating ALI patients.

Abbreviations

ARDS, Acute respiratory distress syndrome; ALI, acute lung injury; CCR4-NOT, carbon catabolite repression 4–negative on TATA-less, WT, wild type; Hetz, heterozygous; MEF, mouse embryonic fibroblast; LPS, lipopolysaccharide; ARE, AU-rich element; SL, stem-loop; 4-OHT, 4-hydroxytamoxifen.

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Disclosure

The authors report no conflicts of interest in this work.

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