



CELL INJURY, REPAIR, AGING, AND APOPTOSIS

# Inhibition of a Microbiota-Derived Peptide Ameliorates Established Acute Lung Injury



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Acute lung injury (ALI) is a clinical syndrome characterized by a diffuse lung inflammation that commonly evolves into acute respiratory distress syndrome and respiratory failure. The lung microbiota is involved in the pathogenesis of ALI. Corisin, a proapoptotic peptide derived from the lung microbiota, plays a role in ALI and acute exacerbation of pulmonary fibrosis. Preventive therapeutic intervention with a monoclonal anticorisin antibody inhibits ALI in mice. However, whether inhibition of corisin with the antibody ameliorates established ALI is unknown. Here, the therapeutic effectiveness of the anticorisin antibody in already established ALI in mice was assessed. Lipopolysaccharide was used to induce ALI in mice. After causing ALI, the mice were treated with a neutralizing anticorisin antibody. Mice treated with the antibody showed significant improvement in lung radiological and histopathologic findings, decreased lung infiltration of inflammatory cells, reduced markers of lung tissue damage, and inflammatory cytokines in bronchoalveolar lavage fluid compared with untreated mice. In addition, the mice treated with anticorisin antibody showed significantly increased expression of antiapoptotic proteins with decreased caspase-3 activation in the lungs compared with control mice treated with an irrelevant antibody. In conclusion, these observations suggest that the inhibition of corisin is a novel and promising approach for treating established ALI. (*Am J Pathol* 2023, 193: 740–754; <https://doi.org/10.1016/j.ajpath.2023.03.003>)

Acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS), are diffuse injury of alveolar epithelial cells and pulmonary capillary endothelial cells that disrupts the alveolar epithelial-endothelial barrier, leading to impaired gas exchange and acute hypoxic respiratory failure.<sup>1,2</sup> The presence of hyaline membrane, edema, neutrophil infiltration, fibrin deposition, alveolar collapse, and increased apoptosis of alveolar epithelial cells and capillary endothelial cells are characteristic histopathologic findings in ALI/ARDS.<sup>1,2</sup> The most frequent causative factors of ALI/ARDS are pulmonary infection (35%; eg, bacteria, viruses, and fungi), nonpulmonary sepsis (30%; eg, peritonitis and urinary tract or skin infection), aspiration (10%; eg, gastric juice), and trauma (10%; eg, burning, blunt injury).<sup>3</sup> Newly emerging causes of ALI/ARDS are

lung injury associated with an e-cigarette, vaping, chemotherapy, immunotherapy (eg, checkpoint inhibitors), and periodic outbreaks of infection by new strains of virus, including the severe acute respiratory syndrome coronavirus

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V.F.D., C.N.D.-G., and T.Y. contributed equally to this work.

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(SARS-Co-V), H1N1 influenza, Middle East respiratory syndrome coronavirus, and SARS-Co-V2.<sup>3</sup> This latter is the cause of the ongoing coronavirus disease 2019 (COVID-19) pandemic. Under nonoutbreak conditions, the annual number of patients with ALI/ARDS worldwide varies between 1 and 5 million and underlies approximately 10% of all admissions to the intensive care unit.<sup>3–7</sup> The number of annual deaths for ALI/ARDS is approximately 200,000 in the United States.<sup>6</sup> The mainstay of treatment for ALI/ARDS is lung-protective mechanical ventilation. Adjunctive therapies include treatment of the underlying cause, prone positioning, lung recruitment maneuvers, corticosteroids, extracorporeal membrane oxygenation, and CO<sub>2</sub> removal.<sup>2,5,8,9</sup> However, despite these recent advances in therapeutic modalities, the mortality rate of patients with ALI/ARDS remains high. The intrahospital mortality is 34.9% in patients with mild ALI/ARDS, 40% in those with moderate disease, and 46.1% in patients with severe disease.<sup>4</sup> Survivors of ALI/ARDS also continue to have a poor health-related quality of life; very few of them return to their original work several months after hospital discharge.<sup>10–12</sup> Therefore, there is an urgent need to develop new therapeutic strategies for patients with ALI/ARDS.

Experimental and clinical evidence in the literature suggests that perturbation or dysbiosis of the microbial population within the human body or microbiota is involved in the pathogenesis of critical illness, including ALI/ARDS.<sup>13</sup> Lipopolysaccharide (LPS)—induced acute lung injury provokes significant changes in the microbial body composition of rodents, and alteration of the microbiota composition by therapy (eg, antibiotics and diet) exerts a significant impact on disease outcome in rodents with sepsis or LPS-induced septic shock.<sup>14–19</sup> In humans, changes in the lung microbiota by smoking are associated with the development of ARDS following severe trauma.<sup>20</sup> In addition, translocation of gut bacteria to the lungs (gut-lung axis) has been demonstrated in murine models of sepsis and patients with ARDS, and enrichment of the lung microbiome with gut bacteria and respiratory tract dysbiosis is associated with worse clinical outcomes in critically ill patients.<sup>20–25</sup> However, despite advances made in understanding the role of the lung microbiome in the mechanism of ALI/ARDS, a molecular target with inherent therapeutic potential has not been identified so far. A proapoptotic peptide called corisin was recently identified and characterized in the lung microbiota of mice with lung fibrosis.<sup>26</sup> Corisin is a fragment of bacterial transglycosylase released after the degradation of the protein by a putative serine protease.<sup>26</sup> Corisin induces acute exacerbation of pulmonary fibrosis in mice, and its bronchoalveolar lavage fluid (BALF) and serum levels are dramatically increased in patients with idiopathic pulmonary fibrosis with acute exacerbation.<sup>26,27</sup> Interestingly, i.p. administration of a neutralizing monoclonal anticorisin antibody before the intratracheal instillation of LPS to wild-type mice significantly prevented LPS-induced lung inflammation in mice, suggesting the role of corisin in acute lung injury.<sup>27</sup>

The present study tested the hypothesis that inhibition of corisin will ameliorate established LPS-induced lung injury. To interrogate this hypothesis, ALI was induced in mice by intratracheal instillation of LPS, and then the mice were treated with anticorisin monoclonal antibody.

## Materials and Methods

### Animals

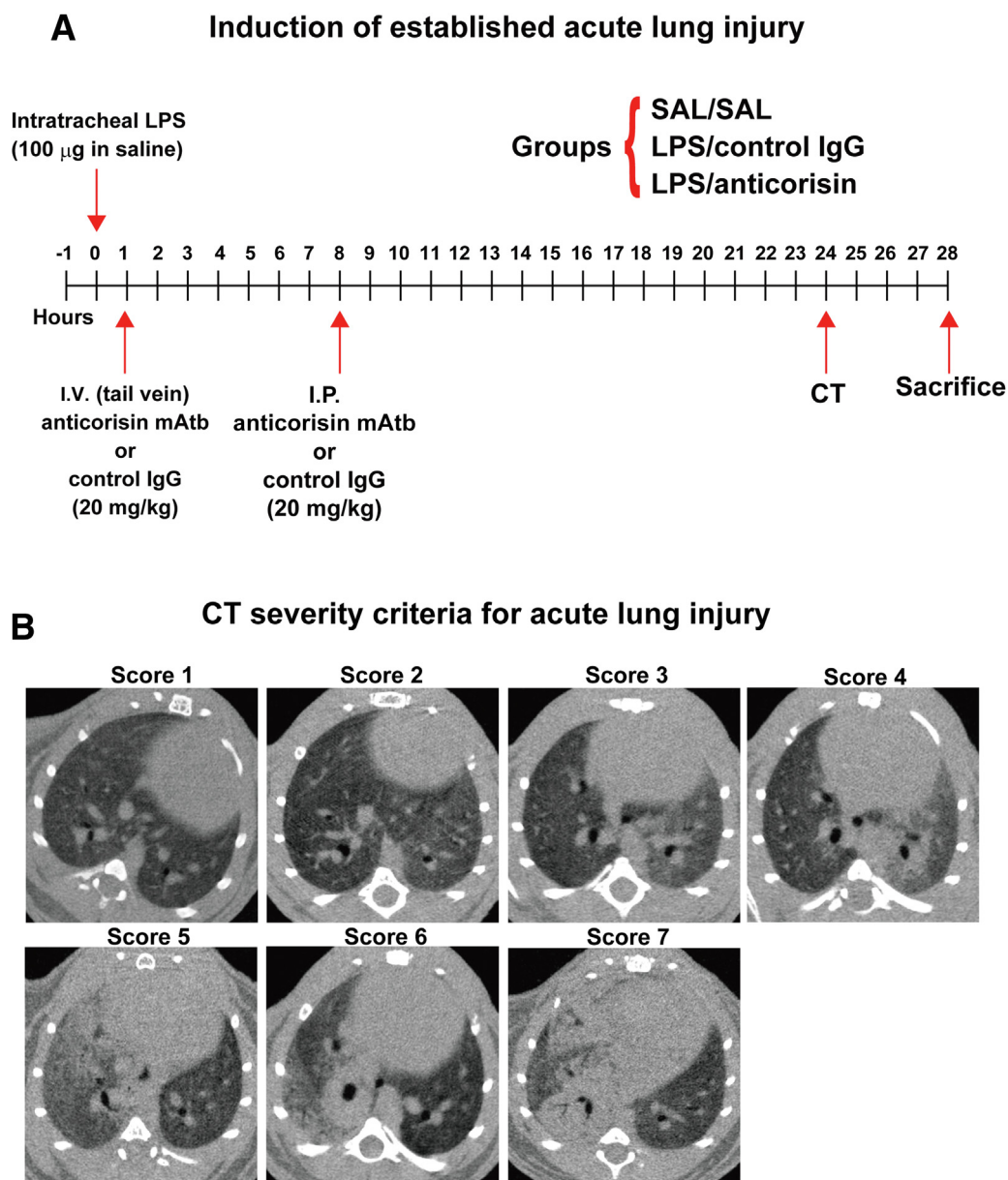
Male wild-type C57BL/6J mice were purchased from Nihon SLC (Hamamatsu, Japan). The weight of the mice was 20 to 22 g, and their age was 8 to 9 weeks. The mice were kept in the new environment for 2 weeks before using them in the experiments. The animals were maintained in a specific pathogen-free environment at a temperature of 21°C and under a 12-hour light/dark cycle in the Experimental Animal Center of Mie University (Tsu, Japan). The mice were allocated in cages containing wood-wool nesting material with free access to water and food *ad libitum*.

### Ethical Statement

The Committee for Animal Investigation of Mie University approved the experimental protocols (approval number: 2021-5; September 6, 2021). All experimental procedures were performed following internationally approved laboratory animal care principles published by the NIH.<sup>28</sup> In addition, the research followed the Animal Research: Reporting of In Vivo Experiments (ARRIVE) Guidelines for animal investigation, and variables were measured blindly of the treatment groups (<https://arriveguidelines.org>, last accessed March 8, 2023).

### Experimental Model of Acute Lung Injury

Mice that received intratracheal instillation of LPS (100 µg dissolved in 75 µL of saline) under normal air condition were treated with monoclonal anticorisin antibody (LPS/anticorisin group) or control IgG (LPS/control IgG) at a dose of 20 mg/kg of mouse weight by i.v. route 1 hour after and by i.p. route 8 hours after LPS instillation.<sup>29,30</sup> Control mice received intratracheal instillation of physiological saline (75 µL) and then were treated with saline (SAL/SAL) by i.v. route 1 hour after and by i.p. route 8 hours after intratracheal saline instillation (Figure 1A). Previous studies have shown that inflammatory markers in the lungs increase as early as 1.5 hours after lung injury and that early initiation of treatment for lung injury may improve clinical outcomes.<sup>31,32</sup> Therefore, in the present study, the treatment with anticorisin neutralizing monoclonal antibody (mAtb) was started by i.v. route 1 hour after inducing lung injury with LPS. The mice received the same dose of anticorisin neutralizing mAtb or control IgG by i.p. route 8 hours after inducing lung injury to maintain an effective circulating antibody concentration before euthanasia. Chest computed



**Figure 1** Experimental plan and computed tomography (CT) criteria for acute lung injury. **A:** Wild-type C57BL/6 mice were intratracheally instilled with lipopolysaccharide [LPS; 100 µg in 75 µL of saline (SAL)] and then treated with monoclonal anticorisin antibody (LPS/anticorisin) or control IgG (LPS/control IgG; 20 mg/kg of mouse weight) by i.v. route 1 hour after and by i.p. route 8 hours after LPS instillation. Control mice were intratracheally instilled physiological SAL (75 µL) and then treated with SAL by i.v. route 1 hour after and by i.p. route 8 hours after intratracheal SAL instillation. **B:** Chest CT was performed, and acute lung injury was scored, as described in *Materials and Methods*. mAtb, monoclonal antibody.

tomography (CT) was performed 24 hours after the intratracheal instillation of LPS or SAL. Mice were sacrificed 28 hours after LPS or saline administration to collect BALF, blood, and both lungs for biochemical and histopathologic studies.

### Evaluation of ALI Severity

The severity of ALI was assessed using a scoring system based on previously reported histopathologic findings of ALI, including neutrophil accumulation, hyaline membrane, interstitial thickening, formation of microthrombi,

atelectasis, and hemorrhage.<sup>33,34</sup> The presence of each of these findings was scored as follows: 0, no finding; 1, focalized finding; and 2, diffuse finding.

### CT Study

A micro-CT Latheta LCT-200 from Hitachi Aloka Medical (Tokyo, Japan) was used for the radiological study. After anesthesia, mice anesthetized by isoflurane inhalation were placed in a prone position, as previously described.<sup>35</sup> The radiological findings of LPS-induced lung injury were evaluated using a CT score as follows: 1, normal radiological lung

**Table 1** Primers for RT-PCR

Gene	Direction	Sequence	Length (no. of nucleotides)	Tm	Reference*	Location	Product size, bp
<i>Gapdh</i>	Sense	5'-TGGCCTTCCGTGTTCTCTAC-3'	19	61.3	NM_008084	686-704	178
	Antisense	5'-GAGTTGCTGTGAAGTCGCA-3'	20	60.9		863-844	
<i>Birc1a</i> ( <i>Naip1</i> )	Sense	5'-TGCCCAGTATATCCAAGGCTAT-3'	22	60.2	NM_008670	708-729	116
	Antisense	5'-AGACGCTGTCGTTGCAGTAAG-3'	21	62.6		823-803	
<i>Birc1b</i> ( <i>Naip2</i> )	Sense	5'-AGCTTGGTGTCTGTTCTCTGT-3'	21	61	NM_001126182	1204-1224	180
	Antisense	5'-GCGGAAAGTAGCTTTGGTGTAG-3'	22	61.2		1383-1362	
<i>Birc2</i> ( <i>c-IAP1</i> )	Sense	5'-TGTGGCCTGATGTTGGATAAC-3'	21	60	NM_007465	256-276	164
	Antisense	5'-GGTGACGAATGTGCAAATCTACT-3'	23	60.9		419-397	
<i>Birc3</i> ( <i>c-IAP2</i> )	Sense	5'-ACGCAGCAATCGTGCATTTTG-3'	21	62.9	NM_007464	1073-1093	181
	Antisense	5'-CCTATAACGAGGTCACCTGACGG-3'	22	61.6		1253-1232	
<i>Birc4</i> ( <i>Xiap</i> )	Sense	5'-CGAGCTGGGTTTCTTTATACCG-3'	22	60.7	NM_009688	145-166	126
	Antisense	5'-GCAATTTGGGGATATTCTCCTGT-3'	23	60.4		270-248	
<i>Birc5</i> ( <i>Survivin</i> )	Sense	5'-GAGGCTGGCTTCATCCACTG-3'	20	62.6	NM_009689	118-137	250
	Antisense	5'-CTTTTGTGCTTGTGTTGGTCTCC-3'	23	60.7		367-345	
<i>Birc6</i> ( <i>Apollon</i> )	Sense	5'-ACAGATTGTCTTACCTCTTGCCC-3'	23	61.9	NM_007566	695-717	120
	Antisense	5'-GCCACGAAGTGAAGGTCTCC-3'	20	62.5		814-795	
<i>Birc7</i> ( <i>ML-IAP</i> )	Sense	5'-AGCCTCCTTCTACGACTGG-3'	19	60.1	NM_001163247	291-309	245
	Antisense	5'-GCAAAGGGGTGTAGGTCTGG-3'	20	62.2		535-516	
<i>Bcl2</i>	Sense	5'-AGCTGCACCTGACGCCCTT-3'	19	67.6	NM_177410	344-362	194
	Antisense	5'-GTTTCAGGTACTCAGTCATCCAC-3'	22	60.1		537-516	
<i>Bcl2l1</i> ( <i>Bcl-xL</i> )	Sense	5'-AGGTTCCCTAAGCTTCGCAATTC-3'	22	64.4	NM_001289739.1	128-149	248
	Antisense	5'-TGTTTAGCGATTCTCTCCAGG-3'	22	64.2		375-354	
<i>Bax</i>	Sense	5'-CGGCGAATTGGAGATGAACTG-3'	21	68.7	NM_007527	190-210	161
	Antisense	5'-GCAAAGTAGAAGAGGGCAACC-3'	21	63.8		350-330	

\*NM accession numbers from NIH National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov/nucleotide>).

*Bax*, Bcl-2-associated X protein; *Bcl-2*, B-cell lymphoma 2; *Bcl-xL*, B-cell lymphoma—extra large; *Bcl2l1*, B-cell lymphoma 2 like 1; *Birc*, baculoviral inhibitor of apoptosis repeat containing protein; *c-IAP*, human protein, cellular inhibitor of apoptosis protein; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase; *ML-IAP*, inhibitor of apoptosis protein livin; *Naip*, neuronal apoptosis inhibitory protein; Tm, melting temperature; *Xiap*, X-linked inhibitor of apoptosis protein.

findings; 2, intermediate findings; 3, mild ground-glass opacity; 4, intermediate findings; 5, moderate ground-glass opacity or focalized or unilateral opacity; 6, intermediate findings; and 7, severe ground-glass opacity, bilateral opacity, consolidation, intralobular pleural septal thickening, or pleural effusion (Figure 1B). Several experts (including T.Y., A.Ta., T.K., H.F., K.N., and A.To.) in the field blinded to the treatment groups evaluated and scored the CT findings.

### Collection of Bronchoalveolar Lavage Fluid

Mice were anesthetized with isoflurane to collect BALF, as previously described.<sup>36</sup> The BALF samples were centrifuged (1000 × g, 10 minutes, 4°C) for cell and supernatant separation. The supernatant was stored at −80°C until use. The total cell number was determined using a nucleocounter from ChemoMetec (Allerød, Denmark). BALF cells were dispersed on a glass slide using cytospin and stained with a May-Grünwald Giemsa (Merk, Darmstadt, Germany) for differential cell counting.

### Collection of Lung Samples

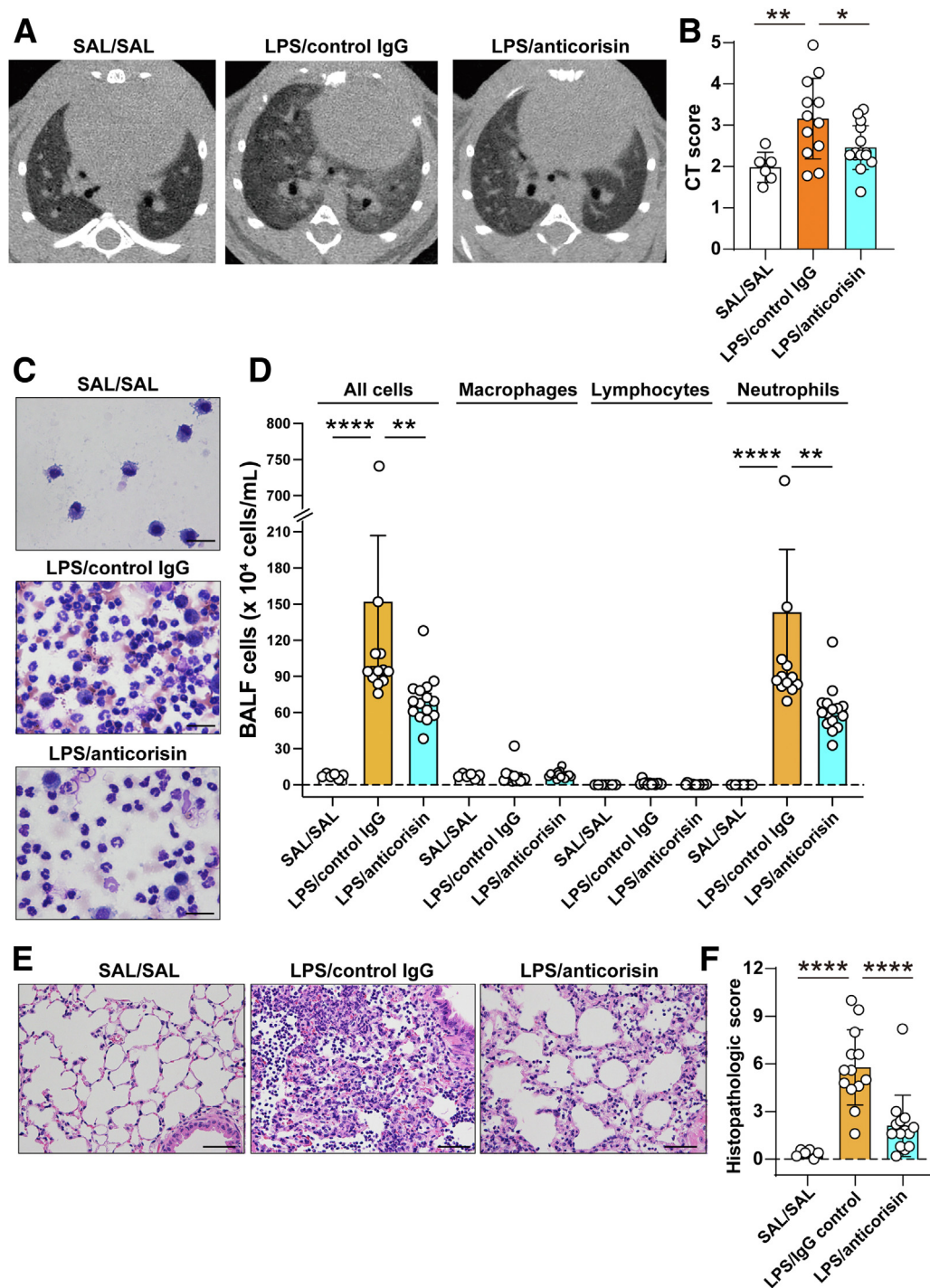
Mice were euthanized by an overdose of isoflurane before resectioning the lungs, as previously described.<sup>37</sup> A lung was fixed in formalin, then embedded in paraffin, and

subsequently used for hematoxylin-eosin staining. Lung histopathologic findings were observed using an Olympus BX50 microscope with a plan objective combined with an Olympus DP70 digital camera (Tokyo, Japan). Another lung was stored at −80°C until use for immunoassay and PCR studies.

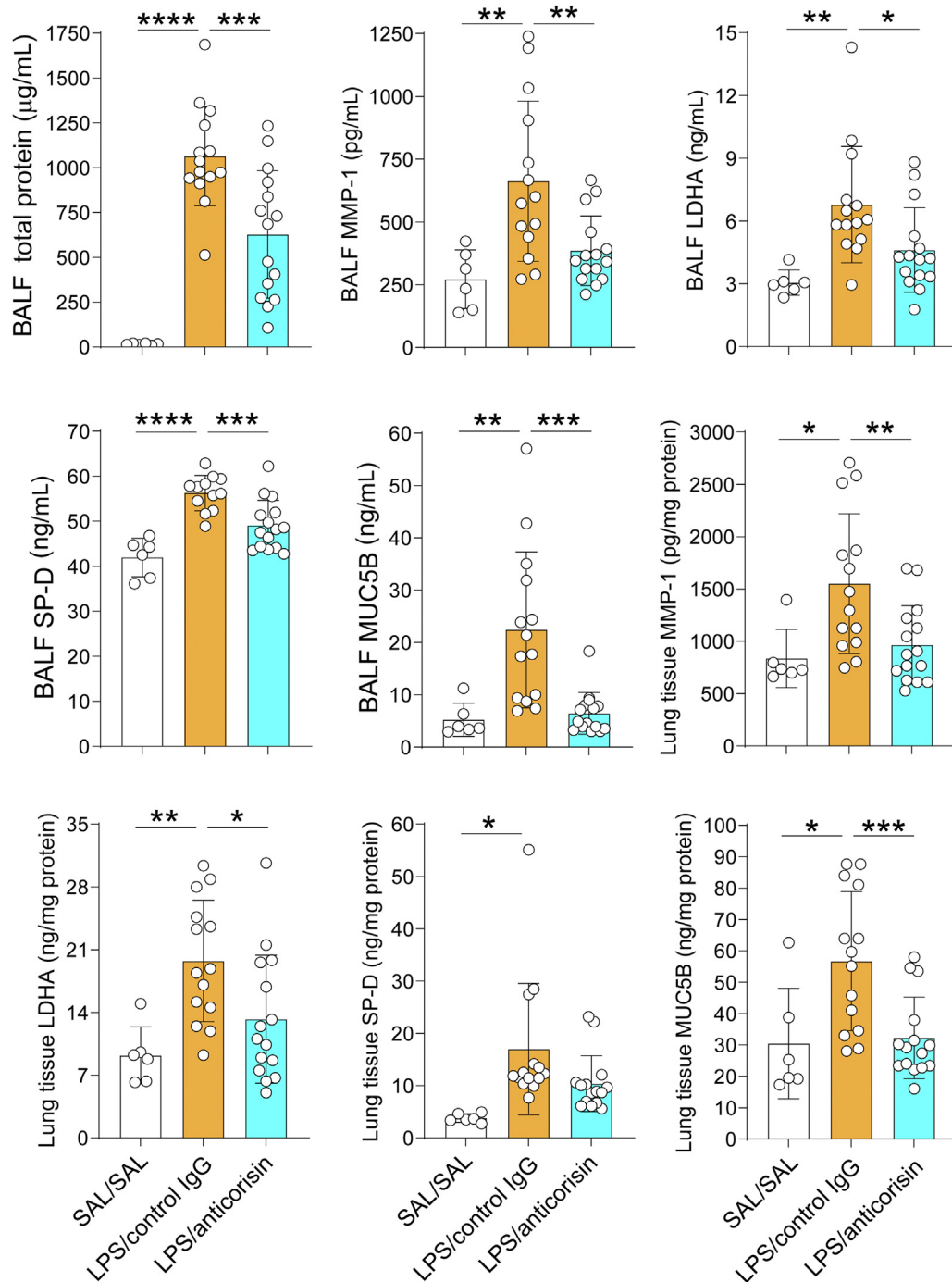
### Evaluation of Apoptosis

DNA fragmentation in lung tissue was evaluated by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay and cleavage of caspase-3 by Western blot analysis. The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay was performed at Morpho-Technology Corp. (Sapporo, Japan) following canonical methods. To perform Western blot analysis, the lung tissue from each group was minced with scissors into a homogenate paste, washed twice with ice-cold phosphate-buffered saline, and then lysed using radioimmunoprecipitation assay buffer containing protease/phosphatase inhibitors (1 mmol/L orthovanadate, 50 mmol/L β-glycerophosphate, 10 mmol/L sodium pyrophosphate, 5 μg/mL leupeptin, 2 μg/mL aprotinin, and 5 mmol/L sodium fluoride). The samples were then centrifuged at 17,000 × g for 10 minutes at 4°C, and the sample protein level was measured using the Pierce bicinchoninic acid protein assay kit (Thermo Fisher Scientific Inc.,





**Figure 2** Inhibition of corisin ameliorates computed tomography (CT) findings and lung inflammation in established acute lung injury. **A** and **B**: CT was performed 18 hours after the intratracheal instillation of lipopolysaccharide (LPS) or saline (SAL) in mice with established lipopolysaccharide-induced acute lung injury treated with anticorin antibody or control IgG. Several experts (including T.Y., A.Ta., T.K., H.F., K.N., and A.To.) in the field who were blinded to the treatment groups evaluated and scored the CT findings. **C** and **D**: Bronchoalveolar lavage fluid (BALF) was collected, and cells were counted using a nucleocounter. Differential count was done after Giemsa staining. **E** and **F**: Hematoxylin-eosin staining and acute lung injury score. Statistical analysis was performed by one-way analysis of variance with a Neuman-Keuls test. Data are expressed as means  $\pm$  SD (**B**, **D**, and **F**).  $n = 6$  in SAL/SAL group (**B**, **D**, and **F**);  $n = 12$  in LPS/control IgG group (**B** and **D**);  $n = 15$  in LPS/anticorin group (**B** and **D**);  $n = 13$  in LPS/control IgG group (**F**);  $n = 14$  in LPS/anticorin group (**F**). \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\*\* $P < 0.0001$ . Scale bars: 20  $\mu$ m (**C**); 50  $\mu$ m (**E**).



**Figure 3** Amelioration of markers of acute lung injury in mice with established acute lung injury after treatment with anticorisin antibody. Mice received intratracheal instillation of lipopolysaccharide (LPS; 100 μg) or saline (SAL) and then treatment with anticorisin monoclonal antibody or control IgG. Mice receiving intratracheal SAL and treated with control IgG were the control mice. The concentrations of surfactant protein-D (SP-D), mucin-5B (MUC5B), lactate dehydrogenase A (LDHA), and matrix metalloproteinase-1 (MMP-1) were measured using commercial enzyme immunoassay kits following the manufacturer's instructions. Statistical analysis was performed by analysis of variance with the Neuman-Keuls test.  $n = 6$  in SAL/SAL group;  $n = 14$  in LPS/control IgG group;  $n = 15$  in LPS/anticorisin group. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$ . BALF, bronchoalveolar lavage fluid.

Waltham, MA). The samples were mixed with Laemmli buffer and run on SDS-PAGE. Nitrocellulose membranes, anti-cleaved caspase-3, anti-noncleaved caspase-3, or anti-β-actin antibodies from Cell Signaling (Danvers, MA)

were used to perform Western blot analysis. The public domain NIH ImageJ version 1 program (NIH, Bethesda, MD; <https://imagej.net>, last accessed February 1, 2023) was used for quantification.

## Biochemical Analysis

The total protein level in samples was measured using a commercial kit from Thermo Fisher Scientific Inc. The concentrations of tumor necrosis factor- $\alpha$ , monocyte chemoattractant protein-1, IL-6 (BD opt-EIA kits; BD Bioscience, San Diego, CA), surfactant protein-D (Sino Biologicals, Beijing, China), IL-1 $\beta$ , osteopontin, IL-17 (R&D Systems, Minneapolis, MN), mucin-5B (MUC5B), lactate dehydrogenase A, and matrix metalloproteinase-1 (LSBio, Seattle, WA) were measured using commercial enzyme immunoassay kits following the manufacturer's instructions. Corisin was measured by an enzyme-linked immunoassay using anti-transglycosylase 351 polyclonal antibody as a capture antibody and biotinylated anticorisin 9A mAb as a detection antibody, as previously described.<sup>27</sup> Briefly, the anti-transglycosylase 351 polyclonal antibody was coated on a 96-well plate at a final concentration of 2  $\mu$ g/mL in phosphate-buffered saline at 4°C overnight. Next, blocking was performed with 1% bovine serum albumin in phosphate-buffered saline, followed by washing with phosphate-buffered saline in Tween. Then, varying concentrations of corisin as standards and samples were added to the wells and incubated at 4°C overnight. The wells were washed, and then horseradish peroxidase-conjugated streptavidin (R&D Systems) in a phosphate-buffered saline solution was added. After washing, substrate solution was added for color development and measurement of absorbance at 450 nm.

## Gene Expression Analysis

Sepasol RNA-I Super G reagent (Nacalai Tesque Inc., Kyoto, Japan) was used to extract the total RNA from lung tissues. cDNA was then synthesized using 2  $\mu$ g of total RNA with oligo-dT primer and ReverTra Ace Reverse Transcriptase (Toyobo Life Science Department, Osaka, Japan). Standard PCR was performed using primers described in Table 1. PCR was performed with 26 to 35 cycles, depending on the gene, denaturation at 94°C for 30 seconds, annealing at 65°C for 30 seconds, and elongation at 72°C for 1 minute, followed by a further extension at 72°C for 5 minutes. The mRNA expression was normalized against the glyceraldehyde 3-phosphate dehydrogenase mRNA expression.

## Statistical Analysis

The data are expressed as the means  $\pm$  SD. The sample size was calculated using the G\*Power software version 3.1.9.7.<sup>38</sup> Statistical analyses were performed using one-way analysis of variance with *post hoc* analysis using the Newman-Keuls test to assess differences among three or more variables with normal distribution. In addition, the Kruskal-Wallis one-way analysis of variance with the Dunn test was used to determine the statistical difference among three or more variables with skewed distribution. GraphPad

Prism version 7 (GraphPad Software, Inc., San Diego, CA) was used for analysis.

## Results

### Inhibition of Corisin Ameliorates CT Findings and Lung Inflammation in Established ALI

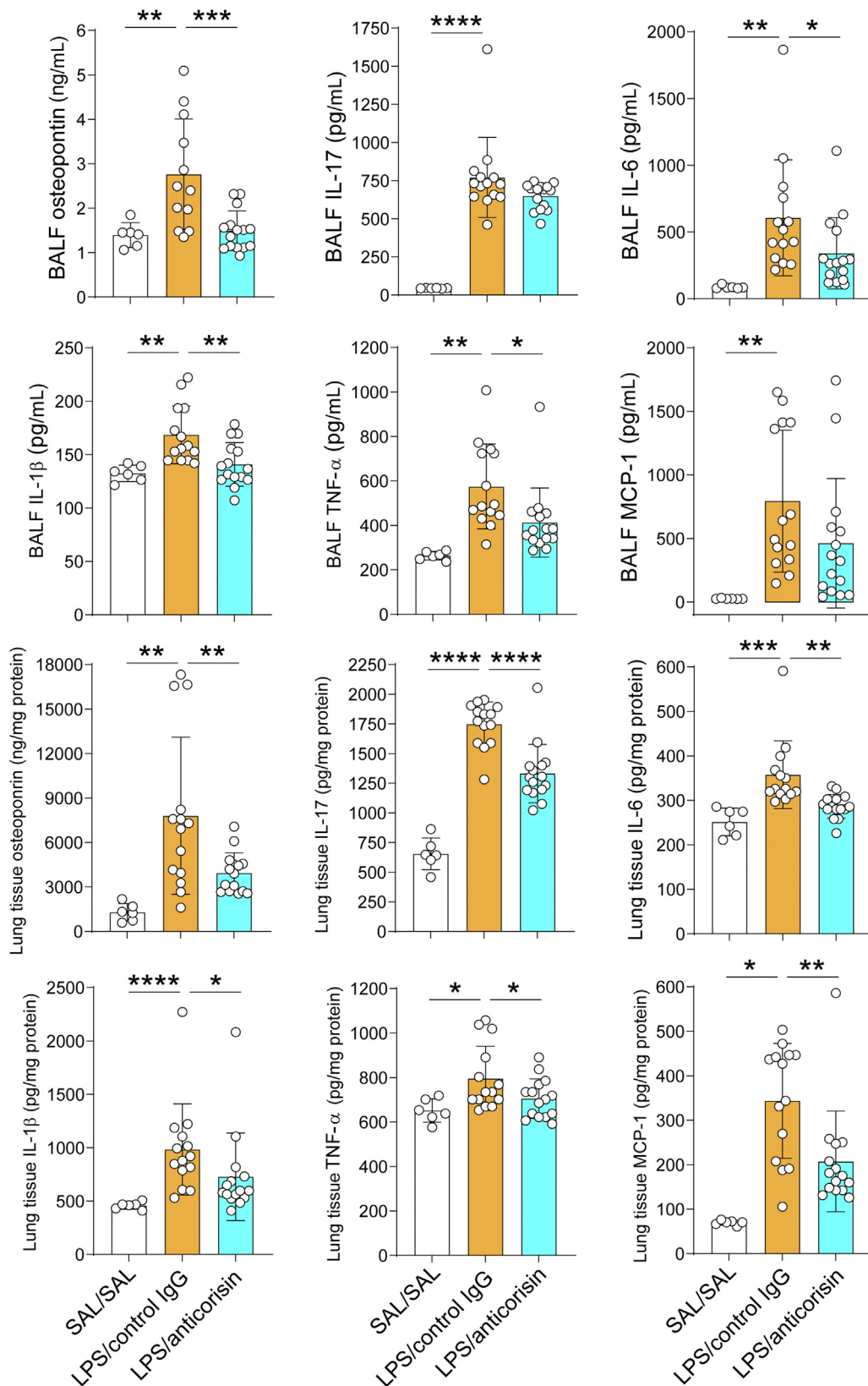
CT was performed under profound anesthesia 24 hours after intratracheal instillation of LPS and treatment with anticorisin mAb. Mice from the LPS/control IgG group showed diffuse lung opacity with typical ground-glass findings, which were less extensive and milder in the LPS/anticorisin group. Scoring of CT findings by experts in the field showed that the score of acute lung injury was significantly increased in the LPS/control IgG group compared with SAL/SAL group. However, the CT score was significantly reduced in mice treated with the anticorisin mAb (LPS/anticorisin group) compared with mice (LPS/control IgG) treated with control IgG (Figure 2, A and B). BALF was collected, and the number of inflammatory cells was counted. The total number of cells, the total number of neutrophils in BALF, and the histopathologic score of ALI were significantly increased in the LPS/control IgG group compared with SAL/SAL group, but they were significantly decreased in mice treated with the anticorisin mAb compared with mice treated with control IgG (Figure 2, C–F).

### Amelioration of Markers of Acute Lung Injury in Mice with Established ALI after Treatment with Anticorisin Antibody

The concentrations of total protein, matrix metalloproteinase-1, lactate dehydrogenase A, surfactant protein-D, and MUC5B are markers of the severity of ALI/ARDS.<sup>39,40</sup> Here, whether inhibition of corisin reduces the increased secretion of these markers in established ALI was evaluated. As expected, all these markers were increased in BALF and lung tissue from LPS/control IgG mice compared with mice without the disease (SAL/SAL). However, mice with ALI treated with the anticorisin mAb showed significantly decreased levels of BALF total protein and reduced levels of matrix metalloproteinase-1, lactate dehydrogenase A, surfactant protein-D, and MUC5B in BALF and lung tissue compared with mice with ALI treated with the control IgG (Figure 3).

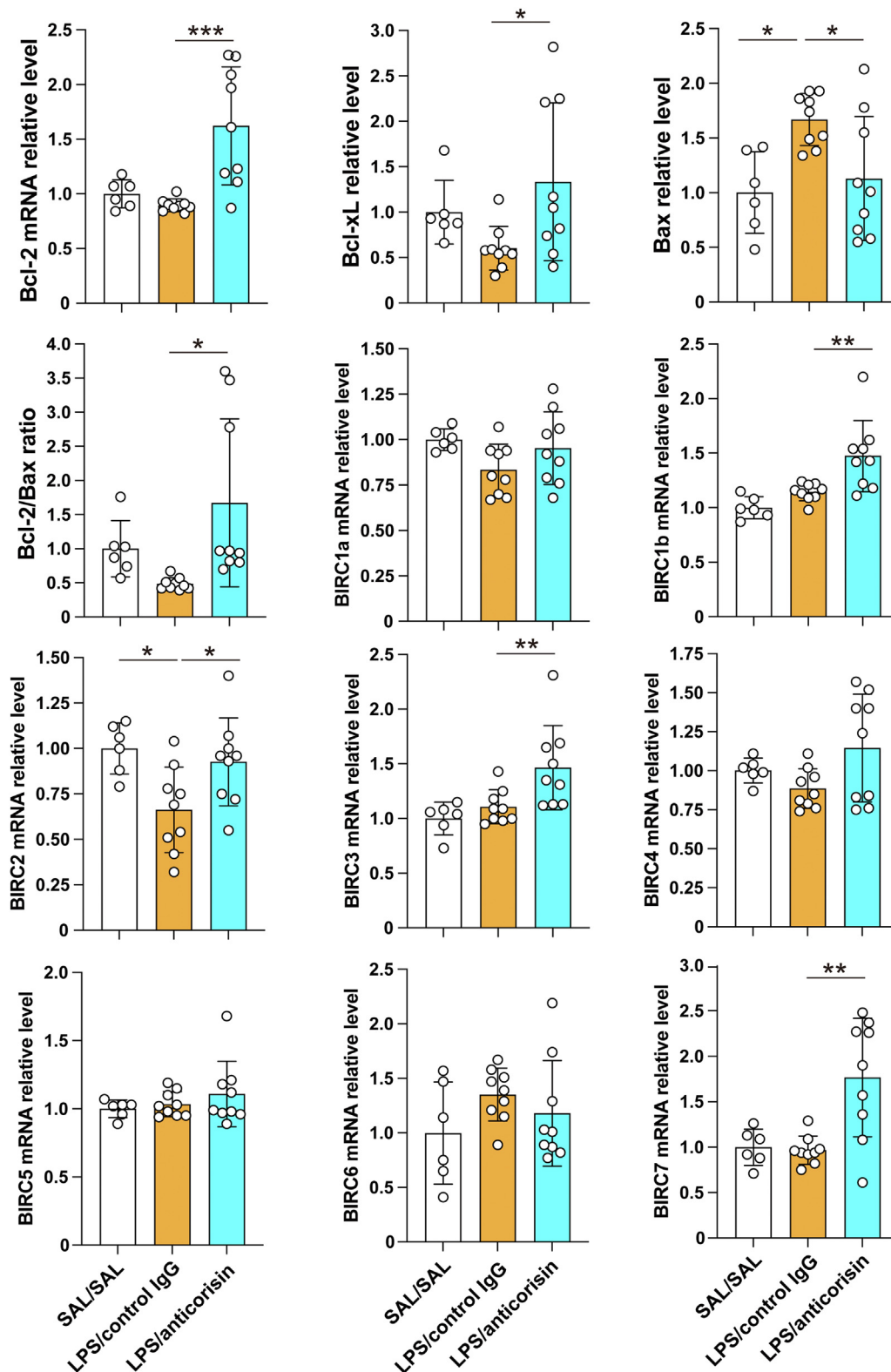
### Inhibition of Inflammatory Cytokines and Chemokines by Treating Mice with Established Acute Lung Injury with Anticorisin Antibody

Inflammatory cytokines and chemokines, including osteopontin, IL-17, IL-6, IL-1 $\beta$ , tumor necrosis factor- $\alpha$ , and monocyte chemoattractant protein-1, play critical roles in the pathogenesis of ALI/ARDS.<sup>41,42</sup> As expected, all these

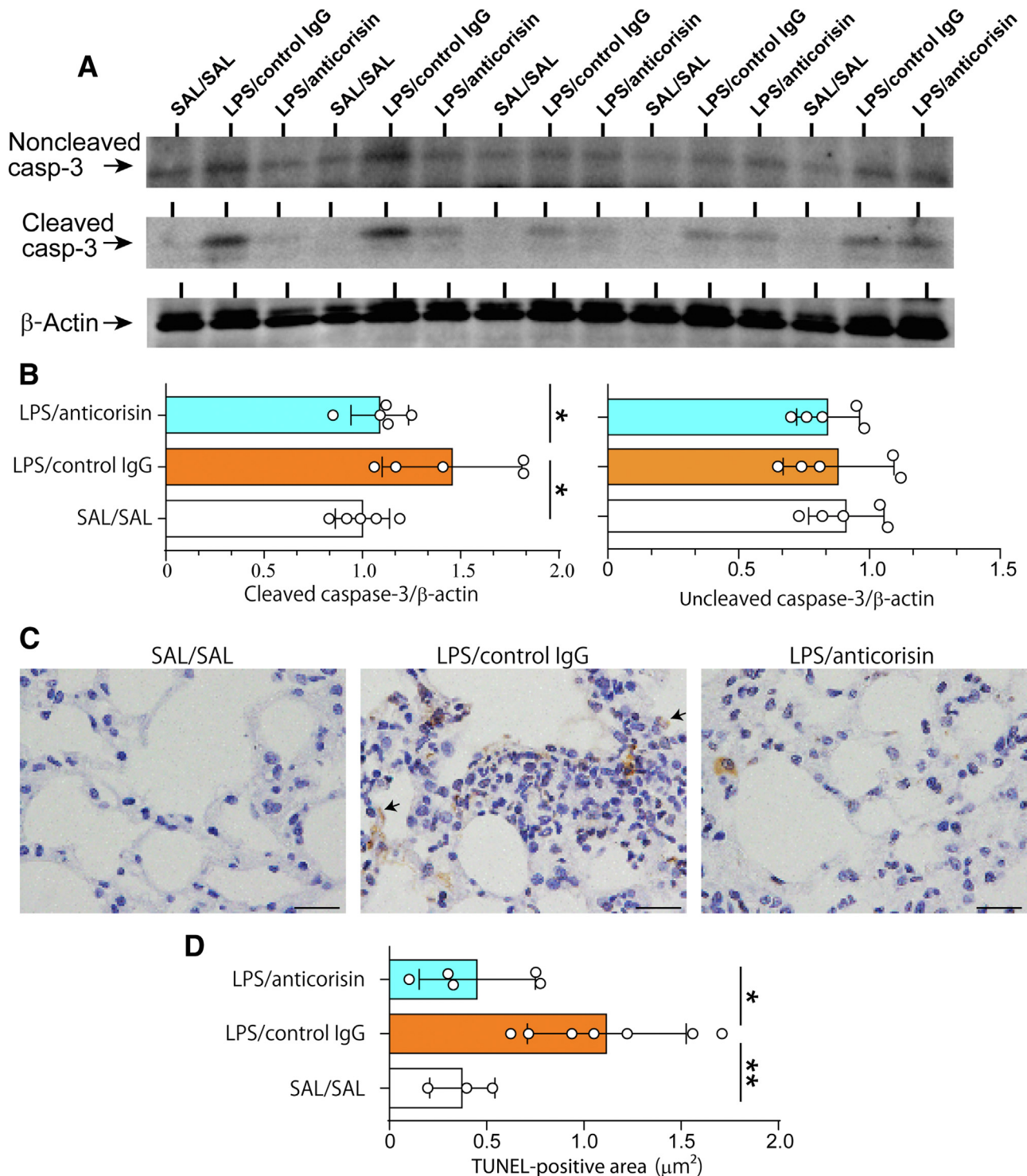


**Figure 4** Inhibition of inflammatory cytokines and chemokines by treating mice with established acute lung injury with anticorin antibody. Mice received intratracheal instillation of lipopolysaccharide (LPS; 100  $\mu$ g) or saline (SAL) and then treatment with anticorin monoclonal antibody or control IgG. Mice receiving intratracheal SAL and treated with control IgG were the control mice. The concentrations of osteopontin, IL-17, IL-1 $\beta$ , tumor necrosis factor (TNF)- $\alpha$ , and monocyte chemoattractant protein-1 (MCP-1) were measured using commercial enzyme immunoassay kits following the manufacturer's instructions. Statistical analysis was performed by analysis of variance with the Neuman-Keuls test.  $n = 6$  in SAL/SAL group;  $n = 14$  in LPS/control IgG group;  $n = 15$  in LPS/anticorin group. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$ . BALF, bronchoalveolar lavage fluid.





**Figure 5** Inhibition of corisin increases the mRNA expression of antiapoptotic factors in mice with established acute lung injury. Mice received intratracheal instillation of lipopolysaccharide (LPS; 100  $\mu$ g) or saline (SAL) and then treatment with anticorin monoclonal antibody or control IgG. Mice receiving intratracheal SAL and treated with control IgG were the control mice. Total RNA was extracted from lung tissue, and the mRNA expression of proapoptotic and antiapoptotic factors was evaluated by RT-PCR. Statistical analysis was performed by analysis of variance with the Neuman-Keuls test. Data are expressed as means  $\pm$  SD.  $n = 6$  in SAL/SAL group;  $n = 9$  in LPS/control IgG group;  $n = 9$  in LPS/anticorin group. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ . BIRC1a [neuronal apoptosis inhibitory protein 1 (NAIP1)], 1b (NAIP2), 2 [cellular inhibitor of apoptosis protein 1 (c-IAP1)], 3 (c-IAP2), 4 (X-linked inhibitor of apoptosis protein), 5 (survivin), 6 (apollon), or 7 (melanoma inhibitor of apoptosis), baculoviral IAP (inhibitor of apoptosis) repeat-containing 1a, 1b, 2, 3, 4, 5, 6, or 7. Bax, Bcl-2-associated X; Bcl-2, B-cell lymphoma 2; Bcl-xL, B-cell lymphoma-extra large.



**Figure 6** Inhibition of apoptosis in mice with established acute lung injury treated with anticorisin monoclonal antibody. Mice received intratracheal instillation of lipopolysaccharide (LPS; 100  $\mu\text{g}$ ) or saline (SAL) and then treatment with anticorisin monoclonal antibody or control IgG. Mice receiving intratracheal SAL and treated with control IgG were the control mice. **A** and **B**:  $\beta$ -Actin, noncleaved caspase-3 (casp-3), and the activation of caspase-3 were evaluated using specific antibodies by Western blot analysis. **C** and **D**: DNA fragmentation was evaluated by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method, as described in *Materials and Methods*. **Black arrows** indicate TUNEL (+) cells. Statistical analysis was performed by analysis of variance with the Neuman-Keuls test. Data are expressed as means  $\pm$  SD (**B** and **D**).  $n = 5$  in SAL/SAL group, control LPS/control IgG group, and LPS/anticorisin group (**A** and **B**);  $n = 3$  in SAL/SAL group (**C** and **D**);  $n = 7$  in LPS/control IgG group (**C** and **D**);  $n = 5$  in LPS/anticorisin group (**C** and **D**). \* $P < 0.05$ , \*\* $P < 0.01$ . Scale bars = 50  $\mu\text{m}$  (**C**).

markers were significantly increased in mice with ALI treated with control IgG compared with the nondisease group. However, mice with ALI treated with anticorisin mAtb had significantly decreased BALF and lung tissue levels of osteopontin, IL-6, IL-1 $\beta$ , tumor necrosis factor- $\alpha$ , and monocyte chemoattractant protein-1 compared with mice with ALI treated with control IgG. The BALF level of IL-17 was also decreased in the LPS/anticorisin groups compared with the LPS/control IgG groups, but the reduction did not lead to a statistically significant difference (Figure 4).

### Inhibition of Corisin Increases the mRNA Expression of Antiapoptotic Factors in Mice with Established ALI

Cell apoptosis is regulated by the B-cell lymphoma 2 (BCL-2) family proteins and by members of the baculoviral inhibitor of apoptosis repeat-containing (BIRC) family of proteins. PCR analysis of these factors was evaluated to assess the effect of corisin inhibition on their expression. The mRNA expressions of the anti-apoptotic factors Bcl-2, B-cell lymphoma extralarge, BIRC1b or neuronal apoptosis inhibitory protein-2, BIRC2 or cellular inhibitor of apoptosis protein-1, BIRC3 or cellular inhibitor of apoptosis protein-2, and BIRC7 or melanoma inhibitor of apoptosis were significantly increased in the lung tissue from mice with ALI treated with anticorisin mAtb compared with mice treated with the control IgG. On the other hand, the pro-apoptotic protein Bcl-2-associated X protein (Bax) was significantly decreased, whereas the Bcl-2/Bax ratio was significantly increased, in mice treated with the anticorisin mAtb compared with mice treated with control IgG (Figure 5).

### Inhibition of Apoptosis in Mice with Established Acute Lung Injury Treated with Anticorisin Monoclonal Antibody

Apoptosis can be induced by the extrinsic or intrinsic pathway.<sup>43</sup> Activation of either of these pathways leads to activation and cleavage of caspase-3. The present study evaluated the caspase-3 cleavage in mice with ALI treated with or without anticorisin mAtb. Compared with control mice, cleavage of caspase-3 was significantly increased in mice with ALI treated with control IgG. However, mice with ALI treated with the anticorisin mAtb showed significantly decreased caspase-3 cleavage compared with counterpart mice with ALI treated with control IgG (Figure 6, A and B). Noncleaved caspase-3 was not different between groups. In addition, the degree of DNA fragmentation by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay was evaluated. The area of DNA fragmentation was significantly enhanced in mice with ALI treated with irrelevant IgG compared with control mice without ALI treated with saline. However, the area of DNA fragmentation was significantly decreased in mice with ALI

treated with anticorisin mAtb compared with mice with ALI treated with irrelevant IgG (Figure 6, C and D).

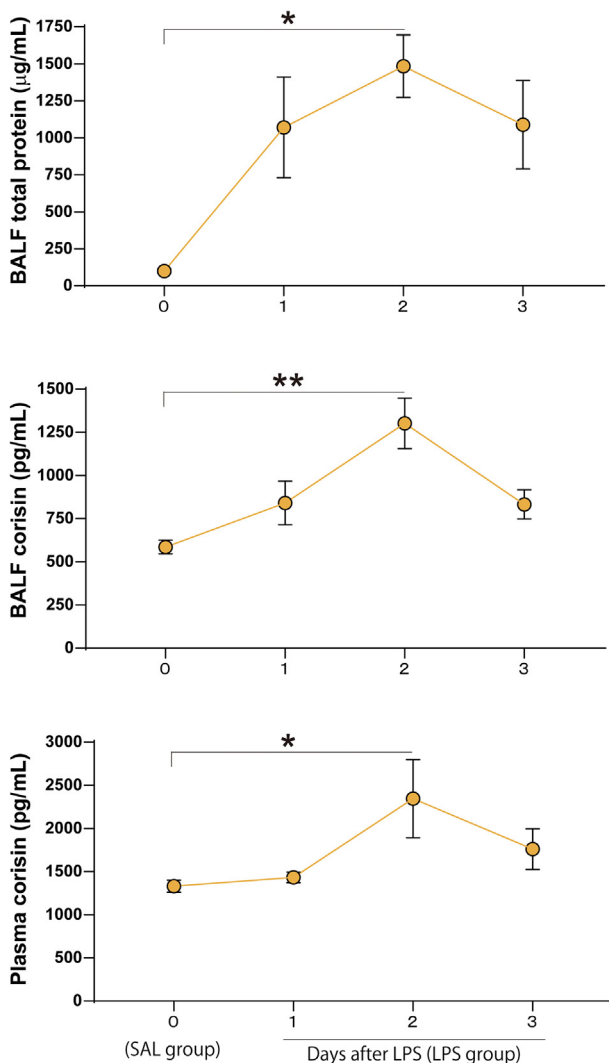
### Corisin Storm Occurs on the Second Day after LPS-ALI

The current study tested the hypothesis that LPS injury remodels the lung microbiota by favoring the proliferation of corisin-producing bacteria with concomitant release of high amounts of corisin or corisin-like peptides. To interrogate this hypothesis, the plasma level of corisin and the plasma and BALF levels of total protein and corisin were measured several days after intratracheal instillation of LPS. BALF and plasma samples collected from mice receiving intratracheal instillation of SAL were used as controls. The BALF level of total protein and the plasma and BALF levels of corisin significantly increased on the second day after LPS intratracheal instillation. The BALF level of total protein and the plasma and BALF levels of corisin also increased on the first and third days after LPS instillation, but the increase did not reach statistical significance (Figure 7).

## Discussion

This study shows that inhibition of corisin, a lung microbiota-derived proapoptotic peptide, ameliorates radiological findings, inflammatory response, and apoptosis in established LPS-induced ALI in mice.

A large body of evidence implicates the microbiome in the pathogenesis of ALI/ARDS.<sup>44</sup> Culture-independent methods have demonstrated disruption of the lung microbiome in patients with critical lung illness.<sup>20,23</sup> The lung microbiota becomes less diverse and contains an unbalanced proportion of commensals and pathogens, with the enrichment of bacteria originating from other ecosystems (eg, gut).<sup>20,23</sup> However, no specific microbiome-derived factor that may be therapeutically targetable has been demonstrated so far. Corisin is a microbiota-derived peptide originally discovered in the culture supernatant of *Staphylococcus nepalensis* strain CNDG isolated from the fibrotic lung tissue of transgenic mice overexpressing the human transforming growth factor- $\beta$ 1 in the lungs.<sup>26,27</sup> Phylogenetic analysis showed that the corisin sequence is conserved in a large group of staphylococci and different strains of bacteria.<sup>26</sup> For example, the corisin or corisin-like sequence has been identified in commensal bacteria, including *Staphylococcus xylosus*, *Staphylococcus cohnii*, and *Staphylococcus hominis*, and pathogenic bacteria, including *Staphylococcus haemolyticus*, *Mycobacteroides abscessus*, and *Listeria monocytogenes*.<sup>26,27</sup> Corisin triggers acute exacerbation of pulmonary fibrosis by inducing apoptosis of lung epithelial cells. The corisin sequence is a segment of bacterial transglycosylase, which, by itself, does not induce cell death.<sup>26</sup> Thus, this killer peptide is a hidden bullet in a transglycosylase. The high concentration of this



**Figure 7** Significant increase in corisin levels on the second day after inducing acute lung injury in mice. Twelve mice received intratracheal instillation of lipopolysaccharide (LPS; 100 µg), and after 24, 48, or 72 hours, four mice were anesthetized with isoflurane to collect bronchoalveolar lavage fluid (BALF) and plasma before sacrifice. Statistical analysis was performed by analysis of variance and one-way Kruskal-Wallis with Dunn test.  $N = 4$  in each group. \* $P < 0.05$ , \*\* $P < 0.01$ . SAL, saline.

killer peptide detected in BALF and serum from patients with idiopathic pulmonary fibrosis with acute exacerbation and inhibition of lung epithelial cell apoptosis induced by BALF with anticorisin neutralizing antibody suggests the clinical implication of corisin in human disease.<sup>26,27,45</sup> Interestingly, corisin has also been detected in body fluids from normal mice and healthy subjects, suggesting that corisin may also play a role in the pathogenesis of diseases other than lung fibrosis.<sup>26,27</sup> In the current study, the hypothesis that corisin may be a potential therapeutic target in ALI/ARDS was tested. A previous study reported that pretreatment of mice with an anticorisin monoclonal antibody before inducing ALI by intratracheal instillation of LPS is associated with significant mitigation of radiological

changes and inflammatory response in the lungs, implicating corisin in the mechanism of ALI.<sup>27</sup> However, whether the anticorisin mAtb is effective in already established ALI/ARDS is unclear. Patients usually come or are brought to the clinic when the disease has already developed. In the present study, ALI in mice was induced by intratracheal instillation of LPS, and thereafter treatment with anticorisin mAtb was initiated. The score of radiological findings, the infiltration of neutrophils, the expression of inflammatory cytokines, chemokines, and the expression of markers of acute tissue injury were significantly ameliorated in mice with ALI treated with the anticorisin mAtb compared with their mouse counterparts treated with an irrelevant antibody. These findings reinforce the pathogenic role of corisin in ALI and point to corisin as a potential target for treating this life-threatening disease.

Experimental and clinical studies support the role of apoptosis of lung epithelial and endothelial cells in the pathogenesis of ALI/ARDS.<sup>46–48</sup> Apoptosis by the extrinsic and intrinsic pathways has been described in ALI/ARDS.<sup>43</sup> There are reports of increased DNA fragmentation, enhanced expression of proapoptotic proteins [eg, Bax and Bcl-2 homologous antagonist killer (Bak)], and activation of the proapoptotic Fas/FasL pathway in the alveolar epithelium from patients with ARDS.<sup>47,49–52</sup> Apoptosis of endothelial cells also occurs in patients with ARDS and animal models of ALI.<sup>46,47,53</sup> The BALF from patients with ARDS is cytotoxic to vascular endothelial cells.<sup>54</sup> Injury and apoptosis of endothelial cells increase the permeability of the pulmonary microvascular endothelium, leading to abnormal protein leakage and edema formation.<sup>2</sup> The major cause of ARDS is bacterial pneumonia.<sup>55</sup> Corisin is a bacterial peptide that induces apoptosis of epithelial cells from different organs. It was previously reported that pretreatment of mice with mAtb against corisin before induction of ALI with LPS mitigates the inflammatory response in the lungs.<sup>27</sup> However, whether inhibition of corisin ameliorates apoptosis in LPS-induced ALI remains unclear. In the current research, anticorisin mAtb was administered to mice after inducing ALI by intratracheal instillation of LPS, and DNA fragmentation and the mRNA expression of apoptotic and proapoptotic factors in lung tissue were assessed. The expression of antiapoptotic factors, including Bcl-2, B-cell lymphoma extralarge, and inhibitor of apoptosis, was significantly increased, whereas the proapoptotic factor Bax, the cleaved caspase-3, and DNA fragmentation were significantly decreased, in mice treated with anticorisin mAtb compared with untreated mice, suggesting the inhibitory activity of the anticorisin mAtb against the apoptosis likely induced by corisin. This inhibition of apoptosis with the anticorisin mAtb was associated with a reduced lung inflammatory response. The results, therefore, agree with a previous report showing that treatment with a caspase inhibitor is protective in a murine model of ALI.<sup>56</sup> Overall, these observations suggest that the anticorisin mAtb protects ALI by inhibiting apoptosis of lung cells and that the



bacterial apoptotic factor corisin and related peptides likely play an important role in the disease.

## Conclusion

The results of this study suggest that corisin is a potential therapeutic target, that the anticorisin neutralizing antibody is a promising therapeutic approach, and that inhibition of excessive apoptosis is beneficial in established ALI/ARDS. However, interpatient variability in corisin sequence and corisin variant-dependent efficacy of anticorisin mAtb may occur. Therefore, further studies are needed to identify other potentially pathogenic microbiome-derived proapoptotic peptides in patients with ALI/ARDS and to develop and evaluate the efficacy of humanized mAtb against different corisin variants. In addition, future studies should also be performed to clarify why enhanced intrapulmonary instillation of LPS elicits a significant release of corisin peptides from the microbiota.

## Author Contributions

E.C.G. and I.C. conceptualized the study; A.To., T.Y., K.N., S.T., and M.T. validated the study; V.F.D., C.N.D.-G., M.T., and T.Y. analyzed the data; T.K., H.F., M.T., A.To., A.Ta., and V.F.D. performed experiments; T.K., I.O.L., I.C., and R.Y.H. obtained resources; K.N. and T.Y. curated data; V.F.D., C.N.D.-G., and E.C.G. wrote the manuscript; E.C.G. and I.C. reviewed and edited the manuscript; I.C. and A.Ta. visualized the data; T.K. and C.N.D.-G. supervised the study; C.N.D.-G. administered the project; and E.C.G., C.N.D.-G., and T.Y. acquired funding. All authors have read and agreed to the published version of the manuscript.

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