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Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) prevents lipopolysaccharide (LPS)-induced, sepsis-related severe acute lung injury in mice

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Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an energy metabolism-related enzyme in the glycolytic pathway. Recently, it has been reported that GAPDH has other physiological functions, such as apoptosis, DNA repair and autophagy. Some *in vitro* studies have indicated immunological aspects of GAPDH function, although there is no definite study discussing the advantage of GAPDH as a therapeutic target. Here, we show that GAPDH has an anti-inflammatory function by using a lipopolysaccharide (LPS)-induced, sepsis-related severe acute lung injury (ALI) mouse model, which is referred to as acute respiratory distress syndrome (ARDS) in humans. GAPDH pre-injected mice were protected from septic death, and their serum levels of proinflammatory cytokines were significantly suppressed. In lung tissue, LPS-induced acute injury and neutrophil accumulation were strongly inhibited by GAPDH pre-injection. Pulmonary, proinflammatory cytokine gene expression and serum chemokine expression in GAPDH pre-injected mice were also reduced. These data suggest the therapeutic potential of GAPDH for sepsis-related ALI/ARDS.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is constitutively expressed in the cytosol, is a glycolytic pathway-related enzyme and is best known as a housekeeping molecule. GAPDH reversibly catalyzes the phosphorylation of glyceraldehyde-3-phosphate into 1,3-bisphosphoglycerate, which is essential for energy metabolism. In addition to catalyzing phosphorylation, GAPDH has various physiological functions, and it locates at not only the cytosol but also the cell membrane, nucleus, polysomes, ER and Golgi[1–5]. For instance, Yamaji *et al.* have reported that extracellular GAPDH inhibits cell spreading and that the cysteine residue at position 151 of GAPDH is involved in its binding to the cell[6].

Recent investigations have also demonstrated that GAPDH is translocated to nuclei and links to many fundamental cellular functions, including apoptosis[7], DNA repair[8], telomere protection[9], nuclear tRNA export[10], histone biosynthesis[11] and autophagy[12]. In HeLa cells, GAPDH was identified as P_1, P_4-di(adenosine-5′) tetraphosphate (Ap4A) binding protein[13], and diadenosine phosphates (Ap3A, Ap4A, Ap5A and Ap6A) have been reported to interact with granulocyte-macrophage colony-stimulation factor (GM-CSF) for the regulation of neutrophil apoptosis[14]. These lines of evidence strongly suggest that GAPDH has immunomodulatory activity though its interaction with diadenosine phosphates, resulting in the induction of neutrophil apoptosis. Thus, it has been speculated that GAPDH acts on immune cells and regulates their immune and inflammatory responses. However, little is known about the potential of GAPDH as a therapeutic strategy.

Acute lung injury (ALI) and its severe manifestation, acute respiratory distress syndrome (ARDS), are well-known as lethal diseases in intensive care units. Despite the fact that effective therapies for ALI/ARDS have been developed, ALI/ARDS still has a high mortality rate (35–45%), which has been unchanged since 1994[15]. ARDS is easily developed in patients with a systemic inflammatory response, such as sepsis, major trauma and aspiration pneumonia, with sepsis being the most common cause of ARDS[16–19]. Therefore, it is of great importance to develop an effective treatment for sepsis. Recently, it has been reported that activated protein C (APC) has an anti-inflammatory effect by protecting against the production of proinflammatory cytokines, such as TNF-α, IL-1 and IL-6, in monocytes[20]. For application in human sepsis therapy, a recombinant human APC (Xigris (drotrecogin alfa), Lilly, Germany) was developed, but Ranieri *et al.* revealed that drotrecogin alfa does not have a significant therapeutic effect for sepsis during the PROWESS-SHOCK trial[21]. Thus, it is necessary to find other targets for sepsis therapy. Some novel targets in sepsis have indeed been identified, such as complement C5a[22], its receptor[23], macrophage migration inhibitory factor (MIF)[24], high-mobility group box 1 protein (HMGB1)[25] and histones[26]; therapeutic drugs against each target are being developed, but effective drugs have not yet been achieved.

Lipopolysaccharide (LPS) is a glycolipid that constructs the outer membrane of gram-negative bacteria and is well-known as an important mediator of sepsis. In several animals, the administration of LPS has been used as a model of sepsis-related ALI[27–32]. In this study, therefore, we have investigated the therapeutic potential of GAPDH by using a mouse model of LPS-induced, sepsis-related severe ALI, as a model of ARDS in humans.

Results

Prevention of LPS-induced, sepsis-related severe ALI/ARDS by pre-treatment with GAPDH. To evaluate the therapeutic significance of GAPDH for LPS-induced, sepsis-related severe ALI/ARDS mice, 10 mg/kg of GAPDH was pre-injected before LPS administration. As shown in Fig. 1, GAPDH pre-treatment significantly increased the survival rate (80%) at 48 h after LPS administration, while all PBS pre-treated mice died at the same time point.

Effect of GAPDH pre-treatment on serum cytokine profiles. To explore the effect of GAPDH on serum cytokine profiles, serum samples were collected from GAPDH or PBS pre-treated mice at 6 and 12 h after LPS injection (n = 5 per group). All cytokine (IL-6, TNF-α and IFN-γ) levels at 6 h after LPS injection had no definite difference between GAPDH and PBS pre-treated mice, while those cytokine levels (with the exception of IFN-γ ($P = 0.0597$)) at 12 h after LPS injection were significantly decreased by GAPDH pre-treatment (Fig. 2).

Amelioration of LPS-induced fatal lung injury and neutrophil accumulation by pre-treatment with GAPDH. To explore the onset of ALI/ARDS, lungs were collected at 12 h after LPS injection and stained with hematoxylin and eosin. As shown in Figs. 3a–c, LPS-induced lung injury was significantly ameliorated by GAPDH pre-treatment. One of the characteristics of ALI/ARDS is a potent pulmonary infiltration of neutrophils[33]. Therefore, we next evaluated the extent of neutrophil infiltration in the lung by Giemsa staining (Figs. 3d–f). In PBS pre-treated mice, we observed large-scale infiltration of neutrophils in the lung. However, less neutrophil infiltration was observed in the GAPDH pre-treated lung tissue, which was comparable with normal lung tissue. Additionally, we counted Giemsa-stained cell number in each image. The stained cell number in GAPDH pre-treated lung was lower than that in PBS pre-treated lung (Fig. 3g). Furthermore, we also investigated neutrophil accumulation in the lung by using a specific antibody against Ly-6G, which is known as a cell-surface marker highly expressed on neutrophils[34]. As shown in Figs. 3h–j, PBS pre-treated lung tissue revealed Ly-6G positive neutrophil accumulation, while no such accumulation was found in the lung obtained from GAPDH pre-treated mice as well as naïve mice.

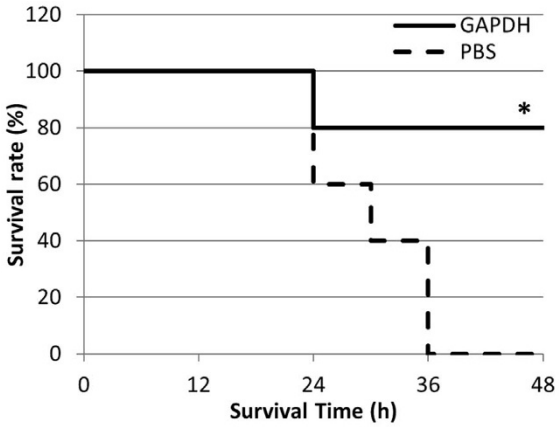

Figure 1 | Survival curve after LPS injection. BALB/c mice (n = 15, each group) were pre-treated with GAPDH (i.p., 10 mg/kg) or control PBS 90 minutes before LPS (i.p., 20 mg/kg) injection. Survival at 48 h after LPS injection was monitored. At 6 (n = 5 per group) and 12 h (n = 5 per group) after LPS injection, surviving mice were sacrificed to collect serum and lung tissue. Full and broken lines show GAPDH and PBS pre-treated groups, respectively. Survival data were analyzed using the Kaplan-Meier method and log-rank test (SPSS 16.0). *, $P < 0.05$ versus the PBS pre-treated group.

Effect of GAPDH pre-treatment on pulmonary cytokine expression. Histopathological analysis of the lung clearly revealed the therapeutic impact of GAPDH on ALI/ARDS (Fig. 3). To further explore the local protective effect of GAPDH in the lung, we next checked LPS-induced gene expression of proinflammatory cytokines (IFN-γ, IL-6 and TNF-α) in the lung. As shown in Fig. 4a–c, GAPDH significantly suppressed pulmonary gene expression of IL-6 and IFN-γ at 12 h after LPS administration, while GAPDH did not affect pulmonary TNF-α gene expression. Protein level of each cytokine was not fully consistent with mRNA level mainly due to the different peak of expression, while IL-6 and TNF-α expression in the lung extracts at 12 h after LPS injection were slightly ($P = 0.0579$) and significantly ($P < 0.05$) reduced by GAPDH pre-treatment, respectively (Fig. 4d–f).

Effect of GAPDH pre-treatment on chemokine profiles. GAPDH pre-treatment significantly suppressed neutrophil accumulation in the lung caused by LPS-induced severe ALI/ARDS. To demonstrate the effect of GAPDH pre-treatment on serum chemokine profiles, we next performed a cytokine array for the global evaluation of cytokines and chemokines. Serum was obtained from each mouse 12 h after LPS administration (n = 5 per group), and the serum was pooled. The profile of serum chemokine expression was determined, and we checked the difference between PBS and GAPDH pre-treated groups. As shown in Fig. 5a and b, the expression levels of MIG (CXCL9), MIP-2 (CXCL2) and RANTES (CCL5) were significantly suppressed in the serum by GAPDH pre-treatment. Notably, neutrophil-chemoattractant chemokine MIP-2 expression[35] was suppressed in the lung (Fig. 5c). Taken together, our present data suggest that GAPDH pre-treatment may suppress both chemokines and proinflammatory cytokines, resulting in decreased accumulation of neutrophils in the lung.

Discussion

GAPDH, which is known as a housekeeping protein, is an energy metabolism-related enzyme in the glycolytic pathway. However,

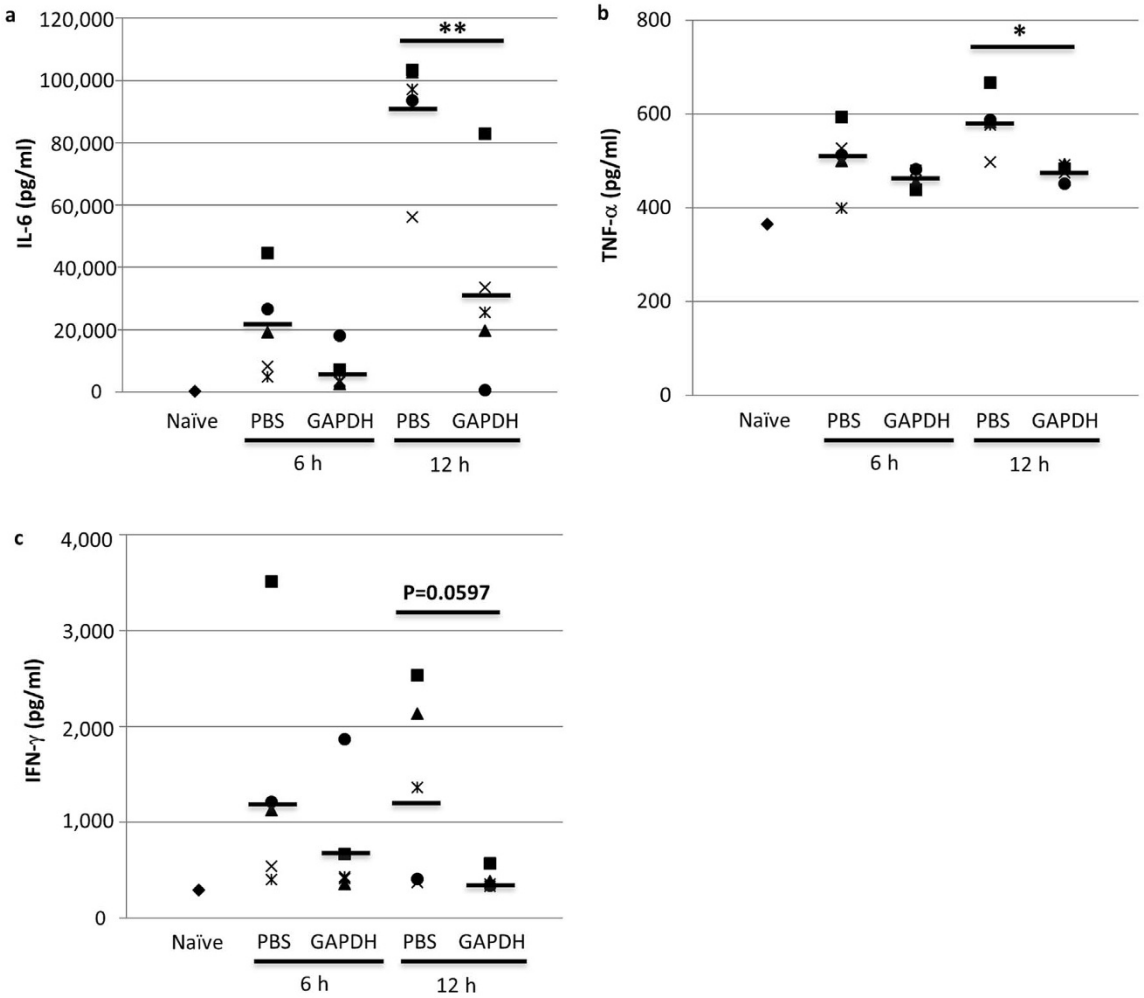

Figure 2 | Serum cytokine levels after LPS injection. Peripheral blood was obtained from GAPDH or PBS pre-treated mice at 6 or 12 h after LPS injection. Serum cytokine levels of IL-6 (a), TNF-α (b) and IFN-γ (c) were determined by using specific ELISA kits. Each symbol in all graphs indicates individual mouse, and bars show mean value. *, $P < 0.05$; **, $P < 0.01$ versus the PBS pre-treated group.

accumulating evidence suggests other physiological functions of GAPDH. In particular, some *in vitro* studies have suggested that GAPDH might inhibit inflammatory and immune responses[13,14,36–39], although the advantage of GAPDH as a therapeutic target based on *in vivo* research has not previously been explored either experimentally or clinically. In the present study, we report for the first time on the therapeutic significance of GAPDH in a LPS-induced, sepsis-related severe ALI model.

We clearly demonstrate that GAPDH pre-treated mice are protected from septic death caused by LPS, resulting in significant prolongation of survival (Fig. 1). One of the possible explanations for this mechanism is that GAPDH pre-treatment of mice with LPS-induced sepsis significantly suppresses their serum and pulmonary levels of proinflammatory cytokines, such as IFN-γ, IL-6 and TNF-α (Figs. 2 and 4). Pathological evidence also demonstrates that the therapeutic significance of GAPDH for LPS-induced lung injury is well-supported, based on the evidence of decreased numbers of neutrophils (Fig. 3) in the lungs of GAPDH pre-treated mice compared to the lungs of naïve mice, due to the suppression of several chemokines including neutrophil-chemoattractant chemokine MIP-2 by GAPDH pre-treatment (Fig. 5). Additionally, we also checked neutrophil number in blood after LPS injection to explore the effect of GAPDH on the induction of neutrophils, and Ly-6G positive cell population was evaluated by flow cytometry. Circulating neutrophils were increased by LPS injection, but there was no definite difference of neutrophil number in the blood between PBS and GAPDH pre-treated group (data not shown). Therefore, these results suggested that GAPDH does not affect neutrophil number in the blood, while GAPDH inhibits neutrophil migration into the lung, leading to suppress chemokine expression in the lung and serum. Because our model of LPS-induced sepsis and acute pulmonary inflammation in mice mimicked the state of ALI/ARDS[27,28], we conclude that GAPDH might protect against the onset of not only ALI but also ARDS by suppressing pulmonary neutrophil infiltration following inhibition of proinflammatory cytokine and chemokine expression. Since there is no effective therapeutic care against ARDS, the mechanism of suppressive activity of GAPDH against the influx of neutrophils into the lung following septic status will be of great importance.

ARDS, the more severe form of ALI in humans, was first reported by Ashbaugh *et al.* in 1967[40]. As for the cause of ARDS, there are direct (pneumonia, aspiration, contusion) and indirect (sepsis, trauma, pancreatitis) lung insults[16]. Notably, sepsis is the main cause of ARDS, and 30 to 40% of patients with sepsis will eventually develop ARDS[41]. ARDS has some major features, such as loss of the alveolar-capillary barrier[42], alveolar epithelial injury[43], inflammatory cell influx[44], activation of coagulation and inhibition of fibrinolysis[45]. Inflammatory cell influx occurs as a result of direct lung injury or as the response to systemic inflammation and cytokine production[46]. The accumulated inflammatory cells mainly consist

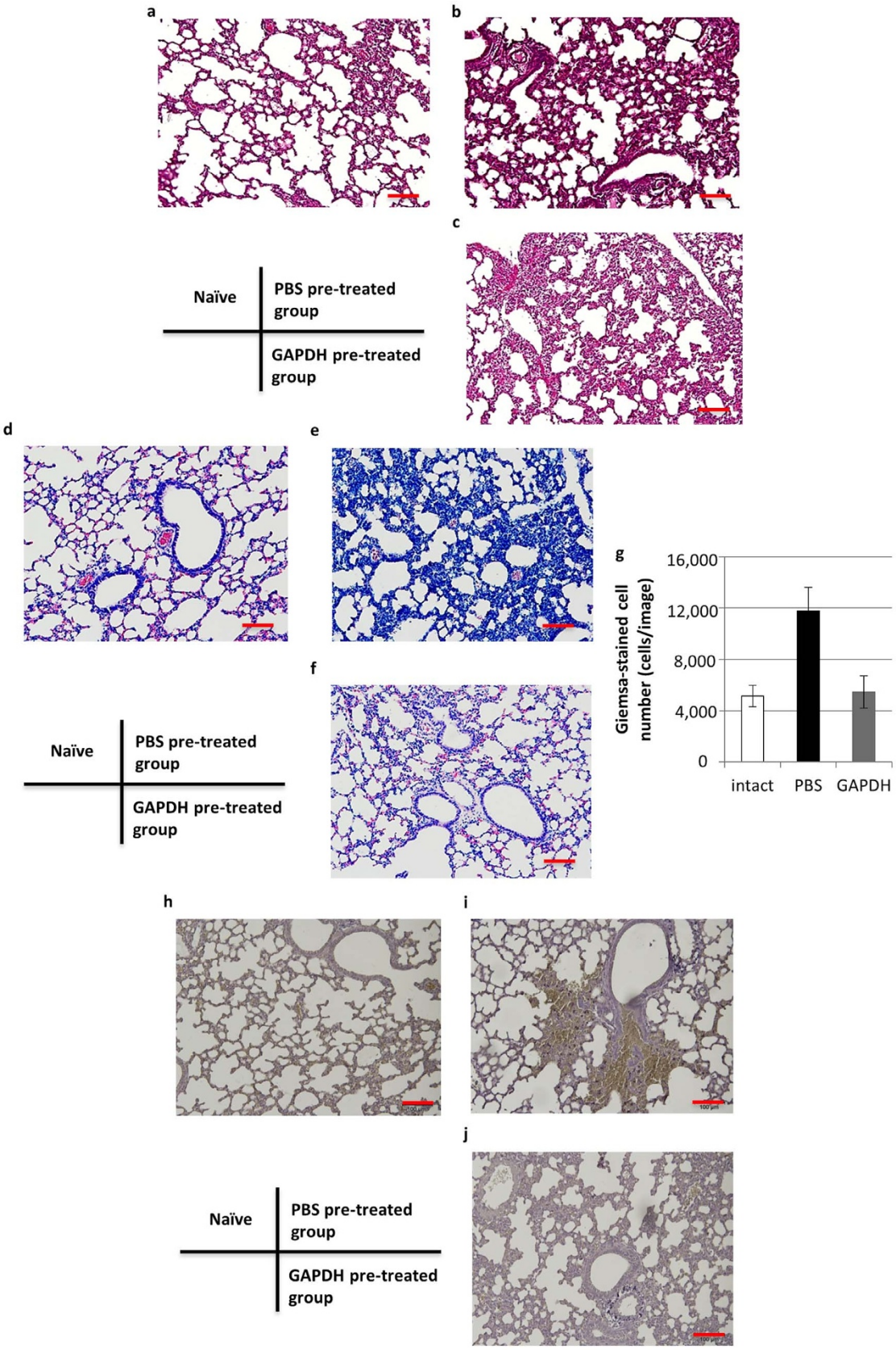

Figure 3 | **Histopathological analysis of lung tissues after LPS injection.** Lungs were obtained from GAPDH or PBS pre-treated mice 12 h after LPS administration and embedded in paraffin. Embedded lungs were sectioned and the sections were stained with hematoxylin and eosin (a–c), Giemsa (d–f) or anti-Ly-6G antibody (h–j). The images of stained lung sections were taken using a light microscope (Olympus Corporation, ×100). Each panel represents naïve (a, d, h), PBS (b, e, i) and GAPDH pre-injected groups (c, f, j), respectively. The data are representative examples of three individual lung sections. Scale bars in all pictures show 100 μm. Giemsa-stained cell number in each image was counted (g). White, black and gray bars show naïve, PBS and GAPDH pre-treated groups, respectively. Values are presented as the mean ± S.D.

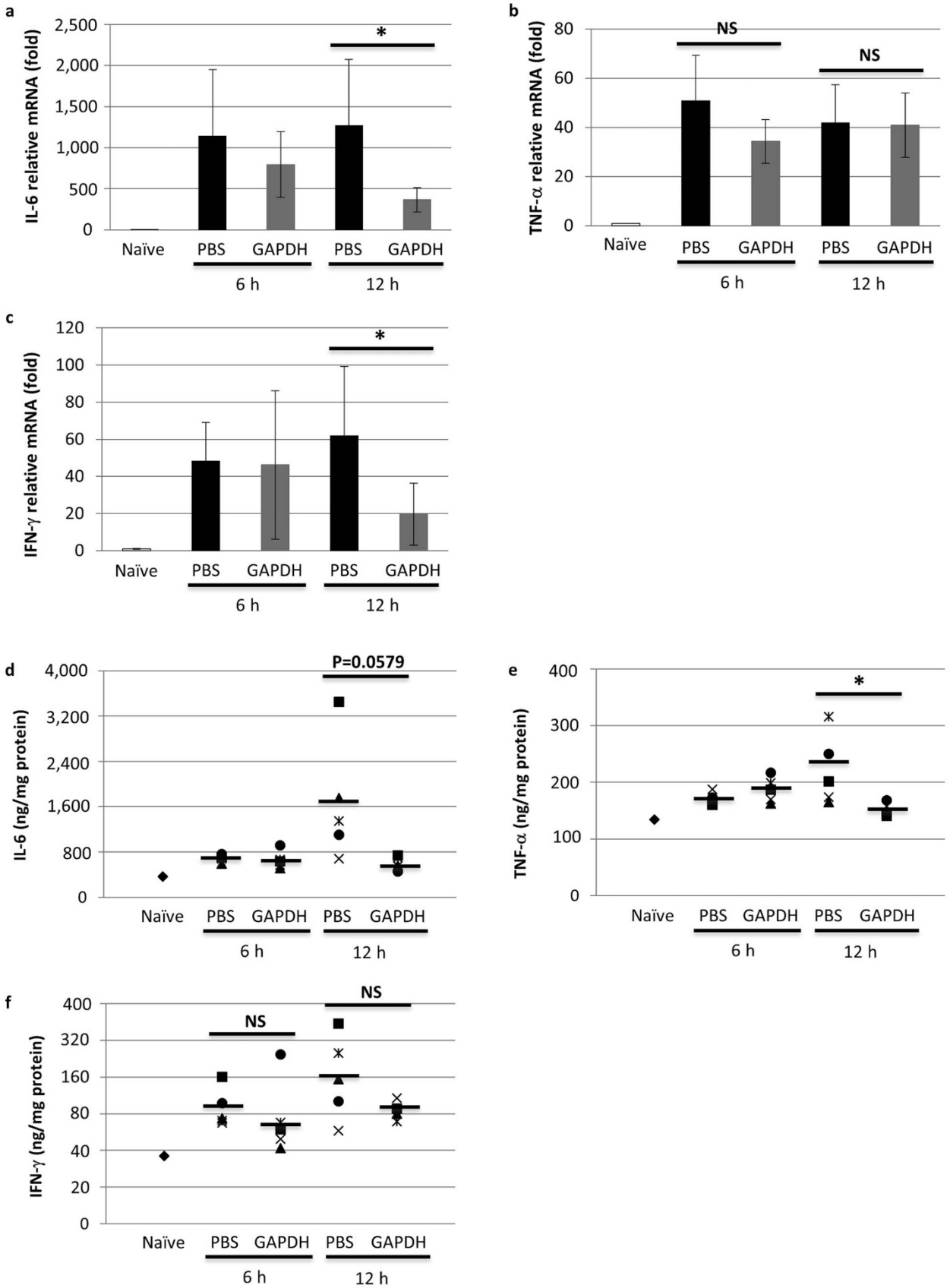

Figure 4 | **Proinflammatory cytokine expression in the lung after LPS injection.** Lungs were obtained from GAPDH or PBS pre-treated mice at 6 or 12 h after LPS administration, and pulmonary mRNA and protein were extracted. Gene expression of cytokines (IL-6 (a), TNF-α (b) and IFN-γ (c)) in the lung was determined by real-time PCR. White, black and gray bars show naïve, PBS and GAPDH pre-treated groups, respectively. Values are presented as the mean ± S.D. Cytokine level (IL-6 (d), TNF-α (e) and IFN-γ (f)) in the lung was measured by using specific ELISA kit. Each symbol in all graphs indicates individual mouse, and bars show mean value. NS: not significant. *, $P < 0.05$ versus the PBS pre-treated group.

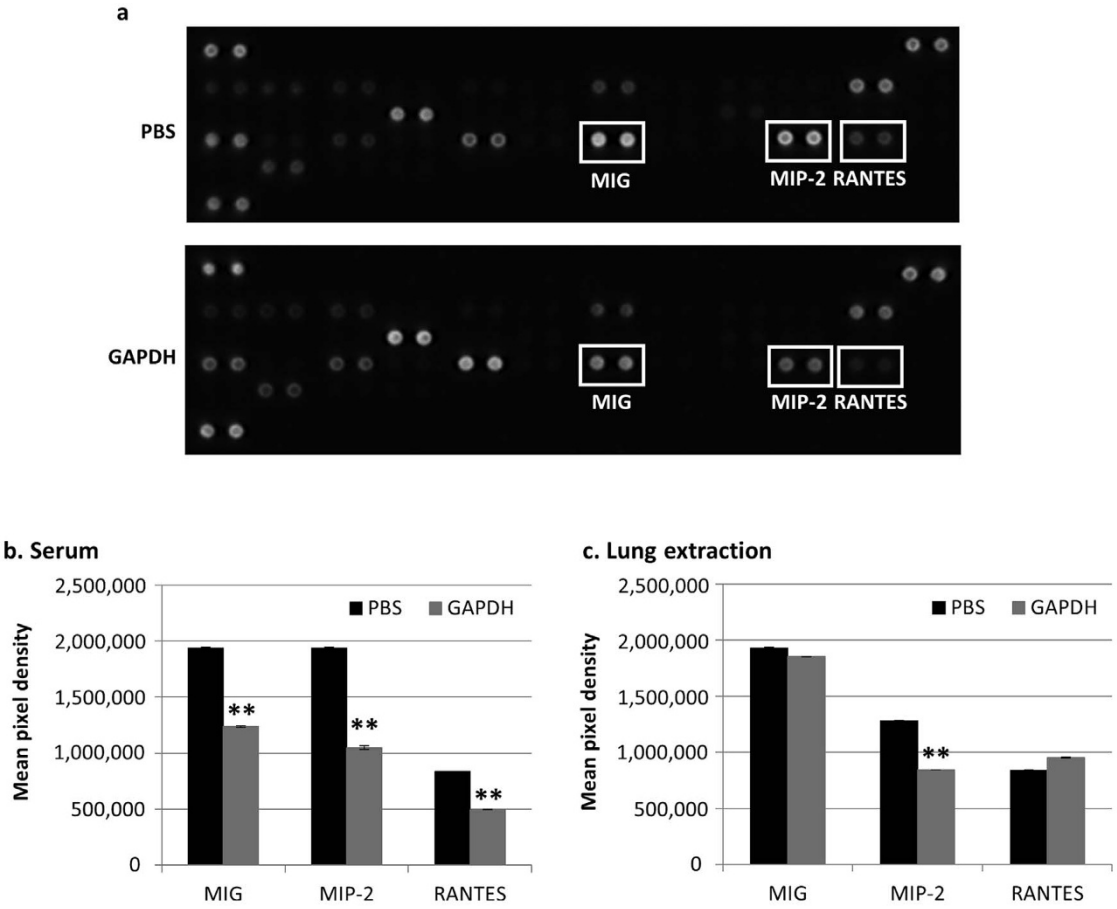

Figure 5 | Serum chemokine levels after LPS injection. Serum and lung were obtained at 12 h after LPS injection, and lung proteins were extracted. Pooled serum or lung extraction (n = 5, each) was used for a cytokine array. Top and bottom images show serum sample of PBS and GAPDH pre-treated groups, respectively (a). Bar graph presents mean pixel density of each spot (b: serum sample, c: lung extraction). Black and gray bars show PBS and GAPDH pre-treated groups, respectively. Values are presented as the mean ± S.D. **, $P < 0.01$ versus the PBS pre-treated group.

of neutrophils[33], and lung edema and endothelial and epithelial injury are also associated with the influx of neutrophils into the broncheoalveolar space[47]. Hence, neutrophils play a key role in the onset and progression of ARDS[48]. Accumulated neutrophils release some injurious mediators, such as elastases[49], oxidants[50] and cationic peptides (ex. LL-37)[48], which contribute to lung injury. For the clinical therapy of ARDS, a neutrophil elastase inhibitor (sivelestat sodium) has been developed as an effective drug of ARDS and is widely used in Japan. However, Iwata *et al.* have demonstrated that treatment with sivelestat was not associated with decreased mortality[51]. Thus, because there is no intensive care medicine for ARDS currently, ARDS still exhibits high mortality rates[15,52].

In this study, we showed that GAPDH remarkably increases survival rate in a mouse severe ALI model, suggesting that GAPDH might be used as therapeutic target for ARDS. However, in the present study, we investigated only the preventative effect of GAPDH against the onset of ARDS by the pre-administration of GAPDH. Therefore, we need to study the therapeutic effect of GAPDH. Additionally, Glare *et al.* have reported that pulmonary GAPDH expression levels in asthmatic patients are lower than in healthy donors, but this reduction in asthmatic patients disappeared after administering inhaled corticosteroids[53]. These reports also support the possibility of GAPDH as a novel therapeutic target for ARDS. To further apply GAPDH to clinical use, it is necessary to elucidate the anti-inflammatory mechanism of GAPDH. As the first step, we need to identify the target cell of GAPDH in the mouse severe ALI model. It is known that neutrophils and macrophages have a quite important role against the onset of sepsis and ALI/ARDS[16,54]. Neutrophils are recruited to eliminate pathogens and damaged tissues, such as epithelial and endothelial cells, before finally undergoing apoptosis[55–57]. Because neutrophils release some proinflammatory components, such as IL-6 and nitric oxide[58], macrophages and other lymphocytes are also recruited to the injured lung and produce large amounts of pro- and anti-inflammatory cytokines[59]. In the recovery stage, the clearance of apoptotic neutrophils via phagocytosis by macrophages, which is named efferocytosis, is crucial for the restoration and maintenance of an anti-inflammatory and tolerogenic milieu[16,60]. In addition, some previous *in vitro* studies have suggested that GAPDH might act on macrophages and modify their function. For instance, GAPDH on the macrophage surface works together with transferrin receptor 1/2 to supply iron to the cell to maintain normal immune function[36]. Mookherjee *et al.* have shown that GAPDH works as the intracellular receptor for anti-infective peptide (LL-37) in monocytes, and GAPDH also has a critical function for the LL-37-induced innate immune response[39]. Therefore, the interaction between GAPDH and neutrophils or macrophages using *in vitro* assays is currently being investigated.

In conclusion, we revealed that GAPDH pre-injection prolongs survival in a LPS-induced, sepsis-related severe ALI mouse model and that GAPDH strongly inhibits the accumulation of neutrophils in the lung following sepsis, suggesting that it might be a potential target for establishing a novel preventative or therapeutic strategy against ARDS.

Methods

Ethics statement. Our experimental design was reviewed and approved by the Institutional Animal Care and Use Committee, and the Committee recognizes that the proposed animal experiment follows the Animal Protection Law by the Council of

Table 1 | Primer sequence

	Forward primer	Reverse primer
IL-6	5′-GAGGATACCACTCCCAACAGACC-3′	5′-AAGTGCATCATCGTTGTTCATACA-3′
TNF-α	5′-ATGAGCACAGAAAGCATGATC-3′	5′-TACAGGCTTGTCACTCGAATT-3′
IFN-γ	5′-ATGAACGCTACACACTGCATC-3′	5′-CCATCCTTTTGCCAGTTCCTC-3′
β-actin	5′-GGCTGTATTCCCCTCCATCG-3′	5′-CCAGTTGGTAACAATGCCATGT-3′

Agriculture, Executive Yuan, R.O.C. and the guideline as shown in the Guide for the Care and Use of Laboratory Animals as promulgated by the Institute of Laboratory Animal Resources, National Research Council, USA.

Reagents and mice. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, derived from rabbit muscle) and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (St Louis, MO, USA). BALB/c mice (female, five-week-old) were obtained from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). These mice were maintained under specific pathogen-free conditions, and fed the CRF-1 diet (Oriental Yeast, Tokyo, Japan) and sterile distilled water *ad libitum*.

LPS-induced, sepsis-related ALI/ARDS mouse model. BALB/c mice were randomly divided into two groups with similar mean body weight and were intraperitoneally injected with GAPDH (10 mg/kg, n = 15) or phosphate-buffered saline (PBS, 100 μl, n = 15). After 90 min, LPS (20 mg/kg) was intraperitoneally administered into pre-injected mice. The survival rate 48 h (n = 5 per group) after LPS administration was monitored, and serum samples and lung tissues were collected from surviving mice at 6 (n = 5 per group) and 12 h (n = 5 per group) after LPS administration.

Measurement of cytokine production. Serum levels of TNF-α, IL-6 and IFN-γ were measured using DuoSet ELISA Development kits (R&D systems, Minneapolis, MN, USA) according to the manufacturer's protocols. Briefly, capture antibodies diluted in PBS were coated on 96-well microplates (Nalgene Nunc International, Roskide, Denmark) and incubated for overnight at room temperature (RT). The plates were washed three times with 0.05% (v/v) Tween 20-PBS (PBST) and blocked with 1% (w/v) bovine serum albumin (BSA, Sigma-Aldrich)/PBS for 1 h at RT. After washing, samples (x25 dilution) and standard were added to each well and incubated for 2 h at RT. Next, detection antibodies diluted in 1% BSA/PBS were added and incubated at RT for 2 h. Streptavidin-horseradish peroxidase was then added and incubated at RT for 20 minutes, followed by the addition of 1-Step Ultra TMB substrate solution (Thermo Fisher Scientific Inc., Rockford, IL, USA). Lastly, the reactions were stopped using 2N HCl (Sigma-Aldrich), and the absorbance at 450 nm was determined using a Victor X4 Multilabel Plate Reader (PerkinElmer, Shelton, CT, USA).

Histopathological analysis. Lung tissue was taken 12 h after LPS injection, fixed in 10% formaldehyde and then embedded in paraffin. After embedding, lungs were sectioned (3-μm thickness), and the sections were stained with hematoxylin and eosin or Giemsa. In Giemsa-stained tissue, the number of stained cells in each image of three individual lung sections was counted. For immunohistochemistry, sections were incubated with ChemMate Target Retrieval Solution (Dako, Glostrup, Denmark) for 1 h at 95°C after paraffin deletion. After washing with PBST, sections were treated with 3% (v/v) H_2O_2 (Sigma-Aldrich, diluted in PBST) for 10 minutes at RT. Non-specific proteins were then blocked using Universal Blocking Reagent (BioGenex, Fremont, CA, USA) for 30 minutes at RT. After blocking, sections were incubated for overnight at 4°C with 1 μg/ml of purified anti-mouse Ly-6G (Gr-1) antibody (eBioscience, Inc., San Diego, CA, USA) diluted in Antibody Diluent with Background Reducing Components (Dako). After washing, sections were treated with Super Enhancer (BioGenex) for 20 minutes at RT, followed by labeling for 1 h at RT with HRP conjugated Goat anti-rat IgG antibody (x1,000, Biosource International, Camarillo, CA, USA) diluted in Antibody Diluent with Background Reducing Components. After rinsing, DAB signal was detected by Super Sensitive Polymer-HRP IHC Detection system/DAB (BioGenex) according to the manufacturer's protocols. All images of stained sections were captured using a light microscope (Olympus Corporation, Tokyo, Japan).

RNA collection and real-time PCR. Total RNA in the lung 6 and 12 h after LPS injection was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed using the High Capacity Reverse Transcription Kit (Applied Biosystems, Foster, CA, USA) according to the manufacturers' instructions. To evaluate the expression of each gene, cDNA was amplified by real-time PCR using Fast SYBR® Green Master Mix (Applied Biosystems) and gene-specific primer mixtures in the 7500 Real-time PCR System (Applied Biosystems). Each gene-specific primer was made by Genomics (New Taipei, Taiwan), and the sequences of each primer are described in Table 1. The real-time PCR program was as follows: enzyme activation step: at 95°C for 20 sec; amplification step: 40 cycles of 3 sec denaturation at 95°C, 30 sec of annealing and extension at 60°C. Using the comparative CT method (ΔΔCT), the real-time PCR data were analyzed. All results were normalized using β-actin as an internal standard.

Protein extraction from lung tissue. Total proteins in the lung tissue at 6 and 12 h after LPS injection were extracted using T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific Inc.). In brief, the weight of lung tissue was measured, and the reagent was added (20 μl-reagent/1 mg-tissue). To protect the protease activity, cOmplete, Mini, EDTA-free (Roche Diagnostics GmbH, Mannheim, Germany) was also added into the reagent (1 tablet/10 ml), and lung tissue was then homogenized. After centrifuging, the supernatant was collected. Protein concentration was determined by BCA Protein Assay Kit (Thermo Fisher Scientific Inc.) according to the manufacturer's methods. To quantify the pulmonary levels of TNF-α, IL-6 and IFN-γ, lung extracts (x10 dilution) were used with DuoSet ELISA Development kits (R&D systems) and detail method is described above. All data are expressed as ng of cytokine per mg total protein.

Cytokine array. Serum and pulmonary chemokine profiles at 12 h after LPS injection were determined using the Mouse Cytokine Array Panel A (R&D systems) according to the manufacturer's protocols. Briefly, 50 μl of pooled serum or 300 μg of pooled lung extraction (n = 5 per group) was incubated with the Mouse Cytokine Array Panel A Detection Antibody Cocktail for 1 h at RT. After blocking, the membrane was incubated with the sample/antibody mixture overnight at 4°C. After washing, Streptavidin-horseradish peroxidase was added and incubated for 30 minutes at RT. Next, the Chemi Reagent Mix was added and incubated for 1 minute at RT, and the X-ray signal was then detected using a G:BOX iChemi XL (Syngene, Frederick, MD, USA). Mean pixel density of each spot was measured using Gene tools (Syngene). The data on different arrays were normalized using the density of the reference spots.

Statistical analysis. All data are presented as the mean ± S.D. of three independent experiments. Student's *t* test was used to judge statistical significance against the negative control. Survival data were analyzed by the Kaplan-Meier method and log-rank test using SPSS 16.0 software (IBM, Armonk, NY, USA). A p value less than 0.05 was defined as statistically significant.

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Author contributions

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Additional information

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