IL-35 interferes with splenic T cells in a clinical and experimental model of acute respiratory distress syndrome

Chuan-jiang Wang[a,c], Mu Zhang[c], Hua Wu[b], Shi-hui Lin[c], Fang Xu[a,c,*]

[a] *The Laboratory of Lipid & Glucose Metabolism, the First Affiliated Hospital of Chongqing Medical University, Chongqing, China*
[b] *Center for Cognitive and Neurobiological Imaging, Stanford University, Stanford, CA, USA*
[c] *Department of Emergency and Critical Care Medicine, the First Affiliated Hospital of Chongqing Medical University, Chongqing, China*

ARTICLE INFO

ABSTRACT

Acute respiratory distress syndrome (ARDS) is a life-threatening critical care syndrome with uncontrolled inflammation that is a central issue. Its main characteristic is inflammatory mediators and cytokines as well as agglutinating chemokines that injure target cells. Interleukin (IL)-35 is a newly identified IL-12 cytokine family member with structural similarities to other IL-12, IL-23, and IL-27 cytokines but unique immunological functions. How IL-35 functions in ARDS is unclear. The purpose of our study was to determine what role IL-35 played in the development of ARDS. Here we found serum IL-35 concentrations were significantly elevated in patients with ARDS relative to healthy people. Moreover, we established a mouse model of lipopolysaccharide- and cecal ligation and puncture-induced ARDS treated with neutralizing antibodies (anti-IL-35 Ebi3 or anti-IL-35 P35); the results showed that lung injury occurred more often than in untreated models and the inflammatory cytokines CXCL-1, tumor necrosis factor alpha, IL-6, and IL-17A increased significantly after neutralizing antibody treatment in bronchoalveolar lavage fluid and serum. Therefore IL-35 can protect against the development of ARDS. Even more interesting in our study was that we discovered IL-35 expression differed between lung and spleen across different ARDS models, which further demonstrated that the spleen likely has an important role in extrapulmonary ARDS model only, improving the ratio of $CD4^+/CD4^+CD25^+Foxp3^+$(Tregs). Meanwhile in our clinical work, we also found that the concentration of IL-35 and the ratio of $CD4^+/Treg$ in the serum are higher and the mortality is lower than those with the spleen deficiency in patients with extrapulmonary ARDS. Therefore, IL-35 is protective in ARDS by promoting the ratio of splenic $CD4^+/Tregs$ in extrapulmonary ARDS, and as such, may be a therapeutic target.

1. Introduction

Acute respiratory distress syndrome (ARDS) is a life-threatening critical care syndrome characterized by alveolar-capillary membrane injury and hypoxemic respiratory failure [1–3]. It is a common cause of admission to the intensive care unit (ICU) because of hypoxemic respiratory failure requiring mechanical ventilation [4]. Despite decades of research, few therapeutic strategies for ARDS have emerged, and current options for treatment are limited [5–7]. Currently, uncontrolled inflammation is thought to be the physiological mechanism underlying ARDS [8]. Research shows that upregulation of adhesion molecules and chemokines, and an imbalance in proinflammation/anti-inflammation are needed for the development and progression of ARDS [9]. Thus, we sought to understand how inflammatory cytokines contribute to ARDS and attempted to identify a therapeutic target for treatment.

The IL-12 family has unique structural, functional, and immunological characteristics [10]. Studies suggest that IL-12 cytokines have immune-regulatory roles in suppressing the development of T helper (Th)1, Th2, and Th17 cell subsets in ARDS [11, 12]. IL-35, a newly identified IL-12 cytokine, shares structural similarities with other IL-12, IL-23, and IL-27 cytokines but mediates different immunological functions [13]. Research suggests that IL-35 is a potent anti-inflammatory and immunosuppressive cytokine; therefore, increased IL-35 may be a biomarker for inflammation and disease severity in sepsis [14]. We hypothesized that IL-35 may have anti-inflammatory roles in the development of ARDS, but studies are lacking to validate this hypothesis.

ARDS is due to direct pulmonary (severe pneumonia, aspiration pneumonia, and pulmonary contusion) or indirect (extrapulmonary) insults to the lungs (abdominal infection, multiple trauma, severe acute

---

pancreatitis, and septic shock) [15]. Thus far, studies have not determined the mechanistic differences between each type of insult. IL-35 can regulate Tregs to achieve its anti-inflammatory and immunosuppressive effects [16] and a positive feedback loop exists between IL-35 and Tregs, including inhibiting effector T cells (CD4$^+$CD25$^-$Teff) proliferation, blocking Th1 and Th17 cell synthesis and downregulating IL-17 [17,18]. Studies show that increased Th17/Treg ratios in the blood of ARDS patients is positively correlated with poor prognosis [19].

Therefore, to clarify the role of IL-35 in ARDS, we collected serum from patients with pulmonary and extrapulmonary ARDS and measured IL-35 and inflammatory factors. Then we created lipopolysaccharide (LPS)-induced pulmonary and cecal ligation and puncture (CLP)-induced extrapulmonary mouse models and treated each with IL-35 neutralizing antibodies to determine the different roles and mechanisms of IL-35. We also constructed a spleen-free ARDS model to illustrate the effects of the spleen on IL-35 in ARDS.

2. Materials and methods

2.1. Study population

Twenty-seven adult patients with ARDS were recruited from the ICU of the First Affiliated Hospital of Chongqing Medical University from December 2015 to February 2016. ARDS diagnosis was based on the Berlin standard [20]. Study patients were admitted to the ICU while in the acute phase of the disease (onset within 24 h). Patients with massive transfusion or hemofiltration within the preceding 24 h, those undergoing immunosuppressive or immune-enhancing therapy, or those with chronic lung diseases were excluded. Patient data are presented in Table 1. Control samples were obtained from healthy donors (n = 11). The study protocol was approved by the Clinical Research Ethics Committee of the University and informed consent was obtained from all participants.

2.2. Human serum cytokine measurements

Blood was collected as described in Methods and kept at −80 °C until analysis. IL-35 was measured using enzyme-linked

Table 1
Characteristics of the study population.

Characteristic	Controls (n = 11)[a]	ARDS[b] (n = 27)
Age (years)	57 ± 6	60 ± 8
Male/female gender (proportion of male)	6/5 (54.5%)	16/11 (59.2%)
Smoker	5 (45.6%)	13 (48.1%)
Major surgery	–	1 (3.7%)
Multiple trauma	–	2 (7.4%)
Diabetic ketoacidosis	–	1 (3.7%)
HELLP syndrome	–	1 (3.7%)
Severe acute pancreatitis	–	4 (14.8%)
Severe pneumonia	–	8 (29.6%)
Aspiration pneumonia	–	2 (7.4%)
Pulmonary contusion	–	2 (7.4%)
Necrotizing fasciitis	–	2 (7.4%)
Abdominal infection	–	1 (3.7%)
Urinary tract infection	–	1 (3.7%)
Chronic obstructive pulmonary disease (COPD)	–	2 (7.4%)
PaO$_2$/FiO$_2$ ratio	392.16 ± 43.82	194.08 ± 76.64
APACHE II[c] score	–	20.13 ± 7.86
Ventilator free days	–	7.11 ± 7.76
ICU free days	–	8.73 ± 9.34
Survival	100% (31)	59.3% (16/27)

[a] Data as a percentage of patients or mean ± SEM.
[b] ARDS acute respiratory distress syndrome.
[c] APACHE II Acute Physiology and Chronic Health Evaluation II.

immunosorbent assay (ELISA) as described (MyBioSource, USA) and tumor necrosis factor (TNF)-α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-17α, IL-27, interferon (IFN)-γ, CXCL1, CXCL8, and CXCL10 were measured using a Human Cytokine/Chemokine Magnetic Bead Panel Kit (Merck Millipore, Germany) [21].

2.3. Animals

Male mice (C57BL/6, 8–12 w old, 20–24 g; Laboratory Animal Center of Chongqing Medical University, Chongqing, China) were pathogen free, kept under a 12-h light/dark cycle and given food and water ad libitum. All experimental protocols were approved by the Institutional Animal Care and Use Committee at Chongqing Medical University.

2.4. LPS-induced pulmonary ARDS model

Mice were anesthetized with chloral hydrate (3.5%, intraperiotoneally) and then 50 μg LPS intranasally (Escherichia coli, serotype 055: B5; Sigma-Aldrich, St. Louis, MO, USA) in 50 μL phosphate buffered saline (PBS) to induce lung injury. Control mice received 50 μL of PBS intranasally. Mice were euthanized at 6, 12, and 24 h after the treatment and lung and spleen tissues were collected. Blood was collected retro-orbitally. Bronchoalveolar lavage fluid (BALF) was collected by ligating blood vessels leading to lungs and the trachea leading to left lungs and injecting them with 200 μL PBS into the right lungs via the trachea. Next (10 s later), PBS was collected. This was repeated with 400 μL BALF and collected [21].

2.5. CLP-induced extrapulmonary ARDS model

Mice were anesthetized as before and an extrapulmonary ARDS model was established using CLP [22]. At the end of the experiment, mice were killed on the same timetable and the same fluids and tissues were collected.

2.6. Splenectomy model

Mice were anesthetized as before and then placed in a flat position. After shaving and aseptic preparation of the surgical site, a transverse incision was made into the abdominal cavity along the left side of the abdominal line. The splenic artery and vein were ligated and the spleen was removed. The abdominal incision was closed. A sham operation was performed in control mice, the abdominal wall was cut, and the spleen was not resected. Mice were given ceftriaxone (20 g/g, subcutaneously) for 3 days after surgery. Fourteen days later, a splenectomized mouse model was confirmed [23].

2.7. Histopathology

Lung tissues were fixed, sectioned, and stained with hematoxylin and eosin (H&E) for morphological analysis. Lung injury scores were estimated using a method by Mikawa (1 Alveolar hyperemia, 2 Hemorrhage, 3 Interstitial or neutrophil infiltration or aggregation, 4 Alveolar septal thickening or hyaline membrane formation). According to the severity of lesions in each indicator, 0 to 4 points indicate semiquantitative analysis. The total score for each score is used as the pathological score for ARDS. Higher scores indicate greater injury.

2.8. Antibody-mediated neutralization

Neutralization assays were performed by giving 50 μg anti-mouse IL-35 EBI3 (Rockland Immunochemicals) or anti-mouse IL-12A p35 (Abcam Systems) 30 min after CLP or LPS. Normal goat isotype immunoglobulin G (IgG) (Bioss, China) was used as a control. Blood, BALF, spleen, and lung tissues were then collected as indicated [14].
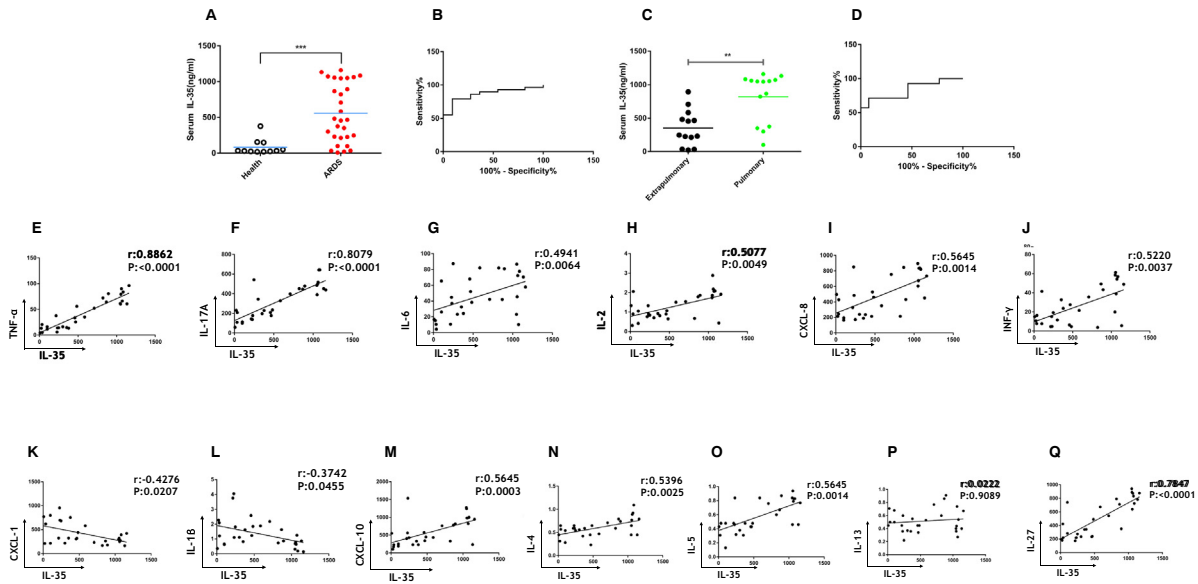
Fig. 1. IL-35 was elevated in those with pulmonary ARDS compared to those with extrapulmonary ARDS and was correlated with immune mediators but negatively associated with CXCL-1 and IL-1β. (A) IL-35 from healthy donors and patients with ARDS. Each sample was tested in duplicate and concentrations were determined from a standard curve (***p < 0.0001). (B) ROC curve analysis of serum IL-35 in patients with ARDS and control patients. (C) IL-35 measured by ELISA from patients with extrapulmonary and pulmonary ARDS. Each sample was tested in duplicate and a standard curve was used (**p < 0.001). (D) ROC curve analysis and area under the curve for serum IL-35 concentration in patients with extrapulmonary and pulmonary ARDS. (E–Q) IL-35 was positively and significantly correlated with immune mediators but negatively associated with CXCL-1 and IL-1β when compared between healthy individuals and those with ARDS.
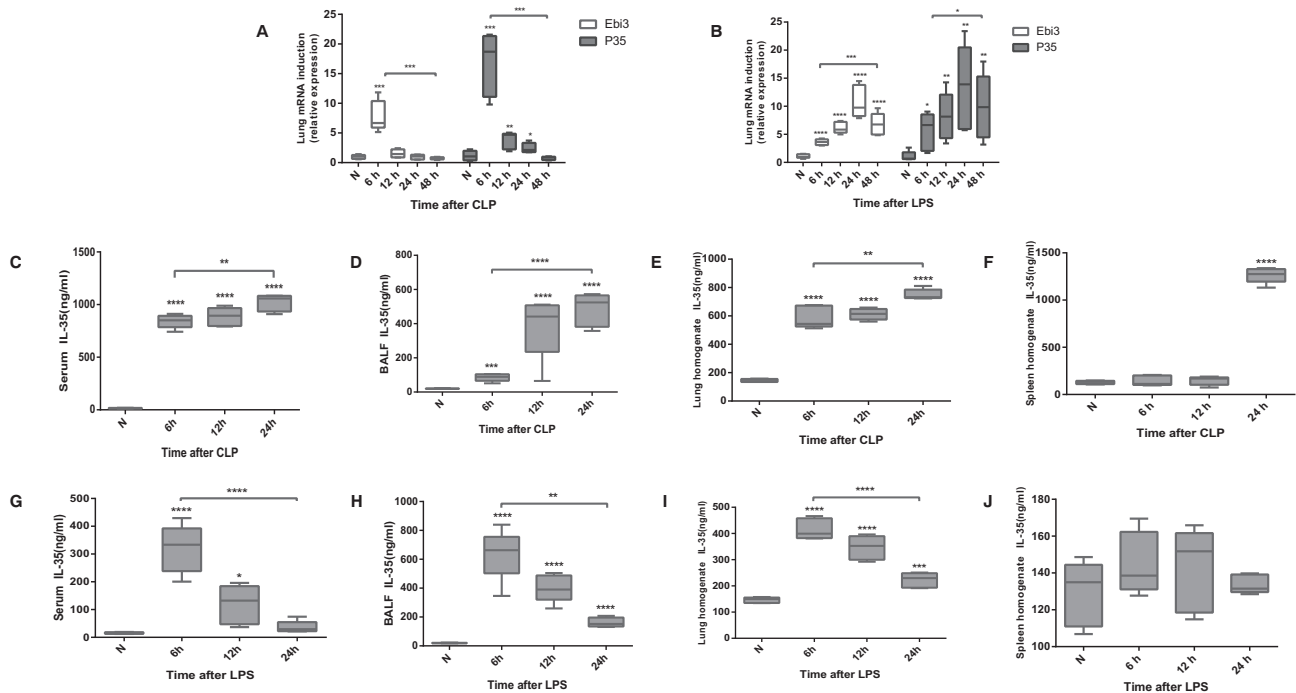

Fig. 2. IL-35 expression differed in lungs and spleens of different ARDS models. C57BL/5 mice (5/group) were subjected to LPS or CLP. (A, B) IL-35 mRNA levels in the lungs were measured with qRT-PCR. Relative expression levels of the genes were expressed with the GAPDH housekeeping gene as an internal reference. (C–J) Organs were removed at the indicated time points, Blood specimens were collected from mice under anesthesia via the ophthalmic vein. Bronchoalveolar lavage fluid was obtained by washing the bronchus three times with 0.2 mL of sterile PBS each time, and the homogenate was obtained by mixing tissue and PBS in a ratio of 0.5 g:1 mL. Samples were assayed for IL-35 content by enzyme-linked immunosorbent assays.*p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001, by the one-way ANOVA followed by LSD multiple comparisons test, compared with normal mice.

2.9. Mice Serum, BALF, lung homogenate, and spleen homogenate cytokines

Blood was collected from mice under anesthesia via the ophthalmic vein and saved to EDTA tubes and centrifuged at 3000 rpm for 15 min at 4 °C. Spleen and lungs were homogenized with PBS (10 mg tissues/

100 μL PBS). Fluids and tissues were aliquoted and kept frozen at −80 °C until analysis. IL-35 (MyBioSource, USA) in fluids and tissues were assayed with ELISA kits. TNF-α, IL-2, IL-6, IL-10, IL-13, IL-17-A, IFN-γ, and CXCL1 were measured using a Mice Cytokine/Chemokine Magnetic Bead Panel Kit (eBioscience, USA).
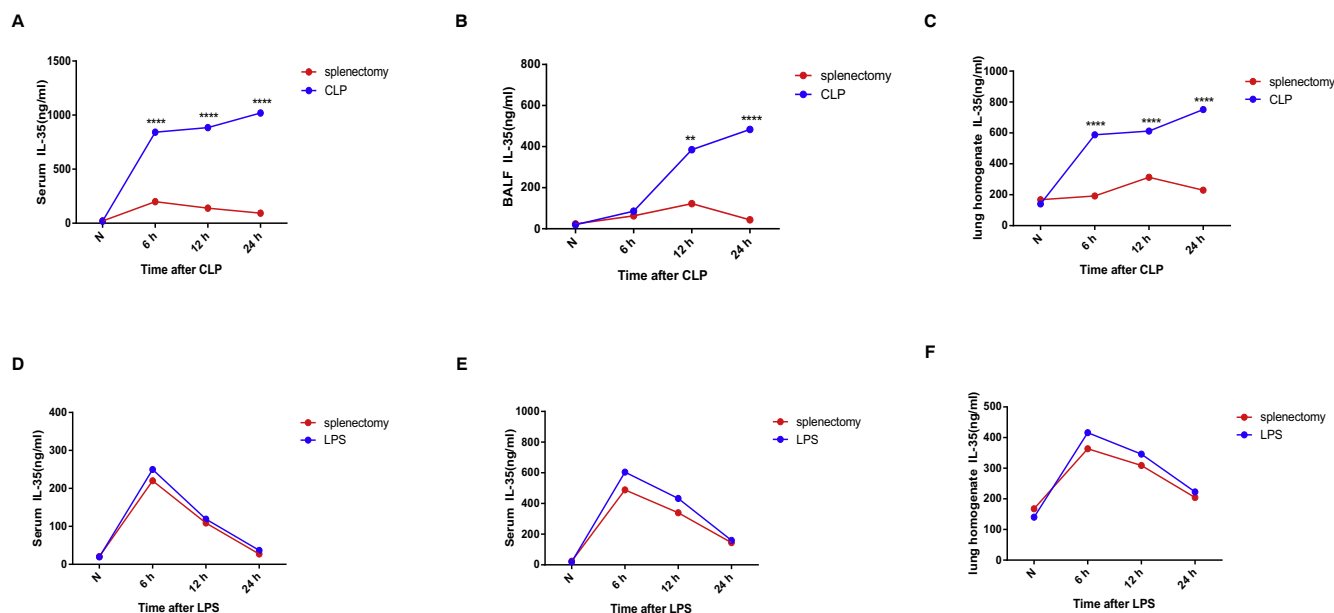
Fig. 3. Splenic function was tied to IL-35 in a CLP-induced ARDS model. C57BL/5 mice (5/group) were subjected to LPS, LPS (splenectomy), CLP or CLP (sple-nectomy). The sample was extracted and detected by referring to the aforementioned method. IL-35 in serum, BALF, and lung homogenates in the splenectomized CLP group were significantly lower than those in the nonsplenectomized CLP group. In the LPS and splenectomized LPS groups IL-35 in serum, BALF, and lung homogenate did not differ significantly. **p < 0.01, and ****p < 0.0001, by the two-way ANOVA followed by LSD multiple comparisons test, compared with the no-splenectomy group.

2.10. RNA extraction and quantification

Total cellular RNA was extracted from lung tissue using TRIzol reagent (TakaraBio, Tokyo, Japan) and DNaseI digestion. Quantitative real-time polymerase chain reaction (qRT-PCR) for mouse IL-35 EBI3 and p35 was performed using specific primers and sequences for IL-35 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were: EBI3 sense 5′-CGGTGCCCTACATGCTAAAT-3′; antisense 5′-GCGGAGTCGGT ACTTGAGAG-3′; P35 sense 5′-CATCGATGAGCTGATGCAGT-3′; anti-sense 5′-CAGATAGCCCATCACCCTGT-3′; GAPDH sense 5′-TTCACCAC CATGGAGAAGGC-3′; antisense 5′-GGCATGGACTGTGGTCATGA-3′.

Quantitative real-time PCR was performed in a 25-L volume with 2 L cDNA, 400 nM each sense and antisense primer, and 12.5 L Brilliant SYBR Green QPCR Master Mix (Takara Bio) on ABIPRISM7000 (Applied Biosystems, Foster City, CA, USA). The action was performed for 40 cycles with denaturation at 95 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 10 s. Gene expression normalized to GAPDH was used to determine relative target gene expression and the $^{2\Delta\Delta}C(t)$ method was used.

2.11. Flow cytometry

Cells were washed in PBS, pelleted, and subsequently stained for flow cytometry. Mouse peritoneal cells were characterized accordingly with monoclonal antibodies against CD4, CD25, and Foxp3. Cells were counted and flow cytometry was performed using antibodies purchased from eBioscience. For Foxp3 staining, extracellular staining was performed followed by intracellular staining using the manufacturer's recommendations. At least 10^4 cells were collected with a FACScan flow cytometer (Becton Dickinson) and analyzed with Flow Jo software 7.6.

2.12. Statistical analyses

SPSS 19.0 was used for all statistical analyses and data are reported as means ± standard error of the mean. Differences between groups were analyzed using Student's t-test. Statistical analysis was performed using one- or two-way analysis of variance (ANOVA) followed by a least

significant difference (LSD) multiple comparison test or a Dunnett test for multiple groups. Correlation analysis was done using a nonpara-metric Spearman rank correlation coefficient (p < 0.05).

3. Results

3.1. Baseline characteristics of the study population

According to the inclusion and exclusion criteria, 27 patients with ARDS and 11 healthy control patients were involved in this experiment. The baseline characteristics of the study population are detailed in Table 1.

IL-35 is abnormally elevated in patients with ARDS and associated with other cytokines.

Because IL-35 is a newly identified IL-12 cytokine family member its function in ARDS is unclear. We first compared the serum IL-35 levels in patients with ARDS and healthy control patients. The results showed that IL-35 of patients with ARDS were greater than in control patients (Fig. 1A). Based on our receiver operating characteristic (ROC) analysis, a cutoff level of serum IL-35 for the diagnosis of ARDS was set at 185.2 pg/mL. The specificity, sensitivity, negative predictive value, and positive predictive value were 91%, 79%, 81%, and 79%, respectively (Fig. 1B). IL-35 for pulmonary ARDS was greater than for patients with extrapulmonary ARDS (Fig. 1C). Based on our ROC analysis, a cutoff level of serum IL-35 for the diagnosis of pulmonary ARDS was set at 765.3 pg/mL. The specificity, sensitivity, negative predictive value, and positive predictive value were 92%, 71%, 71%, and 92%, respectively (Fig. 1D). In addition, as others have reported a variety of inflammatory mediators involved in initiating, amplifying, and maintaining in-flammation during ARDS, we therefore measured serum indices including TNF-α, CXCL-1, IL-1β, CXCL-8, CXCL-10, IL-6, IFN-γ, IL-2, IL-4, IL-5, IL-17A, and IL-27 to determine their correlation with IL-35. The level of IL-35 in serum was positively and significantly correlated with TNF-α, CXCL-8, CXCL-10, IL-6, IFN-γ, IL-2, IL-4, IL-5, IL-17A, and IL-27 (Fig. 1E–J, M–O, Q, p < 0.01) but negatively correlated with CXCL-1 and IL-1β during ARDS (Fig. 1K, L, p < 0.01).
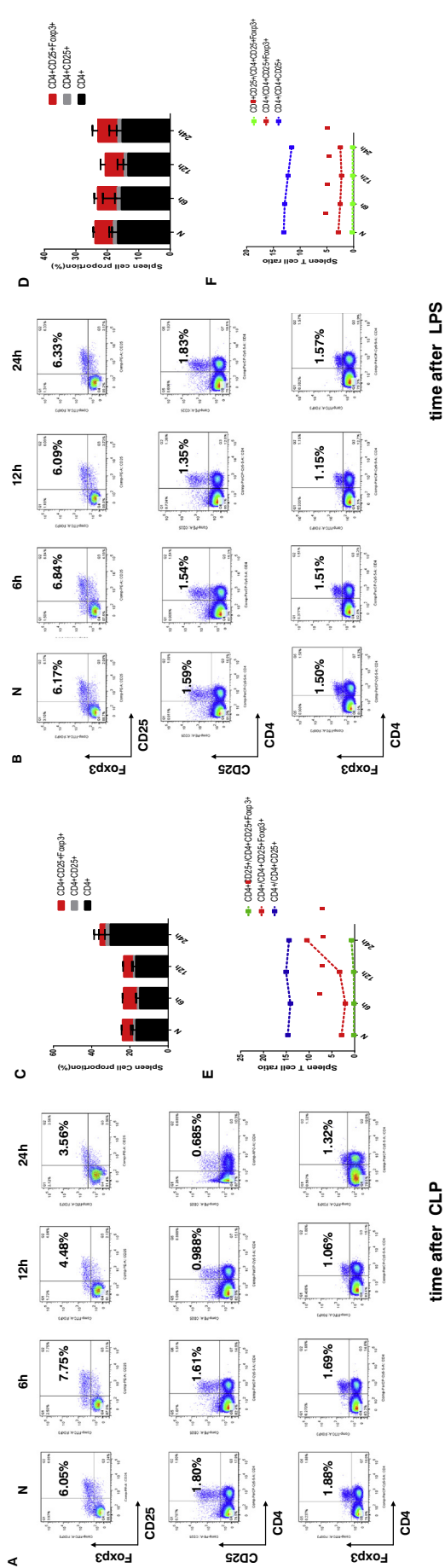
Fig. 4. ARDS was induced in wild-type mice via CLP or LPS. T cells were isolated from mice spleen and the frequency of Tregs was subsequently determined by flow cytometry, five mice per time point. A and B were representative dot plots as shown. (A–D) Treg cells gradually decrease with the prolongation of time in CLP-induced ARDS, but not in LPS. (E–F) Ratio of CD4 + /Treg in spleen was significantly increased in CLP-induced ARDS, but not in LPS. Ratio of CD4 + /CD4 + CD25 + and CD4 + CD25 + /Treg in spleen showed no significant change in the two ARDS models. *p < 0.05 ****p < 0.0001, by two- way ANOVA followed by LSD multiple comparisons test, compared with the LPS + IgG group.

3.2. Expression of IL-35 in CLP- and LPS-induced murine ARDS models

We next analyzed local, systemic, and organ IL-35 concentrations, using our well-established LPS- or CLP-induced ARDS model. qRT-PCR and ELISA data show that IL-35 did what in CLP-induced or LPS-in-tranasal instillation ARDS respectively. *EBI3* and *P35* mRNA in lungs decreased 6 h after CLP and peaked (Fig. 2A). IL-35 protein in serum, BALF, lung, and spleen homogenate were increased at 24 h with CLP and peaked (Fig. 2C–F). *EBI3* and *P35* mRNA in the lung increased at 6 h and peaked at 24 h after LPS administration (Fig. 2B). IL-35 protein in serum, BALF, and lung homogenate decreased (Fig. 2G–I), but the expression in spleen homogenate did not differ after LPS administration (p > 0.05, Fig. 2J).

3.3. Expression of IL-35 in CLP and LPS-induced murine ARDS splenectomy models

Having observed that the IL-35 expression differed between the lung and spleen across different ARDS models, we used our splenectomy ARDS model to analyze changes in IL-35. Compared with the CLP-induced ARDS model after splenectomy, IL-35 in serum, BALF, and lung homogenate were significantly lower in a CLP-induced ARDS model (no splenectomy) (Fig. 3A–C). In the LPS-induced ARDS model, IL-35 did not differ between splenectomized and nonsplenectomized groups (Fig. 3D–F).

3.4. Regulatory T lymphocytes ratios differ across ARDS models

Furthermore, because IL-35 can promote the proliferation of Treg cells, Treg cells can also be elevated by IL-35 and have anti-inflammatory and immunosuppressive effects. We used a flow cytometric method to determine whether Treg cells in spleen are involved in different ARDS models. The outcomes showed that Treg cells in spleen were not significantly different after LPS (p > 0.05, Fig. 4B, D). In the CLP-induced ARDS model, Treg cells in spleen showed a transient increase at 6 h and then there was a downward trend during 6 h to 24 h (Fig. 4A, C). What is undefined and more interesting is the ratio of CD4 + /Treg was significantly higher in the CLP-induced extra-pulmonary ARDS model, and the percentage of CD4+ /CD4+ CD25+ and CD4+ CD25+ /Treg did not increase significantly. The CD4+ / CD4+ CD25+, CD4+ /Treg, CD4+ CD25+ /Treg ratios were not increased in the LPS-induced pulmonary ARDS model (Fig. 4E–F).

3.5. Neutralization of IL-35 affects histopathology expression in spleens across ARDS models

In order to verify whether the antibody is effective, we detected IL-35 levels in serum, BALF, and lung homogenate after given anti-EBI3 or anti-p35 in both ARDS models. The results showed that IL-35 protein level was significantly lower after administration of neutralizing antibody (Supplementary Fig. 2). From scoring the histologic examination of lung sections stained with H&E, CLP-induced ARDS have a higher Mikawa score than the IgG group after treatment with anti-IL-35 (p35 or EBI3) at 12 h. In addition, the increase in the Mikawa score lasted for 24 h (Fig. 5A–B). Furthermore, the LPS-induced ARDS model had the same result after treatment with IL-35 neutralizing antibody (p35 or EBI3) (Fig. 5C–D).

3.6. Neutralization of IL-35 affects CD4 + /Treg ratio expression in spleens across ARDS models

By performing flow cytometry detection, we found that there was a significant difference in the CD4+ /Treg of spleen at different times in CLP-induced ARDS after treatment with neutralizing antibody (Fig. 6A–B). Also, there were similar results for the CD4+ /Treg of spleen in LPS-induced ARDS (Fig. 6C–D). Thus, either in CLP- or LPS-
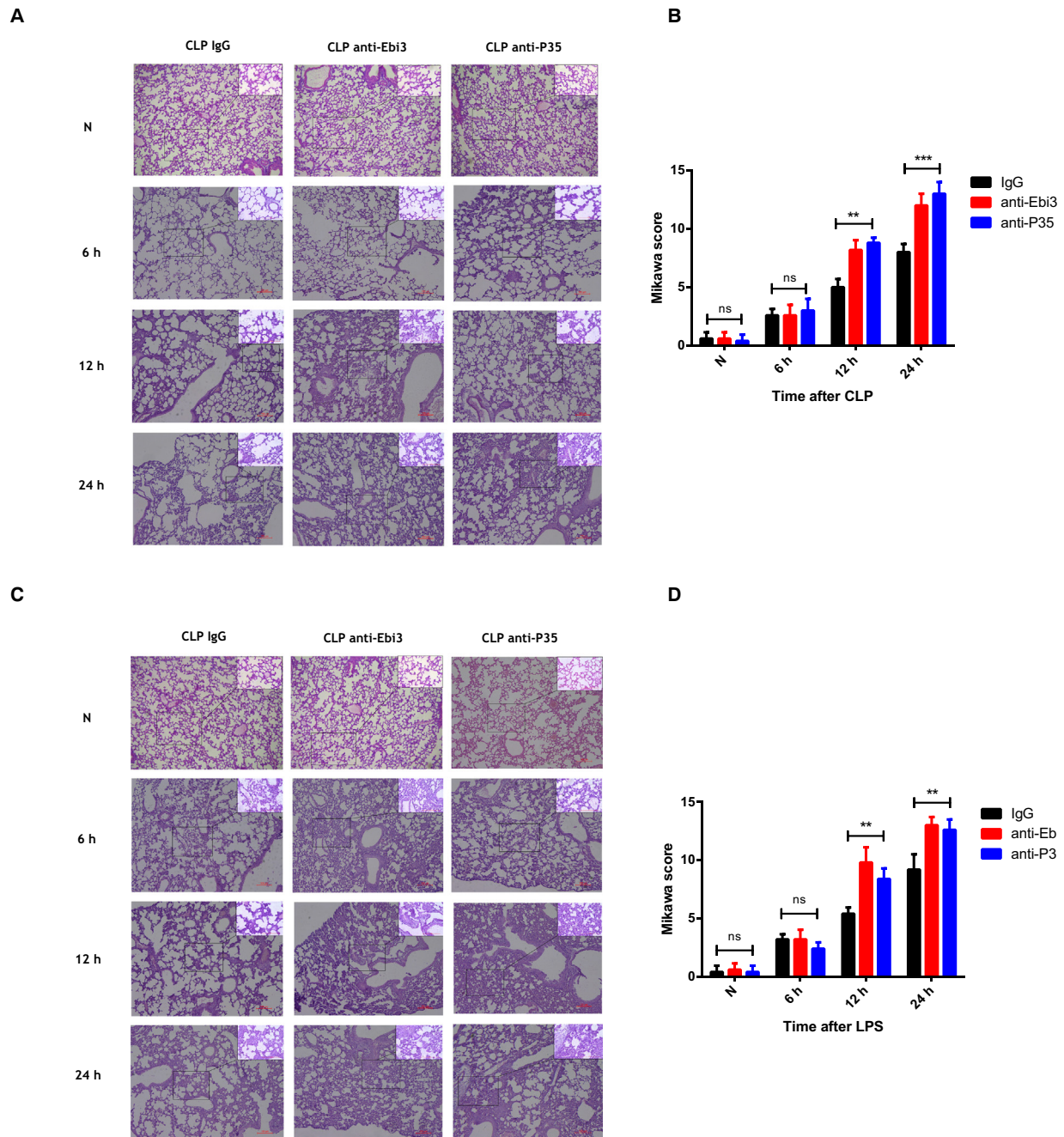
Fig. 5. (A, E) Pretreatment with IL-35 neutralizing antibodies (anti-P35 or anti-Ebi3) aggravated LPS- and CLP-induced ARDS. Lungs from each experimental group were processed for histological examination after H&E staining. Compared with the LPS + IgG group, thickened alveolar wall, alveolar hemorrhage and collapse, inflammatory cells in filtration were more severe and pretreated with IL-35 neutralizing antibodies prior to LPS challenge. (B, D) Lung injury scores were estimated by the method of Mikawa, which is from the following four indicators of lung injury score: alveolar congestion; bleeding; gap or vascular wall neutrophil infiltration or aggregation; alveolar septal thickening or transparent membrane formation. 0 marks: no or very slight damage, 1 marks: mild injury, 2 marks: moderate injury, 3 marks: severe injury, 4 marks: very severe damage, the cumulative increase in the number of lesions of the total score is the pathological score of the ARDS. Pretreatment with IL-35 neutralizing antibodies lungs injury were more severe than LPS + IgG group. **p < 0.01, ***p < 0.001, by the two-way ANOVA followed by LSD multiple comparisons test, compared with the LPS + IgG group.

induced ARDS, IL-35 can regulate the transformation of CD4$^+$ T lymphocyte subsets in spleen, and it should lead to the decrease in the CD4$^+$/Treg ratio of the spleen.

3.7. Neutralization of IL-35 and cytokine expression across ARDS models

As shown in Fig. 7, the concentrations of inflammatory cytokines and chemokines, including TNF-α, IL-6, IL-17A, CXCL1 in BALF, and blood specimens obtained 24 h after CLP or LPS were significantly

upregulated in ARDS mice that underwent therapeutic IL-35 treatment. However, for IFN-γ, IL-10, IL-2, IL-13, these concentrations were significantly downregulated.

4. Discussion

ARDS is not an isolated, fragmented disease but a serious injury caused by a systemic inflammatory cascade [24]. An "uncontrolled inflammatory reaction" is a manifestation of excessive inflammation in
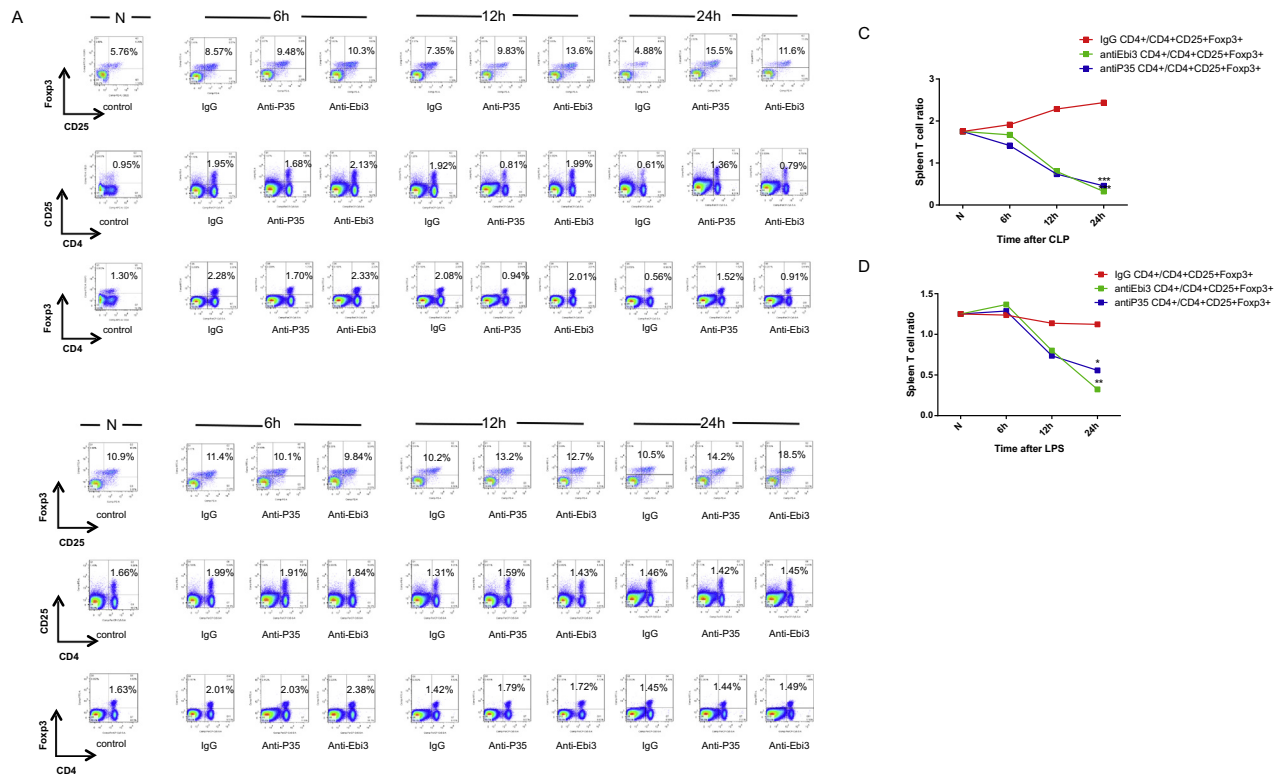
Fig. 6. Pretreatment with IL-35 neutralizing antibodies prior to CLP or LPS challenge. T cells were isolated from mice spleen, and the frequency of Tregs was subsequently determined by flow cytometry, five mice per time point. A and C were representative dot plots as shown. Either in CLP- or LPS-induced ARDS, ratio of the CD4 + /Treg in spleen was decreased gradually after treatment with IL-35 neutralizing antibodies. *p < 0.05 ****p < 0.0001, by the two-way ANOVA followed by LSD multiple comparisons test, compared with the LPS + IgG group.

the lung and a central link to ARDS. At its initiation, inflammatory mediators and cytokines, as well as agglutinated chemokines, release ROS and damage target cells to cause ARDS.

IL-12 is an inflammatory mediator along with IL-23, IL-27, and IL-35 [13], which helps to regulate immunity against infectious and autoimmune diseases and cancers [25,26]. IL-23 and IL-27 may have pathogenic roles in inflammatory diseases of the lungs, such as asthma [27–29] and ARDS [22]. IL-35 has significant anti-inflammatory and immunosuppressive effects and can improve the symptoms of mice with collagen-induced arthritis [30], preventing multiple sclerosis in mice with central nervous system demyelination [31] and improving mouse T cell-dependent colitis [32]. Recently, IL-35 was reported to reduce the severity of ARDS by decreasing IL-17 [33]. We noted increased IL-35 in patients with ARDS (Fig. 1A), and an association between IL-35 and other cytokines with inflammation was observed in patients with ARDS (Fig. 1E–Q). IL-35 may affect other cytokines and contribute to the immunopathogenesis of ARDS. Neutralization antibodies (anti-IL-35 EBI3 or anti-IL-35 p35) applied to both ARDS models lead to profound alveolar hyperemia, neutrophil infiltration, alveolar hemorrhage, and transparent film formation (Fig. 5A, C). Both ARDS models had greater Mikawa scores than CLP/LPS groups at 12 and 24 h (Fig. 5B, D). CXCL-1, TNF-α, IL-6, and IL-17A were increased significantly, and IFN-γ, IL-10, IL-2, and IL-13 were significantly reduced after IL-35 neutralizing antibody treatment in BALF and serum (Fig. 7). Multiple studies have shown that IL-35 can play a cross-talk role in regulating the expression of various cytokines based on lymphocyte cells and related signaling pathways. IL-35 could inhibit the expression of TNF-α and IL-6 [43]. It can promote apoptosis by activating JAK1/STAT1 and shifting activation from TNF receptor-associated death domain (TRADD)-TRAF2/RIP1-NF-κB to TRADD-FADD-caspase 3 signaling [44]. Binding of IL-35 with IL-12 receptor subunit beta 2 (IL-12RB2) and IL-6 signal transducer (IL-6ST) occupies the binding sites of IL-6 and reducing the

proinflammatory effects [45]. IL-35 can upregulate IFN-γ and inhibit phosphorylation smad3 (transforming growth factor beta [TGF-β] receptor downstream effector), then impede the differentiation of Th17 cells [46]. Further, IL-17, an important cytokine secreted by Th17 cells, could be suppressed by IL-35 in pulmonary inflammatory disease [47]. Another study showed that the Notch/Notch ligands play a role in maintaining balance of Th17/Tregs cells (IL-17/IL-35) [48]. Regarding Th2-related cytokine, one study showed that IL-35 can reduce IL-13 to induce eosinophils infiltration in asthma [49]. However, the interacted mechanism between IL-35 and IL-10 is quite specific. IL-35 promoted the phosphorylation of STAT3 and IL-10 production in B cells. It may have potent effects in regulating immunoreactivity via IL-10-dependent mechanisms in autoimmunity [50] because of a possibility for a feed-forward loop whereby regulatory B cells utilize IL-10 to enhance their other mechanisms of immune regulation [51]. IL-35 blockade may increase inflammation during ARDS. The mutual regulation mechanism of cytokines involved is complex and diverse.

IL-35 expression was greater in pulmonary than in extrapulmonary ARDS (Fig. 1C). In LPS-induced direct pulmonary ARDS and CLP-induced indirect extrapulmonary ARDS models, abnormal expression of IL-35 was similar, but IL-35 in serum, BALF, and lung homogenate increased in the extrapulmonary ARDS model (peaked at 24 h; Fig. 2C–E). The opposite trend was observed for pulmonary ARDS (Fig. 2G–I). *EBI3* and *P35* mRNA in the lung also showed similar results in the corresponding models (Fig. 2A, B). Thus, different pathological mechanisms may underpin each type of ARDS.

Our unusual extrapulmonary ARDS case had less serum IL-35 and different inflammatory factors than other ARDS cases (Supplemental Table 2), and ratios of CD4 + /Treg cells were also lower than in other ARDS cases (Supplementary Fig. 1B). The spleen, which is the largest peripheral lymphoid organ and contains numerous immunologically active cells and immune factors, may explain differences in these data
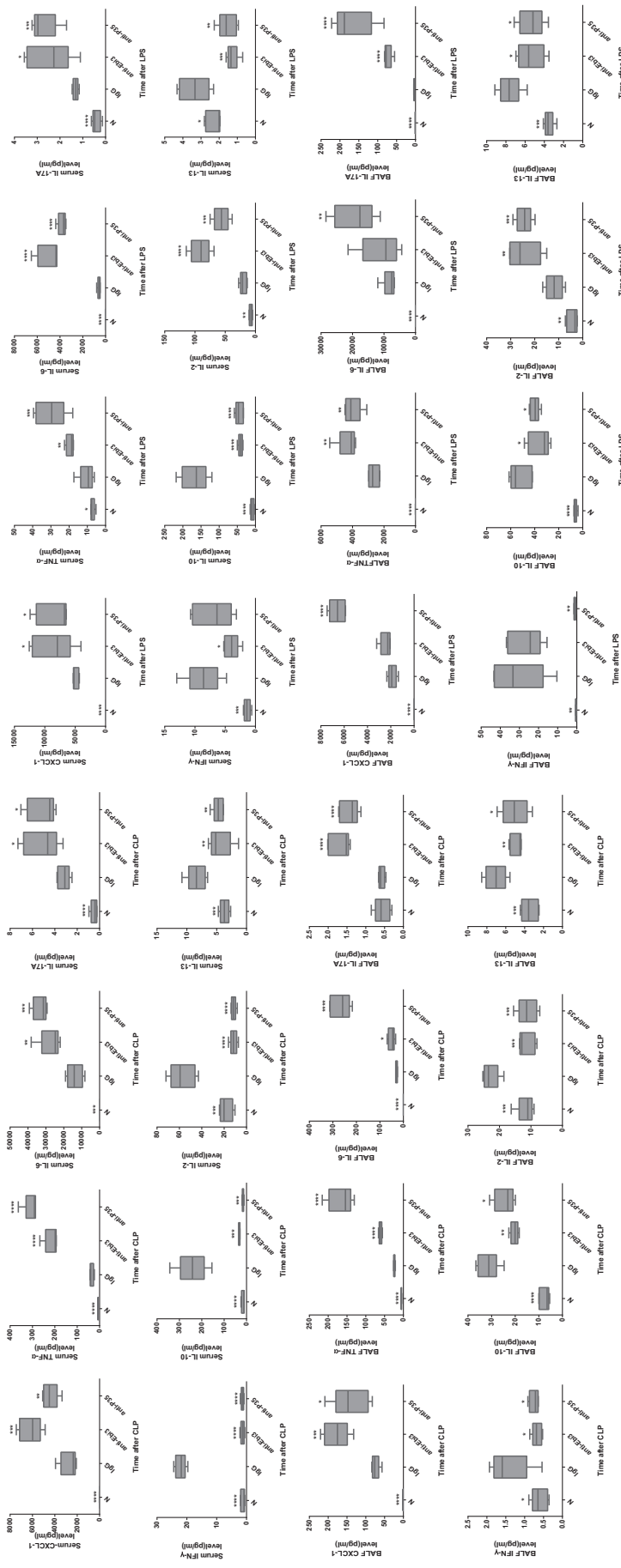
Fig. 7. Interleukin 35 (IL-35) blockade upregulated the production of proinflammatory cytokines and downregulated anti-inflammatory cytokines during ARDS models. Cytokine and chemokine concentrations in BALF and blood specimens from five mice treated with or without anti–Ebi3 or anti–P35 blocking antibodies were determined by mice cytokine/chemokine magnetic bead panel kit assays 24 h after onset of ARDS. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, by the one-way ANOVA followed by LSD multiple comparisons test, compared with mice treated with isotypical IgG control. ARDS featured increased CXCL-1, TNF-α, IL-6, and IL-17A and reduced IFN-γ, IL-10, IL-2, and IL-13 after neutralizing antibody treatment (anti-IL-35 Ebi3 or anti-IL-35 P35) in BALF and serum.

[34–36]. Sepsis occurred rapidly in splenectomized mice, and they had reduced pneumococcal clearance [37]. Patients with greater mortality due to fulminant infection were seen in sepsis-induced ARDS and required prolonged mechanical ventilation after splenectomy [38]. Treg expression increased in spleen and peripheral blood, so its immunoregulation should be enhanced after administration of exogenous IL-35 gene expression vector [19]. In a mouse endotoxin model, Tregs were significantly reduced in the spleen [39]. Thus, the regulatory mechanism of spleen-IL-35-T lymphocytes may contribute to extrapulmonary ARDS.

ELISA data show that splenic IL-35 did not differ across LPS-induced ARDS models (Fig. 2J) but gradually increased at 24 h after CLP-induced (Fig. 2F). After splenectomy, IL-35 in lung homogenate, serum, and BALF was significantly lower than after no splenectomy in an extrapulmonary ARDS model (Fig. 3A–C). There was no significant difference in pulmonary ARDS model (Fig. 3D–F). Thus, splenic production of IL-35 differs between extrapulmonary and pulmonary ARDS. The immune and barrier functions of the lung may be less than that of the spleen, and IL-35 expression is reduced in the pulmonary ARDS model.

Tregs are anti-inflammatory due to contact-dependent suppression or release of cytokines, IL-10, and TGF-β1 in other immune cells, such as CD4 + and CD8 + T cells, B cells, natural killer cells, and dendritic cells [42]. Data show that reduced generation or deficient function of Tregs is associated with disease severity and activity, as this is documented in patients with various inflammatory and autoimmune diseases [40]. In mice and patients with ALI, alveolar recruitment of Tregs, specifically mediated by leukotriene B4 (LTB4)-leukotriene B4 receptor (BLT1) pathway [41], contributed to the resolution of lung inflammation [42]. IL-35 and Tregs are reported to interact, but whether IL-35 affects differentiation of Tregs in the spleen and affects progression of ARDS is not known. We found that splenic Tregs increased at 6 h and decreased at 24 h (Fig. 4A) in extrapulmonary ARDS, and this was associated with IL-35 expression in the spleen. However, this was not observed in pulmonary ARDS (Fig. 4B). Also, the CD4 + /Treg ratio increased significantly in the extrapulmonary ARDS model (Fig. 4E) and was correlated with splenic IL-35 expression, but this was not observed in the pulmonary ARDS model (Fig. 4F). IL-35 secretion is linked to Tregs and associated with the ratio of CD4 + /Tregs. Treatment with neutralizing antibodies decreased splenic CD4 + /Treg ratios, and this followed IL-35 trends in the ARDS models (Fig. 6B, D). Thus, IL-35 can regulate the transformation of CD4 + T lymphocyte subsets in the spleen to elicit immunosuppression of Tregs and increased inflammatory amplification of ARDS.

5. Conclusion

IL-35 is protective against the development of ARDS, and different underlying pathophysiological mechanisms occur with different etiologies of ARDS. The spleen was not involved in IL-35 secretion in pulmonary ARDS but had a role in extrapulmonary ARDS. IL-35 may promote the ratio of splenic CD4 + /Tregs in extrapulmonary ARDS only, and this may offer a promising therapeutic avenue for ARDS.

Acknowledgment

Patient consent: Obtained.

Ethics approval: Human subjects: This study was carried out in accordance with the recommendations of the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University. The number is Lot 2016-34.

Animal subjects: This study was carried out in accordance with the recommendations of The Institutional Animal Care and Use Committee at Chongqing Medical University. All experimental protocols were approved by the Institutional Animal Care and Use Committee at Chongqing Medical University.

Provenance and peer review: Not commissioned; externally peer reviewed.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2018.12.024.

References

[1] S.E. Erickson, G.S. Martin, J.L. Davis, M.A. Matthay, M.D. Eisner, N.N.A. Network, Recent trends in acute lung injury mortality: 1996–2005, Crit. Care Med. 37 (5) (2009) 1574–1579, https://doi.org/10.1097/CCM.0b013e31819fefdf (published Online First: Epub Date).

[2] G.D. Rubenfeld, E. Caldwell, E. Peabody, et al., Incidence and outcomes of acute lung injury, N. Engl. J. Med. 353 (16) (2005) 1685–1693, https://doi.org/10.1056/NEJMoa050333 (published Online First: Epub Date).

[3] G. Bellani, J.G. Laffey, T. Pham, et al., Epidemiology, patterns of care, and mortality for patients with acute respiratory distress syndrome in intensive care units in 50 countries, JAMA 315 (8) (2016) 788–800, https://doi.org/10.1001/jama.2016.0291 (published Online First: Epub Date).

[4] J. Phua, J.R. Badia, N.K. Adhikari, et al., Has mortality from acute respiratory distress syndrome decreased over time?: a systematic review, Am. J. Respir. Crit. Care Med. 179 (3) (2009) 220–227, https://doi.org/10.1164/rccm.200805-722OC (published Online First: Epub Date).

[5] E. Crimi, A.S. Slutsky, Inflammation and the acute respiratory distress syndrome, Best Pract. Res. Clin. Anaesthesiol. 18 (3) (2004) 477–492.

[6] D. Mehta, J. Bhattacharya, M.A. Matthay, A.B. Malik, Integrated control of lung fluid balance, Am. J. Phys. Lung Cell. Mol. Phys. 287 (6) (2004) L1081–L1090, https://doi.org/10.1152/ajplung.00268.2004 (published Online First: Epub Date).

[7] D. Zolnoun, K. Hartmann, G. Lamvu, S. As-Sanie, W. Maixner, J. Steege, A conceptual model for the pathophysiology of vulvar vestibulitis syndrome, Obstet. Gynecol. Surv. 61 (6) (2006) 395–401, https://doi.org/10.1097/01.ogx.0000219814.40759.38 (quiz 23, published Online First: Epub Date).

[8] G. Graziani, M. Buskermolen, S. Oldani, G. Brambilla, Sepsis, acute renal failure and multiple organ dysfunction syndrome, G. Ital. Nefrol. 23 (Suppl. 36) (2006) S13–S21.

[9] M.P. Mortelliti, H.L. Manning, Acute respiratory distress syndrome, Am. Fam. Physician 65 (9) (2002) 1823–1830.

[10] P. Behzadi, E. Behzadi, R. Ranjbar, IL-12 family cytokines: general characteristics, pathogenic microorganisms, receptors, and signalling pathways, Acta Microbiol. Immunol. Hung. 63 (1) (2016) 1–25, https://doi.org/10.1556/030.63.2016.1.1 (published Online First: Epub Date).

[13] L.W. Collison, C.J. Workman, T.T. Kuo, et al., The inhibitory cytokine IL-35 contributes to regulatory T-cell function, Nature 450 (7169) (2007) 566–569, https://doi.org/10.1038/nature06306 (published Online First: Epub Date).

[14] J. Cao, F. Xu, S. Lin, et al., IL-35 is elevated in clinical and experimental sepsis and mediates inflammation, Clin. Immunol. 161 (2) (2015) 89–95, https://doi.org/10.1016/j.clim.2015.08.016 (published Online First: Epub Date).

[15] L. Gattinoni, P. Pelosi, P.M. Suter, A. Pedoto, P. Vercesi, A. Lissoni, Acute respiratory distress syndrome caused by pulmonary and extrapulmonary disease. Different syndromes? Am. J. Respir. Crit. Care Med. 158 (1) (1998) 3–11, https://

doi.org/10.1164/ajrccm.158.1.9708031 (published Online First: Epub Date).

[16] X.G. Xiang, Q. Xie, IL-35: a potential therapeutic target for controlling hepatitis B virus infection, J. Dig. Dis. 16 (1) (2015) 1–6, https://doi.org/10.1111/1751-2980.12218 (published Online First: Epub Date).

[17] X. Li, J. Mai, A. Virtue, et al., IL-35 is a novel responsive anti-inflammatory cytokine—a new system of categorizing anti-inflammatory cytokines, PLoS One 7 (3) (2012) e33628, https://doi.org/10.1371/journal.pone.0033628 (published Online First: Epub Date).

[18] Z. Ning-Wei, Interleukin (IL)-35 is raising our expectations, Rev. Med. Chil. 138 (6) (2010) 758–766.

[19] G. Karimi, M. Mahmoudi, M. Balali-Mood, et al., Decreased levels of spleen tissue CD4(+)CD25(+)Foxp3(+) regulatory T lymphocytes in mice exposed to berberine, J. Acupunct. Meridian Stud. 10 (2) (2017) 109–113, https://doi.org/10.1016/j.jams.2016.10.003 (published Online First: Epub Date).

[20] A.D.T. Force, V.M. Ranieri, G.D. Rubenfeld, et al., Acute respiratory distress syndrome: the Berlin definition, JAMA 307 (23) (2012) 2526–2533, https://doi.org/10.1001/jama.2012.5669 (published Online First: Epub Date).

[21] S.H. Lin, J. Fu, C.J. Wang, et al., Inflammation elevated IL-33 originating from the lung mediates inflammation in acute lung injury, Clin. Immunol. (2016), https://doi.org/10.1016/j.clim.2016.10.014 (published Online First: Epub Date).

[22] F. Xu, Q. Liu, S. Lin, N. Shen, Y. Yin, J. Cao, IL-27 is elevated in acute lung injury and mediates inflammation, J. Clin. Immunol. 33 (7) (2013) 1257–1268, https://doi.org/10.1007/s10875-013-9923-0 (published Online First: Epub Date).

[23] J. Higashijima, M. Shimada, M. Chikakiyo, et al., Effect of splenectomy on anti-tumor immune system in mice, Anticancer Res. 29 (1) (2009) 385–393.

[24] J.E. Schroeder, Y.G. Weiss, R. Mosheiff, The current state in the evaluation and treatment of ARdS and SIRS, Injury 40 (Suppl. 4) (2009) S82–S89, https://doi.org/10.1016/j.injury.2009.10.041 (published Online First: Epub Date).

[25] C. Beadling, M.K. Slifka, Regulation of innate and adaptive immune responses by the related cytokines IL-12, IL-23, and IL-27, Arch. Immunol. Ther. Exp. 54 (1) (2006) 15–24, https://doi.org/10.1007/s00005-006-0002-6 (published Online First: Epub Date).

[26] L.L. Jones, D.A. Vignali, Molecular interactions within the IL-6/IL-12 cytokine/receptor superfamily, Immunol. Res. 51 (1) (2011) 5–14, https://doi.org/10.1007/s12026-011-8209-y (published Online First: Epub Date).

[27] Y. Li, M. Sun, H. Cheng, et al., Silencing IL-23 expression by a small hairpin RNA protects against asthma in mice, Exp. Mol. Med. 43 (4) (2011) 197–204, https://doi.org/10.3858/emm.2011.43.4.024 (published Online First: Epub Date).

[28] H. Wakashin, K. Hirose, Y. Maezawa, et al., IL-23 and Th17 cells enhance Th2-cell-mediated eosinophilic airway inflammation in mice, Am. J. Respir. Crit. Care Med. 178 (10) (2008) 1023–1032, https://doi.org/10.1164/rccm.200801-086OC (published Online First: Epub Date).

[29] G. Ciprandi, C. Cuppari, A.M. Salpietro, et al., Serum IL-23 strongly and inversely correlates with FEV1 in asthmatic children, Int. Arch. Allergy Immunol. 159 (2) (2012) 183–186, https://doi.org/10.1159/000336418 (published Online First: Epub Date).

[30] Y. Li, S. Wu, Y. Li, et al., Interleukin-35 (IL-35) inhibits proliferation and promotes apoptosis of fibroblast-like synoviocytes isolated from mice with collagen-induced arthritis, Mol. Biol. Rep. 43 (9) (2016) 947–956, https://doi.org/10.1007/s11033-016-4034-7 (published Online First: Epub Date).

[31] M. Zandian, K.R. Mott, S.J. Allen, O. Dumitrascu, J.Z. Kuo, H. Ghiasi, Use of cytokine immunotherapy to block CNS demyelination induced by a recombinant HSV-1 expressing IL-2, Gene Ther. 18 (7) (2011) 734–742, https://doi.org/10.1038/gt.2011.32 (published Online First: Epub Date).

[32] R.J. Xavier, D.K. Podolsky, Unravelling the pathogenesis of inflammatory bowel disease, Nature 448 (7152) (2007) 427–434, https://doi.org/10.1038/nature06005 (published Online First: Epub Date).

[33] K. Risso, G. Kumar, M. Ticchioni, et al., Early infectious acute respiratory distress syndrome is characterized by activation and proliferation of alveolar T-cells, Eur. J. Clin. Microbiol. Infect. Dis. 34 (6) (2015) 1111–1118, https://doi.org/10.1007/s10096-015-2333-x (published Online First: Epub Date).

[34] W.H. Crosby, An historical sketch of splenic function and splenectomy, Lymphology 16 (2) (1983) 52–55.

[35] J.S. Chen, L.C. Chang, S.J. Huang, C.W. Cheng, Targeting spleen tyrosine kinase-Bruton's tyrosine kinase axis for immunologically mediated glomerulonephritis, Biomed. Res. Int. 2014 (2014) 814869, https://doi.org/10.1155/2014/814869 (published Online First: Epub Date).

[36] A. Khalilnezhad, E. Mahmoudian, N. Mosaffa, A. Anissian, M. Rashidi, D. Amani, Effects of Chlorella vulgaris on tumor growth in mammary tumor-bearing Balb/c mice: discussing association of an immune-suppressed protumor microenvironment with serum IFNgamma and IgG decrease and spleen IgG potentiation, Eur. J. Nutr. (2017), https://doi.org/10.1007/s00394-017-1387-1 (published Online First: Epub Date).

[37] R.J. Holdsworth, A.D. Irving, A. Cuschieri, Postsplenectomy sepsis and its mortality rate: actual versus perceived risks, Br. J. Surg. 78 (9) (1991) 1031–1038.

[38] M. Crandall, M.B. Shapiro, M.A. West, Does splenectomy protect against immune-mediated complications in blunt trauma patients? Mol. Med. 15 (7–8) (2009) 263–267, https://doi.org/10.2119/molmed.2009.00029 (published Online First: Epub Date).

[39] L. Tao, J. Zhu, Y. Chen, et al., IL-35 improves Treg-mediated immune suppression in atherosclerotic mice, Exp. Ther. Med. 12 (4) (2016) 2469–2476, https://doi.org/10.3892/etm.2016.3649 (published Online First: Epub Date).

[40] M. Noack, P. Miossec, Th17 and regulatory T cell balance in autoimmune and inflammatory diseases, Autoimmun. Rev. 13 (6) (2014) 668–677, https://doi.org/10.1016/j.autrev.2013.12.004 (published Online First: Epub Date).

[41] L. Wang, L. Zhao, J. Lv, et al., BLT1-dependent alveolar recruitment of CD4(+)CD25(+) Foxp3(+) regulatory T cells is important for resolution of acute lung injury, Am. J. Respir. Crit. Care Med. 186 (10) (2012) 989–998, https://doi.org/10.1164/rccm.201202-0261OC (published Online First: Epub Date).

[42] F.R. D'Alessio, K. Tsushima, N.R. Aggarwal, et al., CD4+CD25+Foxp3+ Tregs resolve experimental lung injury in mice and are present in humans with acute lung injury, J. Clin. Invest. 119 (10) (2009) 2898–2913, https://doi.org/10.1172/JCI36498 (published Online First: Epub Date).

[43] S. Wu, Y. Li, L. Yao, et al., Interleukin-35 inhibits angiogenesis through STAT1 signalling in rheumatoid synoviocytes, Clin. Exp. Rheumatol. 36 (2) (2018) 223–227.

[44] M. Peng, Y. Wang, L. Qiang, et al., Interleukin-35 inhibits TNF-alpha-induced osteoclastogenesis and promotes apoptosis via shifting the activation from TNF receptor-associated death domain (TRADD)-TRAF2 to TRADD-Fas-associated death domain by JAK1/STAT1, Front. Immunol. 9 (2018) 1417, https://doi.org/10.3389/fimmu.2018.01417 (published Online First: Epub Date).

[45] Z. Zhu, Y. Zhang, J. Ye, et al., IL-35 promoted STAT3 phosphorylation and IL-10 production in B cells, but its production was reduced in patients with coronary artery diseases, Hum. Immunol. (2018), https://doi.org/10.1016/j.humimm.2018.10.009 (published Online First: Epub Date).

[46] R.K. Gurram, W. Kujur, S.K. Maurya, et al., Caerulomycin A enhances transforming growth factor-beta (TGF-beta)-Smad3 protein signaling by suppressing interferon-gamma (IFN-gamma)-signal transducer and activator of transcription 1 (STAT1) protein signaling to expand regulatory T cells (Tregs), J. Biol. Chem. 289 (25) (2014) 17515–17528, https://doi.org/10.1074/jbc.M113.545871 (published Online First: Epub Date).

[47] S. Lin, H. Wu, C. Wang, et al., Regulatory T cells and acute lung injury: cytokines, uncontrolled inflammation, and therapeutic implications, Front. Immunol. 9 (2018) 1545, https://doi.org/10.3389/fimmu.2018.01545 (published Online First: Epub Date).

[48] W. Cao, X. Wang, T. Chen, et al., The expression of notch/notch ligand, IL-35, IL-17, and Th17/Treg in preeclampsia, Dis. Markers 2015 (2015) 316182, https://doi.org/10.1155/2015/316182 (published Online First: Epub Date).

[49] Y. Li, X. Pan, X. Peng, et al., Adenovirus-mediated interleukin-35 gene transfer suppresses allergic airway inflammation in a murine model of asthma, Inflamm. Res. 64 (10) (2015) 767–774, https://doi.org/10.1007/s00011-015-0858-1 (published Online First: Epub Date).

[50] J. Choi, P.S. Leung, C. Bowlus, et al., IL-35 and autoimmunity: a comprehensive perspective, Clin. Rev. Allergy Immunol. 49 (3) (2015) 327–332, https://doi.org/10.1007/s12016-015-8468-9 (published Online First: Epub Date).

[51] A. Ray, L. Wang, B.N. Dittel, IL-10-independent regulatory B-cell subsets and mechanisms of action, Int. Immunol. 27 (10) (2015) 531–536, https://doi.org/10.1093/intimm/dxv033 (published Online First: Epub Date).