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Potential therapeutic effects of interleukin-35 on the differentiation of naïve T cells into Helios^{+}Foxp3^{+} Tregs in clinical and experimental acute respiratory distress syndrome

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ABSTRACT

Regulatory T lymphocytes are important targets for the treatment of acute respiratory distress syndrome (ARDS). IL-35 is a newly identified IL-12 cytokine family member that plays an important protective role in a variety of immune system diseases by regulating Treg cell differentiation; however, the role of IL-35 in the pathogenesis of ARDS is still unclear. Here, we found that IL-35 was significantly elevated in adult patients with ARDS compared to controls. Additionally, IL-35 was positively and significantly correlated with IL-6, IL-10 and the oxygenation index (PaO2/FiO2 ratio) but negatively correlated with TNF-α, IL-1β and APACHE II score during ARDS. Moreover, the proportion of Treg/CD4^{+} cells in the peripheral blood of ARDS patients and the expression of NF-κB in PMBCs were significantly higher than in healthy individuals. Recombinant IL-35 improved survival in a murine model of CLP-induced ARDS. Additionally, IL-35 administration decreased the inflammatory response, as reflected by lower levels of cytokines (including IL-2, TNF-α, IL-1β and IL-6) and less lung damage in CLP-induced ARDS. Furthermore, recombinant IL-35 reduced the apoptosis of lung tissue and the expression of NF-κB signalling in a CLP-induced ARDS model and increased the proportion of Treg cells in spleen and peripheral blood. In vitro experiments revealed that IL-35 can affect the phosphorylation of STAT5 during differentiation of naïve CD4^{+} T lymphocytes into Foxp3^{+}Helios^{+} Tregs. Our findings suggest that IL-35 attenuates ARDS by promoting the differentiation of naïve CD4^{+} T cells into Foxp3^{+}Helios^{+} Tregs, thereby providing a novel tool for anti-ARDS therapy.

1. Introduction

Acute respiratory distress syndrome (ARDS) is a high-risk, high-mortality clinical syndrome caused by a variety of factors (McNicholas et al., 2018). In recent years, although clinical management by intensive care unit (ICU), mechanical ventilation and other measures have made great progress, its mortality rate has remained at 30 %–40 % (Laffey and Matthay, 2017). Therefore, investigation of its prevention and treatment has been the focus of critical medicine and respiratory sciences. ARDS is characterized by increased pulmonary capillary permeability and imbalance of intrapulmonary ventilatory blood flow (Mancini et al., 2001). Its pathogenesis is complex, involving uncontrolled inflammatory responses, cell damage and repair, tissue energy metabolism and immune cell regulation (Rezoagli et al., 2017). Recent studies have confirmed that uncontrolled inflammation is the central link of the systemic pathophysiological process of ARDS and that its effects occur directly or indirectly (Lin et al., 2016). Among pulmonary and/or extrapulmonary factors, inflammatory cells (neutrophils/PMN, macrophages, T cells, and B cells) are activated and recruited, releasing a large number of cytokines and chemokines to exert biological effects (Reiss et al., 2018). These factors also act as signal molecules that can activate various inflammatory signalling pathways, leading to the amplification of inflammation cascades (Huang et al., 2018). A large number of studies have found that the dynamic balance of pro-inflammatory and/or anti-inflammatory cytokines plays a key role in ARDS (He et al., 2017).

Interleukin (IL)-35, which is a recently identified member of the IL-

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12 cytokine family that was originally named by Niedbala et al., consists of an IL-12/p35 and IL27/EBI3 chain (Choi et al., 2015). IL-35 signalling occurs through either a unique heterodimer of IL-12Rb2 and gp130 or homodimers of each chain (Behzadi et al., 2016). Compared with other members (IL-12, IL-23, and IL-27), which are primarily produced by activated antigen-presenting cells (APCs), IL-35 is mainly secreted by regulatory T cells, regulatory B cells, and plasma cells and is unique in that it strictly functions as an immune suppressive cytokine in the IL-12 family (Hu et al., 2017). In human research, IL-35 secreted by plasma cells ($CD138^+CD38^+$ subgroup) can treat chronic periodontitis by regulating the relationship between oral microbes and the host immune system (Jing et al., 2019). However, IL-35 secreted by $CD4^+CD25^+FOXP3^+$ regulatory T lymphocytes (Tregs) has a significant immunosuppressive effect in colon cancer and pancreatic cancer and is negatively related to the prognosis of patients (Zeng et al., 2013; Nicholl et al., 2014). On the other hand, IL-35 secreted by Breg cells can negatively regulate the immune response of human systemic lupus erythematosus, inhibit the release of a variety of pro-inflammatory cytokines, and slow down the progression of the disease (Wang et al., 2019b). In animal experiments, IL-35 produced by activated Breg cells can significantly improve T cell-mediated encephalomyelitis and inhibit the growth of Salmonella in mice (Shen et al., 2014). Activated $CD278^+$ Treg cells selectively expressing IL-35 can play a protective role in mouse airway inflammatory diseases by downregulating the cytokines IL-4, IL-5, and IL-13 and chemokines CCL2, CXCL-1, and CXCL-5 (Zhang et al., 2019a). IL-35 secreted by iTregs can promote the secretion of IL-10 and inhibit the expression of the pro-inflammatory cytokines IL-6, TNF-α, and IL-17 to improve inflammatory bowel disease in mice (Barnett et al., 2016). To date, IL-35 has been linked to various diseases (Su et al., 2018), For example, Samuel S Duffy et al. proved that spinal delivery of Treg cells and IL-35 reduces pain associated with experimental autoimmune encephalomyelitis by decreasing neuroinflammation and increasing myelination independently of motor symptoms (Duffy et al., 2019). Fatima Manzoor et al. found that IL-35-mediated suppression is sufficiently robust to block established β-cell autoimmunity, and support the use of IL-35 to treat type 1 diabetes and other T-cell-mediated autoimmune diseases (Manzoor et al., 2017). Regarding inflammatory bowel disease, Zhen Nan et al. suggested modification of Bone marrow-derived mesenchymal stem cells by dual expression of CXCR4 and IL-35 can provide an effective therapeutic strategy (Nan et al., 2018). And in collagen type II-induced arthritis (CIA), Mirjana Dimitrijević. et al. found this disease is related to the low expression of IL-35 in female rat dLN cells. IL-35 can regulate the Treg/Th17 inflammatory response axis and improve the outcome of CIA (Dimitrijevic et al., 2019). Not only that, in asthma and allergic asthma, IL-35 can effectively reduce the overexpression of IL-5, IL-13 and IL-17 in alveolar lavage fluid, promote $CD4^+CD25^+Foxp3^+$regulatory T in lungs, and improve lung inflammation and excessive mucus secretion (Hu, 2017; Wang et al., 2015]. In terms of periodontitis, IL-35 also can inhibit the progression of the disease by reducing the levels of IL-6 and IL-8 induced by IL-17A (Shindo et al., 2018).IL-35 also impacts colorectal cancer progression and prognosis and is highly expressed in tumour tissue in lung and colon cancer and oesophageal, hepatocellular, and cervical carcinomas (Teymouri et al., 2018). However, the role and mechanism of IL-35 in ARDS are still unclear.

NF-κB is a multidirectional nuclear transcription factor that regulates multiple physical functions in the body (Catrysse and van Loo, 2017). As a constitutive nuclear transcription factor, NF-κB has a low level of sustained activity in tissue cells and plays an important role in the growth and development of immune cells and their function (Jha and Das, 2017). Under the stimulation of trauma, poisoning, and microbial infection, NF-κB activity is abnormally increased, resulting in excessive release of a large number of pro-inflammatory cytokines, which causes systemic inflammatory response syndrome (SIRS, which was defined according to standard criteria:, i.e., 2 or more of the following parameters during hospitalization: body temperature <36 °C or >38 °C, respiratory rate >20 per minute, heart rate >90 per minute, or white blood cell count <4000/μL or >12,000/μL in the absence of infection) and even multiple organ dysfunction syndrome (MODS, which was defined as the development of potentially reversible physiologic derangement involving two or more organ systems not involved in the disorder that resulted in ICU admission and arising in the wake of a potentially life threatening physiologic insult) (Wang et al., 2018a). NF-κB plays an important role in the immune response (Hayden et al., 2006). The proliferation of immune cells and the formation, differentiation, maturation and survival of T and B lymphocytes and natural killer cells must involve NF-κB protein (Liu et al., 2017). Some studies have shown that rats lacking NF-κB may have multiple immunodeficiencies. For example, rats lacking NF-κB1 (P50/P105) may exhibit B-cell proliferation, antibody secretion, and B-cell-mediated immune response defects (Jacque et al., 2014). Lack of NF-κB2 (P52/P100) is manifested by B-cell maturation and impaired T-cell activity (Beinke and Ley, 2004). In the absence of IKB protein (IκB, which interacts with the RHD structural region on NF-κB, making NF-κB exist in an inactive state), various forms of immunodeficiency disease also occur (Mulero et al., 2019). In addition, IKK (IκB kinase, which can specifically phosphorylate inhibitory IκB protein, and this phosphorylation leads to the dissociation of IκB from NF-κB) activity can influence lymphocyte generation, maturation and function (Guo et al., 2019). In the inflammatory response, NF-κB plays a central role. In all types of inflammatory reactions, increases in NF-κB activity can be observed, and inhibition of NF-κB activity can prevent or reduce the pathological response of inflammation (Esposito et al., 2016). Other studies have found that activation of the NF-κB signalling pathway is involved in the expression of multiple inflammatory factors that regulate the occurrence and development of ARDS and influence the prognosis of ARDS (Lopez et al., 2015). Blocking and inhibiting the NF-κB signalling pathway can effectively alleviate ARDS-related inflammatory responses and other related pathological changes (Xie et al., 2019). According to references, there are few reports on the correlation between IL-35 and NF-κB. Previous studies showed that IL-35 could promote the serum level of the anti-inflammatory cytokine IL-10, which could potentially inhibit tissue inflammation by inhibiting the NF-κB pathway (Hu et al. (2017). Osamu Yoshie and his colleagues demonstrated that IL-35 possessed the ability to inhibit the production of CCL11 and CCL24 in lung epithelial cells. As CCL11 is under the control of the NF-κB signalling pathway, in their LPS-induced acute airway inflammation model, it is possible that IL-35 might suppress this chemokine production by inhibiting NF-κB (Kanai et al., 2017). In addition, recent studies have confirmed that IL-35 can play a protective role in the prevention of rheumatoid arthritis and acute kidney injury by inhibiting the NF-κB signalling pathway (Hu et al. (2017). However, no studies have investigated whether IL-35 can inhibit the inflammatory response of ARDS by coordinating the NF-κB signalling pathway.

Sepsis, trauma, and shock are the most common causes of ARDS (Cutts et al., 2017). In sepsis, the lung is the vulnerable target organ, and it appears the earliest and has the highest incidence (De Freitas Caires et al., 2018). Caecal ligation and perforation (CLP) is the most commonly used model for the construction of murine sepsis, and it is also a classic model for ARDS (Villar et al., 2011). Therefore, to elucidate the role of IL-35 in ARDS, we created CLP-induced ARDS mouse models and treated them with recombinant IL-35 to determine the roles and mechanisms of IL-35.

2. Materials and methods

2.1. Human study subjects

Seventy-three adult patients from February 2018 to February 2019 and June 2019 to January 2020 who met the Berlin standard (the Berlin definition identified patients as having ARDS when they fulfilled all of the following criteria: 1. Onset within seven days of a known clinical

insult, 2. Bilateral opacities on chest imaging, 3. PaO_2/FiO_2 ratio less than 300 mmHg, 4. Hypoxaemia not fully explained by cardiac failure or fluid overload) were screened for eligibility within the first 24 h of being admitted to the ICU of the First Affiliated Hospital of Chongqing Medical University. Patients with massive transfusion or haemofiltration within the preceding 24 h, immunosuppressive or immune-enhancing therapy, or chronic lung diseases were excluded. Patient data are showed in Table 1. Control samples were obtained from healthy donors (n = 46) with no medical problems in the medical examination centre of The First Affiliated Hospital of Chongqing Medical University. This protocol was approved by the Clinical Research Ethics Committee of Chongqing Medical University, and informed consent was obtained from all participants according to the Declaration of Helsinki.

2.2. Human serum cytokine and peripheral blood Treg/$CD4^+$ measurements

At the time of admission, blood was drawn by venipuncture from the admitted ARDS patients. And among them, we used the same method to collect the blood of twenty-seven ARDS patients on the admission, 1 day, 3 day, 5 day, and 7 day. The blood was centrifuged at 1000 *g* for 10 min at 4 °C immediately after collection. The serum was then aliquoted into Eppendorf (EP) tubes and kept frozen at −80 °C until analysis. IL-35 was measured using an ELISA test kit (MyBioSource, California, USA, #MBS2511987) as previously described (Wang et al., 2019a), and the levels of IL-1β, IL-6, IL-10, TNF-α in the serum and Treg/$CD4^+$ (the percent of $CD4^+CD25^{high}CD127^{low}$ regulatory T lymphocytes in $CD4^+$ T cells) in the peripheral blood were analysed at the First Affiliated Hospital of Chongqing Medical University Clinical Testing Center for determination.

2.3. Isolation of human peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from fresh blood of healthy volunteers or ARDS patients by density gradient (Histopaque, Sigma) centrifugation. Isolated human cells were washed twice with PBS and reconstituted in RPMI-1640 containing 1% L-glutamine, 100 μg/mL penicillin G, 100 μg/mL streptomycin, and 5 μg/mL Fungizone (Gibco) supplemented with 10 % heat inactivated foetal calf serum (Sigma) at a final concentration of 10^6 cells/mL. This cell suspension (50 μL) was aliquoted into the wells of a 96-well round bottom tissue culture plate. This was followed by the addition of either PBS or 10 μL of heat-killed *E. coli* (Aldrich, Sigma) at a final concentration of 2×10^4 bacteria/mL. The plate was incubated for 6 h. At the indicated time after culture, cell-free supernatants were stored at −80 °C until use. Additionally, peripheral blood mononuclear cells (PBMCs) from 46 patients and 23 healthy people were isolated by density gradient centrifugation and stored at −80 °C for further analysis and functional study.

Table 1
Characteristics of the study population.

Characteristic	Controls(n = 46)	ARDS(n = 73)
Age(years)	54 ± 5	56 ± 4
Biological sex (Proportion of male)	30/16(65.2 %)	47/26(64.4 %)
WBC, 10^9/L	6.62 ± 1.36	16.81 ± 7.56
PCT, ng/mL	0.05 ± 0.02	35.09 ± 16.43
CRP, mg/mL	NA	69.71 ± 27.68
pathogen, no. of patients		
Necrotizing fasciitis	NA	7
Intestinal infection	NA	15
Biliary tract infection	NA	5
Urinary tract infection	NA	11
Peripancreatic infection	NA	10
Severe pneumonia	NA	19
Aspiration pneumonia	NA	6
PaO2/FiO2 ratio	NA	204 ± 39
APACHE II score	NA	14 ± 6
Ventilator Free Days	NA	5.08 ± 2.55
ICU free days	NA	10 ± 3
Survival	NA	10/63

Table a) Data as a percentage of patients or median ± interquartile range. b) ARDS acute respiratory distress syndrome. c) APACHE II score: Acute Physiology and Chronic Health Evaluation II score. The APACHE II score system includes: (1) acutephysiology score, (2) agepoints, (3) chronichealthpoints(McNicholas et al., 2018). d) The patient's white blood cell(WBC), procalcitonin(PCT), C-reactive protein(CRP), PaO2/FiO2 ratio and APACHE II score were collected within 24 h of entering our ICU unit. e) The survival indicates the patient's survival on the 28th day.
(McNicholas et al., 2018) Knaus WA, Draper EA, Wagner DP, et al. APACHE II: a severity of disease classification system. Critical care medicine 1985;**13** (10):818−29.

2.4. Real-time quantitative PCR detection

First, PBMCs were washed in PBS, pelleted and subsequently added to 1 mL of TRIzol solution for RNA isolation or 100 mg of mouse lung tissue to 1 mL of RNAiso solution. The lung tissue was pulverized thoroughly with a homogenizer, shaken and allowed to stand at room temperature for 5 min to completely lyse the tissue. Then, total RNA from PBMCs or mouse lung tissue was isolated using TRIzol reagent (TakaraBio, Tokyo, Japan). The Prime Script RT Reagent kit was employed to construct first-stand complementary DNA (cDNA)-isolated total RNA. The cDNA was used as template to run polymerase chain reactions (PCRs). SYBR Premix Ix Taq II (Takara, Dalian, China) was used to quantify the target gene in the Bio-Rad CFX-96 Real-Time System (Bio-Rad, Hercules, CA, USA). The following primers (Takara, Dalian, China) were used as shown in Table 2. Analyses were performed in a 25 μL volume with 2 μL of cDNA, each sense and antisense primer at 400 nM, and 12.5 μL of Brilliant SYBR Green QPCR Master Mix (Takara Bio) using an ABI PRISM 7000 (Applied Biosystems, Foster City, CA, USA). Samples were subjected to 40 cycles of 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 the relative target gene expression, and the $^{2\Delta\Delta}C(t)$ method was used.

2.5. Animals

Pathogen-free male (at present, in the study of disease animal models, it is an important step to include both male and female animals. In the study of ARDS/ALI, the protective effect of oestrogen was clearly confirmed (Hsu et al., 2009). Previous studies have shown that oestrogen is involved in the pathological process of ALI. Studies have also found that the incidence of ALI in male patients is higher than that in female patients, and the mortality rate is also significantly higher than

Table 2
The primers for PCR.

Human Gene Name	Forward primer	Reverse primer
NF-κB	5′-TCCAGACCAACAACAACCCC-3′	5′-GATCTTGAGCTCGGCAGTGT-3′
GAPDH	5′-GCTCTCTGCTCCTCCTGTTC-3′	5′-GTTGACTCCGACCTTCACCT-3′
Mouse Gene Name	Forward primer	Reverse primer
EBI3	5‘-CGGTGCCCTACATGCTAAAT-3′	5′-GCGGAGTCGGTACTTGAGAG-3′
P35	5′-CATCGATGAGCTGATGCAGT-3′	5′-CAGATAGCCCATCACCCTGT-3′
NF-κB p65	5′-AAGATCAATGGCTACACAGG-3′	5′-CCTCAATGTCTTCTTTCTGC-3′
GAPDH	5′-TTCACCACCATGGAGAAGGC-3′	5′-GGCATGGACTGTGGTCATGA-3′

that in female patients (Lemos-Filho et al., 2013; Han et al., 2011). Therefore, if the role of hormones is not a special research goal in ARDS, the male animal model is mainly selected in the current research (Yang et al., 2004)) mice (C57BL/6, 8–12 weeks old, 20–24 g; Laboratory Animal Center of Chongqing Medical University, Chongqing, China) were kept under a 12 h light/dark cycle and given food and water *ad libitum*. A total of 30 mice were randomly assigned to the untreated group, CLP (6 h) group, CLP (24 h) group, CLP (48 h) group, CLP + IgG (24 h) group and CLP + IL-35 (24 h) group, with 5 mice in each group. Indicators were tested after modelling, intervention, and specimen collection under the same conditions. The indicators of each mouse were repeatedly tested 3 times, and the mean of these technical replicates was calculated and used for analysis. All experimental protocols were approved by the Institutional Animal Care and Use Committee at Chongqing Medical University.

2.6. Caecal ligation and puncture (CLP)-induced extrapulmonary ARDS model

Intraperitoneal administration of chloral hydrate (3.5 %) was used to anaesthetize the mice, after which they were positioned in the dorsal position. Following shaving and aseptic preparation of the surgical site, a ventral midline incision (1 cm) was made to allow exteriorization of the caecum. The caecum was found and penetrated with a 21-gauge needle and 3−0 silk suture 75 % from the tip. After puncturing, the caecum was pinched to produce a small amount of faeces, after which it was returned to the abdominal cavity, and the abdominal incision was closed. Mice were euthanized at 6 and 24 h after the treatment, and lung and spleen tissues were collected. In addition, blood was collected retro-orbitally (Wang et al., 2019a).

2.7. Morphological evaluation

Lung tissues were fixed, sectioned, and stained with haematoxylin and eosin (H&E) for morphological analysis. Lung injury scores were estimated using the method described by Mikawa, which was as follows: 1. alveolar hyperemia; 2. haemorrhage; 3. interstitial or neutrophil infiltration or aggregation; 4. alveolar septal thickening or hyaline membrane formation. According to the severity of lesions for each indicator, a score of 0–4 points was assigned, and the total for each score was used as the pathological score for ARDS. Higher scores indicate greater injury. The histological abnormalities were evaluated under a light microscope (Olympus, Japan).

2.7.1. TUNEL staining

The TUNEL assay was accomplished using the In-Situ Cell Apoptosis Detection Kit I, POD. In brief, tissue sections (4 μm) were deparaffinized in xylene and then rehydrated in decreasing concentrations of ethanol. Endogenous peroxidase was blocked in 3% hydrogen peroxide for 10 min and digested in proteinase K for 15 min at 37 °C. The dilution (1:20) of terminal deoxynucleotidyl transferase in a reaction buff ;er (containing a fixed concentration of digoxigenin-labelled nucleotides) was applied to serial sections for 2 h at 37 °C. The slides were then washed with stop/wash buff ;er for 2 min three times. Following washes, the slide sections were incubated with the prediluted antidigoxin antibody (dilution 1:100) for 30 min at 37 °C, followed by ABC for 30 min at 37 °C. Apoptotic cells were detected by incubation with the 3,3′-diaminobenzidine chromogen for approximately 20 min. Slides were counterstained with haematoxylin. The In Situ Cell Death Detection Kit (Roche, Switzerland) was used to detect the apoptosis rate in lung tissue sections according to the manufacturer's instructions. Each section in 5 random fields under ×400 magnification was shot, and the percentage of TUNEL-positive cells was calculated.

2.8. Estimating pulmonary oedema

The amount of extravascular lung water was calculated as an index of lung oedema. The left lung was removed, and the wet weight was measured and recorded, after which the lungs were incubated for 3–4 days at 60 °C to remove all moisture and then re-weighed. Finally, the ratio of wet to dry weight was obtained by dividing the wet weight by the dry weight (Lin et al., 2016).

2.9. In vivo administration of recombinant IL-35

Recombinant murine IL-35 protein (0.3 μg/g; Adipogen International) (Sha et al., 2015) was injected 30 min before CLP for preventive treatment, and 1 dose of IL-35 was injected 30 min after CLP challenge for therapeutic treatment. Normal goat isotype IgG (Bioss, China) was used as a control. Blood and lung tissues were then collected as indicated.

2.10. Mouse serum and spleen homogenate cytokines

Blood was collected from mice under anaesthesia via the ophthalmic vein into EDTA tubes and then centrifuged at 3000 rpm for 15 min at 4 °C. Spleen tissues were homogenized with PBS (10 mg of tissue/100 μL of PBS). Fluids and tissues were aliquoted and kept frozen at −80 °C until analysis. IL-35 (MyBioSource, USA) in serum and spleen homogenate were assayed with ELISA kits. TNF-α, IL-1β, IL-2, IL-6, IL-10, and TGF-β in serum were measured using a Mice Cytokine/Chemokine Magnetic Bead Panel Kit (eBioscience, USA).

2.11. Western blot analysis

Total proteins from T lymphocytes or lung samples were prepared according to the protein extract kit instructions (Beyotime, China), after which the concentration of protein was determined using a BCA protein assay kit (Pierce, USA). The protein extracts were fractionated on a 10 % polyacrylamide-sodium dodecyl sulfate (SDS) gel and then transferred to a nitrocellulose membrane. The membrane was blocked with 5% (w/v) nonfat milk in Tris-buffered saline containing 0.05 % Tween-20, after which the membranes were then incubated overnight at 4 °C with an anti-phospho-P65 antibody (Cell Signaling Technology, USA, #3039), anti-phospho-Stat5 antibody (Cell Signaling Technology, USA, #9356), anti-phospho-Stat3 antibody (Cell Signaling Technology, USA,#9138), or β-actin antibody (Cell Signaling Technology, USA,#3700). The densitometric analyses of bands were performed by the use of an Imager system (BIO-RAD).

2.12. Flow cytometry

Cells were washed in PBS, pelleted, and subsequently stained for flow cytometry. Treg cells were characterized accordingly with monoclonal antibodies against CD4, CD25, Foxp3 or CD4, Helios, Foxp3. To stain CD4, CD25, Foxp3 or CD4, Helios, Foxp3, anti-CD4-FITC (eBioscience, USA, #11-0042-81), anti-CD25-PE (eBioscience, USA, #35-0251-82), anti-Foxp3-APC (eBioscience, USA, #17-5773-82) or anti-CD4-Percp-cy5-5 (eBioscience, USA, #45-0042-82), anti-Helios-PE (eBioscience, USA, #12-9883-42), anti-Foxp3-FITC (eBioscience, USA, #11-4776-42), and a Fixation/Permeabilization kit (eBioscience, USA, 00-5123-43) were used according to the manufacturer's instructions. At least 10^5 cells were collected with a FACScan flow cytometer (Becton Dickinson) and analysed with Flow Jo software 7.6. Animal and cell flow experiments showed off the full gating once respectly and each performed the same gating for all analyses.

2.13. Cell purification and culture

Peripheral blood mononuclear cells (PBMCs) were isolated from

mouse spleens using Lymphocyte Separation Medium (GE healthcare, USA). Naïve CD4 + T cells (naïve CD4 + T Cell Isolation Kit II, StemCell, Canada) were isolated from PBMCs by magnetic activated cell sorting (Miltenyi Biotec) according to the manufacturer's instructions. The purity of naïve CD4 + T cells was > 90 % as measured by flow cytometry. All cells were cultured in complete RPMI 1640 medium (Gibco, Grand Island, NY, USA) containing 10 % foetal bovine serum (FBS) at 37 °C under 5% CO2.

2.14. Treg cell subset generation

Treg cell subtypes were generated by the addition of relevant exogenous cytokines. Tregs were generated by exposure to 2 mM L-glutamine, 50 mM β-mercaptoethanol, 5 μg/mL anti-CD3, 2 μg/mL anti-CD28, 50 U/mL IL-2, and 2.5 ng/mL TGF-β for 3 days (Zabransky et al., 2012). To determine whether IL-35 is involved in the induction process, we added recombinant IL-35 (20 ng/mL, Sigma, USA) and NF-κB molecular inhibitor (100 ng/mL, R&D, USA, #2743/10) to the culture. The expression of surface markers and intracellular staining were assessed by flow cytometry.

2.15. ELISAs

The concentrations of IL-10(Human, R&D, USA, #D1000B; Mouse, R&D, USA, #M1000B) and TGF-β(Mouse, R&D, USA, #MB100B) in the culture supernatants were analysed using ELISA kits according to the manufacturers' instructions. The plates were read at 450 nm.

2.16. Statistical analyses

SPSS 19.0 was used for all statistical analyses, and data are reported as the median ± interquartile range. Differences between groups were identified by Mann-Whitney U tests. Statistical analysis was performed using one- or two-way ANOVA followed by an LSD multiple comparisons test or Dunnett's test for multiple groups. Correlation analysis was conducted using a non-parametric Spearman's rank correlation coefficient ($p < 0.05$).

3. Results

Circulating IL-35, the ratio of Treg/CD4$^+$ cells, the total number of treg cell in blood and the expression of NF-κB in PBMCs were elevated in

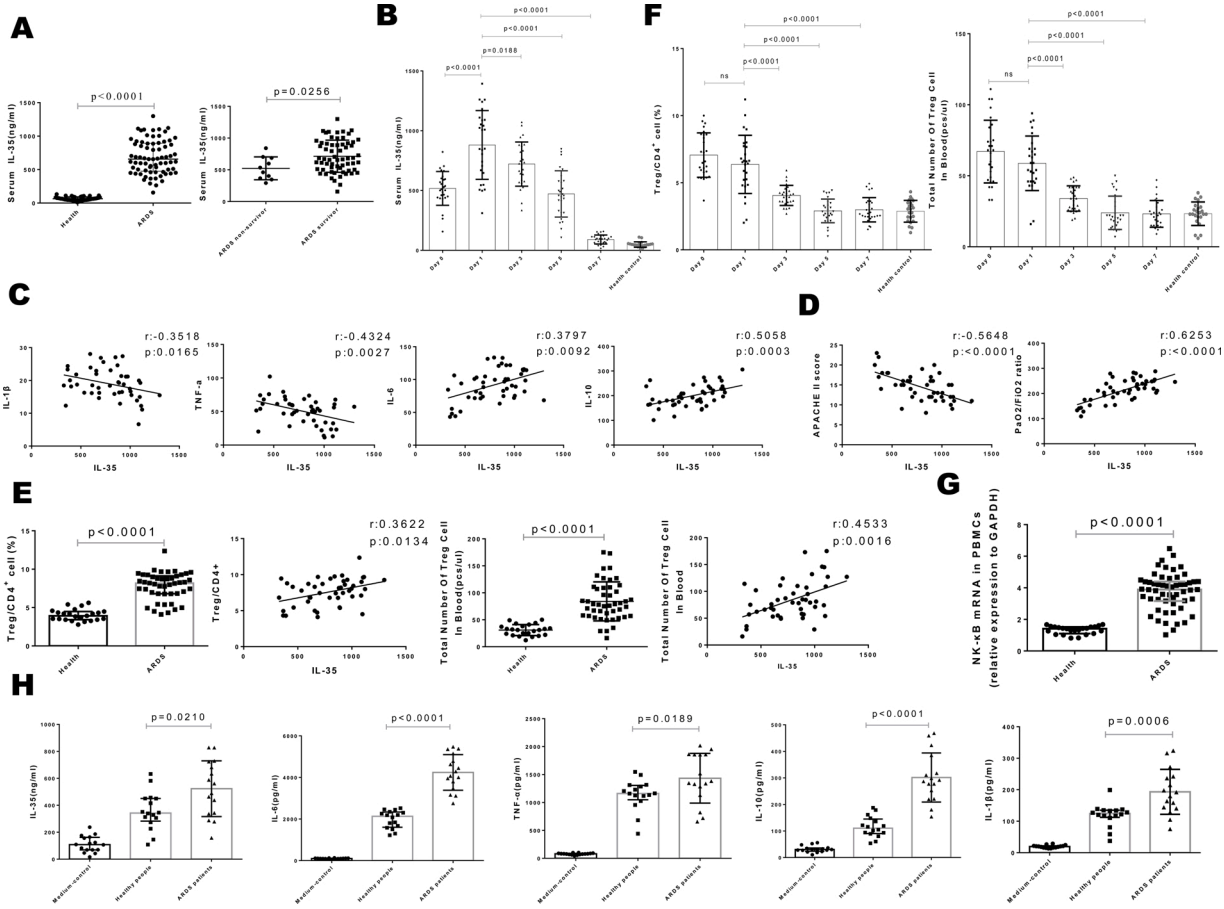

Fig. 1. Circulating IL-35, the ratio of Treg/CD4+ cells and the expression of NF-κB in PBMCs were elevated in human ARDS patients, and IL-35 was associated with the severity of ARDS and other cytokines. (A, B) IL-35 concentrations were measured by ELISA in serum samples collected from 73 adult patients with ARDS and from 46 healthy control subjects. (C, D) IL-35 was positively and significantly correlated with IL-6, IL-10 and the oxygenation index (PaO2/FiO2 ratio) but negatively correlated with TNF-α, IL-1β and APACHE II score during ARDS. (E, F) The ratio of Treg/CD4$^+$ cells or the total number of treg cell in the blood was determined by the First Affiliated Hospital of Chongqing Medical University Clinical Testing Center. The proportion of Treg/CD4$^+$ cells and the total number of treg cells were significantly elevated in ARDS patients. Its ratio and total number were reached a peak, and gradually decreased after 1 day. (G) PMBCs were isolated from ARDS patients and healthy volunteers. NF-κB concentrations were measured by qRT-PCR in PBMCs. Relative expression levels of the genes were expressed with the GAPDH housekeeping gene as an internal reference. (H) PBMCs were isolated from fresh blood of healthy volunteers or ARDS patients by density gradient centrifugation and incubated with heat-killed *E. coli*. After stimulation with heat-killed *E. coli* for 6 h, IL-1β, TNF-α, IL-6, IL-10 and IL-35 were significantly elevated in ARDS patients. Horizontal bars represent median values, and dots represent individual participants. p < 0.0001, significant difference between groups (denoted by horizontal brackets; Mann-Whitney U tests).

human ARDS patients, and IL-35 was associated with the severity of ARDS and other cytokines.

First, IL-35 was quantified in a total of 73 serum samples from adult ARDS patients and 46 serum samples from healthy controls. The general characteristics of the study groups are summarized in Table 1. The IL-35 levels in ARDS patients were greater than those in healthy people. However compared with ARDS survivors, its levels were significantly lower in ARDS non-survivors (Fig. 1A). In order to further explore the potential role of IL-35 in ARDS, IL-35 was quantified by time gradient in total of 27 serum samples from these adult ARDS patients. The IL-35 levels in ARDS patients were greater than those in healthy people. And reached a peak on the first day of admission, and then gradually decreased (Fig. 1B). In addition, the level of IL-35 in serum was positively and significantly correlated with IL-6, IL-10 and the oxygenation index (PaO_2/FiO_2 ratio) but negatively correlated with TNF-α, IL-1β and APACHE II score during ARDS (Fig. 1C and D). Furthermore, the proportion of Treg/$CD4^+$ cells or the total number of treg cell in the peripheral blood of ARDS patients were significantly higher than those in healthy individuals (Fig. 1E). Its ratio and total number were reached a peak, and gradually decreased after 1 day (Fig. 1F).

Next, in vitro experiments, we found the expression of NF-κB in PMBCs of ARDS patients were significantly higher than those in healthy individuals (Fig. 1G). To determine whether heat-killed *E. coli* could induce IL-35 release in human cells in vitro, peripheral blood mononuclear cells (PBMCs) were isolated from healthy people or ARDS patients and incubated with heat-killed *E. coli*. IL-1β, TNF-α, IL-6, IL-10 and IL-35 were significantly elevated in ARDS patients 6 h after stimulation with heat-killed *E. coli* (Fig. 1H).

3.1. Expression of IL-35, regulatory T lymphocytes, cytokines and lung injury in CLP-induced murine ARDS models

IL-35 is a newly identified IL-12 cytokine family member (Choi et al., 2015), and its function in ARDS is unclear. Therefore, we first analysed

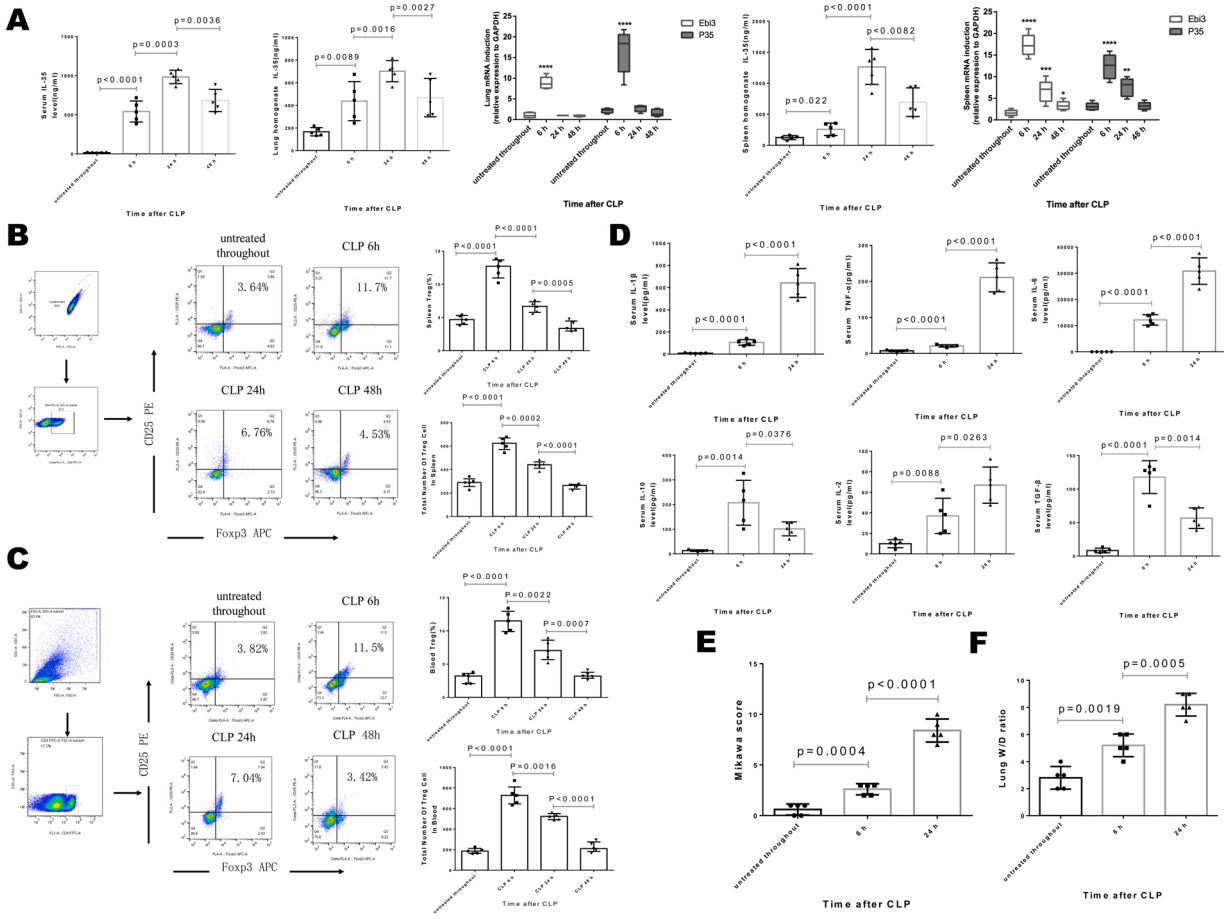

Fig. 2. IL-35 was significantly elevated in sera, lungs, and spleens in the CLP-induced ARDS mouse model. C57BL/5 mice were subjected to no treatment, the CLP (6 h) group, CLP (24 h) group, or CLP (48 h) group. Each group had n = 5, the indicators of each mouse were repeatedly tested 3 times, and the best data were selected as the representative data. (A) Organs were removed at the indicated time points. Blood specimens were collected from mice under anaesthesia via the ophthalmic vein. The lung or spleen homogenate was obtained by mixing tissue and PBS at a ratio of 0.5 g:1 mL. Samples were assayed for IL-35 content by enzyme-linked immunosorbent assays. IL-35 mRNA levels in the lungs or spleen were measured with qRT-PCR. Relative expression levels of the genes were expressed with the GAPDH housekeeping gene as an internal reference. (B, C) T cells were isolated from mouse spleens or peripheral blood. The frequency of Tregs was subsequently determined by flow cytometry, and the results reflect five mice per time point. B and C are representative dot plots as shown. Treg cells gradually decreased in blood and spleen with time in CLP-induced ARDS. (D) TNF-α, IL-1β, IL-2, IL-6, IL-10, and TGF-β in serum were measured using a Mice Cytokine/Chemokine Magnetic Bead Panel Kit. As the time of the CLP-induced ARDS model was prolonged, the cytokines in serum, including TNF-a, IL-1β, IL-6 and IL-2, gradually increased, and IL-10 and TGF-β decreased. (E, F) Compared with the untreated group, lung injury, which is expressed by the Mikawa score (lung injury was estimated using the method described by the Mikawa score based on the following four indicators of lung injury: alveolar congestion; bleeding; gap or vascular wall neutrophil infiltration or aggregation; alveolar septal thickening or transparent membrane formation, where 0 indicates no or very slight damage, 1 mild injury, 2 moderate injury, 3 severe injury, and 4 very severe damage. The cumulative increase in the number of lesions of the total score is the pathological score of the ARDS) or pulmonary oedema (W/D ratio) is gradually increased with the prolongation of the modelling time. The comparison between the two groups was performed by one-way ANOVA followed by LSD multiple comparisons test.

local, systemic, and organic IL-35 concentrations using our well-established CLP-induced ARDS model. qRT-PCR and ELISA data show that IL-35 is involved in CLP-induced ARDS. IL-35 protein in serum, lung and spleen homogenates increased by 6 h and peaked at 24 h post CLP. *EBI3* and *P35* mRNA in the lungs and spleen decreased and peaked at 6 h after CLP (Fig. 2A). 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 (Wang et al., 2019a). We used a flow cytometric method to determine whether Treg cells in sera and spleens were involved in ARDS models. The outcomes showed that Treg cells in sera and spleens increased transiently at 6 h and then decreased from 6 h to 48 h (Fig. 2B and C). As the time of the CLP-induced ARDS model was prolonged, the cytokines in serum, including TNF-a, IL-1β, IL-6 and IL-2, gradually increased, and IL-10 and TGF-β decreased (Fig. 2D). The lung injury and wet to dry ratio (W/D) were the same as the above results, and they gradually increased with prolonged modelling time (Fig. 2E–F).

3.2. Recombinant IL-35 affects Treg cells, cytokine levels in serum, histopathology, and lung apoptosis across CLP-induced ARDS models

To demonstrate the therapeutic role of IL-35 in sepsis-related ARDS, we administered intraperitoneal injections of recombinant IL-35 30 min before CLP for preventive treatment, as well as 1 dose of IL-35 30 min after CLP challenge. Histological examination of lung sections stained with H&E revealed that CLP-induced ARDS had a lower Mikawa score than the IgG group after treatment with recombinant IL-35 (Fig. 3A). Additionally, pulmonary oedema improved significantly after treatment (Fig. 3B). Moreover, recombinant IL-35 treatment efficiently reduced the production of IL-1β, TNF-α, IL-2, and IL-6 and upregulated IL-10 and TGF-β in serum (Fig. 3C). Flow cytometry revealed a significant increase in serum and spleen Treg cells at 24 h in CLP-induced ARDS after treatment with recombinant IL-35 (Fig. 3D and E). Consistently, the survival of mice treated with IL-35 was higher than that of the CLP and CLP + IgG groups (Fig. 3F).

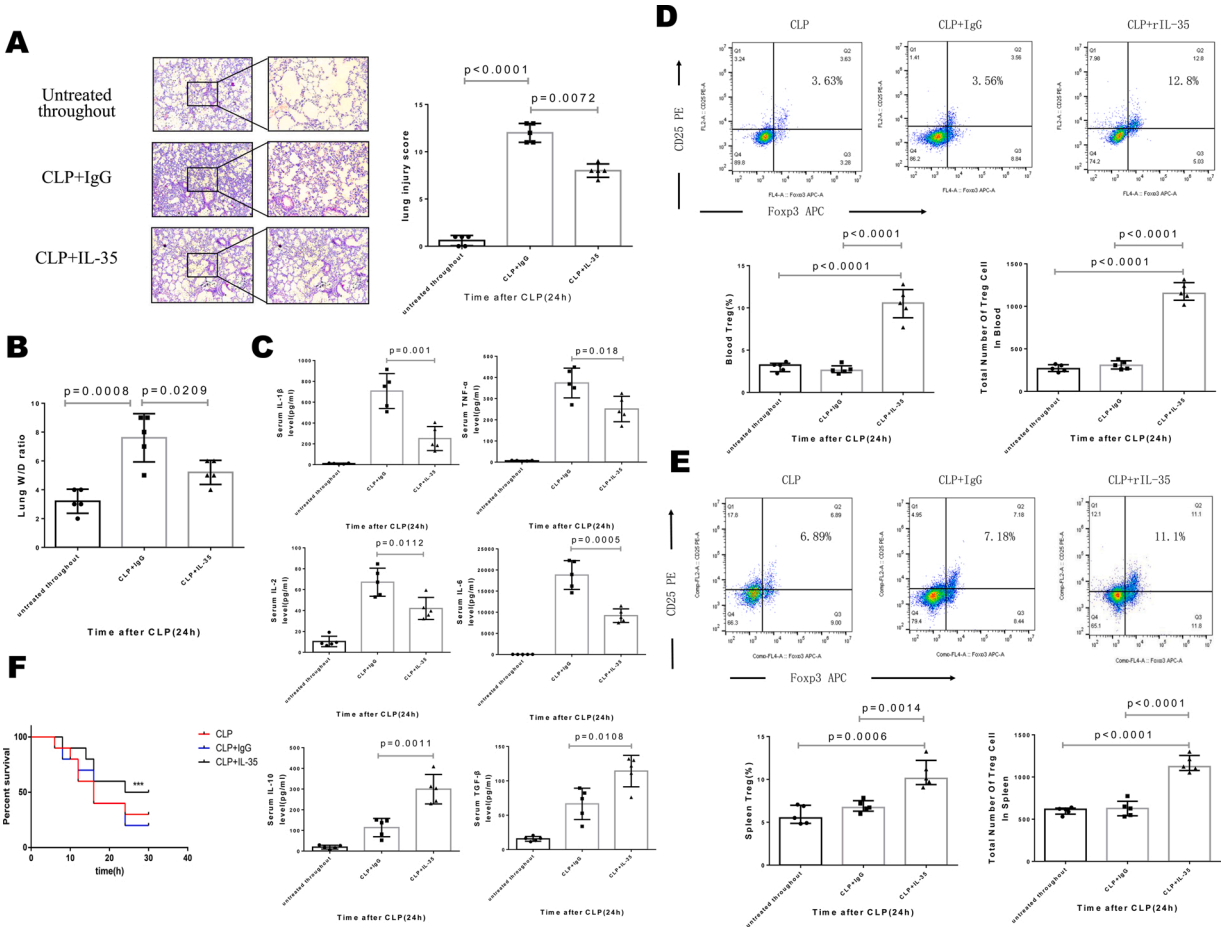

Fig. 3. C57BL/6 mice were randomly divided into untreated, CLP + IgG and CLP + IL-35 groups (n = 5 mice/time point). The indicators of each mouse were repeatedly tested 3 times, and the best data were selected as the representative data. (A) Treatment with IL-35 attenuated CLP-induced ARDS. Lungs from each experimental group were processed for histological examination after H&E staining. Compared with the LPS + IgG group, thickened alveolar wall, alveolar haemorrhage and collapse, and inflammatory cells in filtration were less severe and treated with IL-35 prior to CLP challenge. (B) Treatment with recombinant IL-35 attenuated CLP-induced lung injury, and the lung wet:dry weight ratios from each experimental group 24 h after CLP administration were determined. (C) IL-35 blockade upregulated the production of proinflammatory cytokines and downregulated anti-inflammatory cytokines in ARDS models. Cytokine concentrations in serum from mice treated with IL-35 were determined by mouse cytokine magnetic bead panel kit assays 24 h after the onset of ARDS and compared with those of mice treated with isotypical IgG controls. ARDS featured increased IL-10 and TGF-β and reduced IL-1β, TNF-α, IL-2, and IL-6 after recombinant IL-35 treatment in serum. (D, E) Treatment with recombinant IL-35 after CLP challenge. T cells were isolated from mouse spleens and peripheral blood. The frequency of Tregs was subsequently determined by flow cytometry. D and E are representative dot plots as shown. After 24 h of CLP-induced ARDS, Tregs in the blood and spleens increased significantly after treatment with IL-35. The comparison between the two groups was performed by one-way ANOVA followed by LSD multiple comparisons test. (F) C57BL/6 mice were subjected to CLP, CLP + IgG and CLP + IL-35. Mice were administered 0.3 μg/g recombinant IL-35 followed by a booster dose of 0.3 μg/g 0.5 h before CLP. Survival of ARDS mice (n = 10 per group) following recombinant IL-35 treatment. Groups were compared by Kaplan–Meier analysis followed by log-rank tests. ***p < 0.001 when compared with ARDS mice treated with isotypical IgG control.

3.3. The nuclear translocation of NF-κB changed in the lung after treatment with recombinant IL-35 in ARDS models

As recent studies have confirmed, IL-35 exerts a protective effect by inhibiting the NF-κB signalling pathway (Hu et al., 2017). Therefore, we measured the nuclear translocation of NF-κB in lung tissue. qRT-PCR showed that P65 mRNA in the lungs decreased relative to the CLP + IgG group at 24 h (Fig. 4A). Next, we further measured the expression of the NF-κB-associated activation protein P65 using Western blotting. After IL-35 treatment, P65 protein expression was lower relative to the CLP + IgG group (Fig. 4B). Furthermore, the number of TUNEL-positive cells in CLP mice also decreased after recombinant IL-35 treatment (Fig. 4C). These data suggest that IL-35 may play a protective role in ARDS by inhibiting the NF-κB signalling pathway.

3.4. IL-35 promotes the differentiation of naïve $CD4^+$T lymphocytes into Treg cells, this effects of IL-35 were increased through inhibiting NF -κB pathway

To confirm whether IL-35 can affect Naïve $CD4^+$ T lymphocytes differentiation into Treg cells, we extracted naïve $CD4^+$ T lymphocytes from the spleens of mice for in vitro cell culture. Next, recombinant IL-35 was added to the medium for intervention. After 3 days of training, naïve $CD4^+$ T lymphocytes differentiated more Treg cells after treatment with recombinant IL-35. Furthermore, the degree of differentiation of Treg cells increased significantly after administration of the NF-κB molecule inhibitor into the medium (Fig. 5A). As phosphorylation of STAT5 is a necessary signal for the differentiation of naïve $CD4^+$ T lymphocytes into Treg cells. To further illustrate whether IL-35 can affect the phosphorylation of STAT3 or STAT5 which affect the differentiation of naive T lymphocytes into Treg cells, we used the WB method to detect the expression of p-STAT3 or p-STAT5 in T lymphocytes. The results showed that as the concentration of recombinant IL-35 gradually increased, the level of p-STAT5 in T lymphocytes gradually increased, while the level of p-STAT3 did not change significantly (Fig. 5B). Next after 3 days of training, the expression of IL-10 and TGF-β in the supernatant was highest after administration of recombinant IL-35 and NF-κB molecule inhibitor compared with the other two groups (Fig. 5C). Taken together, these data demonstrate that IL-35 can promote the differentiation of naïve $CD4^+$ T lymphocytes into Treg cells and this effects of IL-35 were increased through inhibiting NF—KB pathway.

3.5. IL-35 promotes the differentiation of naïve $CD4^+$ T lymphocytes into $Helios^+$ Treg cells

Helios is a member of the IKaros transcription factor family (Akimova et al., 2011). Studies have shown that Helios can enhance the function of Foxp3. It is a marker of activated Treg cells (Verhagen and Wraith, 2010). Compared with $Foxp3^+Helios^-$ Treg cells, $Foxp3^+Helios^+$ Treg cells have a stronger immunosuppressive function (Alexander et al., 2013). Therefore, in our further study, we found that in the CLP-induced ARDS model, $Foxp3^+Helios^+$ Tregs in the spleen or peripheral blood of mice gradually decreased (Fig. 6A–B). After administration of recombinant IL-35, the proportion of $Foxp3^+Helios^+$ Treg cells was restored (Fig. 6C–D), and lung injury was also significantly improved (Fig. 3A–B). To clarify whether IL-35 affects the differentiation of $Foxp3^+Helios^+$ Tregs, we extracted naïve $CD4^+$ T lymphocytes from the spleens of mice for in vitro cell culture. After 3 days of training, naïve $CD4^+$ T lymphocytes differentiated more $Foxp3^+Helios^+$ Treg cells after treatment with recombinant IL-35 or

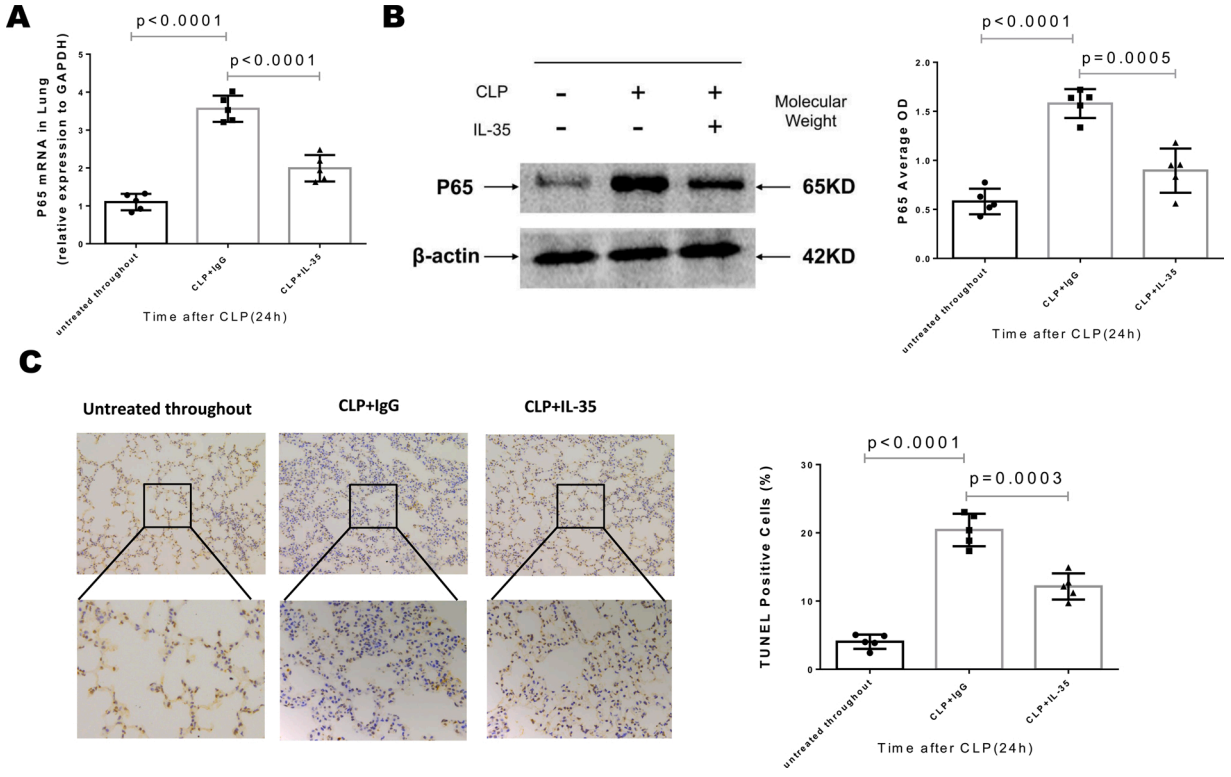

Fig. 4. (A—B) Expression of the NF-κB-related activation protein *P65* in the lung was lower after IL-35 treatment than in the CLP + IgG group. *P65* expression in the lung was determined by Western blot and qRT-PCR at 24 h. C57BL/5 mice (5/group) were divided into a CLP, CLP + IgG and LPS + IL-35 group. (C) IL-35 suppressed lung cell apoptosis in the CLP-induced ARDS model. The ARDS model was induced by CLP, and 15 mice were divided into three groups (n = 5 per group). We administered intraperitoneal injections of recombinant IL-35 30 min before CLP for preventive treatment, and 1 dose of IL-35 was injected 30 min after CLP challenge for therapeutic treatment. Lung samples were harvested at 24 h. The apoptotic cells were determined by a TUNEL assay, and the TUNEL-positive cells showed a dark-brown nucleus. Representative lung sections of each group are shown (original magnification 200×). The comparison between the two groups was performed by one-way ANOVA followed by LSD multiple comparisons test.

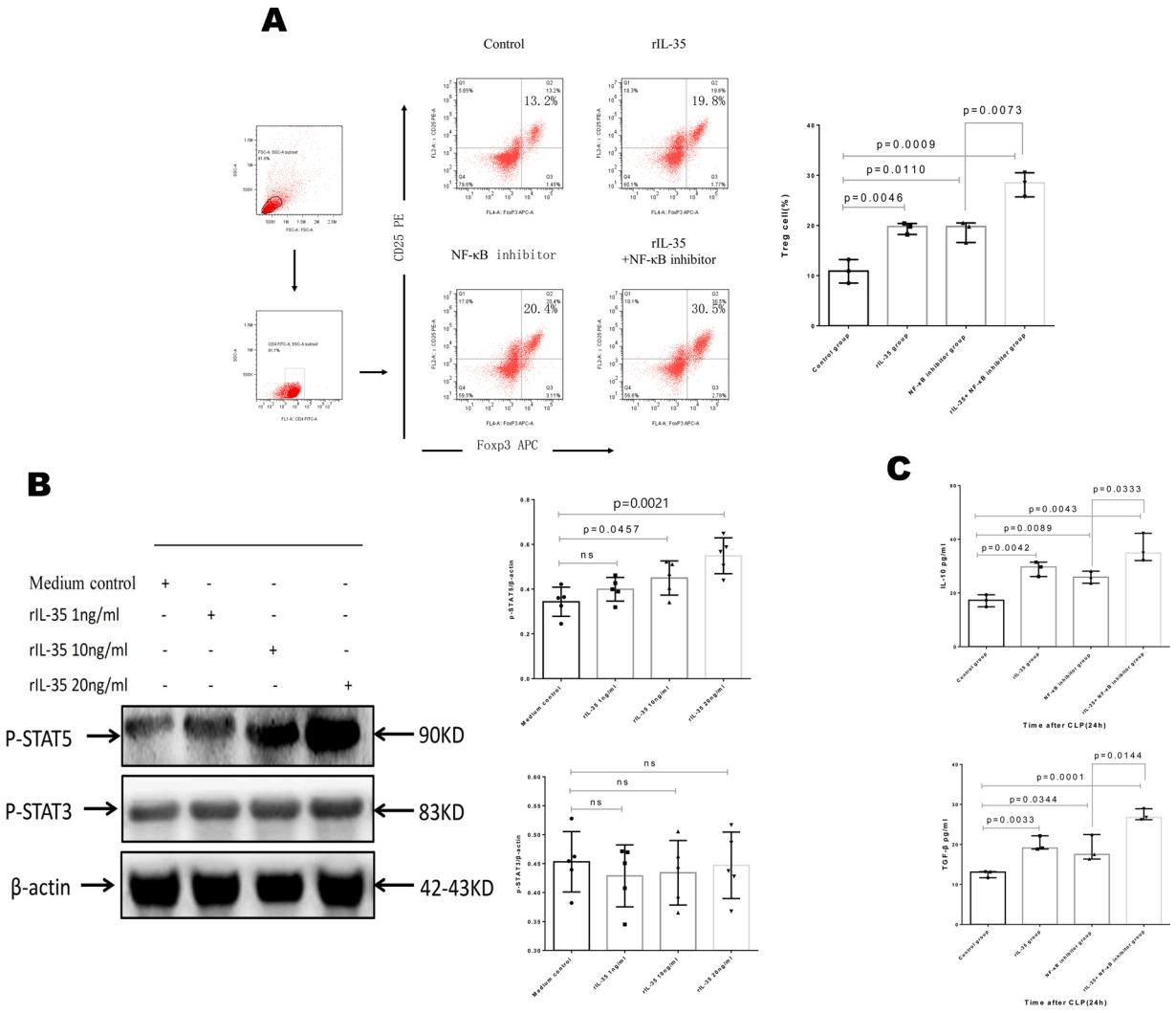

Fig. 5. IL-35 can promote the differentiation of naïve $CD4^+$ T lymphocytes into Treg cells. (A) The proportion of Tregs increased compared with that in the untreated group after the addition of recombinant IL-35. Furthermore, naïve CD4 + T lymphocytes differentiated into more Tregs after administration of NF-κB molecule inhibitors. (B) The expression of p-STAT3 or p-STAT5 in CD4 + T lymphocytes was analysed by Western blot. (C) Concentrations of IL-10 and TGF-β in cell culture supernatant were analysed by ELISA. The comparison between the two groups was performed by one-way ANOVA followed by LSD multiple comparisons test.

NF-κB molecule inhibitor (Fig. 6E).

4. Discussion

The theory of uncontrolled inflammation can explain the relationship between coagulation and fibrinolysis imbalance theory, redox imbalance theory, apoptosis theory, and water channels in ARDS (Yadav et al., 2017). Excessive inflammation increases the apoptosis of alveolar epithelial cells and destroys the integrity of the respiratory membrane, while the release of elastase-degrading pulmonary surfactant-associated protein A (SP-A) by intravascular inflammatory cells promotes alveolar collapse and atelectasis, which aggravates lung injury, resulting in dysregulation of ventilated blood flow and uncorrectable hypoxemia (Lin et al., 2018). At present, both clinical studies and animal experiments indicate that inflammatory cell infiltration, free radical production and excessive expression of pro-inflammatory mediators in lung tissue are the early major pathological features of ARDS (Lin et al., 2016; Patel et al., 2018; Mason et al., 2017). Although excessive inflammation is the main cause of organ damage in ARDS, excessive immunosuppression also can lead to serious secondary infections. Both of these are high-risk factors leading to death in patients with ARDS (Martin-Loeches et al., 2019). Early clinical studies by Donnelly and Armstrong found that the immunosuppressive cytokine IL-10 was expressed at high levels in the alveolar lavage fluid of ARDS survivors, while IL-10 was at low levels in non-survivors (Donnelly et al., 1996). The decrease of IL-10 in bronchoalveolar lavage fluid in patients with acute respiratory distress syndrome is related to mortality (Stein, 1997). Recent studies have shown that the decrease in the accumulation of regulatory T cells and the decrease in interleukin-10 in a mouse model of acute respiratory distress syndrome can lead to increased tissue damage (Toyama et al., 2018). Interleukin-10-overexpressed mesenchymal stromal cells can promote the survival of mice with acute lung injury induced by endotoxin. In fact, the anti-inflammatory response is a compensatory response to an overactive systemic inflammatory response (Wang et al., 2018b). In summary, insufficient secretion of anti-inflammatory cytokines is the important factors for organ damage caused by excessive inflammation (Bone, 1996). Cytokines and inflammatory mediators involved in ARDS have been found to include tumour necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), IL-4, IL-6, IL-8, IL-10, IL-13, IL-17A, transforming growth factor-β (TGF-β), and interferon-γ (IFN-γ). Among these, TNF-α, IL-1, IL-6, IL-8 and IL-17A trigger inflammatory reactions, while IL-4, IL-10, IL-13, and TGF-β reduce inflammation, and the dynamic balance between them is the key role of ARDS in pathogenesis (Lin et al., 2016; Xie et al., 2019; Yang et al.,

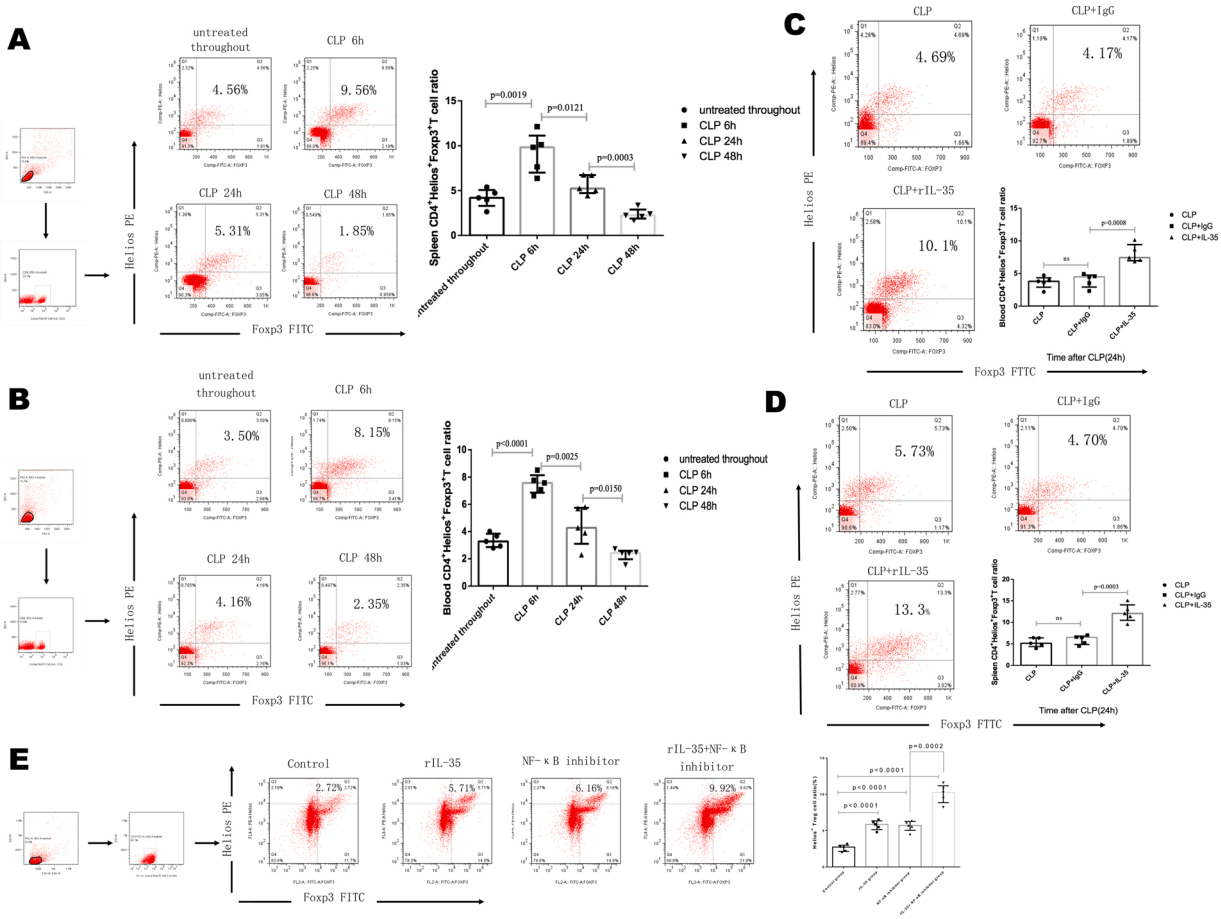

Fig. 6. C57BL/5 mice were assigned to the untreated group, CLP (6 h) group, CLP (24 h) group, CLP (48 h) group, CLP + IgG group and CLP + IL-35 group. Each group had n = 5, the indicators of each mouse were repeatedly tested 3 times, and the best data were selected as the representative data. (A, B) T cells were isolated from mouse spleens or peripheral blood. The frequency of Foxp3+Helios + Treg cells was subsequently determined by flow cytometry, and the results reflect five mice per time point. Foxp3+Helios + Treg cells gradually decreased in the blood and spleen with time in CLP-induced ARDS. (C, D) Treatment with recombinant IL-35 after CLP challenge restored Foxp3+Helios + Treg cells in the blood and spleens. (E) Naïve CD4 + T lymphocytes induce the differentiation of CD4 + Foxp3+ Helios + T cells (Tregs) via the IL-35/NF-κB signalling pathway. The comparison between the two groups was performed by one-way ANOVA followed by LSD multiple comparisons test.

2018a; Mazzocchi et al., 2017). As an organic whole, when the body is in a state of systemic inflammatory response, anti-inflammatory cytokines and pro-inflammatory cytokines may increase at the same time. As a new member of the IL-12 family, IL-35 plays an important immunosuppressive role in various inflammatory diseases (Zhang et al., 2019b). Therefore, although in our clinical studies, we investigated the high expression of IL-35 in ARDS, compared with ARDS survivors, its levels were significantly lower in ARDS non-survivors (Fig. 1A). And it gradually decreases during the uncontrolled inflammation process of ARDS (Fig. 1B). This indicates that maintaining IL-35 levels during acute inflammation may be a useful approach to dampen inflammation and prevent lung injury. In addition to the effects of pro-inflammatory factors, the insufficient secretion of anti-inflammatory factors (for example, IL-10, TGF-β) is also a factor in the progression of inflammation. Therefore, although the levels of IL-1β, TNF-α, IL-6 and IL-10 were elevated in clinical ARDS, elevated IL-35 had a weak negative correlation with IL-1β and TNF-α (r=-0.35 and -0.43 respectively) in human patients with ARDS, suggesting the anti-inflammatory potential of IL-35 in clinical ARDS. But surprisingly among these elevated cytokines, IL-6 plays an important role of pro-inflammatory factor in ARDS, which is currently recognized internationally (Meduri, 2002). As an anti-inflammatory cytokine, IL-35 should be negative correlated with IL-6 and other pro-inflammatory cytokines in an uncontrolled inflammatory disease such as ARDS. However, we observed a weak positive correlation between IL-35 and IL-6 (r = 0.38), which may indicate that IL-35 is unable to regulate the high levels of IL-6 being produced at this early timepoint of ARDS. And this needs to be further confirmed in future research. Thus, we hypothesized that there was a deficiency of IL-35 in ARDS. Supplementation with exogenous IL-35 could compensate for the deficiency and improve the anti-inflammatory effect. In addition, IL-35 is positively correlated with Treg/CD4+ cells in the peripheral blood of patients with ARDS, indicating that there is a correlation between IL-35 and Treg cells in ARDS (Fig. 1C). Recent animal studies have shown that the administration of IL-35 antibody can significantly increase the degree of lung injury in the ARDS mouse model (Wang et al., 2019a). Moreover, various pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IL-17A, were significantly increased, while the inhibitory inflammatory cytokines IL-4, IL-10, and IL-13 were significantly decreased (Wang et al., 2019a). In this study, we found that IL-35 was abnormally highly expressed in the CLP-induced ARDS mouse model (Fig. 2A). Moreover, after administration of recombinant IL-35, the expression of various pro-inflammatory factors, such as TNF-α, IL-1β, IL-2 and IL-6, was significantly reduced (Fig. 3C), the degree of lung injury in mice was improved (Fig. 3A) and the survival rate of ARDS mice was increased (Fig. 3F). Overall, the results of this study further confirm that IL-35 has important protective effects in the ARDS model.

Tregs are anti-inflammatory because of contact-dependent suppression or release of cytokines, IL-10, and transforming growth factor

(TGF)-β1 in other immune cells, such as CD4+ and CD8 + T cells, B cells, natural killer (NK) cells and dendritic cells (Jing et al., 2019). Previous studies have shown that reduced generation or deficient function of Tregs is associated with disease severity and activity (Zeng et al., 2013). In mice and patients with ALI, alveolar recruitment of Tregs contributed to the resolution of lung inflammation (Jing et al., 2019). Therefore, we further examined the change in Treg cells in the CLP-induced ARDS model. The results revealed that with the prolongation of modelling time, Treg cells gradually decreased in peripheral blood and spleen (Fig. 2B and C), and the severity of lung injury in mice gradually increased (data not shown in article). These findings are consistent with those of previous studies. Although IL-35 and Tregs have been reported to interact, it is not known whether IL-35 affects the differentiation of Tregs and further affects the progression of ARDS. Therefore, after treatment with recombinant IL-35 in mice, the proportion of Treg cells in the spleen and peripheral blood increased significantly (Fig. 3D and E), and lung injury decreased (Fig. 3A and B). These findings suggest that IL-35 can play a protective role in ARDS by altering the differentiation of Tregs.

Some studies have confirmed that IL-35 can play a protective role in the prevention of rheumatoid arthritis and acute kidney injury by inhibiting the NF-κB signalling pathway (Hu et al., 2017; Alunno et al., 2017). However, studies of ARDS have not yet shown whether IL-35 can exert its function through the NF-κB signalling pathway. Therefore, we investigated whether the expression of NF-κB increased significantly in PBMCs from patients with ARDS (Fig. 1D). We then investigated whether the NF-κB-associated protein P65 gradually increased in the CLP-induced ARDS mouse model. In this study, qRT-PCR and Western blotting showed that administration of recombinant IL-35 could significantly downregulate the expression of NF-κB in lung tissue and reduce the inflammatory response in ARDS model mice (Fig. 4A↓B). In fact, nuclear factor kappa B (NF-κB), which is an inducible nuclear transcription factor that can increase its activity under the stimulation of trauma and microbial infection, results in excessive release of a large number of pro-inflammatory cytokines (Sun, 2017). Studies have confirmed that there is overexpression of NF-κB in bronchial mucosa in animal models of inhalation lung injury, suggesting that the NF-κB signalling pathway plays an important role in the promotion of pulmonary ARDS, especially inflammatory factors (Cui et al., 2018). Other studies have shown that lipopolysaccharide-induced lung injury activates NF-κB and initiates TNF-α expression (Niu et al., 2017). The released TNF-α and lipopolysaccharide further activate the NF-κB signalling pathway in effector cells and initiate the expression of other cytokines and adhesion molecules involved in acute lung injury (Niu et al., 2017). In clinical trials, the role of NF-κB activation in the pathogenesis of acute lung injury has been validated. For example, Schwartz et al. showed that endotoxin activates NF-κB and induces the production of pro-inflammatory factors, which is the key to the pathogenesis of acute lung injury. NF-κB activation may be a control point for the large expression of inflammatory response genes (Piegeler et al., 2014). Therefore, we further confirmed that IL-35 may block the uncontrolled inflammatory response of ARDS by downregulating the signal expression of NF-κB in lung tissue. Interestingly, after the administration of recombinant IL-35, we found that it could reduce the degree of apoptosis in addition to downregulating the NF-κB inflammatory signalling pathway (Fig. 4C).

Animal experiments revealed that IL-35 can change the degree of Treg cell differentiation and play an important protective role in ARDS and that these effects may be related to the NF-κB signalling pathway. Therefore, we investigated the mechanism of the interaction between IL-35 and Tregs in cell experiments. It is well known that cellular immunity is the main force behind host anti-infection and immune protection; however, it is also an important factor leading to immunopathology and plays an important role in a variety of inflammatory diseases (Halle et al., 2017). T lymphocytes constitute the major cellular components of the adaptive immune response. More than any other cellular system, cell-mediated immune responses are largely controlled by T cells (Zygmunt and Veldhoen, 2011). $CD4^+$ T lymphocytes are the most active subset of immune cells that regulate ARDS, and their innate and adaptive immune integration leads to characteristic pathological stages such as ARDS exudation, a proliferative phase and a fibroproliferative phase (Yang et al., 2018b). Tregs are a subtype of $CD4^+$ T cells known to play important roles in immune homeostasis and maintenance of self-tolerance that were originally identified as suppressor cells (Josefowicz et al., 2012). Recent studies have shown that Treg cells interact with cytokines such as TGF-β, IL-6, IL-10, IL-18, and IL-33 by altering neutrophil clearance, lung vascular endothelial cell damage, and sodium channel activity involved in the regulation of ARDS (Togashi and Nishikawa, 2017; Zhong et al., 2016; Miao et al., 2017). Moreover, the degree of differentiation of Treg cell subsets in the spleen plays an important protective role in the CLP-induced ARDS model (Wang et al., 2019a). Recent studies have demonstrated a core role of Tregs in the alleviation or treatment of ALI/ARDS, in that they orchestrate a complex series of therapeutic events (Lin et al., 2018). IL-35 is a relatively newly identified cytokine obligatory for the regulatory role of Tregs that plays a vital function in the prevention and treatment of autoimmune diseases (Zhang et al., 2019a). One study showed that the degree of lung injury mitigation was associated with an increase in Tregs, as well as the Foxp3 transcription level in the lungs and IL-35 levels from BALF in rats (Bai et al., 2012), but there is no direct evidence of the role and mechanism of Tregs and IL-35. However, the in vitro experiments conducted in the present study indicated that recombinant IL-35 protein promotes the differentiation of Naïve $CD4^+$ T cells into Tregs by promoting P-Stat5, while also affecting the secretion of IL-10 and TGF-β. And this effects of IL-35 were increased through inhibiting NF -KB pathway (Fig. 5).

Foxp3 is an important transcription factor that affects the development and immunosuppression of Treg cells (Wan and Flavell, 2007; Zheng and Rudensky, 2007). In the past, it has been thought that it is specifically expressed in $CD4^+CD25^+$ T cells, and it is a specific indicator of Treg cells. However, as it is known that non-Treg cells can transiently upregulate Foxp3 expression upon activation, a combination of markers is required to identify Tregs in the context of activation (Lipscomb et al., 2010). With further research, it was found that Helios, as a member of the IKaros transcription factor family, is expressed at a high level in Treg cells (Akimova et al., 2011). Earlier studies believed that Helios existed only in thymically-derived Treg (tTreg) cells and could distinguish peripherally-derived Treg (pTreg) cells (Thornton et al., 2010; Zabransky et al., 2012). However, later studies found that with IL-2 and TGF-β cytokine co-stimulation, activated effector T cells can also differentiate into $Foxp3^+Helios^+$Treg cells (Verhagen and Wraith, 2010). The Helios gene overexpressed in mice can enhance the function of Foxp3. Compared with $Foxp3^+Helios^-$T cells, $Foxp3^+Helios^+$T cells have a stronger immunosuppressive function (Alexander et al., 2013). Together, Foxp3 and Helios represent the key functional stages of Treg cells. Therefore, Helios is currently considered to be a marker of activated Treg cells. In our further study, we found that the IL-35/NF-κB signalling pathway can promote the differentiation of naïve CD4 + T lymphocytes into Foxp3+Helios + T cells and play an important protective role in ARDS mouse models (Fig. 6).

5. Conclusion

Our data indicate that IL-35 plays an important protective role in ARDS by regulating the dynamic balance of pro-inflammatory and anti-inflammatory factors. IL-35 could promote the differentiation of naïve CD4 + T cells into Foxp3+Helios + Tregs, which help control inflammation in ARDS, and these effects of IL-35 were increased through inhibiting NF -κB pathway. However this is our data from the mouse CLP-induced ARDS model are preliminary (n = 5 mice per group from a single experiment) and need to be replicated in a larger study.

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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 and 2020-846.

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.

Declaration of Competing Interest

The authors report no declarations of interest.

Contributor

Conception hypothesis and design: Fang xu, Chuanjiang wang. Data acquisition and analysis: Fang xu, Shihui lin, Kefeng Li. Manuscript preparation: Fang xu and Chuanjiang wang. Revised manuscript: Chuanjiang wang, Ke Xie, Fang xu. Searched and collected bibliography: Fang xu, Ke Xie. We are very grateful to the laboratory of Lipid & Glucose Metabolism at The First Affiliated Hospital of Chongqing Medical University for providing laboratory facilities.

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