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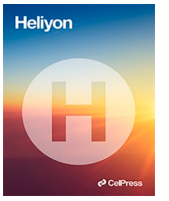

Research article

The effect of adoptive transferring myeloid-derived suppressor cells in ventilator-induced lung injury mice

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ABSTRACT

The effects of adoptive transferring myeloid-derived suppressor cells (MDSCs) to mice with ventilator-induced lung injury (VILI) are unclear. Our objective was to investigate the effects of adoptively transferring MDSCs in VILI. The mouse model was created by introducing mechanical ventilation through a high tidal volume of 20 ml/kg for 4 h. Inflammation-induced MDSCs (iMDSCs) were collected from the bone marrow of mice with cecal ligation and puncture. iMDSCs were administrated through retrobulbar angular vein 1 h before the mechanical ventilation. The control group was anesthetized and maintained spontaneous respiration. After the termination of mechanical ventilation, bronchoalveolar lavage fluid (BALF) and lung samples 6 h were collected. The concentrations of BALF protein, levels of inflammatory mediators, and white blood cells were all significantly decreased in mice treated with iMDSCs. Histological examinations indicated reduced lung damage after iMDSCs treatment. Moreover, adoptive transfer of iMDSCs could reduce $CD4^+$ T-cell counts and inhibit its inflammatory cytokine secretion. iMDSCs treatment was found to had no immunostimulatory effects or cause secondary infections in mice. In conclusion, MDSCs might be a potential targeted therapy for alleviating the inflammatory response of VILI mice in a T-cell dependent manner.

1. Introduction

Positive pressure mechanical ventilation is a crucial life support method for patients with acute respiratory distress syndrome (ARDS). However, studies have revealed that mechanical ventilation can cause or worsen acute and chronic lung injuries, even when the ventilator parameters are optimized or the treatment strategies are adjusted. ventilator-induced lung injury (VILI) is characterized by inflammatory cell infiltration, transparent membrane formation and increased vascular permeability in lung tissue [1]. According to studies, the incidence of VILI is up to 50 %, which significantly impacts on the safety and efficacy of the mechanical ventilation [2].

Excessive mechanical stretch induced by mechanical ventilation results in mechanical injury that can lead to biological injury, in

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the form of an excessive inflammatory response in the lungs. Biological damage is a critical contributing factor that promotes VILI and is closely linked to the prognosis of mechanically ventilated patients [1]. In VILI, excessive mechanical stretch could cause the edema of alveolar wall endothelium and epithelium, as well as epithelium and interstitium. This leads to swelling and necrosis of the alveolar epithelial cells, followed by the release of substances that draw in and activate inflammatory cells such as macrophages and neutrophils, which release a large amount of inflammatory mediators, further worsening lung injury [3,4]. Research has proven that recruiting inflammatory cells and releasing of inflammatory cytokines in response to mechanical injury in the lung causes a "secondary injury" to the lung tissue. Inflammatory cells like macrophages, widely distributed in the lung, recognize signals released by dead cells, [such as interleukin-1β (IL-1β), IL-6, tumor necrosis factor-α (TNF-α), or high mobility group protein 1 (HMGB-1)] and secrete a mass of inflammatory cytokines, becoming an important source causing acute lung injury in VILI [5–7]. Lung protective ventilation strategies aimed at reducing lung injury are related to the inhibition of lung inflammatory cell infiltration and inflammatory activation [8]. It is therefore essential to reduce or hinder pulmonary inflammation to alleviate lung injury resulting from mechanical ventilation, which is significant in improving the safety and efficacy of positive pressure mechanical ventilation.

Given the significant economic and health burden of VILI, a lot of research has been focused on developing effective treatment strategies. Cell therapy, a promising treatment strategy using hematopoietic stem cells, has been investigated recently [9,10]. In recent years, myeloid-derived suppressor cells (MDSCs) have been identified as an important negative regulator of the adaptive immune system. It is a heterogeneous population derived from bone marrow, including immature myeloid cells, myeloid progenitor cells or precursor cells, macrophages and dendritic cells [11,12]. MDSCs present powerful immunosuppressive functions through several mechanisms. They could efficiently inhibit the activation and expansion of effector T lymphocytes by upregulating inducible nitric oxide synthase (iNOS), Arginase-1 (Arg-1), reactive oxygen species, a series of inflammatory mediators, or inducing regulatory T lymphocytes (Tregs) [13]. In addition to regulating the differentiation of myeloid cells, MDSCs could suppress immune system activation by regulating the differentiation of myeloid cells locally and respond to a range of inflammatory stimulation signals [14,15]. Studies have demonstrated the crucial role this population of cells play in a variety of inflammatory diseases, including autoimmune diseases and infectious conditions; and it might be useful in treating asthma or rheumatoid arthritis [16]. However, the treatment role of MDSCs in VILI have not been investigated.

Recently, a significant number of inflammation-induced MDSC (iMDSCs) were detected in the bone marrow and spleen of septic mouse models [17,18]. Thus, in our experiment, we used iMDSCs from cecal ligation and puncture (CLP) mouse model to investigate their therapeutic potential on VILI mouse model. Our study demonstrated that adaptive transferring of MDSCs effectively attenuated inflammation response in VILI without causing any immunostimulatory effects and secondary infections. In conclusion, MDSCs show promise as a viable therapeutic approach for alleviating the inflammatory injure caused by VILI.

2. Materials and methods

2.1. Establishment of VILI animal model

The animal model of VILI induced by high tidal volume was established according to the operation method as reported previously [19,20]. Specific pathogen-free (SPF)-grade, 6-8 week-old male C57BL/6 mice were intraperitoneally anesthetized with sodium pentobarbital, intubated with a 20G catheter, and ventilated in volume control mode with an FiO_2 of 0.21, inspiratory and expiratory time ratio of 1:1, tidal volume of 20 ml/kg, positive end expiratory pressure (PEEP) of 0 cmH_2O, and respiratory rate of 40 breaths/min. Mice in the control group were intubated and kept breathing autonomously. The mice were removed from the ventilator after 4 h of continuous mechanical ventilation. Based on our previous study [21], lung injury was most severe 6h after removing the mice from the ventilator, which is when samples were collected for further experiments. Animal experimental procedures were approved by the animal ethics committee of China-Japan Friendship Hospital (NO.13009). Animals were randomly divided into three groups: control group, VILI group, and adoptive transferring MDSCs from CLP bone marrow in VILI mice (iMDSCs + VILI group).

2.2. CLP model

The CLP animal model was established according to previous study [22]. An intraperitoneal injection of pentobarbital sodium was administered to the animals to induce anesthesia before making a midabdominal incision. After puncturing the cecum with an 18G needle, a small amount of intestinal content was squeezed out to maintain an open puncture hole. The cecum was then carefully repositioned within the abdominal cavity, and the abdominal layers were closed. Post-operation, 1 ml of normal saline was injected subcutaneously to help the animals recover.

2.3. Specimen collection and procession

After mechanical ventilation, mice were anesthetized abdominally with sodium pentobarbital, and a suitable volume of peripheral blood was collected for analysis. Mice were then carefully positioned in supine position on the operating table and sterilized with 75 % alcohol cotton ball. Subsequently, the anterior neck skin was incised in the middle expose the trachea after bluntly separating the subcutaneous tissue. A 20G catheter was inserted into the trachea, and PBS was used to retrieve bronchoalveolar lavage fluid (BALF) via repeated aspiration with a syringe for three cycles. The BALF recovery rate was above 80 %. The recovered BALF was mixed thoroughly, centrifuged, and the supernatant was retained for BALF analysis. The lower layer cells were also collected and used for flow cytometry analysis. Finally, a thoracotomy was performed, and the lungs were excised and preserved in 10 % formalin.

2.4. The determination of wet/dry weight ratio (W/D) in lung

After mechanical ventilation, mice were anesthetized via intraperitoneal injection of sodium barbitone at various time intervals. The mice were sterilized with 75 % alcohol cotton balls, and thoracotomy was performed to fully expose the lungs. The left main bronchus was tied off with a silk thread, and the left lung was completely excised and weighed for wet weight using a precision electronic analytical balance. The lung tissue was then dried in an incubator at 60 °C for 72 h and weighed for dry weight. Finally, the wet-to-dry weight ratio was calculated for each specimen [23].

2.5. Flow cytometry analysis

Peripheral blood and BALF were collected, lysed with red blood cell lysate (BD Biosciences, USA), and then incubated with mixed fluorescent anti-mouse antibody for 20 min. The following antibodies were used: anti-mouse CD45-FITC, Gr-1-PE, CD11b-PerCP, CD48-APC, CD3-PE, CD4-Percp, CD8-APC, TNF-α-APC, F4/80-PE, and absolute count fluorescent microspheres (BD Biosciences, USA). All samples were examined using a FACS Calibur flow cytometer (BD Biosciences, USA), and data were analyzed using FlowJo software (Tree Star, Ashland, OR).

2.6. Sorting and adoption of iMDSCs

Single cell suspension was prepared from bone marrow of CLP mice and iMDSC was labeled with anti-mouse Gr-1 and CD11b (BD Biosciences, USA). Cells were sorted using FACS Aria (BD Biosciences, USA), achieving a purity of over 95 %. Each 5 × 10ˆ6 iMDSCs was resuspended in 100 μl PBS and were injected into mice through the retrobulbar medial canthus venous plexus. The control group received an equal volume of phosphate buffer saline (PBS).

2.7. Immunosuppressive function of iMDSCs

The CD3e antibody (5 μg/ml, eBioscience) was coated in 96-well plates at 100 μl per well. $CD4^+$ T cells were isolated from normal mouse spleens using a mouse $CD4^+$ T-cell isolation kit (Miltenyi, Germany). The sorted $CD4^+$ T cells were stained with 1 μM 5, 6-carboxycein diacetate succinimidyl ester (CFSE, Invitrogen). Next, the CD28 antibody (5 μg/ml, eBioscience) was then added. Different ratios of $CD4^+$ T cells (1×10^5/well) and sorted iMDSCs were co-cultured in 96-well plates for 72h. After co-culture, anti-CD4-APC and 7-AAD (eBioscience) were used to stain the cells, and the degree of CFSE decay in 7-AAD-$CD4^+$ T cells was determined by flow cytometry. In the meantime, the proliferation index (PI) of $CD4^+$ T cells was calculated as the ratio of the percentage of cells co-cultured with iMDSCs to cells co-cultured without iMDSCs, multiplied by 100 %.

2.8. Detection of protein concentration and inflammatory cytokines

Total protein concentration in BALF was detected using a protein detection kit (Thermo, Germany). The levels of inflammatory cytokines IL-6, TNF-α and MCP-1 (monocyte chemotactic protein-1) were detected by cytometric bead array (CBA) kit (BD Biosciences, USA).

2.9. Real-time PCR

Total RNA was extracted by using TRIzol reagent (Invitrogen, USA) from 1×10^4-1×10^5 sorted cells, followed by reverse transcription of RNA to cDNA using PrimeScript™ II 1st Strand cDNA Synthesis Kit (Qiagen, Germany). Quantitative real-time PCR (qRT-PCR) was performed on a CFX96 Touch™ system (Bio-Rad, USA) by using UltraSYBR Mixture (Cwbio, China). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as the internal reference. The sequences of primers used are as follows: iNOS, F: 5′-ACATCGACCCGTCCACAGTAT-3′, R: 5′-CAGAGGGGTAGGCTTGTCTC-3’; Arg-1, F: 5′- TGTCCCTAATGACAGCTCCTT-3′, R: 5′-GCATCCACCCAAATGACACAT-3’; IL-10, F: 5′- GCTCTTACTGACTGGCATGAG-3′, R: 5′- CGCAGCTCTAGGAGCATGTG-3’.

2.10. Cell proliferation assay

The $CD4^+$ T cells were isolated using magnetic CD4 microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) from the spleen of mice that were either treated with iMDSCs or served as sham-controls. 1×10^6 CFSE-labeled $CD4^+$ T-cells were used in cell proliferation assays and stimulated with anti-CD3/CD28 antibody (eBioscience, San Diego, CA, USA) for 72 h. The proliferation of $CD4^+$ T cells were determined by flow cytometry. The proliferation rate was calculated by the following formula: (the percentage of stimulus-induced proliferating $CD4^+$ T cells – the percentages of proliferating T cells in medium alone)/(100 − % proliferating T cells in medium alone) × 100.

2.11. Histopathological evaluation of lung

The formalin-fixed lung tissue was dehydrated using gradient ethanol, embedded in paraffin, sectioned into 5 μm sections. Hematoxylin and eosin (HE) stain was subsequently used for the standard procedure. The changes in lung tissue structure were observed

under light microscope, with 10 random visual fields selected. Two pathologists independently performed the lung injury evaluation using high-power fields to observe the images [24,25].

2.12. Statistical analysis

SPSS 20.0 software (SPSS Inc., Chicago, IL, USA) and Graphpad Prism 7.0 software (San Diego, CA) was used for statistical analysis. Kolmogorov-Smirnov test was applied to test the normality of data. Two independent sample *t*-test or nonparametric analysis was used to compare measurement data between two groups. One-way analysis of variance (ANOVA) or Kruskal-Wallis ANOVA was used to analyze multiple comparisons with Bonferroni's test as appropriate. Data are reported as mean ± standard deviation (SD) for normally distributed data or as median (interquartile range). $P < 0.05$ was considered statistically significant.

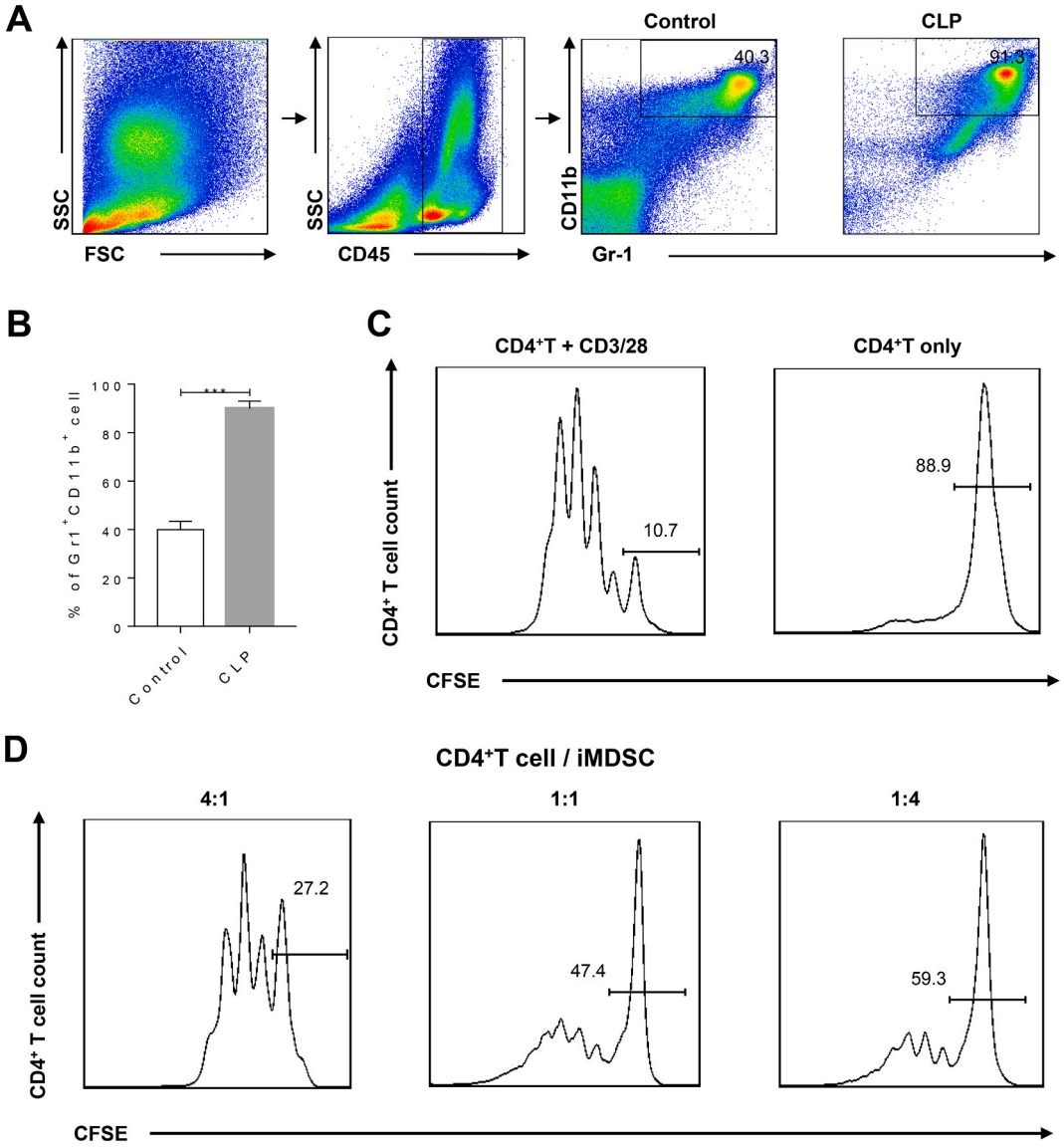

Fig. 1. Identification of iMDSCs immunosuppressive function. (A) Flow cytometry presentation of $CD11b^+Gr1^+$ cells in bone marrow of normal and CLP mice. (B) $CD11b^+Gr1^+$ cells were expanded in bone marrow of CLP mice. (C) The proliferation of $CD4^+$ T cells in the spleen of normal mice stimulated with or without CD3/CD28 antibody. (D) iMDSCs suppressed the proliferation of CFSE-stained $CD4^+$ T cells, and the proportions of $CD4^+$ T cells: iMDSC ($CD11b^+Gr1^+$) cells were 4:1, 1:1 and 1:4, respectively. Data were presented as mean ± standard deviation and analyzed using the Student's t-test. $P < 0.05$ was considered statistically significant. n = 6. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Abbreviations: CLP, cecal ligation and puncture; CFSE, 5, 6-carboxycein diacetate succinimidyl ester; iMDSCs, inflammation-induced myeloid-derived suppressor cells; VILI, ventilator-induced lung injure.

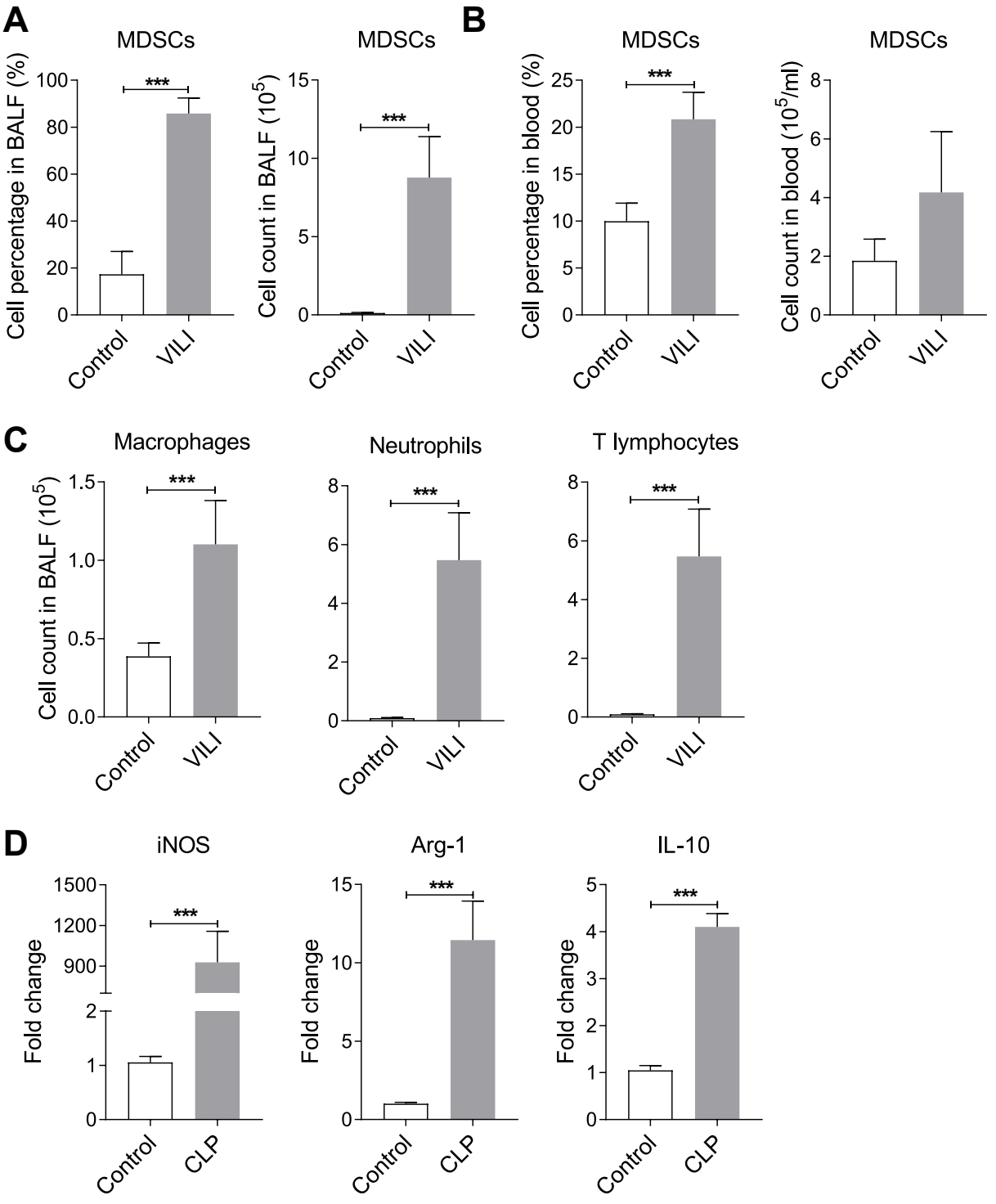

Fig. 2. Differential cell counts in BALF of VILI and molecular expression of MDSC suppression. (A–B) Percentages and numbers of $CD11b^{+}Gr1^{+}$ cells in lung (A) and circulation (B) of VILI mice. (C) Cell counts of macrophages, neutrophils and T lymphocytes in BALF from VILI mice. (D) mRNA expression levels of iNOS, Arg-1 and IL-10 in iMDSCs from CLP mice. Data are presented as mean ± standard deviation and were analyzed using the Student's t-test. $P < 0.05$ was considered statistically significant. n = 6. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Abbreviations: Arg-1, Arginase-1; BALF, bronchoalveolar lavage fluid; CLP, cecal ligation and puncture; IL-10, Interleukin-10. iNOS, inducible nitric oxide synthase (iNOS); iMDSCs, inflammation-induced myeloid-derived suppressor cells; VILI, ventilator-induced lung injure.

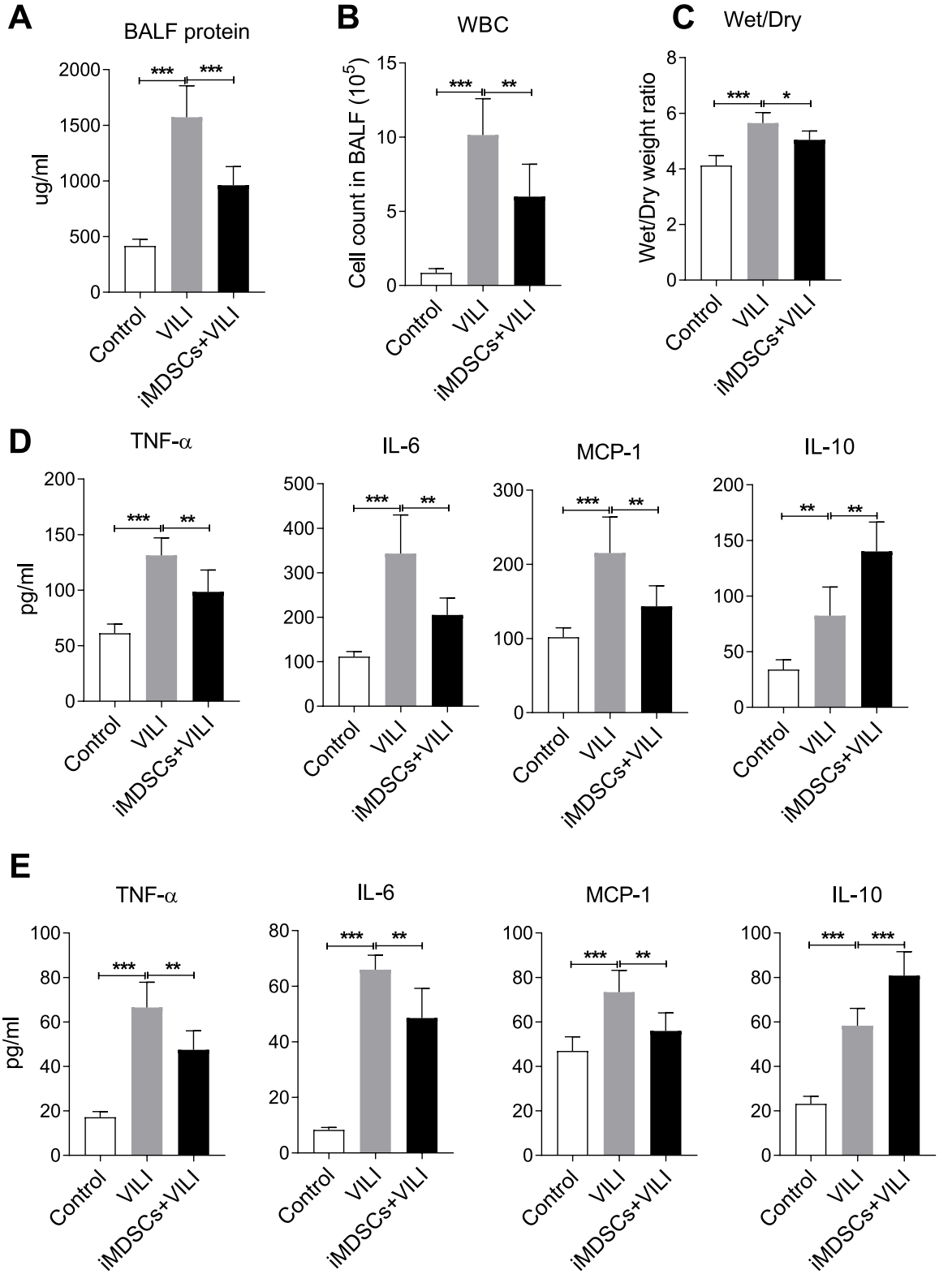

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Fig. 3. Adoptive transfer of iMDSCs reduced the pulmonary inflammation in VILI. (A) Total protein concentration in BALF in each group; (B) Numbers of WBC in BALF; (C) Lung wet/dry weight ratio; (D–E) Comparison of the levels of inflammatory cytokines TNF-α, IL-6, MCP-1 and IL-10 in BALF (D) and circulation (E). Comparison between multiple groups were determined by one-way analysis of variance on ranks flowed by Bonferroni's post hoc test, and data are presented as the mean ± standard deviation. $P < 0.05$ was defined significant. n = 6. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Abbreviations: BALF, bronchoalveolar lavage fluid; IL-6, interleukin-6; IL-10, interleukin-10; iMDSCs, inflammation-induced myeloid-derived suppressor cells; MCP-1, monocyte chemotactic protein-1; TNF-α, tumor necrosis factor-α; VILI, ventilator-induced lung injure; WBC, white blood cells.

3. Results

3.1. Identification of immunosuppressive function of iMDSCs from CLP mice

The flow cytometry of $CD11b^{+}Gr1^{+}$ cells isolated from the bone marrow of both control and CLP mice was shown in Fig. 1A. The fluorescence minus one (FMO) control for CD11b and Gr-1 staining were presented in Supplementary Fig. S1. As reported previously, bone marrow of CLP mice showed a significant increase in the proportion of $CD11b^{+}Gr1^{+}$ cells when compared to control mice (Fig. 1B). In order to evaluate the suppressive capacity of iMDSCs on T-cell proliferation, we sorted the cells from the bone marrow of CLP mice and treated them with CD3/CD28 antibody. We conducted co-cultures of iMDSCs with CFSE-labeled $CD4^{+}$ T cells from normal mouse spleen to quantify the proliferation rate of T cells, which was measured by flow cytometry. The data showed that the presence of $CD11b^{+}Gr1^{+}$ cells from CLP mice significantly suppressed the proliferation of $CD4^{+}$ T cells. An increased in the ratio of iMDCs observed in the co-culture led to significant improvement in their ability to inhibit the proliferation of $CD4^{+}$ T cells. These results demonstrate that iMDSCs had the capability to suppress the proliferation of T cells (Fig. 1C–D).

3.2. The mechanisms of iMDSCs suppression

We detected the number of MDSCs in the lungs and peripheral blood of both control and VILI mice. Mechanical ventilation increased the accumulation of MDSCs in the lungs and circulation of VILI mice when compared to controls, indicating the MDSCs expansion (Fig. 2A–B). Moreover, we also counted the differential cell numbers in BALF. Notably, the counts of macrophages, neutrophils and T lymphocytes in the BALF of VILI mice showed a significant increase (Fig. 2C). To investigate the specific mechanism by which sepsis-induced MDSCs inhibit T lymphocyte function, we determined mRNA expression levels in MDSCs from CLP mice. The gene expression analysis revealed an up-regulation of iNOS, Arg-1, and IL-10 in the CLP mice at 7 days, indicating that iMDSCs from CLP might exert suppression ability through these molecular expressions (Fig. 2D).

3.3. Adoptive transfer of iMDSCs could reduce VILI pulmonary inflammation

Changes in total protein concentration in BALF serve as an indicator of the blood gas barrier integrity within the lung. The total protein concentration in BALF of VILI group was significantly higher ($P < 0.05$) when compared to the control group. In VILI mice receiving adoptive transfer of iMDSCs, the protein concentration in BALF was significantly decreased when compared to the VILI group ($P < 0.05$, Fig. 3A). The cell counts in BALF was utilized to evaluate the extent of inflammatory cell infiltration in small airways and alveolar lumen. In contrary to the control group, the numbers of white blood cells (WBC) in BALF of the VILI group were significantly increased ($P < 0.05$). When compared to the VILI group, the number of WBCs in BALF of mice in VILI model group with adoptive iMDSCs was significantly decreased ($P < 0.05$, Fig. 3B). The W/D ratio of lung tissue in the adoptive group was significantly lower as compared to the VILI group ($P < 0.05$, Fig. 3C). These data indicate that adoptive transfer of iMDSCs reduces the migration or recruitment of inflammatory cells into the lungs, thus reducing protein exudation and pulmonary edema in VILI.

Inflammatory cytokine concentration in BALF is a direct indicator of the degree of inflammatory reaction in the alveolar space. The levels of inflammatory cytokines TNF-α, IL-6, MCP-1 and IL-10 in BALF of VILI mice were significantly elevated when compared to the control group (all $P < 0.05$, Fig. 3D). Compared to the VILI group, adoptive transfer of iMDSCs significantly reduced the concentrations of the proinflammatory mediators TNF-α, IL-6, MCP-1 in BALF of VILI mice (all $P < 0.05$). Additionally, the concentration of anti-inflammatory cytokine IL-10 was significantly increased in the MDSCs adaption group ($P < 0.05$, Fig. 3D). We also detected the levels of inflammatory cytokines in the circulation for each group. The results shown that adoptive transfer of iMDSCs could adoptive transfer reduce the levels of proinflammatory cytokines in peripheral blood of VILI mice. Moreover, our data demonstrated that the concentration of IL-10 was elevated in the MDSCs + VILI group (all $P < 0.05$, Fig. 3E). These findings indicate that iMDSCs may mitigate the pulmonary inflammatory response of VILI.

3.4. Adoptive transfer of iMDSCs could reduce the counts and function of T cells

The inhibitory effects of MDSCs on T lymphocyte proliferation and activation were evaluated by quantifying the T cell subsets, including $CD4^{+}$ T cells and $CD8^{+}$ T cells in the lung and peripheral blood of each group. The date showed that adoptive transfer of iMDSCs significantly decreased the counts of T cells and $CD4^{+}$ T cells in the pulmonary (all $P < 0.05$, Fig. 4A), but had no significant effect on T cells, $CD4^{+}$ T cells or $CD8^{+}$ T cells counts in the circulation (Fig. 4B). Subsequently, the effect of iMDSC adoptive transfer on the function of $CD4^{+}$ T cells that secreted inflammatory cytokines was examined. The results revealed that the percentage of $CD4^{+}$ T cells secreting TNF-α in both lungs and peripheral blood was significantly lower in the group that received iMDSCs adoptive transfer

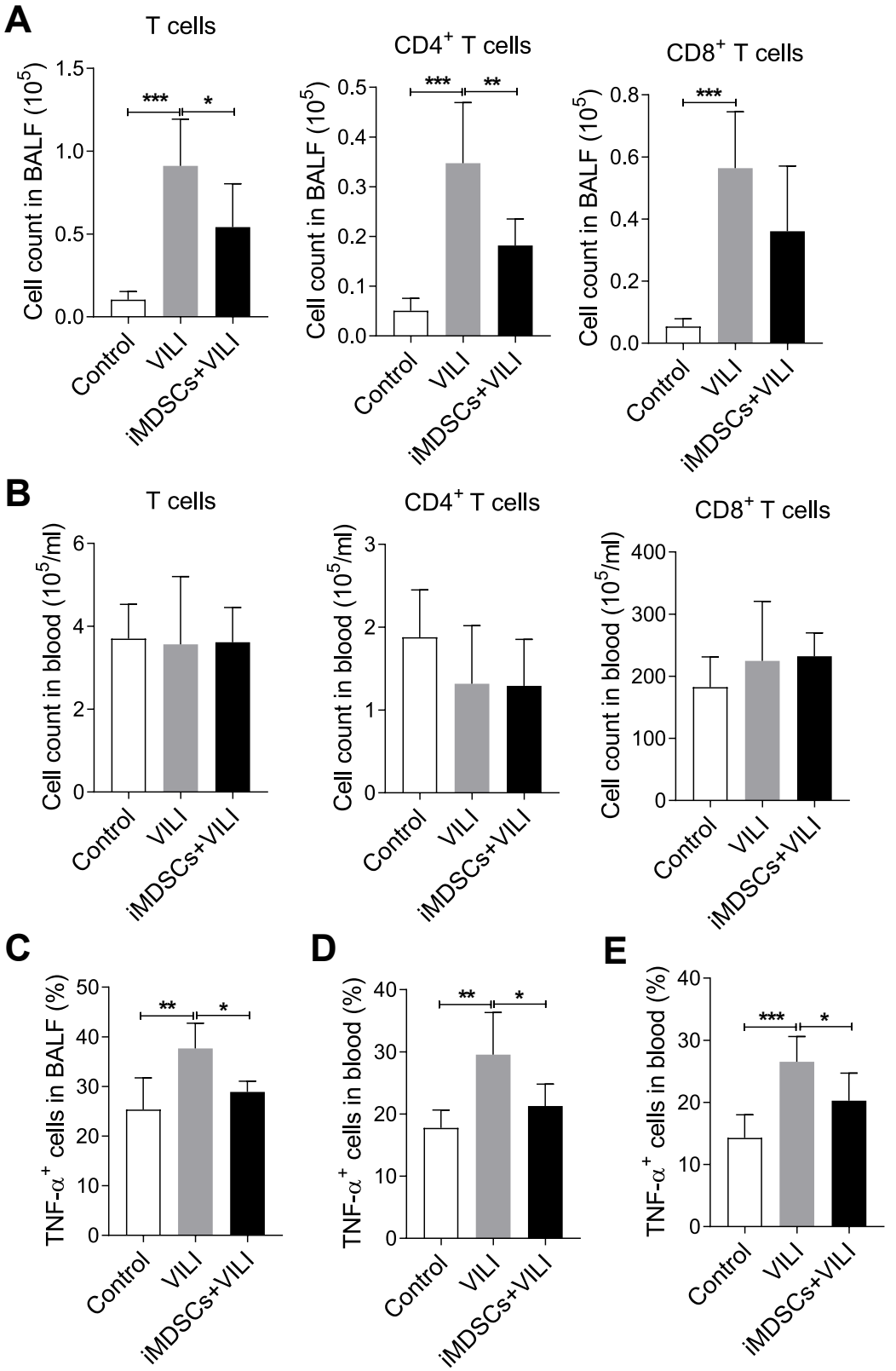

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Fig. 4. Adoptive transfer of iMDSCs effected the counts and function of CD4$^+$ T cells. (A) The numbers of T cells and CD4$^+$ T cells in the pulmonary of each group; (B) The numbers of T cells and CD4$^+$ T cells in peripheral blood; (C–D) Detection of TNF-α cytokines secreted by CD4$^+$ T cells in the lung (C) and the peripheral blood (D). (E) Determination of TNF-α production for monocytes in blood. One-way analysis of variance on ranks was used for multiple comparisons. And data are presented as the mean ± standard deviation. $P < 0.05$ denotes significance. n = 6, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Abbreviations: BALF, bronchoalveolar lavage fluid; iMDSCs, inflammation-induced myeloid-derived suppressor cells; TNF-α, tumor necrosis factor-α.

(all $P < 0.05$, Fig. 4C and D). Furthermore, we also detected the effect of iMDSC adoptive transfer on TNF-α secretion by myeloid cells. The data indicated that the use of iMDSCs significantly reduced TNF-α production by monocytes in blood (Fig. 4E). In summary, the data suggest that adoptive transfer of iMDSCs may alleviate lung injury by suppressing TNF-α production in CD4$^+$ T cells and in monocytes.

3.5. The pathological injury of lung tissue in each group

Analysis of HE-stained lung pathological sections showed that the control group had normal alveolar structure without septal thickening or inflammation, while the VILI model group exhibited increased inflammatory cell infiltration, thickened alveolar septum, and aggravated alveolar destruction compared to the control group. In contrast, the VILI mice that underwent iMDSC adoptive transfer showed reduced inflammatory cell infiltration, alveolar septum thickening, and alveolar destruction (Fig. 5A). The VILI injury score was assessed in each group, revealing a significant decrease in VILI injury score following iMDSC adoptive transfer ($P < 0.05$, Fig. 5B). These data indicated that iMDSCs adoption may relieve lung tissue injury in VILI mice.

3.6. MDSCs treatment had no immunostimulatory effect and secondary infections

To clarify any potential immunostimulatory effect of MDSCs in mice, we subjected the sham control mice to iMDSCs. After 7 days, no significant difference was observed in T cells counts, including CD4$^+$ T cells or CD8$^+$ T cells, in the MDSC-treated sham control group compared to the control group based on the analysis of the BALF and blood (Fig. 6A–B). Additionally, CD4$^+$ T cells from the

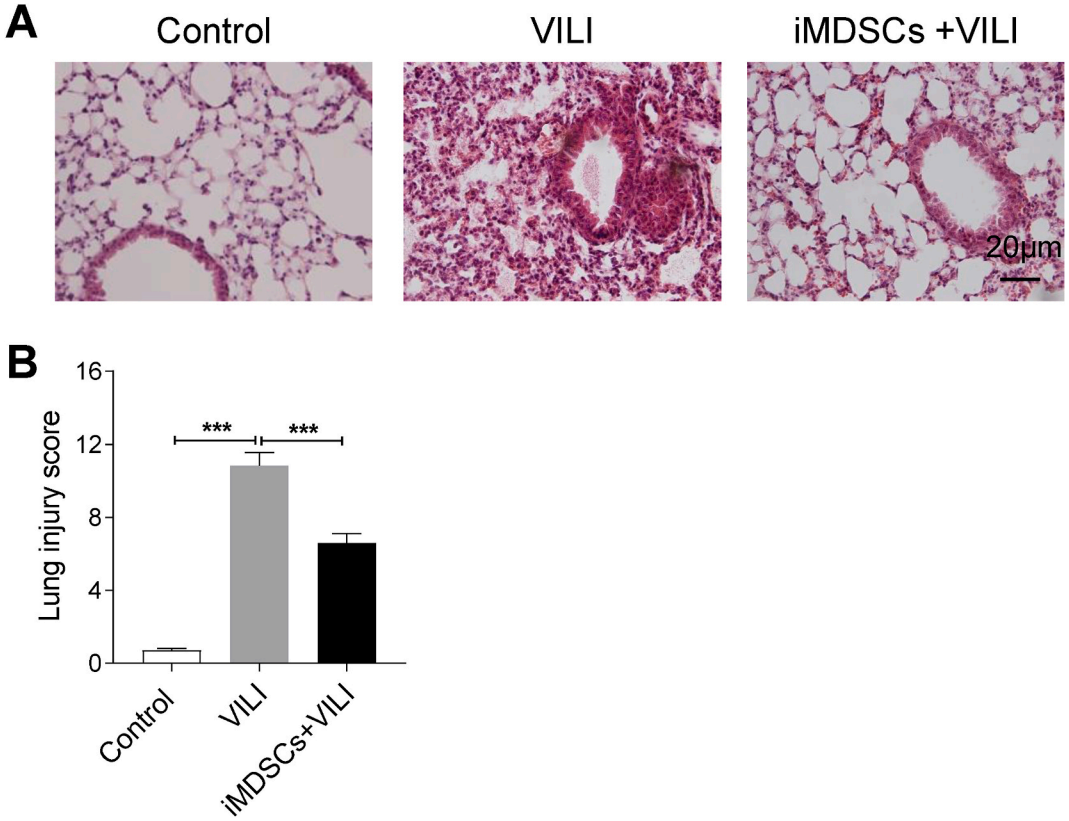

Fig. 5. Lung histopathology of mice in each group. (A) HE pathological staining of lung tissue, × 400, original magnification; (B) Comparation of lung tissue injury score. One-way analysis of variance on ranks was used for multiple comparisons. $P < 0.05$ was significant different. n = 6, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Abbreviations: HE, Hematoxylin and eosin.

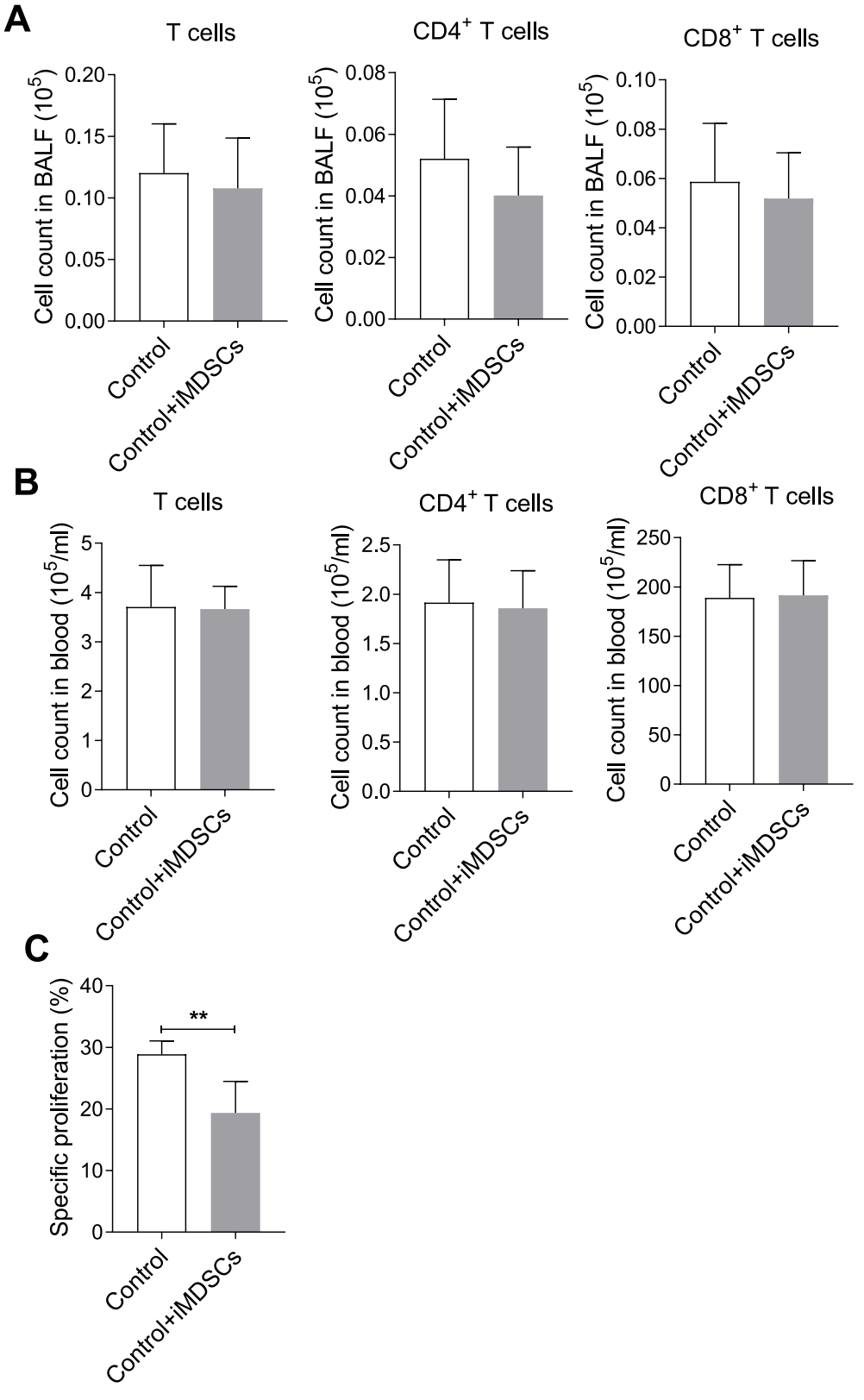

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Fig. 6. T cell counts and functions were not affected after adoptive transfer of iMDSCs into sham-treated mice. Sham-treated mice were adopted with iMDSCs from CLP mice or untreated, and were analyzed one week after the adoption. (A) Cell counts of T cells in BALF. (B) Cell numbers of T cells, $CD4^+$, and $CD8^+$ T cells in the blood. (C) $CD4^+$ T cells from splenocytes of iMDSC-treated or untreated sham mice were CFSE-labeled and stimulated with medium, anti-CD3/28 antibodies. The proliferation of $CD4^+$ T cells was analyzed and percentage of specific proliferation was calculated after 72 h. Data are were analyzed using the Student's t-test and presented as mean ± standard deviation. $P < 0.05$ was considered significant. n = 6, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Abbreviations: BALF, bronchoalveolar lavage fluid; CFSE, 5, 6-carboxycein diacetate succinimidyl ester; CLP, cecal ligation and puncture; iMDSCs, inflammation-induced myeloid-derived suppressor cells.

splenocytes showed no significant increase in proliferative capacity after CD3/CD28 stimulation (Fig. 6C). To assess the susceptibility to secondary infections in mice, we sacrificed the mice at 3 days and 7 days after iMDSCs transplantation. The results revealed that the BALF protein, WBC, and wet/dry ratio did not increase at VILI 7days (Fig. 7A–C). Moreover, the analysis of inflammation mediators in the lungs and circulation showed no significant elevations as the mice were weaned off the ventilator (Fig. 7D–E).

4. Discussion

ARDS is a condition characterized by acute and progressive aggravation of respiratory failure caused by various internal and external pulmonary pathogenic factors other than cardiogenic factors. It is primarily characterized by local inflammatory reaction mediated by a variety of inflammatory cells and alveolar-capillary membrane injury arising from uncontrolled inflammatory reaction, with a mortality rate exceeding 40 % [26,27]. Mechanical ventilation is currently an essential and crucial supportive treatment strategy for ARDS; however, it may cause VILI [28]. Therefore, exploring effective treatments for ADRS has become a significant concern [29]. In this study, we found that iMDSCs could alleviate the inflammatory injury of VILI induced by high tidal volume by suppressing the immune response of $CD4^+$ T cells.

In this study, a mouse model of VILI was induced by continuous mechanical ventilation with a high tidal volume of 20 ml/kg for 4 h [19,20]. Mechanical stimuli produced by mechanical ventilation with high tidal volume, such as excessive stretch and shear force, transduce biochemical signals into alveolar epithelial cells, resulting in the activation of local inflammatory cells and the expansion of inflammatory reaction. This leads to the release of massive inflammatory mediators which causes pulmonary injury, namely biological injury [4,30]. The release of inflammatory mediators mediates the systemic inflammatory response syndrome (SIRS) during mechanical ventilation and can also lead to bacteria and toxins entering the circulation from the lungs, causing SIRS [31]. It causes septal congestion, hemorrhage, inflammatory cell infiltration and alveolar wall thickening, and increased pulmonary capillary permeability, leading to pulmonary edema, hyaline membrane formation, pulmonary capillary hemorrhage and thrombosis, inflammatory cell infiltration and a series of structural changes in the lungs. Mechanical ventilation with high tidal volume in mice showed significant VILI, including destruction of pulmonary barrier integrity, severe pulmonary inflammation, and pulmonary edema due to increased alveolar capillary permeability [32]. Analysis of inflammatory cells and total protein concentrations in the pulmonary demonstrated that high tidal volume could lead to VILI, including increased pulmonary WBC counts caused by increased endothelial and epithelial permeability, increased inflammatory cytokine levels, and increased total protein concentrations in BALF, which is a marker of lung barrier dysfunction [33]. In brief, these indicated that mice suffering the mechanical ventilation with high tidal volume could progress into typical VILI. Although hematopoietic stem cells and mesenchymal stem cells show promise in treating VILI, their application is limited in clinical settings due to challenges such as stem cell source and accession, and function impairment after cryopreservation [34,35]. Given the various limitations of stem cell therapy, this study investigates the therapeutic potential of iMDSCs on VILI mice. MDSCs are vital regulatory immune cells that regulate inflammation in many inflammatory diseases, autoimmune diseases or organ transplantations [36]. It was found that intravenous administration of iMDSCs significantly reduced the lung inflammation induced by high-tidal-volume mechanical ventilation in VILI mice. These findings demonstrated that all lung injury indicators exhibited a downward trend after adoptive transfer iMDSCs into VILI mice, including protein concentration, inflammatory cell counts and inflammatory cytokine levels in BALF. Moreover, the adoptive transfer of iMDSCs also dampen histopathological injure. Our findings indicated that iMDSCs adoptive transfer can significantly alleviate the local pulmonary inflammation and damage to the alveolar capillary membrane integrity caused by high-tidal-volume mechanical ventilation, demonstrating its therapeutic effect.

Previous studies have shown that MDSCs effectively inhibit the proliferation and cytokine production of effector T cells through various mechanisms [13]. We investigated the number and function of T cells in the lungs and circulation of the adoptive transfer cells group. Our study found that adoptive transfer of iMDSCs significantly reduced the number of $CD4^+$ and $CD8^+$ T cells in the lungs of VILI mice and suppressed their function of secreting TNF-α cytokines, which may alleviate lung injury in VILI mice through the above mechanisms. MDSCs have been reported to skew the polarization of T cells towards the anti-inflammatory Th2 phenotypes and Th2 activation pathways, as well as impair the viability and activity of natural killer cells and dendritic cells [17,35,37]. Although the concept of differentially expressed genes in $CD4^+$ T cells of MDSCs-treated mice is interesting, it was a limitation of our study and warrants further investigation. Compared with other immune regulatory cells, iMDSCs have the ability to suppress the adaptive immune system, making them clinically advantageous. For instance, bone marrow precursor cells have a greater proliferation ability than lymphoid precursor cells, allowing for a large number of MDSCs to be produced *in vitro*. This study demonstrated that sufficient MDSCs could be generated from bone marrow hematopoietic stem cells or bone marrow precursor cells *in vitro*, indicating that MDSCS could be used to regulate immune response in VILI clinically. MDSCs have a relatively short life span because of their granulocyte-like properties [38,39]. Thus, they could greatly reduce the excessive immunosuppressive response of the body long-term after adoption without the addition of other immunomodulators. Moreover, MDSCs could promote immune tolerance by inducing Treg cell differentiation [40,41], thereby inhibiting the overactivation of immune state *in vivo*.

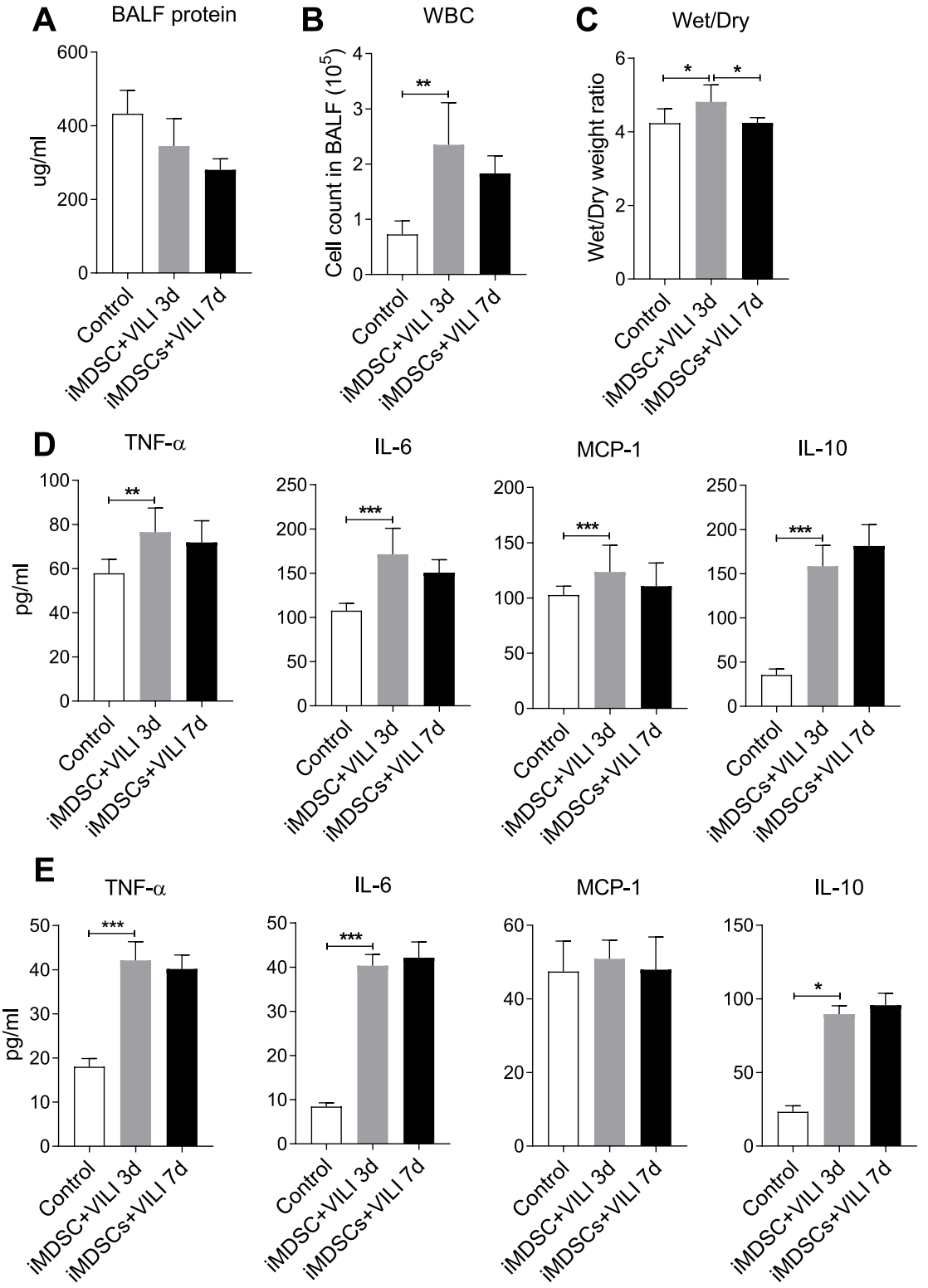

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Fig. 7. No secondary infections after iMDSCs adaption in VILI mice. (A) Protein concentration in BALF; (B) Cell counts of WBC in BALF; (C) Comparations of lung wet/dry weight ratio among the three groups; (D–E) Levels of inflammatory cytokines TNF-α, IL-6, MCP-1 and IL-10 in BALF (D) and circulation (E). *P*-values were determined by one-way analysis of variance on ranks was used for multiple comparisons flowed by Bonferroni's post hoc test. Data are presented as mean ± standard deviation. $P < 0.05$ was defined significant. n = 6, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Abbreviations: BALF, bronchoalveolar lavage fluid; IL-6, interleukin-6; IL-10, interleukin-10; iMDSCs, inflammation-induced myeloid-derived suppressor cells; MCP-1, monocyte chemotactic protein-1; TNF-α, tumor necrosis factor-α; VILI, ventilator-induced lung injure; WBC, white blood cells.

MDSCs are considered detrimental since they create an immunosuppressive environment in sepsis through inducing Arg-1 and increasing iNOS production [17]. The elevated activity of Arg-1 and iNOS leads to depletion of L-arginine deposits, which inhibits T-cell proliferation and function [42]. Our findings showed that the increased expressions of iNOS, Arg-1 and IL-10 might be the mechanisms by which MDSCs exert immunosuppression ability in VILI model. However, to confirm the anti-inflammatory effect through Arg-1, the selective Arg-1 inhibitor needs to be used in VILI mice model. Moreover, the dramatically elevated production of anti-inflammatory mediators in the treated mice of VILI might increase their susceptibility to secondary infection. Our data demonstrated that the inflammatory cytokines or BALF protein did not significantly increase in the 7 days after initial administration, indicating no susceptibility to the secondary infections. However, there may be secondary infections or immune imbalance with further adoptive periods, necessitating further investigation. In addition, the activity of MDSCs mainly depends on the environment [43], which means that the immunosuppressive activity and marker profile expressions of in vitro-generated MDSCs might have been changed after adoptive transfer into vivo, adjusting to the complex *in vivo* environment and leading to functional differences compared with endogenously induced MDSCs [44]. Nowadays, the combination of CD11b and Gr-1 markers to characterize MDSCs has been standardized in mice [43]. However, MDSCs are highly heterogenous, and the expression of certain markers largely depends on the disease state and the cell types, making MDSC characterization more complex [44]. Therefore, the in vitro-generated MDSCs might exhibited differences from in vivo-induced MDSCs; nonetheless, our results are consistent with previous evidence on murine MDSCs.

Cytokines or pharmacological compounds have been reported to promote the activation or generation of MDSCs *in vitro* or *in vivo* [45]. Recombinant granulocyte-macrophage colony-stimulating factor or granulocyte colony-stimulating factor has been demonstrated to increase leucocytes and MDSCs counts, resulting in the highest efficiency when producing MDSCs from CD34 human umbilical cord blood cells *in vitro* [46]. Additionally, IL-1 has been found to increase not only the numbers but also the functions of MDSCs in sepsis [47,48]. IL-1β has been shown to trigger the expansion of circulating granulocytic MDSCs subset that is dependent on Erk1/2 activation [49]. Furthermore, mammalian target of rapamycin inhibitors such as rapamycin could increase the numbers and activity of MDSCs in heart failure or graft-versus-host disease models [50,51]. Adoptive transfer of dexamethasone-induced MDSCs has been shown to prolonged heat allograft survival by increasing the levels of iNOS and Treg numbers [52]. Moreover, rapamycin-induced MDSCs could improve the outcomes of acute kidney injury, resulting in elevated Tregs counts and decreased inflammatory cytokines [53]. Therefore, more effective strategies for MDSCc expanding *in vitro* or *in vivo* need to be explored in the future research.

In the study, we demonstrated that adoptive transfer of iMDSCs could decrease inflammatory injury caused by high tidal volume in VLII. Although a traditional VILI model was used, there are limitations that need to be considered. In the animal model of tidal volume VILI created for this study, different ventilator parameters, such as tidal volume and PEEP, were not set. Nevertheless, different parameter settings have been shown to affect the degree of VILI in the lungs, without altering the mechanism of lung injury [54]. Additionally, a volume-controlled ventilation mode was utilized, rather than a pressure-controlled ventilation mode. The study mainly focused on early VILI, after continuous mechanical ventilation for 4h, and the duration of mechanical ventilation warrants further investigated. Further study is necessary to clarify whether the protective effect of iMDSCs on VILI is related to the above factors.

In summary, the findings of this study demonstrated that adoptive transfer of iMDSCs alleviates lung inflammatory injury caused by VILI, which may have potential clinical applications for the treatment of VILI.

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Ethics approval

This study was approved was approved by the animal ethics committee of China-Japan Friendship Hospital (NO.13009).

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Fangzhen Shan: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Writing – original draft. **Fenglian Tang:** Investigation, Data curation. **Yuan Liu:** Investigation, Data curation. **Xiao Han:** Investigation, Data curation. **Wei**

Wu: Data curation, Investigation. **Yanhua Tang:** Investigation. **Qingyuan Zhan:** Resources, Writing – review & editing. **Nannan Zhang:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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