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Regulatory B cells attenuate sepsis-associated pancreatic injury by regulating T cell homeostasis

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Sepsis is a common and life-threatening syndrome resulting from systemic and dysregulated immune response to severe infection, which contributes to morbidity and mortality in critically ill patients. This work aimed to evaluate the regulatory function of Breg cells in sepsis-associated pancreatic injury. We established mice model of sepsis-associated pancreatic injury by cecal ligation and puncture (CLP). Pancreatic injury was assessed by measuring the levels of amylase activity and histologic pancreatic injury scores. The proportions of Breg cells and T cell subsets were analyzed by flow cytometry, their secreted cytokines were detected by ELISA. The expressions of T-bet, RORγt and Foxp3 in spleen were determined by RT-PCR. The apoptosis of pancreatic cells was examined by LDH assay and Tunel, and the cell viability was detected by MTT assay. Compared to the sham group, a significantly lower percentage of Breg cells was observed in model mice. Anti-CD22 treatment exacerbated pancreatic injury, and significantly increased the percentages of Th1, Th17 cells along with the levels of IFN-γ, IL-17 in CLP-induced sepsis model, but did not affect the differentiation of Treg cells and expression of IL-10. Anti-CD22 administration promoted the expressions of T-bet and RORγt, but did not affect the Foxp3 expression. Adoptive transfer Breg cells remarkably alleviated pancreatic injury, and significantly decreased the percentages of Th1, Th17 cells along with the levels of IFN-γ, IL-17 and further promoted the percentage of Treg cells and expression of IL-10 in CLP-induced sepsis model. Moreover, adoptive transfer Breg cells inhibited the expressions of T-bet and RORγt, and promoted Foxp3 expression in model mice. Lipopolysaccharide (LPS) promoted the apoptosis in pancreatic acinar cells, which was inhibited after culturing with Breg cells in vitro. LPS remarkably upregulated the differentiation of Th1 and Th17 cells, and downregulated the differentiation of Treg cells, which could be significantly reversed by Breg cells in vitro. In conclusion, Breg cells may exhibit the protective effects by modulating T cell responses along with the cytokines in sepsis-associated pancreatic injury.

Keywords Breg cells, Sepsis, Pancreatic injury, T cell

Sepsis is an acute systemic dysregulated responses caused by pathogens and releasing toxins in the blood circulation. The host immune dysfunction of pro- and anti-inflammatory mechanisms is disturbed in sepsis and fails to return to normal homeostasis, which is associated with high mortality worldwide[1]. The symptoms of sepsis range from mild infection to septic shock, disseminated intravascular coagulation and multiple organ failure[2]. The prognosis of sepsis depends to a large extent on the host's immune status, because extreme inflammation and immune dysfunction are the main reasons for the failure of pathogen clearance[3]. Organ dysfunction in sepsis is the consequence of tissue aggression mediated by the inflammation and causative pathogen and no organ is exempt from sepsis induced injury and dysfunction[4]. Although the most common dysfunction in sepsis implies respiratory, cardiovascular, gastrointestinal, renal, neuronal and metabolic involvement[5], the pancreas is also vulnerable to injury and may go unnoticed in its all stages of sepsis[6].

Due to the dysfunction of T cell subsets, including T helper 1 cell (Th1), Th17 and regulatory T cell (Treg), immune dysfunction is usually accompanied with the progression of sepsis[7]. Th1 cells secrete pro-inflammatory cytokine interferon-γ (IFN-γ) and Th17 cells release their related cytokines interleukin-17 (IL-17) to participate in inflammation and multiple organ failure in sepsis model[8]. Moreover, Th1 and Th17 cells are increased in patients with sepsis, and Th17 cell proportion correlates with higher inflammation level, severity of disease and unfavorable prognosis[9]. A previous study demonstrated that Treg cells played an important role in immune response of sepsis due to the increase of functional enhancement and number after the onset of sepsis or septic

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shock[10]. The activation of Treg cells mediated suppression of immune response could inhibit the host's excessive inflammation by activating TCR signaling pathway, making Treg cells closely related to pathogenesis of sepsis[11]. Our recent study showed the important role of Th1, Th17 and Treg responses in sepsis-associated pancreatic injury and baicalin was capable of ameliorating sepsis-associated pancreatic injury by suppressing Th1 and Th17 responses and promoting Treg response[12].

Besides these classical $CD4^+$ T cells, other cells also have immunosuppressant function, such as regulatory B cell (Breg). The major surface markers of regulatory B cells are $CD19^+CD5^+CD1d^{hi}$. $CD19^+$ B lymphocytes could predict organ failure during the early phase of acute pancreatitis[13]. Mesenteric adipose tissue B lymphocytes aggravated local inflammation in acute pancreatitis and circulating B cells were associated with the severity of acute pancreatitis[14,15]. Taken together, these evidences potently suggested a crucial role of B cells in pancreatic diseases. In the study by Tao et al., the decreased numbers and impaired function of Breg cells were observed in endotoxic shock mice and involved in the development and immune dysfunction in septic shock[16]. A recent study showed Breg cells attenuated cardiac inflammation by regulating Th1 and Th17 cells in acute viral myocarditis induced by coxsackie virus B3[17]. Moreover, Breg cells is crucial for the suppression of Th1/Th17 responses, induction of Treg cells and reduction of collagen-induced arthritis[18].

Based on the important role of Breg cells and Th1, Th17 and Treg responses in sepsis, we investigated the role and regulatory function of Breg cells on Th1, Th17 and Treg responses to further confirm the precise function of Breg cells involved in the pathogenesis of sepsis-associated pancreatic injury.

Materials and methods

Mice and treatment

Male C57BL/6 mice weighing 23–26 g (8–10 weeks of age) were purchased from animal experiment center of Xiangya Medical College (Changsha, China). All the experiments adhered strictly to the ARRIVE guidelines for the Care and Use of Laboratory Animals. This animal study consists of two parts as follows. Thirty-two mice were randomly divided into four groups (n = 8): sham group, sham + anti-CD22 group, model group, and model + anti-CD22 group. Another 24 mice were randomly divided into three groups (n = 8): sham group, model group, and model + AT Breg group (adoptive transfer of Breg cells). Sepsis-associated pancreatic injury mouse models were constructed by cecal ligation and puncture (CLP) according to our recent report[12]. Following CLP surgery, mice in model + AT Breg group were administered intravenously through tail vein with 1×10^6 Breg cells daily. Mice in sham + anti-CD22 group and model + anti-CD22 group were intraperitoneally injected 300 μg anti-CD22 antibody (Sangon Biotech, China) daily. Mice in sham group and model group were received the same dose of normal saline. All mice were housed in a pathogen-free barrier facility with free access to water ad libitum and regular chow diet under a 12 h light–dark cycle. Three days later, all the mice were sacrificed with sodium pentobarbital to collect spleen, blood samples and pancreatic tissues.

Ethical approval

Mice experiments were approved by the ethics committee of the Hunan Children's Hospital (KYSQ2021-194), accordance with the Basel Declaration.

Serum amylase measurement

The grading of pancreatic injury was assessed by serum amylase activity. The activity of serum amylase was determined with the corresponding assay kit (Sigma-Aldrich) according to the manufacturer's protocols.

Histopathological examination

Hematoxylin and eosin (H&E) staining analysis was to assess pancreatic pathological changes. The pancreatic tissues were collected and fixed with 4% paraformaldehyde for 24 h at room temperature. Subsequently, the tissues were dehydrated in ethanol, embedded in paraffin and sliced at about 4-μm sections. The tissue sections were then stained with hematoxylin and eosin solution at 37 °C according to manufacturer's protocol. Pancreatic histological injury was observed under a light microscope and scored according to the method described previously.

Flow cytometric analysis

Briefly, the spleen tissues were collected, washed and homogenized. Individual lymphocytes were separated by using lymphocyte separation solution. For the detection of Breg cells, cells were stained with PEcy7-conjugated CD19, PE- conjugated CD1d and PerCP-CY5.5-conjugated CD5. For Th1 cell staining, APC-conjugated CD3, FITC-conjugated CD4 and PE-conjugated IFN-γ antibodies were used. For identification of Th17 cells, APC-conjugated CD3, FITC-conjugated CD4 and PE-conjugated IL-17 antibodies were used. The cells stained with APC-conjugated CD25, FITC-conjugated CD4 and PE-conjugated Foxp3 for detecting Treg cells. $CD19^+CD5^+CD1d^{hi}$ cells were recognized as the Breg cells. The cells labeled $CD3^+CD4^+IFN\text{-}\gamma^+$ represent Th1 and $CD3^+CD4^+IL\text{-}17^+$ represent Th17 cell. $CD4^+CD25^+Foxp3^+$ cells were recognized as the Treg cells. All samples were detected by flow cytometer (FC500, Beckman, USA) and results were analysed with FlowJo 7.6.1 software (Tree Star, USA). Total protein from spleen of each group.

RT-PCR

Real-time quantitative PCR was detected to quantify mRNA levels of the T-bet, RORγt and Foxp3 according to our previous study. RNA was extracted from the spleen of each group with the Trizol reagent (Invitrogen, USA) and the purity was analyzed by spectrophotometer. RT-qPCR was performed by using MasterMix (SYBR Green) (Roche, Switzerland). The sequences of the primer pairs used for amplification were listed as follows. All data were normalized to β-actin and expressed as a relative ratio. Primer pairs sequences used as follows.

T-bet: 5′-AAGTTTAATCAGCACCAGACAG-3′, 5′-AGACCACGTCCACAAACATCCA-3′;
RORγt: 5′-CTCCCTGGATGAATAGAATGGC-3′, 5′-GCAGAGGCAGAAAATGTAAAGG-3′;
Foxp3: 5′-CAATGAAGACCCTGATAGCCAG-3′, 5′-CTTGCTGGATGAGAACAGAATC-3′.

ELISA

IFN-γ, IL-17 and IL-10 in cell culture medium and serum were measured by using commercially murine ELISA kits (R&D, USA) and the OD values were detected with microplate reader (MD SpectraMax M3, USA), and the concentrations of the above cytokines were calculated.

Primary pancreatic acinar cell isolation and culturing

The primary pancreatic acinar cells (PACs) from C57BL/6 mice were isolated by using a specific collagenase digestion procedure as previously described[19]. In brief, the pancreas was meticulously dissected and rinsed thrice with PBS. Subsequently, collagenase IV (200 U/mL) was gently infused into the pancreas. The sample was then subjected to a 37 °C water bath for digestion period. Following digestion, the sample was immersed in an extracellular solution with a composition of 140 mM NaCl, 1.13 mM MgCl2, 4.7 mM KCl, 10 mM D-glucose, 1 mM CaCl2 and 10 mM HEPES, maintaining a pH of 7.30. The digested sample was subjected to repetitive pipetting in this solution. The resultant suspension underwent sterile filtration through a cell strainer. Subsequent to a mild centrifugation at 700 rpm for 2 min. The cells were maintained in a 37 °C incubator with 5% carbon dioxide and plated in 6-well cluster dishes with a density of 1×10^5 cells/ml. This PACs study consists of four groups as follows. (1) PACs group, (2) PACs + LPS (10 μg/ml), (3) PACs + LPS (10 μg/ml) + Breg (10^6/ml), (4) PACs + LPS (10 μg/ml) + Breg (LPS) (10^6/ml, cultured with 10 μg/ml LPS for 24 h). PACs in each group were fostered for 24 h.

Cell viability assay

The viability of PACs was performed by MTT assay. Briefly, PACs were planted in 96-well plate at a density of 1×10^4 cells/well. Following treatment and culture of the cells for 24 h, 100 μl of MTT solution (Beyotime, China) was added to each well and incubated for 4 h with 5% CO_2 at 37 °C. Then, the OD value was measured with the enzyme-labeled instrument (Thermo Fisher Scientific, USA).

LDH assay

Cell death of PACs was detected by lactate dehydrogenase (LDH) release. The concentrations of LDH of the supernatants in cultured cells were measured with the corresponding cytotoxicity kit (Promega, USA) according to the manufacturer's instructions. Cell death was quantitated via measuring the lactate dehydrogenase activity in the supernatants at the end of the incubation period.

TUNEL assay

Pancreatic acinar cell apoptosis was detected by using a TdT-mediated dUTP nick end labeling (TUNEL) assay following the manufacturer's instructions (Beyotime Institute of Biotechnology). Briefly, the cells were rinsed with PBS and fixed with 4% paraformaldehyde. Then, the cells were incubated with TdT reaction cocktail followed by treatment of Click-iT reaction cocktail as instructed. Cell nuclei were stained with DAPI.

Breg cells and CD4⁺ T cell isolation

$CD19^+$ B cells were magnetically isolated and stimulated with 10 μg/ml LPS for 24 h, and $CD19^+CD5^+CD1d^{hi}$ Breg cells were sorted by flow cytometry according to previously described methodology[16]. The naïve $CD4^+$ T cells were isolated from spleen by using immunomagnetic beads (MACS beads, USA). The naïve $CD4^+$ T cells were added anti-IL-4 (10 μg/ml) and IL-12 (5 ng/ml) for inducing Th1 differentiation. The cells were cultured for Th17 differentiation with anti-IL-4 (5 mg/ml), IL-6 (20 ng/ml), TGF-β (1 ng/ml) and anti-IFN-γ (10 μg/ml), and anti-IL-4 (5 mg/ml) and TGF-β (5 ng/ml) for Treg differentiation. This naïve $CD4^+$ T cells study consists of three parts as follows. Part I: (1) $CD4^+$ T cells (Th1 polarization, 10^6/ml), (2) $CD4^+$ T cells (Th1 polarization, 10^6/ml) + LPS (10 μg/ml), (3) $CD4^+$T cells (Th1 polarization, 10^6/ml) + LPS (10 μg/ml) + Breg (10^6/ml), (4) $CD4^+$ T cells (Th1 polarization, 10^6/ml) + LPS (10 μg/ml) + Breg (LPS) (10^6/ml, cultured with 10 μg/ml LPS for 24 h). Part II: (1) $CD4^+$ T cells (Th17 polarization, 10^6/ml), (2) $CD4^+$ T cells (Th17 polarization, 10^6/ml) + LPS (10 μg/ml), (3) $CD4^+$ T cells (Th17 polarization, 10^6/ml) + LPS (10 μg/ml) + Breg (10^6/ml), (4) $CD4^+$ T cells (Th17 polarization, 10^6/ml) + LPS (10 μg/ml) + Breg (LPS) (10^6/ml, cultured with 10 μg/ml LPS for 24 h). Part III: (1) $CD4^+$ T cells (Treg polarization, 10^6/ml), (2) $CD4^+$ T cells (Treg polarization, 10^6/ml) + LPS (10 μg/ml), (3) $CD4^+$ T cells (Treg polarization, 10^6/ml) + LPS (10 μg/ml) + Breg (10^6/ml), (4) $CD4^+$ T cells (Treg polarization, 10^6/ml) + LPS (10 μg/ml) + Breg (LPS) (10^6/ml, cultured with 10 μg/ml LPS for 24 h). Each well was fostered for 24 h.

Statistical analysis

All the data processing were accomplished by using SPSS version 26.0 statistical software. All the results were expressed as mean ± standard deviation. Comparisons among multiple groups were evaluated by One-way analysis of variance (ANOVA) and the Student's t-test was selected for two group comparisons. A value of $P < 0.05$ was considered statistically significant.

Results

Insufficient Breg cells exacerbated pancreatic injury and dysregulated T cell responses in CLP-induced sepsis model

Firstly, CLP induced pancreatic injury, characterized by the pathological injury of edema, hyperemia, vacuolization and necrosis. However, anti-CD22 treatment exacerbated pancreatic injury in CLP-induced sepsis model (Fig. 1A, B). The amylase activity and pancreas weight/body weight were increased in the model group, which further promoted by anti-CD22 (Fig. 1C, D). We investigated the percentages of $CD19^{+}CD5^{+}CD1d^{hi}$ Breg cells subpopulation in the circulating blood, lung, spleen and bone marrow. Intriguingly, the percentages of $CD19^{+}CD5^{+}CD1d^{hi}$ Breg cells of circulating blood (Fig. 2A, B), lung (Fig. 2C, D), spleen (Fig. 2E, F) and bone marrow (Fig. 2G, H) were much lower in the model mice than in the control mice (Representative dot plots depict the gating strategy for Breg cells is shown in Supplementary Figure S1A). Furthermore, we performed flow cytometry to further evaluate whether insufficient Breg cells were related to T cell responses. Flow cytometry analyses showed the percentages of Th1, Th17 and Treg cells increased significantly compared with those in the sham group. Anti-CD22 treatment significantly increased the percentage of Th1 (Fig. 2I, J) and Th17 cells (Fig. 2K, L) in CLP-induced sepsis model (Representative dot plots depict the gating strategy for Th1 and Th17 cells is shown in Supplementary Figure S1B, C). However, there were no statistical differences of Treg cells in model mice and model mice treated with anti-CD22 (Fig. 2M, N) (Representative dot plots depict the gating strategy for Treg cells is shown in Supplementary Figure S1D). It has been proved that activated T-bet, RORγt and Foxp3 were the key transcription factors of Th1, Th17 and Treg cells, respectively. In this study, the expressions of T-bet, RORγt and Foxp3 were significantly increased in the model group, and anti-CD22 administration promoted the expressions of T-bet and RORγt, but there were no statistical differences of Foxp3 expression in model mice and model mice with anti-CD22 treatment (Fig. 3A–C). Consistent with these changes, the cytokines of IFN-γ, IL-17 and IL-10 in serum of model group were significantly increased, while anti-CD22 administration promoted the expressions of IFN-γ and IL-17, but did not affect the expression of IL-10 (Fig. 3D–F). These results suggested that insufficient Breg cells exacerbated pancreatic injury and dysregulated T cell responses in CLP-induced sepsis model.

Adoptive transfer of Breg cells alleviated pancreatic injury and restored the dysregulated T cell responses

As shown in Fig. 4A, B, HE staining showed the intact architecture in the sham group, while significant pathological injury including edema, hyperemia, vacuolization and necrosis exhibited in the model group, which remarkably alleviated after adoptive transferring of Breg cells. The amylase activity and pancreas weight/body weight were increased in the model group, which could be inhibited by adoptive transferring of Breg cells (Fig. 4C, D). Moreover, our results showed the percentages of Th1, Th17 and Treg cells increased significantly compared with those in the sham group, adoptive transfer Breg cells significantly decreased the percentages

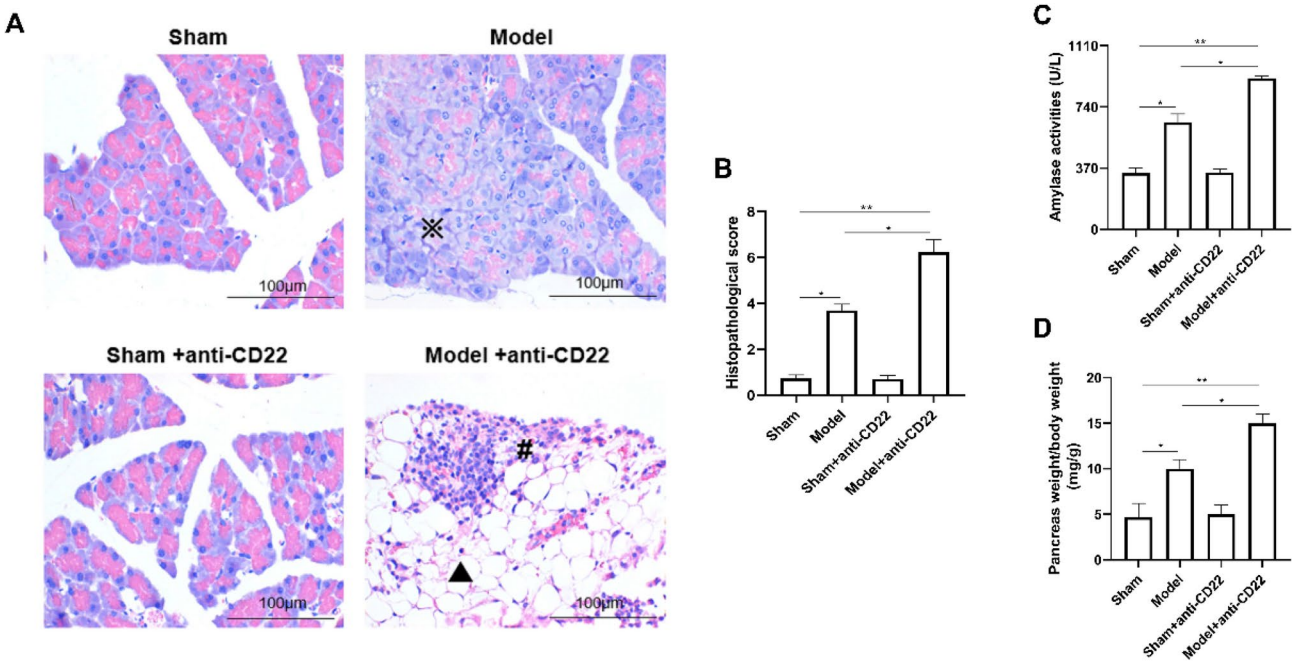

Fig. 1. Insufficient Breg cells exacerbated pancreatic injury in CLP-induced sepsis model. (**A**) Representative HE pathological staining of pancreatic tissues (orginal magnification × 400). (※, representing edema), (▲, representing vacuolization), (#, representing necrosis) (**B**) The histopathological scores of pancreatic injury. (**C**) Amylase activity in the serum was detected. (**D**) pancreas weight relative to body weight. Values were means ± SD, n = no. of animals. $^{*}P < 0.05$, $^{**}P < 0.01$.

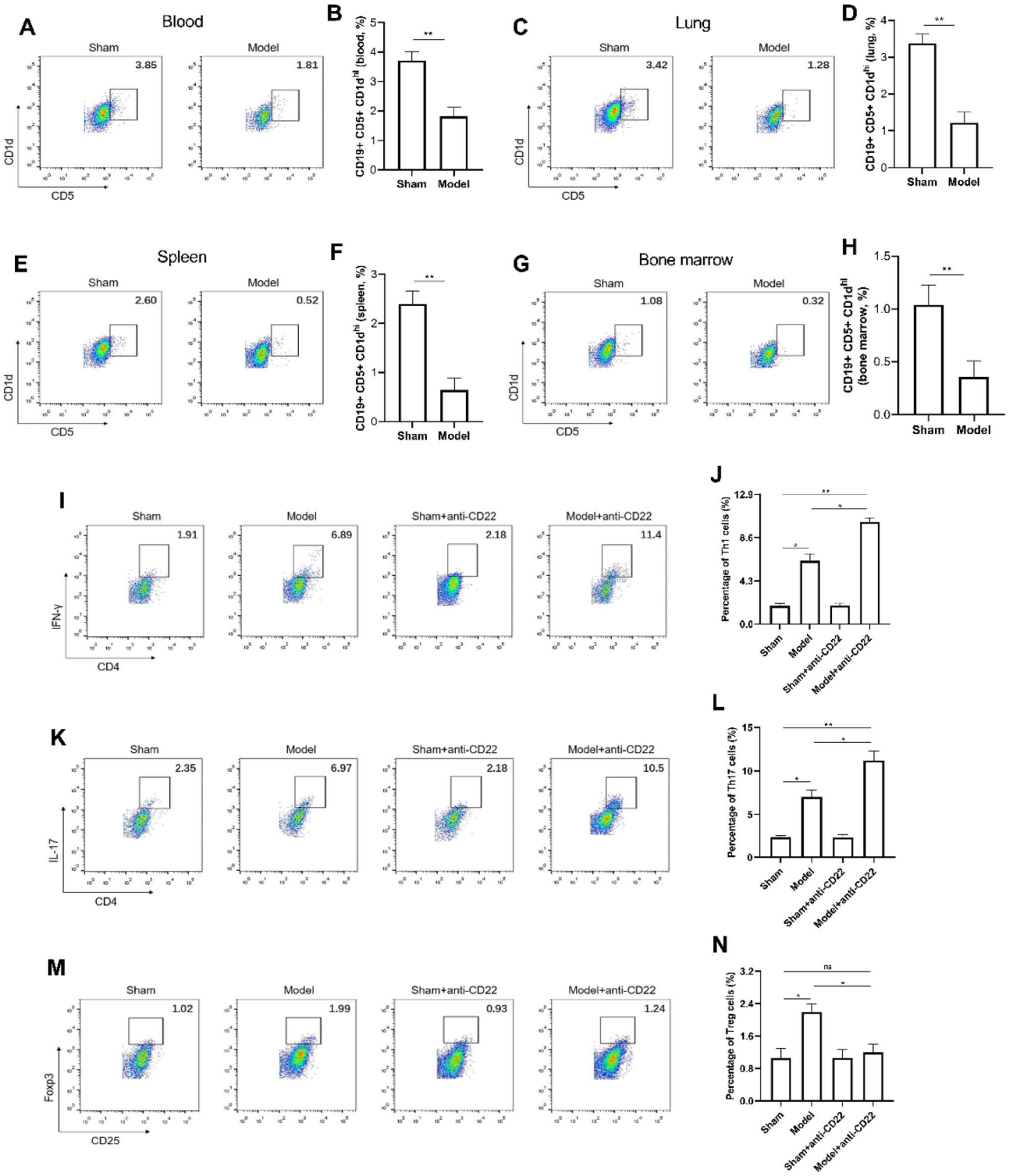

Fig. 2. Insufficient Breg cells regulated the Th1, Th17 and Treg immune responses during sepsis-associated pancreatic injury. $CD19^{+}CD5^{+}CD1d^{hi}$ Breg cell subpopulation in the circulating blood (**A**), lung (**C**), spleen (**E**) and bone marrow (**G**). (**B, D, F, H**) Percentage of Breg cells was analyzed by histogram. (**I**), (**K**), (**M**) Percentages of Th1, Th17 and Treg cells in the spleen. (**J**), (**L**), (**N**) Percentages of Th1, Th17 and Treg cells were analyzed by histogram. Values were means ± SD, n = no. of animals. $^{*}P < 0.05$, $^{**}P < 0.01$.

of Th1 (Fig. 5A, B) and Th17 cells (Fig. 5C, D) and further promoted the percentage of Treg cells in CLP-induced sepsis model (Fig. 5E, F). Consistent with these changes, the expressions of T-bet, RORγt and Foxp3 were significantly increased in the model group, and adoptive transfer Breg cells inhibited the expressions of T-bet and RORγt, and promoted Foxp3 expression in model mice (Fig. 6A–C). At the same time, the expression

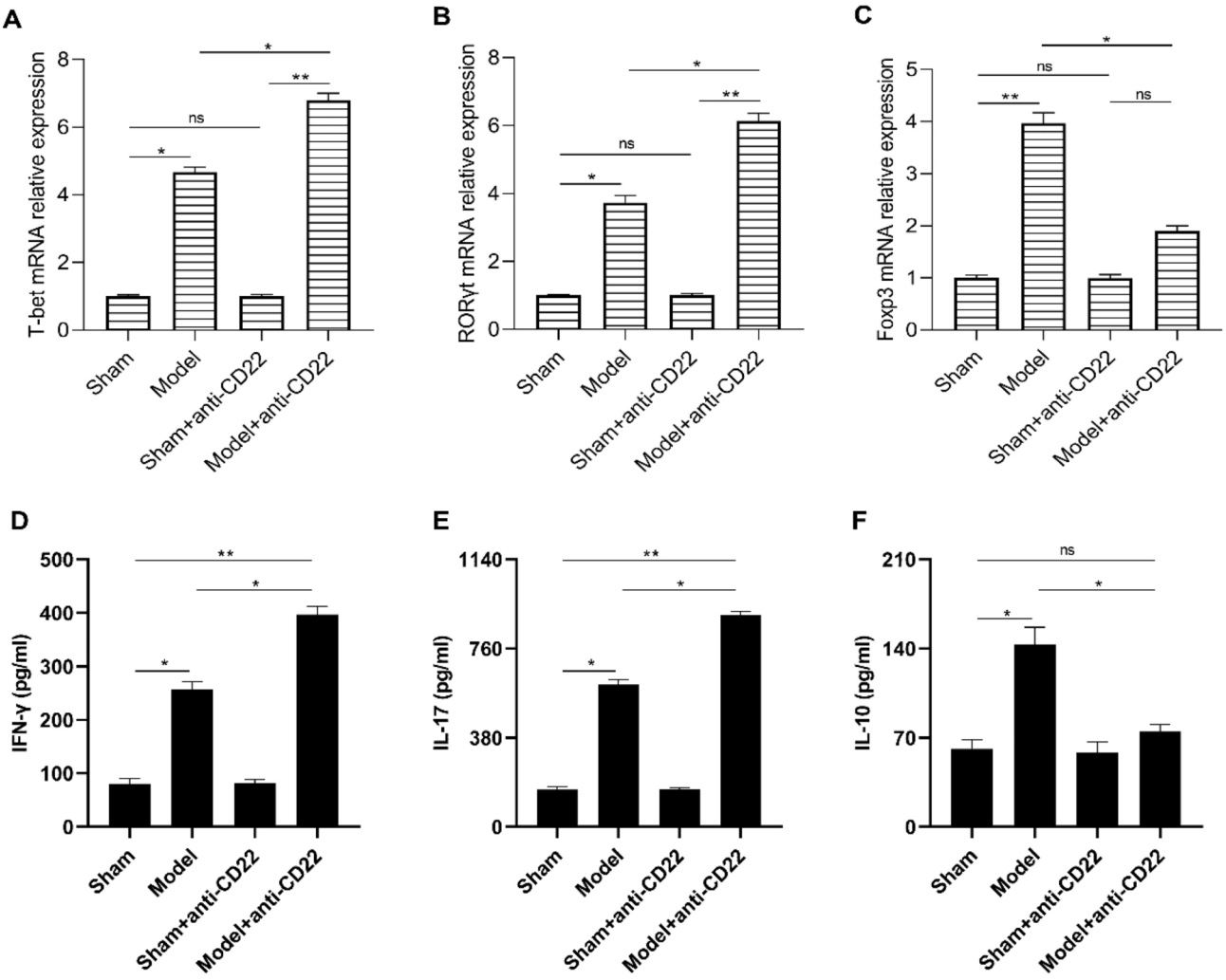

Fig. 3. Insufficient Breg cells regulated the expressions of T-bet, RORγt, Foxp3 and cytokines during sepsis-associated pancreatic injury. (**A**), (**B**), (**C**) Expressions of T-bet, RORγt and Foxp3 in the spleen analyzed by RT-PCR. (**D**), (**E**), (**F**) Serum IFN-γ, IL-17 and IL-10 were analyzed by ELISA. Values were means ± SD, n = no. of animals. *$P < 0.05$, ** $P < 0.01$.

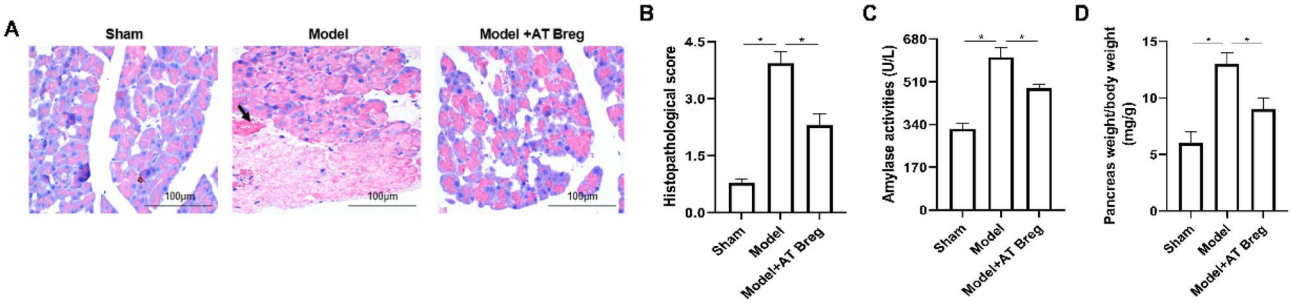

Fig. 4. Adoptive transfer of Breg cells alleviated pancreatic injury in CLP-induced sepsis model. (**A**) Representative HE pathological staining of pancreatic tissues (orginal magnification × 400). (arrow, representing hyperemia). (**B**) The histopathological scores of pancreatic injury. (**C**) Amylase activity in the serum was detected. (**D**) pancreas weight relative to body weight. Values were means ± SD, n = no. of animals. *$P < 0.05$.

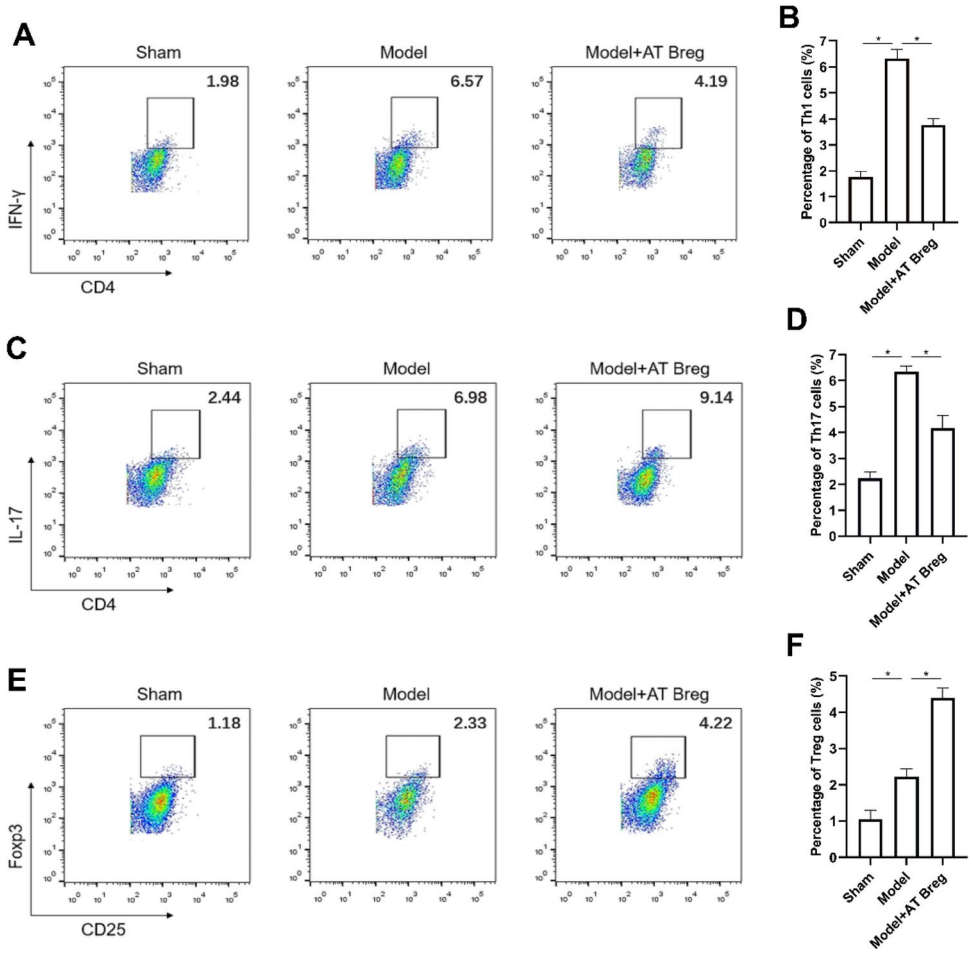

Fig. 5. Adoptive transfer of Breg cells regulated the Th1, Th17 and Treg immune responses during sepsis-associated pancreatic injury. (**A**) (**C**) (**E**) Percentages of Th1, Th17 and Treg cells in the spleen. (**B**) (**D**) (**F**) Percentages of Th1, Th17 and Treg cells were analyzed by histogram. Values were means ± SD, n = no. of animals. $^{*}P < 0.05$.

levels of IFN-γ, IL-17 and IL-10 in serum of model group were significantly increased, while adoptive transfer Breg cells inhibited the expressions of IFN-γ and IL-17, but promoted the expression of IL-10 (Fig. 6D–F). These results suggested that adoptive transfer of Breg cells alleviated pancreatic injury and and restored the dysregulated T cell responses in model mice induced by CLP.

Effect of Breg cells on apoptosis of pancreatic acinar cells in vitro

In coculture, LPS promoted the apoptosis in pancreatic acinar cells, which was inhibited after culturing with Breg cells in vitro (Fig. 7A, B). In order to investigate if Breg cells enhanced the cell viability of LPS-stimulated pancreatic acinar cells, MTT method was used to detect the cell viability, the results showed the cell viability was significantly decreased after co-incubating with LPS. However, Breg cells remarkedly increased the cell viability (Fig. 7C). Then, we detected LDH activity in the culture medium by using the LDH kit assay. A significant increase of LDH release was observed in the LPS treatment group, and co-incubated with Breg cells decreased the LDH activity (Fig. 7D). There were no statistically differences between PACs + LPS + Breg group and PACs + LPS + Breg (LPS) group in terms of cell apoptosis, cell viability and LDH. These results indicated that the effects of Breg cells on reducing the apoptosis of pancreatic acinar cells in vitro.

Breg cells regulated dysregulated T cell responses in vitro

To determine the effect of Breg cells on T cell responses in vitro. The coculture systems of $CD4^+$ T cells (Th1, Th17, Treg polarization) and LPS treated with/without Breg cells in vitro were constructed. In the co-culture system, LPS remarkably upregulated the differentiation of Th1 cells and expression of IFN-γ (Fig. 8 A-C), differentiation of Th17 cells and expression of IL-17 (Fig. 8D–F), and downregulated the differentiation of Treg cells and expression of IL-10 (Fig. 8G–I), which could be significantly reversed by Breg cells. There were no statistically differences in $CD4^+$ T cells differentiation and cytokine levels between $CD4^+$ T cells + LPS + Breg group and $CD4^+$ T cells + LPS + Breg (LPS) group. These results further demonstrated that the effect of Breg cells on dysregulated T cell responses in vitro.

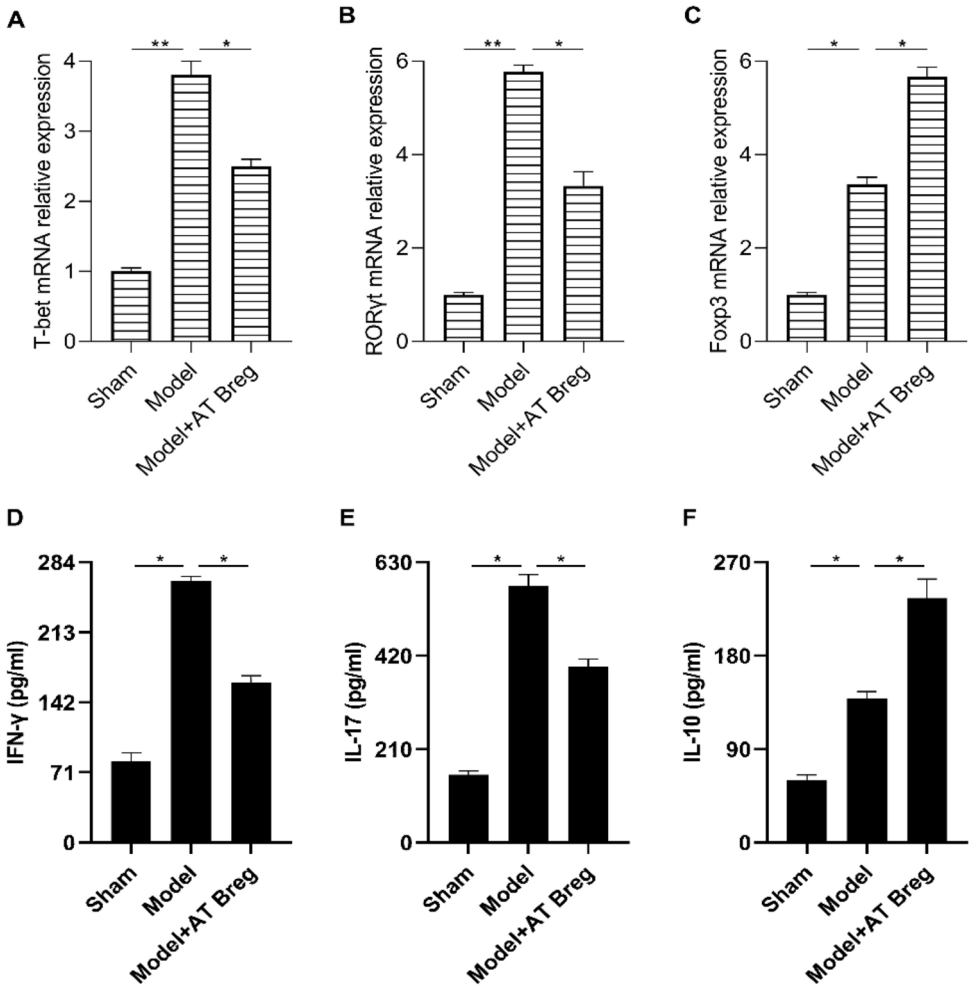

Fig. 6. Adoptive transfer of Breg cells regulated the expressions of T-bet, RORγt, Foxp3 and cytokines during sepsis-associated pancreatic injury. (**A**), (**B**), (**C**) Expressions of T-bet, RORγt and Foxp3 in the spleen analyzed by RT-PCR. (**D**), (**E**), (**F**) Serum IFN-γ, IL-17 and IL-10 were analyzed by ELISA. Values were means ± SD, n = no. of animals. $^{*}P < 0.05$, $^{**}P < 0.01$.

Discussion

Sepsis arises from systemic dysregulated responses caused by infection, which leads to high incidence and mortality. Sepsis is significantly associated with multiple organ failure, and the pancreas is particularly vulnerable. Sepsis-associated pancreatic injury is common, which increases the mortality in critically ill patients. In our recent clinical study, acute pancreatic injury could occur at early stage of sepsis, which with high incidence in sepsis and septic shock patients. Moreover, the level of serum amylase was significantly associated with the incidence of pancreatic injury, which could serve as a significant biomarker to predict pancreatic injury in critically ill children[20]. In this study, our results showed the intact architecture exhibited in the sham group, while significant pathological injury including edema, hyperemia, vacuolization and necrosis exhibited in the model group. At the same time, the amylase activity was significantly increased in the model group, which is an important biomarker used to detect pancreatic injury[21]. However, the pathogenesis of sepsis-associated pancreatic injury remains unclear.

It is well known that deregulation of the immune homeostasis played the key role in the pathogenesis of multiple organ failure in sepsis. Recently, B cell subpopulation could either prompt or attenuate sepsis and septic shock, and the regulatory properties of B cells have attracted increasing attention[22]. Breg cells, a subpopulation of immunosuppressive B cells, as the negative regulator of the immune diseases, could protect from infection and negatively regulate immune responses. A previous study by Li and colleagues demonstrated that the frequency of Breg cells was significantly increased in peripheral blood of neonatal sepsis and contributed to the immunoprotective function of the disease[23]. Similarly, another study also showed the elevation of Breg cells could regulate immune function and may participate in the pathogenesis of neonatal sepsis[24]. In other published data, there were opposite variations in Breg cells in septic patients and models. In elderly patients with sepsis, the percentage of Breg cells was reported to be decreased and negatively correlated with acute physiology and chronic health evaluation II score. Furthermore, Breg cells in response to lipopolysaccharide was dramatically decreased in severe septic shock model mice, and adoptive transfer of Breg cells could protect against severe

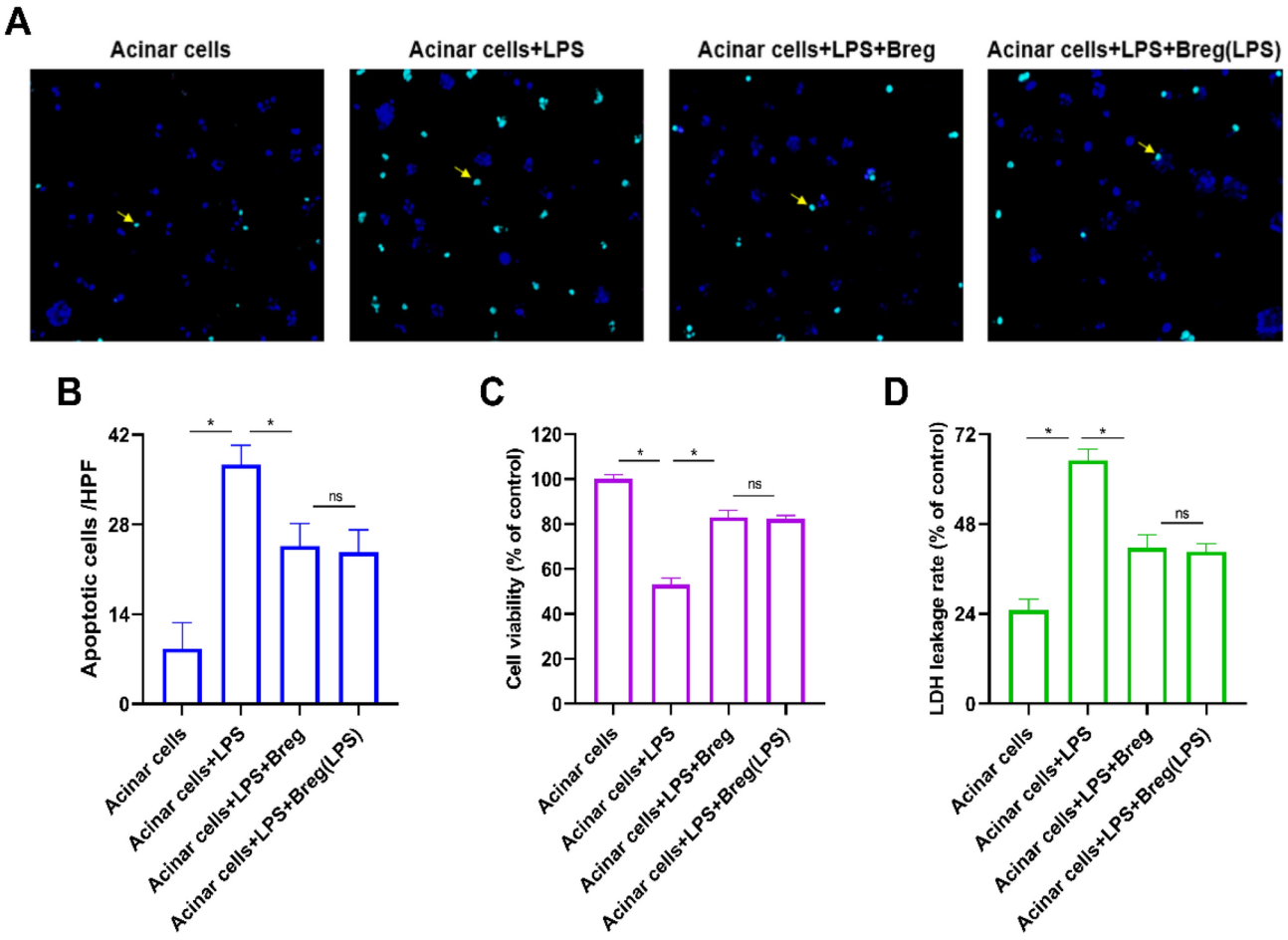

Fig. 7. Breg cells inhibited the apoptosis of pancreatic acinar cells in vitro. (**A**) Apoptotic cells were examined by Tunel. (**B**) Apoptotic cells were quantified by histogram (high power fields at × 400 magnification). (**C**) Cell viability was measured using the MTT assay. (**D**) Cell death was detected by LDH release assay. Values were means ± SD, n = 6. *$P < 0.05$.

septic shock in vivo[16]. Our findings suggested that a significantly lower percentage of $CD19^+CD5^+CD1d^{hi}$ Breg cells was observed in model mice compared with sham mice. Therefore, these results indicated that Breg cells may exhibit diverse responses in sepsis of septic shock in accordance with different disease grades and were involved in the immune disturbance of the disease. Pancreatic injury may be associated with the severity of sepsis and impaired number and function of Breg cells.

T-lymphocyte dysfunction is the most important pathophysiologic feature of immune disturbance in sepsis or septic shock, especially subpopulation of $CD4^+$ T lymphocyte is confirmed to be related to the severity of disease. $CD4^+$ T lymphocyte plays the key role in the development of humoral and cellular immune responses in sepsis. To investigate the impacts of sepsis on $CD4^+$ T lymphocyte is crucial for informing efforts to develop treatments and restore immune dysfunction in sepsis[25–27]. It has also been demonstrated that dysregulated Th1 cell is involved in methicillin-resistant staphylococcus aureus-induced sepsis, pneumonia-induced sepsis and experimental sepsis model of mice[28–30]. Numerous studies have reported the vital role of Th17 cells in the development of sepsis. For example, Luo et al. found treatment with mesenchymal stem cells could protect against sepsis-associated acute kidney injury by inhibiting Th17 cells[31]. Additionally, Taxifolin ameliorated sepsis-induced lung capillary leak by regulating the balance between Th17 and Treg cells[32]. Intravenous calcitriol treatment could elicit more-balanced Th17 subsets and alleviate sepsis-associated intestinal inflammation and injury in sepsis[27]. Our data also revealed that the percentages of Th1 and Th17 cells increased significantly in model group compared with those in the sham group. In the co-culture system, LPS remarkably upregulated the differentiation of Th1 and Th17 cells in vitro. These results suggested that targeting of Th1 and Th17 responses might be a potential strategy for the treatment of sepsis-associated pancreatic injury.

Over the past 10 years, Treg cells have been an important topic of focus in different stages of sepsis research. Several previous studies have shown that the number and function of Treg cells in sepsis. For instance, sepsis caused a significant increase in number and suppressive function of Treg cells in the early stage of sepsis[33]. Furthermore, Treg cells contributed to the positive prognosis in the early stage of sepsis[34]. In the study by Qiu et al., berberine treatment promoted the percentage of Treg cells and exerted the protective effect on sepsis-associated intestinal injury[35]. Consistently, In our study, the results showed that the pancreatic injury model

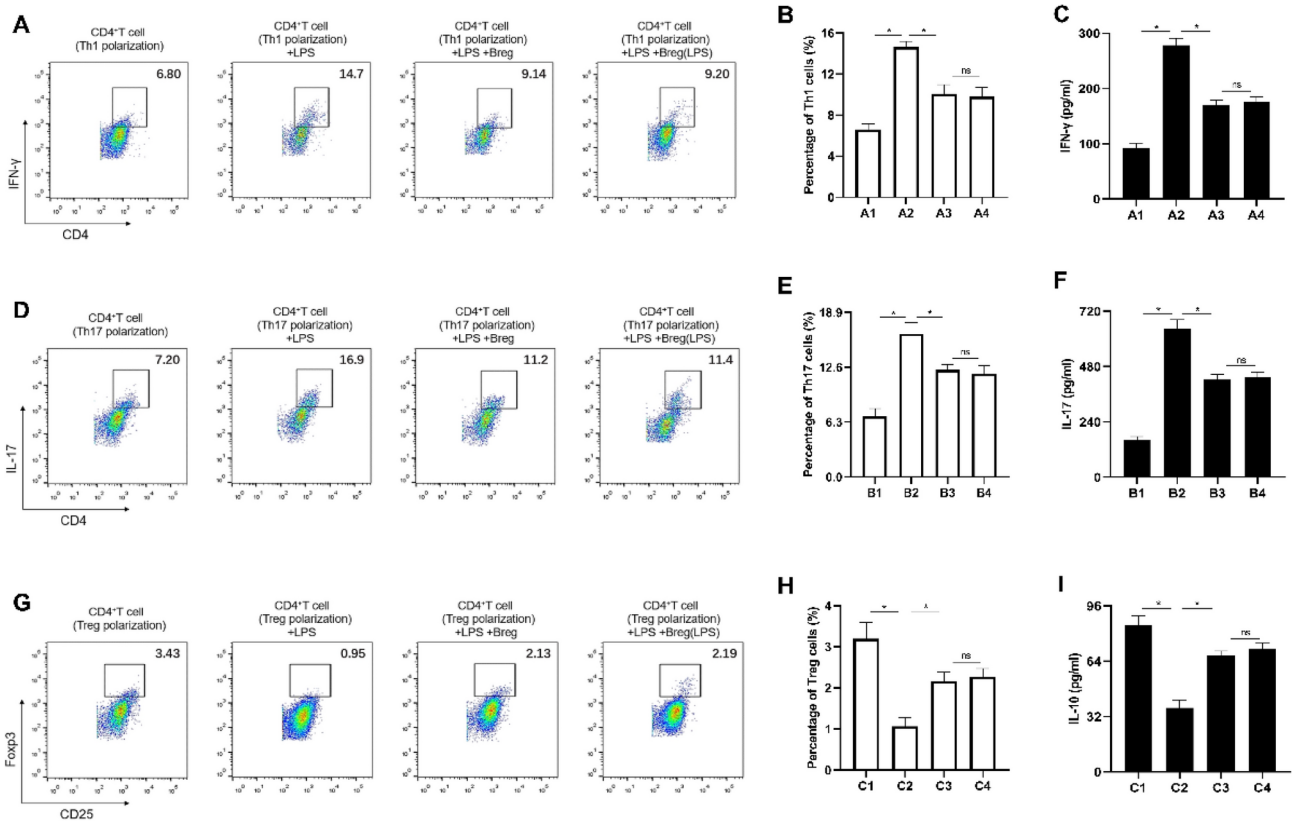

Fig. 8. Breg cells regulated the Th1, Th17 and Treg immune responses in vitro. (**A**), (**D**), (**G**) Percentages of Th1, Th17 and Treg cells in the spleen. (**B**), (**E**), (**H**) Percentages of Th1, Th17 and Treg cells were analyzed by histogram. (**C**), (**F**), (**I**) IFN-γ, IL-17 and IL-10 in cell culture medium were analyzed by ELISA. Values were means ± SD, n = 6. $^{*}P < 0.05$.

induced by CLP caused a obvious increase of Treg cells in spleen, while the differentiation of Treg cells was remarkably downregulated by LPS in vitro, which indicated that Treg cells may exhibit the anti-inflammatory and protective effects in sepsis-associated pancreatic injury in vivo.

Breg cells as a potential regulator exhibited extensive impacts on multiple immune cells in immune responses. Recent studies have shown the key role of Breg cells in the regulation of T cells differentiation. For instance, Breg cells took part in modulating local immune responses and alleviating inflammatory injury in periodontitis by downregulating Th17 cells and upregulating Treg cells[36]. Similarly, Breg cells played a crucial role in regulating of Th1/Th17 cells responses in pneumocystis infection[37]. In addition, Breg cells with regulatory function may prevent the development of rheumatoid arthritis by maintaining Treg cells and limiting Th1 and Th17 cells responses[38]. To clarify the immunoregulatory effect of Breg cells on T cells responses, a Breg cell-deficiency model and model with adoptive transfer of Breg cells were generated. In published data, anti-CD22 antibody preferentially depleted Bregs in mice, and this approach has been used in numerous studies[39,40]. Our data demonstrated that anti-CD22 treatment significantly increased the percentages of Th1 and Th17 cells in CLP-induced sepsis model, but did not affect the differentiation of Treg cells. Thus, we speculated that the depletion of Breg cells further expanded the pro-inflammatory effect of Th1 and Th17 cell responses, but lost the regulatory function on the anti-inflammatory effect of Treg cell response. However, in the present study, adoptive transfer Breg cells significantly decreased the percentage of Th1 and Th17 cells and further promoted the percentage of Treg cells in CLP-induced sepsis model. In vitro, Breg cells could inhibit the apoptosis in pancreatic acinar cells induced by LPS, downregulate the differentiation of Th1 and Th17 cells, and upregulate the differentiation of Treg cells, which indicated that Breg cells might protect pancreatic injury via expanding Treg cell response and restraining Th1 and Th17 cell responses. These results elucidated the protective roles of Breg cells in sepsis-associated pancreatic injury.

It is known that the transcription factor T-bet, RORγt and Foxp3 play the key roles in the Th1, Th17 and Treg lineage commitment, respectively[41,42]. Th1 cells primarily produce IFN-γ and IL-17 is mainly produced by Th17 cells. IL-10 is produced from Treg cells which demonstrated anti-inflammatory effect[43–45]. In this current research, we also found the expressions of T-bet, RORγt and Foxp3 were significantly increased in the model group, and anti-CD22 administration promoted the expressions of T-bet and RORγt, but did not affect the expression of Foxp3. Adoptive transfer Breg cells inhibited the expressions of T-bet and RORγt, and promoted Foxp3 expression in model mice. The expressions of cytokines IFN-γ, IL-17 and IL-10 were consistent with those

of transcription factors. Therefore, our findings demonstrated that Breg cells modulated T cell responses along with the levels of associated cytokines by regulating the transcription factors.

In summary, we undertook this research that decreased proportion and impaired function Breg cells were observed in sepsis-associated pancreatic injury mice. Breg cells may exhibit protective effects by modulating T cell responses along with the levels of associated cytokines in sepsis-associated pancreatic injury mice. Our study also provided new clues to understand the function of Breg cells and raised the possibility of promising target for the treatment of sepsis-associated pancreatic injury.

Data availability

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Received: 24 June 2024; Accepted: 15 April 2025
Published online: 25 April 2025

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Acknowledgements

This work was supported by grant from the Natural Science Foundation of Hunan Province (No. 2022JJ30319), Hunan Provincial Key Laboratory of Emergency Medicine for Children (No. 2018TP1028), Hunan Provincial Health Commission Grant (No. 202106010519).

Author contributions

PPL, ZHX, and XLL designed the study and contributed to discussion. PPL wrote the manuscript and ZHX reviewed and edited the manuscript. PPL and ZHX were the guarantor of this work and the accuracy of the data analysis. PPL, XPZ, and JTH collected and researched data. PPL and ZHX conducted experiments and measured the experimental results. All authors read and approved the final manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-98884-2.

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