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ORIGINAL ARTICLE

Aquaporin 4 Blockade Attenuates Acute Lung Injury Through Inhibition of Th17 Cell Proliferation in Mice

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Abstract— Acute lung injury (ALI) is a syndrome characterized by damage to the alveolar-capillary wall, pulmonary edema and recruitment of inflammatory cells. Previous studies have indicated that aquaporin 4 (AQP4) plays a key role in brain edema formation and resolution. However, the role of AQP4 in the development and progression of ALI is not clear and needs to be resolved. In our current study, mouse ALI was induced by intratracheal instillation of lipopolysaccharide (LPS) at a concentration of 30 mg/kg. For the inhibition of AQP4, 200 mg/kg of TGN-020 (Sigma, USA) was administered intraperitoneally every 6 h starting at 30 min before intratracheal instillation of LPS. The results of the present work indicate, for the first time, that mice treated with the AQP4 inhibitor TGN-020 had attenuated LPS-induced lung injury, reduced proinflammatory cytokine release (including IL-1α, IL-1β, IL-6, TNF-α, IL-23, and IL-17A), and an improved survival rate. Additionally, we found that the attenuated lung injury scores, increased survival rate, and decreased BALF total protein concentration in TGN-020-treated mice were all abrogated by rIL-17A administration. Furthermore, TGN-020 treatment downregulated the phosphorylation of PI3K and Akt, increased the expression of SOCS3, and decreased the expression of p-STAT3 and RORγt. In conclusion, inhibition of AQP4 by TGN-020 has a detectable protective effect against lung tissue injury induced by LPS, and this effect is associated with inhibition of IL-17A through the downregulation of the PI3K/Akt signaling pathway and upregulation of SOCS3 protein.

KEY WORDS: aquaporin 4; acute lung injury; TGN-020; Th17 cells; PI3K/Akt; SOCS3.

INTRODUCTION

Acute lung injury (ALI) is a syndrome characterized by damage to the alveolar-capillary wall (ACW), pulmonary edema, and recruitment of inflammatory cells [1, 2]. When ALI occurs, the lung endothelium is damaged, leading to high permeability of the lung capillaries and clinical pulmonary edema [3, 4]. To date, many studies have investigated the treatment of ALI. However, the results also need to be further investigated.

Aquaporins (AQPs) are a family of membrane water-transporting proteins [5]. Recent investigations have indicated that AQPs contribute to several clinical diseases [5, 6]. AQP4 is a member of a family of bidirectional, high-capacity water channels [7]. AQP4 is expressed in the foot processes of astrocytes, skeletal muscle, and epithelial cells in the lung, gastrointestinal organs, and kidney [8]. Recent reports have indicated that the upregulated expression of AQP4 was associated with the formation and elimination of edema [9]. Previous studies have shown an important role for AQP4 in brain and lung edema formation [10, 11]. Li Y et al. showed that AQP4 inhibition alleviated the

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development and severity of irradiated lung damage. This was associated with attenuated infiltration of inflammatory cells, decreased production of proinflammatory cytokines, and inhibited activation of M2 macrophages [12]. However, the role of AQP4 in the development and resolution of pulmonary edema needs to be further investigated.

Th17 cells are proinflammatory effector T cells that can produce large amounts of interleukin 17 (IL-17), IL-6, and other cytokines [13]. IL-6 appears to be the major cytokine associated with AQP4 autoimmunity. The PI3K/Akt signaling pathway is an important signaling pathway for the regulation of cell survival, growth, and proliferation and has an important role in inflammatory diseases [14]. In our current study, we showed that mice with intratracheal instillation of lipopolysaccharide (LPS) exhibited increased lung injury scores accompanied by lung edema. The expression of AQP4 was significantly upregulated in the lung tissues of these mice. AQP4 inhibition by TGN-020 attenuated LPS-induced lung injury, reduced proinflammatory cytokine release (including IL-1α, IL-1β, IL-6, TNF-α, IL-23 and IL-17A), and improved survival rates in mice. Additionally, the attenuated lung injury scores, prolonged survival rate, and decreased BALF total protein concentration in TGN-020-treated mice were all abrogated by additional rIL-17A administration. Furthermore, TGN-020 treatment downregulated the phosphorylation of PI3K and Akt, increased the expression of SOCS3, and decreased the expression of p-STAT3 and RORγt.

MATERIALS AND METHODS

Animals and Experimental Protocols

Male C57BL/6 mice and BALB/c mice (8–10 weeks old, weight 25–30 g) were from the Center of Experimental Animals of Hubei Province Academy of Traditional Chinese Medicine. All mice were housed in a specific pathogen-free facility with regular food and water *ad libitum*. Experiments were approved by the Institutional Animal Care and Use Committee at Hubei Province Academy of Traditional Chinese Medicine. Intratracheal instillation of LPS (Sigma, St. Louis, MO, USA) was performed to induce mouse ALI. LPS was dissolved in 40 μl of PBS at a concentration of 30 mg/kg [15]. The control group mice were given 40 μl of PBS after endotracheal intubation. For inhibition of AQP4, 200 mg/kg of TGN-020 (Sigma, USA) was administered by i.p. injection every 6 h starting at 30 min before intratracheal instillation of LPS [16]. For inhibition of endogenous IL-17A *in vivo*, 0.2 mg anti-IL-17A blocking antibody (BioLegend, San Diego, CA, USA) was injected intravenously (i.v.) 1 h before the mice were instilled [17]. To study the role of IL-17A, mice were injected i.v. with 1 μg of recombinant mouse IL17A (rIL-17A) diluted in PBS containing 0.1% albumin 5 min prior to instillation [18]. For inhibition of the PI3K/Akt signaling pathway, wortmannin (0.2 μM, 1 μl) dissolved in saline was injected intravenously (i.v.) 1 h before the mice were instilled [19]. After 12, 24, or 48 h, the mice were sacrificed, and the lungs were removed and immediately stored at − 80 °C until being assayed.

Histological Staining

HE staining was performed, and the lung injury score was calculated as previously published [20]. The severity of the lung injury was quantified by adding up the individual scores of each category. For immunohistochemical staining for RORγt, 5-μm sections of cryostat-frozen tissue were applied to poly-L-lysine microscope slides and fixed with cold acetone. Endogenous peroxides were blocked with 3% H_2O_2 in PBS (pH 7.6) for 15 min. To prevent nonspecific binding of primary antibodies, the sections were incubated with 1% BSA in PBS for 60 min. Then, the sections were incubated overnight (at 4 °C) with primary Ab specific for RORγt (Abcam, 1:300). Next, the sections were washed and incubated with biotinylated IgG (Abcam) (at 37 °C) for 15 min following reaction with avidin–biotin–peroxidase complex (Sigma) for 15 min. Subsequently, the sections were stained with DAB (ZSGB-BIO) for 5 min, followed by hematoxylin staining for 2 min.

Observation of Lung Edema

At 12 h, 24 h, and 48 h post intratracheal instillation of LPS, the lung tissues were immediately weighed and wet weight determined. To determine the dry weight, the lung tissues were dried in a 90 °C oven for 24 h and then weighed. Next, we calculated the wet/dry weight ratio [11].

Western Blotting

Mice were sacrificed at 12 h, 24 h, or 48 h post instillation, and the protein levels of AQP4, p-PI3K, p-Akt, p-STAT3, SOCS3, and RORγt were examined by Western blotting analysis as previously published [21].

Immunofluorescent Staining

Immunofluorescent staining for APQ4 in the lung tissues was performed as previously described [19]. The

number of positively stained cells from three fields of each section was quantitatively analyzed. Three independent experiments were performed.

Quantitative Real-Time PCR

Total RNA was isolated from the lung tissues using a Qiagen Mini Kit (Qiagen, Hilden, Germany). The integrity of isolated RNA was verified by analytical agarose gel electrophoresis. All PCRs were performed using an ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA). The ΔΔCt method was used to quantify the data, with β-actin as a reference gene.

Collection of Bronchoalveolar Lavage Fluid

For the collection of bronchoalveolar lavage fluid (BALF), the left lungs were obtained, and the airways were washed three times with 2 ml of PBS. BALF was harvested and centrifuged at 1500 rpm for 10 min at 4 °C. The supernatant was harvested and prepared for further analysis.

ELISA and MPO Measurement

IL-1α, IL-1β, IL-6, IL-23, IL-17A, and TNF-α ELISA kits and MPO assay kits were obtained from Beijing Yuanda Company (Beijing, China). The cytokine levels in the BALF and the MPO activity in the lung tissue were measured according to the manufacturer's instructions.

Data Analysis

All values are expressed as arithmetic means ± SEM. Data were evaluated with GraphPad Prism version 5.04 software. Differences were evaluated using unpaired Student's *t* test between two groups and one-way ANOVA for multiple comparisons, followed by the Newman–Keuls test. The survival curves were compared using the log-rank (Mantel–Cox) test. P values < 0.05 were considered statistically significant.

RESULTS

LPS Instillation Induced Lung Injury and Increased AQP4 Expression

The pulmonary edema of mice after LPS instillation was demonstrated by HE staining. The results indicated that there was congestion, interstitial edema, and neutrophil invasion in the lung tissues in the mice with ALI compared to the sham group mice (Fig. 1a). The lung injury scores and the lung wet/dry weight ratio were also markedly increased in the ALI group at 12 h, 24 h, and 48 h post LPS instillation compared with the sham group (Fig. 1b, c). After LPS instillation, the AQP4 protein levels in lung tissues were markedly increased compared with those in the sham group lung tissues (Fig. 1d). Immunofluorescence staining of AQP4 in lung tissues also indicated that the number of positive cells in the ALI group was significantly increased when compared with the lung tissues of the sham group at 12 h, 24 h, and 48 h post LPS instillation (Fig. 1e).

AQP4 Blockade Ameliorated ALI and Improved the Survival Rate

To investigate the role of AQP4 in ALI, AQP4 was inhibited with TGN-020. The results showed that the lung tissues from mice treated with TGN-020 exhibited less severe alveolar wall collapse and decreased inflammatory cell infiltration when compared to lung tissues in control mice. The lung injury scores and wet/dry weight ratio were also significantly decreased in the TGN-020-treated group at 12 h, 24 h, and 48 h after LPS instillation compared to the control group (Fig. 2a–c). In addition, the survival rate of TGN-020-treated mice was higher than that of the control mice (Fig. 2d).

AQP4 Blockade Decreased Inflammatory Cytokine Production and Neutrophil Infiltration in ALI

ALI is accompanied by the release of proinflammatory cytokines [22, 23]. Therefore, the protein levels of proinflammatory cytokines, including TNF-α, IL-1α, IL-6, IL-1β, IL-23, and IL-17A, were measured in the BALF. As shown in Fig. 3, when compared with the control mice, the levels of IL-1α, IL-1β, IL-6, IL-23, and IL-17A were markedly decreased in TGN-020-treated mice at 12 h, 24 h, and 48 h after LPS instillation. The TNF-α levels were significantly decreased by TGN-020 treatment at both 24 h and 48 h post intratracheal instillation of LPS. The ALI process was also accompanied by neutrophil infiltration into the lung tissues, which resulted in pulmonary protein leakage and then increased microvascular permeability in the lung tissues [24, 25]. In our current study, the TGN-020-treated mice exhibited a marked reduction in BALF neutrophil infiltration and tissue MPO activity at 12 h, 24 h, and 48 h after LPS instillation

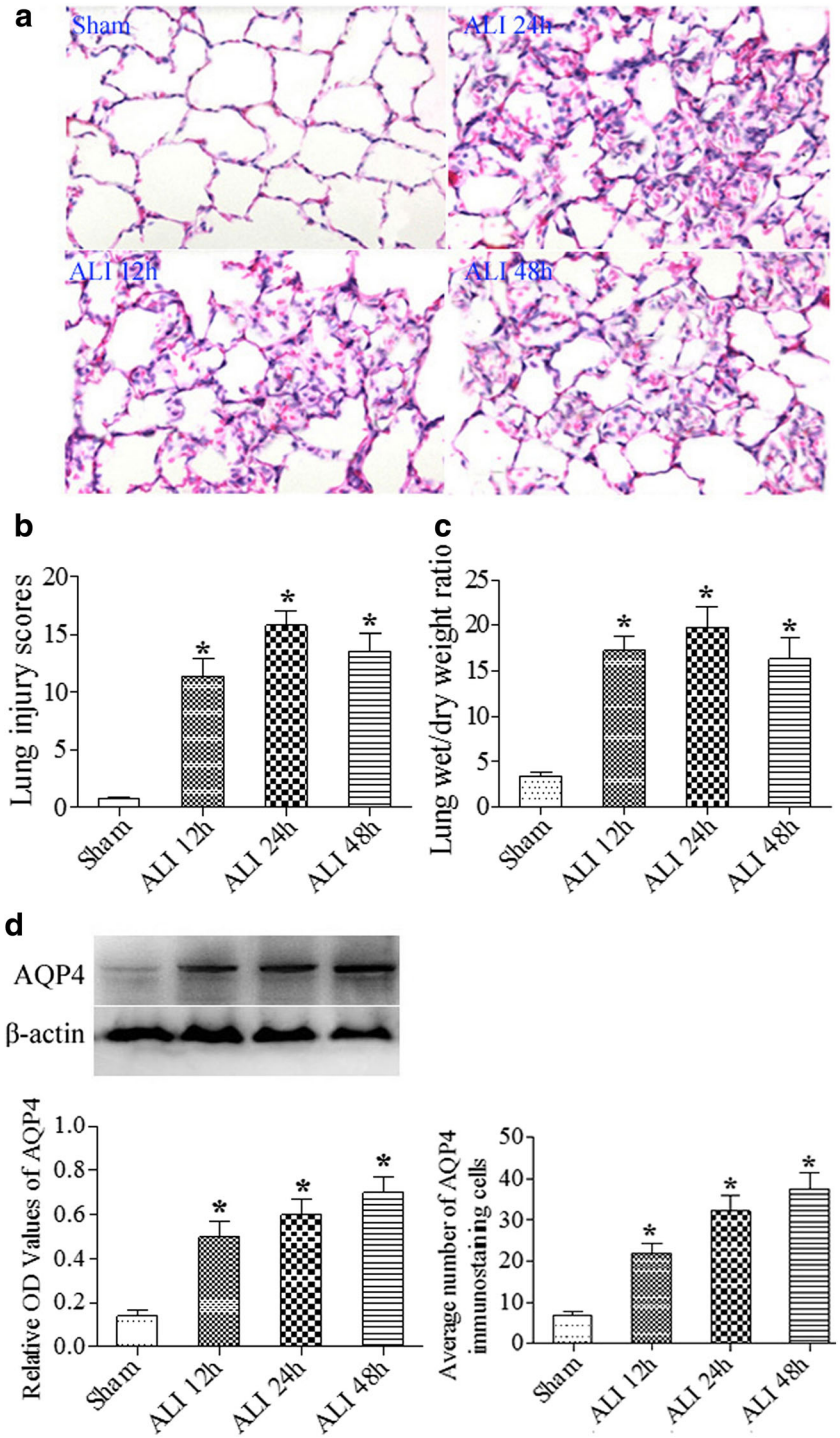

Fig. 1. Lung edema and morphology after LPS instillation. **a** HE staining for lung morphology in the sham and ALI groups (12 h, 24 h, 48 h ALI). **b** Lung injury scores of mice in the sham and ALI groups. **c** The lung wet/dry weight ratio in the sham and ALI groups. **d** The expression level of AQP4 protein. **e** Quantitative analysis of AQP4-positive cells. The assays were performed in triplicate on samples from each animal. The data are presented as the mean ± SEM ($N = 6$ animals in each group). $^*P < 0.05$.

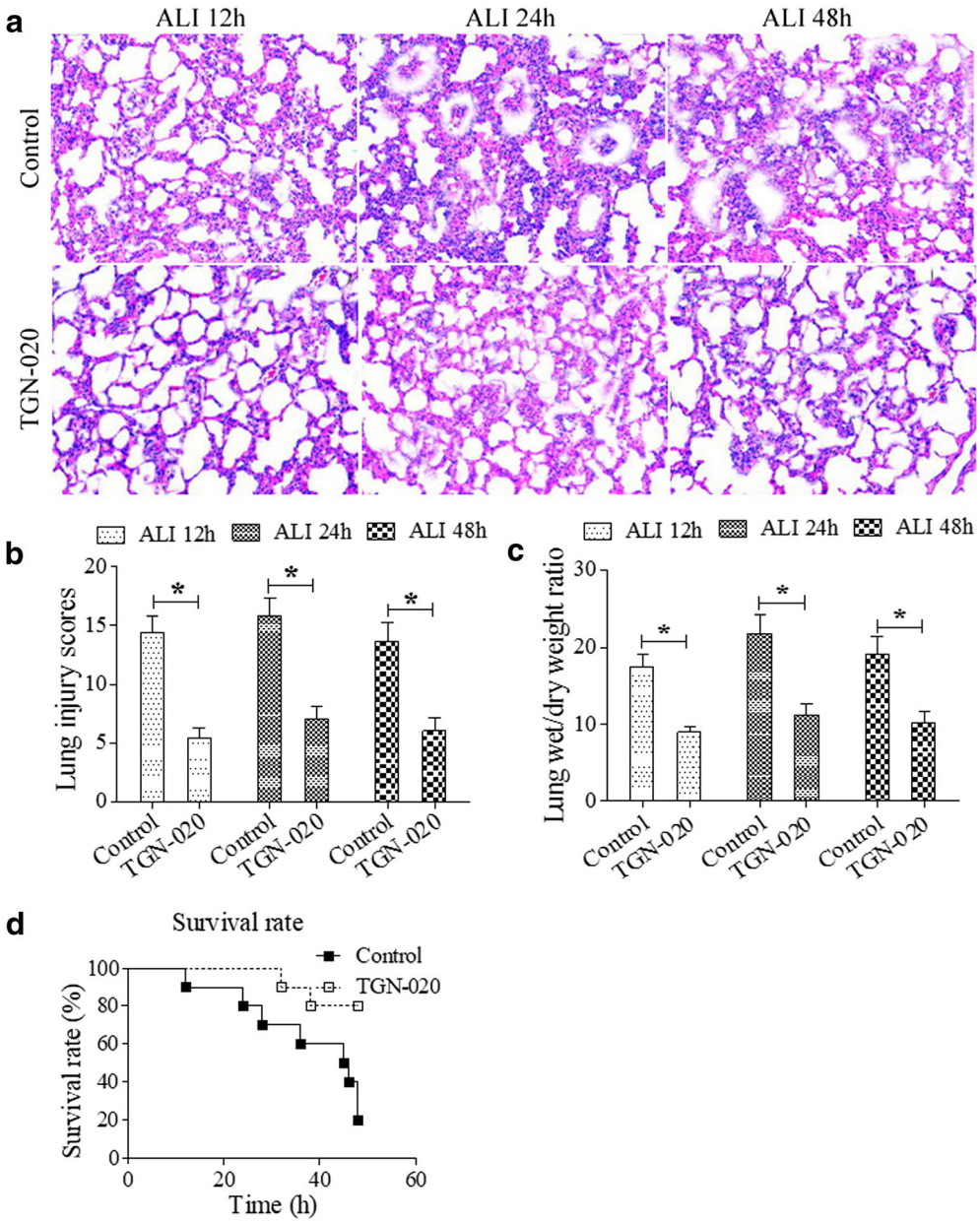

Fig. 2. AQP4 blockade ameliorated ALI and improved the survival rate. **a** HE staining for lung morphology in the control and TGN-020 groups (12 h, 24 h, 48 h). **b** Lung injury scores of mice in the control and TGN-020 groups (12 h, 24 h, 48 h). **c** The lung wet/dry weight ratio in the control and TGN-020 groups (12 h, 24 h, 48 h). **d** The survival rate of TGN-020-treated mice and control mice ($N = 10$ in each group). The assays were performed in triplicate on samples from each animal. The data are presented as the mean ± SEM ($N = 6$ animals in each group). *$P < 0.05$.

compared with control mice (Fig. 4a, b). TGN-020-treated mice also showed a significant reduction in the BALF total protein concentration at 12 h, 24 h, and 48 h compared to that of the control mice (Fig. 4c).

AQP4 Blockade Inhibited Th17 Cells and Was Associated with Ameliorated Lung Injury

Blockade of AQP4 with TGN-020 inhibited IL-17A expression post LPS instillation in our current study.

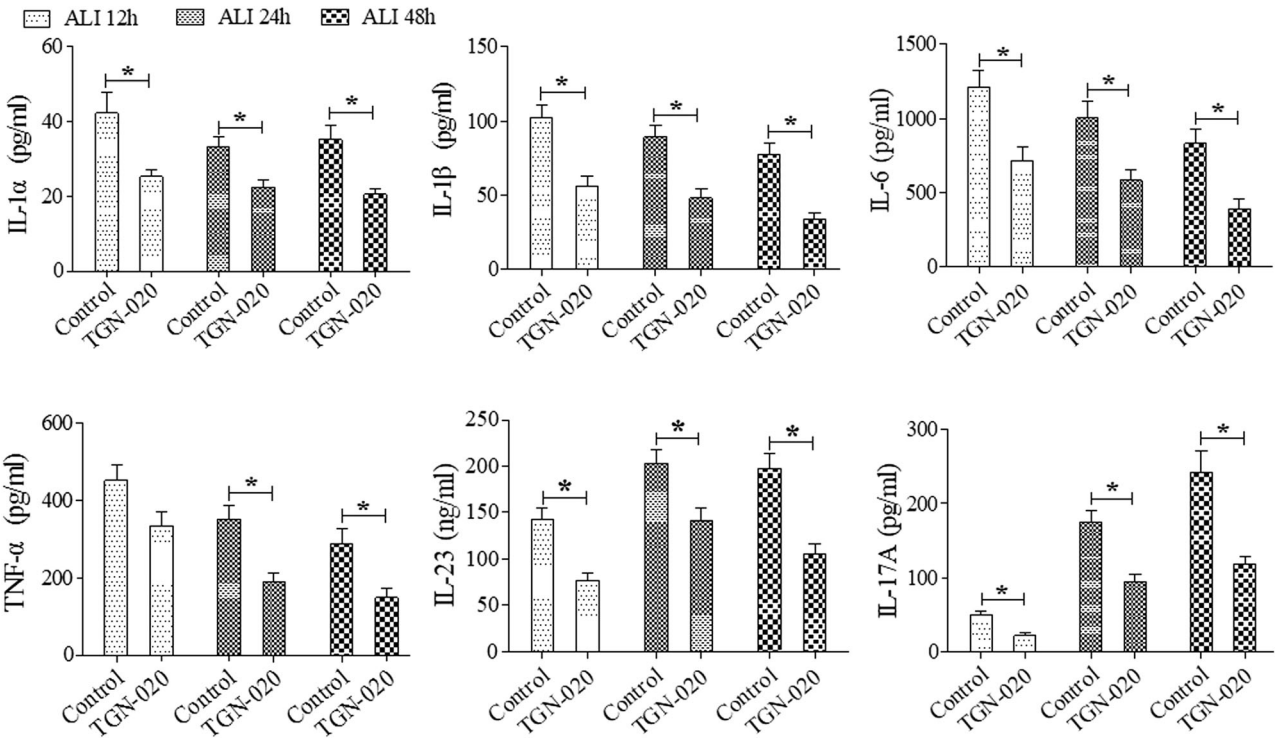

Fig. 3. AQP4 blockade decreased the levels of proinflammatory cytokines and Hmgb1 in ALI. The concentrations of IL-1α, IL-1β, IL-6, TNF-α, IL-23, and IL-17A are shown. The assays were performed in triplicate on samples from each animal ($N = 6$ animals in each group). The data are presented as the mean ± SEM. $^*P < 0.05$.

Previous studies have shown that ALI is accompanied by a high level of Th17 cell activation and proliferation [26, 27]. Therefore, we detected the expression of RORγt, which is the specific nuclear transcription factor for Th17 cells. In the present study, both the gene and protein levels of RORγt were markedly decreased in the lung and spleen tissues from TGN-020-treated mice when compared with those from the control mice at 12 h,

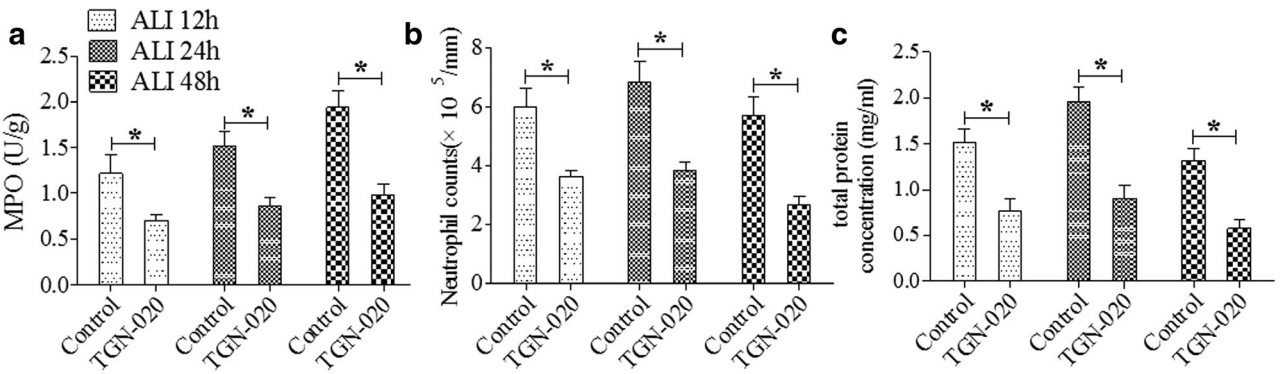

Fig. 4. AQP4 blockade reduced the tissue MPO activity, neutrophil counts, and total protein concentration in ALI. **a** MPO activity in lung tissue. **b** The neutrophil counts in the BALF. **c** The total protein concentration in the BALF. The assays were performed in triplicate on samples from each animal ($N = 6$ animals in each group). The data are presented as the mean ± SEM. $^*P < 0.05$.

24 h, and 48 h post LPS instillation (Fig. 5a, b). The immunohistochemical analysis also indicated that there were fewer RORγt-positive cells in lung tissues from mice treated with TGN-020 (Fig. 5c). Next, we evaluated the role of IL-17A in the ameliorated lung injury induced by AQP4 blockade. We found that the lung injury scores were markedly decreased with inhibition of IL-17A (Fig. 5d). The addition of recombinant IL-17A (rIL-17A) increased the lung injury score, and the decreased lung injury scores induced by TGN-020 were reversed by the addition of rIL-17A (Fig. 5d). The BALF total protein concentration was significantly decreased in the anti-IL-17A group and markedly increased in the rIL-17A group. Additionally, the decreased BALF total protein concentration induced by TGN-020 was upregulated by additional administration of rIL-17A (Fig. 5e). The increased survival rate in TGN-020-treated mice was also abrogated by rIL-17A administration (Fig. 5f).

AQP4 Blockade Impaired Th17 Differentiation but Not Through the Upregulation of Treg Cells

Several studies have highlighted the interaction between Th17 cells and Treg cells in ALI [28–30]. Our current results are not consistent with previous reports, as Treg cell proliferation was not effectively amplified by TGN-020 treatment *in vivo* and *in vitro*. The Foxp3 levels in lung tissues from mice treated with TGN-020 were significantly decreased compared with those from the control group (Fig. 6a, b). In the mixed lymphocyte reaction, both the mRNA and protein levels of Foxp3 were lower in the TGN-020-treated group than in the LPS-treated group (Fig. 6c, d).

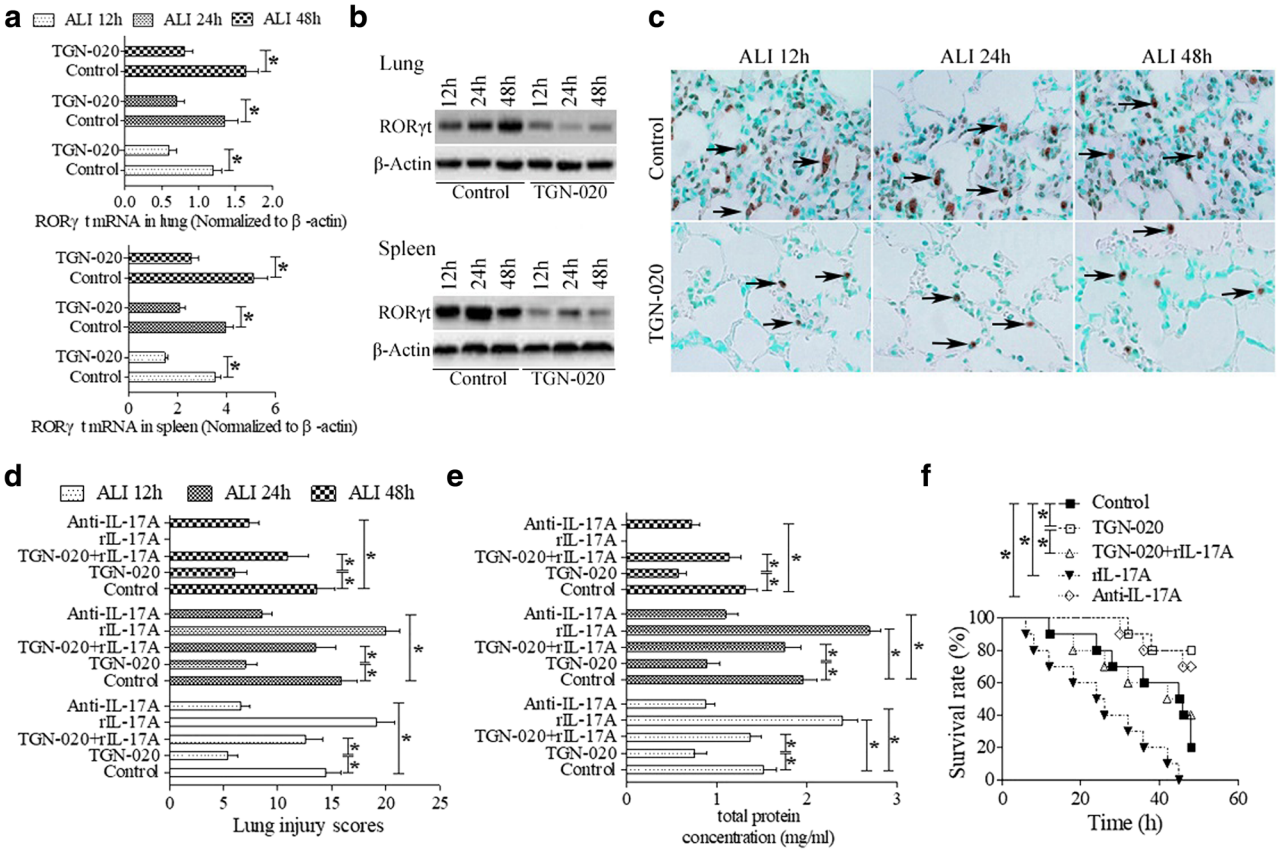

Fig. 5. AQP4 blockade inhibited Th17 cells, and this inhibition was associated with ameliorated lung injury. **a** The expression of RORγt mRNA in the lung and spleen tissue at different points. **b** Western blot analysis was used to evaluate the expression of RORγt protein in the lung and spleen tissues. **c** Immunohistochemistry study of RORγt in lung tissues. **d** Lung injury scores of mice (12 h, 24 h, 48 h) ($N = 5$). **e** The total protein concentration in BALF. **f** The survival rate of mice ($N = 10$ animals in each group). The assays were performed in triplicate on samples from each animal ($N = 6$ animals in each group). The data are presented as the mean ± SEM. $^*P < 0.05$.

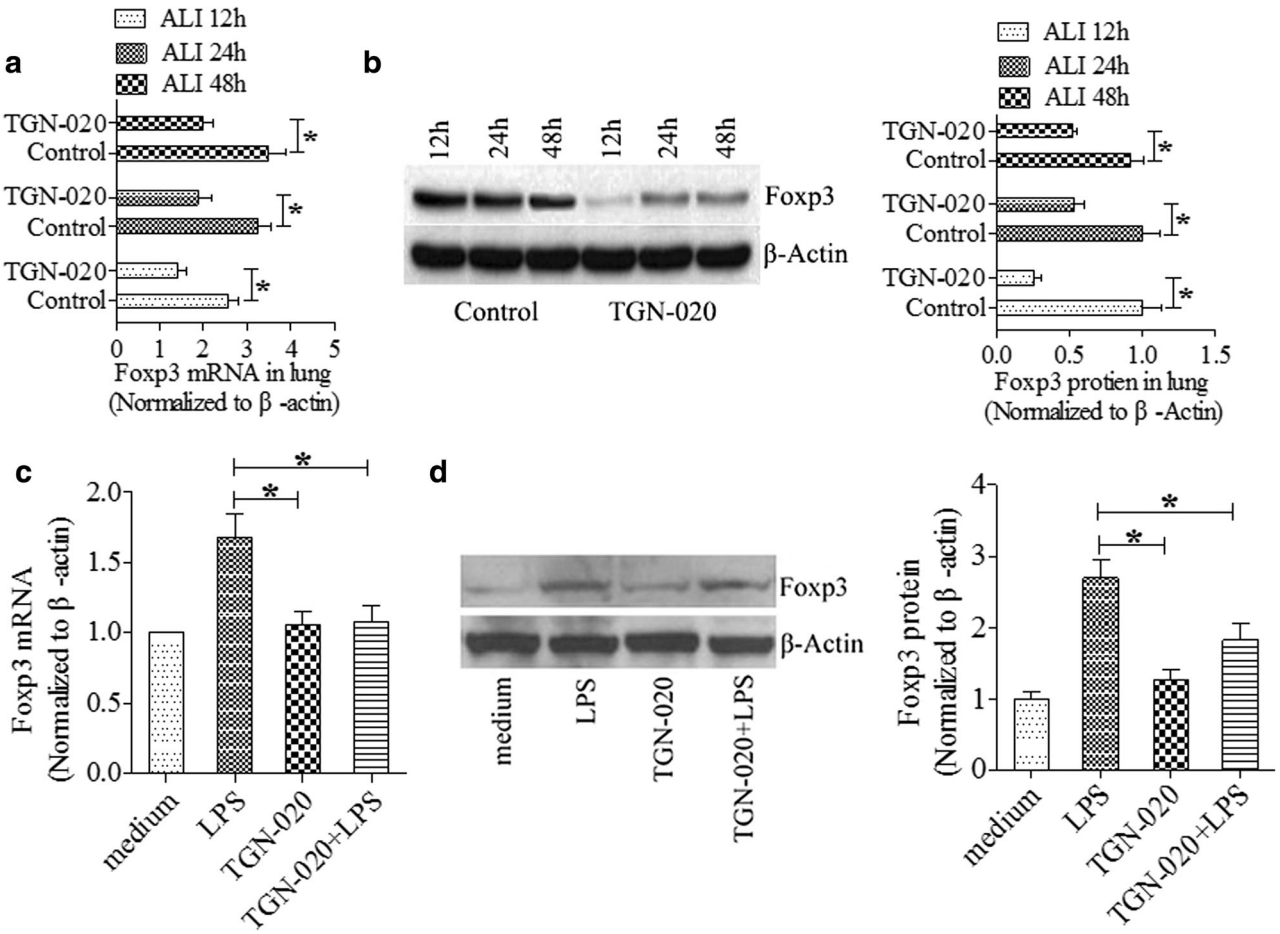

Fig. 6. AQP4 blockade impaired Th17 differentiation but not through the upregulation of Treg cells. **a** The expression of Foxp3 mRNA in the lung tissues. **b** The protein level of Foxp3 in the lung tissue. **c** The expression of Foxp3 mRNA in mixed lymphocyte reactions. **d** The expression of Foxp3 protein in mixed lymphocytes reactions. For the mixed lymphocyte reaction, bone marrow cells were obtained from the femurs and iliac bones of BALB/c mice and cultured with completive medium (DC-CM; RPMI 1640 containing 10% heat-inactivated fetal calf serum [FCS], 50 mM 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin) (GIBCO), 10 ng/ml recombinant mouse GM-CSF, and 10 ng/ml recombinant mouse IL-4 (R&D systems). On day 6, DCs were purified using $CD11c^+$ microbeads (Miltenyi Biotec), pulsed with TGN-020 or LPS for 24 h, and washed three times with PBS. As a control, DCs were cultured only with medium. $CD4^+$ naive T cells were generated from the splenic cells of C57BL/6 mice and purified using microbeads from Miltenyi Biotec. $CD4^+$ naive T cells and 7-day differentiated DCs were cocultured with ConA (Sigma) for 6 h. Then, cells were collected for WB and qPCR testing. The assays were performed in triplicate with samples from each animal ($N = 6$ animals in each group). The data are presented as the mean ± SEM. $^*P < 0.05$.

AQP4 Blockade Inhibited Th17 Cell Proliferation Through the Downregulation of PI3K and the Upregulation of SOCS3

PI3K/Akt has been reported to play an important role in oxidative injury and regulating cell proliferation and survival [31]. Therefore, we determined the effect of the AQP4 inhibitor on the PI3K/Akt signaling pathway. The results of our current study indicated that TGN-020 treatment significantly downregulated the phosphorylation of PI3K and Akt at 12 h, 24 h, and 48 h post LPS instillation when compared to the control treatment (Fig. 7a).

STAT3 has been demonstrated to be a positive regulatory factor for Th17 polarization. A previous report indicated that SOCS3 could block STAT3 phosphorylation. In our present study, TGN-020 or wortmannin (a PI3K inhibitor) treatment decreased the expression of p-STAT3 and increased the expression of SOCS3 at 12 h, 24 h, and 48 h post LPS instillation when compared to the no inhibitor controls (Fig. 7b). As a result, the RORγt level was significantly reduced in the mice treated with TGN-020 or wortmannin (Fig. 7b).

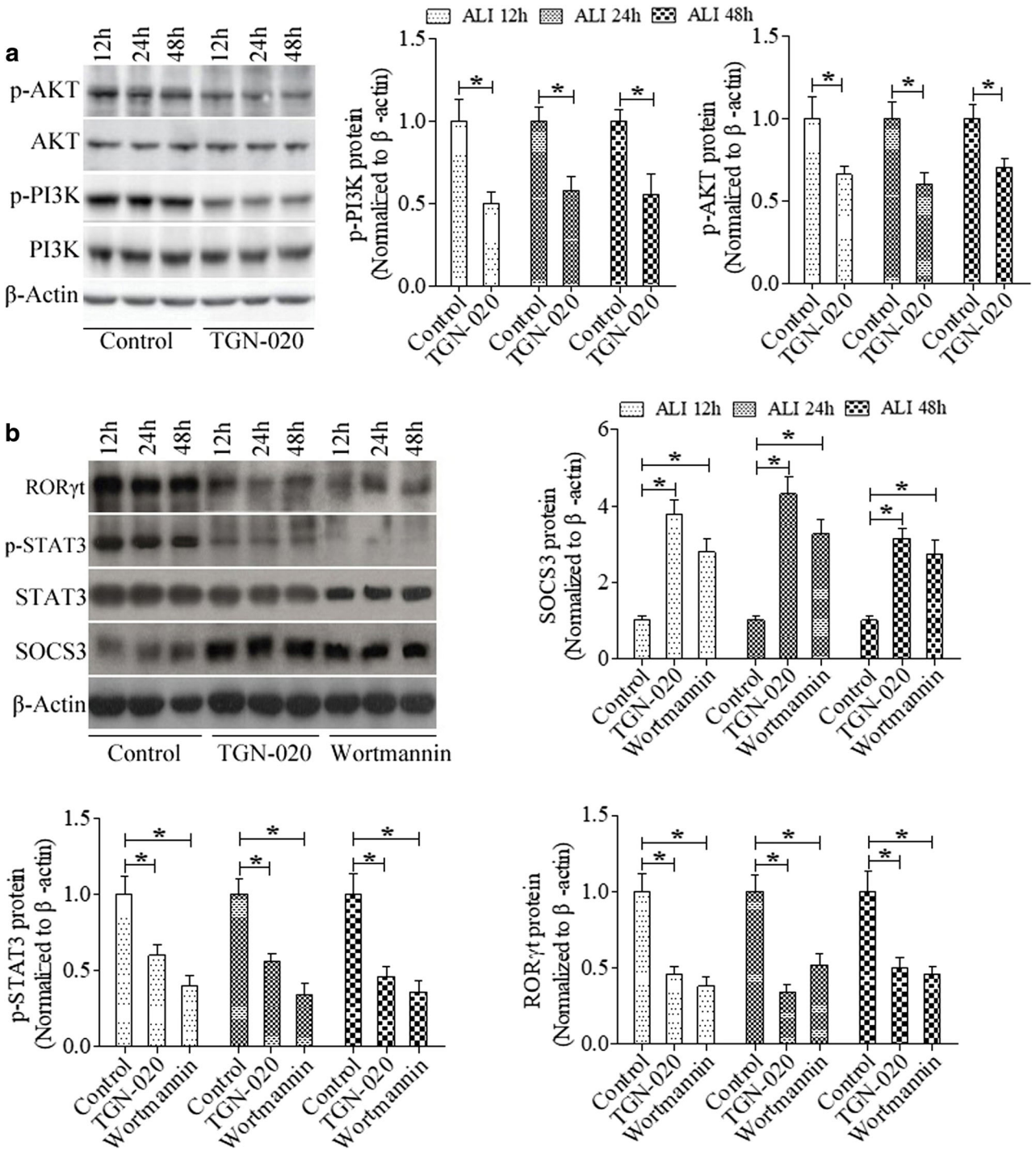

Fig. 7. AQP4 blockade inhibited Th17 cell proliferation through the downregulation of PI3K and upregulation of SOCS3. **a** The protein levels of p-PI3K and p-Akt in the lung tissue. **b** The protein level of RORγt, p-STAT and SOCS3 in the lung tissue. The assays were performed in triplicate with samples from each animal ($N = 6$ animals in each group). The data are presented as the mean ± SEM. $^*P < 0.05$.

DISCUSSION

The results of the present work indicate, for the first time, that mice treated with the AQP4 inhibitor TGN-020 had attenuated LPS-induced lung injury, reduced proinflammatory cytokine secretion, and an improved survival rate. Inhibition of AQP4 by TGN-020 had a detectable, protective effect against LPS-induced lung injury, and this effect was associated with the inhibition of IL-17A through the downregulation of the PI3K/Akt signaling pathway and upregulation of SOCS3 protein.

Pneumonia is the single most common feature of patients with ALI [32]. Inflammatory factors contribute to pneumonia and aggravate the condition of patients with ALI [33]. Despite pneumonia being identified as the most common risk factor for acute lung injury, there is limited understanding of the molecular mechanism of inflammatory factors in ALI [34]. Therefore, investigating the role of inflammatory factors in the progression of ALI within the alveolar space in mice is essential. In our current study, the levels of IL-1α, IL-1β, IL-6, IL-23, and IL-17A were markedly decreased in TGN-020-treated mice at 12 h, 24 h, and 48 h after LPS instillation when compared with the control mice. TGN-020 treatment also decreased the levels of TNF-α at both 24 h and 48 h post intratracheal instillation of LPS. Recent papers have demonstrated the key role of invading neutrophils in causing tissue damage and increasing microvascular permeability in the lung [24, 25]. The results of our current study indicated that both neutrophil infiltration into lung tissue and protein leakage into the BALF were decreased at 12 h, 24 h, and 48 h in TGN-020-treated mice. These results suggested that the attenuated lung injury induced by TGN-020 treatment partly prevented inflammatory cytokine expression and lung neutrophil infiltration in mice.

Interleukin 17 A (IL-17A) has been demonstrated to be a proinflammatory cytokine that regulates host defense against multiple pathogens [35]. Previous research has shown that Th17 cells play a key role in the progression of ALI and that IL-17 contributes to the increased permeability of human alveolar epithelial monolayers [36, 37]. $CD4^+CD25^+Foxp3^+$ Tregs exert anti-inflammatory effects mainly through the release of inhibitory cytokines such as IL-10 and TGF-β [38]. A previous study suggested the potential role of Tregs in treating ALI by modifying the innate immune response [39]. In the present study, both the levels of IL-17A and RORγt were markedly decreased in lung tissues from TGN-020-treated mice when compared with those from the control group at 12 h, 24 h, and 48 h post LPS instillation. The Foxp3 levels were also downregulated by TGN-020 treatment. These results suggested that TGN-020 treatment functioned through Th17 cells, but not Treg cells. To further investigate the role of IL-17A in the attenuated lung injury induced by the AQP4 inhibitor, we administered additional rIL-17A in the presence of TGN-020. The results showed that the attenuated lung injury scores, increased survival rate, and decreased BALF total protein concentration in TGN-020-treated mice were all abrogated by additional rIL-17A administration. We proposed that the inhibition of Th17 cell proliferation and inhibition of the release of IL-17A by TGN-020 treatment contribute to the attenuated lung injury induced by LPS instillation.

The PI3K/Akt signaling pathway has been reported to regulate LPS-induced acute inflammatory responses *in vitro* and *in vivo* [40]. A recent study found that inhibition of the PI3K/Akt signaling pathway exerts a protective role in ALI [41]. The PI3K/AKT pathway has been shown to regulate the cell proliferation regulation, anti-apoptosis, anti-senescence, cancer generation and migration, tissue development, and memory formation functions of the JAK2/STAT3 signaling cascade [42, 43]. STAT3 has also been shown to promote the expansion of Th17 cells [44, 45]. It was previously reported that SOCS3 gene expression was upregulated in ALI [46]. The increased SOCS3 could suppress proinflammatory cytokine-induced tissue damage *in vivo* by inhibiting the JAK/STAT3 signaling pathway. A recent report also indicated that a lack of SOCS3 induced more severe ALI and increased Th17 cell differentiation [47]. In our current study, TGN-020 treatment significantly downregulated the phosphorylation of PI3K and Akt in mice at 12 h, 24 h, and 48 h post LPS instillation when compared to those in the control group. TGN-020 or wortmannin (a PI3K inhibitor) also increased the expression of SOCS3 and decreased the expression of p-STAT3 and RORγt. These results demonstrated that TGN-020 could inhibit Th17 cell proliferation through downregulation of the PI3K/Akt signaling pathway and upregulation of SOCS3 protein.

In conclusion, our current study demonstrated, for the first time, that administration of the AQP4 inhibitor TGN-020 attenuated LPS-induced lung injury, reduced proinflammatory cytokine release, and improved the survival rate in mice. These findings support the notion that AQP4 blockade may represent an attractive strategy for ALI therapy.

COMPLIANCE WITH ETHICAL STANDARDS

Experiments were approved by the Institutional Animal Care and Use Committee at Hubei Province Academy of Traditional Chinese Medicine.

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