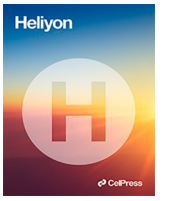
Heliyon 10 (2024) e31747

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Combination therapy with probiotics and anti-PD-L1 antibody synergistically ameliorates sepsis in mouse model

Leiming Sun *, Kun Fang, Zheng Yang **

Department of Critical Care Medicine, Hangzhou Red Cross Hospital, Hangzhou, 310003, Zhejiang Province, China

ARTICLE INFO

Keywords:
Sepsis
Probiotics
Anti-PD-L1 antibody
Combination
Immune regulation

ABSTRACT

The study investigated the protective effects and mechanisms of probiotics in conjunction with an anti-PD-L1 antibody on the immune functions of septic mice. Sixty-four mice were assigned to sepsis groups receiving vehicle, probiotics, and anti-PD-L1 antibody individually or in combination, with healthy mice as controls. Sepsis was induced by cecal ligation and puncture (CLP), followed by intraperitoneal Lipopolysaccharide (LPS) injection. Blood and tissues were collected one day post-injection for detecting inflammation-related cytokines, Treg, PI3K/Akt pathway-related protein expression, and lung tissue pathology. The survival time of the remaining ten mice was recorded over seven days. Compared to healthy mice, septic mice given PBS exhibited significantly different serum levels of IL-6, IL-8, IL-17, IL-10, and IFN-γ (all $p < 0.001$). Treatment with anti-PD-L1 antibody combined with probiotics significantly increased the 7-day survival rate in septic mice, accompanied by decreased pro-inflammatory cytokines, increased anti-inflammatory cytokines, improved oxidative stress, reduced lung injury, and enhanced Th17/Treg balance. This combined therapy demonstrated superior efficacy compared to antibodies or probiotics alone. Additionally, it facilitated peripheral blood polymorphonuclear neutrophil apoptosis, enhancing protection by blocking PD-L1 function and inhibiting PI3K-dependent AKT phosphorylation. In conclusion, combining probiotics with an anti-PD-L1 antibody enhances protective effects in septic mice by reducing serum inflammatory factors, promoting neutrophil apoptosis, regulating Th17/Treg balance, and inhibiting the PI3K/Akt pathway.

1. Introduction

Sepsis is a dysregulated immune response to infection, which can result in life-threatening organ dysfunction. It is also a leading cause of death in intensive care units. Recent epidemiological analyses suggest that mortality from severe sepsis and septic shock is still increasing by approximately 30 % in Europe and the United States [1]. Septic shock induces an inflammatory response secondary to sepsis-induced immunosuppression [2]. Despite significant advancements in understanding the pathophysiology of sepsis and the development of comprehensive bundle treatment regimens, including antibiotics, aggressive fluid resuscitation, vasopressor administration, and supportive care, no specific sepsis treatment strategy has emerged as widely used in clinical practice [3]. The gut microbial composition undergoes complex changes in sepsis and is considered a potential therapeutic option [4].

While the pathogenesis of sepsis remains multifactorial and not fully understood, there is mounting evidence suggesting that

* Corresponding author.
** Corresponding author.
E-mail addresses: nightsun16@outlook.com (L. Sun), yzhilson@163.com (Z. Yang).

https://doi.org/10.1016/j.heliyon.2024.e31747
Received 29 October 2023; Received in revised form 21 May 2024; Accepted 21 May 2024
Available online 22 May 2024

disruption of the gut microbiome predisposes patients to sepsis and adversely affects sepsis outcomes [5,6]. The breakdown of the epithelial barrier and increased intestinal permeability are frequently observed in critically ill patients and are considered to play a central role in the development of bacterial translocation and systemic infections. The gut microbiota has long been recognized as a key component of the gut barrier. The gastrointestinal tract has played a crucial role in the pathophysiology of sepsis by serving as a mechanism for the initiation and perpetuation of multiple organ dysfunction. Relevant studies on the mechanisms of action of probiotics have primarily focused on the intestine. However, the role of probiotics is not confined to the initial site of infection; they can exert systemic effects through the immune system and play a significant immunoregulatory role [7].

Persistent neutrophil activation due to delayed apoptosis contributes to nonspecific tissue damage in patients with sepsis. The upregulation of PD-L1, a ligand of the PD-1 protein, in sex-specific granulocytes during sepsis is associated with sepsis-induced immunosuppression [8]. Previous studies have demonstrated that neutrophil apoptosis is regulated through the PI3K/AKT signaling pathway. Moreover, PD-L1 has been reported to maintain the stemness of cancer stem cells through AKT signaling [9].

In gut-associated lymphoid tissue (GALT), M cells phagocytose or internalize probiotics and antigenic material from their metabolites to form endosomes [10,11]. Antigens within M cells are rapidly released and absorbed by dendritic cells (DCs), which then transport antigens to regional lymph nodes. This process activates immature T and B cells, leading to distinct immune responses characterized by the release of specific cytokines [12,13]. While the immunomodulatory effects of probiotics are known, there is a lack of studies on their combined treatment with immunosuppressive checkpoint drugs for sepsis.

The objective of this study was to evaluate the potential of probiotics in conjunction with anti-PD-L1 antibodies to modulate inflammatory levels and immune status in septic mice, along with investigating the underlying mechanisms. We postulated that the combination therapy involving probiotics and an anti-PD-L1 antibody holds significant promise for synergistically ameliorating sepsis in murine models.

2. Materials and methods

2.1. Experimental materials

Inverted microscope (Nikon model ECLIPSE Ts2), low-speed centrifuge (Eppendorf model 5702R), flow cytometer (BECKMAN model CytoFLEX); FITC rat anti-mouse CD4 monoclonal antibody (BD, RUO-561831), APC rat anti-CD25 monoclonal antibody (BD, RUO-557192), PE rat anti-mouse Foxp3 monoclonal antibody (BD, RUO-560408), anti-mouse/rat IL-17APE monoclonal antibody (Biogems, 12-7177-81). Peifeikang probiotics (*Bifidobacterium*, *Lactobacillus acidophilus*, *Enterococcus* triple-viable bacteria capsules, each capsule containing 210 mg of powder, with no less than $1.0 \times 100{,}000$ CFU of viable bacteria, Shanghai Xinyi Pharmaceutical Co., Ltd.), and LPS (SIGMA, L2880). Anti-murine PD-L1 antibody (Abcam, ab269674). ICAM-1, MDA, and SOD activity ELISA kits were obtained from R&D Systems Co., Ltd. (Minneapolis, MN, USA). Primary antibodies against cleaved caspase-3 (ab231289), PD-L1 (ab213480), Akt (ab38513), *p*-Akt (ab131443), or β-actin (ab115777), and horseradish peroxidase-conjugated secondary antibody (ab288151), were purchased from Abcam (Cambridge, UK).

2.2. Experimental animals

Healthy male Balb/c mice, weighing 18–22 g and aged 6–8 weeks, were provided by Shandong San Francisco Bass Biotechnology Co., Ltd. (animal license No. SCXK (Lu) 20160001). Six mice were group-housed, with six per cage, under standard conditions and maintained on a regular 12-h light/dark cycle. All animal procedures were reviewed and approved by the BIOFAVOR BIOTECH Experimental Animal Ethics Committee (approval no. BFE-2006021).

2.3. Grouping of experimental animals and modeling of sepsis

All animals were adaptively fed for 7 days and were free of water and food. The mice were randomly divided into five groups according to body weight: a normal control group and four sepsis model groups receiving treatment with PBS, probiotics, anti-PD-L1 antibody alone, or combined treatment. Each group included 16 mice, of which 6 mice were collected for detection 24 h after modeling. Probiotics were intragastrically administered to the corresponding animals at a dose of 200 mg/kg for two consecutive days before modeling. The anti-PD-L1 antibody was intravenously injected into the mice in the two groups at a dose of 10 mg/kg 0.5 h before modeling, and the other groups were intragastrically administered the same amount of saline for two consecutive days. In the cecal ligation and puncture sepsis model, a 1.5 cm-long incision was made along the midline of the abdomen after anesthesia with 50 mg/kg pentobarbital sodium. The cecum was ligated at the root of the cecum, formed by penetrating the cecum three times with an 18-gauge needle. The cecum was then returned to the abdominal cavity, and the abdominal wall incision was sutured layer by layer. At the end of surgery, 50 mL/kg of normal saline was immediately administered for anti-shock treatment. The abdomen of the mice in the sham group was closed after flipping the bowel using laparotomy only. At the beginning of the modeling, mice in each sepsis group were intraperitoneally injected with LPS at a dose of 15 mg/kg, and the serum, lungs, and spleen were collected 24 h after injection from six mice.

2.4. Sample collection and testing

Survival status, including postoperative spirit, activity, and coat color, as well as the 7-day survival rate of mice after surgery, were

strictly recorded. Finally, surviving mice were euthanized. Specimens were collected 24 h after modeling in all mice, except for those whose survival rates were recorded. After the isolation and purification of mouse spleen lymphocytes, cell sample labeling, cell surface antigen, and intracellular staining were performed. The proportion of Th17 and Treg cells in total Th cells was detected by flow cytometry. Mouse blood was extracted from the eyeball, serum was separated, and IL-6, IL-8, IL-10, and IL-17 levels were measured by ELISA according to the manufacturer's instructions. Venous blood was drawn from mice, heparin was used for anticoagulation, and Histopaque 1119 and Histopaque 1083 were added. The mixture was then centrifuged at 700×*g* for 30 min, the polymorphonuclear leukocytes (PMN) enrichment layer was drawn, washed twice with PBS, dissolved red blood cells were removed, washed once with PBS, and stained with Raynaud's-Apodemus. The purity of PMNs was more than 95 % before they could be used in subsequent experiments. ELISA, flow cytometry, and Western blot detection methods are described in sections 2.5 and 2.6, respectively.

2.5. Flow cytometry detection of Treg and Th17 cells

Treg and Th17 cells were analyzed by flow cytometry, and the methods were briefly described as follows: 1) the spleens of each group were ground and passed through a 200-mesh screen; 2) 3 mL of red blood cell lysate was added to each group, and the mixture was blown and lysed for 2 min; 3) 9 mL of PBS was added, mixed well, and centrifuged at 2000 rpm for 3 min; 4) the supernatant was removed and washed with PBS twice; 5) the supernatant was discarded, and the cells were resuspended in 1640 medium; 6) PMA was added at a final concentration of 20 ng/mL, ionomycin at a final concentration of 1 μg/mL, monensin at a final concentration of 2 μmol/L, and incubated in a 5 % CO2 incubator at 37 °C for 5h; 7) after the end of incubation, 1 mL of PBS was added, centrifuged at 2000 rpm for 5 min, washed twice, and resuspended with 100 μL of PBS; 8) the primary antibody to surface antigen was added and incubated in the dark at 4 °C for 30 min; 9) centrifuged at 2000 rpm for 3 min and washed with PBS twice; 10) 1 mL of buffer/perm buffer was added to each tube and incubated at 4 °C for 50 min; 11) 1 mL of perm/wash buffer was added to each tube, centrifuged at 2000 rpm for 3 min, and the supernatant was discarded; 12) 1 mL of perm/wash buffer was added to each tube, and the cells were resuspended with 100 μL of perm/wash buffer; 13) the primary antibody to intracellular antigen was added, centrifuged at 4 °C, and protected from light for 30 min; 14) centrifuged, the supernatant was discarded, centrifuged at 2000 rpm for 3 min, and washed with PBS twice; 15) resuspended with 200 μL PBS; 16) Detected with a flow cytometer.

2.6. Western blot analysis

Lung tissues were ground and lysed on ice, centrifuged, and the supernatant protein lysates were used to measure protein concentrations using the BCA method. Equal amounts of protein samples (10 μg) were electrophoresed by SDS-PAGE, transferred onto a PVDF membrane, blocked with 5 % skimmed milk powder for 1 h, incubated with a primary antibody (1:1000) overnight at 4 °C, followed by incubation with a horseradish peroxidase-labeled secondary antibody for 2 h at room temperature. The proteins were then detected using an ECL luminescence kit.

2.7. Histopathologic analysis

Histopathological analysis was performed as previously described [14]. Briefly, the lung tissues of animals in each group were fixed in a 4 % aqueous paraformaldehyde solution, embedded in paraffin, and stained with hematoxylin and eosin (H&E). Three slices of each lung tissue were cut with a slice thickness of 5 μm. Inflammatory changes in the lungs of the mice were observed under a light

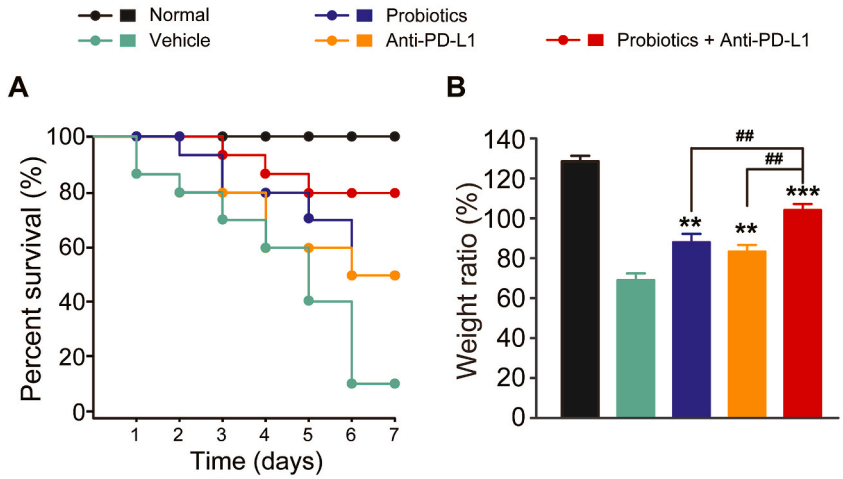


Fig. 1. Observation of the survival curve and body weight change of healthy or sepsis model mice. (A) Survival curve and (B) body change ratio of the mice. All data showed as Mean ± SD (n = 10) and analyzed via One-way ANOVA, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs PBS group. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$.

microscope. Pathological grading was performed based on alveolar dilatation, hemorrhage, leukocyte infiltration, neutrophil accumulation in the vascular wall of the airways, and alveolar wall thickness, which were scored from 0 to 4: 0 for normal lung tissue, 1 for 25 % lung tissue involvement, 2 for 25%–50 % lung tissue involvement, 3 for 50%–75 % lung tissue involvement, and 4 for >75 % lung tissue involvement.

2.8. Statistical analysis

Data were analyzed using GraphPad Prism 5 and expressed as mean ± SD and incidence. Differences between means and Fisher's exact tests were analyzed using analysis of variance (ANOVA). $p < 0.05$ was considered statistically significant.

3. Results

This study aimed to assess whether probiotics combined with anti-PD-L1 antibodies could modulate inflammatory levels and immune status in septic mice and the underlying mechanisms.

3.1. Combination therapy with probiotics and anti-PD-L1 antibody improve the survival of sepsis mice

The activity of the septic mice was observed after establishing the model. As depicted in Fig. 1A, mice in the control group exhibited normal activity without any noticeable shivering, resulting in a 7-day survival rate of 100 %. However, a majority of mice in the sepsis group huddled in the cage corner and exhibited shivering. The survival rate in the PBS group was 10 %, while mice receiving either probiotics or anti-PD-L1 antibody showed increased rates at 50 %. The combined group demonstrated the highest survival rate, reaching 80 %, significantly surpassing the rates observed in the two monotherapy groups. The trend in body weight improvement was consistent with the survival rate, collectively illustrating that combination therapy can offer promising protection in mice (Fig. 1B).

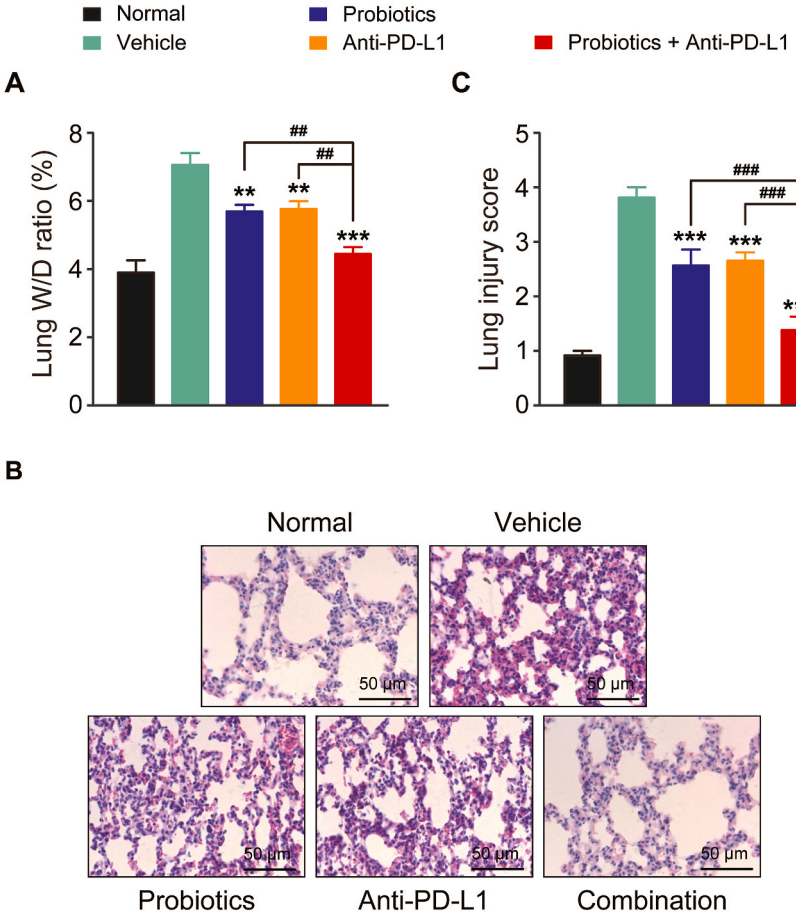


Fig. 2. Combination therapy alleviated the lung W/D ratio and lung tissue damages induced by LPS in sepsis model mice. The (A) lung wet/dry ratio, (B) lung tissue histopathological changes and (C) lung injury score in sepsis mice. Data are expressed as the Mean ± SD (n = 6). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$.

3.2. Combination therapy with probiotics and anti-PD-L1 antibody improves lung histopathological characters

The lung W/D ratio was further assessed to evaluate pulmonary edema, and the results are shown in Fig. 2. The combination therapy significantly reversed the increase in lung W/D ratio-induced sepsis, and the protective effect was significantly better than that of saline, indicating that the combination therapy exhibited improved effects on lung injury (Fig. 2A). Significant inflammatory changes were observed in the sepsis model group, including inflammatory cell infiltration, alveolar wall thickening, pulmonary congestion, and fibrous hyperplasia (Fig. 2B), whereas no obvious pathological changes were observed in the normal group. Moreover, the combination therapy showed better protective effects against pathological damage to lung tissues than saline. Similarly, the combination therapy intervention significantly reduced the increase in the mean pathological score in response to LPS stimulation and showed a significant dose response (Fig. 2C); the protective effect of the combination therapy was significantly better than that of PBS or both monotherapies.

3.3. Combination therapy with probiotics and anti-PD-L1 antibody improves the release of inflammatory factors in sepsis model mice

As shown in Table 1, LPS stimulation significantly increased the levels of pro-inflammatory factors in the blood, including IL-6, IL-8, IL-17, IFN-γ, and also down-regulated the levels of IL-10. Prophylactic administration of probiotics and anti-PD-L1 antibodies reversely inhibited the changes in some cytokines, with significant differences observed in IL-6, IL-17, IL-8, and IFN-γ (all $p < 0.05$). Compared with monotherapy, combination therapy significantly reversed the increase or decrease in the aforementioned cytokines, and all of them exhibited significant differences (all $p < 0.001$). Furthermore, the effects of combination therapy on LPS-induced oxidative stress were evaluated in a mouse model of sepsis. As shown in Table 2, combination therapy significantly prevented sepsis-induced changes in SOD activity, MDA, and ICAM-1 levels compared to those in sepsis model mice prophylactically or instantly administered any monotherapy, suggesting a better reversal of oxidative stress under sepsis.

3.4. Combination therapy with probiotics and anti-PD-L1 antibody regulated the Treg/Th17 balance in sepsis model mice

As shown in Fig. 3A–D, the proportions of Th17 and Treg cells were up-regulated in septic mice compared to the control group (all $p < 0.001$). Notably, both Th17 and Treg cells exhibited a decreasing trend in septic mice administered probiotics compared to the septic control group. However, although the Treg/Th17 ratio significantly improved, it remained higher than that in the PBS-treated sepsis mice group ($p < 0.05$). The proportions of Th17, Treg, and Treg/Th17 cells in the combination treatment group were significantly lower than those in the sepsis control group and other treatment groups (all $p < 0.05$). These results suggest that treatment with probiotics combined with an anti-PD-L1 antibody can effectively balance the expression ratio of Tregs and Th17 and promote the normalization of immune regulation.

3.5. Combination therapy with probiotics and anti-PD-L1 antibody inhibited PI3K/Akt signaling pathway to promote peripheral blood polymorphonuclear neutrophil apoptosis

As shown in Fig. 4A–D, the expression of cleaved caspase-3 in the sepsis model group was significantly lower than that in the control group, and PD-L1 expression was significantly upregulated, accompanied by a significant increase in AKT phosphorylation. The probiotic group showed no significant inhibitory effect on the upregulation of PD-L1 and AKT phosphorylation but exhibited a significant upregulation of cleaved caspase-3 expression. Moreover, the phosphorylation of PD-L1 and AKT was significantly down-regulated by the combined administration of probiotics and an anti-PD-L1 antibody, and the upregulation of cleaved caspase-3 expression was still observed, indicating a certain combined effect relative to the two.

As shown in Fig. 5A, the apoptotic rate of polymorphonuclear neutrophils (PMN) in septic mice receiving PBS only at 24 h was 11.2 ± 2.3 %, significantly down-regulated compared with healthy controls (57.9 ± 5.2 %), and the difference had statistical significance ($p < 0.05$). After PD-L1 antigen silencing using antibodies, the apoptotic rates of PMNs in the peripheral blood of septic mice increased to 29.2 ± 3.9 % and 31.2 ± 2.2 % in the antibody alone and combination groups, respectively, compared with septic control mice, and the differences were statistically significant (both $p < 0.05$).

We further analyzed the correlation between the granulocyte apoptosis rate and PD-L1 expression in the sepsis group, and the results shown in Fig. 5B demonstrated that the PMN apoptosis rate was significantly negatively correlated with the PD-L1 expression

Table 1
Combination therapy alleviated LPS-induced inflammatory and oxidative stress of sepsis mice. Data are expressed as the Mean ± SD (n = 6). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs.* the PBS group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ *vs.* the probiotics group. $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$ and $^{\wedge\wedge\wedge}p < 0.001$ *vs.* the anti-PD-L1 antibody group.

Group	IL-6 (pg/mL)	IL-8 (pg/mL)	IL-10 (pg/mL)	IL-17 (pg/mL)	IFN-γ (pg/mL)
Normal control	21.4 ± 1.8	18.4 ± 1.8	92.7 ± 8.2	14.1 ± 1.0	10.2 ± 1.3
PBS	53.5 ± 1.5	73.1 ± 6.1	48.0 ± 5.4	34.8 ± 2.1	158.0 ± 14.6
Probiotics	46.9 ± 1.6*	51.2 ± 6.5*	57.7 ± 5.1	29.1 ± 2.0*	97.2 ± 10.5***
Anti-PD-L1 antibody	41.4 ± 2.2**	38.4 ± 4.1***	66.0 ± 8.4*	25.0 ± 3.2*	136.0 ± 20.6
Combination	30.4 ± 3.2***,##,^	35.9 ± 7.2***,##,	67.2 ± 7.2**	18.7 ± 2.9***,#,^	107.2 ± 18.6**,^

Table 2
Combination therapy alleviated LPS-induced oxidative stress of sepsis mice. Data are expressed as the Mean ± SD (n = 6). $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs.* the PBS group. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *vs.* the probiotics group. $^{\wedge}p < 0.05$ *vs.* the anti-PD-L1 antibody group.

Group	MDA (mol/mL)	SOD (U/mL)	ICAM-1 (ng/mL)
Normal control	13.2 ± 2.8	20.4 ± 2.9	45.2 ± 7.4
PBS	48.8 ± 7.6	8.2 ± 1.1	242.6 ± 26.2
Probiotics	45.2 ± 5.3	12.3 ± 0.7	158.2 ± 15.4
Anti-PD-L1 antibody	35.2 ± 4.5	11.0 ± 1.9	126.5 ± 9.5
Combination	26.3 ± 3.4$^{**,\#}$	16.9 ± 1.2$^{***,\#,\wedge}$	96.1 ± 16.6$^{***,\#\#,\wedge}$

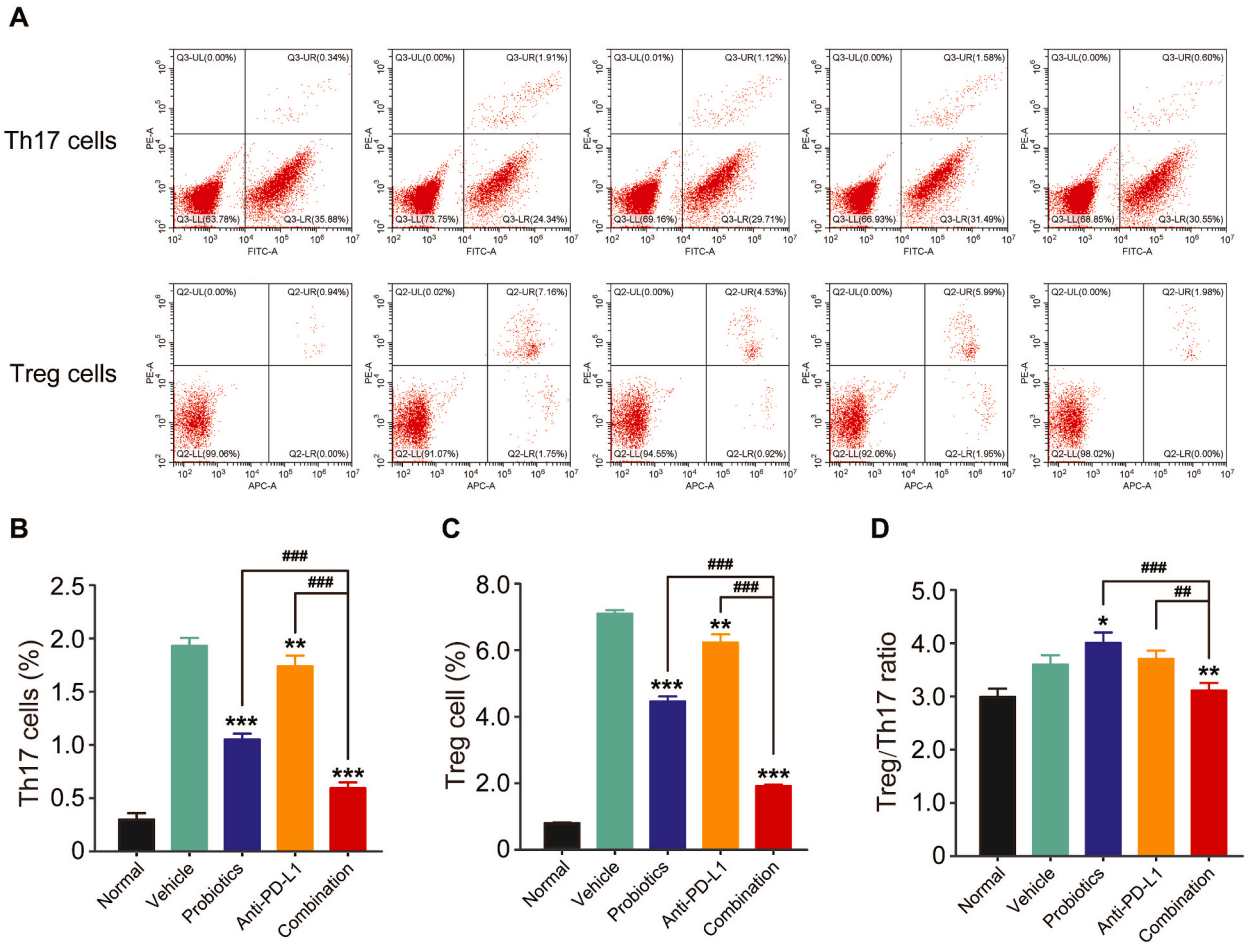


Fig. 3. Combination therapy with probiotics and anti-PD-L1 antibody regulated the Treg/Th17 balance in sepsis model mice. The (A) flow cytometry image and the analysis of (B) Th17 cells%, (C) Treg cells% and (D) Treg/Th17 ratio. All data showed as Mean ± SD (n = 6), $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs.* PBS group. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$.

level (r = - 0.555, $p = 0.015$). The above data collectively suggest that the combination therapy enhances the protection of septic mice by blocking the function of PD-L1 and inhibiting PI3K-dependent AKT phosphorylation to promote peripheral blood PMN apoptosis, thereby realizing the protection of sepsis mice.

4. Discussion

Recent studies have found that sepsis induces immunosuppression, leading to more challenging treatments [15,16]. Therefore, improving immunosuppression is one of the hotspots of current research on sepsis. Previous studies have demonstrated that monocytes from septic mice highly express PD-L1, and the results of sample analysis have also shown the upregulation of PD-L1 on dendritic cells and PD-1 on $CD8^{+}$ T lymphocytes, indicating that the PD-L1/PD-1 negative immunoregulatory pathway may be involved in the development of sepsis [9]. It has also been reported that, compared with septic mice with wild-type and knockout PD-L1 genes, the

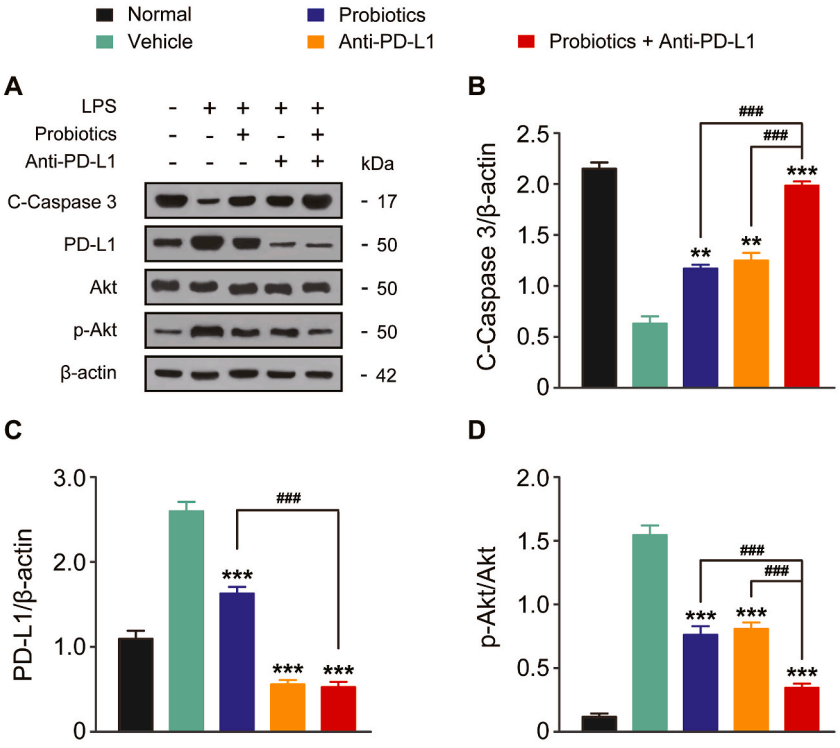


Fig. 4. Combination therapy inhibited PI3K/Akt signaling pathway. The (A) representative Western blot image and the calculated ratio of (B) cleaved caspase-3, (C) PD-L1, (D) *p*-Akt/Akt. All the data were showed as Mean ± SD (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, $^{\#\#\#}p < 0.001$. The uncropped versions of Western blot images were shown in Figs. S1–5.

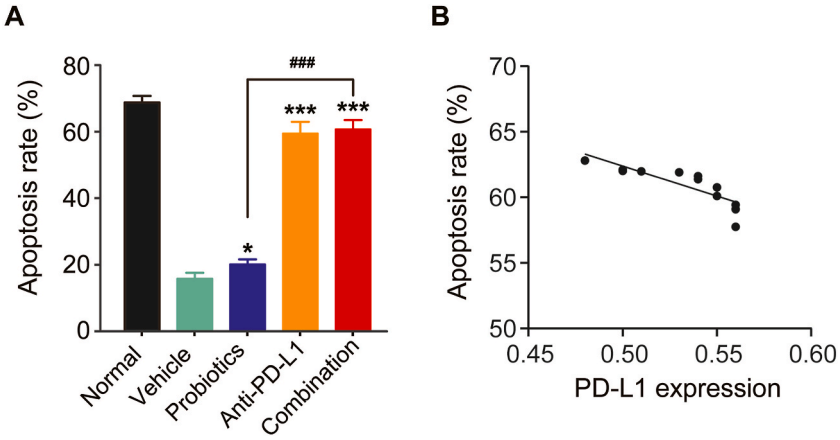


Fig. 5. Anti-PD-L1 antibody combined with probiotic therapy promotes neutrophil apoptosis in sepsis. (A) Apoptosis rate of peripheral blood PMN; (B) correlation between apoptosis rate of granulocytes and PD-L1 expression in sepsis group received anti-PD-L1 antibody. All the data were showed as Mean ± SD (n = 6). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, $^{\#\#\#}p < 0.001$.

latter had significantly improved survival and bacterial clearance rates, and the degree of organ tissue injury and circulating cytokine levels were also significantly lower [17]. Therefore, the PD-1/PD-L1 pathway has been suggested as a potential target for immunotherapy of sepsis.

Neutrophils are innate immune cells that play a role in the migration of tissue lesions, phagocytosis, and pathogen killing during acute infection. Some studies have used cecal ligation and puncture (CLP) in this model. Flow cytometry was used to observe changes in the expression of PD-1 and PD-L1 in the peripheral blood neutrophils of septic mice, and the results suggested that the expression of PD-L1 in neutrophils was increased in the early stage of sepsis in mice, but the expression of PD-1 was significantly unchanged [17,18]. As immune cells, neutrophils play a key role in the body in the face of infection. However, in patients with severe sepsis, neutrophils

often exhibit abnormal function and release various enzymes and inflammatory mediators, ultimately leading to multiple organ failure. While neutrophils rely on PD-L1 to achieve immunosuppression and avoid removal, the control of upregulated PD-L1 on the surface of neutrophils in the septic state may be a potential strategy to control the development of sepsis [19].

In sepsis, intestinal homeostasis is disrupted by both endogenous and exogenous factors, including intestinal ischemia and hypoxia. This disruption leads to weakened intestinal peristalsis and the multiplication of pathogenic bacteria in the intestine. Several studies have shown that a reduction in the number of dominant intestinal bacteria and bacterial diversity is positively correlated with the risk of death in patients with systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) [20–23]. At the same time, the imbalance of intestinal microecological disorders can lead to intestinal mucosal cell damage and intestinal mucosal barrier damage, resulting in submucosal dendritic cell dysfunction. Thus, Treg cells are reduced, and the body cannot produce immune tolerance to various intestinal bacterial antigens and toxins, ultimately causing immune diseases. Therefore, promoting Treg cell growth by regulating the intestinal microecological balance, correcting immune tolerance defects, and restoring immune balance is also key to the treatment of sepsis. Fay KT et al. reported that mice with increased gut microbiome α-diversity have improved sepsis survival, mediated by a distinct immunophenotype characterized by an increased $CD4^+$ T cell response. Enhancing microbiome α-diversity in mice through co-housing not only led to increased sepsis survival but also altered the immune response to sepsis [24].

Therefore, in the present study, we aimed to investigate the effect and mechanism of action of probiotics combined with an anti-PD-L1 antibody on the inflammatory immune function in septic mice. We first observed that both probiotics and PD-L1 alone or in combination therapy significantly improved the survival rate of septic mice (all $p < 0.05$). Compared with the treatment group, the combination therapy group showed a substantial advantage in improving the survival rate and effectively mitigated lung injury in mice (Figs. 1 and 2). Therefore, we investigated the possible mechanisms at the protein and cellular levels. From the perspective of inflammatory factors, the results of this study showed that the prophylactic administration of probiotics may improve the prognosis of sepsis by decreasing the inflammatory factors IL-6 and IL-17 and increasing the inflammatory factor IL-10, thereby inhibiting the inflammatory response. Mice with sepsis treated with a placebo were in a hyperinflammatory state, showing elevated levels of various inflammatory factors. Consistent with previous predictions, the prophylactic administration of probiotics has been shown to be effective in controlling inflammatory response of the body. Moreover, the improvement in the release of proinflammatory factors was more significant than that of probiotics or anti-PD-L1 antibodies alone. This enhancement may be because anti-PD-L1 antibodies inhibit the upregulation of PD-L1 indicated by neutrophils and synergize with probiotics to control inflammation-related signaling pathways, thereby improving the overall inflammatory response.

The $CD4^+$ $CD25^+$ Treg population in the serum of septic mice from each group was further monitored. Compared with PBS-treated septic mice, the proportion of $CD4^+$ T cells and Th17 cells increased in septic mice treated with prophylactic probiotics, whereas the proportion of Th17 cells decreased, and the Treg/Th17 ratio increased significantly. One of the main functions of Tregs is to suppress the activation and proliferation of inflammatory effector T cells. Compared to the probiotic group, the anti-PD-L1 antibody group showed a more significant decrease in the proportion of Tregs, suggesting that its use can reduce T-cell immunosuppression and enhance T-cell proliferation and Th1. As shown in Table 1, the increase in IFN-γ levels and the decrease in IL-6 and IL-8 levels secreted by neutrophils collectively demonstrate the effects in improving immune imbalance. Th17 cells are T helper cells composed of Th0 cells differentiated under the stimulation of IL-6 and IL-23, which mainly secrete proinflammatory factors such as IL-17 and IL-22. The balance of Th17/Treg plays an important role in autoimmunity [25]. Intestinal microorganisms can trigger a variety of signals and induce $CD4^+$ T cell differentiation; for example, Klebsiella ectopic colonization and other invasive bacteria can induce dendritic cell phagocytosis and the release of proinflammatory cytokines (IL-6, IL-12, and TNF), but also induce $CD4^+$ T cells to differentiate into Treg cells. Further secretion of IL-10 and other molecules has an inhibitory effect on immune cells [26,27].

In the current study, probiotics appeared to suppress the proinflammatory response that leads to sepsis by inhibiting Th17 cells. The $CD4^+$ T helper cells have long been thought to differentiate only into Th1 and Th2. However, it is now shown that $CD4^+$ T cells can also differentiate into Th17 cells, which are characterized by production of IL-17 rather than IFN-γ or IL-4. IL-17 is a potent chemical agonist of neutrophils, and elevated IL-17 levels have been found in the serum of patients with sepsis. Th17 cells have a different developmental program and produce cytokines that differ from those of Th1 or Th2 cells. Induction of Th17 differentiation is independent of IL-12 or IL-4; in contrast, IL-6 and TGF-β1 (in mice) and IL-1 (in humans) mediate Th17 differentiation of naïve $CD4^+$ T cells [28,29]. However, the role of Th17 cells in intestinal pathology and homeostasis remains poorly understood. Some of their roles in gut-associated tissues include the promotion of microbial defense, regulation of T cell differentiation and cellular production of inflammatory mediators, and regulation of neutrophil migration and function; however, the mechanisms by which probiotics modulate Th17 cell differentiation and production in the intestine remain to be elucidated. In addition, Lin et al. prepared dendritic cells highly expressing Jagged1 using adenoviral transduction and found that their surface PD-L1 expression was increased. Co-culture of these dendritic cells with naïve T cells promoted their differentiation into $FOXP3^+$ Tregs, and Treg expansion could be partially arrested after blocking PD-L1 using specific antibodies, suggesting that PD-L1 is involved in promoting Treg differentiation and proliferation [30]. In addition, Dong et al. showed that PD-L1 expressed by acute myeloid leukemia cells can directly promote Treg proliferation and maintain a high expression of FOXP3 and PD-1 on their surfaces, whereas anti-PD-L1 mAb promotes the differentiation of naive $CD4^+$ T cells into Th17 cells. Briefly, these reports support the above results [31].

Furthermore, the activation of PI3K and downstream AKT phosphorylation are indispensable for the anti-apoptotic activity of LPS and protein transcription, which inhibits apoptosis. Previous studies have shown that PD-L1 interacts with the p85 subunit of PI3K to phosphorylate the downstream AKT and delay neutrophil apoptosis, thereby exacerbating sepsis [9]. Therefore, the effects of sepsis and treatment with probiotics and anti-PD-L1 antibodies alone or in combination on changes in this signaling pathway were further evaluated. In the current study, we found that the expression of cleaved caspase-3 in the sepsis model group was significantly lower than that in the control group using the WB method, whereas PD-L1 was significantly upregulated, accompanied by a significant

increase in AKT phosphorylation. Only significant upregulation of cleaved caspase-3 expression was observed in the probiotic group. Accordingly, we found that the combination therapy significantly upregulated cleaved caspase-3 expression and downregulated PD-L1 and AKT phosphorylation. Wang et al. reported that PD-L1 expression is inversely correlated with the rate of apoptosis in human neutrophils collected from septic patients and that increased PD-L1 expression in human neutrophils drives lung injury by triggering PI3K-dependent AKT phosphorylation, increasing mortality, and delaying apoptosis during clinical and experimental sepsis [9]. This is largely consistent with our results, suggesting that combination therapy may enhance protection in mice by blocking the function of PD-L1 and inhibiting PI3K-dependent AKT phosphorylation.

5. Conclusion

The combination of probiotics and the anti-PD-L1 antibody can complement their therapeutic advantages. On one hand, it relies on probiotics to achieve a reduction in serum inflammatory factors and regulate the balance of Th17 and Treg cells. On the other hand, it depends on the anti-PD-L1 antibody to promote neutrophil apoptosis and inhibit the PI3K/Akt pathway, thereby enhancing the protective effect in septic mice.

Ethics statement

All animal procedures were reviewed and approved by the BIOFAVOR BIOTECH experimental Animal Ethics Committee with the approval No. BFE-2006021.

Funding

This study was supported by Medical Health Science and Technology Project of Zhejiang Provincial Health Commission (2018KY608).

Data availability

Data included in article/supp. material/referenced in article.

CRediT authorship contribution statement

Leiming Sun: Writing – original draft, Supervision, Methodology, Data curation, Conceptualization. **Kun Fang:** Writing – review & editing, Validation, Software, Methodology. **Zheng Yang:** Writing – review & editing, Validation, Methodology, Data curation.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31747.

References

[1] P.V. Van Heerden, A. Abutbul, S. Sviri, et al., Apoptotic cells for therapeutic use in cytokine storm associated with sepsis- A phase ib clinical trial, Front. Immunol. 12 (2021) 718191.
[2] R.S. Hotchkiss, G. Monneret, D. Payen, Immunosuppression in sepsis: a novel understanding of the disorder and a new therapeutic approach, Lancet Infect. Dis. 13 (3) (2013) 260–268.
[3] L. Evans, A. Rhodes, W. Alhazzani, et al., Surviving sepsis campaign: international guidelines for management of sepsis and septic shock 2021, Intensive Care Med. 47 (11) (2021) 1181–1247.
[4] C. Wang, Q. Li, J. Ren, Microbiota-immune interaction in the pathogenesis of gut-derived infection, Front. Immunol. 10 (2019) 1873.
[5] M. Niu, P. Chen, Crosstalk between gut microbiota and sepsis, Burns & trauma 9 (2021) tkab036.
[6] E. Arribas-López, N. Zand, O. Ojo, et al., The effect of amino acids on wound healing: a systematic review and meta-analysis on arginine and glutamine, Nutrients 13 (8) (2021) 2498.
[7] P. Szychowiak, K. Villageois-Tran, J. Patrier, et al., The role of the microbiota in the management of intensive care patients, Ann. Intensive Care 12 (1) (2022) 3.
[8] A. Endo, M. Okamura, S. Yoshikawa, et al., Multilateral functional alterations of human neutrophils in sepsis: from the point of diagnosis to the seventh day, Shock 48 (6) (2017) 629–637.
[9] J.F. Wang, Y.P. Wang, J. Xie, et al., Upregulated PD-L1 delays human neutrophil apoptosis and promotes lung injury in an experimental mouse model of sepsis, Blood 138 (9) (2021) 806–810.
[10] M.A.K. Azad, M. Sarker, D. Wan, Immunomodulatory effects of probiotics on cytokine profiles, BioMed Res. Int. 2018 (2018) 8063647.
[11] Y. Zhao, Y. Zeng, D. Zeng, et al., Probiotics and MicroRNA: their roles in the host-microbe interactions, Front. Microbiol. 11 (2020) 604462.

[12] E. Owaga, R.H. Hsieh, B. Mugendi, et al., Th17 cells as potential probiotic therapeutic targets in inflammatory bowel diseases, Int. J. Mol. Sci. 16 (9) (2015) 20841–20858.
[13] Y. Mishima, R.B. Sartor, Manipulating resident microbiota to enhance regulatory immune function to treat inflammatory bowel diseases, J. Gastroenterol. 55 (1) (2020) 4–14.
[14] C. Promputta, V. Anupunpisit, B. Panyarachun, et al., Effect of Vernonia cinerea in improvement of respiratory tissue in chronic nicotine treatment, J. Med. Assoc. Thai. 95 (Suppl 12) (2012) S47–S55.
[15] M. Singer, C.S. Deutschman, C.W. Seymour, et al., The third international consensus definitions for sepsis and septic shock (Sepsis-3), JAMA 315 (8) (2016) 801–810.
[16] J.L. Vincent, J.C. Marshall, S.A. Namendys-Silva, et al., Assessment of the worldwide burden of critical illness: the intensive care over nations (ICON) audit, Lancet Respir. Med. 2 (5) (2014) 380–386.
[17] X. Huang, F. Venet, Y.L. Wang, et al., PD-1 expression by macrophages plays a pathologic role in altering microbial clearance and the innate inflammatory response to sepsis, Proc. Natl. Acad. Sci. U. S. A. 106 (15) (2009) 6303–6308.
[18] J. Xu, X. Ma, K. Yu, et al., Lactate up-regulates the expression of PD-L1 in kidney and causes immunosuppression in septic Acute Renal Injury, J. Microbiol. Immunol. Infect. 54 (3) (2021) 404–410.
[19] J.F. Wang, J.B. Li, Y.J. Zhao, et al., Up-regulation of programmed cell death 1 ligand 1 on neutrophils may be involved in sepsis-induced immunosuppression: an animal study and a prospective case-control study, Anesthesiology 122 (4) (2015) 852–863.
[20] S. Liao, J. Luo, T. Kadier, et al., Mitochondrial DNA release contributes to intestinal ischemia/reperfusion injury, Front. Pharmacol. 13 (2022) 854994.
[21] P. Chen, T. Billiar, Gut microbiota and multiple organ dysfunction syndrome (MODS), Adv. Exp. Med. Biol. 1238 (2020) 195–202.
[22] B.W. Haak, W.J. Wiersinga, The role of the gut microbiota in sepsis, Lancet Gastroenterol Hepatol. 2 (2) (2017) 135–143.
[23] S.F. Assimakopoulos, C. Triantos, K. Thomopoulos, et al., Gut-origin sepsis in the critically ill patient: pathophysiology and treatment, Infection 46 (6) (2018) 751–760.
[24] K.T. Fay, N.J. Klingensmith, C.W. Chen, et al., The gut microbiome alters immunophenotype and survival from sepsis, Faseb. J. 33 (10) (2019) 11258–11269.
[25] N. Qu, M. Xu, I. Mizoguchi, et al., Pivotal roles of T-helper 17-related cytokines, IL-17, IL-22, and IL-23, in inflammatory diseases, Clin. Dev. Immunol. 2013 (2013) 968549.
[26] X. Zhang, P. Zheng, T.R. Prestwood, et al., Human regulatory dendritic cells develop from monocytes in response to signals from regulatory and helper T cells, Front. Immunol. 11 (2020) 1982.
[27] S. Bhowmick, D. Chatterjee, K. Chaudhuri, Human epithelial cells stimulated with Vibrio cholerae produce thymic stromal lymphopoietin and promote dendritic cell-mediated inflammatory Th2 response, Int. J. Biochem. Cell Biol. 44 (11) (2012) 1779–1790.
[28] F. Cristofori, V.N. Dargenio, C. Dargenio, et al., Anti-inflammatory and immunomodulatory effects of probiotics in gut inflammation: a door to the body, Front. Immunol. 12 (2021) 578386.
[29] A. Shetty, S.D. Bhosale, S.K. Tripathi, et al., Interactome networks of FOSL1 and FOSL2 in human Th17 cells, ACS Omega 6 (38) (2021) 24834–24847.
[30] C.L. Lin, H.M. Huang, C.L. Hsieh, et al., Jagged1-expressing adenovirus-infected dendritic cells induce expansion of Foxp3(+) regulatory T cells and alleviate T helper type 2-mediated allergic asthma in mice, Immunology 156 (2) (2019) 199–212.
[31] Y. Dong, Y. Han, Y. Huang, et al., PD-L1 is expressed and promotes the expansion of regulatory T cells in acute myeloid leukemia, Front. Immunol. 11 (2020) 1710.