

Original Article

Oral administration of *Lactobacillus acidophilus* strain SW1 suppresses tumor necrosis factor (TNF)-alpha and increases transforming growth factor (TGF)-beta in mice

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Abstract: In the present study, the effect of the *L. acidophilus* strain SW1 on the systemic immune response was evaluated in BALB/c mice. Upon oral administration of SW1, tumor necrosis factor (TNF)- α production was strongly inhibited compared to the control group, whereas transforming growth factor (TGF)- β was significantly increased. Changes in the relative abundance of serum immunoglobulin isotypes were also observed *via* oral intake of SW1. Increased frequencies of CD4⁺CD25⁺Foxp3⁺ regulatory T cells in peripheral blood was further obtained in mice following SW1 treatment. These results demonstrated profound immunomodulatory properties of this bacterial strain in healthy mice upon oral administration. In conclusion, strain SW1-induced suppression of pro-inflammatory cytokine TNF- α might occur in a Treg-dependent manner.

Keywords: Lactobacillus acidophilus, tumor necrosis factor- α , regulatory T cells

Introduction

Probiotic bacteria, such as lactic acid bacteria (LAB), have been shown to regulate intestinal immune responses in humans and mice by inhibiting or augmenting cytokines. There is growing interest in probiotics for treating inflammatory bowel disease, diarrhea, and atopic dermatitis as well as for adjusting the T helper (Th) 1/Th2 balance that is tightly associated with allergic, autoimmune, and chronic inflammatory diseases [1-4]. Recently, *Lactobacillus reuteri* was revealed to inhibit TNF- α production by preventing the activation of c-Jun and the mitogen-activated protein kinase (MAPK)-regulated transcription factor activator protein (AP)-1 in mammals [5]. An *in vitro* study demonstrated that co-culture of mucosal explants from Crohn's disease patients with either *Lactobacillus casei* or *Lactobacillus bulgaricus* inhibited TNF- α release by the inflamed mucosa [6]. This finding illustrates that probiotic-influenced immune responses can occur in a strain-dependent manner. In this regard, confirming that results obtained in animal models translating into human models will be critical to the selection of strains and treatment strategies that are most

likely to meet with success in preventing or treating human diseases such as chronic inflammatory disease.

Lactobacillus acidophilus is a species of gram positive bacteria in the genus *Lactobacillus*, some strains have been studied extensively for health effects [7]. In our previous study, we demonstrated that *L. acidophilus* strain SW1 could function as an immune adjuvant for a DNA vaccine against the foot-and-mouth disease virus (FMDV) [8]. In this study, increased viral-specific antibody titers, T cell proliferation responses were observed. These observed changes were likely mediated in part by SW1-mediated alterations in systemic cytokine production, although this was not formally tested.

Based on these previous findings, we hypothesized that oral administration of *L. acidophilus* SW1 could affect cytokine production in healthy animals. We therefore tested the serum cytokines in SW1-treated mice, which are well known to reflect the overall systemic cytokine profile in the host as a meaningful evaluation of host immunity. Overall, this work aimed to provide insight into how the probiotic bacterial

strain SW1 exerts its immune regulation in mice by assessing the impact of this probiotic strain on the cytokine profiles *in vivo*. We demonstrated that *Lactobacillus acidophilus* could balance inflammatory response in a favor of Treg cell status in health mice.

Materials and methods

Bacterial strains

L. acidophilus strain SW1 was described previously [8]. Bacteria were grown in overnight culture at 37°C with 5% CO₂ in sterile MRS broth (BD Biosciences, San Jose, CA, USA). When the OD value reached approximately 0.6, bacteria were harvested by centrifugation at 6000 rpm for 10 min, washed three times with fresh phosphate-buffered saline (PBS), and then resuspended in fresh PBS.

Animals and experimental design

Male BALB/c mice (6-8 weeks old) were purchased from Hunan SJA Laboratory Animal Co., Ltd and acclimatized for 1 week before the start of the experiment. They were fed with a standard diet and allowed free access to distilled water throughout the experimental period. Animal care was performed according to the Animal Ethics Procedures and Guidelines of the People's Republic of China, and the experimental protocol was approved by the local committee on animal use and protection. For the animal experiments, a total of 30 mice were divided into two groups. Mice (n=15) in the experimental group daily received approximately 2.5×10⁹ CFU of SW1 in 300 μ L of MRS by oral administration for consecutive 5 days. The control group (n=15) received 300 μ L of MRS. Serum samples were collected from the mice from the tail and stored in -80°C until use.

Cytokine detection

All serum cytokines were measured using commercial ELISA kits (eBioscience, San Diego, CA, USA) strictly according to the manufacturer's instructions. Cytokine concentrations in each serum sample were calculated from the equations derived from the standard curve.

Quantification of mouse Ig isotypes by flow cytometry

For quantifying the effect of SW1 on the concentration of Ig isotypes in response to oral administration of SW1, the total concentration

of IgA, IgG1, IgG2a, IgG2b and IgM in the sera samples were measured using a Mouse Immunoglobulin Isotyping Panel 6 plex FlowCytomix Multiplex Kit (eBioscience) according to the manufacturer's instructions. Flow Cytomix Pro Analysis software (eBioscience) was used to calculate and analyze the data against a standard curve.

Flow cytometry (FCM)

For analyzing the T regulatory cell populations in response to oral administration of SW1, mouse blood were collected from the tail vein into the EDTA-Na₂-containing tubes prepared for subsequent processing on days 6. For staining, 110 μ L aliquot of peripheral blood (containing EDTA-Na₂) was incubated at room temperature (RT) for 10 min in the dark with the following mAbs: APC-anti-CD4 and PE-anti-CD25 (eBioscience). Red blood cell (RBC) lysis solution (1 \times , 5 mL) (BD Biosciences) was then added to the sample and incubated for 15 min at RT in the dark. T cells were collected by centrifugation (350 \times g for 10 min) at RT, and the supernatant was discarded. For Foxp3 expression, cells were fixed, permeabilized, and stained with FITC-anti-Foxp3 (eBioscience) according to the manufacturer instruction. T cells were collected by centrifugation (350 \times g for 10 min) at RT, and the supernatant was discarded. The pellet was then washed with 4 mL PBS (350 \times g for 7 min) at RT, and resuspended in 300 μ L of PBS after aspirating off the supernatant. To discard cell debris, cells were filtered into BD falcon tubes (BD Biosciences) using a 300- μ m nylon mesh filter and fixed with 4% paraformaldehyde (300 μ L). The stained cells were then analyzed by flow cytometry, and cells in the lymphocyte gate were used for analysis.

Statistical analysis

Each specimen was tested at least twice. All analyses were performed using the IBM SPSS Statistics 19.0 software for Windows, and one-way ANOVA was used to calculate significance. Differences were considered statistically significant if $P < 0.05$. Graphs were created using GraphPad software 5.0.

Results

Oral administration of SW1 suppresses TNF- α production

We showed in a previous study that SW1 had an adjuvant effect to increase immune respons-

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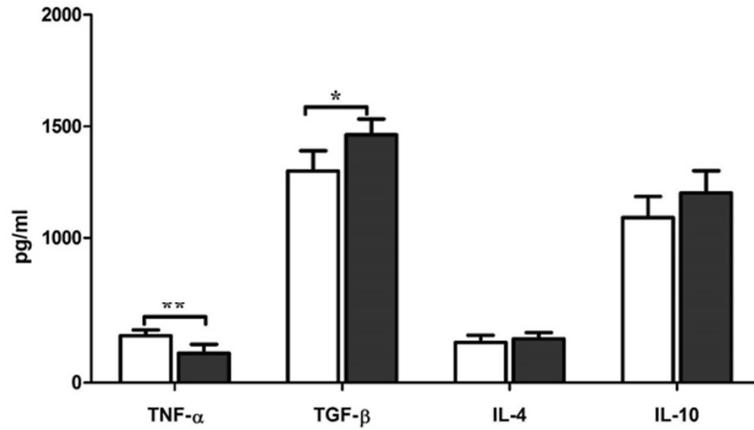


Figure 1. Serum cytokine pattern of BALB/c mice receiving oral administration of SW1. TNF- α , TGF- β , IL-4 and IL-10 levels in sera were measured by ELISA on day 6 after receiving oral administration of SW1. Data are expressed as the mean \pm standard deviation (SD). The assay was repeated twice. Statistical significance was calculated using the one-way ANOVA method. * $P < 0.05$, ** $P < 0.01$.

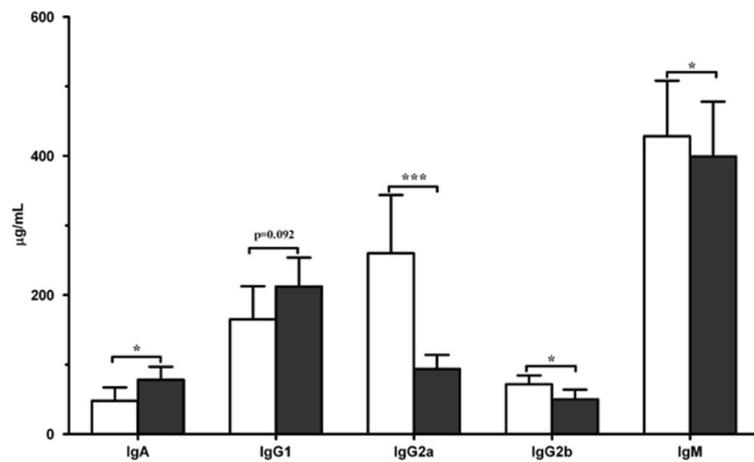


Figure 2. Quantitative detection of mouse serum immunoglobulin isotypes following probiotic treatment by flow cytometry. Sera were collected 6 days after mice received a daily dose ($2 \sim 5 \times 10^9$ CFU) of SW1 for consecutive 5 days. A Mouse Immunoglobulin Isotyping Panel 6plex FlowCytomix Multiplex Kit was used to measure immunoglobulin isotypes IgA, IgG1, IgG2a, IgG2b and IgM. Data are expressed as the mean \pm SD ($n=6$). Statistical significance was calculated using the one-way ANOVA method. * $P < 0.05$, *** $P < 0.01$.

es for a vaccine against FMDV. However, the underlying reason for this increase remained unknown, and we speculated that the orally administered SW1 induced cytokine changes within the mice. To address this question, BALB/c mice were orally administrated with SW1 and mouse serum cytokine levels were evaluated over time. As shown in **Figure 1**, serum levels of TNF- α significantly decreased on day 6 post-administration, while levels of TGF- β were increased in sera from mice treated

with SW1 compared to those in the control mice. The concentrations of IL-4 and IL-10 were not affected.

SW1 modifies the systemic concentration of immunoglobulin isotypes

Next, we evaluated whether SW1 activated adaptive immune responses and influenced the systemic concentration of different Ig isotypes. For this, we collected the sera from the various groups of mice following oral administration with SW1, and the production of Ig isotypes was quantified by flow cytometry.

As expected, SW1 significantly altered the systemic concentration of many of the Ig isotypes as compared to mock treatment. As shown in **Figure 2**, IgA production was significantly increased in mice receiving SW1 treatment compared to control mice. These data indicated that oral intake of SW1 can enhance the production of serum IgA in mice. The concentration of IgG1 showed a trend towards being increased after SW1 treatment ($P=0.092$). Conversely, oral administration of SW1 strongly suppressed the production of serum IgG2a and IgG2b. Moreover, IgG3 production showed a trend towards being decreased after SW1 treatment ($P=0.19$) (data not shown). Interestingly, IgM secretion into the

serum was decreased after SW1 treatment. These results demonstrated that consuming probiotic SW1 induces profound changes in humoral immune responses.

SW1 treatment increases the frequency of systemic regulatory T cells

We further questioned whether the blood Treg cell population was changed in mice after orally administrated with SW1. CD4⁺ Tregs were de-

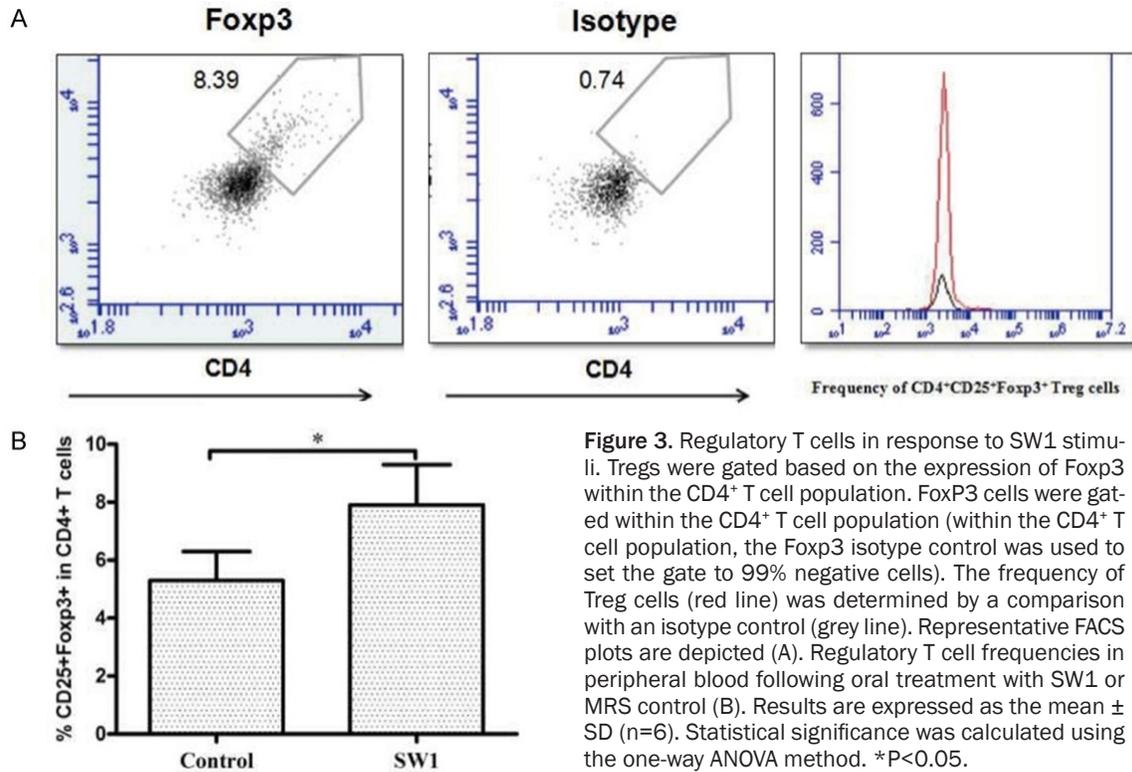


Figure 3. Regulatory T cells in response to SW1 stimuli. Tregs were gated based on the expression of Foxp3 within the CD4⁺ T cell population. FoxP3 cells were gated within the CD4⁺ T cell population (within the CD4⁺ T cell population, the Foxp3 isotype control was used to set the gate to 99% negative cells). The frequency of Treg cells (red line) was determined by a comparison with an isotype control (grey line). Representative FACS plots are depicted (A). Regulatory T cell frequencies in peripheral blood following oral treatment with SW1 or MRS control (B). Results are expressed as the mean \pm SD (n=6). Statistical significance was calculated using the one-way ANOVA method. *P<0.05.

fined on the basis of Foxp3 (Figure 3) and CD25⁺ expression compared to the total CD4⁺ T cell population. As shown in Figure 3B, the frequency of CD4⁺CD25⁺Foxp3⁺ Tregs increased after treatment with SW1 compared to treatment with MRS alone.

Discussion

Immunomodulation by probiotics have been widely investigated during the last several decades. In particular, probiotics, including some LABs, were able to alleviate inflammatory symptoms and modulate immune response [9, 10]. In this study, the ability of a strain SW1 to regulate murine immune responses was evaluated by analyzing systemic cytokine profiles and peripheral blood T lymphocytes, and the changes in systemic concentration of immunoglobulin isotypes. We demonstrated that consumption of this probiotic bacterial strain induced profound changes in both the humoral and cellular adaptive immune responses *in vivo*.

High TNF- α levels are correlated with severe diarrhea in children [11] and disease progression of many inflammatory conditions [12, 13]. In some cases, inflammation can cause inflam-

matory disease in the intestine. Therefore, the inhibition of inflammation or inflammatory cytokines such as TNF- α is critical for maintaining intestinal homeostasis. One well-known method to inhibit TNF- α activity in TNF- α -mediated diseases is administration of TNF- α antagonist, such as etanercept and golimumab, some of which are currently in clinical practice, as previously reviewed [14]. Our data demonstrated that oral administration of SW1 strongly inhibits systemic TNF- α production (Figure 1). As shown in our study, administration of *L. acidophilus* might present an alternative method to inhibit TNF- α production, which is consistent with previous studies. For instance, oral administration of *Lactobacillus delbrueckii subsp. Bulgaricus OLL1073R-1* to DBA mice inhibits TNF- α and IL-6 secretion [15]. Our data suggested that SW1 may be a promising probiotic to prevent allergy and/or chronic inflammation in healthy individuals.

CD4⁺CD25⁺Foxp3⁺ regulatory T cell is a subpopulation of T cells which modulate the immune system and maintain tolerance to self-antigen. Recent studies revealed that oral intake of *Lactobacillus rhamnosus* suppresses Th1 and Th2 cytokines in asthma mouse model by

CD4⁺CD25⁺Foxp3⁺ Tregs cell-mediated mechanism [16]. In our study, the increased population of CD4⁺CD25⁺Foxp3⁺ Tregs was also observed in probiotic-treated mice, and high TGF- β levels were also obtained. TGF- β is a powerful pleiotropic cytokine and produced by Tregs, with immune-suppressing and anti-inflammatory properties [13, 17]. Collectively, the suppression of TNF- α production in this study might be a consequence of the anti-inflammatory properties of Tregs [18], as demonstrated by the simultaneous increase of Tregs and TGF- β and decrease of TNF- α in our experiments (**Figures 1 and 3**).

Probiotics have been reported to modulate systemic Ig isotypes in the host [19]. Ingesting yogurt containing *L. acidophilus* and *Bifidobacterium* have been shown to potentiate the IgA response [20]. In this study, we further showed that systemic IgA and IgG1 levels were enhanced in mice treated with SW1 (**Figure 2**). Therefore, oral intake of *Lactobacillus acidophilus* can modulate systemic Ig responses in mice, which might also occur in a Treg-dependent manner.

In conclusion, we demonstrated in the current study that systemic immunomodulation occurs following oral administration of SW1 in healthy mice. This study also showed that oral administration of SW1 strain predominately inhibits TNF- α and induces Tregs in mice, suggesting that the suppression of inflammatory response might be resulted from the increases in CD4⁺CD25⁺Foxp3⁺ regulatory T cell population.

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Disclosure of conflict of interest

None.

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