



Research article

The effects of oral administration of *Aureobasidium pullulans*-cultured fluid containing β -glucan on concanavalin A injected miceZhidan Wang^{a,b}, Kuai Ma^a, Masayuki Fujino^a, Kisato Kusano^c, Shuang-Qin Yi^b, Atsushi Iwai^{c,d,*}, Xiao-Kang Li^{a,b,**}^a Division of Transplantation Immunology, National Research Institute for Child Health and Development, Tokyo, Japan^b Laboratory of Functional Morphology, Graduate School of Human Health Sciences, Tokyo Metropolitan University, Tokyo, Japan^c Aureo Co., Ltd., Chiba, Japan^d Aureo Science Co., Ltd., Hokkaido, Japan

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ABSTRACT

A black yeast, *Aureobasidium pullulans*, extracellularly produces β -(1,3), (1,6)-D-glucan (β -glucan) under certain conditions. The β -glucan is known to be an immunomodulatory agent, and β -glucan enriched *A. pullulans* cultured fluid (AP-CF) is used in supplements to maintain human health. Concanavalin A (ConA) is a lectin, and when injected it is known to cause T cell mediated autoimmune hepatitis in mice. The present study investigated the effects of oral administration of AP-CF on ConA injection in mice. The results demonstrated that increases in serum alanine transaminase (ALT) levels after ConA injection were significantly suppressed in an AP-CF administered group of mice. To understand the mechanism of the ALT lowering effects of AP-CF, we used Foxp3 (forkhead box P3) knock-in mice which express the green fluorescent protein (GFP) in Foxp3 induced cells, and the effects of AP-CF on the regulatory T cell (T_{reg}) populations were investigated. The results show that the basal level of Foxp3⁺ T_{reg} populations in peripheral blood lymphocytes, liver infiltrating lymphocytes, and splenocytes was decreased after 7 days of administration of AP-CF. These findings suggest that oral administration of AP-CF suppresses the basal level of inflammation, and that it may be postulated to be involved in the ALT lowering effects of AP-CF.

1. Introduction

Regulatory T cells (T_{reg}) are immunosuppressive T cells [1, 2, 3], initially identified as CD4⁺ T cell populations expressing the interleukin-2 (IL-2) receptor alpha-chain (CD25) molecules on the cell surface [4]. When CD25⁺ cells were depleted from CD4⁺ T cells, and the cells inoculated into athymic nude mice, the mice spontaneously developed autoimmune diseases. The results indicate that CD4⁺ CD25⁺ T cells contribute to maintaining self-tolerance through the suppression of the immune response. Later, Foxp3 (forkhead box P3) was found to be a master regulator of T_{reg} development [5], and CD25 negative T_{reg} populations were identified [6]. The T_{reg} function is crucial for the maintenance of homeostasis. As mentioned above, dysfunction of T_{reg} is thought to be involved in the onset of autoimmune diseases, while excess T_{reg} activity causes suppressed tumor immunity and is related in the progress of cancer [7, 8].

A black yeast, *Aureobasidium pullulans* extracellularly produces β -(1,3), (1,6)-D-glucan (β -glucan) under certain conditions [9]. The *A. pullulans* produced β -glucan is obtained as a viscous solution in a culture medium, and consists of a β -(1, 3)-linked glucose main chain and β -(1, 6)-linked glucose branches [9, 10]. β -glucan enriched *A. pullulans*-cultured fluid (AP-CF) is used in supplements to maintain human health. The *A. pullulans* produced β -glucan is known to act as a dietary fiber as well as an immune modulating agent, and is thought to exhibit beneficial effects on health through these functions. Several beneficial effects of AP-CF and its derived β -glucan, such as anti-atherosclerosis [11], anti-tumor [12, 13, 14], anti-allergy [15,16, 17] and anti-infectious disease effects [18, 19], have been reported.

Concanavalin A (ConA) is a lectin which induces T-cell mediated liver injury in mice [20, 21]. The Con A induced hepatitis is a well-established and widely used mouse model for autoimmune hepatitis. We previously reported that AP-CF has a liver protecting effect with high fat diet

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induced liver injury [22]. The mechanism of the protective effects of orally administered AP-CF is thought to be dependent on the function of AP-CF as a dietary fiber as well as on the immune modulation effects of AP-CF. Considering this, we thought possibility that oral administration of AP-CF modulates the ConA induced autoimmune reaction.

This study used Foxp3 knock-in mice (Foxp3^{GFP} mice) which express the green fluorescent protein (GFP) in Foxp3 induced cells [23] to determine Foxp3⁺ T_{reg} cells, and the effects of ConA injection and AP-CF administration on the T_{reg} populations in peripheral blood lymphocytes, liver infiltrating lymphocytes, and splenocytes were investigated.

2. Results

2.1. The effects of ConA injection on the T_{reg} populations in peripheral blood lymphocytes, liver infiltrating lymphocytes, and splenocytes

To evaluate the effects of ConA injection on Treg populations, Foxp3GFP mice were intravenously injected with ConA, and Treg populations in peripheral blood lymphocytes were monitored. As shown in Figure 1A, the results indicate that the Foxp3⁺ T_{reg} population (CD4⁺ Foxp3⁺ cells) in the total T cell (CD3⁺ cells) population was transiently increased at 24 h after the ConA injection. Similarly, the Foxp3⁺ T_{reg} (Figure 1B) and CD25⁺ Foxp3⁺ T_{reg} populations in CD4⁺ T cells (Figure 1C) were also transiently increased at 24 h, and had decreased at 48 h after the ConA injection.

To further investigate the effects of ConA injection on the T_{reg} populations, the liver and spleen were isolated from the ConA injected Foxp3^{GFP} mice and the T_{reg} populations in the liver infiltrating lymphocytes and splenocytes were determined. As shown in Figure 2A, B, C, the results indicate that the Foxp3⁺ T_{reg} population in the total T cell population, and the Foxp3⁺ T_{reg} and CD25⁺ Foxp3⁺ T_{reg} populations in CD4⁺ T cells were increased at 24 h and the increments were reduced at 48 h after the ConA injection. These observations resembled the data on T_{reg} populations in the peripheral blood lymphocytes shown in Figure 1A, B, C. In splenocytes, the Foxp3⁺ T_{reg} population in the total of the T cells (Figure 3A) and the Foxp3⁺ T_{reg} population in the CD4⁺ T cells (Figure 3B) were not significantly changed, and only the CD25⁺ Foxp3⁺ T_{reg} population in the CD4⁺ T cells was increased at 24 and 48 h after the ConA injection (Figure 3C).

2.2. The effects of oral administration of AP-CF on ConA injection and T_{reg} populations

To assess the effect of oral administration of AP-CF on ConA injection, C57BL/6J mice were daily administered AP-CF by gavage for 7 days, and then intravenously injected with ConA. As shown in Figure 4A, the serum ALT level after the ConA injection was significantly decreased in the AP-CF administered group of mice (n = 7) compared with the control group (n = 10). The serum ALT level of untreated male C57BL/6J mice has been reported as 18–94 Unit/L [24], and the serum ALT level decreased to the normal range in 6 of the 7 AP-CF administered group of mice. We

previously demonstrated that serum ALT levels are not influenced after oral administration of β-glucan produced by *A. pullulans* [22]. These results suggest that oral administration of AP-CF ameliorates the ConA induced autoimmune reaction.

To understand the mechanism of the serum ALT lowering effects of the orally administered AP-CF on the ConA injection, the T_{reg} populations in the peripheral blood lymphocytes, liver infiltrating lymphocytes, and splenocytes were determined by flow cytometry. The results show that compared with the control group of mice, the Foxp3⁺ T_{reg} population in the total T cells (Figure 4B) and in the CD4⁺ T cells (Figure 4C) after ConA injection were not significantly changed in the peripheral blood lymphocytes, liver infiltrating lymphocytes, and splenocytes in the AP-CF administered group of mice. The CD25⁺ Foxp3⁺ T_{reg} population in CD4⁺ T cells, however, were significantly lower in the AP-CF administered group of mice compared with that of the control group (Figure 4D), and the findings were similar to those observed in peripheral blood lymphocytes, liver infiltrating lymphocytes, and splenocytes.

2.3. The effects of oral administration of AP-CF on the T_{reg} populations in peripheral blood leukocytes

To evaluate the effects of oral administration of AP-CF on the T_{reg} populations, Foxp3^{GFP} mice were daily administered AP-CF by gavage for 7 days, and the T_{reg} populations in the peripheral blood leukocytes were analyzed by flow cytometry. The results show that the Foxp3⁺ T_{reg} population in the total T cells (Figure 5A), and in CD4⁺ T cells (Figure 5B) were moderately decreased after 7 days of administration of AP-CF. In comparison with these, the CD25⁺ Foxp3⁺ T_{reg} population in the CD4⁺ T cells (Figure 5C) was more strongly decreased after the AP-CF administration.

3. Discussion

In this study, the effects of oral administration of AP-CF on T_{reg} populations were investigated. To maximize the effects of oral administered AP-CF, the doses of AP-CF in this study were set to nearly limit of the daily gavage. However, any negative effects on the health of mice were not observed during the experimental periods. In ConA injected mice, 7 days of administration of AP-CF before the injection reduced serum ALT levels significantly, suggesting the possibility that oral administration of AP-CF may ameliorate ConA induced hepatitis (Figure 4A). After the ConA injection, the CD4⁺ CD25⁺ Foxp3⁺ T_{reg} population was significantly lower in the orally AP-CF administered group of mice than the PBS administered group of mice (Figure 4D). These observations would suggest that none of CD4⁺ CD25⁺ Foxp3⁺ T_{reg} is involved in the efficacy of the oral administration of AP-CF on the serum ALT lowering effects in ConA injected mice. The CD4⁺ CD25⁺ Foxp3⁺ T_{reg} population as well as the CD4⁺ Foxp3⁺ T_{reg} population in the total T cells or the CD4⁺ T cells decreased after the daily oral administration of AP-CF for 7 days (Figure 5). This would suggest that the basal level of inflammation in the mice was suppressed after the oral

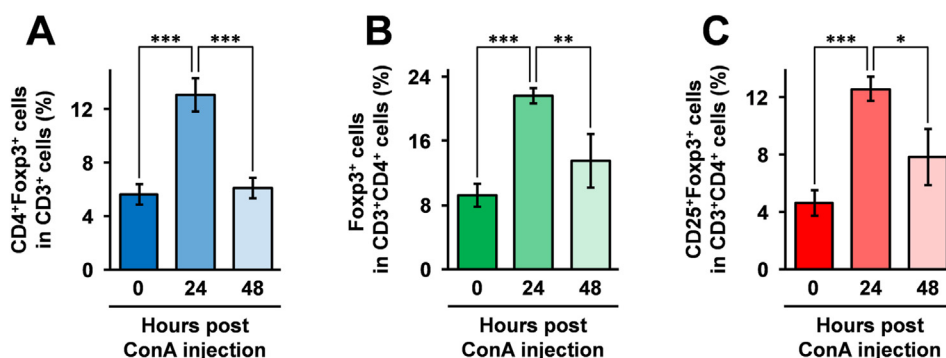


Figure 1. Effect of ConA injection on the T_{reg} populations in peripheral blood lymphocytes of Foxp3^{GFP} mice. The Foxp3^{GFP} mice were injected with ConA (15 mg/kg) via the tail vein. At the indicated time post ConA injection, serum samples were collected from the tail vein. Isolated lymphocytes from the serum were immunofluorescently stained with anti-CD3, anti-CD4, and anti-CD25 antibodies, and analyzed by flow cytometry. The 0 time shows data before the ConA injection, and error bars indicate standard deviations. The asterisk (*), double asterisks (**), and triple asterisks (***) indicate that the difference is statistically significant; *: p < 0.05, **: p < 0.01, ***p < 0.001.

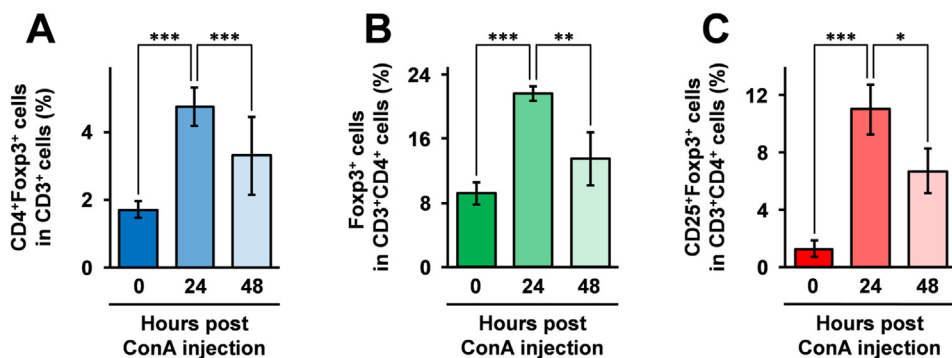


Figure 2. Effects of ConA-injection on the T_{reg} populations in liver infiltrating lymphocytes of Foxp3^{GFP} mice. The Foxp3^{GFP} mice were injected with ConA (15 mg/kg) via the tail vein. At the indicated time points, lymphocytes isolated from the liver were immunofluorescently stained with anti-CD3, anti-CD4, and anti-CD25 antibodies, and analyzed by flow cytometry. The 0 time shows data before the ConA injection, and error bars indicate standard deviations. The asterisk (*), double asterisks (**), and triple asterisks (***) indicate that the difference is statistically significant; *: p < 0.05, **: p < 0.01, ***p < 0.001.

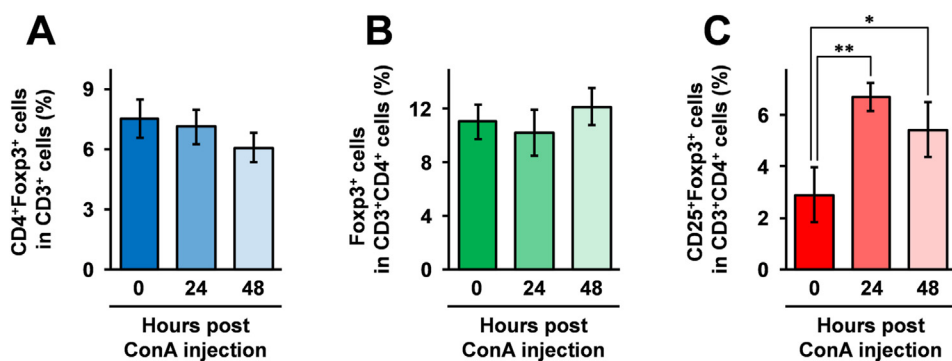


Figure 3. Effects of ConA-injection on the T_{reg} populations in splenocytes of Foxp3^{GFP} mice. The Foxp3^{GFP} mice were injected with ConA (15 mg/kg) via the tail vein. At the indicated time points, lymphocytes isolated from the splenocytes were immunofluorescently stained with anti-CD3, anti-CD4, and anti-CD25 antibodies, and analyzed by flow cytometry. The 0 time shows data before the ConA injection, and error bars indicate standard deviations. The asterisk (*), and double asterisks (**) indicate that the difference is statistically significant; *: p < 0.05, **: p < 0.01.

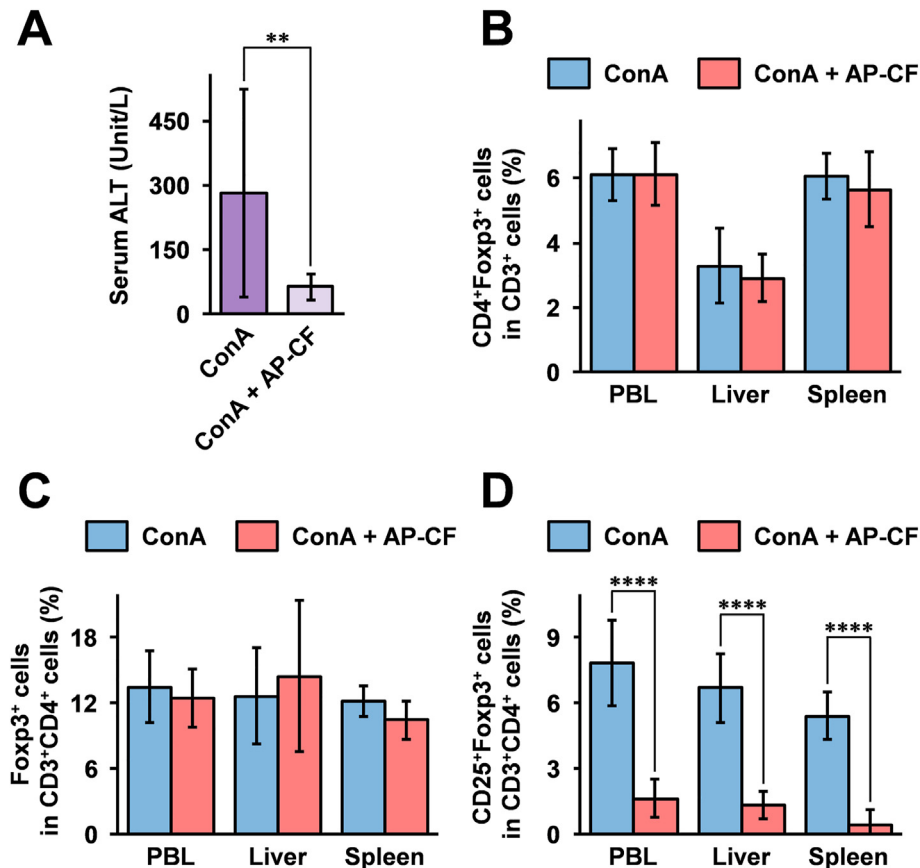


Figure 4. Effects of oral administration of AP-CF with ConA injection on the T_{reg} populations. (A) C57BL/6J mice were orally administered with AP-CF (50 mg/kg) or PBS by gavage, daily for 7 days, and then injected with ConA (15 mg/kg) via the tail vein; 48 h after this, the serum ALT levels were determined. (B–D) Foxp3^{GFP} mice were treated with AP-CF (1.44 mg/mouse, 47.9–57.6 mg/kg) and injected with ConA (15 mg/kg) as in panel A; 48 h after this, peripheral blood lymphocytes (PBL), liver infiltrating lymphocytes (liver), and splenocytes were isolated, and immunofluorescently stained with anti-CD3, anti-CD4, and anti-CD25 antibodies. The immunofluorescently stained lymphocytes were analyzed by flow cytometry. Error bars indicate standard deviations. The double asterisks (**), and quad asterisks (****) indicate that the differences are statistically significant; **: p < 0.01, ****: p < 0.0001.

administration of AP-CF. Intestinal microbiota are closely associated with in the permeability of the small intestine and circulating lipopolysaccharide (LPS), and involved in increasing the basal level of inflammation [25, 26]. The CD4⁺ CD25⁺ Foxp3⁺ T_{reg} cells express Toll-like receptors (TLRs) including TLR4, a receptor for innate immune response recognizing LPS, and is activated and proliferates after stimulation with LPS [27]. We previously reported that oral administration of AP-CF influences bacterial flora in the intestines of Japanese Black calves [28]. Similarly, β-glucans are known to be a prebiotic which promotes growth of probiotic bacteria [29, 30], and the effects of AP-CF as a prebiotic could be involved in reducing the CD4⁺ CD25⁺ Foxp3⁺ T_{reg} population.

A previous study has demonstrated that sublethal doses of ConA injected into C57BL/6 mice induces tolerance against ConA-induced hepatitis [31]. The results of cell depletion studies using the anti-CD25 antibody suggest that the tolerance involves the CD4⁺ CD25⁺ T_{reg} cells. As shown in Figures 1, 2, 3, our results indicate that overall, the CD4⁺ Foxp3⁺ T_{reg} population in the total numbers of T cells, and the Foxp3⁺ or CD25⁺ Foxp3⁺ T_{reg} populations among the CD4⁺ T cells were transiently increased after ConA injection. The results in this study suggest that the increased T_{reg} populations would be decreased to the basal level at a later time point than the time points presented here, and the increment of T_{reg} population may not be involved in the ConA induced tolerance. The T_{reg} cells isolated from the mice injected with sublethal doses of ConA showed more effectively suppressed polyclonal T cell responses than T_{reg} cells isolated from control mice *in vitro*. This suggests that functional changes in the T_{reg} cells may be hypothesized to be involved in the tolerance against ConA induced liver injury by the sublethal doses of ConA injection. To understand the mechanism of the serum ALT level lowering effects of orally administered AP-CF with ConA injection, further study of the functional changes in T_{reg} cells will be required. Additionally, it has been reported that the tolerance induced by sublethal doses of ConA injection is involved in type 1 regulatory (Tr1) cells [32]. The Tr1 cells are known to be CD4⁺ CD49b⁺ LAG-3⁺ CD226⁺ T cells, and this type of T_{reg} cells do not consistently express Foxp3 [33]. For this reason, the Tr1 population may remain undetected in the experimental condition of the present study, and it could still be involved in the serum ALT lowering effect of orally administered AP-CF against ConA injection.

The present study demonstrates that oral administration of AP-CF ameliorates increased serum ALT levels caused by the ConA injection, and no involvement of T_{reg} cells on the serum ALT lowering effect of AP-CF was identified in the experiments performed in this study. Further investigations are required to understand the mechanism of the serum ALT lowering effect of orally administered AP-CF against ConA injection in more detail.

4. Materials and methods

4.1. Mice

The C57BL/6J mice (10–12 weeks old) were obtained from the Shizuoka Laboratory Animal Center (Hamamatsu, Japan), and Foxp3-GFP knock-in C57BL/6 (Foxp3^{GFP}) mice [19] were purchased from the Jackson Laboratory (Bar Harbor, ME). The mice were maintained under standard conditions and fed rodent food and water. All animal experiments performed in this study were approved by the Committee on the Care and Use of Laboratory Animals at the National Research Institute for Child Health and Development (Permission number: A2009-010-C11) and performed according to their recommendations.

4.2. Preparation of AP-CF

The AP-CF containing β-glucan as a main compound was prepared as described elsewhere [9]. Briefly, *A. pullulans* was grown at 24.5 °C for 4 days, in a medium containing 2% sucrose, 0.3 % rice bran, 0.08% sodium L-ascorbate, 0.02% L-ascorbic acid. After the cultured medium was heated at 90 °C for 30 min, this heat-sterilized cultured medium was diluted to the concentration of 3.6 mg/ml β-glucan with sterilized water, and then used as the AP-CF in this study. The amount of remaining low molecular weight sugars in AP-CF used in this study was 0.33 mg/ml by measurement of a phenol-sulfuric acid method.

4.3. ConA injection

To assess the effects of oral administration of AP-CF on the serum ALT level after ConA injection, male 8–12 week-old C57BL/6J mice were orally administered with AP-CF by gavage at the dosage of 50 mg β-glucan/kg or an equivalent amount of PBS for 7 days, and then ConA (15 mg/kg; Sigma-Aldrich, St Louis, MO) was intravenously injected into the mice. To evaluate the effects of the oral administration of AP-CF on the T_{reg} populations after the ConA injections, Foxp3^{GFP} were orally administered by gavage at the dosage of 400 μl of AP-CF (containing 1.44 mg β-glucan) daily for 7 days, and then ConA (15 mg/kg) was intravenously injected into the mice. The control group of mice was administered the same amount of phosphate buffered saline (PBS) by gavage. The daily administration of AP-CF or PBS was maintained until the end of the experiments.

4.4. Measurement of serum ALT

The mice were anesthetized, and about 500 μl of blood samples were collected from the crural vein using a 21G needle. After incubation for 30

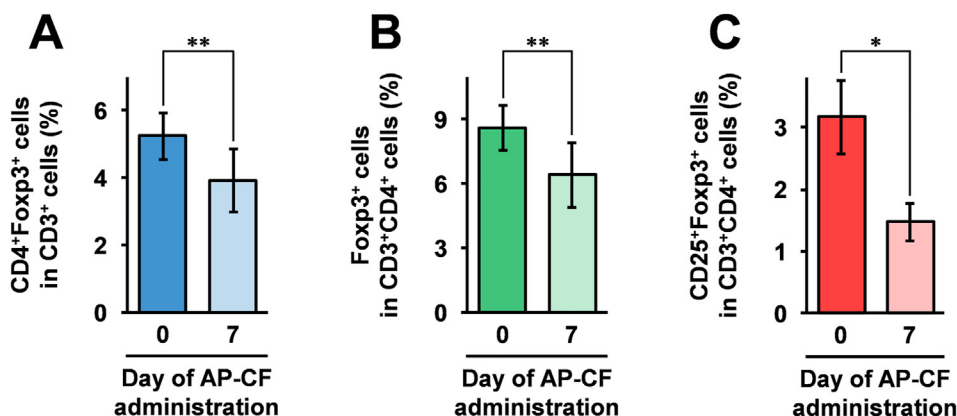


Figure 5. Effects of oral administration of AP-CF on T_{reg} populations in the peripheral blood leukocytes of Foxp3^{GFP} mice. (A–C) Foxp3^{GFP} mice were orally administered with AP-CF (1.44 mg/mouse, 47.9–57.6 mg/kg) or PBS by gavage for the indicated number of days, after which peripheral blood leukocytes were isolated. Then the isolated lymphocytes were immunofluorescently stained with anti-CD3, anti-CD4, and anti-CD25 antibodies, and analyzed by flow cytometry. The 0 time shows data before the AP-CF administration, and error bars indicate standard deviations. The asterisk (*), and double asterisks (**) indicate that the differences are statistically significant; *: p < 0.05, **: p < 0.01.

min at 37 °C, the blood samples were centrifuged at 700 × g at room temperature, and the supernatant was used as the serum samples. A 10 µl serum sample was subjected to the monitoring of the ALT activity, and the ALT activity was measured using Fuji DRI-CHEM slides of GPT/ALT-PIII (Fujifilm, Tokyo, Japan) and an automatic analyzer (DRI-CHEM3500i, Fujifilm).

4.5. Analysis of mouse peripheral blood lymphocytes

The 10–15 µl of peripheral blood samples were collected from the tail vein using a 21G needle. The collected blood samples were suspended in 1 ml of sodium heparin solution (40 units/ml in PBS; Novo-Heparin®, Mochida Pharmaceutical, Tokyo, Japan). The samples were centrifuged at 1,500 rpm for 5 min at 4 °C, and the supernatant was removed by decantation. Sample pellets were resuspended in the remaining solution by tapping, and the red blood cells were hemolyzed by addition of 1.8 ml distilled water. Immediately after this, 0.2 ml of 10×PBS was added to the samples and suspended, then centrifuged 1,500 rpm for 5 min at 4 °C. This hemolysis procedure was repeated twice, and the supernatant of the samples was removed by decantation. The samples were stained with 1.0 µl of phycoerythrin (PE) conjugated anti-mouse CD3, PE-Cyanine5 (Cy5) tandem conjugated anti-mouse CD4, and Allophycocyanin (APC) conjugated anti-mouse CD25 (BioLegend, San Diego, CA) for 20 min at 4 °C. After staining, the samples were washed twice with 1 ml of PBS, and subjected to flow cytometry analysis (Attune, Thermo Fisher Scientific, Waltham, MA).

4.6. Analysis of mouse liver infiltrating lymphocytes

Mice were chosen randomly from each group. The liver was mashed and passed through a 70 µm nylon cell strainer on ice. Then, the tissue suspension was centrifuged at 4 °C for 1 min 60 × g, and the floating cells were collected from the supernatant. The procedure for the collection of floating cells was repeated twice by washing with PBS. Non-parenchymal liver cells were isolated by centrifugation at room temperature for 25 min over a 40% discontinuous Percoll gradient (Sigma-Aldrich). The isolated cells were suspended in PBS and filtered with 100 µm filter, and then the cells were washed with PBS. The 1.0×10⁶ cells were stained with 1.0 µl of PE conjugated anti-mouse CD3, PE-Cy5 tandem conjugated anti-mouse CD4, and APC conjugated anti-mouse CD25 (BioLegend) for 20 min at 4 °C. After washing twice with 1 ml of PBS, the cells were subjected to flow cytometry analysis (Attune, Thermo Fisher Scientific).

4.7. Analysis of mouse splenocytes

The spleens extracted from the mice were cut into fragments, and suspended in PBS. After the cell suspension was centrifuged at 1,200 rpm for 5 min, at 4 °C, the supernatant was removed by decantation. The cells were resuspended into the remaining supernatant by tapping, and hemolyzed with 4.5 ml of distilled water. Immediately following this, 0.5 ml of 10×PBS was mixed into the samples, and then the samples were centrifuged at 1,200 rpm for 5 min, at 4 °C. Next, 1.0×10⁶ cells were stained with 1.0 µl of PE conjugated anti-mouse CD3, PE-Cy5 tandem conjugated anti-mouse CD4, and APC conjugated anti-mouse CD25 (BioLegend) for 20 min at 4 °C. After washing twice with 1 ml of PBS, the cells were subjected to flow cytometry analysis (Attune, Thermo Fisher Scientific).

4.8. Statistical analysis

The results were expressed as the mean and standard deviation (mean ± SD). All data were analyzed using the GraphPad Prism software program (version 7.0, GraphPad Software, San Diego, CA). One-tailed unpaired Student's t-tests were used to compare two groups. In the case of normal distributions, one-way ANOVA analysis with the Tukey's multiple comparison test were used to compare multiple groups. In non-normal

distributions, the Kruskal-Wallis test analysis with Dunn's multiple comparison test were used to compare multiple groups. In the results, P values of <0.05 were considered to indicate statistically significant differences.

Declarations

Author contribution statement

Zhidan Wang: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Shuang-Qin Yi: Conceived and designed the experiments.

Masayuki Fujino and Kisato Kusano: Conceived and designed the experiments; Analyzed and interpreted the data.

Kuai Ma: Performed the experiments; Analyzed and interpreted the data.

Atsushi Iwai: Analyzed and interpreted the data; Wrote the paper.

Xiao-Kang Li: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

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

Declaration of interests statement

The authors declare the following conflict of interests: Atsushi Iwai and Kisato Kusano are employees of Aureo Co., Ltd., and β-glucan-containing *Aureobasidium pullulans*-cultured fluid and its derivatives are marketed by Aureo Co., Ltd.

Additional information

No additional information is available for this paper.

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