Induction of Regulatory T Cells as a Novel Mechanism Underlying the Therapeutic Action of Kakkonto, a Traditional Japanese Herbal Medicine, in a Murine Food Allergy Model

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Key Words
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Abstract
Background: The number of patients with food allergy (FA) has dramatically increased. Although satisfactory drug therapies for FA are not available, we have found that kakkonto, a traditional Japanese herbal medicine, suppressed the occurrence of allergic symptoms in an FA mouse model. Thus, we investigated whether kakkonto could regulate the activation and differentiation of T cells in the colon. Methods: BALB/c mice were systemically sensitized and then orally challenged with ovalbumin. FA mice were orally treated with kakkonto. Lamina propria (LP) cells from their colons were isolated and analyzed. Results: Kakkonto significantly reduced the proportion of CD69+ cells and the elevated helper T cell type 2-specific transcription factor GATA-3 mRNA expression in the LP CD4+ T cells, showing that kakkonto has a suppressive effect on the activation and Th2 differentiation of LP effector CD4+ T cells of the FA mouse colon. Furthermore, kakkonto significantly increased the proportion of Foxp3+CD4+ regulatory T cells in the LP CD4+ T cells of the FA mouse colon. Similarly, the number of Foxp3-positive cells was dramatically increased in the colonic mucosa of kakkonto-administered FA mice. However, the pharmacological effect and Foxp3+CD4+ regulatory T cell-inducing ability of kakkonto were not attenuated by the administration of an anti-CD25 monoclonal antibody in the FA model. Conclusions: The induction of Foxp3+CD4+CD25− regulatory T cells in the colon as a novel mechanism underlying the therapeutic action of kakkonto could be utilized for the development of a novel anti-FA drug.

Introduction
The number of patients with food allergy (FA) has significantly increased over the last several decades. However, effective drug therapies for FA are not available. We have previously suggested that kakkonto is a potential therapeutic drug for FA, using our mouse FA model with gastrointestinal symptoms [1]. Kakkonto is a traditional Japanese herbal medicine that originated in China and is composed of the following 7 species of medicinal plants:
In this study, we tested our working hypothesis that kakkonto has the potential to induce regulatory T cells, thereby suppressing the occurrence of allergic symptoms using an experimental FA mouse model.

**Materials and Methods**

**Animals**

Five-week old male BALB/c mice were purchased from Japan SLC Inc. (Shizuoka, Japan) and were housed in the experimental animal facility at the University of Toyama. This study was approved by the Animal Experiment Committee at the University of Toyama (authorization No. S-2009INM-9) in accordance with the Guide for the Care and Use of Laboratory Animals at the University of Toyama, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology, Japan.

**Experimental Protocol of the Murine FA Model**

Induction of FA and treatment with kakkonto were performed, as previously described [1]. Briefly, mice were sensitized twice at 2-week intervals with 50 μg ovalbumin (OVA, Fraction V; Sigma-Aldrich, St. Louis, Mo., USA) in the presence of 1.3 mg aluminum hydroxide gel (Sigma-Aldrich) as an adjuvant by intraperitoneal injection. Two weeks after systemic priming, the mice were repeatedly given 50 mg OVA dissolved in water with intragastric feeding needles 3 times per week. Diarrhea was assessed by visually monitoring the mice for up to 1 h following the intragastric challenge. For the kakkonto treatment in the FA model, kakkonto (100, 200 or 500 mg/kg) was orally administered to mice 1 h before the oral OVA challenge. Kakkonto extracted with hot water from a mixture of 7 medicinal herbs was obtained from Tsumura Co. (Tokyo, Japan) as a dried powder with the 3D-HPLC data (online suppl. fig. S1; see www.karger.com/doi/10.1159/000445433 for all online suppl. material). The dose of kakkonto was extrapolated from the conventional dose for humans. The administration of kakkonto had no adverse effects on body weight, stool state, behavior and pelage.

**Isolation of LP Cells**

One hour after the 6th oral OVA challenge, the colon was excised and flushed with ice-cold saline. LP cells were isolated from the colon as previously described [17]. In brief, the colon was dis-
sected into short segments and stirred at 37°C in RPMI 1640 containing 2% FCS and 0.5 mM EDTA for 20 min. LP cells were isolated by an enzymatic dissociation procedure using collagenase IV (Nitta Gelatin, Osaka, Japan) or collagenase (Wako, Osaka, Japan). Discontinuous Percoll density gradient centrifugation was performed to purify the LP cells. The single-cell suspensions from the spleen and mesenteric lymph node (MLN) were prepared by passing them through a 70-μm mesh filter.

**Flow Cytometry Analysis**

To analyze cell surface markers by flow cytometry, LP cells were stained for 30 min at 4°C with the following monoclonal antibodies (mAbs) from BD Pharmingen, San Diego, Calif., USA: FITC-conjugated anti-mouse CD3ε (clone 145-2C11), PE-conjugated anti-mouse CD4 (clone RM4-5), APC-conjugated anti-mouse CD4 (clone RM4-5), APC-conjugated anti-mouse CD8 (clone 53-6.7), FITC-conjugated anti-mouse CD25 (clone 3C7), FITC-conjugated anti-mouse CD69 (clone H1.2F3), and PE-conjugated anti-mouse CTLA4 (clone UC10-4B9) from Biologie, San Diego, Calif., USA.

LP cells were gated on lymphocytes by analysis of forward- and side-scatter (FSC/SSC). Dead lymphocytes were excluded by negative gating of a BD Pharmingen viaprobe-positive fraction, and live lymphocytes were gated on CD4+ or CD3+ cells. Staining for intracellular Foxp3 was performed according to the manufacturer’s instructions using the Foxp3 staining buffer set (eBioscience, San Diego, Calif., USA) and APC-conjugated anti-mouse/rat Foxp3 (clone FJK-16s; eBioscience). Cells were then washed with PBS-BSA and permeabilized for 1 h in fixation/permeabilization working solution (eBioscience). Cells were then preincubated with a mouse Fc receptor-blocking reagent (MACS, Auburn, Ala., USA) for 5 min before staining to prevent antibody binding to the Fc receptor. A viaprobe was used to discriminate between dead and live cells. Staining with isotype control antibodies was performed in all experiments, and discrimination of positively stained cells was based on data obtained with isotype control antibodies. Flow cytometry analysis was performed using FACS Calibur or FACS Canto II (BD Biosciences, Franklin Lakes, N.J., USA). All data are representative of at least 3 independent experiments.

**Quantitative Real-Time PCR Analysis**

CD4+ T cells were separated from LP cells using the Mouse CD4+ T-Lymphocytes Enrichment Set and IMagnet (BD Pharmingen) according to the manufacturer’s instructions. The purity of CD4+ T cells in the separated cells typically ranged from 90 to 95%. Total RNA was isolated from CD4+ T cells using RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer’s instructions. Reverse transcription was performed using the PrimeScript RT reagent kit (Takara Bio, Shiga, Japan) and random primers. Quantitative real-time PCR was then performed using SYBR Premix Ex Taq (Takara Bio) and the Thermal Cycler Dice real-time system (Takara Bio). The following primer pairs were used: GATA-3, forward 5′-AGAGATTTCAGATCTGGGATTGGGGGTAG-3′ and reverse 5′-GACGGACAC-ATTGGGGGTAG-3′. The target mRNA levels were normalized to that of GAPDH mRNA as an internal control in each sample. The results are expressed as the ratio relative to CD4+ T cells in the LP cells of normal mice.

**Immunohistochemistry**

Immunohistochemistry was performed according to the method previously described [1]. Briefly, the excised proximal colon was fixed in 4% paraformaldehyde (w/v) in 0.1 M sodium phosphate buffer (pH 7.3) at 4°C for 12–18 h. Frozen sections (15 μm) were cut at –20°C using a cryostat microtome (Leica Microsystems, Nussloch, Germany). The sections were exposed to anti-mouse Foxp3 antibody (1:1,000; Abcam, Cambridge, UK) for 1 h and incubated with Alexa 488-conjugated donkey anti-rabbit IgG (1:400; Jackson Immunoresearch Laboratories, West Grove, Pa., USA) for 30 min. The primary and secondary antibodies were diluted with Can Get Signal immunostain solution A (TOYOBO, Osaka, Japan). Finally, the sections were mounted in Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, Calif., USA). The preparations were observed using confocal laser-scanning microscopy (LSM700; Carl Zeiss, Oberkochen, Germany). Quantitative image analysis was performed using open-source software ImageJ (a Java-based image processing program developed at the NIH).

**Depletion of CD25+ Cells**

Hybridoma clone PC61.5.3 (anti-mouse CD25 IL-2Rα; TIB-222) was purchased from American Type Culture Collection (Manassas, Va., USA). Hybridoma (106 cells per mouse) was intraperitoneally injected with incomplete Freund’s adjuvant (Wako) to athymic nude mice (BALB/c nu/nu, Japan SLC). Two weeks after the injection, the ascites fluid of the athymic nude mice was harvested. The anti-mouse CD25 mAb was purified using an Ab-Capcher affinity column (Protenova, Tokushima, Japan), and an antibody concentration was measured using the BCA protein assay reagent (Pierce, Rockford, Ill., USA). A total of 500 μg anti-mouse CD25 mAb was intraperitoneally administered to the mice 5 h before each kakkonto treatment. Mock-treated mice were treated with 500 μg normal rat IgG isotype control (MBL, Nagoya, Japan). After the 6th oral OVA challenge, LP cells, splenocytes and lymphocytes in mesenteric lymph node were analyzed by flow cytometry.

**Statistical Analyses**

The data are expressed as mean ± SE. Statistical comparisons were made with the two-tailed, Student unpaired t test, the χ2 test or one-way ANOVA followed by the Dunnett post hoc test for multiple comparisons. p values <0.05 were assumed to be statistically significant.

**Results**

**Effect of Kakkonto on the Mouse Model of FA**

Repeated OVA administrations resulted in a significant increase in the occurrence of allergic diarrhea. In FA mice, the incidence of allergic diarrhea reached 89.0 ± 5.1% after the 6th OVA challenge (5 independent ex-
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Experiments, total n = 46;  fig. 1 a). Administration of 500 mg/kg of kakkonto significantly reduced the incidence of allergic diarrhea and this incidence markedly decreased to 31.2 ± 11.3% after the 6th OVA challenge (p < 0.01; 5 independent experiments, total n = 41;  fig. 1a). Furthermore, kakkonto suppressed the occurrence of diarrhea in a dose-dependent manner. After the 6th OVA challenge, the incidence of allergic diarrhea was significantly reduced by administration of 200 and 500 mg/kg of kakkonto (p < 0.05; 3 independent experiments, total n = 20–28;  fig. 1 b). No kakkonto-related adverse effects were detected in the kakkonto-administered mice.

Effect of Kakkonto on T Cell Function in the Intestinal LP

In our previous study, the administration of kakkonto reduced the enhanced transcription of Th2-related cytokine genes in the proximal colon of FA mice [1]. Therefore, we examined whether kakkonto has a suppressive effect on T cell functions in the colon of FA mice.

We investigated whether 500 mg/kg of kakkonto affects the expression of surface markers on CD3+ T cells in the intestinal LP. The proportion of CD4+ T cells in CD3+ T cells was significantly increased in FA mice (p < 0.01; 69.8 ± 1.5%; n = 4) compared with normal mice (58.7 ± 1.5%; n = 4), but kakkonto treatment did not affect the increased proportion of CD4+ T cells (67.6 ± 1.5%; n = 4;  fig. 2a, b).

In our previous investigation into the mechanism underlying the therapeutic effects of kakkonto in the FA mouse model, we examined the global mRNA expression patterns in the proximal colon of normal mice, FA mice and FA + kakkonto mice using the Affymetrix Genechip mouse genome 430 2.0 array. The results from our previous report revealed that the mRNA expression of cytotoxic T lymphocyte-associated 4 (CTLA-4), a marker of activated T cells, was enhanced in the proximal colon of FA mice; this increased expression was suppressed by kakkonto [1]. Therefore, we further investigated whether kakkonto influences the activation status of CD4+ T cells in the intestinal LP. We analyzed the expression of CD69, an early activation marker antigen of effector T cells, in CD4+ T cells using flow cytometry ( fig. 2c). The proportion of CD69+ CD4+ T cells in the LP CD4+ T cells of FA mice (30.9 ± 2.0%; p < 0.01, n = 9) was much higher than in normal mice (17.6 ± 1.6%; n = 8). Administration of kakkonto significantly reduced the increased proportion of CD69+ CD4+ T cells (p < 0.05; 22.5 ± 1.3%; n = 7;  fig. 2c; online suppl. fig. S2A). Furthermore, we analyzed the expression of CTLA4 and CD25, activation markers of effector T cells, in CD4+ T cells ( fig. 2d, e; online suppl. fig. S2B, C). The proportion
of CTLA4+CD4+ T cells in the LP CD4+ T cells of FA mice was higher than in normal mice, i.e. 7.5 ± 0.9% (n = 7) and 3.5 ± 0.4% (n = 4), respectively (p < 0.01). Administration of kakkonto significantly reduced the increased proportion of CTLA4+CD4+ T cells (p < 0.01; 3.6 ± 0.8%; n = 5). The proportion changes of CD25+ CD4+ T cells were similar to those of CD69+CD4+ T cells and CTLA4+CD4+ T cells. The proportion of CD25+ CD4+ T cells significantly increased in the LP CD4+ T cells of FA mice (normal mice: 11.4 ± 0.5%; n = 4 and FA
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**Fig. 3.** Effect of kakkonto on Foxp3 expression in CD4⁺ T cells. Representative flow cytometric data (a) and combined data from all mice (b) show that the proportion of Foxp3⁺ CD4⁺ T cells is increased in FA + kakkonto mice. Kakkonto (500 mg/kg) was orally administered to FA mice. Data are gated on live CD4⁺ T cells and the proportions of Foxp3⁺ cells of total CD4⁺ T cells are indicated. * p < 0.05 (n = 3–7). c Foxp3 mRNA expression in the CD4⁺ T cells of LP cells is measured by real-time PCR. The level of Foxp3 mRNA expression is upregulated in FA + kakkonto mice. * p < 0.05 (4 independent experiments, total n = 8–12). Representative photographs of immunohistochemical staining with anti-Foxp3 antibody (green; d) and summarized data (e) show that the number of Foxp3⁺ cells is increased in FA + kakkonto mice. Colors refer to the online version only. Arrows indicate Foxp3⁺ and DAPI-positive cells. Scale bar = 50 μm. ** p < 0.01 (n = 4–8).
mice: 19.8 ± 2.6%; n = 4; p < 0.01) and significantly decreased after kakkonto treatment (p < 0.01; 11.2 ± 0.7%; n = 4). These results demonstrated that the administration of kakkonto prevented the activation of CD4+ T cells in the intestinal LP of FA mice.

To further elucidate the CD4+ T cell profile in the LP, the mRNA expression level of the Th2-specific transcription factor GATA-3 in the LP CD4+ T cells was examined by real-time PCR. As shown in figure 2f, the expression level of GATA-3 mRNA in the LP CD4+ T cells of FA mice was almost 10-fold higher than that of normal mice, i.e. 9.7 ± 2.6 and 1.0 ± 0.4, respectively (for both: 6 independent experiments, total n = 12). Treatment with 500 mg/kg of kakkonto significantly reduced the increased GATA-3 mRNA expression to almost one third in the LP CD4+ T cells of FA mice (p < 0.05; 21.5 ± 2.2%; n = 7) compared with normal mice (Foxp3+ CD4+ CD25– T cells: 9.3 ± 0.2% and Foxp3+ CD4+ CD25+ T cells: 11.8 ± 0.9%; n = 4). Administration of kakkonto slightly increased the proportion of Foxp3+ CD4+ CD25– T cells (p < 0.01; 12.1 ± 0.8%; n = 6) compared with FA mice. On the other hand, administration of kakkonto was not attenuated by the depletion of CD25+ cells. Thus, we next investigated if the therapeutic effect of kakkonto was affected by the depletion of CD25+ cells. As shown in figure 4a, repeated OVA administrations resulted in allergic diarrhea in the FA model. Oral administration of kakkonto significantly suppressed the occurrence of allergic diarrhea, but the suppressive effect of kakkonto was not attenuated by the depletion of CD25+ cells by the anti-CD25 mAb injection.

To examine whether Foxp3+ regulatory T cells in the LP were depleted by the anti-CD25 mAb injection, we assessed the proportion of Foxp3+ CD4+ T cells with flow cytometry. Anti-CD25 mAb treatment did not affect the proportion of Foxp3+ CD4+ T cells in the LP CD4+ T cells of FA + kakkonto mice (FA + kakkonto mice: 16.8 ± 2.8%, n = 4 and FA + kakkonto + anti-CD25 mAb mice: 14.3 ± 3.1%, n = 4; fig. 4b). In contrast, the proportion of CD25+ T cells in CD4+ T cells was markedly decreased by the anti-CD25 mAb administration, i.e. from 13.1 ± 1.4 to 5.4 ± 0.2% in the splenocytes (n = 4; p < 0.01; fig. 4c) and from 12.0 ± 0.3 to 4.0 ± 0.0% in MLN cells (n = 4; p < 0.01; fig. 4d).

We further assessed the proportions of Foxp3+ CD4+ CD25+ T cells and Foxp3+ CD4+ CD25+ T cells in the LP CD4+ T cells (fig. 4e). The proportions of Foxp3+ CD4+ CD25+ T cells and Foxp3+ CD4+ CD25+ T cells in LP CD4+ T cells were slightly reduced in FA mice (Foxp3+ CD4+ CD25+ T cells: 8.1 ± 0.8% and Foxp3+ CD4+ CD25+ T cells: 10.3 ± 0.9%; n = 4) compared with normal mice (Foxp3+ CD4+ CD25+ T cells: 9.3 ± 0.2% and Foxp3+ CD4+ CD25+ T cells: 11.8 ± 0.9%; n = 4). Administration of kakkonto significantly increased the proportion of Foxp3+ CD4+ CD25+ T cells (p < 0.01; 12.1 ± 0.8%; n = 6) compared with FA mice. On the other hand, administration of kakkonto slightly increased the proportion of Foxp3+ CD4+ CD25+ T cells (12.3 ± 1.2%; n = 6).

**Discussion**

This study demonstrated that kakkonto increased the proportion of LP Foxp3+ CD4+ CD25+ regulatory T cells in the FA model and thereby suppressed the activation and Th2 differentiation of LP effector CD4+ T cells, which attenuated the development of Th2-mediated FA in mice. Therefore, the present findings suggest that the induction of regulatory T cells in the intestine may provide a novel strategy in the treatment of FA.
Effect of Kakkonto on the Activation Status of CD4+ T Cells in the Intestinal LP

Th2-dominant immune status in the intestinal LP is associated with the development of allergic symptoms. CD69 is expressed on the surface of activated effector CD4+ T cells and regarded as one of surface markers of activated effector CD4+ T cells. The proportion of CD69+ T cells increases as a result of the development of symptoms in mouse models of arthritis [18] and allergic airway inflammation [19], and CD69+ mice displayed a marked attenuation of these inflammatory responses in these animal models. In this study, the proportion of CD69+ T cells significantly increased in the LP CD4+ T cells of FA mice, and significantly decreased after kakkonto treatment (fig. 2c). Similarly, the increased proportions of CD25+ T cells and CTLA4+ T cells in FA mice were markedly decreased after kakkonto treatment (fig. 2d, e). Furthermore, the expression of GATA-3 mRNA in LP CD4+ T cells was downregulated by kakkonto treatment (fig. 2f). Therefore, it is indicated that kakkonto treatment sup-

**Fig. 4.** Effects of kakkonto on allergic diarrhea following anti-CD25 mAb treatment. 

- Repeated oral challenges with 50 mg OVA result in allergic diarrhea. FA + kakkonto mice were treated with the injection of anti-CD25 mAb to deplete CD25+ T cells. The occurrence of allergic diarrhea is significantly suppressed by kakkonto administration with or without the injection of anti-CD25 mAb. *p < 0.05, **p < 0.01 (n = 8–10). The suppressive effect of kakkonto on allergic diarrhea is not attenuated by anti-CD25 mAb compared with FA + kakkonto mice (n = 8–10). 

- Anti-CD25 mAb administration has no effect on the proportion of Foxp3+CD4+ T cells compared with FA + kakkonto mice. Data are gated on live CD4+ T cells, and the proportions of Foxp3+ cells of total CD4+ T cells are indicated (n = 4). Flow cytometric data in splenocytes (c) and MLN cells (d) reveal that anti-CD25 mAb administration greatly reduces the proportion of CD4+CD25+ T cells in the spleen and MLN of FA + kakkonto mice. Data are gated on live CD4+ T cells and the proportions of CD25+ cells of total CD4+ T cells are indicated. **p < 0.01 (n = 4). 

- Flow cytometric data in LP cells reveal that the proportion of Foxp3+CD4+CD25+ T cells is increased in FA + kakkonto mice. Data are gated on live CD4+ T cells and the proportions of Foxp3+CD4+CD25+ and Foxp3+CD4+CD25- cells of total CD4+ T cells are indicated. *p < 0.05, **p < 0.01 (n = 4–6).
presses the differentiation of naïve T cells into effector Th2 cells in the LP of FA mice.

Taken together, these results reveal that the suppression of activated effector CD4+ T cells and the differentiation of naïve T cells by kakkonto treatment induce the attenuation of Th2-mediated mucosal immune responses in the intestine, thereby ameliorating the allergic symptoms.

**Induction of Regulatory T Cells by Kakkonto in the Murine FA Model**

Foxp3+CD4+ regulatory T cells maintain intestinal immune homeostasis through the regulation of various effector T cell functions [10]. This study showed that kakkonto administration increased the proportion of Foxp3+CD4+ regulatory T cells in the LP in our FA model (fig. 3a, b). Kakkonto contains many bioactive components because it is extracted from 7 medicinal herbs. There are several reports that the bioactive components contained in kakkonto exert the antiallergic effects and have immunosuppressive effects. Li et al. [20] reported that glycyrrhizin, a component of *G. radix*, has therapeutic effects and reduces IL-4 levels in the nasal mucosa of allergic rhinitis mice. Dong et al. [21] reported that puerarin, a component of *P. radix*, has therapeutic effects on the development of allergic asthma in a rat model, reduces the release of IL-4 in the serum and suppresses the transcription of IL-4 mRNA in the lungs of rats. These components might increase the proportion of regulatory T cells in the allergic models and thereby reduce IL-4 levels. Furthermore, Wang et al. [22] reported that several bioactive components contained in medicinal herbs induce Foxp3+CD4+ regulatory T cells via an aryl hydrocarbon receptor-dependent pathway. It was therefore assumed that the induction of Foxp3+CD4+ regulatory T cells might be involved in the underlying mechanisms of kakkonto.

Regulatory T cells play a key role in the treatment of Th2-dominant inflammatory diseases. The induction of regulatory T cells ameliorates the symptoms of Th2-type diseases such as human skin inflammation [23], mouse airway inflammation [24] and mouse diarrhea caused by food allergies [25]. In addition, it has been already reported that these cells suppress not only the differentiation of naïve T cells to effector T cells, but also the activation of effector T cells [26]. Our results indicated that kakkonto treatment suppresses the activation and differentiation of effector Th2 cells in the LP through the induction of Foxp3+CD4+ regulatory T cells. Our FA model thus suggests that the induction of Foxp3+CD4+ regulatory T cells by kakkonto ameliorates allergic diarrhea.

**Involvement of Foxp3+CD4+CD25+ Regulatory T Cells in the Therapeutic Effect of Kakkonto**

The proportion of CD25+ T cells in CD4+ T cells was markedly decreased by the anti-CD25 mAb administration in the splenocytes (fig. 4c) and MLN cells (fig. 4d), but the anti-CD25 mAb administration did not affect the proportion of Foxp3+CD4+ regulatory T cells in the LP CD4+ T cells of the FA + kakkonto mice (fig. 4b) and the therapeutic effect of kakkonto in the FA model (fig. 4a). Furthermore, kakkonto treatment significantly increased the proportion of Foxp3+CD4+CD25+ T cells (fig. 4e). Thus, Foxp3+CD4+ regulatory T cells induced by kakkonto administration were assumed to exhibit a CD25-negative phenotype, suggesting that this CD25-negative phenotype of Foxp3+CD4+ T cells can exert the therapeutic effect in the FA model.

In immunological studies over the past decade, Foxp3+CD4+CD25+ regulatory T cells have been the most actively investigated, and numerous studies have demonstrated the importance of Foxp3+CD4+CD25+ regulatory T cells in immune regulation [10]. However, several studies have recently revealed that regulatory T cells have several phenotypes [10]. Regarding intestinal immunity, Hauert-Broere et al. [27] reported that functional mucosal regulatory CD4+ T cells are induced within both the CD25+ and CD25- population of MLN and Peyer’s patches in their oral tolerance study. Furthermore, Sun et al. [28] demonstrated that CD25- regulatory CD4+ T cells as well as CD25+ regulatory CD4+ T cells in the MLN can strongly suppress effector T cell responses to antigens and that both these cell populations express the Foxp3 gene. Additionally, in the systemic immunity, Foxp3+CD4+CD25- T cells are detected in systemic lupus erythematosus [29] and in aged mice [30]. However, the function of these Foxp3+CD4+CD25- regulatory T cells is not yet well understood. Moreover, mice lacking CD25 display a slight decrease in the proportion of Foxp3+CD4+ T cells within the thymus, whereas the peripheral proportions are unchanged [31]. These data support our present findings, but the classification of Foxp3+CD4+CD25- T cells and their functions remain under investigation, especially in the intestine.

It has been reported that Foxp3+CD4+CD25+ regulatory T cells exert immunosuppressive effects through the secretion of soluble factors (IL-10, TGF-β, IL-35 and galectin-1), cell contact and IL-2 depletion [10, 32]. It has also been reported that Foxp3+CD4+CD25+ regulatory T cells have suppressive effects on the immune response in several immune diseases [27, 28, 33–35]; however, the molecular mechanisms underlying these effects remain under investigation.
Microarray analysis of CD4+Foxp3+ T cells reveals that the CD25+ and CD25− phenotypes of CD4+Foxp3+ T cells share a regulatory T cell gene expression profile [33], suggesting that these phenotypes cells might have several common mechanisms for their immunosuppressive functions. Recently, Coleman et al. [35] reported that CD4+Foxp3+CD25− regulatory T cells from the lungs of mice suppress the proliferation of CD4+ effector T cells in vitro. Furthermore, they demonstrated that the release of IL-10 is one of the underlying mechanisms of the immunosuppressive effect of CD4+Foxp3+CD25− regulatory T cells in a cell contact-independent manner. Fontenot et al. [33] reported that both the CD25+ and CD25− phenotypes of CD4+Foxp3+ T cells express high levels of IL-10 mRNA. Thus, it was assumed that the expression of IL-10 mRNA might be upregulated in the colon of FA + kakkonto mice. However, we previously demonstrated that kakkonto reduces the expression of IL-10 mRNA in the proximal colon of FA mice. Several kinds of immune cells have potentials for IL-10 secretion in the mouse colon and relatively few Foxp3+CD4+CD25− T cells are distributed in the colon. Therefore, the change in IL-10 mRNA expression in the proximal colon may not reflect that in the Foxp3+CD4+CD25− regulatory T cells, even if the IL-10 secretion from these cells is increased in the proximal colon. Therefore, IL-10 secretion from intestinal Foxp3+CD4+CD25− regulatory T cells remains unclear. The immunosuppressive function of Foxp3+CD4+CD25+ regulatory T cells is attributed to the secretion of TGF-β [10, 32], but it remains unclear whether Foxp3+CD4+CD25− regulatory T cells secrete TGF-β. In addition, similar to IL-10 mRNA expression, kakkonto treatment did not affect the expression of TGF-β mRNA in the proximal colon of FA mice (FA mice: 1.6 ± 0.1; n = 5 and FA + kakkonto mice: 1.4 ± 0.2; n = 5; online suppl. fig. S3). Therefore, it remains unclear whether IL-10 and TGF-β secretion from the intestinal Foxp3+CD4+CD25+ regulatory T cells is involved in their immunosuppressive function.

Our data suggest that Foxp3+CD4+CD25− regulatory T cells induced by kakkonto have immunosuppressive effects on the allergic responses in the FA mouse colon.

**Kakkonto Treatment for the FA**

There is currently no effective medicine for the treatment of FA. The clinical treatment comprises only the use of preventative elimination diets to avoid food antigens. Recent clinical trials have demonstrated that oral immunotherapy (OIT) can induce oral tolerance and thereby inhibit allergic reactions in some population of patients with FA [36]. However, it is necessary for OIT to administer food antigens in the long term. Thus, there is a risk that the administration of food antigens may induce severe adverse reactions including anaphylactic shock. Our data indicate that the administration of kakkonto can induce regulatory T cells during food antigen administration, thereby reducing food-allergic responses. Therefore, kakkonto has potential as a therapeutic medicine for ensuring the induction of oral immune tolerance by OIT, reducing various risks caused by OIT and shortening the therapeutic period of OIT in humans.

In conclusion, kakkonto ameliorates the gastrointestinal symptoms in the mouse FA model; this is attributed to the induction of Foxp3+CD4+CD25− regulatory T cells. It therefore has potential as a therapeutic drug for the treatment of immune diseases induced by the disruption of intestinal mucosal tolerance. The mechanism of the induction of Foxp3+CD4+CD25− regulatory T cells in the colon could be utilized for the development of a novel anti-FA drug.

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**References**
