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Feeding Administration of Daikenchuto Suppresses Colitis Induced by Naive CD4⁺ T Cell Transfer into SCID Mice

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Short title: DKT suppresses experimental colitis

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Abstract

Background and Aims Daikenchuto, a traditional Japanese herbal medicine, suppresses bacterial translocation by improvement of gastrointestinal motility and blood flow. As Daikenchuto reportedly reduces gastrointestinal inflammatory activity by these mechanisms, we analyzed whether Daikenchuto suppresses experimental colitis and reduces inflammatory cytokine expression in a mouse model.

Methods Colitis was induced by transfer of naive CD4⁺ T cells of BALB/c mice into SCID mice, and mice were given either control or 2.7% Daikenchuto-containing feed. We investigated body weight, clinical symptoms, histological changes and Th1- and Th17-cytokine expression. Cytokine mRNA expression was analyzed using real-time RT-PCR. The ratio of IL-17⁺ and IFN- γ ⁺ CD4⁺ T cells were analyzed by flow cytometry.

Results Daikenchuto delayed the development of colitis and significantly reduced the histological inflammation scores. Analyses of cytokine mRNA revealed that Th17 cytokines were significantly decreased in colons of mice that received Daikenchuto. Absolute numbers of IL-17⁺ or IFN- γ ⁺ CD4⁺ T cells per colon were less in mice receiving Daikenchuto than in mice that received control feed, as both groups received naive CD4⁺ T cells to induce colitis.

Conclusions We demonstrated that feeding administration of Daikenchuto suppresses colitis induced by naive CD4⁺ T cell transfer into SCID mice. Daikenchuto may show clinical benefit in the treatment of human inflammatory bowel disease and further studies are warranted.

Keywords: Colitis • Inflammatory bowel disease • treatment • Daikenchuto

Introduction

Inflammatory bowel diseases (IBDs), such as Crohn's disease (CD) and ulcerative colitis (UC), are characterized by chronic relapsing inflammation of the gastrointestinal tract. Although the precise etiology of IBDs is still obscure, several researchers have reported that IBDs are multifactorial diseases caused by immunological, genetic and various environmental factors [1–3]. IBD is presumed to be caused by excess immune response to luminal commensal bacteria in genetically susceptible individuals. The aims of treatment are induction of remission and prevention of relapses. Aminosalicylates and immunosuppressive agents such as immunomodulators and anti-tumor necrosis factor (TNF)- α antibodies are commonly used for induction as well as maintenance treatment [4], but there are still a substantial number of patients in whom inflammation recurs. Therefore, in addition to these immunosuppressive agents, there is a strong need for the development of novel efficacious medications.

Daikenchuto (DKT), a traditional Japanese herbal medicine, is integrated in Japan's modern medical system as a pharmaceutical grade ethical drug and widely prescribed for patients with gut-dysfunctional disorder. DKT, consisting of extract powders from zanthoxylum fruit, processed ginger, ginseng, and maltose syrup, has been used to improve gastrointestinal motility, postoperative adhesion, and paralytic ileus after abdominal surgery [5–7]. Several studies show that DKT increases intestinal blood flow [8, 9]. Increased intestinal blood flow may result in improvement of gastrointestinal motility, which suppresses bacterial overgrowth [5, 8, 10]. Another mechanism also has been reported that DKT has an anti-inflammatory effect. Yoshikawa et al. reported that DKT prevented bacterial translocation and inhibited the production of inflammatory cytokines, including IFN- γ , IL-6 and TNF- α , and alleviated intestinal epithelial apoptosis [11]. Kono et al. reported that DKT

suppress colitis by anti-inflammatory effect via up-regulation of endogenous adrenomedullin [12].

Based on these observations, we hypothesize that DKT holds possibility as a new treatment option for IBD. However, further studies are necessary before they can be introduced into clinical practice. In the present study, we investigated whether DKT suppresses murine colitis and reduces inflammatory cytokines related to Th1/Th17 immune responses.

Methods

Animals

BALB/c mice and C.B-17 SCID mice were obtained from CLEA Japan (Tokyo, Japan). All mice were maintained in the specific pathogen-free facilities at Kyushu University. Mice were used at 6 weeks of age. All experiments were conducted with the approval of the Committee on Animal Research of Kyushu University.

Materials

DKT extract powder was obtained from Tsumura & Co. (Tokyo). DKT extract powder was used in the form of a powdered extract obtained by spray-drying a hot water extract of the following three crude drugs: processed ginger (*Zingiber Officinale* ROSCOE, rhizome), ginseng (*Araliaceae*, *Panax ginseng* C.A. MEYER, radix), and zanthoxylum fruit (*Rutaceae*, *Zanthoxylum piperitum* DE CADOLLE) in the ratio of 5:3:2. DKT is prepared by mixing DKT extract powder and maltose syrup powder (Tsumura & Co.) at a ratio of 1:8. Other reagents used for analysis were purchased from commercial source.

Experimental design

C.B.-17 SCID mice were divided into the following four groups. Naive CD4⁺ T cells from BALB/c mice were injected intraperitoneally (i.p.) into SCID mice with or without feeding administration of DKT (n=5, respectively). Phosphate-buffered saline (PBS) instead

of naïve CD4⁺ T cells was injected i.p. with or without feeding administration of DKT (n=5, respectively). Cell isolation of naïve CD4⁺ T cells from BALB/c mice and transfer to SCID mice are described in detail later. Mice were sacrificed at the end of the experimental period, and the removed colons were evaluated in various ways.

Cell Isolation

CD4⁺ T cells were isolated from splenocytes of BALB/c mice using a CD4⁺ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells were isolated from the CD4⁺ T cells with a CD25 microbead kit (Miltenyi Biotec), and CD4⁺CD25⁻CD62L⁺ T cells were subsequently obtained by positive selection with CD62L microbeads (Miltenyi Biotec). All populations were confirmed to be > 95% pure by flow cytometry.

T-Cell Transfer Experiments

Colitis was induced in immunocompromised SCID recipients by transfer of CD4⁺ T cells as previously described [13]. A modified protocol was used in which CD4⁺CD25⁻CD62L⁺ T cells rather than CD4⁺CD45RB^{high} T cells were transferred as a naïve CD4⁺ T cell subset [14, 15]. Briefly, 0.5×10^6 CD4⁺CD25⁻CD62L⁺ T cells were intraperitoneally transferred into SCID mice. Mice were fed standard laboratory food (F-1, Oriental Yeast, Tokyo, Japan) or F-1 mixed with 2.7% DKT (Tsumura & Co., Tokyo, Japan). Mice were weighed weekly for signs of colitis. At various time points, stool consistency was scored on a scale of 0–5 (0–4: normal to severe diarrhea; 5: bloody stools). Mice were euthanized after completion of the experiments, and colon specimens were collected.

Colonic Sample Preparation

We used colon weight/length ratio as a parameter of colonic inflammation as previously described [13]. After the colon had been measured, parts of the distal and proximal colon

were removed for histological analysis; remaining parts were used for mRNA extraction and isolation of lamina propria mononuclear cells (LPMCs).

Histological Analysis

The removed colons were fixed in 10% buffered formalin and embedded in paraffin. Thin sections (6 µm) were cut and stained with hematoxylin-eosin. Histological scoring was performed regarding the grade of infiltration of inflammatory cells, destruction of intestinal structure, and change of crypt structure, in which the methods of Siegmund et al. [16] were modified. For inflammatory cell infiltration, normal numbers of inflammatory cells in the lamina propria were counted as 0, increased numbers of inflammatory cells in the lamina propria were counted as 1, infiltration to the submucosa was counted as 2, and transmural infiltration was counted as 3. For destruction of intestinal structure, no damage of intestinal structure was counted 0, focal lymphoepithelial lesions were counted as 1, mucosal erosion or ulceration was counted as 2, and transmural destruction was counted as 3. For changes in crypt structure, normal structure was counted as 0, goblet cell hyperplasia without reduction was counted as 1, reduction of goblet cells was counted as 2, and crypt abscess was counted as 3. The points of 3 items were added and histological score ranged from 0 to 9, which represented no change to severe changes.

mRNA Expression Assay Using Real-Time RT-PCR

RT-PCR was conducted as previously described [13]. Total RNA was extracted from colonic tissues using RNeasy[®] Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. An aliquot (1 µg) of the total RNA was reverse-transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Cytokine mRNA expressions were analyzed by real-time RT-PCR using TaqMan Gene Expression Master Mix (Applied Biosystems). Real-time RT-PCR was monitored using an ABI Prism 7900HT (Applied Biosystems) with

TaqMan gene expression assay probes. The probe IDs were TNF- α : Mm00443258_m1, IFN- γ : Mm00801778_m1, IL-17A: Mm00439619_m1, IL-17F: Mm00521423_m1.

Isolation of LPMCs

LPMCs were isolated as previously described [17]. Briefly, to remove epithelial cells and intraepithelial lymphocytes, the large intestines were taken out, washed and cut into small pieces. The pieces were incubated with calcium- and magnesium-free Hanks' balanced salt solution supplemented with 2% bovine calf serum, 30 mM HEPES Buffer, 0.170 g/ml DTT and 5 mM EDTA (Sigma-Aldrich, Irvine, UK) on a magnetic stirrer at 37 °C for 20 min. The tissues were then incubated with RPMI 1640 containing 2% bovine calf serum, antibiotics, 25 mM HEPES (Sigma-Aldrich), 0.89 mg/ml collagenase D solution and 0.01 mg/ml DNase (Roche Diagnostics, Indianapolis, IN) for 60 min at 37 °C with stirring. The tissues were squeezed through nylon gauze filters (100 μ m followed by 40 μ m). After washing, the cells were used as LPMCs.

Intracellular Cytokine Staining of LPMCs

Intracellular cytokine staining was performed as previously described [13]. Cells were stimulated with phorbol myristate acetate (50 ng/ml) and ionomycin (1 mg/ml). After surface staining for CD4, the cells were fixed, permeabilized and stained for IFN- γ and IL-17 using FITC-conjugated anti-IFN- γ and PE-conjugated anti-IL-17 Abs (eBioscience, San Diego, CA, USA). The cells were analyzed using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA). FITC-conjugated rat IgG1 and PE-conjugated rat IgG2a were used as isotype matched controls.

Statistical Analysis

The results were expressed as means \pm SEM. The significance of differences between means was determined by Tukey–Kramer test. Differences were considered statistically significant at values of $P < 0.05$.

Results

DKT Prevents Development of Experimental Colitis

Yoshikawa et al. reported that the suppression of bacterial translocation by DKT reduced inflammatory activity in rats [11]. Therefore, we investigated whether DKT could suppress experimental colitis. We chose a model of colitis in C.B-17 SCID mice created by transfer of naive CD4⁺ T cells from normal BALB/c mice, which is associated with augmented Th1- and Th17-responses [13]. Naive CD4⁺ T cells were injected intraperitoneally into SCID mice with or without feeding administration of DKT. Mice that received naive CD4⁺ T cells showed a weight reduction from 7 weeks after the cell transfer, but control mice showed no weight loss at this point. Mice that received naive CD4⁺ T cells with feeding administration of DKT showed little weight loss ($P < 0.05$) (Fig. 1A). The stool consistency score of the mice that received naive CD4⁺ T cells was significantly higher than that of control mice and mice that received naive CD4⁺ T cells with feeding administration of DKT (Fig. 1B). The colonic weight/length ratios of mice that received naive CD4⁺ T cell transfer showed significant increases, but mice that received naive CD4⁺ T cells with feeding administration of DKT showed little increase ($P < 0.05$; Fig. 1C). Colonic histological findings of the mice that received naive CD4⁺ T cells showed severe hypertrophy of the colonic mucosa, infiltration of inflammatory cells and destruction of the crypt structure. The mice that received naive CD4⁺ T cells with feeding administration of DKT showed significantly reduced levels of colonic inflammation compared with those that received only naive CD4⁺ T cells (Fig. 2A). The average histological score of mice that received naive CD4⁺ T cells with feeding administration of DKT was 2.8—significantly lower than 6, which was the score of mice that received naive CD4⁺ T cells ($P < 0.05$; Fig. 2B). These results demonstrate that DKT suppresses the severity of experimental colitis.

Prevention of Development of Experimental Colitis by DKT is Associated with Downregulation of Th1- and Th17-Responses

We then evaluated whether Th1- and Th17-responses are downregulated when DKT suppresses colitis. Analyses of cytokine mRNA expression by real-time RT-PCR revealed that Th17 cytokines (IL-17a and IL-17f) were significantly decreased in colons of mice that received naive CD4⁺ T cells with feeding administration of DKT compared with mice that received naive CD4⁺ T cells alone ($P < 0.05$; Fig. 3A). Levels of IFN- γ (Th1 cytokine) and TNF- α (proinflammatory cytokine) were lower in mice that received naive CD4⁺ T cells with feeding administration of DKT than those in mice that received naive CD4⁺ T cells alone. However, the differences were not statistically significant (Fig. 3B). We investigated IL-17 and IFN- γ expressions by LPMC at the protein level. Flow cytometric analyses revealed that the percentages of both IL-17⁺ and/or IFN- γ ⁺ CD4⁺ T cells among LPMC were not altered between the mice that received naive CD4⁺ T cells alone and the mice that received naive CD4⁺ T cells with feeding administration of DKT (Fig. 4A). However, because the numbers of LPMC of mice that received naive CD4⁺ T cells with feeding administration of DKT were lower than those of mice that received naive CD4⁺ T cells alone, the absolute numbers of Th1 cells (IFN- γ -producing CD4⁺ T cells) or Th17 cells (IL-17-producing CD4⁺ T cells) per colon were decreased in mice that received naive CD4⁺ T cells with feeding administration of DKT compared with mice that received naive CD4⁺ T cells alone (Fig. 4B). These results show that feeding administration of DKT prevents the development of colonic inflammation, in association with downregulation of both Th1- and Th17-responses.

Discussion

This study has proven for the first time that the feeding administration of DKT has a suppressive effect on development of colonic inflammation, in association with downregulating both Th1 and Th17 responses. As Th1- and Th17-responses are implicated in

IBD pathogenesis, we consider that DKT may be potentially useful for treating such disorders.

IBDs are multifactorial, with immunological, genetic and various environmental aspects. With regard to environmental factors, disordered epithelial barrier function and imbalances in intestinal-dwelling commensal bacteria promote bacterial translocation and play important roles in IBD pathogenesis [18, 19]. Bacterial translocation is defined as the passage of bacteria or bacterial products from the gastrointestinal tract to normally sterile tissues, such as the mesenteric lymph nodes or other extra-intestinal sites [20, 21]. As IBD is caused by an unrestrained inflammatory response to bacterial agents, IBD treatment requires control over both environmental factors and immunological factors.

In previous studies, several researchers have reported that DKT increases intestinal blood flow [8, 9] and enhances gastrointestinal propulsive motility [5–7]. Increased intestinal blood flow may result in improvement of gastrointestinal motility, which suppresses bacterial overgrowth [5, 8, 10]. Wang et al. reported that decreased intestinal blood flow and gastrointestinal dysmotility increase intestinal mucosal permeability [22], which causes bacterial translocation. Schultz et al. reported that BT was observed in a murine transfer colitis model [23]; Yoshikawa et al. reported that DKT prevented bacterial translocation by maintaining intestinal barrier [11], which suggests that DKT presents a therapeutic approach to IBD. Additionally, DKT increases *Bacteroides fragili* in the intestine, which was reported to suppress IBD [24]. Sarkis et al. showed that *Bacteroides fragili* protected the host from IBD in an animal model of experimental colitis. These mechanisms indicate that DKT can control epithelial barrier function and keep commensal bacteria within the lumen of the intestine. Therefore, DKT may be able to suppress colitis; and present data shows that DKT suppresses severity of inflammation in a murine transfer colitis model.

Probiotic treatment can also restore a predominance of beneficial *Lactobacillus* and *Bifidobacterium* species in the intestine [25]. Kruis et al. reported that probiotic *E. coli* Nissle 1917 can maintain remission of UC [26]. But probiotic treatment is not effective to maintain CD remission. One reason is that microvascular dysfunction of the intestine in CD is etiologically related to CD recurrence. Blood flow is an important factor in CD pathogenesis. Kono et al. reported that DKT improves decreased blood flow in ischemia that complicates TNBS-induced colitis [12], suggesting that it could be a new therapeutic target for CD by promoting mucosal healing of the intestinal microvasculature.

We originally considered that DKT suppressed colitis because it controls commensal bacteria by accelerating gastrointestinal transit and increasing blood flow. Recently, however, another mechanism of DKT has been reported. Surprisingly, DKT also has an anti-inflammatory effect. Yoshikawa et al. reported that DKT has an anti-inflammatory effect by inhibiting production of inflammatory cytokines, including IFN- γ , IL-6 and TNF- α , and alleviating intestinal epithelial apoptosis [11]. Kono et al. reported that DKT is not immunomodulatory, but may suppress colitis by anti-inflammatory effect via up-regulation of endogenous adrenomedullin [12]. It is not clear whether the suppression of inflammatory cytokines is the result of reduced colonic inflammation or direct effect of DKT on T cells; DKT may have both direct and indirect effects. Further study is needed to clarify this point.

Our examinations showed that DKT downregulated both Th1 and Th17 cytokines. Differences in TNF- α and IFN- γ levels were not statistically significant, because DKT could not suppress the inflammation completely. However, DKT reduced the inflammation and tended to suppress TNF- α and IFN- γ ; therefore we believe differences in IFN- γ and TNF- α levels might be significantly pronounced if a larger number of mice were used. Previous concepts of the Th1/Th2 paradigm in chronic inflammatory and autoimmune diseases have been challenged by the discovery of Th17 cells [27]. Several disorders originally thought to

be Th1-mediated, such as experimental autoimmune encephalomyelitis and collagen-induced arthritis, have been reclassified as Th17-mediated inflammation [28, 29]. Similarly, among experimental colitis models, Th17 cells rather than Th1 cells have been shown to be major effector cells in IL-10-deficient mice [30] and C3H/HeJBir mice [31]; several papers report the involvement of IL-17 in human IBD [32, 33]. Although the Th1/Th17 balance in IBD is unclear, Th17 cells do seem to affect IBD. As DKT suppress both Th1 and Th17 reactions, DKT is expected to be efficacious in treating IBD.

In summary, we have shown for the first time that DKT suppresses experimental colitis by downregulating Th1- and Th17-responses. The results of our study suggest that further investigation into the possibility of DKT as a therapeutic agent in treating human IBDs is warranted.

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Figure legends

Fig. 1. Naive CD4⁺ T cells (0.5×10^6) were injected intraperitoneally (i.p.) into SCID mice with or without feeding administration of Daikenchuto (DKT). Control SCID mice were injected i.p. with phosphate-buffered saline (PBS) instead of naive CD4⁺ T cells. (A) Changes in body weight. Mice that received naive CD4⁺ T cells showed significant weight loss from 7 weeks after the cell transfer. Control mice showed no reduction and mice that received naive CD4⁺ T cells with feeding administration of DKT showed little weight loss. Data represent mean \pm SEM ($n = 5$ per group). (B) Stool consistency scores evaluated at 11 weeks after the cell transfer. Mice that received naive CD4⁺ T cells transfer showed significant score elevation compared with scores of mice that received naive CD4⁺ T cells with feeding administration of DKT, which showed little elevation. (C) Colonic weight/length ratio determined at 11 weeks after the cell transfer. Mice that received naive CD4⁺ T cell transfer showed significantly elevated ratios compared with ratios of mice that received naive CD4⁺ T cells with feeding administration of DKT, which showed little elevation. * $P < 0.05$. ** $P < 0.01$. Representative data of two independent experiments are shown.

Fig. 2. Mice that received naive CD4⁺ T cells with feeding administration of DKT had significantly suppressed levels of colonic inflammation. (A) Colonic histological findings. Mice that received CD4⁺ T cells showed severe hypertrophy, severe mucosal damage and crypt abscess. Mice that received naive CD4⁺ T cells with feeding administration of DKT showed some infiltration of inflammation cells, but suppressed mucosal damage and changes of crypt structure. (B) Average histological score of mice that received naive CD4⁺ T cells was 6; that of mice that received naive CD4⁺ T cells with feeding administration of DKT was

2.8, which was significantly lower than that of mice that received naive CD4⁺ T cells alone.

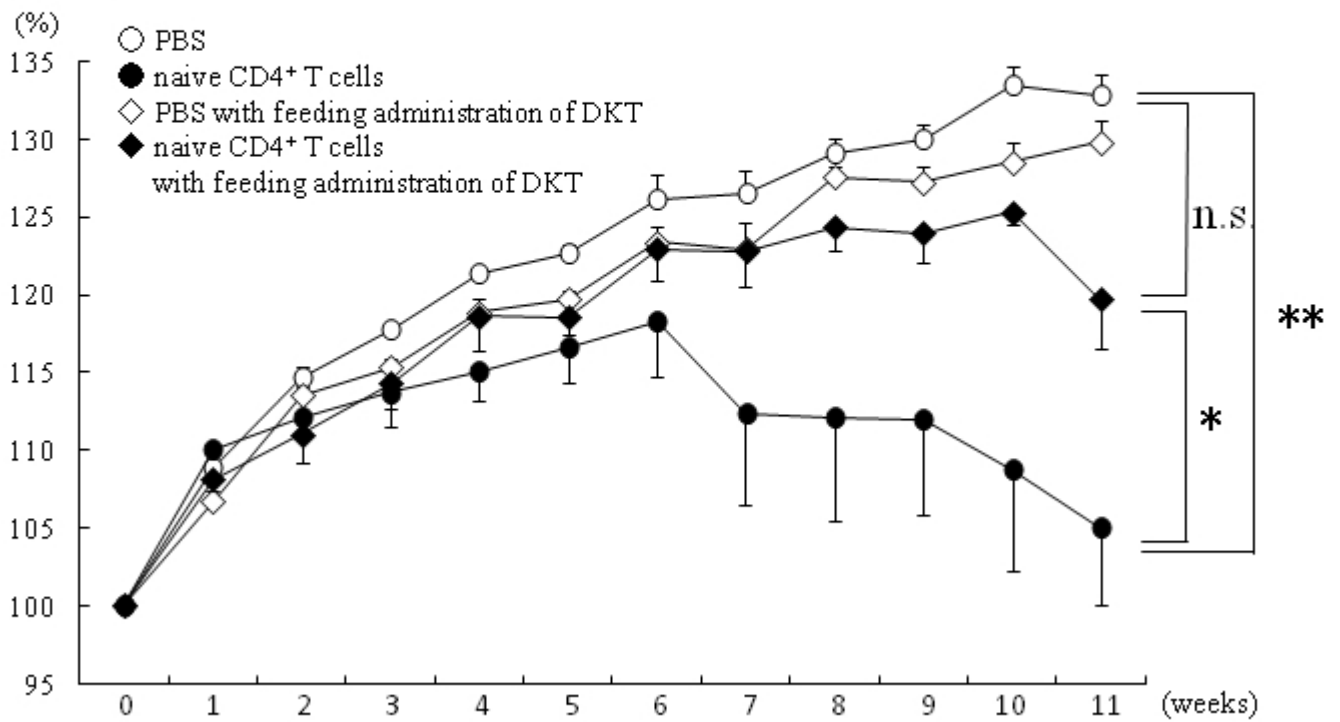
**P* < 0.05. Representative data of two independent experiments are shown.

Fig. 3. Feeding administration of DKT downregulates Th1, Th17 and proinflammatory cytokine mRNA expressions by real-time RT-PCR in the colonic tissues. (A) Th17 cytokines (IL-17a and IL-17f) of mice that received naive CD4⁺ T cells with feeding administration of DKT were significantly decreased compared with mice that received naive CD4⁺ T cells alone. **P* < 0.05. ***P* < 0.01. (B) While levels of IFN- γ of Th1 cytokine and TNF- α of mice that received naive CD4⁺ T cells with feeding administration of DKT were lower than those of mice that received naive CD4⁺ T cells alone, the difference were not statistically significant. Representative data of two independent experiments are shown.

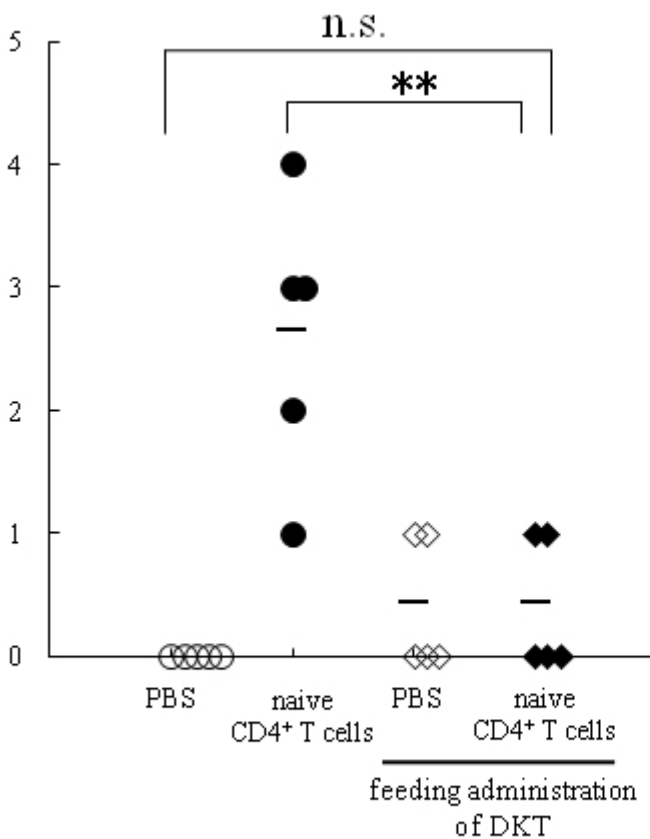
Fig. 4. Feeding administration of DKT suppresses production of colon IL-17⁺ and IFN- γ ⁺ CD4⁺ T cells per colon by LPMC. (A) Flow cytometric analyses of intracellular IFN- γ and IL-17 levels in CD4⁺ T cells among LPMC. Dot plots of the CD4⁺-gated populations are shown. The analyses revealed that the percentages of both IL-17⁺ and IFN- γ ⁺ CD4⁺ T cells among LPMC did not differ between mice that received naive CD4⁺ T cells and those that received naive CD4⁺ T cells with feeding administration of DKT. (B) Absolute numbers of Th1 cells (IFN- γ -producing CD4⁺ T cells) and Th17 cells (IL-17-producing CD4⁺ T cells) per colon were calculated, and were found to be decreased in mice that received naive CD4⁺ T cells with feeding administration of DKT compared with mice that received naive CD4⁺ T cells. Representative data of two independent experiments are shown.

Figure 1

(A) Body weight



(B) Stool consistency



(C) Colon weight / length ratio

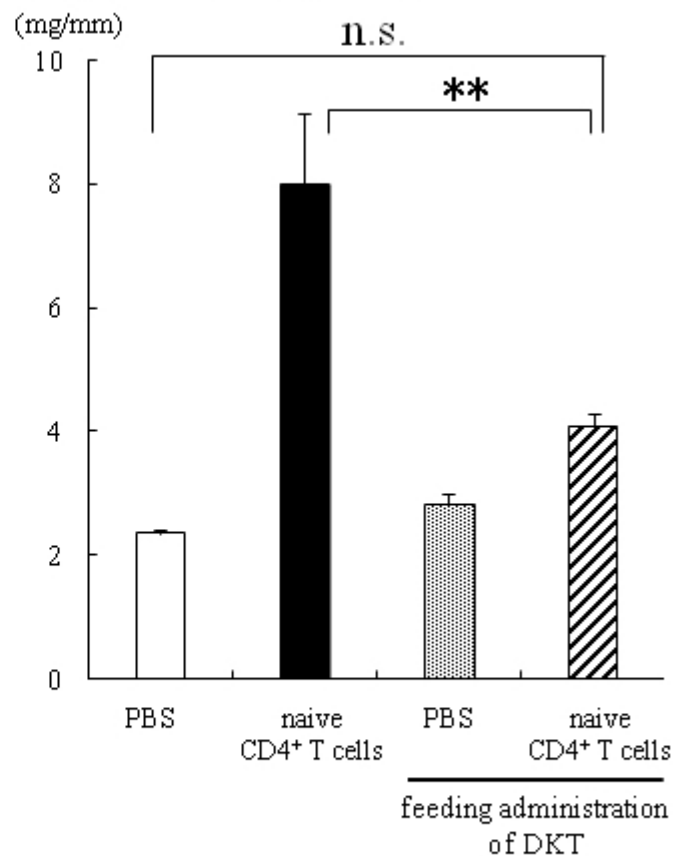
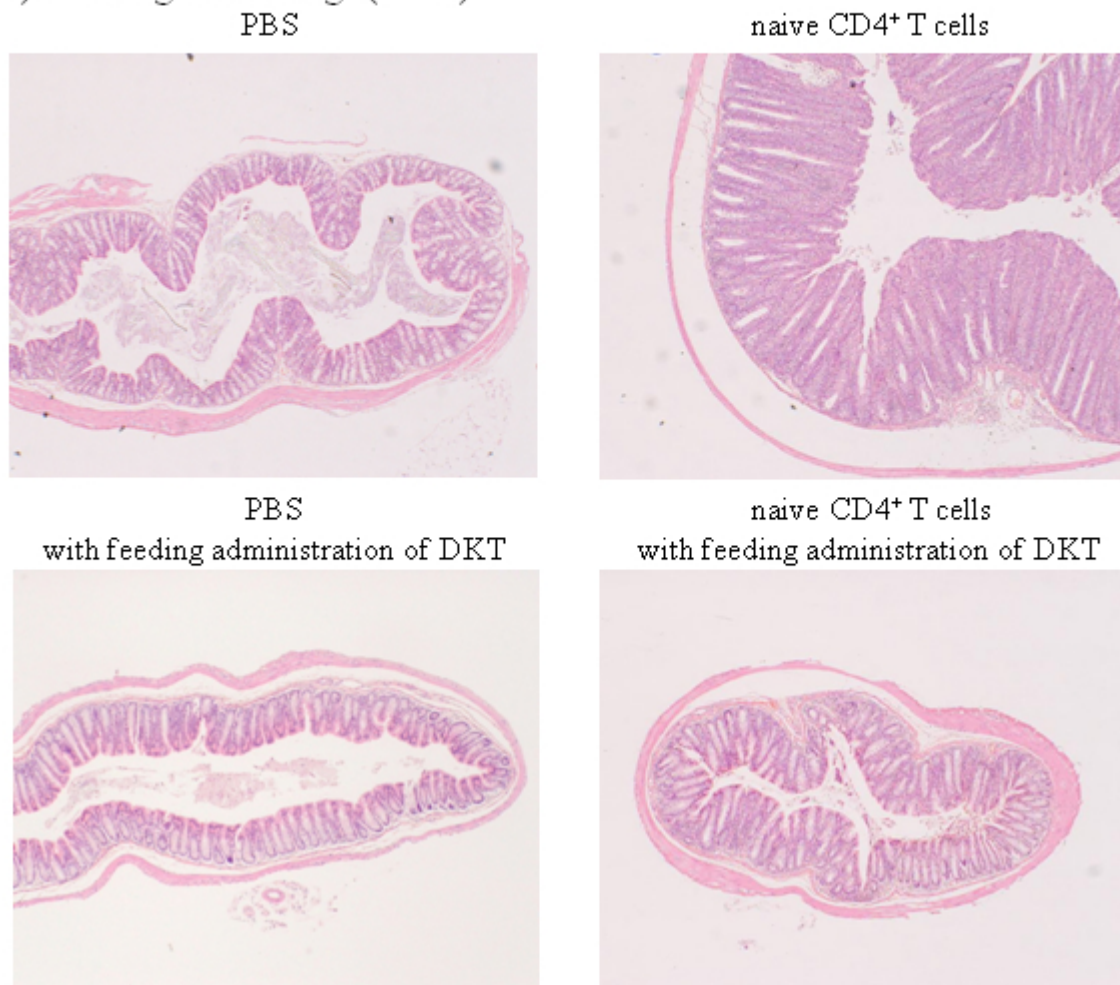


Figure 2

(A) Histological findings (x 40)



(B) Histological score

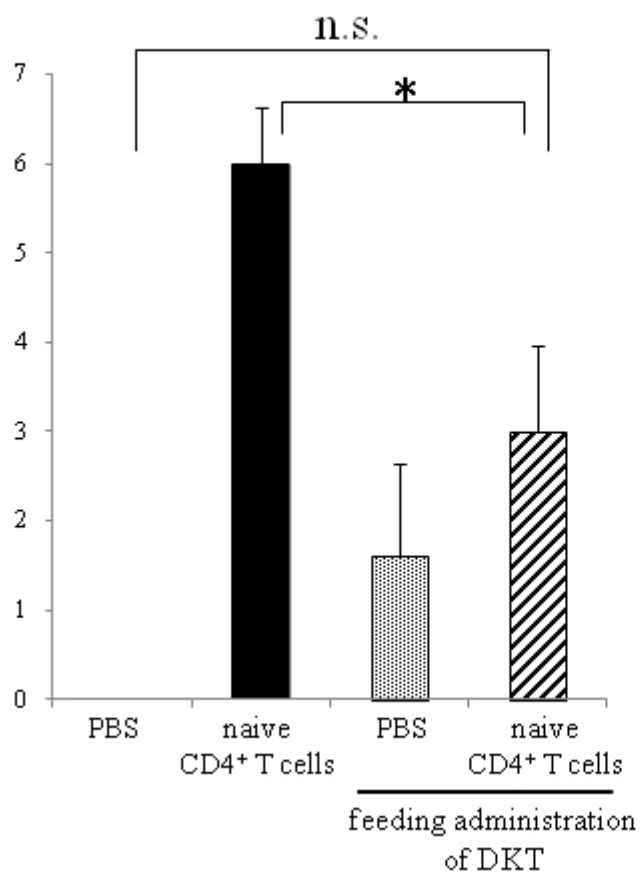
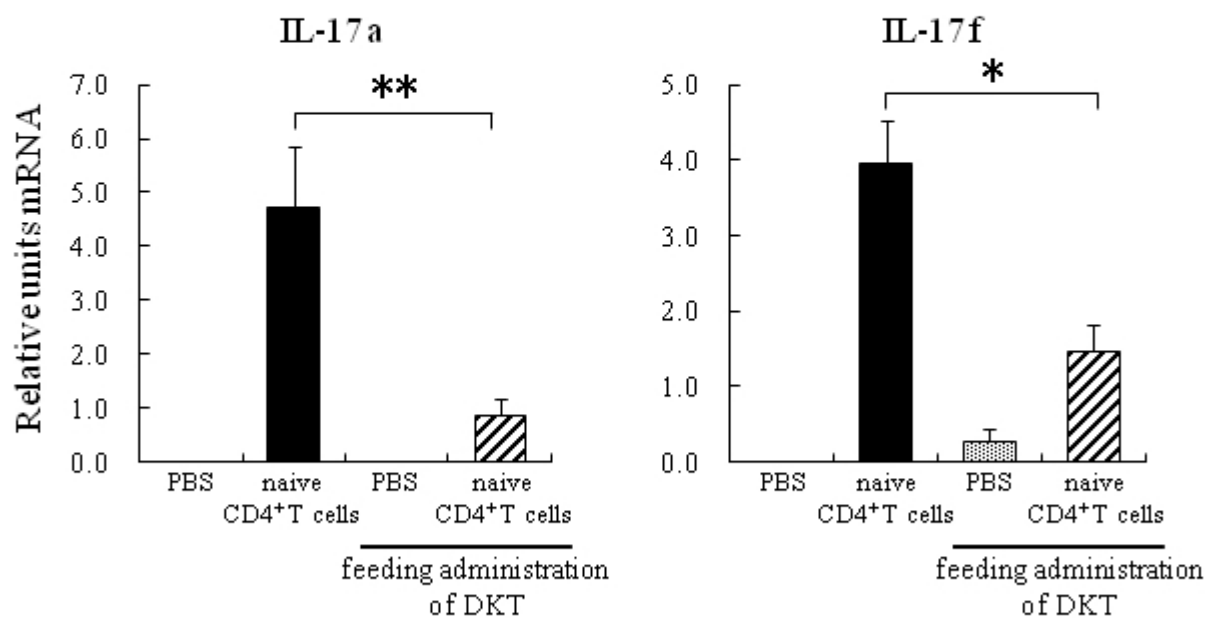


Figure 3

(A) Th17 cytokines



(B) TNF α / IFN γ

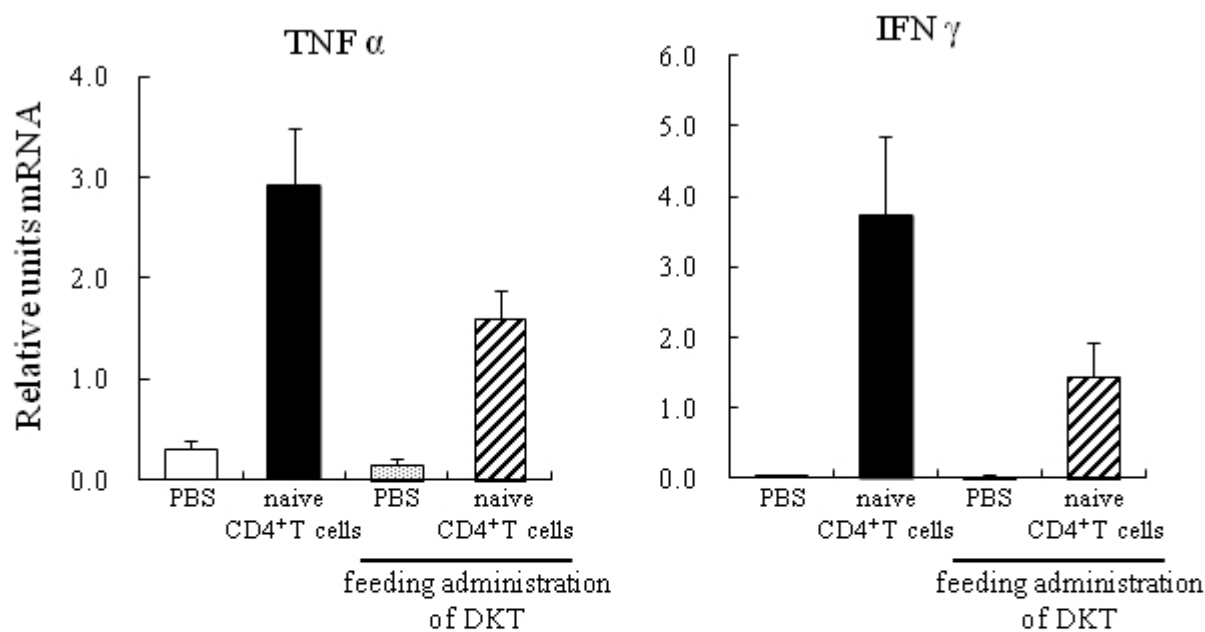
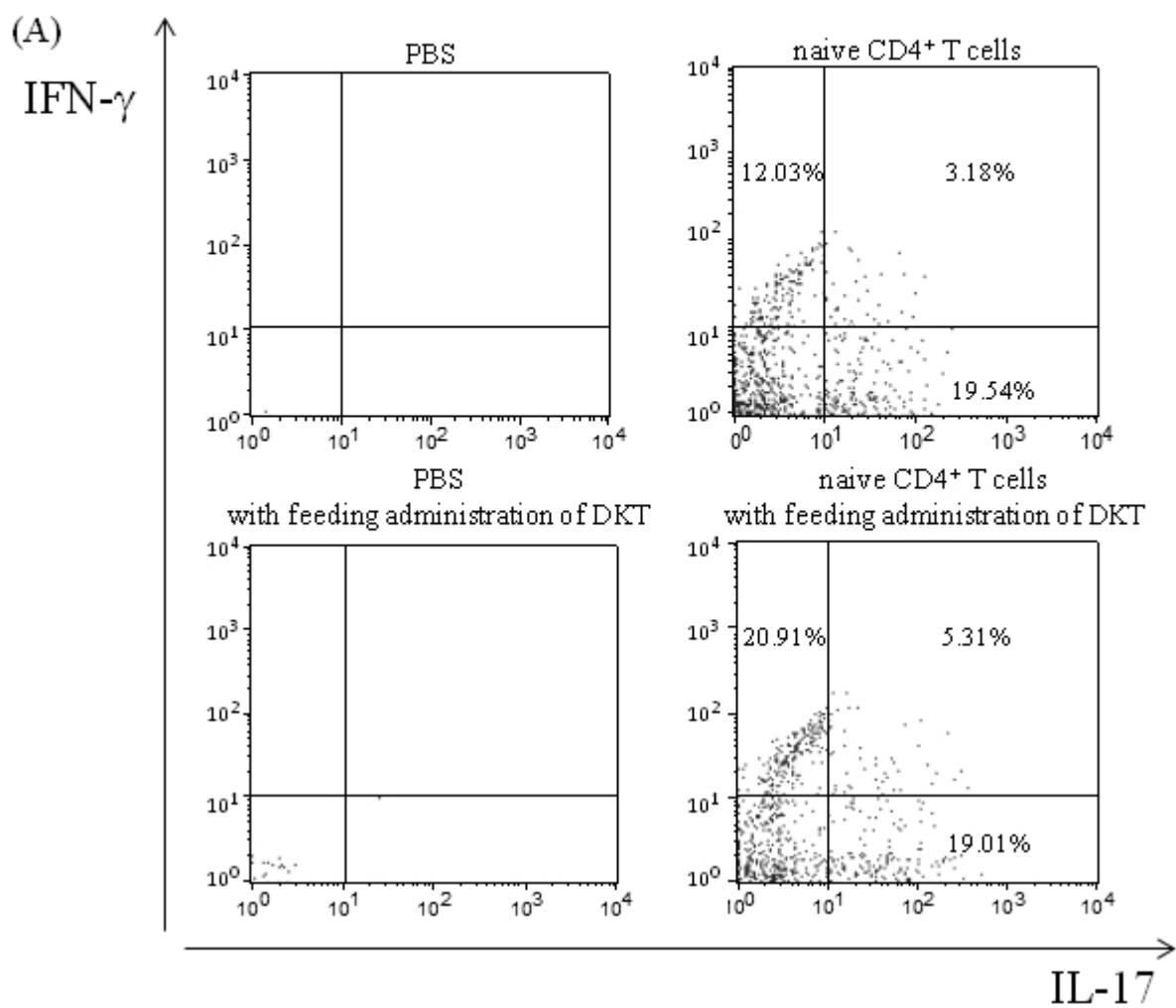


Figure 4



(B)

	Absolute number of CD4 ⁺ T cells
naive CD4 ⁺ T cells	95,040
naive CD4 ⁺ T cells with feeding administration of DKT	37,350

