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RESEARCH ARTICLE

Dietary Yeasts Reduce Inflammation in Central Nerve System via Microflora

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Abstract

Objectives: The intestinal microflora affects the pathogenesis of several autoimmune diseases by influencing immune system function. Some bacteria, such as lactic acid bacteria, have been reported to have beneficial effects on immune function. However, little is known about the effects of yeasts. Here, we aimed to investigate the effects of various dietary yeasts contained in fermented foods on experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS), and to elucidate the mechanisms underlying these effects. **Methods**: The effects of eight yeasts selected from 18 types of yeasts contained in fermented foods were examined using an EAE model. Of these, *Candida kefyr* was investigated by analyzing the intestinal microflora and its effects on intestinal and systemic immune states. **Results**: Administration of *C. kefyr* ameliorated the severity of EAE. Reduced numbers of Th17 cells, suppressed interleukin (IL)-6 production by intestinal explants, and increased Tregs and CD103-positive regulatory dendritic cells in mesenteric lymph nodes (MLNs) were observed. Analysis of 16s-rDNA from feces of *C. kefyr*-treated mice demonstrated increased *Lactobacillales* and decreased *Bacteroides* compared to control flora. Transfer of intestinal microbiota also resulted in decreased *Bacteroides* and ameliorated symptoms of EAE. Thus, oral administration of *C. kefyr* ameliorated EAE by altering the microflora, accompanied by increased Tregs and CD103-positive regulatory dendritic cells in MLNs and decreased Th17 cells in the intestinal lamina propria. **Interpretation**: Oral ingestion of *C. kefyr* may have beneficial effects on MS by modifying microflora. In addition, our findings also suggested the potential health benefits of dietary yeasts.

Introduction

Food habits and intestinal microflora have been shown to modulate the intestinal and systemic immune states, thereby affecting human health.[1,2] Th17 cells are induced by intestinal segmented filamentous bacteria and have been implicated in the pathogenesis of autoimmune diseases, including experimental autoimmune encephalomyelitis (EAE).[3–5] On the other hand, certain groups of commensal bacteria and their metabolites play critical roles in the induction of Foxp3-positive regulatory T cells in the colon.[6] Furthermore, the intestine itself has a mechanism to control excessive inflammation by eliminating or suppressing pro-inflammatory Th17 cells.[7]

These data highlight the importance of immune responses in the intestine.

Indeed, intestinal microflora and related intestinal immune mechanisms affect the susceptibility of humans and animals to inflammatory autoimmune diseases. For example, fermented foods and lactic acid bacteria are thought to have healthful effects, and recent studies have shown that modification of intestinal microflora ameliorates clinical symptoms of experimental disease models such as EAE and inflammatory bowel disease.[8,9] Although the effects of lactic acid bacteria on various autoimmune diseases have been reported,[10,11] few studies have investigated the effects of yeasts, such as *Saccharomyces*, *Candida*, and *Aspergillus* species, which are found in fermented foods.

Kefir is an acidic, mildly alcoholic fermented milk originating from the Caucasus mountains. Kefir grains represent a natural symbiosis of yeasts and lactic acid bacteria.[12] Importantly, in a mouse model of bronchial asthma, kefir has been reported to have anti-inflammatory and anti-allergic effects.[13]

In the current study, we sought to determine whether yeasts found in fermented foods have beneficial effects on EAE. Our results suggested that ingestion of *Candida kefyr*, one of the yeasts examined in this study, is a novel therapeutic strategy for overcoming autoimmune disease.

Materials and Methods

Reagents and animals

All yeasts (Table S1) were purchased from the National Institute of Technology and Evaluation (NITE) Biological Resource Center (NBRC, Chiba, Japan). They were cultured according to the manufacturer's protocols. The use of viable yeast is restricted in our animal facility because of the requirement for maintenance of specific pathogen-free conditions, yeasts were dissolved in 0.2 g/mL double distilled water (DDW), and all yeasts were heat-killed at 120°C for 15 min and stored at −80°C. C57BL/6 mice were administered water containing 8 mg/mL heat-killed yeasts in water bottles beginning at 14 days before immunization.

Induction of EAE

All experimental procedures were approved by the Animal Care and Use Committee of Osaka University Graduate School of Medicine. C57BL/6 mice were obtained from Oriental Yeast Corp. (Tokyo, Japan). EAE was induced as described previously.[10] In brief, after administration of heat-killed yeasts for 14 days, as described above, C57BL/6 mice were subcutaneously injected with 100 μg myelin oligodendrocyte glycoprotein (MOG) 35–55 (MEV GWYRSPFSPVVHLYRNGK) peptide ($MOG_{35–55}$) emulsified in complete Freund's adjuvant (CFA) containing 200 μg of *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, Detroit, MI). Mice were concurrently injected twice with 200 ng of pertussis toxin (List Laboratories, Campbell, CA) on days 0 and 2. All mice were monitored daily for clinical signs and were scored as described previously.[10]

Histology and semiquantification of data

Mice were sacrificed on day 22 postimmunization followed by transcardiac perfusion with 4% paraformaldehyde in PBS. Spinal cords were fixed in 4% paraformaldehyde in PBS and prepared for histological analysis. Cryosections (10-μm thick) were stained with hematoxylin and eosin (H&E). Semiquantitative histological analysis of inflammatory cellular infiltration was performed as previously described.[14]

Isolation of MNCs and lymphocytes

MLNs, inguinal lymph nodes (ILNs), and cervical lymph nodes (CLNs) were harvested and homogenized. Cells were centrifuged and the resulting pellets were used as lymphocytes. Lamina propria (LP) lymphocytes were isolated as previously described.[10] The detailed method to isolate LP lymphocytes is described in the Data S1.

Cytokine assay

For the assessment of antigen-specific cytokine production, mononuclear cells (MNCs) were isolated from draining ILNs and cervical LNs of mice on day 8 after immunization with $MOG_{35–55}$. Cells were restimulated with the peptide for 72 h, and interleukin (IL)-17, interferon (IFN)-γ, and IL-10 were assayed by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Intracellular cytokine staining

Intracellular expression of IL-17 and IFN-γ in $CD4^+$ T cells was analyzed using an Intracellular Fixation and Permeabilization Buffer Set (eBioscience, San Diego, CA) according to the manufacturer's instructions. Surface staining was performed with anti-CD4-APC-H7 antibodies (BD Biosciences, Franklin Lakes, NJ, USA). The cells were then stained with Fixable Viability Dye eFluor 450, fixed with fixation solution, and then washed with permeabilization diluent. Intracellular cytokine staining was performed with anti-IL-17A Alexa Fluor 647 (BD Biosciences), anti-IL-10-PE (BD Biosciences), and anti-IFN-γ-FITC (fluorescein isothiocyanate) (BioLegend,

San Diego, CA) antibodies. For intracellular staining of Foxp3, cells were stained using a Foxp3 Staining Buffer set (eBiosciences).

Flow cytometry

The following antibodies were used for flow cytometry: anti-CD4-APC/H7, anti-CD11c-PE/Cy7, anti-major histocompatibility complex (MHC) class II-Pacific Blue, and anti-CD103-APC antibodies (BD Biosciences). Anti-Foxp3-Alexa Fluor 647 antibodies (eBioscience) were also used; conditions were set according to the manufacturer's instructions. Data were acquired using a FACS Cant-2 instrument with Diva software (Becton Dickinson, Franklin Lakes, NJ, USA).

Intestinal tissue explant cultures

Explant culture was performed according to previously published methods with some modifications.[15,16] Briefly, large intestines were collected, opened longitudinally, washed in PBS to remove contents, and shaken at 110 rpm in RPMI 1640 containing 50 mg/mL gentamicin, 100 U/mL penicillin, 100 mg/mL streptomycin (GIBCO, Carlsbad, CA, USA), and 5 mmol/L ethylenediaminetetraacetic acid for 20 min at 37°C. After removing epithelial cells and fat tissue, intestinal tissue was cut into 10-mm fragments. Tissue fragments were incubated in 0.5 mL RPMI is abbreviation of Roswell Park Memorial Institute medium. Normally, RPMI is used. 1640 supplemented with 50 mg/mL gentamicin, 100 U/mL penicillin, 100 mg/mL streptomycin, and 5% heat-inactivated fetal bovine serum. Supernatants from the tissue fragment incubations were collected after 24 h for cytokine ELISAs (IL-6 and IL-10; R&D Systems), and tissue dry weights were measured.

Intestinal microflora analysis (T-RFLP method)

Analysis of intestinal bacterial flora using mouse fecal specimens was outsourced to Techno Suruga Laboratory (Shizuoka, Japan), where the T-RFLP (terminal restriction fragment length polymorphism) method was used.[17] The details of this method are described in the Data S1.

Microflora transfer

Microflora transfer was performed according to previously published methods, with modifications.[18] Briefly, 6-week-old female mice were treated with a cocktail of antibiotics (0.5 mg/mL vancomycin [Duchefa Biochemie, Haarlem, the Netherlands], 1 mg/mL ampicillin, 1 mg/mL metronidazole, 1 mg/mL neomycin, and 1 mg/mL gentamicin [Nacalai Tesque, Kyoto, Japan]) in drinking water for 2 weeks. Diluted cecal contents were collected from 8-week-old mice treated with *C. kefyr* or water for 2 weeks. The ceca of control mice or *C. kefyr*-treated mice were dissected and opened, and the contents were transferred to a sterile tube and resuspended in 50 volumes of sterile water. Next, 200 μL of this suspension was administered to each recipient by oral gavage using a gavage needle for five consecutive days. At 2 days after the final oral gavage, feces were collected for T-RFLP analysis, and mice were immunized for EAE.

Statistical analysis

Statistical analysis of the results was performed by one-way analysis of variance (ANOVA). Repeated measures ANOVA was used to compare the ratio of bacteria in T-RFLP analysis. Differences were considered significant when P values were less than 0.05. The data were analyzed using SPSS 14.J. (SPSS, Chicago, IL, USA)

Results

Candida kefyr decreased the susceptibility of mice to EAE

Eighteen types of yeasts that are found in common fermented foods were investigated in this study (Table S1). Because TNF-α is involved in the pathogenesis of intestinal autoimmune diseases[19,20] and IL-10 is a key anti-inflammatory cytokine involved in the maintenance of intestinal homeostasis,[21,22] the effects of yeasts on the production of these cytokines by MNCs from intestinal LP were examined. The yeasts were then classified into four groups depending on the pattern of relative cytokine production: high TNF-α/high IL-10, high TNF-α/low IL-10, low TNF-α/high IL-10, and low TNF-α/low IL-10 (data not shown). Eight yeasts representing the four groups were arbitrarily selected, and their effects on EAE model mice were examined. When administered beginning 14 days before immunization with $MOG_{35–55}$, only *C. kefyr*, which belonged to the low TNF-α/low IL-10 group, significantly ameliorated the clinical severity of EAE symptoms (Fig. 1A). Pathological examinations revealed that the number of infiltrated MNCs into the spinal cords of mice treated with *C. kefyr* was apparently lower than that observed in the control group (Fig. 1B). The significant decrease in the number of infiltrating cells in the *C. kefyr*-treated group was confirmed by semiquantitative analysis (*C. kefyr*: 1.16 ± 0.24 vs. control: 2.07 ± 0.22; $P = 0.010$; Fig. 1C). To investigate the effects of *C. kefyr* on systemic inflammation, draining inguinal LNs and cervical LNs harvested on day 8 after

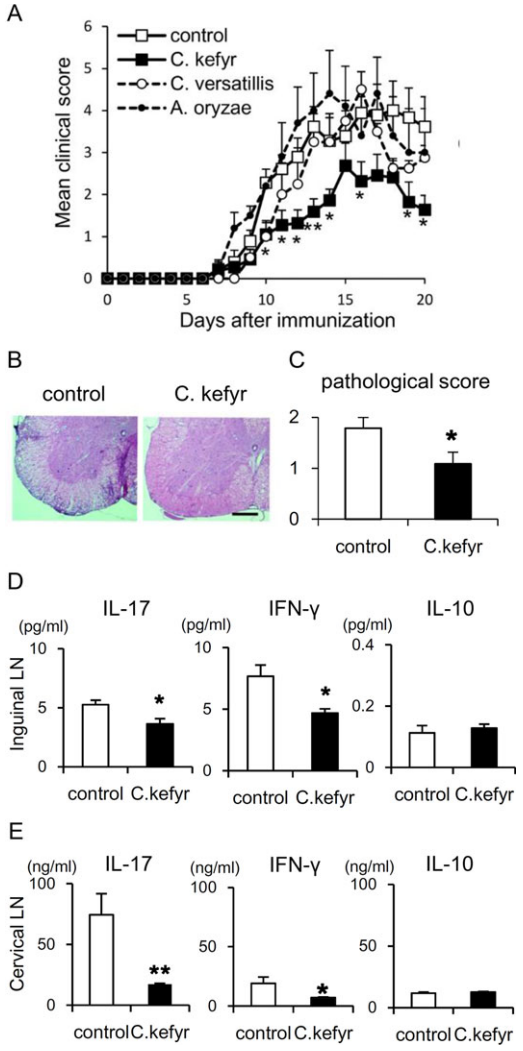

Figure 1. *Candida kefyr* ameliorates symptoms of EAE. The effects of *C. kefyr* (n = 11), *C. versatilis* (n = 8), *A. oryzae* (n = 6), and control (water, n = 9) on the clinical severity of EAE are shown. (A) The three yeasts listed above are shown because the other five yeasts did not differ significantly from the control. Yeasts were administered from 14 days before immunization until the end of the study. Data represent the mean clinical score +SEM. (*$P < 0.05$, **$P < 0.01$ compared to the control group using ANOVA). (B) Spinal cord sections obtained from control or *C. kefyr*-treated C57BL/6 mice on day 22 after immunization were analyzed by hematoxylin and eosin (H&E) staining. Scale bar = 250 μm. (C) Semiquantitative evaluation of the pathological scores was performed as described in the Materials and Methods section. Each bar indicates the mean pathological score +SEM of six mice from each group. Lymphocytes were isolated from draining lymph nodes (D) and cervical lymph nodes (E) on day 8 after immunization and then restimulated with $MOG_{35–55}$ for 72 h. IL-17, IFN-γ, and IL-10 in the culture supernatants were assayed by ELISA. Data are means + SEMs and are representative of three independent experiments (n = 5–8 each). EAE, experimental autoimmune encephalomyelitis; ANOVA, analysis of variance; MOG, myelin oligodendrocyte glycoprotein; IL, interleukin; IFN, interferon; ELISA, enzyme-linked immunosorbent assay.

immunization were restimulated with $MOG_{35–55}$. Both inguinal and cervical LNs obtained from the *C. kefyr*-treated mice produced significantly less IL-17 and IFN-γ than those obtained from the control group. The production of IL-10 did not differ significantly between the two groups (Fig. 1D and E). Although we assayed IL-4 to examine the effects of *C. kefyr* on Th2-skewing, the levels were below the sensitivity of the assay system. These data suggested that treatment with *C. kefyr* inhibited the induction of antigen-specific Th17 and Th1 cells.

Next, the effects of *C. kefyr* were examined in a model of dextran sulfate sodium (DSS)-induced colitis because inflammatory bowel disease is known to be directly affected by intestinal microflora and intestinal immunity.[23] In this colitis model, prophylactic oral administration of *C. kefyr* significantly inhibited body weight loss, reduced colon length, and increased relative colon weights (Fig. S1A–D). The effects of other *Candida* species were less prominent than those of *C. kefyr*, and no significant differences were observed compared to the control. The effects of *C. kefyr* were also examined in a toluene-2, 4-diisocyanate (TDI) contact dermatitis model, another model of autoimmune dysfunction. However, *C. kefyr*, as well as the other yeasts examined (*C. versatilis*, *C. valida*, and *Saccharomyces cerevisiae*), had no effects on TDI-induced dermatitis (Fig. S2). Thus, our data supported that *C. kefyr* ameliorated symptoms of EAE and DSS-induced colitis, but did not affect TDI-induced dermatitis, suggesting that the efficacy was disease specific.

When *C. kefyr* administration was initiated on day 8 after immunization of mice with EAE, clinical severity was not affected (Fig. S3A). Moreover, in the DSS-induced colitis model, disease deterioration was observed when *C. kefyr* was administered after DSS induction (data not shown). Thus, *C. kefyr* was not effective as a therapeutic agent, but exhibited efficacy in the prophylactic/preventive setting.

Candida kefyr suppressed generation of Th17 cells and induced production of regulatory T cells (Tregs) and dendritic cells

In order to elucidate the mechanism through which *C. kefyr* suppressed intestinal and systemic inflammation, we analyzed $CD4^+$ T cells from mice treated with *C. kefyr*. Intracellular staining of $CD4^+$ T cells from LP and MLNs of mice treated with *C. kefyr* for 2 weeks revealed that $CD4^+$ IL-17-producing cells were downregulated in intestinal LP in both small and large intestines (Fig. 2A). The production of IL-6 by intestinal tissue explants was also downregulated in both small and large intestines, and IL-10 was significantly upregulated in the colon (Fig. 2B). Significantly increased percentages of $CD4^+$ $Foxp3^+$ iTregs

were observed in *C. kefyr*-treated mice (*C. kefyr*: 7.5 ± 0.4% vs. control: 9.8 ± 0.5%), although the ratio of Th17 cells was not altered in MLNs (Fig. 2C). No significant differences in the ratios of iTregs in intestinal LP were observed (data not shown). The percentage of $CD103^+$ dendritic cells was significantly increased in MLNs (Fig. 2D) and ILNs (data not shown) on day 8 postimmunization in *C. kefyr*-treated mice, although differences were not observed between the two groups before immunization. These data suggested that *C. kefyr* induced the production of Tregs and dendritic cells and suppressed the production of Th17 cells. Additionally, decreased IL-6 and increased IL-10 levels may contribute to these effects.

Ingestion of *C. kefyr* altered the intestinal microflora

Because intestinal immune cells are affected by intestinal microbiota,[24] the intestinal microflora of mice treated with *C. kefyr*-treated mice for 2 weeks was analyzed using the T-RFLP method. There were no differences in the patterns of microflora between the control and *C. kefyr* groups at baseline (Fig. 3A). One week after

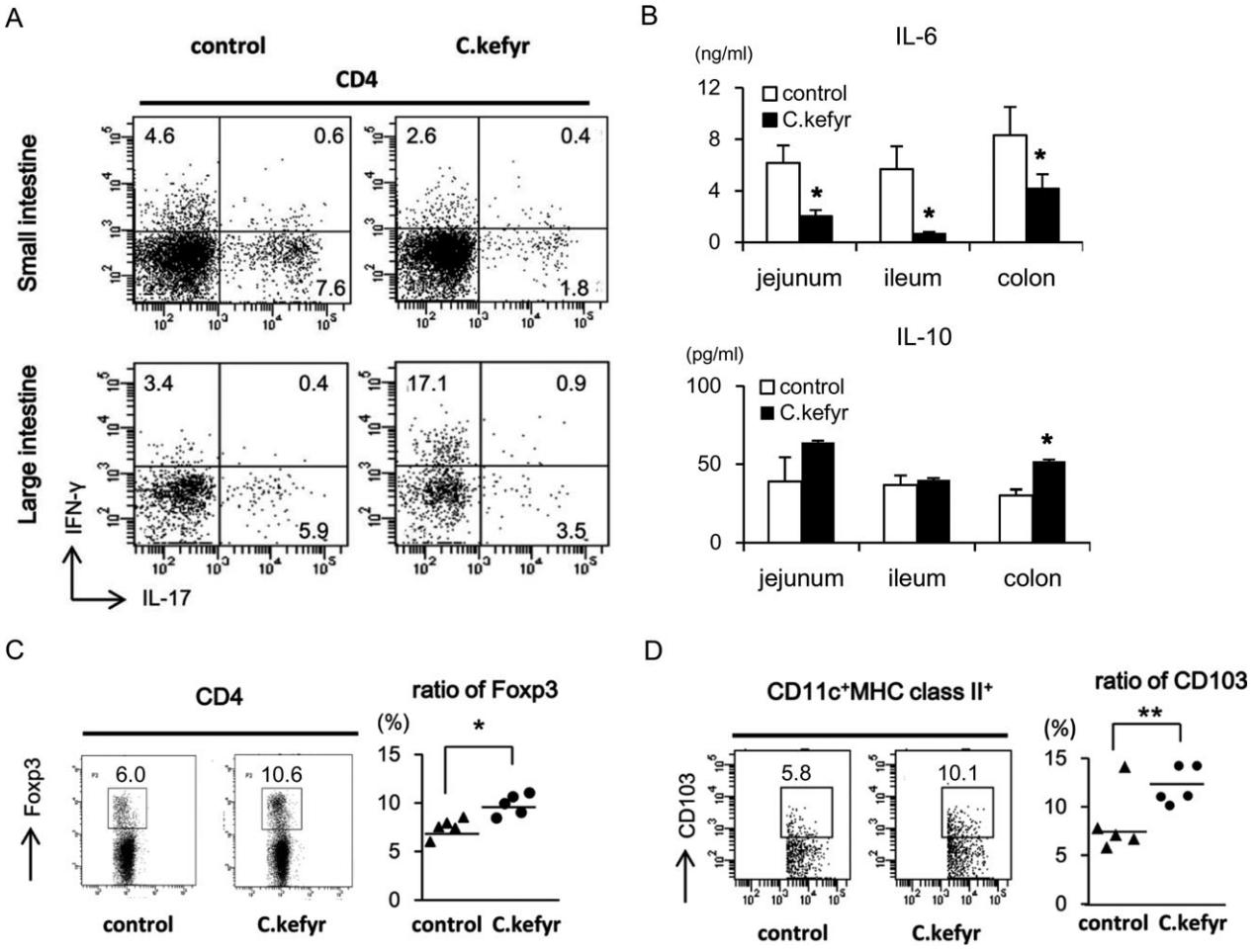

Figure 2. Oral administration of *Candida kefyr* suppresses intestinal Th17 cells and induces regulatory T cells and dendritic cells. (A) Lamina propria lymphocytes from small and large intestines were isolated from *C. kefyr*-treated nonimmunized mice. Intracellular staining of IL-17 and IFN-γ in $CD4^+$ T cells was analyzed by flow cytometry. Data are representative of three independent experiments. (B) Tissue explants of small and large intestines from control mice and mice treated with *C. kefyr* for 14 days were cultured for 24 h, and IL-6 and IL-10 in supernatants were assayed by ELISA. (*$P < 0.05$, **$P < 0.01$ using ANOVA). (C) Lymphocytes from MLNs isolated from *C. kefyr*-treated nonimmunized mice were stained with anti-CD4 and anti-Foxp3 antibodies and analyzed by flow cytometry. Dotplots showed one of five representative experiments, and the graphs show the ratios of Foxp3 cells in $CD4^+$ T cells. (D) Lymphocytes from MLNs isolated from *C. kefyr*-treated mice on day 8 postimmunization were stained with anti-CD11c, anti-MHC class II, and anti-CD103 antibodies and analyzed by flow cytometry. Dotplots show one of five representative experiments, and the graphs show the ratio of $CD103^+$ cells in $CD11c^+$ and MHC class 2^+ dendritic cells. IL, interleukin; IFN, interferon; ELISA, enzyme-linked immunosorbent assay; MLNs, mesenteric lymph nodes; ANOVA, analysis of variance.

administration, the ratio of *Bacteroides* was decreased in the *C. kefyr*-treated group, while the ratio of *Lactobacillales* remained higher (Fig. 3B). The decrease in the ratio of *Bacteroides* was not observed when administered after immunization (Fig. S3B). In addition to decreased *Bacteroides* and increased *Lactobacillales*, the ratio of *Prevotella* tended to be increased 2 weeks after administration (Fig. 3C). Statistical analysis revealed significantly increased *Lactobacillales* (*C. kefyr*: 49.5 ± 0.2% vs. control: 24.2 ± 0.3%, $P = 0.005$; Fig. 3D) and significantly decreased *Bacteroides* (*C. kefyr*: 12.6 ±5.1% vs. control: 35.6 ± 6.3%, $P = 0.039$; Fig. 3E). *Prevotella* tended to be increased, although the difference was not significant (*C. kefyr*: 16.7 ± 2.2% vs. control: 10.4 ± 3.7%, $P = 0.325$; Fig. 3F). The percentages of total *Clostridium*, which have been reported to induce regulatory T cells,[25] were not different between the two groups (Fig. 3G).

Microflora transferred from *C. kefyr*-treated mice ameliorated symptoms of EAE in recipients

Because *C. kefyr* altered the intestinal microflora, as described above, and therapeutic administration of *C. kefyr* was not effective in either the EAE model or the DSS-induced colitis model, we hypothesized that modified intestinal microbiota would ameliorate disease pathogenesis and progression. Then, we examined the effects of prophylactic *C. kefyr* administration from day −14 to day 0 postimmunization. Interestingly, this prophylactic administration was still effective, although the effect was less than that of *C. kefyr* administration from day −14 to the end of the study (Fig. 4A). The microflora on day 8 postimmunization exhibited a pattern similar to that observed before EAE induction, as shown in Figures 3C, 4B. Furthermore, CD103-positive DCs were induced in MLNs (Fig. 4C). These results suggested that microflora altered by the ingestion of *C. kefyr* affected the amelioration of EAE.

Thus, we next examined the effects of altered microflora following ingestion of *C. kefyr*. Diluted cecal contents from mice treated with *C. kefyr* for 2 weeks were transferred to recipient mice, and EAE was then induced (Fig. 4D). Analysis of microbiota before immunization showed that the transfer of feces from *C. kefyr*-treated mice tended to decrease *Bacteroides* (*C. kefyr*-t: 7.2 ± 3.7% vs. control-t: 21.8 ± 3.6%, $P = 0.025$), but did not significantly alter the ratio of *Prevotella* (*C kefyr*-t: 1.7% vs. control-t: 7.7%) and *Lactobacillales* (*C. kefyr*-t: 25.7% vs. control-t: 23.8%; Fig. 4E and F). The clinical

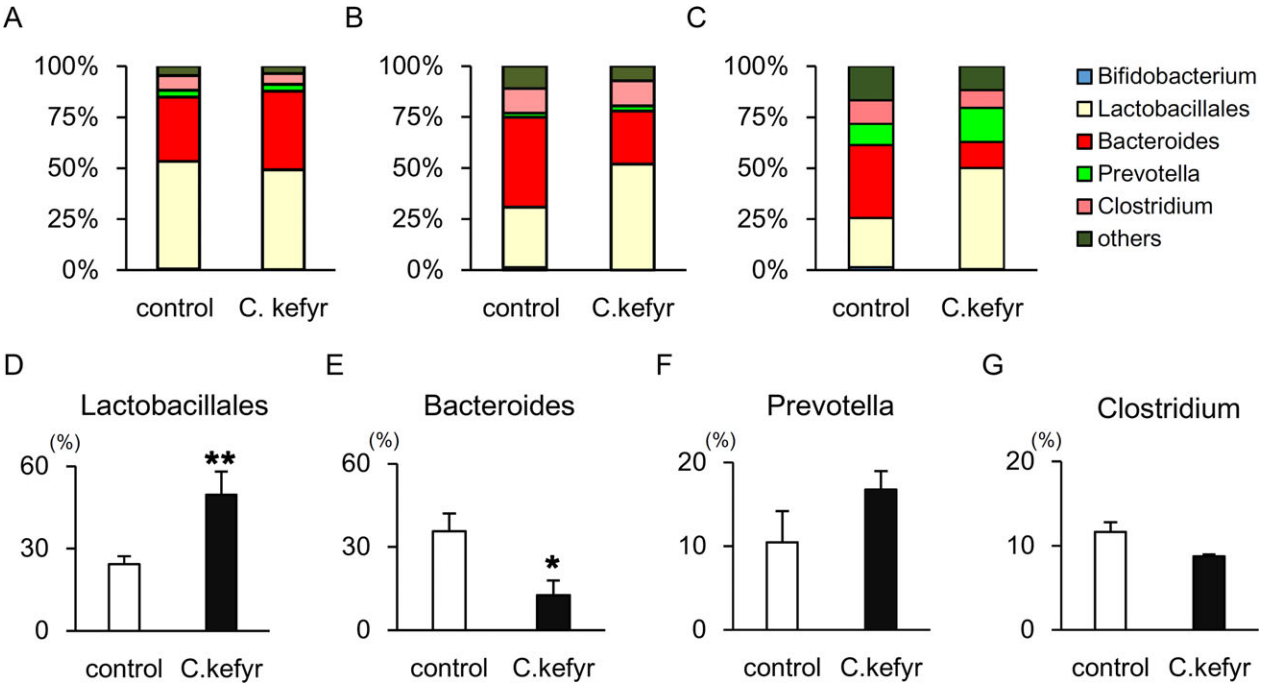

Figure 3. *Candida kefyr* modifies the intestinal microflora. T-RFLP analysis of 16s-rDNA from feces of control mice or mice treated with *C. kefyr*. (A) At baseline (−14 days before immunization [−14 dpi]), (B) 1 week after treatment (−7 dpi), (C) 2 weeks after treatment (day 0). Data show the means of 3–5 mice from two or three independent experiments. (D–G) The ratios of *Lactobacillales*, *Bacteroides*, *Prevotella* and *Clostridium* after a 2-week treatment are shown. Data are the means + SEMs ($n = 5$) (*$P < 0.05$, **$P < 0.01$ using repeated measures analysis of variance [ANOVA]).

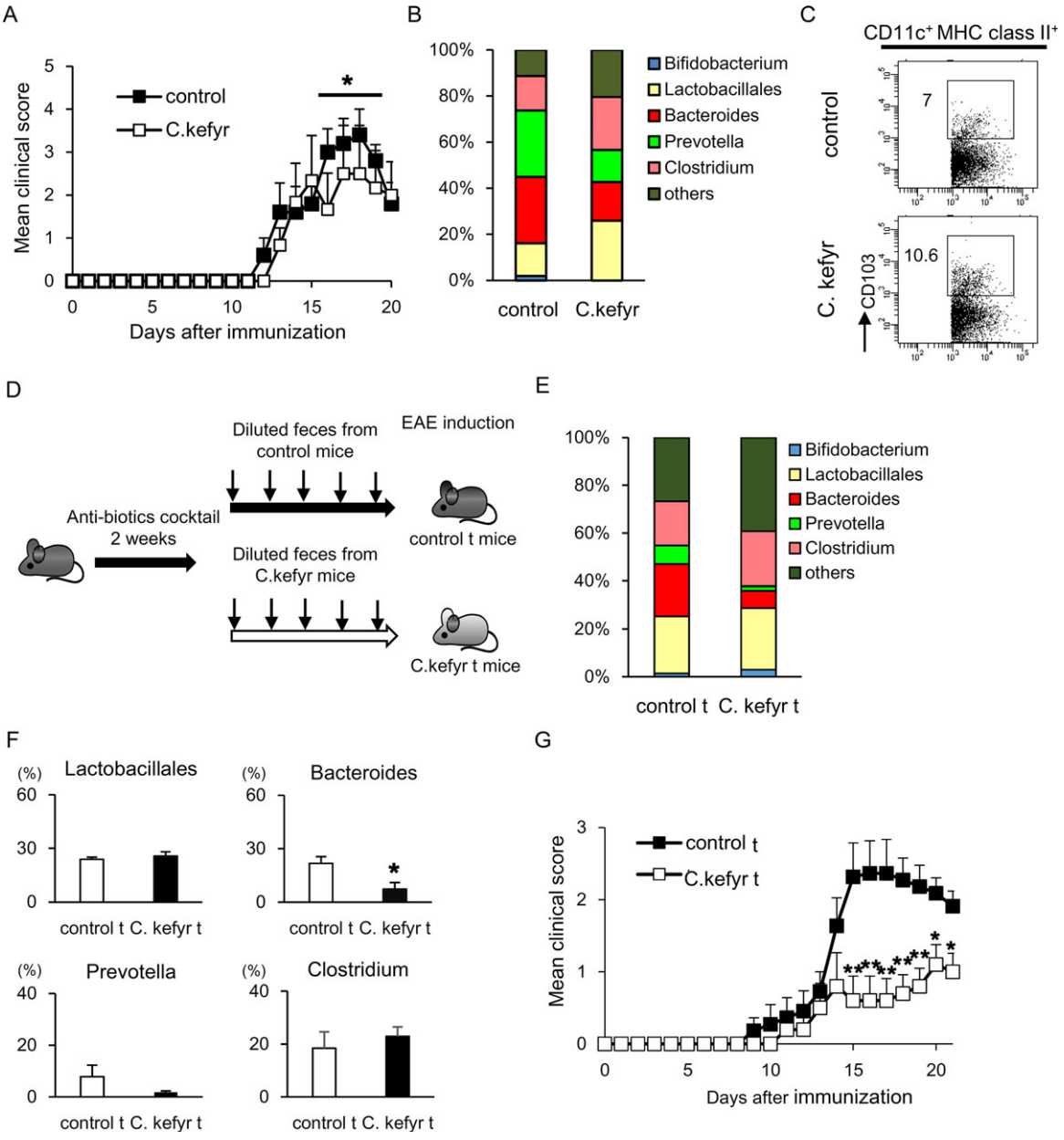

Figure 4. Microflora from *Candida kefyr*-treated mice ameliorates symptoms of EAE. (A) The effects of *C. kefyr* ($n = 6$) administered only prophylactically (from −14 dpi until day 0) and control (water, $n = 6$) on the clinical severity of EAE are shown. Data represent the mean clinical score +SEM. The area under the curve (AUC) under the bar was significantly lower in *C. kefyr*-treated mice (*$P < 0.05$ using ANOVA). (B) T-RFLP analysis of 16s-rDNA from feces of control mice or mice treated with *C. kefyr* (from day −14 to day 0) on day 8 postimmunization. (C) Lymphocytes from MLNs isolated from mice treated prophylactically with *C. kefyr* on day 8 postimmunization were stained with anti-CD11c, anti-MHC class II, and anti-CD103 antibodies and analyzed by flow cytometry. Dotplots show one of three representative experiments (D) Schematic of microflora transfer. Mice were treated with an antibiotic cocktail in their drinking water for 2 weeks and were then fed diluted feces from *C. kefyr*-treated mice or control mice once per day for 5 consecutive days. Following a 2-day rest, mice were immunized with $MOG_{35–55}$ peptide in CFA. (E) T-RFLP analysis of 16s-rDNA of feces from *C. kefyr*-treated mice and control mice before immunization. Data show the means of five mice from three independent experiments. (F) The ratios of *Lactobacillales*, *Bacteroides*, *Prevotella* and *Clostridium* in 16s-rDNA from feces of control-t or *C. kefyr*-t mice on the day of immunization are shown. Data are the means + SEMs ($n = 5$). (*$P < 0.05$, **$P < 0.01$ using repeated measures ANOVA). (G) Clinical scores of EAE mice administered feces from *C. kefyr*-treated (*C. kefyr*-t) or nontreated (control-t) mice. Data show the means + SEMs (*C. kefyr*-t, $n = 10$; control-t, $n = 11$) from two independent experiments (*$P < 0.05$ using repeated measures ANOVA). EAE, experimental autoimmune encephalomyelitis; ANOVA, analysis of variance; MLNs, mesenteric lymph nodes; MOG, myelin oligodendrocyte glycoprotein.

scores of mice administered cecal contents from *C. kefyr*-treated mice were significantly decreased compared with those of mice administered cecal contents from control mice (Fig. 4G). Because the microflora of antibiotic-treated recipients before fecal transfer revealed that these four genera were undetectable using the T-RFLP method (data not shown), reconstituted microflora were thought to reflect the original microflora harvested from control or *C. kefyr*-treated mice. In addition, contamination of *C. kefyr* itself or other metabolites was thought to be minimal since the transfer was performed by oral administration of small amount of diluted feces. Taken together, these results suggested that *C. kefyr*-induced changes in microbiota contributed to the amelioration of EAE.

Discussion

Several studies have provided evidence of the importance of microflora in the pathogenesis of multiple sclerosis (MS) pathology,[2,8,26] and a recent epidemiological analysis conducted in patients living on the island of Crete revealed that modification of microflora due to changes in food habits could be a risk factor for MS.[27] In addition, oral administration of a single type of bacterium or a bacterial mixture has been shown to reduce the susceptibility of model animals to EAE.[10,28–30] However, the effects of yeasts on MS/EAE have not yet been investigated. In the present study, we found that *C. kefyr* had beneficial effects on the symptoms of EAE, suggesting that dietary yeasts prove to be important for the management of immune-mediated diseases.

With regard to the underlying mechanisms, *C. kefyr* treatment was shown to induce $CD103^+$ dendritic cells, which function to regulate the immune response, and $Foxp3^+$ Tregs in MLNs. Intestinal $CD103^+$ dendritic cells are induced by oral administration of polysaccharide A from *Bacteroides fragilis*,[29,31] while Tregs are induced in MLNs.[10] $CD103^+$ dendritic cells migrate towards MLNs in a CCR7-dependent manner.[32] In MLNs, $CD103^+$ dendritic cells induce $Foxp3^+$ Tregs with through a mechanism involving retinoic acid and transforming growth factor (TGF)-β.[33] Our results suggested that induced $CD103^+$ dendritic cells have important roles in reducing susceptibility to EAE.

To analyze whether oral administration of *C. kefyr* was effective in other disease models, *C. kefyr* was administered to mice with DSS-induced colitis and TDI contact dermatitis. In the DSS model, colitis is induced by the inflammatory response to microflora.[34] Although many types of bacteria have been reported to be effective in the DSS-induced colitis model,[35] very few studies have reported the roles of yeasts, such that *Saccharomyces boulardii* that has been shown to reduce the severity of colitis.[36] In the present study, we found that prophylactic administration of *C. kefyr* ameliorated the symptoms of DSS-induced colitis and EAE, but did not affect mice in the TDI dermatitis model, which is induced by a cutaneous delayed-type hypersensitivity response.[37] Thus, it seems likely that *C. kefyr* affects some specific immune-mediated diseases, depending on the underlying pathology.

Microflora analysis revealed that ingestion of *C. kefyr* increased *Lactobacillales* and reciprocally decreased *Bacteroides* and increased *Prevotella*. Thus, changes in microflora were identified at the genus level, and the inter-cage effects were minimal within animals in the same group; changes at the species level were not identified due to the limitations of T-RFLP analysis for evaluation of intestinal microflora. Our experiment involving microflora transfer suggested that the decrease in *Bacteroides* rather than the increase in *Lactobacillales* and *Prevotella* seemed to affect the clinical course of EAE. *Bacteroides* and *Prevotella* consist of three predominant enterotypes with Ruminococcus,[38] and the reciprocal abundance patterns of these two genera have been reported in several other studies of the human gut microbiome.[39–41] Consumption of a high-fat diet is known to induce *Bacteroides*, increase intestinal permeability, and promote Th17 immune responses.[42,43] In our study, ingestion of *C. kefyr* inhibited the production of IL-6 and generation of Th17 cells in intestinal LP in the intestine. Microflora modify local activation of the IL-6 pathway,[44] and commensal *Bacteroides* species can induce spontaneous inflammatory colitis, depending on the genetic backgrounds.[45] The present data suggested that modification of the intestinal microflora by *C. kefyr* reduced susceptibility to inflammation by decreasing IL-6 production.

The relationship between intestinal fungi and bacteria is not well understood. One study reported a correlation between intestinal fungi and bacteria, such as *Prevotella* and *Bacteroides*[46] *Candida* species have been shown to induce production of carbohydrates, which subsequently reduce the ratio of *Bacteroides*.[46] In our study, although both *C. kefyr* and *S. cerevisiae* increased the proportion of *Lactobacillus* species, *Saccharomyces* species did not reduce the ratio of *Bacteroides* (data not shown). Thus, *C. kefyr* may have significant effects on the *Bacteroides* ratio through a mechanism that is distinct from that of *S. cerevisiae*.

In conclusion, *C. kefyr* decreased the ratio of *Bacteroides* and the production of IL-6 in the intestines, which contributed in part to the induction of regulatory dendritic cells and the suppression of EAE. Therefore, modulation of microflora by dietary yeasts may be an option to prevent and treat MS.

Author Contribution

K. T. and T. T. carried out the experiments. K. T. and Y. N. wrote the paper. T. K. and J. A. H. assisted the experiments. T. O., M. K., M. T., and T. S. assisted with interpretations of data. S. S. and Y. N. designed the experiments. K. H., H. M., and S. S. supervised the study.

Conflict of Interest

K. T., T. T., T. O., T. K., M. T., K. H., S. S., and Y. N. has a patent (2013-044430) pending relevant to this work. T. T., M. T., and K. H. are relevant persons of Kyorin Pharmaceutical Co., Ltd.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Dietary yeasts examined in this study.

Figure S1. *Candida kefyr* administration ameliorates DSS-induced colitis. Yeasts (*C. kefyr*, $n = 10$; *C. versatilis*, $n = 10$; *C. valida*, $n = 9$) or water ($n = 10$) were administered to C57BL/6 mice in a water bottle for 14 days before DSS administration. (A) Percent weight change after DSS administration for 5 days. The initial weight of each mouse was defined as 100%. Data are representative of two independent experiments. Each bar indicates the mean body weight (%) +SEM. ($^*P < 0.05$ compared to the control group using ANOVA). (B) Colon length and (C) relative weight of the colon collected on day 20 after DSS treatment. The sums of two experiments are shown. Each bar represents the mean + SEM (*C. kefyr*, $n = 20$; *C. versatilis*, $n = 20$; *C. valida*, $n = 19$; water, $n = 20$). ($^*P < 0.05$, $^{**}P < 0.01$ using ANOVA). (D) Colon sections obtained from control or *C. kefyr*-treated C57BL/6 mice on day 18 after DSS treatment were analyzed by hematoxylin and eosin (H&E) staining. Scale bar = 200 μm. Data are representative of four mice from two independent experiments.

Figure S2. The effects of yeast administration in the TDI model. Seven-week-old BALB/c mice were administered water ($n = 9$) or yeasts (*Candida kefyr*, *C. versatilis*, *C. valida*, and *Saccharomyces cerevisiae* 0.8 mg/mL) in a water bottle beginning 2 weeks before TDI sensitization to the end of the study. Application of TDI to mouse ears was performed 3 weeks after preapplication of TDI to bilateral hind legs. Increases in an ear thickness were measured 22 and 48 h after the second application. Data are representative of two experiments and are presented as the mean clinical score.

Figure S3. Therapeutic administration of *Candida kefyr* does not ameliorate EAE. The effects of therapeutic administration of *C. kefyr* ($n = 6$) and control (water, $n = 6$) on the clinical severity of EAE are shown. (A) *Candida kefyr* was administered from the day of clinical onset until the end of the study. Data represent the mean clinical score +SEM. (B) T-RFLP analysis of 16s-rDNA from feces of control mice or mice treated with *C. kefyr* from the day after immunization to day 7 after treatment. Representative data of three independent experiments are shown.

Data S1. Supplementary methods.

