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

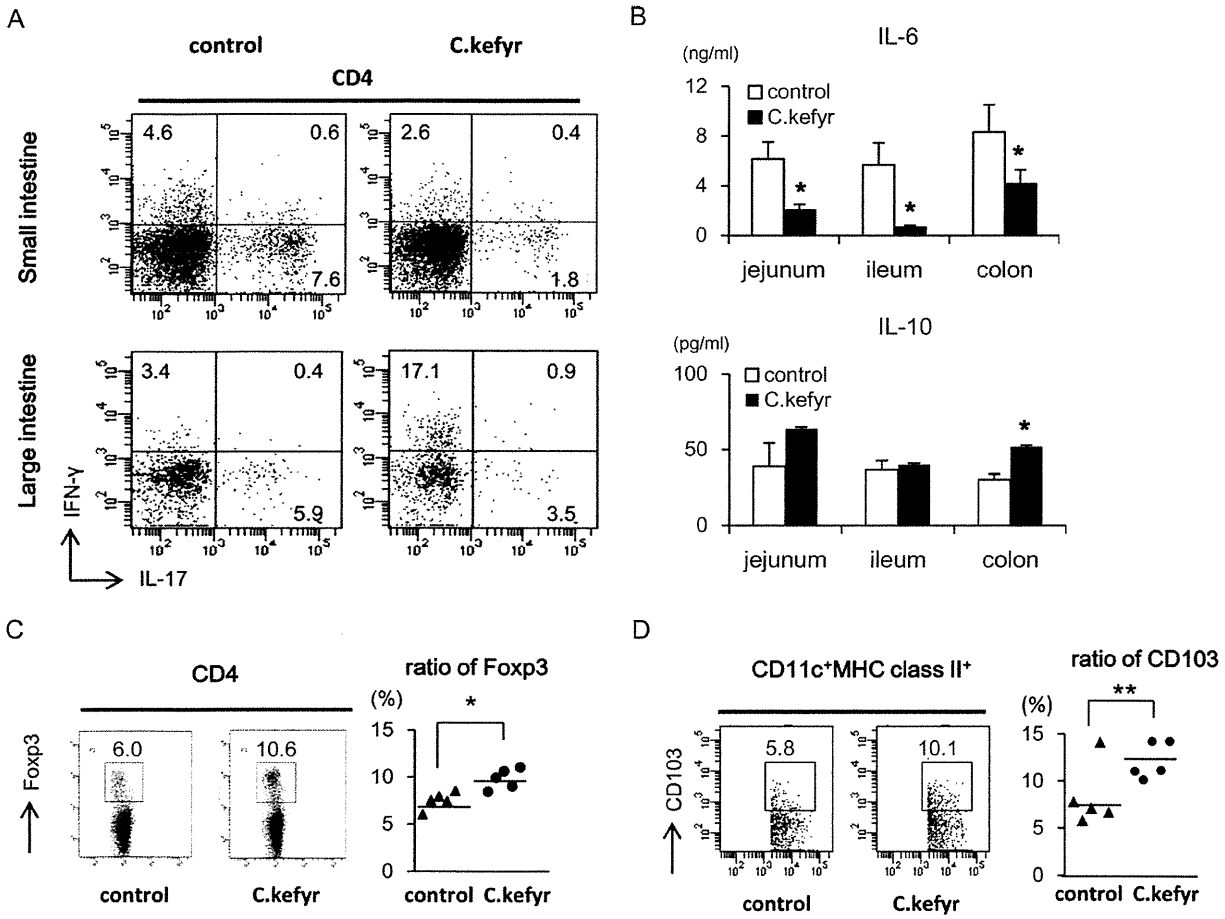

Figure 2. Oral administration of *Candida kefyr* suppresses intestinal Th17 cells and induces regulatory T cells and dendritic cells. (A) Lamina propria lymphocytes from small and large intestines were isolated from *C. kefyr*-treated nonimmunized mice. Intracellular staining of IL-17 and IFN-γ in $CD4^+$ 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

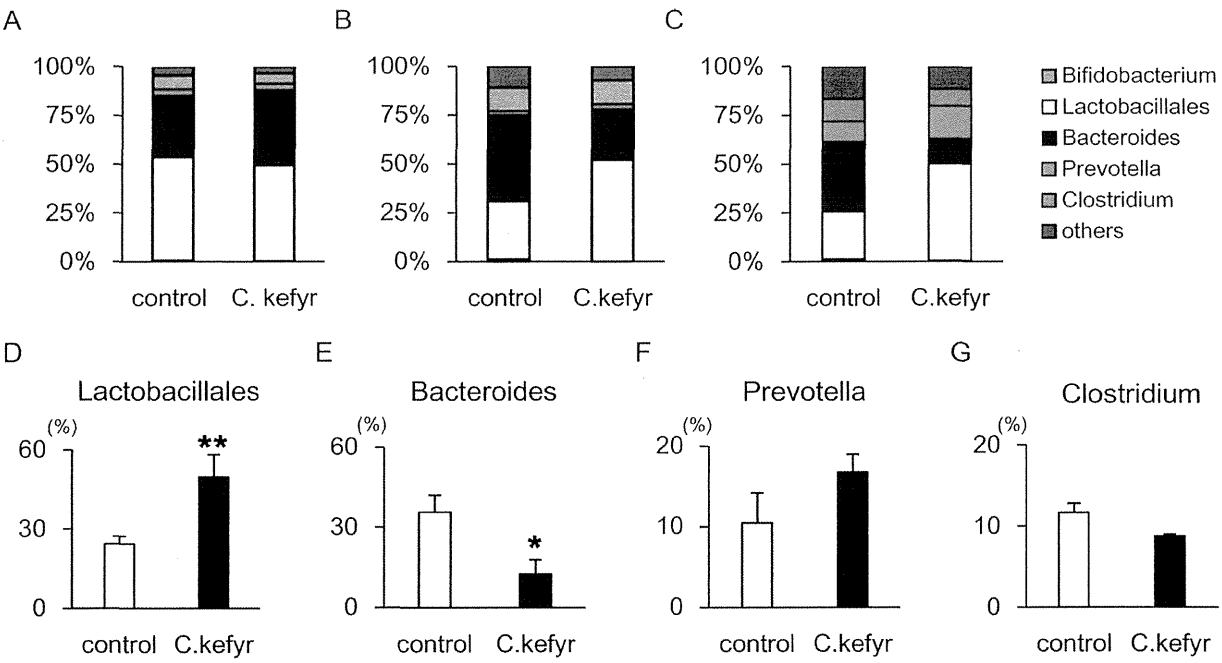

Figure 3. *Candida kefyr* modifies the intestinal microflora. T-RFLP analysis of 16s-rDNA from feces of control mice or mice treated with *C. kefyr*. (A) At baseline (−14 days before immunization [−14 dpi]), (B) 1 week after treatment (−7 dpi), (C) 2 weeks after treatment (day 0). Data show the means 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]).

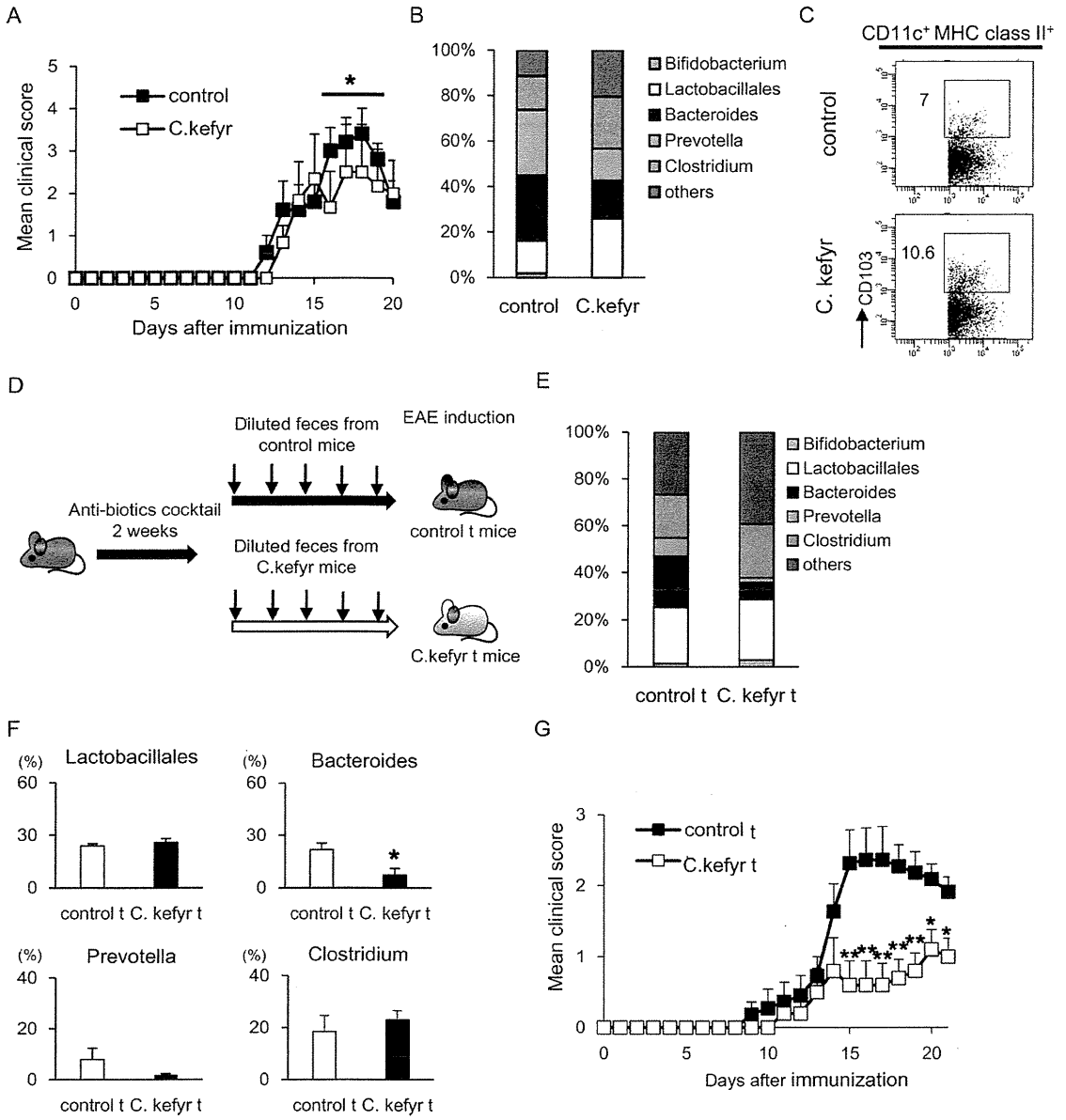

Figure 4. Microflora from *Candida kefyr*-treated mice ameliorates symptoms of EAE. (A) The effects of *C. kefyr* ($n = 6$) administered only prophylactically (from −14 dpi until day 0) and control (water, $n = 6$) on the clinical severity of EAE are shown. Data represent the mean clinical score +SEM. 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.

