

Generation of regulatory dendritic cells and CD4⁺Foxp3⁺ T cells by probiotics administration suppresses immune disorders

Ho-Keun Kwon^a, Choong-Gu Lee^a, Jae-Seon So^a, Chang-Suk Chae^a, Ji-Sun Hwang^a, Anupama Sahoo^a, Jong Hee Nam^b, Joon Haeng Rhee^b, Ki-Chul Hwang^c, and Sin-Hyeog Im^{a,d,1}

^aDepartment of Life Sciences, Gwangju Institute of Science and Technology, Gwangju 500-712, Korea; ^bChonnam National University Medical School, Gwangju 501-749, Korea; ^cCardiovascular Research Institute, Yonsei University College of Medicine, Seoul 120-752, Korea; and ^dCenter for Distributed Sensor Network, Gwangju Institute of Science and Technology, Gwangju 500-712, Korea

Edited by Anjana Rao, Harvard Medical School, Boston, MA, and approved December 8, 2009 (received for review April 15, 2009)

The beneficial effects of probiotics have been described in many diseases, but the mechanism by which they modulate the immune system is poorly understood. In this study, we identified a mixture of probiotics that up-regulates CD4⁺Foxp3⁺ regulatory T cells (Tregs). Administration of the probiotics mixture induced both T-cell and B-cell hyporesponsiveness and down-regulated T helper (Th) 1, Th2, and Th17 cytokines without apoptosis induction. It also induced generation of CD4⁺Foxp3⁺ Tregs from the CD4⁺CD25[−] population and increased the suppressor activity of naturally occurring CD4⁺CD25⁺ Tregs. Conversion of T cells into Foxp3⁺ Tregs is directly mediated by regulatory dendritic cells (rDCs) that express high levels of IL-10, TGF- β , COX-2, and indoleamine 2,3-dioxygenase. Administration of probiotics had therapeutic effects in experimental inflammatory bowel disease, atopic dermatitis, and rheumatoid arthritis. The therapeutic effect of the probiotics is associated with enrichment of CD4⁺Foxp3⁺ Tregs in the inflamed regions. Collectively, the administration of probiotics that enhance the generation of rDCs and Tregs represents an applicable treatment of inflammatory immune disorders.

regulatory T cell | inflammation | atopic dermatitis | inflammatory bowel disease | rheumatoid arthritis

Gastrointestinal microorganisms affect host physiology through diverse mechanisms, including modulation of the host immune system. Probiotics are nonpathogenic microorganisms that confer a number of beneficial effects on the health of the host (1). Among them, species of lactobacilli and *Bifidobacteria* are prominent probiotics with antiinflammatory properties (2). For example, administration of *Lactobacillus casei* suppresses proinflammatory responses by increasing IL-10 levels (3, 4), whereas *Lactobacillus acidophilus* increases T helper 1 (Th1) type cytokines (5). However, many questions remain unanswered, such as which probiotic strains are the most effective in modulation of specific immune disorders and how orally administrated probiotics affect the systemic immune system (6).

In this study, we sought to identify which probiotics or mixture of probiotics could confer potent antiinflammatory effects by increasing CD4⁺Foxp3⁺ regulatory T cells (Tregs). The forkhead family protein Foxp3 is a transcription factor highly expressed in CD4⁺ Tregs. It is a regulator of T-cell tolerance and is necessary for the development and function of Tregs (7). Following in vitro and in vivo functional studies on Treg generation, we developed a probiotics mixture that exhibits potent antiinflammatory properties and investigated its modulation of diverse immune disorders.

The probiotics mixture, designated IRT5, consists of a combination of five probiotic strains. Oral administration of IRT5 induced T- and B-cell hyporesponsiveness without inducing apoptosis. IRT5 administration increased the level of CD4⁺Foxp3⁺ Tregs in mesenteric lymph node (MLN) by augmenting Foxp3⁺ levels in CD4⁺CD25[−] T cells. Conversion of T cells into Foxp3⁺ Tregs is directly mediated by regulatory den-

dritic cells (rDCs) that express increased levels of IL-10, TGF- β , COX-2, and indoleamine 2,3-dioxygenase (iDO). Administration of IRT5 suppressed the progression of experimental inflammatory bowel disease (IBD), atopic dermatitis (AD) and rheumatoid arthritis (RA). In addition, migration of Tregs to inflammatory regions in response to chemokines (CCL1 and CCL22) and their receptors (CCR4 and CCR8) mediated disease suppression.

Results

Probiotics Increase CD4⁺Foxp3⁺ Tregs in a CD4⁺CD25[−] T-Cell Population.

Probiotic bacteria have potent immunomodulatory effects, but their mechanism of action is poorly understood. To identify probiotic strains that could specifically up-regulate CD4⁺Foxp3⁺ Tregs, we developed an ex vivo screening system by coculturing freshly isolated MLN with probiotics. Candidate probiotics, or a mixture of them, were selected based on the criteria that showed high levels (>4) of the IL-10/IL-12 production ratio and enhanced Foxp3 expression (>1.5-fold). These include *L. acidophilus* (LA), *L. casei* (LC), *Lactobacillus reuteri* (LR), *Bifidobacterium bifidum* (BB), and *Streptococcus thermophilus* (ST). After determination of the optimal dosage (5×10^8 cfu/day; *SI Materials and Methods*), candidate probiotic strains, individually or in combination, were fed to normal healthy BALB/c mice for 20 days and the population of CD4⁺Foxp3⁺ T cells in MLN was measured by fluorescence-activated cell sorting (FACS). A mixture of the five strains (LA/LC/LR/BB/ST) significantly ($P < 0.005$) increased the CD4⁺Foxp3⁺ Treg population compared with control, individual strain, or other mixture groups (Fig. S1 A–C). Thereafter, all experiments were performed with the mixture of five probiotics, designated IRT5. Administration of IRT5 significantly ($P < 0.005$) increased Foxp3 mRNA expression (Fig. 1A) and protein level (Fig. 1B) compared with the PBS-treated group. Next, we tested whether increased Foxp3 levels originated from naturally occurring CD4⁺CD25⁺ Tregs (nTregs) (8) or from induced Tregs in the CD4⁺CD25[−] population. Administration of IRT5 [probiotics (Pro)] significantly increased Foxp3 levels (6.5%) compared with the control PBS-fed group (Cont; 2.8%) within the population of CD4⁺CD25[−] Tregs (Fig. 1C, Upper). In addition, although IRT5 did not increase the CD4⁺CD25⁺ population (Fig. S1D) and no difference in the CD4⁺CD25⁺Foxp3⁺ population was observed between the treatment groups (Fig. 1C, Lower), the CD4⁺CD25⁺ Tregs of the Pro group showed increased expression of

Author contributions: S.-H.I. designed research; H.-K.K., C.-G.L., J.-S.S., C.-S.C., A.S., J.H.N., and S.-H.I. performed research; J.H.R. contributed new reagents/analytic tools; H.-K.K., J.-S.H., and K.-C.H. analyzed data; and H.-K.K. and S.-H.I. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

¹To whom correspondence should be addressed at: Department of Life Sciences, Gwangju Institute of Science and Technology, 1 Oryong-dong, Buk-gu, Gwangju 500-712, Korea. E-mail: imsh@gist.ac.kr.

This article contains supporting information online at www.pnas.org/cgi/content/full/0904055107/DCSupplemental.

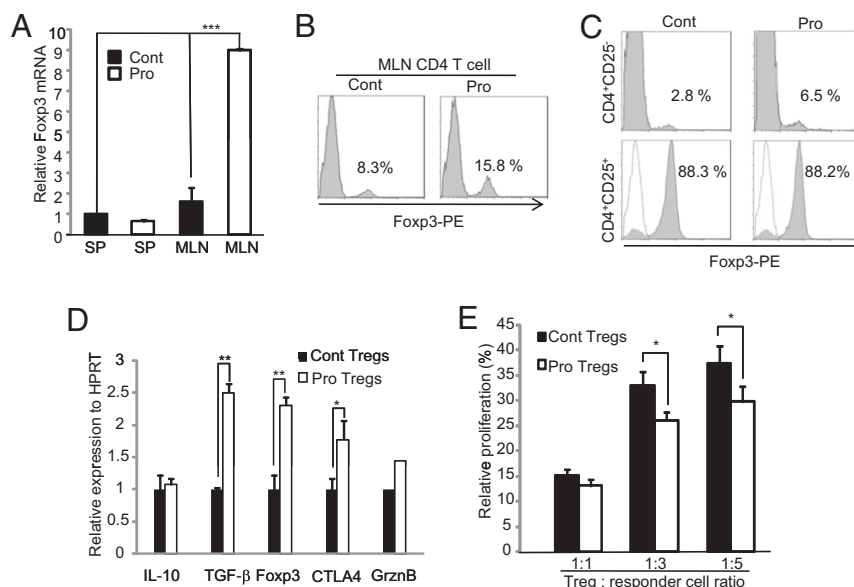


Fig. 1. Probiotics increase CD4⁺Foxp3⁺ Tregs. Foxp3 levels were analyzed by real-time PCR (A) or FACS (B) in SP and MLN CD4⁺ T cells from mice fed either PBS (Cont) or IRT5 (Pro) for 20 days. (C) Levels of Foxp3⁺ cells were analyzed in CD4⁺CD25⁺ and CD4⁺CD25[−] populations. (D) Expression levels of Treg-associated molecules were compared in MLN CD4⁺CD25⁺ Tregs from each group. GrznB, Granzyme B. (E) Suppressor activity of CD4⁺CD25⁺ Tregs was measured by T-cell proliferation assay. Tregs from each treatment group (Cont or Pro) were cocultured with responder cells (CD4⁺CD25[−]) at various ratios of Treg/responder cells. The proliferation level of responder cells alone was assigned a value of 100%. Data are the average of three independent experiments (10 mice per group); error bars indicate SD. **P* < 0.05; ***P* < 0.005; ****P* < 0.001.

Treg-associated molecules [TGF-β and cytotoxic T-lymphocyte-associated (CTLA)-4; Fig. 1D], resulting in significantly (*P* < 0.05) elevated suppressor activity compared with Tregs from the Cont group (Fig. 1E). We also compared the functional difference of CD4⁺CD25[−] effector T cells between PBS- and IRT5-fed groups. Compared with the Cont group, CD4⁺CD25[−] T cells from the Pro group were hypoproliferative (Fig. S24) with slightly increased suppression sensitivity by the CD4⁺CD25⁺ Tregs (Fig. S2B). These observations indicate that administration of IRT5 induced generation of CD4⁺Foxp3⁺ Tregs from the pool of CD4⁺CD25[−] cells and potentiated the suppressor function of CD4⁺CD25⁺ nTregs.

Probiotics Induce Hyporesponsiveness in CD4⁺ T Cells. Next, we tested whether increased CD4⁺Foxp3⁺ Tregs by IRT5 affects the effector function of T cells and B cells. IRT5 (Pro) or PBS (Cont) was fed to normal BALB/c mice for 20 days, and CD4⁺ T cells or B220⁺ B cells isolated from spleen (SP) and MLN were stimulated with phorbol 12-myristate 13-acetone (PMA)/ionomycin. The relative transcript levels of Th1 (IL-2, IFN-γ, and TNF-α), Th2 (IL-4 and IL-10), and Th17 (IL-17) cytokines were measured by real-time PCR. Administration of IRT5 significantly (*P* < 0.05–

0.005) reduced the levels of all tested cytokines in both CD4⁺ T cells (Fig. 2A) and B cells (Fig. S34). In addition, administration of IRT5 induced hyporesponsiveness in both CD4⁺ T cells (Fig. 2B) and B cells (Fig. S3B) without inducing apoptosis (Annexin V⁺/7-aminoactinomycin D (7-ADD)⁺) (Fig. S3C and D).

Probiotics Administration Induces CD11c⁺ rDCs, Which, in Turn, Promote Generation of CD4⁺Foxp3⁺ T Cells. We next investigated the role of CD11c⁺ dendritic cells (DCs) in the generation of CD4⁺Foxp3⁺ Tregs by IRT5. MLN CD11c⁺ DCs isolated from each treatment group were stimulated with PMA/ionomycin, and the levels of tolerogenic DC markers were measured. Administration of IRT5 significantly up-regulated the expression levels of IL-10, TGF-β, iDO, and COX-2 (Fig. 3A) without increasing CD103⁺ DCs, which are recognized as a marker of mucosal DCs generating Foxp3⁺ Tregs (9) (Fig. S44). We further tested whether rDCs generated by IRT5 have the potential to induce Foxp3⁺ Tregs. MLN DCs from each group were cocultured with DO11.10 CD4⁺T cells labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) in the presence of Ova peptide (0.3 μg/mL), with or without exogenous TGF-β, which induces the generation of induced Tregs in vitro. The

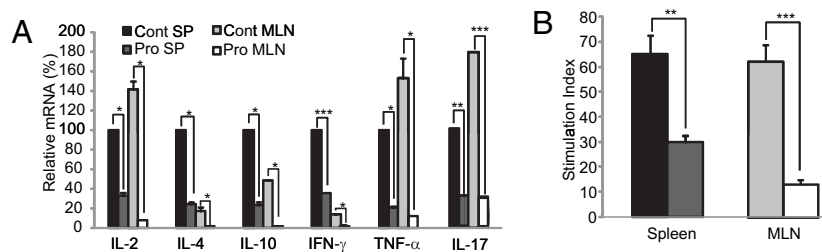


Fig. 2. Probiotics induce hyporesponsiveness in CD4 T cells. (A) Levels of cytokine expression in CD4⁺ T cells were analyzed by real-time PCR. Cytokine expression in spleen CD4⁺ T cells of Cont group mice was set at 100%. (B) Proliferation of CD4⁺ T cells from the Cont or Pro group mice was measured after stimulation with anti-CD3/anti-CD28. Data are from 10 mice per group; error bars indicate SD. Data are representative of three independent experiments. ***P* < 0.005; ****P* < 0.001.

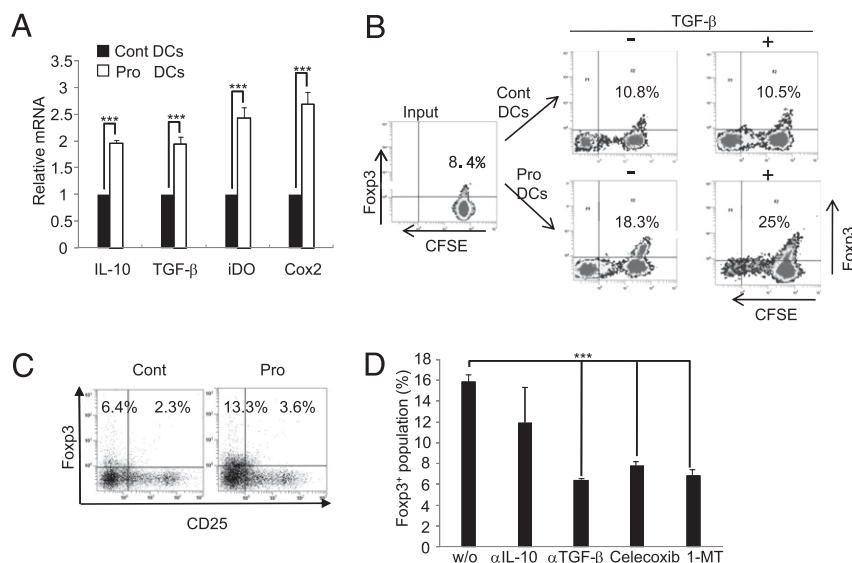


Fig. 3. Probiotics induce CD11c⁺ rDCs. (A) CD11c⁺ DCs isolated from MLN of Cont or Pro group mice were stimulated with PMA/ionomycin. The levels of immunoregulatory molecules were measured by real-time PCR. (B) CD11c⁺ DCs from each group were cocultured with Do11.10 CD4⁺ T cells labeled with CFSE and ovalbumin peptide (0.3 μg/mL) in the absence (–) or presence (+) of exogenous TGF-β. (C) Levels of Foxp3⁺CD25[–] and Foxp3⁺CD25⁺ populations were measured by FACS. (D) MLN CD11c⁺ DCs from IRT5-fed mice were cocultured with CD4⁺ T cells in the presence of ovalbumin peptide and inhibitors for rDCs. w/o, without. After coculture, alteration of the Foxp3⁺ population was measured by FACS. Data are from 10 mice per group and are the average of three independent experiments.

CD4⁺Foxp3⁺ Treg population was then analyzed by FACS. MLN DCs from Pro mice preferentially converted T cells into CD4⁺Foxp3⁺ T cells compared with the Cont group in both the absence (18.3% Pro vs. 10.8% Cont) and presence (25% Pro vs. 10.5% Cont) of TGF-β (Fig. 3B). In addition, CD4⁺ T cells cocultured with IRT5-treated MLN DCs showed significantly reduced proliferation compared with control (30% Pro vs. 61% Cont) (Fig. S4B) because of their increased Treg population (Fig. 3B). Like in vivo-generated Tregs by IRT5 administration (Fig. 1C), up-regulation of Foxp3 levels were mainly observed in the CD4⁺CD25[–] population (13.3% Pro vs. 6.4% Cont) (Fig. 3C). To characterize the functional property of rDCs generated by IRT5 further, we tested whether addition of inhibitors for rDCs could inhibit rDC-dependent Foxp3⁺ Treg generation. CD4⁺ T cells were cocultured with MLN CD11c⁺ DCs in the presence of various types of inhibitors, and Foxp3⁺ populations then were analyzed by FACS. Addition of inhibitors such as anti-TGF-β, celecoxib (a COX-2 inhibitor), or 1-methyl-d-tryptophan (1-MT, an iDO inhibitor) significantly down-regulated Foxp3⁺ generation (Fig. 3D and Fig. S4C and D). Collectively, these results suggest that IRT5 facilitates the generation of rDCs that have the capacity to convert Foxp3[–] T cells into Foxp3⁺ Tregs.

Probiotics Suppress Experimental Immune Disorders. Next, we tested whether IRT5 could ameliorate immune disorders such as IBD, AD, and RA. Trinitrobenzenesulfonic acid (TNBS) colitis is a well-characterized animal model of Th1-mediated IBD, and it mimics human Crohn's disease. Mice were fed with PBS or IRT5 daily for 20 days, and the intestinal colitis was established by an intrarectal injection of TNBS. The prevention effects of IRT5 were monitored by measuring survivalship, weight loss, colitis grade, gross morphology, and histology of the colon (Fig. 4 and Fig. S5). Retardation of IBD progression in the Pro group was associated with reduction of all tested criteria for disease evaluation, including inflammatory cytokines such as IL-17, IFN-γ, and TNF-α (Fig. 4C).

Next, we tested whether IRT5 also has a therapeutic effect on AD, which is known to be a typical Th2-mediated immune disorder. A murine AD model was established by alternate painting of house dust mite extract and dinitrochlorobenzene. Two weeks after AD induction, IRT5 or PBS was administered to mice with AD and the parameters for AD progression were measured. Treatment with IRT5 significantly ($P < 0.05$) inhibited the clinical symptoms of AD progression (Fig. 5A and Fig. S6A) by reducing IgE levels [total IgE level (Fig. 5C) and mite-specific IgE level

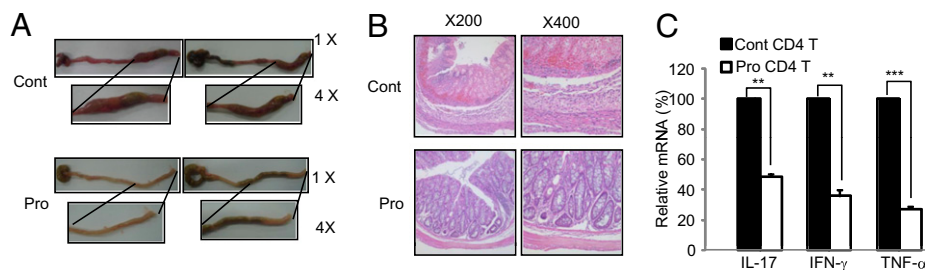


Fig. 4. Probiotics suppress experimental colitis. (A) Changes in gross intestines were evaluated 3 days after induction of colitis. (B) Histological analysis after H&E staining to detect lymphocyte infiltration. (C) Real-time PCR was performed to analyze changes in the expression of proinflammatory cytokines in response to probiotics administration. Data are from 20 mice per group; error bars indicate SD. Data are representative of three independent experiments. *** $P < 0.005$; **** $P < 0.001$.

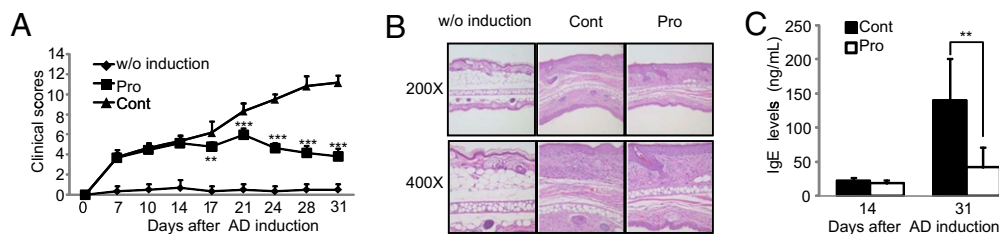


Fig. 5. Probiotics suppress experimental AD. The therapeutic efficacy of IRT5 was measured by the following criteria for AD progression: clinical score (A), lymphocyte infiltration by histological analysis (B), and total IgE levels (C). w/o, without. Bars are from 10 mice per group; error bars indicate SD. Data are representative of three independent experiments. ** $P < 0.005$; *** $P < 0.001$.

(Fig. S6C)], infiltrated lymphocytes and granulocytes (Fig. 5B and Fig. S6B), and levels of AD-associated cytokines, such as IL-4, IL-5, IL-10 and IL-13 (Fig. S6E and F). In addition, administration of IRT5 significantly slowed the progression of collagen-induced experimental RA by reducing clinical symptoms, lymphocyte infiltration into the joint, and levels of inflammatory cytokines, while increasing Foxp3 levels (Fig. S7).

Enriched CD4⁺Foxp3⁺ Treg Cells at Sites of Inflammation Are Associated with Disease Suppression. CD4⁺Foxp3⁺ Tregs suppress pathogenic effector cells through cell-to-cell contact or focal increment at the inflamed sites (10–12). Thereby, we measured the population of CD4⁺Foxp3⁺ Tregs at the sites of disease progression. Mice with AD or IBD were treated with IRT5 or PBS, and the population of CD4⁺Foxp3⁺ T cells at the inflammatory sites (colon in experimental IBD and ear in AD) was determined by FACS and immunohistochemistry (IHC). The Pro group showed significantly increased CD4⁺Foxp3⁺ T cells compared with the Cont group in both IBD (31% vs. 14.2%; Fig. 6A) and AD (32.9% vs. 4.6%; Fig. 6B) mice. Enrichment of Foxp3⁺CD4⁺ Tregs in the IRT5-treated group was also confirmed by IHC in both the inflammatory colon and atopic ear (Fig. 6C and Fig. S8).

Increased Chemoattractants for Treg Induce Migration of CD4⁺Foxp3⁺ Tregs to Inflamed Regions. Next, we checked whether increased CD4⁺Foxp3⁺ Tregs are directly related to the therapeutic effects of IRT5 in multiple types of immune disorders. As in normal mice (Fig. 1), IRT5 administration in disease conditions also significantly increased the CD4⁺Foxp3⁺ Treg populations in MLN (Fig. S9A and B). We further tested whether enriched levels of CD4⁺Foxp3⁺ Tregs in the colon (IBD) or ear (AD) (Fig. 6C) are associated with increased chemoattractants for CD4⁺Foxp3⁺ Tregs, resulting in their migration from MLN to the inflammatory sites. We measured the expression levels of chemokines and their

receptors involved in the recruitment of CD4⁺Foxp3⁺ Tregs in AD mice. Indeed, the group fed with IRT5 (Pro) showed significantly increased expression levels of CCL1 and CCL22 in the ear tissues (Fig. 7A) and their receptors, CCR4 and CCR8 (13–15), in ear residual CD4⁺ T cells (Fig. 7B). We further examined the migratory properties of CD4⁺Foxp3⁺ Tregs generated in MLN by IRT5 administration to the inflammatory region by means of an adoptive transfer experiment. CD4⁺ T cells isolated from MLN of the mice fed PBS or IRT5 for 20 days were labeled with CFSE and then adoptively transferred to AD mice by i.p. injection (Fig. S9C). The CFSE⁺Foxp3⁺ populations in the ear of AD mice were measured by IHC (Fig. 7C and Fig. S10). As a negative control, the same numbers of unlabeled CD4⁺ T cells were transferred (Fig. 7C, Left, and Fig. S10). Ear sections from each group of mice were stained by Hoechst (blue) and anti-Foxp3 antibodies (red). CFSE⁺(CD4⁺)Foxp3⁺ cells were detected by bright green staining as a result of overlap of the colors. The Pro group (Fig. 7C, Right, and Fig. S10) showed much higher numbers of CFSE⁺(CD4⁺)Foxp3⁺ cells compared with the Cont group (Fig. 7C, Middle, and Fig. S10) in the ear regions of AD mice. Conclusively, these results suggest that CD4⁺Foxp3⁺ Tregs generated by administration of IRT5 in MLN migrated to the inflamed sites to mediate suppression of disease progression.

Discussion

In this study, we developed a probiotics mixture (designated IRT5) with potent anti-inflammatory properties and examined its modulation efficacy of experimental immune disorders. Administration of IRT5 induced rDCs, which, in turn, potentiated generation of CD4⁺Foxp3⁺ Tregs in MLN. Enrichment of CD4⁺Foxp3⁺ Tregs at sites of inflammation is associated with up-regulated levels of chemoattractants (CCL1 and CCL22), which inhibited the progression of experimental immune disorders.

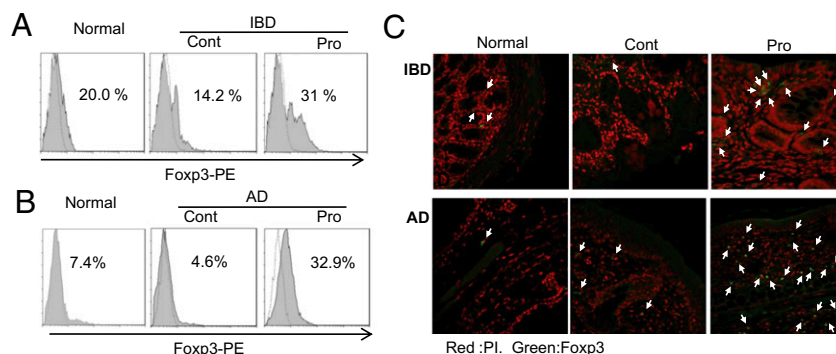


Fig. 6. Enriched CD4⁺Foxp3⁺ Tregs at inflammatory sites are associated with disease suppression. The Foxp3⁺ population from the colon of experimental colitis (A) or ear of AD (B) mice was analyzed by FACS or IHC (C) between the Pro and Cont groups. The arrows in C indicate Foxp3⁺ cells. A control reaction was performed with isotype-matched IgG antibodies (data not shown). Data are from 10 mice per group and represent the average of three independent experiments.

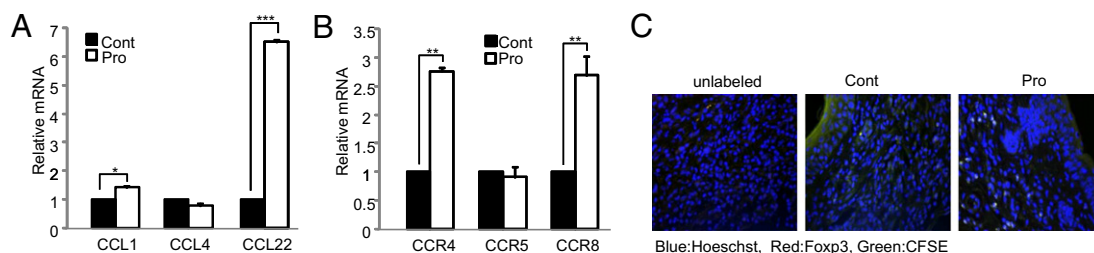


Fig. 7. Increased chemoattractant expression leads to enrichment of Tregs in the inflamed region. The expression levels of chemokines in MHC class II-positive antigen-presenting cells (A) and their receptors in CD4⁺ T cells (B) were analyzed by quantitative real-time PCR. (C) CFSE-labeled T cells obtained from MLN of Cont and Pro groups were adoptively transferred to AD mice. The migration of CFSE⁺(CD4⁺)Foxp3⁺ Tregs to the ear of AD mice was analyzed by IHC. The arrows indicate CFSE⁺(CD4⁺)Foxp3⁺ Tregs. Data are from 10 mice per group; error bars indicate SD. Data are representative of three independent experiments. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$.

Little information is available on the mechanism by which administration of probiotics generates regulatory T-cell populations. In the present study, we present evidence that ingestion of a specific probiotics mixture (IRT5) can generate CD4⁺Foxp3⁺ Tregs in MLN. The immunomodulatory action of IRT5 was not achieved by increasing the expression of immunosuppressive cytokines such as IL-10 or TGF- β but, instead, by suppressing the expression of inflammatory (IL-17, IFN- γ , and TNF- α) and anti-inflammatory (IL-10) cytokines in both T cells (Fig. 2) and B cells (Fig. S3 A and B) without apoptosis induction (Fig. S3 C and D). Administration of IRT5 did not increase the number of nTregs (Fig. 1C) but did increase their suppressor capacity (Fig. 1 D and E). Interestingly, ingestion of IRT5 up-regulated Foxp3 levels more than 2-fold in the CD4⁺CD25⁻ population (Fig. 1C).

How do probiotics promote the generation of CD4⁺Foxp3⁺ cells? They may not directly generate adaptive Tregs but induce rDCs to exhibit immunological tolerance. Indeed, probiotics (IRT5) alone without rDCs failed to induce Foxp3⁺ Tregs when they were cocultured with CD4⁺ T cells up to 3 days (Fig. S4E). Recently, two studies reported that oral administration of a certain probiotic strain could increase Foxp3⁺ Tregs (16, 17) in the SP. However, previous studies did not provide any clue as to how probiotics can enhance the generation of CD4⁺Foxp3⁺ Tregs. In this study, we provided evidence and sequential scenarios for how IRT5 can generate CD4⁺Foxp3⁺ Tregs in MLN. Administration of IRT5 probiotics induced rDCs, which, in turn, promoted generation of CD4⁺Foxp3⁺ T cells in MLN (Fig. 3). DCs can directly present antigens from commensal bacteria to MLN and interact with T and B cells to maintain noninflammatory immune responses (18, 19). Treatment with IRT5 generates CD11c⁺ DCs that express markers of rDCs such as IL-10, TGF- β , COX-2, and iDO (20). COX-2 is one of several active molecules to generate Foxp3⁺ Tregs in various types of systems, including cancer (21, 22). iDO DCs promote the development of Treg cells (23). Therefore, we postulated that IRT5 generated tolerogenic DCs, which, in turn, induced generation of CD4⁺Foxp3⁺ Tregs. Indeed, we demonstrated that rDCs generated by IRT5 could convert CD4⁺CD25⁻ T cells into CD4⁺CD25⁻Foxp3⁺ T cells (Fig. 3 B and C), in which, however, addition of inhibitors for TGF- β , COX-2, or iDO strongly inhibited the generation of Foxp3⁺ Tregs (Fig. 3D and Fig. S4C). Mucosal CD103⁺ DCs play important roles in the generation of Foxp3⁺ Tregs through in a retinoic acid TGF- β -dependent manner (24). However, rDCs generated by IRT5 administration were not CD103⁺CD11c⁺ but CD103c⁻CD11c⁺. IRT5 administration did not up-regulate CD103⁺ (Fig. S4A), and blockade of retinoic acid (LE540, a retinoic acid inhibitor) did not alter the levels of rDC-mediated Foxp3⁺ Tregs (Fig. S4D). How does the immune system recognize to the generation of rDCs in MLN? Currently we are trying to identify and elucidate the role of the pattern recognition receptors

(e.g., Toll-like receptor-2 and other TLRs) (25, 26) and underlying signal transduction pathways (e.g., Myd88 dependency).

Administration of IRT5 increased the numbers of adaptive Tregs (CD4⁺CD25⁻Foxp3⁺) both in healthy mice (Fig. 1) and in IBD and AD mouse models (Fig. S9 A and B). The protective effect of IRT5 is associated with highly up-regulated levels of CD4⁺Foxp3⁺ Tregs in the inflammatory regions (Fig. 6 and Fig. S8). Tregs are able to recirculate through lymphoid tissues and to enter inflamed sites (27) by expressing specific chemokines and their receptors (28). We also found that administration of IRT5 significantly increased the expression of chemokines (CCL1 and CCL22; Fig. 7A) and their receptors (CCR4 and CCR8; Fig. 7B) (13–15). Furthermore, the results of an adoptive transfer experiment revealed that Tregs generated in MLN by IRT5 can migrate to the inflammatory sites of AD mice (Fig. 7C and Fig. S10). Decreased Tregs are associated with inflammatory diseases, including IBD and AD (29, 30). Interestingly, experimental IBD and AD mice also exhibited reduced numbers of CD4⁺Foxp3⁺ Tregs in inflamed sites compared with control mice (Fig. 6 and Fig. S8). Hence, enriched CD4⁺Foxp3⁺ Tregs induced by IRT5 may suppress inflammatory effector T cells at the sites of inflammation, resulting in a protective effect in these disease models.

One of the challenging issues in the probiotic field is how to identify potent immunoregulatory probiotics among the hundreds of probiotic strains. Recently, we developed an ex vivo screening system to identify immune-regulatory probiotics inducing IL-10 and Foxp3⁺ Tregs but reducing proinflammatory cytokines. After coculturing MLN total cells with each probiotic strain for 3 days, probiotic strains that showed a high level (>4) of the IL-10/IL-12 ratio and more than a 1.5-fold increase in Foxp3⁺ levels were selected. A mixture of selected strains (IRT5) showed more potent disease suppression efficacy than single or three- and four-strain combinations (Fig. S11). Interestingly, heat-inactivated IRT5 did not have any significant therapeutical effects. Why does a combination of probiotics show more potent therapeutical effects than a single probiotic strain (Fig. 1 and Fig. S11)? The combination of three (Fig. S11; multistrain combination) and four or five (Fig. S11; multispecies combinations) strains may enhance colonization and adhesion activity (31, 32) compared with a single strain, resulting in increased immune-modulatory activities by which Foxp3⁺ Tregs were generated. How does IRT5 modulate disease progression, regardless of the site of inflammation, in inflammatory diseases? We suggest the following sequence of events. First, DCs recognize and process the administered IRT5 probiotics. During this process, CD11c⁺ DCs are converted into rDCs that are able to convert CD4⁺Foxp3⁻ T cells into CD4⁺Foxp3⁺ Tregs. Next, the generated Tregs in MLN migrate to the inflammatory sites, directed by chemoattractant produced at the inflammatory sites, resulting in an enrichment of Tregs. Finally, Tregs abolish the inflammatory effector function of pathogenic T cells, which leads to suppression of disease progression.

In summary, the combined probiotics in IRT5 exerted potent immunomodulatory effects by up-regulating or potentiating the generation of Tregs by tolerogenic DCs in MLN. In addition, migration of CD4⁺Foxp3⁺ Tregs to sites of inflammation effectively suppressed disease progression. Our results present evidence of the generation of CD4⁺CD25⁺Foxp3⁺ Tregs in response to probiotics, an effect that may be therapeutically useful for the modulation of inflammatory immune disorders.

Materials and Methods

Probiotic Strains. The probiotics mixtures of live bacteria contained 1×10^9 cfus of each of the following strains: BB, LA, ST, LC, and *Lactobacillus leuteri*. To test the effect of single strains, each strain was administered to mice at a concentration of 5×10^8 cfus per 100 μ L. Probiotic strains used in this study were purchased from Cell Biotech Co., Ltd.

DC-Dependent Treg Cell Differentiation Assay. MLN CD11c⁺ DCs were isolated from each group and cultured with splenic CD4⁺ T cells isolated from Do11.10 transgenic mice or WT BALB/c mice labeled with 10 mM CFSE (Invitrogen) in the presence of ovalbumin peptide (0.2 μ g/mL) and soluble anti-CD3 (1 μ g/mL) with or without mouse TGF- β (2 ng/mL, PeproTech; catalog no. 100-21c) for 4 days. To test the roles of rDC-related molecules in rDC-

mediated Treg generation, inhibitors were added such as anti-IL-10 (10 μ g/mL; BD PharMingen), anti-TGF- β (10 μ g/mL; R&D Systems), LE540 (0.1 or 1 μ M; Wako), celecoxib (50 μ M; Biovision) and 1-MT (20 μ M; Sigma). The population of Foxp3⁺ Treg cells was analyzed by FACS.

Adoptive Transfer Experiment. After 20 days of oral administration of PBS or probiotics (IRT5), mice were killed and MLN CD4⁺ T cells were isolated from each group. A total of 5×10^6 CD4⁺ T cells were labeled with 5 μ M CFSE for 15 min at 37°C and adoptively transferred into AD-induced mice by i.p. injection. Twenty-four hours after adoptive transfer, mice were killed and ears were removed to examine the migration of Tregs by IHC (33).

Statistical Analysis. The Student's *t* test was used to determine significance, and *P* < 0.05 was considered significant.

ACKNOWLEDGMENTS. This research was supported by grants from the 21C Frontier Functional Human Genome Project (FG08-21-11); National Research Foundation of Korea (2009-0079438) funded by the Korean government; Regional Technology Innovation Program of the Ministry of Knowledge Economy (RT105-01-01); and Korea Healthcare Technology R&D Project, Ministry for Health, Welfare, and Family Affairs (A080588-5) and by a Systems Biology Infrastructure Establishment grant provided by the Gwangju Institute of Science and Technology in 2008.

- Marteau P (2006) Probiotics, prebiotics, synbiotics: Ecological treatment for inflammatory bowel disease? *Gut* 55:1692–1693.
- Braat H, et al. (2004) Lactobacillus rhamnosus induces peripheral hyporesponsiveness in stimulated CD4⁺ T cells via modulation of dendritic cell function. *Am J Clin Nutr* 80:1618–1625.
- So J-S, et al. (2008) Lactobacillus casei potentiates induction of oral tolerance in experimental arthritis. *Mol Immunol* 46:172–180.
- So J-S, et al. (2008) Lactobacillus casei suppresses experimental arthritis by down-regulating T helper 1 effector functions. *Mol Immunol* 45:2690–2699.
- Sudo N, et al. (2002) An oral introduction of intestinal bacteria prevents the development of a long-term Th2-skewed immunological memory induced by neonatal antibiotic treatment in mice. *Clin Exp Allergy* 32:1112–1116.
- Shanahan F (2003) Probiotics: A perspective on problems and pitfalls. *Scand J Gastroenterol* 38(suppl 237):34–36.
- Ziegler SF (2006) FOXP3: Of mice and men. *Annu Rev Immunol* 24:209–226.
- Jordan MS, et al. (2001) Thymic selection of CD4⁺CD25⁺ regulatory T cells induced by an agonist self-peptide. *Nat Immunol* 2:301–306.
- Coomes JL, et al. (2007) A functionally specialized population of mucosal CD103⁺ DCs induces Foxp3⁺ regulatory T cells via a TGF- β and retinoic acid-dependent mechanism. *J Exp Med* 204:1757–1764.
- Vignali DAA, Collison LW, Workman CJ (2008) How regulatory T cells work. *Nat Rev Immunol* 8:523–532.
- Kim CH (2006) Migration and function of Foxp3⁺ regulatory T cells in the hematolymphoid system. *Exp Hematol (Charlottesville, Va)* 34:1033–1040.
- Huehn J, Hamann A (2005) Homing to suppress: Address codes for Treg migration. *Trends Immunol* 26:632–636.
- Gobert M, et al. (2009) Regulatory T cells recruited through CCL22/CCR4 are selectively activated in lymphoid infiltrates surrounding primary breast tumors and lead to an adverse clinical outcome. *Cancer Res* 69:2000–2009.
- Chen D, Bromberg JS (2006) T regulatory cells and migration. *Am J Transplant* 6:1518–1523.
- Sebastiani S, et al. (2001) Chemokine receptor expression and function in CD4⁺ T lymphocytes with regulatory activity. *J Immunol* 166:996–1002.
- Feleszko W, et al. (2007) Probiotic-induced suppression of allergic sensitization and airway inflammation is associated with an increase of T regulatory-dependent mechanisms in a murine model of asthma. *Clin Exp Allergy* 37:498–505.
- Karimi K, Inman MD, Bienenstock J, Forsythe P (2009) Lactobacillus reuteri-induced regulatory T cells protect against an allergic airway response in mice. *Am J Respir Crit Care Med* 179:186–193.
- Macpherson AJ, Uhr T (2004) Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. *Science* 303:1662–1665.
- Huang F-P, et al. (2000) A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes. *J Exp Med* 191:435–444.
- Morelli AE, Thomson AW (2007) Tolerogenic dendritic cells and the quest for transplant tolerance. *Nat Rev Immunol* 7:610–621.
- Sharma S et al. (2005) Tumor cyclooxygenase-2/prostaglandin E2-dependent promotion of FOXP3 expression and CD4⁺ CD25⁺ T regulatory cell activities in lung cancer. *Cancer Res* 65:5211–5220.
- Sharma S, et al. (2005) Tumor cyclooxygenase-2/prostaglandin E2-dependent promotion of FOXP3 expression and CD4⁺ CD25⁺ T regulatory cell activities in lung cancer. *Cancer Res* 65:5211–5220.
- Mellor AL, Munn DH (2004) IDO expression by dendritic cells: Tolerance and tryptophan catabolism. *Nat Rev Immunol* 4:762–774.
- von Boehmer H (2007) Oral tolerance: Is it all retinoic acid? *J Exp Med* 204:1737–1739.
- Dai J, Liu B, Li Z (2009) Regulatory T cells and Toll-like receptors: What is the missing link? *Int Immunopharmacol* 9:528–533.
- Sutmoller RPM, et al. (2006) Toll-like receptor 2 controls expansion and function of regulatory T cells. *J Clin Invest* 116:485–494.
- Maul J, et al. (2005) Peripheral and intestinal regulatory CD4⁺ CD25^(high) T cells in inflammatory bowel disease. *Gastroenterology* 128:1868–1878.
- D'Ambrosio D, Sinigaglia F, Adorini L (2003) Special attractions for suppressor T cells. *Trends Immunol* 24:122–126.
- Leung DYM, Boguniewicz M, Howell MD, Nomura I, Hamid QA (2004) New insights into atopic dermatitis. *J Clin Invest* 113:651–657.
- Bouma G, Strober W (2003) The immunological and genetic basis of inflammatory bowel disease. *Nat Rev Immunol* 3:521–533.
- Timmerman HM, Koning CJM, Mulder L, Rombouts FM, Beynen AC (2004) Monostrain, multistain and multispecies probiotics—A comparison of functionality and efficacy. *Int J Food Microbiol* 96:219–233.
- Collado MC, Meriluoto J, Salminen S (2007) Development of new probiotics by strain combinations: Is it possible to improve the adhesion to intestinal mucus? *J Dairy Sci* 90:2710–2716.
- Kwon H-K, et al. (2009) Cinnamon extract suppresses tumor progression by modulating angiogenesis and the effector function of CD8⁺ T cells. *Cancer Lett (Shannon, Ire)* 278:174–182.

Supporting Information

Kwon et al. 10.1073/pnas.0904055107

SI Materials and Methods

Experimental Animals and Disease Models. BALB/c mice (6–8 weeks old) and Lewis rats (6–8 weeks old) were purchased from the SLC Co. Do11.10 mice (6–8 weeks old) were purchased from the Jackson Laboratory. Mice were maintained in pathogen-free conditions in the animal facility of Gwangju Institute of Science and Technology (GIST). All animal experiments were approved by the GIST Animal Care and Use Committee.

Determination of Optimal Dosage and Treatment Duration of Probiotics. To determine the optimal dose of probiotics, we tested the protective effect of each probiotic or probiotic combinations in the TNBS-induced colitis model. Probiotics were orally administered with diverse doses (1×10^7 , 5×10^7 , 1×10^8 , 5×10^8 , and 1×10^9 cfu/day) dissolved in 100 μ L of PBS for 20 days, and colitis was then induced by intrarectal injection of TNBS. A minimum dosage of 1×10^8 cfu/day of probiotics was required to exert a protective effect, and a dosage of 5×10^8 cfu/day of probiotics showed the most potent effects in TNBS-induced colitis. To find the minimum duration of probiotics treatment, 5×10^8 cfu/day of probiotics was differentially administered for 5, 10, 20, or 30 days and colitis was induced in each group after the final probiotics treatment. Results indicated that more than 10 days of probiotics treatment was required to see protective effects in TNBS-induced colitis.

Disease Models. To test the prophylaxis effects of probiotics, mice were divided into seven groups and fed either PBS or five individual strains or their mixture in combinations of three, four, or five (IRT5) strains (5×10^8 cfu/day) in PBS for 20 days by oral administration with a catheter and experimental colitis was induced. Briefly, to induce experimental colitis, 50 μ g/g (mouse weight) of TNBS in 50% (vol/vol) ethanol was injected to slightly anesthetized mice through a catheter inserted into the rectum, and 100 μ L of the TNBS-ethanol mixture was carefully poured into the colon. The subsequent course of colitis was evaluated by assessing mortality, body weight decrease, and macroscopic and microscopic observations. After killing mice, lamina propria (LP) and MLN CD4⁺ T cells or B220⁺ B cells were isolated for further experiments.

For the induction of experimental AD in BALB/c mice, the ear surface was stripped three times with surgical tape (Nichiban). Thirty minutes after stripping, 20 μ L of 1% dinitrochlorobenzene (DNCB; Sigma) dissolved in acetone/olive oil solution (1:3 acetone/olive oil ratio) was painted on each ear surface. Four days after DNCB painting, 20 μ L of 10 mg/mL mite extracts (Yonsei University College of Medicine) was repainted after tape stripping. Alternative painting of DNCB or mite antigen was performed. Tape stripping and DNCB/mite extract painting were continued for 4 weeks. Two weeks after AD induction, to confirm the induction of AD, eye breeding was performed to check the serum IgE level. After confirming AD induction, IRT5 or PBS was given daily by oral feeding with a catheter in each group (Pro and Cont) until the end of induction. Ear thickness and clinical scores were measured 24 h after DNCB or mite application. Only tape stripping and painting of PBS were performed in a Cont group of mice.

For the induction of experimental RA, chicken collagen type II (CII; Sigma) was dissolved in 0.1 M acetic acid at a concentration of 4 mg/mL and then emulsified in an equal volume of complete Freund's adjuvant (Difco) containing 4 mg/mL *Mycobacterium tuberculosis* (Difco). One hundred fifty milligrams of this emul-

sion was intradermally injected in Lewis rats (6–8 weeks old). A booster injection was administered 7 days after the first immunization with 300 mg of CII emulsified in an equal volume of incomplete Freund's adjuvant (Difco). After 7 days of disease induction, rats were fed with PBS or IRT5 up to 70 days. RA clinical scores were checked daily. After killing rats, joint regions from each group were isolated for histological analysis and DLN CD4⁺ T cells were collected for additional experiments.

Macroscopic and Histological Evaluation. In experimental colitis (IBD) studies, gross intestines were removed from mice and evaluated as previously described (1). For histological scoring, gross intestines were fixed with 4% (vol/vol) paraformaldehyde in PBS and embedded in paraffin. Paraffin sections were stained with H&E and scored as previously described (2). In experimental AD studies, the clinical condition and symptoms of each mouse were evaluated as previously described, with a slight modification (3). The number of total infiltrated cells stained by H&E in the dermis was counted (3). For the clinical evaluation of experimental RA, clinical scores were counted as described previously (4). Each paw was graded, which could result in one rat having a maximum score of 16. Paw swelling was examined by measuring changes in ankle thickness with calipers. To check histological change, joint tissues were prepared at 10–30 mm and stained with H&E. All clinical and histological evaluations were performed in a blinded manner.

RNA Isolation, cDNA Synthesis, and Quantitative and Standard Real-Time PCR. Total RNA was isolated from the SPs and MLNs of each group using TRIzol reagent (Molecular Research Center). Reverse transcription was performed with reverse transcriptase (Promega) primed with oligo(dT) primer. The synthesized cDNAs were amplified by quantitative real-time PCR (SYBR green; Takara) with CHROMO4 Detector (Biorad) and by standard PCR (SP-Taq, COSMO Genetech; i-Tag, Intron Biotechnology) using an Alpha Unit Block assembly engine system (Biorad) with the following primers: murine L-32 (forward) 5'-GCC CAA GAT CGT CAA AAA GA-3' and (reverse) 5'-ATT GTG GAC CAG GAA CTT GC-3', IL-2 (forward) 5'-CCT GAG CAG GAT GGA GAA TTA CA-3' and (reverse) 5'-TCC AGA ACA TGC CGC AGA G-3', IL-4 (forward) 5'-ACA GGA GAA GGG ACG CCA T-3' and (reverse) 5'-GAA GCC CTA CAG ACG AGC TCA-3', IL-5 (forward) 5'-AGC ACA GTG GTG AAA GAG AC-3' and (reverse) 5'-TCC AAT GCA TAG CTG GTG ATT T-3', IL-10 (forward) 5'-ATA ACT GCA CCC ACT TCC CA-3' and (reverse) 5'-TCA TTT CCG ATA AGG CTT GG-3', IL-12 p40 (forward) 5'-GGA AGC ACG GCA GCA GAA TA-3' and (reverse) 5'-AAC TTG AGG GAG AAG TAG GAA TGG-3', IL-13 (forward) 5'-GCA ACA TCA CAC AGG ACC AGA-3' and (reverse) 5'-GTC AGG GAA TCC AGG GCT AC-3', IL-17A (forward) 5'-TTC ATC TGT GTC TCT GAT GCT-3' and (reverse) 5'-TTG ACC TTC ACA TTC TGG AG-3', IFN- γ (forward) 5'-TCA AGT GGC ATA GAT GTG GAA GAA-3' and (reverse) 5'-TGG CTC TGC AGG ATT TTC ATG-3', TNF- α (forward) 5'-CAT CTT CTC AAA ATT CGA GTG ACA A-3' and (reverse) 5'-TGG GAG TAG ACA AGG TAC AAC CC-3', TGF- β (forward) 5'-GAA GGC AGA GTT CAG GGT CTT-3' and (reverse) 5'-GGT TCC TGT CTT TGT GGT GAA-3', Foxp3 (forward) 5'-CCC ATC CCC AGG AGT CTT G-3' and (reverse) 5'-CCA TGA CTA GGG GCA CTG TA-3', hypoxanthine-guanine phosphoribosyltransferase (HPRT) (forward) 5'-TTA TGG ACA GGA CTG AAA

GAC-3' and (reverse) 5'-GCT TTA ATG TAA TCC AGC AGG T-3', B7.1 (forward) 5'-ACC CCC AAC ATA ACT GAG TCT-3' and (reverse) 5'-TTC CAA CCA AGA GAA GCG AGG-3', B7.2 (forward) 5'-TGT TTC CGT GGA GAC GCA AG-3' and (reverse) 5'-CAG CTC ACT CAG GCT TAT GTT TT-3', inducible costimulatory ligand (ICOSL) (forward) 5'-GAC TGA AGT CGG TGC AAT GGT-3' and (reverse) 5'-TGG GTT TTC GAT TTG CCA ATA GA-3', programmed death-1 ligand (PD1L) (forward) 5'-ATG CTG CCC TTC AGA TCA CAG-3' and (reverse) 5'-TGG TTG ATT TTG CGG TAT GGG-3', IDO (forward) 5'-TGG CGT ATG TGT GGA ACC G-3' and (reverse) 5'-CTG CAT AAG ACA GAA TAG GAG GC-3', CTLA-4 (forward) 5'-AGA ACC ATG CCC GGA TTC TG-3' and (reverse) 5'-CAT CTT GCT CAA AGA AAC AGC AG-3', and GranzymeB (forward) 5'-CTC CTA AAG CTG AAG AGT AAG G-3' and (reverse) 5'-TTT AAA GTA GGA CTC ACA CTC CC-3'. All the PCR experiments were performed under the same condition as follows: 95°C for 5 min, 95°C for 30 s, 62°C for 30 s, and 72°C for 30 s up to 40 cycles.

Isolation of CD4⁺ T Cells, CD4⁺CD25⁺ T Cells, CD4⁺CD25⁻ T Cells, B220⁺ B Cells, and DCs. A single-cell suspension was prepared from the SPs and MLNs of each group. CD4⁺ T cells were isolated with CD4⁺ T-cell isolation beads (Miltenyi Biotech; catalog no. 130-049-201) and columns (Miltenyi Biotech; catalog no. 130-042-401) or with mouse CD4 beads (Dyna; catalog no. 114.45) and DETACHaBEAD (Dyna; catalog no. 124.06). To isolate CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells, CD4⁺ T cells obtained by the latter method were labeled with biotin-conjugated rat anti-mouse CD25 (BD Pharmingen; catalog no. 553069) and incubated with streptavidin microbeads (Miltenyi Biotech; catalog no. 130-048-101) in labeling buffer [PBS (pH 7.2), 2 mM EDTA]. Isolation of CD4⁺CD25⁺ Tregs and a B220⁺ B-cell isolation kit (Miltenyi Biotech; catalog no. 130-091-041) or beads (Miltenyi Biotech; catalog no. 139-049-501) were used, respectively. For the isolation of antigen-presenting cells, an MHC class II-positive population or CD11c⁺ DCs were obtained from each tissue by MHC II magnetic beads (Miltenyi Biotech; catalog no. 130-052-401) or CD11c microbeads (Miltenyi Biotech; catalog no. 130-052-001), respectively.

Cell Culture and Cell Stimulation. Isolated CD4⁺ T cells were cultured in T-cell medium containing DMEM (Invitrogen) supplemented with 10% (vol/vol) FBS (HyClone), 3 mM L-glutamine (Sigma), 10 mM Hepes (Sigma), 100 U/mL penicillin (Sigma), 100 U/mL streptomycin (Sigma), and 0.05 mM 2-β-mercaptoethanol (Sigma). For cytokine analysis, cells were stimulated with PMA (20 nM) and ionomycin (2 μM) for 4 h. For proliferation assays, isolated T cells were stimulated with anti-CD3 and anti-CD28 for 72 h. To check intrinsic effects of probiotics for the generation of Foxp3⁺ Tregs, CFSE-labeled SP CD4⁺ T cells were stimulated with IRT5 alone without DCs for 3 days and the Foxp3⁺ population was then measured by FACS.

In Vitro Lymphocyte Proliferation Assay. CD4⁺ T cells were stimulated with coated anti-CD3 (1–3 μg/mL; BD Pharmingen; catalog no. 553058) alone or together with anti-CD28 (1 μg/mL, BD Pharmingen; catalog no. 553294). B220⁺ B cells were stimulated with 10 μg/mL LPS (Sigma). To compare proliferation capacity of CD4⁺CD25⁻ effector T cells from each group, CD4⁺CD25⁻ T cells were cultured with anti-CD3 (1 μg/mL) alone or together with anti-CD28 (1 μg/mL). Cells were then cultured for 56–72 h in flat-bottomed 96-well plates in T-cell medium containing 10% (vol/vol) heated-inactivated FBS (HyClone). After 56–72 h of culture, 0.5 μCi of [³H]-thymidine (NEN) was added to each well and the cells were incubated for an additional 16 h. Cells were harvested, and [³H]-thymidine uptake was measured by liquid scintillation counting. Stimulation indexes were defined by comparison with [³H]-thymidine incorporation (cpm) by unstimulated cells.

In Vitro T-Cell Suppression Assay. MLN CD4⁺CD25⁺ T cells (Tregs, suppressor cells) were isolated from PBS- or IRT5-fed mice and cocultured with a variable ratio of CD4⁺CD25⁻ T cells (responder cells) isolated from the SP of WT BALB/c mice in the presence of soluble anti-CD3 (1 μg/mL). To test suppression sensitivity of CD4⁺CD25⁻ T cells by CD4⁺CD25⁺ Tregs, WT SP CD4⁺CD25⁺ T cells were cultured with a variable ratio of CD4⁺CD25⁻ T cells from each group in the presence of antigen-presenting cells and soluble anti-CD3 (1 μg/mL). Each sample was triplicated, and culture was maintained up to 72 h. After 56–72 h of culture, 0.5 μCi of [³H]-thymidine was added to each well and the cells were incubated for an additional 6 h. Cells were harvested, and [³H]-thymidine uptake was measured by liquid scintillation counting. Stimulation indexes were defined by comparison with [³H]-thymidine incorporation (cpm) by unstimulated cells.

Flow Cytometric Analysis. To detect apoptotic cells, 1 × 10⁶ CD4⁺ T cells were resuspended in 1 mL of 1× Annexin V binding buffer (BD Bioscience; catalog no. 556454). After incubating for 15 min with 5 μL of Annexin V-PE and 7-ADD at 25°C in the dark, 400 μL of 1× binding buffer was added to each tube and the cells were immediately analyzed by FACS. To measure the CD4⁺CD25⁺ T cells, CD4⁺ T cells were stained with anti-CD4-FITC (BD Bioscience) and anti-CD25-PE (BD Bioscience). To measure the Foxp3⁺CD25⁺ and Foxp3⁺CD25⁻ populations, T cells were stained with anti-CD25-FITC (BD Bioscience) and Foxp3-PE (eBioscience). To detect Foxp3, cells were permeabilized with Foxp3 fixation/permeabilization buffer (eBioscience) and stained with anti-Foxp3-PE (eBioscience; catalog no. 12-5773). IgG isotypes were used as a control in all FACS experiments, and the IgG-positive population was shown to be below 0.2%. To analyze the CFSE⁺Foxp3⁺ population converted by rDCs, T cells prelabeled with CFSE (5 μM) were cocultured with DCs in the presence or absence of probiotics for 7 days. Cells were stained with Foxp3 antibody, followed by the Foxp3 detection protocol. The CFSE⁺Foxp3⁺ population was analyzed by FACS.

Isolation of LP and Ear CD4⁺ T Cells. Intestines (IBD mice) and ears (AD mice) were dissected into 1- to 2-cm segments and placed in Erlenmeyer flasks containing 25 mL of 1.0 mM EDTA/5% (vol/vol) FBS in PBS for 20 min at room temperature, with gentle stirring. The segments were transferred to 50-mL centrifuge tubes containing 15 mL of RPMI medium without serum, minced, and vigorously shaken twice for 15 s. Thereafter, the tissues were transferred to five small vials containing 10 mL of 0.5 mg/mL collagenase type V (Sigma) and incubated for 1 h at 37°C with stirring. After incubation, the suspensions of the cells were washed with ice-cold PBS and used as LP total cells or ear total cells. For further isolation of LP CD4⁺ T cells, the CD4⁺ T-cell isolation procedure was used (5).

Analysis of IgG and IgE Levels in Serum. Blood samples were obtained from mice 2 and 4 weeks after AD induction. Total serum IgE level was measured by an IgE ELISA kit (BD Biosciences). Briefly, serum was diluted 200-fold for measuring total IgE and 250-fold for measuring IgE, with 0.1 M sodium carbonate (pH 9.5) used as the primary antibody. Detection antibody was initially diluted 500-fold in assay diluent (PBS containing 10% (vol/vol) FBS), and streptavidin-conjugated HRP was added in detection antibody diluent at a ratio of 250:1 for ELISA. For the detection of mite-specific IgE, 100 μL of 10 μg/mL mite extract was coated in a 96-well plate and kept at 4°C overnight. For the detection of serum IgG level, serum was analyzed with a mouse IgG ELISA quantitation kit (BETHYL). Briefly, obtained serum was diluted 2,000- to 100-fold for capturing antibody in 0.05 M carbonate-bicarbonate at pH 9.5 and used as the primary antibody. HRP-conjugated detection antibody was diluted 20,000-fold in assay diluent [50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, 1% BSA (pH 8.0)]. The procedure for evaluation

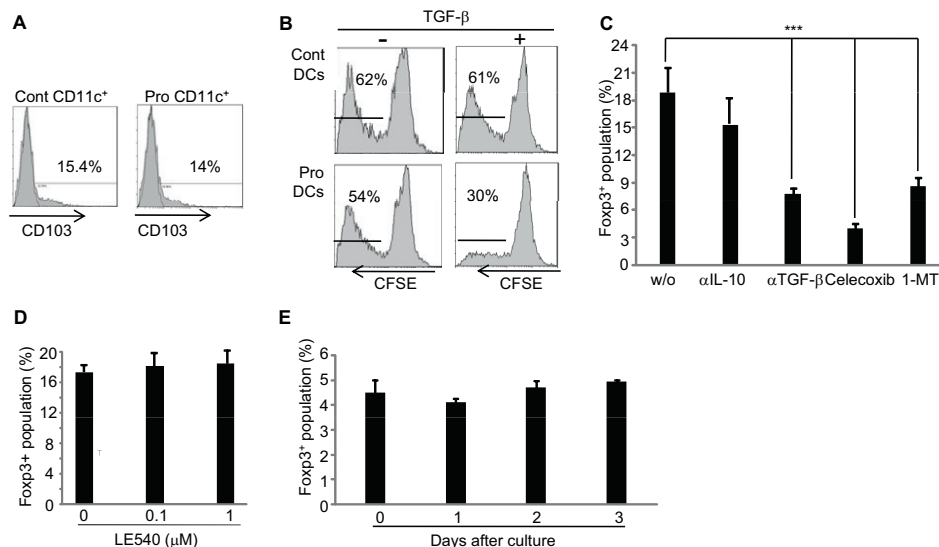


Fig. S4. Characterization of CD11c⁺ rDCs induced by IRT5 administration. (A) No significant changes in the CD103⁺ DC population by IRT5 administration. CD11c⁺ DCs were isolated from the mice fed with either PBS (Cont) or IRT5 (Pro), and the CD103⁺ population was then analyzed by FACS. (B) CD11c⁺ DCs from each group were cocultured with Do11.10 CD4⁺ T cells labeled with CFSE (5 μM) in the absence (–) or presence (+) of exogenous TGF-β. The degree of CD4⁺ T-cell proliferation was analyzed by measuring the CFSE signal by FACS. MLN CD11c⁺ DCs from IRT5-fed mice were cocultured with CFSE-labeled SP CD4⁺ T cells in the presence of soluble anti-CD3 (1 μg/mL) and various inhibitors, including anti-IL-10 (10 μg/mL), anti-TGF-β (10 μg/mL), celecoxib (50 μM), and 1-MT (20 μM) (C) and LE540 (0.1 or 1 μM) (D) for 4 days. On day 5, 100 U/mL of IL-2 was added and cultured for 3 more days; the CFSE⁺Foxp3⁺ population was then analyzed by FACS. ****P* < 0.001. (E) WT SP CD4⁺ T cells labeled with CFSE were cultured with IRT5 at a 1:10 ratio for up to 3 days, and the Foxp3⁺CFSE⁺ population was measured by FACS. Data are from 10 mice per group; error bars indicate SD. Data are representative of three independent experiments.

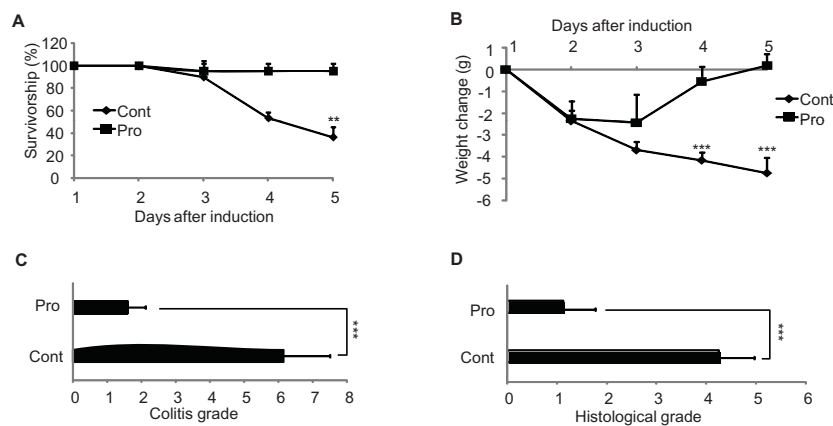


Fig. S5. Probiotics treatment suppresses experimental colitis. Changes in survivorship (A), weight loss (B), colitis grade (C), and histological grade (D) were evaluated after H&E staining. Data are from 20 mice per group; error bars indicate SD. Data are representative of three independent experiments. ***P* < 0.005; ****P* < 0.001.

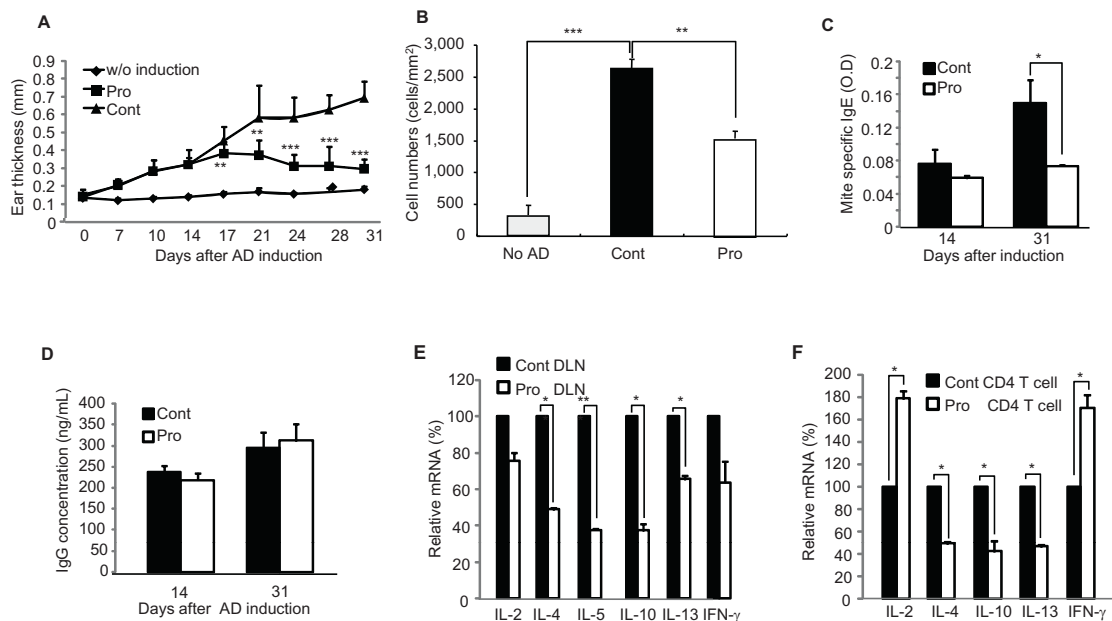


Fig. 56. Probiotics treatment suppresses experimental AD. The therapeutic efficacy of IRT5 was measured by the following criteria for AD progression: ear thickness (A), number of infiltrated lymphocytes based on histological analysis (B), and mite-specific IgE levels (C). (D) No effect was observed on IgG levels. Real-time PCR was used to analyze the levels of cytokine transcript in CD4⁺ T cells of draining lymph nodes (DLNs) (E) and in CD4⁺ T cells isolated from AD ear regions (F). Bars are from 10 mice per group; error bars indicate SD. Data are representative of three independent experiments. **P* < 0.05; ***P* < 0.005; ****P* < 0.001. Interestingly, treatment with IRT5 down-regulated AD-associated cytokine levels in the DLNs (superficial cervical, axillary, and brachial nodes) (E), although inducing phenotypic skewing from Th2 to Th1 cytokines in the CD4⁺ T cells of AD ear (F).

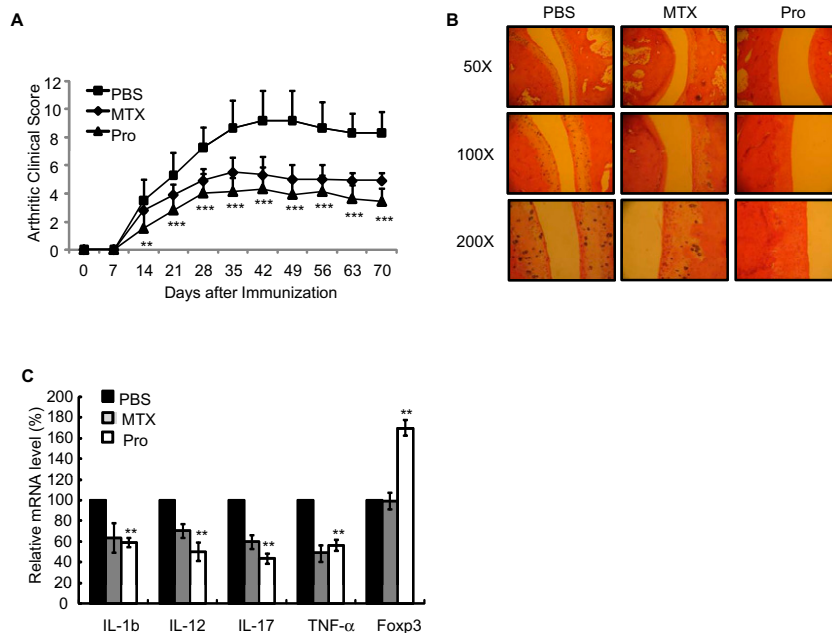


Fig. 57. Probiotics treatment suppresses experimental RA. Experimental RA was induced by immunization of type II collagen with complete Freund's adjuvant. Treatment was started 7 days after disease induction and continued until the end of the experiment. Rats were treated with PBS control (■; negative control), methotrexate (●; MTX; 300 μg/kg twice per week; positive therapeutic control), or IRT5 (▲; Pro; 1 × 10⁹ cfu/day). Pro treatment significantly (*P* < 0.01) protected RA progression, with similar efficacy of MTX, by reducing the clinical score (A), lymphocyte infiltration (B), and inflammatory cytokines (C), through increasing Foxp3 levels (real-time PCR analysis) in draining lymph nodes. Data are representative of three independent experiments. ***P* < 0.005; ****P* < 0.001.

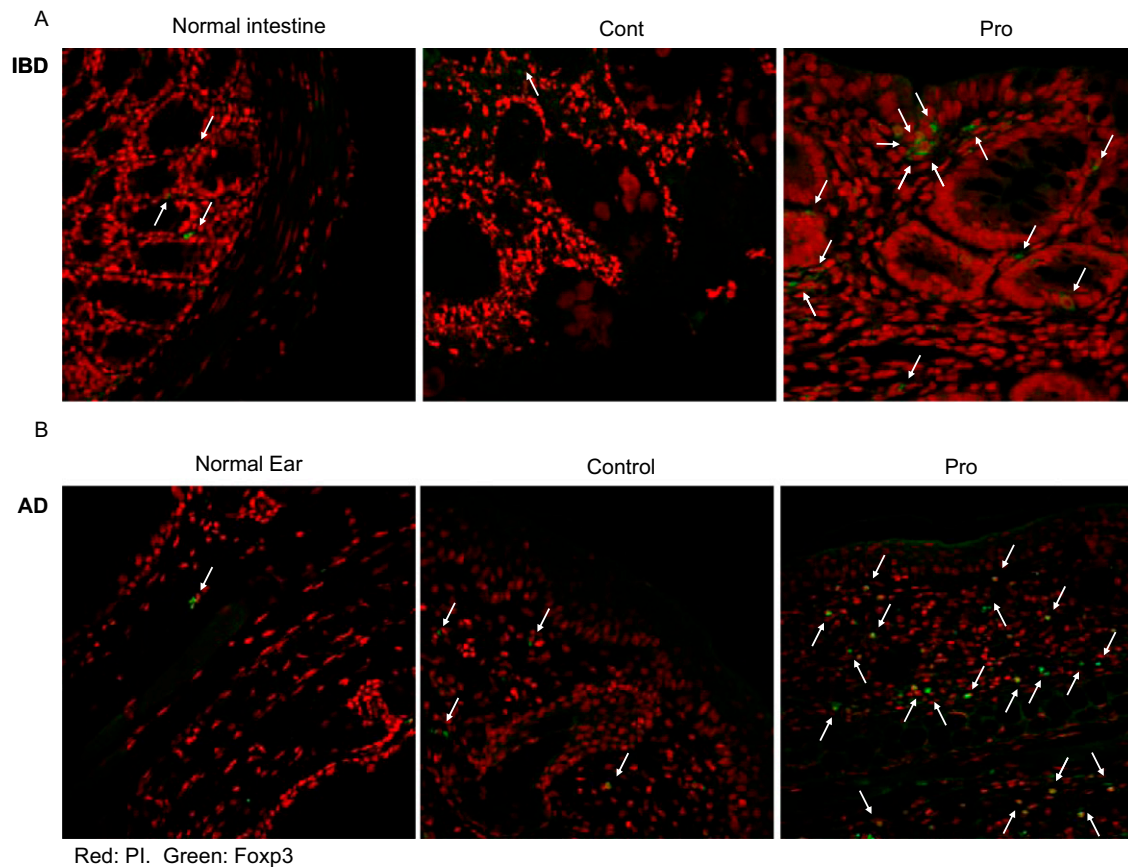


Fig. S8. Enriched CD4⁺Foxp3⁺ Tregs at inflammatory sites are associated with disease suppression. After treatment with probiotics or PBS, the Foxp3⁺ population from the colon of experimental colitis (A) or ear of AD (B) mice was analyzed by IHC between the Pro and Cont groups. Arrows indicate Foxp3⁺ cells. A control reaction was performed with isotype-matched IgG antibodies. Data are from 10 mice per group and are the average of three independent experiments.

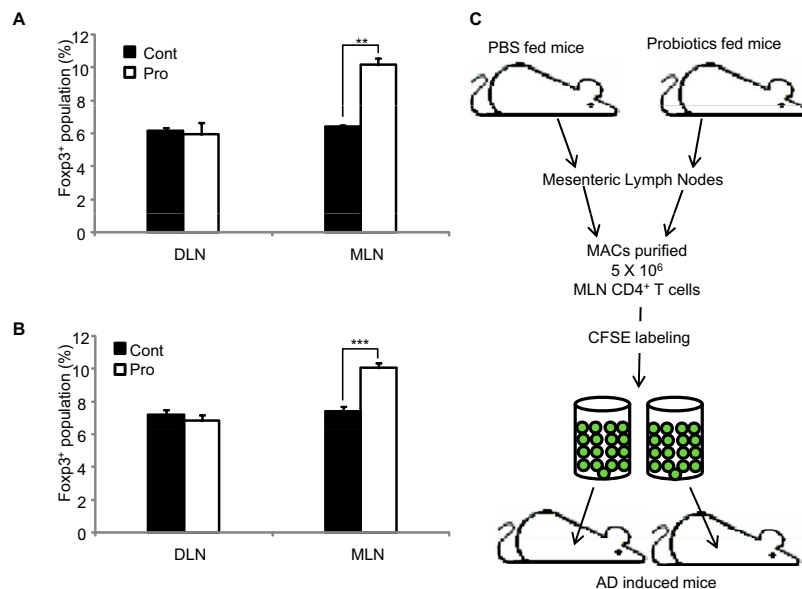


Fig. S9. Administration of probiotics increases Foxp3⁺CD4⁺ Tregs in disease conditions. Draining lymph node (DLN) and MLN were removed from the Cont or Pro group in the IBD (A) or AD (B) condition, and the CD4⁺Foxp3⁺ Treg population was analyzed by FACS. Data are from 10 mice per group; error bars indicate SD. Data are representative of three independent experiments. ** $P < 0.005$; *** $P < 0.001$. (C) Schematic flow chart for the adoptive transfer of CD4⁺ T cells to AD mice. CD4⁺ T cells (5×10^6) obtained from MLN of the Cont or Pro group were labeled with CFSE (green color) and then i.p. transferred to AD mice. CFSE⁺ Foxp3⁺ Tregs were analyzed under fluorescence microscopy (Fig. 7C).

