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Probiotics Ameliorate Recurrent Th1-Mediated Murine Colitis by Inducing IL-10 and IL-10-Dependent TGF-β-Bearing Regulatory Cells[1]

Claudia Di Giacinto,[2]* Mariarosaria Marinaro,[2]* Massimo Sanchez,† Warren Strober,‡ and Monica Boirivant[3]*

Recent studies of murine models of mucosal inflammation suggest that, whereas some kinds of bacterial microflora are inducers of disease, others, known as probiotics, prevent disease. In the present study, we analyzed the regulatory cytokine and cell response to probiotic (VSL#3) administration in the context of the Th1 T cell colitis induced by trinitrobenzene sulfonic acid treatment of SJL/J mice. Daily administration of probiotics for 3 wk to mice during a remission period between a first and second course of colitis induced by trinitrobenzene sulfonic acid, resulted in a milder form of recurrent colitis than observed in mice administered PBS during this same period. This protective effect was attributable to effects on the lamina propria mononuclear cell (LPMC) population, because it could be transferred by LPMC from probiotic-treated mice to naive mice. Probiotic administration was associated with an early increase in the production of IL-10 and an increased number of regulatory CD4$^+$ T cells bearing surface TGF-β in the form of latency-associated protein (LAP) (LAP$^+$ T cells). The latter were dependent on the IL-10 production because administration of anti-IL-10R mAb blocked their appearance. Finally, the LAP$^+$ T cells were essential to the protective effect of probiotics because administration of anti-IL-10R or anti-TGF-β at the initiation of recurrent colitis induction or depletion of LAP$^+$ T cells from LPMC abolished the latter's capacity to transfer protection to naive recipients. These studies show that probiotic (VSL#3) administration during a remission period ameliorates the severity of recurrent colitis by inducing an immunoregulatory response involving TGF-β-bearing regulatory cells. *The Journal of Immunology,* **2005, 174: 3237–3246.**

In Crohn's disease (CD),[4] genetic and environmental factors interact to produce an immunopathogenic process that results in chronic, relapsing intestinal inflammation (1). Recent insights into the nature of this disease, derived mainly from studies of experimental models of colonic inflammation, strongly suggest that it can result from a loss of immune tolerance to Ags in the bacterial microflora. This, in turn, can occur as a consequence of an absolute or relative defect in the function of regulatory T cells or to an excessive immune response to these Ags that cannot be controlled by normal regulatory T cell function (2, 3). Quite appropriately, these immunopathologic mechanisms have focused attention on the bacterial microflora itself and the possibility that, although some bacteria are inducers of disease, others, known as probiotic organisms (probiotics), prevent disease.

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[4] Abbreviations used in this paper: CD, Crohn's disease; IBD, inflammatory bowel disease; TNBS, trinitrobenzene sulfonic acid; hpf, high power field; LPMC, lamina propria mononuclear cell; LAP, TGF-β1 latency-associated peptide.

Based on this latter possibility, therapeutic approaches designed to modulate the local microenvironment with the use of probiotics have been tested both in animal models of colitis as well as in human inflammatory bowel disease (IBD) (4). Thus, in studies of mice, it has been shown that rectal administration of *Lactobacillus reuteri* (5) or oral administration of *Lactobacillus plantarum 299v* (6) to IL-10-deficient mice prevents the development of colitis (5) or attenuates the severity of established colitis (6). Similarly, the administration of the probiotic mixture VSL#3 (a mixture of bifidobacteria, lactobacilli, and *Streptococcus salivarius*) to mice with IL-10 deficiency results in a reduction of microscopic evidence of inflammation along with a reduction in mucosal secretion of TNF-α and IFN-γ (7). In contrast, whereas VSL#3 and *Lactobacillus* strain GG probiotics ameliorated iodoacetamide-induced colitis, they had no effect on dinitrobenzene sulfonic acid-colitis (8). Similarly, *L. plantarum* species 299 had no capacity to improve gut permeability of trinitrobenzene sulfonic acid (TNBS)-induced colitis (TNBS-colitis) and did not reduce the severity of colitis in this model (9).

Studies of the therapeutic effect of probiotics in humans with IBD have usually, but not always, been positive. Thus, it was shown that the oral administration of viable nonpathogenic *Escherichia coli* strain Nissle 1917 is comparable to low-dose mesalazine in maintaining remission and preventing relapse in ulcerative colitis patients (10, 11); similarly, in a small, short-term open-label trial, Guslandi et al. (12) found that CD patients treated with mesalazine plus *Saccharomycetes boulardii* maintained remission better than patients treated with mesalazine alone. In contrast, in another study in which the ability of *Lactobacillus* GC administration to prevent recurrence of CD after surgery was tested, it was found that this probiotic failed to prevent endoscopic recurrence or to reduce the severity of recurrent lesions (13).

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Although there is now a considerable body of information concerning the clinical efficacy of probiotics, knowledge of their mechanism of action is still relatively incomplete. In the present study, we address this lack by conducting studies in which we analyze both the effector and regulatory aspects of the immunologic response of mice undergoing recurrent Th1-mediated colitis induced by TNBS with and without oral administration of a probiotic preparation with known clinical efficacy, VSL#3. We found that daily administration of these probiotics for 3 wk to mice that had recovered their initial weight after a first induction of colitis, results in a milder colitis after the second administration of TNBS that is characterized by earlier weight recovery, reduced mortality, lower level of microscopic inflammation, and reduced mucosal production of IFN-γ. At the same time, this protection is accompanied by the induction of regulatory cells and cytokines.

Materials and Methods

Study design

Colitis studies were performed in 5- to 6-wk-old male SJL mice obtained from Charles River Laboratories (Calco) and from Harlan Laboratories, maintained in the animal facility at the Istituto Superiore di Sanità. The experiments were performed within 7 days from the arrival of the animals. All studies were approved by Animal Care and Use Committee of Istituto Superiore di Sanità and Italian Ministry of Health. For induction of colitis, TNBS (Sigma-Aldrich; Fluka Milan) in 50% ethanol was administered per rectum to lightly anesthetized mice through a 3.5-F catheter inserted into the rectum. The catheter tip was inserted 4 cm proximal to the anal verge; 150 μl of fluid (TNBS/ethanol) was slowly instilled into the colon, and the mouse was held in a vertical position for 30 s. As outlined in Fig. 1, mice were initially administered intrarectally 150 μl containing 0.5–2.5 mg of TNBS in 50% ethanol, and the subsequent course of colitis was evaluated by observing the weight changes of the animals. After 8–10 days, mice that had recovered their initial weight were divided into two groups. One group received VSL#3 (VSL Pharmaceutical), 2 mg in 50 μl of PBS per os, each day for a total of 3 wk, and the other group received 50 μl of PBS only. At the end of the 3-wk period, three to five mice of each group were sacrificed to evaluate cytokine production by lymphocytes isolated from colonic tissue, and the remaining mice of each group received a second intrarectal administration of 150 μl containing 2.5 mg of TNBS in 50% ethanol. In some experiments, additional mice of each group received 0.5 mg of anti-IL-10R mAb (1B1.3; DNAX Research) by i.p. injection at the time of second intrarectal administration of 150 μl containing 2.5 mg of TNBS in 50% ethanol. In some experiments, additional mice from both groups (i.e., PBS treated or VSL-3 treated) received 1 mg of a neutralizing anti-TGF-β 1,2,3 Ab by i.p. injection, and TNBS colitis was induced as described above. Occurrence and course of "recurrent colitis" in the two groups of animals was evaluated by observing the weight changes, mortality, and gross and microscopic appearance of the colons, and by evaluation of isolated colonic mononuclear cell cytokine production.

Probiotic preparation

VSL#3 (VSL Pharmaceutical) is a probiotic mixture containing 3×10^{11}/g of viable lyophilized bacteria including bifidobacteria (*Bifidobacterium longum*, *Bifidobacterium infantis*, and *Bifidobacterium breve*), lactobacilli (*Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus delbrueckii* subsp. *Lactobacillus bulgaricus*, and *L. plantarum*), and *S. salivarius* subsp. *thermophilus.*

Histological assessment of colitis

Tissues removed from mice at indicated times of death were fixed in 10% neutral buffered formalin solution (Sigma-Aldrich) and then embedded in paraffin, cut into tissue sections, and stained with H&E. Stained sections were examined for evidence of colitis using the following as criteria: the presence of lymphocyte infiltration, elongation and/or distortion of crypts, frank ulceration, and thickening of the bowel wall. The degree of inflammation on microscopic cross-sections of the colon was graded semiquantitatively from 0 to 4 as follows: 0, no evidence of inflammation; 1, low level of lymphocyte infiltration with infiltration seen in $<$10% high power fields (hpf), no structural changes observed; 2, moderate lymphocyte infiltration with infiltration seen in 10–25% hpf, crypt elongation, bowel wall thickening, which does not extend beyond mucosal layer, no evidence of ulceration; 3, high level of lymphocyte infiltration with infiltration seen in 25–50% hpf, high vascular density, thickening of bowel wall which extends beyond mucosal layer; and 4, marked degree of lymphocyte infiltration with infiltration seen in $>$50% hpf, high vascular density, crypt elongation with distortion, transmural bowel wall thickening with ulceration.

Isolation of lamina propria mononuclear cells (LPMC)

LPMC were isolated from freshly obtained colonic specimens using a modification of the method described by Van der Heijden and Stok (14). In brief, colon specimens were washed in HBSS-calcium magnesium free (BioWhittaker), cut into 0.5-cm pieces, and incubated twice in HBSS containing EDTA (0.37 mg/ml) and DTT (0.145 mg/ml) at 37°C for 15 min for two cycles. The tissue was then digested further in RPMI 1640 containing collagenase D (400 U/ml) and DNase (0.01 mg/ml) (Boehringer Mannheim, Biochemicals) in a shaking incubator at 37°C. Finally, the released cells were layered on a 40–100% Percoll gradient (Pharmacia) to obtain the lymphocyte-enriched populations at the 40–100% interface.

Adoptive transfer of LPMC

LPMC were isolated from colons of VSL#3 or PBS-fed mice 3 days after completion of the last feeding. LPMC (3.0×10^5) were then injected i.v. into the tail vein of normal SJL mice. TNBS was administered to the recipient mice 5–7 days after the LPMC transfer. In some experiments, additional mice of each group were administered LPMC depleted of LAP$^+$ cells by using a CELLection Biotin Binder kit (Dynal Biotech), where beads coated with goat anti-LAP biotinylated Ab (affinity-purified biotinylated goat anti-latency-associated peptide (LAP) polyclonal Ab; R&D Systems) were used to directly select LAP$^+$ cells from LPMC suspensions in accordance with the manufacturer's instructions; or LPMC depleted of CD4$^+$LAP$^+$cells by cell sorting after staining cells with anti-mouse CD4-allophycocyanin (BD Pharmingen) and anti-LAP-PE using a FACS-ARIA (BD Biosciences). Finally, in some experiments, additional mice of each group were administered an i.p. injection of 0.5 mg of anti-IL-10R mAb (1B1.3; DNAX Research) at the time of intrarectal administration of 150 μl containing 2.5 mg of TNBS in 50% ethanol.

Cell culture of LPMC

Cell culture of LPMC was performed in complete medium consisting of RPMI 1640 (BioWhittaker) supplemented with 2 mM L-glutamine, 10 mM HEPES buffer, 10 μg/ml gentamicin, 100 U/ml penicillin and 100 μg/ml streptomycin, 0.05 mM 2-ME (Sigma-Aldrich), and 10% FCS (HyClone Europe).

Stimulation and measurement of LPMC cytokine production

To measure the capacity of isolated LPMC to produce cytokines, the LPMC populations were cultured in complete medium at 1×10^6 cells/ml in 48-well plates (Costar; Corning) coated or uncoated with anti-CD3ϵ Ab (clone 145-2C11; BD Pharmingen). Coating was accomplished by preexposure of individual wells to 10 μg/ml murine-anti-CD3ϵ Ab in carbonate buffer (pH 9.6) for 1 h at 37°C. Culture fluid for cell populations in coated wells also contained 1 μg/ml soluble anti-CD28 Ab (clone 37.51; BD Pharmingen). After 48 h of culture under these conditions, culture supernatants were removed and assayed for the presence of cytokines (IFN-γ, IL-4, IL-10) by ELISA.

ELISA

Cytokine concentrations were determined by commercially available specific ELISA using duo-paired murine cytokines per the manufacturer's recommendations (BD Pharmingen). Optical densities were measured on a Bio-Rad Novapath ELISA reader at a wavelength of 450 nm. Data were analyzed against the linear portion of the generated standard curve.

LPMC immunofluorescence staining

Freshly isolated LPMC were washed twice in PBS/2% FCS, and FcR was blocked by incubation with anti-CD16/CD32 (Fc Block; BD Pharmingen). Cells were first stained with biotinylated anti-LAP Ab (R&D Systems) or biotinylated normal goat IgG (R&D Systems). After incubation for 30 min, the cells were washed, and streptavidin-PE, anti-mouse-CD3 PerCP (clone 145-2C11), and anti-mouse CD4 allophycocyanin (clone RM4-5) were added and incubated for 30 min. The cells were then washed twice, and the percentage of fluorescent cells was quantified using a FACSCalibur (BD Biosciences). To evaluate the percentage of IL-10-producing cells, LPMC were incubated for 48 h in complete medium without any stimulant or in the presence of anti-CD3 and anti-CD28 mAb. Monensin (GolgiStop; BD Pharmingen) was added during the last 8 h of culture. At the end of incubation period, the cells were washed and labeled with Ab against surface Ags (anti-mouse CD3-PerCP, anti-mouse CD4-allophycocyanin, and

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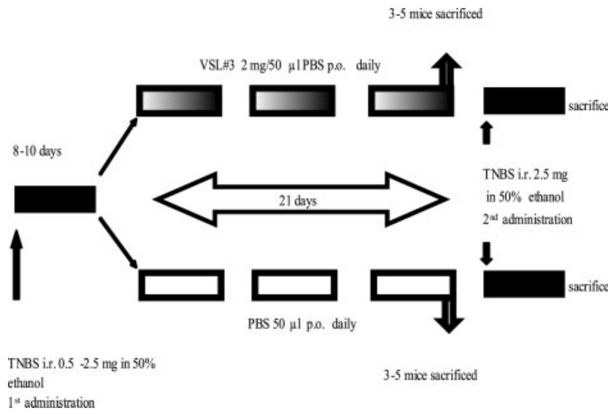

FIGURE 1. Scheme of studies. See *Materials and Methods* for details.

streptavidin-FITC-biotinylated anti-LAP Ab). Cells were then fixed and permeabilized (Cytofix/Cytoperm; BD Pharmingen) for 20 min, washed with WashPerm (BD Pharmingen), and labeled with PE-anti-IL-10 mAb (BD Pharmingen) or isotype-matched Ig control (BD Pharmingen).

Statistical analysis

Assessment of statistical differences was determined by Student's *t* test or χ_c^2 where appropriate.

Results

Probiotic-treated mice manifest less severe recurrent colitis

To emulate the effect of probiotics on recurrent colitis in humans in the context of a mouse model of colitis, we first tested whether such administration after a first course of TNBS-colitis was able to modulate a second course of TNBS-colitis ("recurrent colitis") (see diagram of study protocol, Fig. 1). As shown in Fig. 2, daily VSL#3 administration (hereafter referred to as probiotic administration) during the 3-wk time interval between the two courses of colitis led to a significant reduction in the weight loss and mortality during the recurrent colitis compared with mice that received PBS in the same time interval. In addition, as shown in Fig. 3, probiotic-

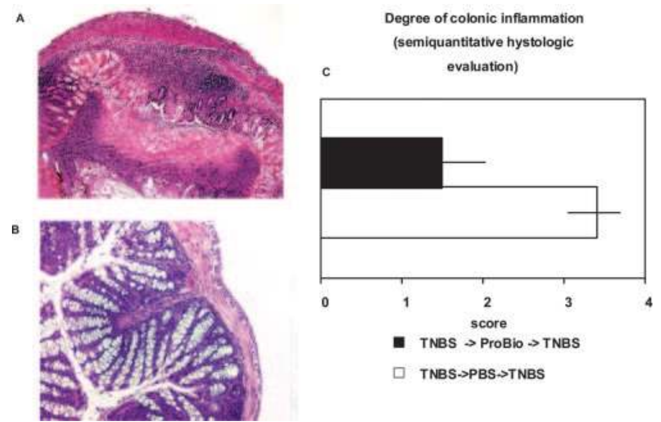

FIGURE 3. Histologic evaluation of recurrent colitis in mice treated with probiotics or PBS. *A*, Photomicrograph (×50) of an H&E-stained paraffin section of a representative colon from a mouse after reinduction of TNBS-colitis following treatment with PBS according to the protocol depicted in Fig. 1. Severe mucosal mononuclear cell infiltrate and disruption of the normal crypt architecture with epithelial crypt ulceration and loss of goblet cells is evident. *B*, Photomicrograph (×50) of H&E-stained paraffin section of a representative colon from a mouse after reinduction of TNBS-colitis following treatment with probiotics according to the protocol depicted in Fig. 1; only small scattered areas of cellular infiltration are present. In the studies shown in *A* and *B*, mice were sacrificed at day 4 after TNBS-colitis reinduction. *C*, Histologic scores (see *Materials and Methods*) of colons of the groups of mice treated with probiotics or PBS after reinduction of TNBS-colitis in mice sacrificed at day 4 after TNBS-colitis reinduction. Histologic scores derived from cumulative microscopic analyses of three cross-sections and longitudinal sections from each mouse. In each of the three experiments performed, each group consisted of five mice. Scoring was performed in a blinded fashion. Difference between TNBS→ProBio→TNBS and TNBS→PBS→TNBS is significant at $p = 0.02$.

treated mouse tissue showed a less severe grade of colonic inflammation compared with PBS-treated mouse tissue, and as shown in Fig. 4, their LPMC isolated from the mice on day 4 after TNBS-colitis reinduction upon in vitro stimulation exhibited a significant

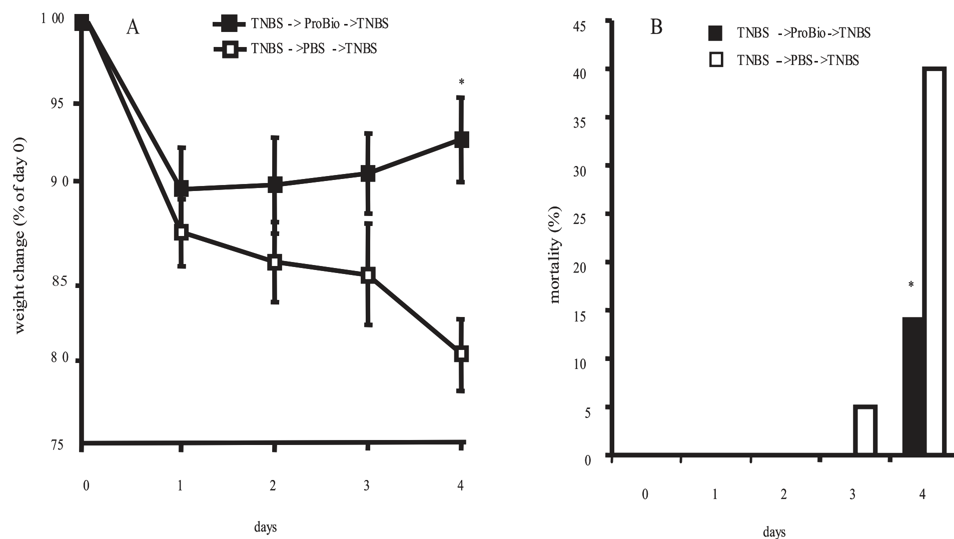

FIGURE 2. Severity of recurrent TNBS-colitis in mice treated with probiotics or PBS. *A*, Weight changes of mice after reinduction of TNBS-colitis in mice following treatment with probiotics (ProBio) (■) or PBS (□) according to the protocol depicted in Fig. 1. Each point represents the cumulative mean weight data from three different experiments. In each experiment, each group consisted of at least five mice. Bars represent SEs. Difference between probiotic-treated mice and PBS-treated mice is significant at $p = 0.01$. Initial weights of the two groups (23.2 ± 0.4 and 23.4 ± 0.4 g) were not significantly different. *B*, Mortality of mice after reinduction of TNBS-colitis in mice treated with probiotics (■) or PBS (□) according to the protocol depicted in Fig. 1. Bars represent the cumulative mortality from three different experiments. In each experiment, each group consisted of at least five mice. Difference between probiotic-treated mice and PBS-treated mice is significant at $p < 0.05$.

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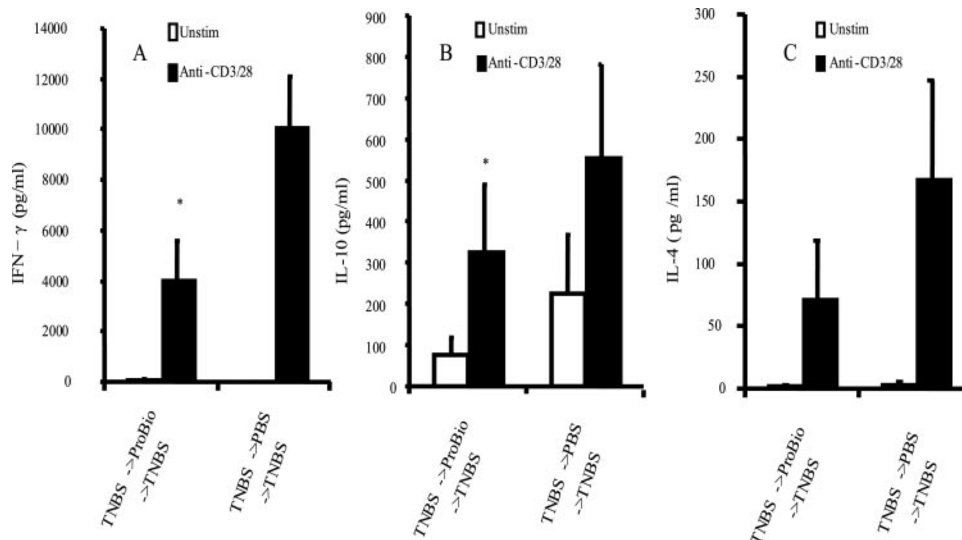

FIGURE 4. In vitro IFN-γ production (*A*), IL-10 production (*B*), and IL-4 production (*C*) by LPMC isolated from the colons of SJL mice on day 4 after TNBS-colitis reinduction with and without previous treatment with probiotics according to the protocol depicted in Fig. 1. Cultures were performed in the absence of any stimulant (□) or in the presence of anti-CD3/CD28 (■). Data represent the mean from four independent experiments. In each experiment, cultures of pooled cells extracted from five mice per group were performed. Percentage of $CD3^+$ cells in LPMC and $CD4^+$ cells in $CD3^+$ cells at the beginning of culture did not differ between TNBS→ProBio→TNBS and TNBS→PBS→TNBS group ($CD3^+$ cells, 49.6 ± 5 and 51.4 ± 9.4%; $CD4^+$ cells in $CD3^+$ cells, 68.9 ± 5.3 and 70.9 ± 6.7%, respectively). Bars represent SEs. TNBS→ProBio→TNBS and TNBS→PBS→TNBS significantly different at the $p < 0.05$ level. Unstim, Unstimulated.

reduction in IFN-γ and IL-10 secretion (but no change in IL-4 secretion). Thus, probiotic administration after a first course of colitis is able to ameliorate the severity of a second (recurrent) course of colitis.

Recipients of LPMC from donor mice administered probiotics exhibit less severe colitis

In further studies, we evaluated whether the protective effect of probiotic administration on TNBS-colitis was transferable to naive mice. To this end, we transferred LPMC isolated from probiotic-treated mice as well as LPMC isolated from PBS-treated mice into groups of naive mice and then, 1 wk after cell transfer, induced TNBS-colitis. As shown in Fig. 5*A*, although recipients of cells from probiotic-treated mice showed only slightly less weight change than recipients of PBS-treated mice, they manifested far less mortality; in addition, as shown in Fig. 5, *B* and *C*, LPMC isolated from the recipient mice at day 4 after TNBS-colitis induction exhibited less IFN-γ and less IL-10 secretion. These data suggest that the protective effect of probiotic treatment resides in large part with the mononuclear cell population and that the latter renders the mouse resistant to colitis induction.

LPMC cytokine production at the end of probiotic administration and during colitis reinduction is characterized by a significant increase of IL-10 production

One possible mechanism of such resistance is that probiotics induce an immunomodulatory effect. To evaluate this possibility, we determined anti-CD3/anti-CD28-induced in vitro cytokine production by colonic LPMC isolated from mice at the end of the 3-wk treatment period with probiotics or PBS, i.e., in the period just before colitis reinduction. As shown in Fig. 6*A*, probiotic administration was associated with a significant increase in LPMC IL-10 production during this period compared with PBS administration.

We then determined anti-CD3/anti-CD28-induced in vitro IL-10 production by LPMC obtained from mice during the first 4 days following colitis reinduction, i.e., in the period just after colitis was reinduced by TNBS administration. As shown in Fig. 6*B*, we found that LPMC isolated from probiotic-treated mice, when compared with PBS-treated mice, exhibited greater IL-10 production in the first several days after colitis reinduction; this was especially evident on day 2 after colitis reinduction, at which time LPMC isolated from probiotic-treated mice produced over four times more IL-10 than PBS-treated mice. However, after this day, the level of IL-10 production declined, correlating with the fact that at day 4 the inflammation had almost subsided, as shown by the weight recovery exhibited by the mice (Fig. 2*A*), the histological appearance of their colons (Fig. 3), and their cytokine production (Fig. 4). In contrast, LPMC isolated from PBS-treated mice exhibited low levels of IL-10 production early after colitis reinduction that only increased on day 4 in association with the onset of spontaneous recovery from colitis, at which point IL-10 production exceeded that in probiotic-treated mice (consistent with IL-10 production at this time point in Figs. 4 and 5). It should be noted that LPMC IFN-γ production did not show the same trend as IL-10 production in that it was lower in cells from probiotic-treated mice than in cells from PBS-treated mice on all days studied (data not shown).

Finally, we investigated the role of IL-10 secretion in the protective effect of probiotic treatment with the use of a mAb against the IL-10R that blocks IL-10 use. As shown in Fig. 7, administration of this Ab at the time of colitis reinduction abrogated the ability of probiotics to ameliorate recurrent colitis in probiotic-treated mice. Thus, in the probiotic-treated mice administered anti-IL10R at the time of colitis reinduction, the extent of colitis (as indicated by percentage of body weight loss at day 3) (84 ± 3%) was not different from that observed in either the PBS-treated mice that were administered anti-IL-10R (85 ± 2%) or in the mice treated with PBS that were not administered anti-IL10R Ab (87 ± 3%). Similar results were obtained in the cell transfer studies described above, in that anti-IL10R administration blocked the protective effect of cells from probiotic-treated mice transferred to untreated recipient mice (data not shown). Taken together, these

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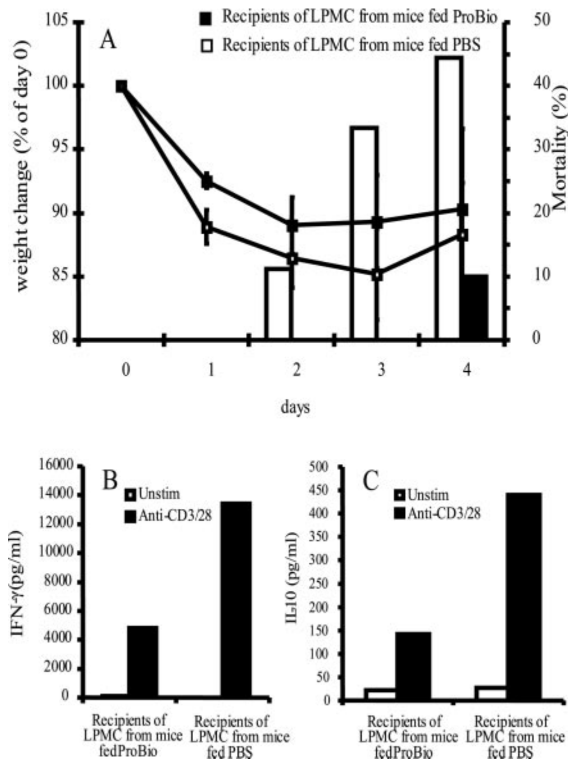

FIGURE 5. Amelioration of TNBS-colitis by the adoptive transfer of LPMC from probiotic-fed mice. *A*, Weight changes and mortality of recipients of LPMC obtained from mice fed probiotics (ProBio) (■) or PBS (□). Recipient mice received TNBS per rectum 5–7 days after adoptive transfer of LPMC (see *Materials and Methods*). Data shown are from one representative experiment of two similar experiments. Each point represents the average weight of five mice, and bars represent SEs. Initial weight did not differ between the groups (20 ± 0.2 vs 19.7 ± 0.3 g; recipients of LPMC obtained from mice fed probiotics vs recipients of LPMC obtained from mice fed PBS). *B* and *C*, Cytokine production by LPMC isolated from recipient mouse colons 4 days after TNBS-colitis reinduction: IFN-γ (*B*) and IL-10 (*C*) secretion by LPMC obtained from recipients of LPMC obtained from mice fed probiotics or PBS; cultures were performed in the absence of any stimulant (□) or in the presence of anti-CD3/CD28 (■). Data shown represent values obtained from one representative experiment of two. In each experiment, culture supernatants from cultures of pooled cells extracted from five mice per group were analyzed. Unstim, Unstimulated.

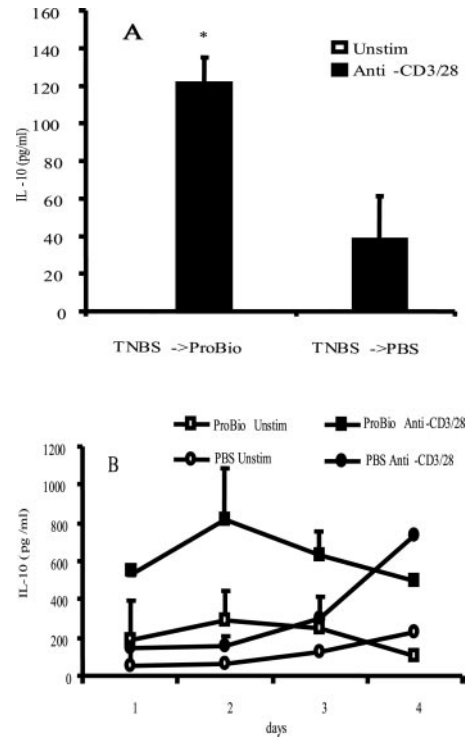

FIGURE 6. Effect of probiotic administration on LPMC IL-10 production. *A*, Cells were isolated from colonic specimens from mice treated, after the first intrarectal administration of TNBS, with probiotics or PBS and sacrificed before the colitis reinduction. IL-10 secretion by cells in unstimulated (□) and anti-CD3/anti-CD28 stimulated (■) cultures. *, $p = 0.01$, probiotic-treated vs PBS-treated mice. Data represent the mean from five independent experiments. In each experiment, cultures of pooled cells extracted from five mice per group were performed. Bars represent SE. Unstim, Unstimulated. *B*, Effect of probiotic administration on LPMC IL-10 production during the course of recurrent TNBS-colitis. Groups of five mice were sacrificed at the indicated time points, and LPMC IL-10 production in supernatants was evaluated. □ and ○, Unstimulated LPMC. ■ and ●, Anti-CD3/anti-CD28-stimulated LPMC. □ and ■, Probiotic-treated mice. ○ and ●, PBS-treated mice.

data provide strong evidence that an immunomodulatory effect involving IL-10 mediates the ability of probiotic treatment to protect against the development of recurrent colitis.

Protection from colitis is associated with an increased number of CD4$^+$LAP$^+$ cells in the lamina propria that is prevented by blockade of IL-10 activity

In a further investigation of possible immunomodulatory effects of probiotics, we determined whether the latter induced regulatory cells. In these studies, we took advantage of the fact that, as recently shown, regulatory T cells can be identified by their capacity to express TGF-β in the form of LAP on their cell surface and the recent demonstration the such LAP$^+$ cells can in fact suppress CD4$^+$CD45Rb^{high}-induced colitis (15, 16). Accordingly, we isolated LPMC from colonic specimens collected both at the end of the probiotic or PBS treatment period and again at 3–4 days after colitis reinduction and stained the isolated cells for detection of LAP expression (see *Materials and Methods*). Although at the end of the treatment period and before colitis reinduction, we found that the percentage of CD3$^+$ and CD4$^+$ as well as CD3$^+$LAP$^+$

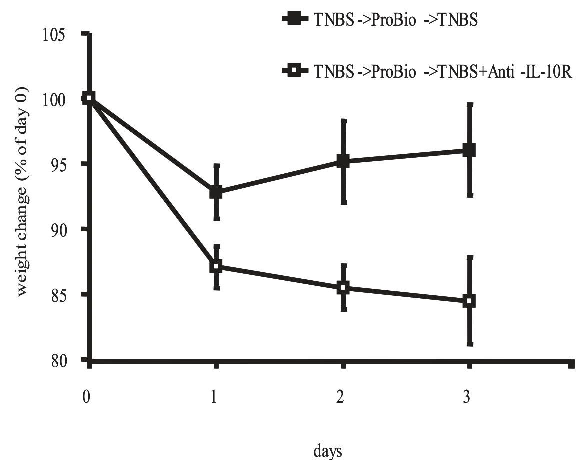

FIGURE 7. Treatment of mice with anti-IL-10R mAb at the time of second TNBS administration abrogates the protective effect of feeding probiotics on the development of TNBS-colitis. Weight changes of mice that were administered TNBS per rectum after probiotic feeding (■), TNBS per rectum and anti-IL-10R i.p. after probiotic feeding (□). Each point represents cumulative mean weight data from two different experiments. In each experiment, the experimental groups consisted of at least five mice. Bars represent SE. Initial weight did not differ among the groups.

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cells in LPMC did not differ between probiotic- and PBS-treated mice ($CD3^+$: 58 ± 8 vs 53 ± 8%; $CD4^+$: 32 ± 11 vs 27 ± 6%; $CD3^+LAP^+$: 6 ± 3 vs 5 ± 2%), there was an increase in the percentage of $CD4^+LAP^+$ cells in probiotic-treated mice when compared with PBS-treated mice (3.4 ± 0.2 vs 1 ± 0.3%) (equivalent to 8.5 ± 3.5 vs 3.5 ± 1.2% LAP^+ positive cells in $CD3^+CD4^+$ gated cells) that was associated to a $p = 0.06$ level. More importantly, as shown in Fig. 8, in a time-course study of the percentage of $CD4^+LAP^+$ cells following colitis reinduction, we observed a persistent increase in the percentage of $CD4^+LAP^+$ cells in probiotic-treated mice vs PBS-treated mice that peaked at day 3 after colitis reinduction, at which point a significant increase in the level of $CD4^+LAP^+$ cells was detected (15 ± 3.3 vs 6 ± 1.1; $p < 0.05$). To further characterize the nature of LAP^+ cells in the lamina propria at the end of the probiotic administration period, we performed flow cytometric intracellular staining studies of unstimulated and stimulated LPMC in these mice to estimate the capacity of the $CD4^+LAP^+$ subpopulation to produce IL-10 (see *Materials and Methods*). We found that before and after stimulation with anti-CD3/anti-CD28, respectively, 1.5 and 3.1% of $CD4^+LAP^+$ cells in LPMC obtained from probiotic-treated mice, whereas 4.4 and 5.4% of $CD4^+LAP^+$ cells in LPMC isolated from PBS-treated mice, contained intracellular IL-10. Thus, $CD4^+LAP^+$ cells from probiotic-treated mice contain, if anything, a lower population of IL-10-producing cells than similar cells from PBS-treated mice, and thus it is unlikely that this population is protective because of a greater capacity to produce IL-10. Finally, we administered anti-IL-10R to probiotic-treated mice after colitis reinduction to determine whether blockade of IL-10 use would affect the level of $CD4^+LAP^+$ cells that develop in such mice. As shown in Fig. 9, we found that mice administered anti-IL-10R manifested $CD4^+LAP^+$ cell levels that were comparable to that observed in PBS-treated mice. Thus, it was evident that expansion

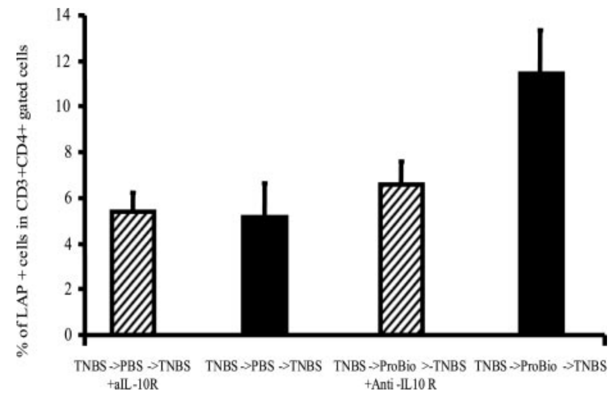

FIGURE 9. Treatment of mice with anti-IL-10R at the time of the second TNBS administration prevents the increase of LAP^+ T cells in mice treated with probiotics. Percentage of LAP^+ cells in $CD3^+CD4^+$ gated cells. Mice were sacrificed at day 3 after TNBS per rectum administration, and freshly isolated LPMC from colons were stained for various surface marker expression (see *Materials and Methods*). Each data point represents mean values from two different experiments. In each experiment, the experimental groups consisted of at least five mice. Bars represent SE.

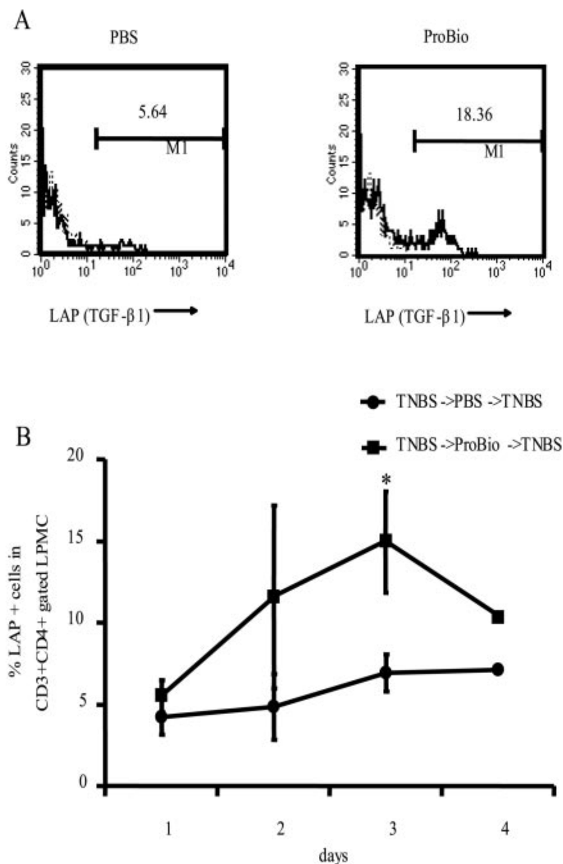

FIGURE 8. Effect of probiotic administration on LAP expression of $CD3^+CD4^+$ lamina propria T cells during the course of recurrent TNBS-colitis. *A*, Expression of LAP on $CD3^+CD4^+$ T cells. Freshly isolated LPMC from PBS or probiotic-treated mice sacrificed at day 3 from colitis reinduction, were stained with PerCP-conjugated anti-CD3, allophycocyanin-conjugated CD4, biotin-conjugated anti-LAP, and PE-conjugated streptavidin. Expression of LAP on $CD3^+CD4^+$ gated cells is shown. *B*, Groups of five mice were sacrificed at the indicate time points, and freshly isolated LPMC from colons were stained for various surface marker expression (see *Materials and Methods*). Each point represents mean of percentage of positive cells in the $CD3^+CD4^+$ gated cells from two different experiments. Bars represent SD. The percentage of $CD3^+CD4^+$ cells did not differ among the groups. $CD4^+LAP^+$ represent 5 ± 2% of the whole LPMC population in probiotic-treated mice and the 2 ± 0.85% of the whole LPMC population in PBS-treated mice. ■, Probiotic-treated mice. ●, PBS-treated mice. Probiotic-treated mice vs PBS-treated mice differ at the $p < 0.05$ level (*).

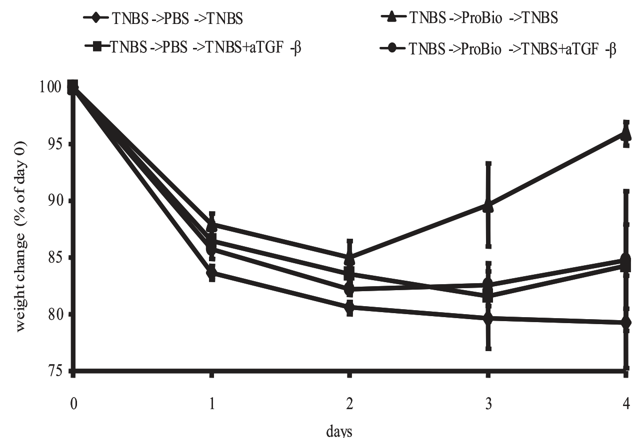

FIGURE 10. Treatment of mice with anti-TGF-β mAb at the time of the second TNBS administration abrogates the protective effect of feeding probiotics on the development of TNBS-colitis. Weight changes of mice that were administered TNBS per rectum after feeding probiotics (▲), TNBS per rectum and i.p. anti-TGF-β after feeding probiotics (●), TNBS per rectum after feeding PBS (◆), and TNBS per rectum and i.p. anti-TGF-β after feeding PBS (■). Each data point represents mean weight. The experimental groups consisted of at least 10 mice. Bars represent SE. Initial weight did not differ among the groups.

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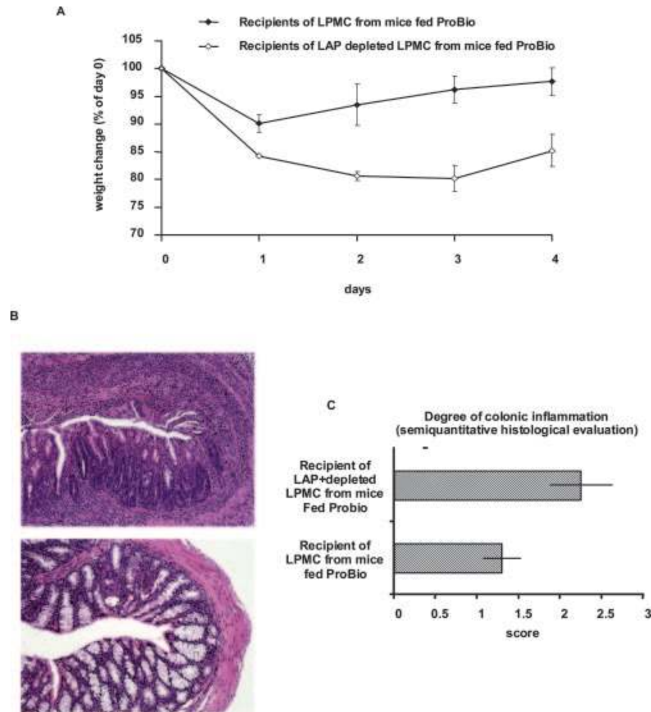

FIGURE 11. Depletion of LAP$^+$ cells from LPMC of probiotic-fed mice before adoptive cell transfer abolishes the ability of LPMC to protect recipient mice from the development of TNBS-colitis. *A*, Weight changes of recipients of LPMC obtained from mice fed probiotics (♦), recipients of LAP$^+$ cell-depleted LPMC obtained from mice fed probiotics (◇). Recipient mice were administered TNBS per rectum 5 days after adoptive transfer of LPMC. The experimental groups consisted of at least five mice. Each data point represents the mean value for each group. Bars represent SE. Initial weight did not differ between the groups. *B*, Histologic analysis of colons of the groups of naive mice transferred LPMC of mice treated with probiotics depleted or not of LAP$^+$ cells. Mice that were administered the undepleted LPMC from donor mice show less inflammation compared with mice that were administered LAP$^+$ cell-depleted LPMCs. *Upper*, Photomicrograph (×50) of an H&E-stained paraffin section of a representative colon at day 6 after intrarectal TNBS administration from a mouse that was administered LAP$^+$ cell-depleted LPMC from donor mice treated with probiotics. Severe cellular infiltration, epithelial crypt ulceration, and loss of goblet cells are seen. *Lower*, Photomicrograph (×50) of an H&E-stained paraffin section of a representative colon at day 6 after intrarectal TNBS administration from a mouse that received undepleted LPMC from donor mice treated with probiotics. Virtually normal histology is seen. *C*, Histologic score (see *Materials and Methods*) of colons of the groups of mice treated as above. Histologic scores were obtained as described in Fig. 3.

of CD4$^+$LAP$^+$ cells during recurrent colitis is dependent on the activity of IL-10.

LAP$^+$ cells induced by probiotic treatment are regulatory cells capable of modulating the course of recurrent TNBS-colitis

To assess the regulatory activity of LAP$^+$ cells appearing after probiotic treatment, we used two different experimental approaches. The first was based on the fact that LAP$^+$ cells have been shown to mediate regulatory function via either cell surface or secreted TGF-β (15, 16) and, in common with CD25$^+$ regulatory cells generally, may require TGF-β for expansion. We therefore reasoned that we could block LAP$^+$ cell regulatory activity during recurrent colitis by administration of anti-TGF-β Ab to mice (see *Materials and Methods*) at the time of colitis reinduction. As shown in Fig. 10, anti-TGF-β administration did indeed prevent the ability of probiotics to ameliorate colitis in probiotic-treated mice.

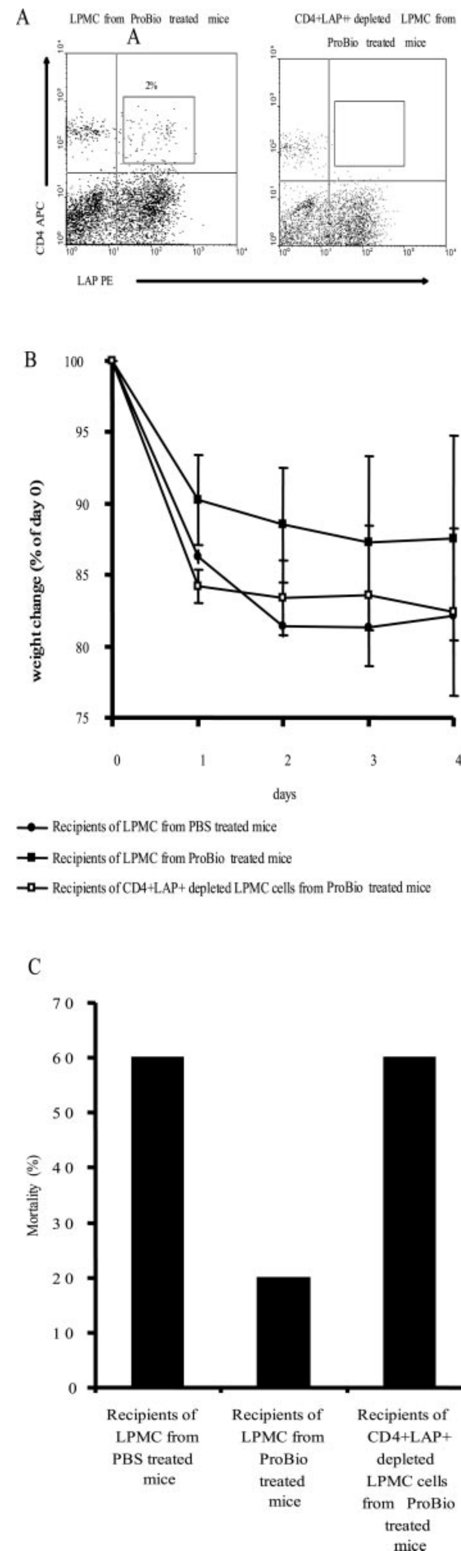

FIGURE 12. Depletion of CD4$^+$LAP$^+$ cells from LPMC of probiotic-fed mice before transfer abolishes the ability of the LPMC to protect recipient mice from the development of TNBS-colitis. *A*, CD4 and LAP expression on LPMC isolated from probiotic-treated mice before and after depletion of CD4$^+$LAP$^+$ cells by flow cytometric cell sorting. *B*, Weight changes of recipients of undepleted LPMC obtained from mice fed probiotics (■), recipients of CD4$^+$LAP$^+$ cell-depleted LPMC obtained from mice fed probiotics (□), and recipients of undepleted LPMC from mice fed PBS (●). *C*, Mortality (%) in the same groups of mice. Recipient mice were administered TNBS per rectum 5 days after adoptive transfer of LPMC. The experimental groups consisted of at least five mice. Each data point represents the mean of value for each experimental group. Bars represent SE. Initial weight did not differ among the groups.

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The second approach was to determine whether LPMC populations derived from probiotic-treated mice and then subjected to selective depletion of LAP$^+$ cells would result in cells that are unable to transfer protection from colitis. We thus adoptively transferred LPMC from mice fed probiotics (or PBS) that had been depleted or not of LAP$^+$ cells using anti-LAP Ab bound to magnetic beads (see *Materials and Methods*) to naive mice and then induced TNBS-colitis in the latter mice. As shown in Fig. 11, *A* and *B*, as assessed by weight changes and histological status of colonic tissue, mice that were administered LPMC depleted of LAP$^+$ cells from donors fed probiotics developed a more severe colitis compared with mice that received nondepleted LPMC. In a second experiment along these lines, we depleted LPMC isolated from probiotic-treated mice of CD4$^+$LAP$^+$ cells by flow cytometric sorting of cells expressing both CD4 and LAP. Thereafter, we transferred undepleted LPMC or CD4$^+$LAP$^+$-depleted LPMC to naive mice and then subjected the latter to TNBS-colitis induction. Naive mice that were transferred LPMC isolated from PBS-treated mice served as additional controls. As shown in Fig. 12*A*, the sorting procedure led to depletion of virtually all CD4$^+$LAP$^+$ cells. In addition, as shown in Fig. 12, *B* and *C*, mouse recipients of LPMC depleted of CD4$^+$LAP$^+$ cells exhibited no protection from TNBS-colitis induction, whereas recipients of undepleted cells were largely protected from such induction. As before, recipients of LPMC isolated from PBS-treated mice were not protected from colitis induction. Taken together, these data provide strong evidence that LAP$^+$ cells (and, more specifically, CD4$^+$LAP$^+$ cells) are directly involved in the protective effect induced by probiotics.

Discussion

In the present study, we demonstrated that daily administration of the probiotic, VSL#3, to mice in the 3-wk interval after recovery from an initial induction of TNBS-colitis and before a second induction of (recurrent) TNBS-colitis resulted in a milder form of recurrent TNBS-colitis. This protective activity was attributable to effects on mononuclear cells in the lamina propria, because it could be transferred by LPMC from probiotic-treated mice to naive mice. Moreover, this activity was due to IL-10-dependent regulatory cells, because it was associated with the production of IL-10, could be blocked by administration of anti-IL-10R, and was associated with the IL-10-dependent appearance of CD4$^+$LAP$^+$ cells that have previously been shown to have regulatory activity in CD4$^+$CD45RB^{high}-induced cell transfer (SCID) colitis model (15, 16). Finally, the relation between the regulatory cells and the protective effect was established by the fact that the protective activity was blocked by the administration of anti-TGF-β and by the depletion of LAP$^+$ cells (CD4$^+$LAP$^+$ cells) from adoptively transferred LPMC otherwise capable of conferring protection against the development of colitis in recipients of such cells.

The concept that probiotics act via the induction of regulatory cells is inherently attractive, because it seems unlikely that they could act by adequately replacing all bacteria in the intestinal microflora that are capable of causing inflammation in experimental mice/individuals susceptible to IBD-like inflammation or IBD. This view is supported by the fact that there is little or no evidence that potentially pathologic bacteria are confined to any one bacterial species, so that if replacement were the mechanism, the probiotics would have to displace the many different bacteria populating the various ecological niches of the mucosal bacterial milieu. In recent studies bearing on this issue, Rachmilewitz et al. (17) have shown that immunostimulatory DNA sequences (DNA containing unmethylated CpG sequences) identical with those used as immunologic adjuvants are paradoxically able to prevent the development of various forms of experimental mucosal inflammation, including induced inflammations such as dextran sodium sulfate-colitis and TNBS-colitis as well as spontaneous colitis associated with IL-10 deficiency. As might be expected, this effect was accompanied by the inhibition of the induction of inflammatory cytokines and chemokines (17). In a more recent study, they showed that both nonprobiotic *E. coli* DNA and probiotic VSL#3 DNA delivered via an intragastric or s.c. route were able to inhibit dextran sodium sulfate-colitis in normal mice but not in TLR9-deficient mice, i.e., mice lacking the TLR receptor that can recognize bacterial DNA. In contrast, they were able to inhibit this colitis in TLR2- and TLR4-deficient mice (18). On this basis, they suggested that probiotic organisms signal dendritic cells via TLR9 to induce regulatory T cells. In recent studies that indirectly speak to this possibility, Drakes et al. have shown that probiotics (VSL#3) display some differences from *E. coli* in the induction of surface markers on human bone marrow-derived dendritic cells and do not enhance the ability of dendritic cells to induce allogeneic T cell proliferation as does *E. coli*. In addition, probiotics tend to induce more IL-10 production by dendritic cells than *E. coli*, although both induce IL-12 when added late in the culture (19). These studies hint at the possibility that probiotics may induce a class of dendritic cells that induce regulatory T cells.

As suggested by other studies of probiotic activity, mechanisms other than those involving effects on regulatory T cells may be at play in bringing about protection from mucosal inflammation. One alternative mechanism suggested by Madsen et al. (7), is that probiotic bacteria (in this case, the same one used in this study, VSL#3) enhance epithelial barrier function in IL-10-deficient mice and secreted a factor that increased resistance to *Salmonella* invasion of epithelial monolayers in vitro. These data offer an explanation of probiotic function in the absence of IL-10, one of the key cytokines implicated in the regulatory cell-dependent mechanism proposed here. A second alternative mechanism is that probiotics inhibit chemokine production (such as IL-8) by epithelial cells and thus inhibit the innate response to mucosal bacteria (20). However, this was seen with one probiotic preparation (VSL#3) but not another (*E. coli* Nissle 1917). Finally, preliminary data suggest the possibility that probiotics might induce defensin production, and thus enhance the control of bacterial growth in the intestinal crypts (21). It should be noted that each of these alternative explanations involve probiotic effects on epithelial cell function, whereas in the present study, it was shown with cell transfer studies that probiotic effects reside with LPMC. Nevertheless, these sets of findings are not mutually exclusive because it remains possible that probiotics have several means of exerting anti-inflammatory effects, and that both effects on epithelial cell function and induction of regulatory cells act synergistically as mechanisms of probiotic activity.

As indicated above, the data presented here quite clearly established that the protective effect of probiotic administration on recurrent TNBS-colitis was dependent on increased IL-10 production in the probiotic-treated mice. This conclusion is consonant with previous studies of recurrent colitis in HLA-B27 rats that had been monoassociated with *Bacteroides vulgatus* and then treated with antibiotics and/or probiotics. These studies showed that probiotic (*Lactobacillus* GG) treatment could prevent recurrent colitis in these rats, and such prevention was associated with increased IL-10 (but no change in TGF-β) secretion as detected in assays of cecal homogenates (22). Similarly, probiotic DNA derived from certain VSL#3 bacterial components induced increased IL-10 production in human PBMCs in vitro (23). Other data bearing on the relation of IL-10 to gut inflammation come from the recent demonstration that IL-10 gene therapy prevents TNBS-colitis (24), and that murine spleen CD4$^+$ lymphocytes transduced with a retroviral

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vector expressing IL-10 are able to prevent the development of colitis in the cell-transfer (SCID)-transfer model of colitis (25). However, it should be noted that the increase in IL-10 production observed in probiotic-treated mice and associated with protection occurred at the end of the probiotic treatment period and during the initial period of recurrent TNBS-colitis. Thus, it occurred before the establishment of full-blown colitis. This is consistent with the fact that, in TNBS-colitis, although IL-10 is able to prevent the development of colitis if given before inflammation develops, it is ineffective if given after inflammation has become established (24–27). This latter observation is not in conflict with studies of SCID-transfer colitis (25), in which it has been shown that IL-10 transduced $CD4^+$ T cells were able to effectively prevent colitis even when administered 14 days after the transfer of the colitogenic $CD45RB^{high}$ T cells, because in this model colitis is not yet fully established before a month after cell transfer.

In further explanation of the significance of the early increase in IL-10 production to probiotic activity, we need to refer to our earlier studies in which we analyzed the prevention of TNBS-colitis achieved by feeding trinitrophenol-haptenated protein before colitis induction (28). In these studies, we first showed that such feeding induced the production of transferable regulatory T cells. We then showed that, although anti-TGF-β administration led to decreased TGF-β production and loss of the regulatory effect, it had no effect on IL-10 production; in contrast, anti-IL-10 administration led to both decreased IL-10 secretion and TGF- β production. These latter findings suggested that the role of IL-10 in this context was to support the development of cells producing TGF-β and that IL-10 had little regulatory effect in and of itself. Finally, in cell transfer studies, we showed that the effect of IL-10 does not occur during the initial induction of TGF-β-producing regulatory cells because these cells may be induced also when IL-10 activity is blocked, but rather during the period of their expansion (28). Thus, these prior studies suggest that the function of IL-10 induced in the lamina propria by probiotics is to support the development of regulatory TGF-β-producing T cells that are the proximal inhibitors of the recurrent colitis. The possibility that IL-10 induced by probiotic treatment also has a direct suppressive effect is not ruled out by the studies reported here, because this can only be ascertained by conducting studies with purified regulatory cells from IL-10-deficient mice that are highly impractical because of the low numbers of regulatory cells in the LPMC, even in probiotic-treated mice. Nevertheless, this possibility appears unlikely because $CD4^+LAP^+$ cell populations obtained from probiotic-treated mice that are protective of naive recipient mice subjected to TNBS-colitis induction contain, if anything, less IL-10-producing cells than a similar population obtained from PBS-treated mice that are not protective; in addition, as mentioned, the increase in IL-10 seen in mice transferred LPMC from probiotic-treated mice is seen early after transfer and is not sustained, as might be expected if it were a major suppressive factor in itself.

Turning our focus from IL-10 to TGF-β, the important observation made here is that probiotic treatment results in an increase in the number of lamina propria IL-10-dependent $CD4^+LAP^+$ T cells, which appeared to be essential to the amelioration of colitis. As mentioned above, $CD4^+LAP^+$ T cells are T cells that bear latent TGF-β on their cell surface. These cells are similar (if not identical) to $CD25^+$ "natural" regulatory cells in that they have previously been shown to inhibit cell-transfer colitis by a TGF-β-dependent mechanism (15, 16). The importance of these cells to the probiotic effect on experimental colitis was strongly supported by the fact that blockade of the function of these cells by administration of anti-TGF-β or, indeed, by depletion of these cells from cell populations, abolishes the protective effect of probiotics on colitis. This effect of anti-TGF-β can be due to two separate but interactive Ab activities. First, the Ab may block the effector function of these cells, by either preventing suppressive cell-cell interactions involving cell surface TGF-β or by preventing suppression mediated by secretion of TGF-β. Second, evidence has recently emerged suggesting that TGF-β can induce naive $CD25^-$ cells to synthesize Foxp3, a forkhead transcription factor that serves as a master switch whose expression results in the de novo induction of regulatory cells in the peripheral tissues (29, 30); in addition, it has been shown that Foxp3 down-regulates Smad7, a protein normally induced by TGF-β that inhibits TGF-β signaling (30). These data suggest that the production of TGF-β by regulatory cells allows for the autocrine or paracrine signals that result in the expansion of regulatory cells, and thus that anti-TGF-β in the present context also acts by blocking the generation of regulatory T cells.

One issue that remains to be discussed is that, as already mentioned, probiotics (including VSL#3) have been shown to ameliorate mucosal inflammation in IL-10 KO mice, which is seemingly at odds with the fact emphasized here that IL-10 is necessary for the regulatory cell response that underlies probiotic action. This apparent paradox can conceivably be explained by a previous study already discussed above in which we showed that the initial generation of TGF-β-producing regulatory cells can occur in the absence of IL-10 (28), and the studies mentioned above showing that the expansion of the regulatory cell population can be mediated by TGF-β itself. Because Th1 cytokines oppose regulatory cell development, this may not occur in the presence of a rapidly developing and intense Th1 response such as that occurring in TNBS-colitis studied here; however, it may well occur in the presence of a slowly developing and initially weak Th1 response characteristic of the colitis seen in IL-10 KO mice. This idea is favored by data from Sheil et al. (31), who have shown that systemic probiotic administration (*Lactobacillus salivarius*) to IL-10 KO mice led to reduced inflammation and reduced inflammatory cytokine production, accompanied by increased TGF-β production by spleen cells. However, we would need to have more information on whether the increased TGF-β production in IL-10 KO mice was the cause of the probiotic effect in these mice to establish this possibility.

In summary, the data obtained here show that probiotic administration, in this case the probiotic VSL#3, leads to less severe recurrent TNBS-colitis and suggest that the induction of IL-10-dependent regulatory LAP^+ T cells is the major mechanism underlying the protective effect. Among the clinically relevant pieces of information to emerge from this study is that probiotic induction of regulatory cells appears to be mainly effective during the remission phase of the disease when inflammatory processes are still not at their peak. Therefore, probiotic administration in CD patients may be most effective during clinical remission and the role of probiotics may be limited to the important task of preventing recurrent inflammation.

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Disclosures

The authors have no financial conflict of interest.

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