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Probiotic *Lactobacillus* spp. Diminish *Helicobacter hepaticus*-Induced Inflammatory Bowel Disease in Interleukin-10-Deficient Mice

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Clinical and experimental evidence has demonstrated the potential role of probiotics in the prevention or treatment of inflammatory bowel disease. Probiotic clones with direct immunomodulatory activity may have anti-inflammatory effects in the intestine. We investigated the roles of tumor necrosis factor alpha (TNF-α)-inhibitory *Lactobacillus* clones with a pathogen-induced murine colitis model. Murine-derived probiotic lactobacilli were selected in vitro for their ability to inhibit TNF-α secretion by *Helicobacter hepaticus*-stimulated macrophages. Interleukin-10 (IL-10)-deficient mice were treated with probiotic *Lactobacillus reuteri* in combination with *Lactobacillus paracasei* and then challenged with *H. hepaticus*. Ten weeks postinoculation, the severity of typhlocolitis was assessed by histologic examination of the cecocolic region. Intestinal proinflammatory cytokine responses were evaluated by real-time quantitative reverse transcriptase PCR and immunoassays, and the quantities of intestinal *H. hepaticus* were evaluated by real-time PCR. Intestinal colonization by TNF-α-inhibitory lactobacilli reduced intestinal inflammation in *H. hepaticus*-challenged IL-10-deficient mice despite similar quantities of *H. hepaticus* in cocolonized animals. Proinflammatory colonic cytokine (TNF-α and IL-12) levels were lowered in *Lactobacillus*-treated animals. In this *H. hepaticus*-challenged IL-10-deficient murine colitis model, lactobacilli demonstrated probiotic effects by direct modulation of mucosal inflammatory responses.

The rational selection of probiotic bacteria provides opportunities for the prevention or treatment of inflammatory bowel disease (IBD) based on accumulated evidence from animal and human studies (12, 40). Selective deficiencies of intestinal lactobacilli and bifidobacteria have been observed in patients with Crohn's disease (9). Supplementation with probiotic *Lactobacillus* species has been effective at ameliorating intestinal inflammation in human patients with IBD. Administration of *Lactobacillus rhamnosus* GG (LGG) to children with Crohn's disease resulted in significant reduction of the Crohn's disease activity index 4 weeks after initiation of therapy (14). Probiotic formulations that included four *Lactobacillus* species have been effective for the prevention (10) or treatment (11) of IBD-related pouchitis.

The murine interleukin-10 (IL-10)-deficient mouse colitis model has provided additional insights into the roles of probiotic *Lactobacillus* spp. as potential prophylactic or treatment modalities in IBD. Introduction of a single *Lactobacillus reuteri* strain into the lower gastrointestinal tracts of IL-10-deficient mice (Crohn's disease model) by intracolonic application restored mucosa-adherent *Lactobacillus* populations and prevented development of colitis in these animals (27). Similarly, the introduction of *Lactobacillus plantarum* strain 299V into IL-10-deficient mice ameliorated colitis in these animals (38). *Lactobacillus salivarius* inhibited proinflammatory cytokine production and attenuated colitis in placebo-controlled trials with IL-10-deficient mice (28, 32). The replenishment of intestinal IL-10 by recombinant lactic acid bacteria ameliorated disease in IL-10-deficient mice, indicating the importance of IL-10 in controlling intestinal inflammation (42). Alternatively, these studies suggest that the absence of IL-10 in the intestine may be compensated by anti-inflammatory activities of probiotic bacteria.

In this study, mechanisms of probiosis were explored in a pathogen-induced mouse IBD model. Mice lacking IL-10 rapidly develop robust IBD-like lesions following infection by *Helicobacter hepaticus* (20), whereas germ- or pathogen-free IL-$10^{-/-}$ mice fail to develop colitis or manifest delayed onset of disease, respectively (39). Intestinal *Lactobacillus* spp. were administered orally to IL-10-deficient mice in order to examine probiotic effects in colitis-susceptible mice challenged with pathogenic *H. hepaticus*. We selected mouse-derived *Lactobacillus paracasei* and *L. reuteri* strains on the basis of in vitro tumor necrosis factor-alpha (TNF-α)-inhibitory activity toward macrophages (33) and investigated probiotic anti-inflammatory effects in IL-10-deficient mice. The effects of probiotic lactobacilli on intestinal quantities of *H. hepaticus* were evaluated in parallel with studies of mucosal proinflammatory cytokine responses.

MATERIALS AND METHODS

Bacterial strains. *Lactobacillus* spp. were grown anaerobically in deMan, Rogosa, Sharpe (MRS) broth (Becton Dickinson, Sparks, Md.) overnight at 37°C. *L. paracasei* 1602 was isolated from a fecal sample of a mouse in our facility, and *L. reuteri* 6798 was isolated from jejunal sample of a different mouse

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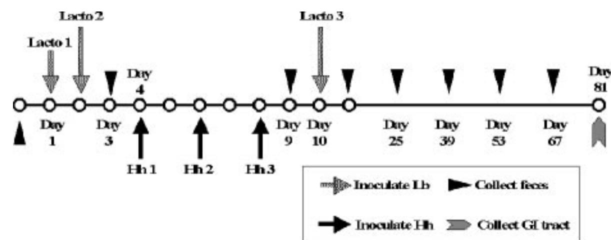

FIG. 1. Timeline of *H. hepaticus* infection and *Lactobacillus* co-colonization study. Bacteria were administered to mice by orogastric gavage. An equal mixture (10^9 cells per dose) of *L. paracasei* 1602 and *L. reuteri* 6798 (Lacto or Lb) was used to antagonize colitis induced by *H. hepaticus* (Hh) (10^7 cells per dose).

in the same facility (33). *H. hepaticus* type strain 3B1 (ATCC 51449) was grown in Brucella broth (Becton Dickinson) supplemented with 5% heat-inactivated fetal bovine serum (FBS) under microaerobic conditions for 48 to 60 h at 37°C. Cultures were assessed for purity by Gram staining prior to animal inoculation.

***Lactobacillus*-macrophage bioassays and evaluation of probiotic antagonism.** Overnight cultures of lactobacilli were diluted to an optical density at 600 nm of 1.0 (representing approximately 10^9 cells/ml), further diluted 1:10, and grown in MRS for an additional 24 h. *H. hepaticus* 3B1 was cultured for 48 h in Brucella broth supplemented with 5% FBS. Cultures were diluted 1:10 and grown for another 24 and 48 h. Bacterial cell-free conditioned medium was collected by centrifugation at 8,500 RCF for 10 min at 4°C. Conditioned medium was separated from cell pellet and filtered through a 0.22-μm-pore-size filter unit (Millipore, Bedford, Mass.).

The mouse monocyte/macrophage cell line RAW 264.7 gamma NO (−) (ATCC CRL-2278) was used as the reporter cell line for studying probiotic antagonism of TNF-α production. RAW 264.7 cells were grown in RPMI medium 1640 (Gibco-Invitrogen, Carlsbad, Calif.) supplemented with 10% FBS and 2% antibiotic (5,000 U of penicillin/ml and 5 mg of streptomycin/ml; Sigma, St. Louis, Mo.) at 5% CO_2, 37°C until 80 to 90% confluent. Approximately 5×10^4 cells were seeded into 96-well cell culture clusters and allowed to adhere for 2 h prior to addition of conditioned medium. Naive RAW 264.7 cells were exposed to a mixture of lactobacillus-conditioned medium and cell-free *Helicobacter*-conditioned medium (1:1 ratio). Cell viability was assessed by the trypan blue (Invitrogen) exclusion assay. Production of TNF-α in macrophage cell culture supernatants was measured with a mouse TNF-α-specific sandwich enzyme immunoassay (Biosource, Camarillo, Calif.).

Animals and experimental infection. IL-10-deficient C57BL/6 mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care-approved facility at the Division of Comparative Medicine, Massachusetts Institute of Technology (Cambridge, Mass.), under specific-pathogen-free conditions in microisolator cages. Animals were provided standard chow and water and allowed to feed ad libitum under a 12-h daylight cycle. Mice were kept free of known murine viruses, *Salmonella* spp., *Citrobacter rodentium*, ecto- and endoparasites, and known murine *Helicobacter* spp.

Research was conducted following protocols approved by the Committee on Animal Care at Massachusetts Institute of Technology. Six- to 13-week-old mice were matched by age and sex and assigned to one of four infection groups. Animals received ~10^9 CFU of lactobacilli or ~10^7 CFU of *H. hepaticus* per dose by gastric gavage. *Lactobacillus* was administered twice, two days in a row; each inoculum included equivalent cell counts of *L. paracasei* 1602 and *L. reuteri* 6798. Forty-eight hours after the second lactobacillus dose, *H. hepaticus* was administered thrice (once per day, every other day). A final dose of lactobacilli was given 48 h after the third *H. hepaticus* dose. Control animals received sterile MRS or Brucella broth (for *Lactobacillus* spp. or *H. hepaticus*, respectively). Ten weeks postinfection, animals were euthanized by CO_2 asphyxiation. A schematic timeline of the infection study is depicted (Fig. 1).

Histologic examination. At necropsy, tissue was collected and, after expression of intestinal contents, fixed in 10% neutral pH-buffered formalin and processed by routine histologic methods. A comparative pathologist (A.B.R.), blinded to sample identity, evaluated hematoxylin-and-eosin-stained sections of the ceco-colic junction. Sections were graded on an ascending scale from 0 to 4 for inflammation, hyperplasia, and dysplasia, using previously defined criteria (6).

Determination of bacterial colonization. Total DNA, RNA, and protein were extracted from the mid-cecum, using Trizol LS (Invitrogen) reagent for subsequent quantitation. Quantities of *H. hepaticus* in the mid-cecum were determined by real-time PCR amplification of the *cdtB* gene, as previously described (8). TaqMan probe-based detection was performed with an ABI Prism 7700 sequence detection system (Applied Biosystems), and murine 18S rRNA gene was used as a correction factor (8).

Cytokine quantitation. IL-4, IL-12 (p40), gamma interferon (IFN-γ), and TNF-α mRNA were measured by real-time reverse transcription-PCR of RNA isolated from cecal tissue samples. Briefly, 1 μg of total RNA was reverse transcribed by using oligo(dT) primers and the Superscript III reverse transcriptase system (Invitrogen) according to the manufacturer's recommendations. All cDNA preparations were normalized by endogenous glyceraldehyde-3-phosphate dehydrogenase, using commercial primer-probe sets (Applied Biosystems, Foster City, Calif.) in an ABI Prism 7700 sequence detection system (Applied Biosystems) according to the manufacturer's recommendations.

Immediately after euthanasia, a 10-mm-long section of proximal colon was harvested and the contents were removed by vigorous agitation in sterile saline. Colons were divided into four sections, weighed, and cultured (mucosa side up) in RPMI 1640 supplemented with 10% heat-inactivated FBS and antimicrobial agents (50 U of penicillin/ml, 50 μg of streptomycin/ml, and 125 ng of amphotericin B/ml) (Invitrogen) for 24 h in transwell inserts (Millicell; Millipore). Two sections from each colon served as duplicates and were cultured with or without purified *Escherichia coli* lipopolysaccharide (LPS) serotype O127:B8 (Sigma) (250 ng/well). TNF-α in colon explant supernatants was measured by quantitative enzyme-linked immunosorbent assay (Biosource). Mouse DNA was extracted from entire explants for 18S rRNA gene quantitation and used to standardize TNF-α output.

Statistical analyses. Statistics were performed by using SPSS for Windows, version 11.0.1 (SPSS Inc., Chicago, Ill.). Histologic, bacterial colonization and cytokine data were analyzed using the Kruskal-Wallis test followed by the Mann-Whitney U test for two independent samples. Results are presented as median and interquartile range. Correlations were assessed by using Spearman's method and reported as the coefficient, rho. Comparisons yielding P values of ≤0.05 were considered significant. Nonparametric statistics were used because histology was the primary endpoint, and cytokine data distribution did not meet the requirements for parametric tests.

RESULTS

Probiotic lactobacilli inhibit *Helicobacter*-mediated stimulation of TNF-α production by macrophages. Since dysregulated inflammatory responses appear to be central to the pathogenesis of IBD in mouse models, we identified mouse-derived *Lactobacillus* strains with anti-inflammatory properties. We screened intestinal murine *Lactobacillus* isolates (33) for probiotic activity as defined by the inhibition of TNF-α production by LPS-stimulated macrophages (34). Approximately 10% of *Lactobacillus* isolates inhibited TNF-α production by LPS-stimulated mouse macrophages (33). *L. paracasei* 1602 and *L. reuteri* 6798 represented two TNF-α-inhibitory strains recovered from mice lacking a predisposition to colitis. Murine macrophages were exposed to cell-free *H. hepaticus*-conditioned medium in order to assess the ability of selected lactobacilli to antagonize innate immune responses to *H. hepaticus*. In the presence of conditioned medium from lactobacilli, TNF-α production was significantly diminished in *H. hepaticus*-conditioned medium-stimulated macrophages. Selected *Lactobacillus* clones inhibited *H. hepaticus*-mediated stimulation of macrophage TNF-α production (Fig. 2).

Probiotic lactobacilli reduce intestinal inflammation in *Helicobacter*-infected female IL-10-deficient mice. *Lactobacillus* clones with TNF-α-inhibitory activity in vitro were evaluated in vivo by studying mucosal protection in a *Helicobacter*-exacerbated colitis model. We performed two infection studies with IL-$10^{-/-}$ mice. In a pilot study, both male ($n = 21$) and female ($n = 18$) mice were precolonized with a combination of two murine lactobacilli, *L. paracasei* 1602 and *L. reuteri* 6798, that had displayed anti-inflammatory activity with cultured macro-

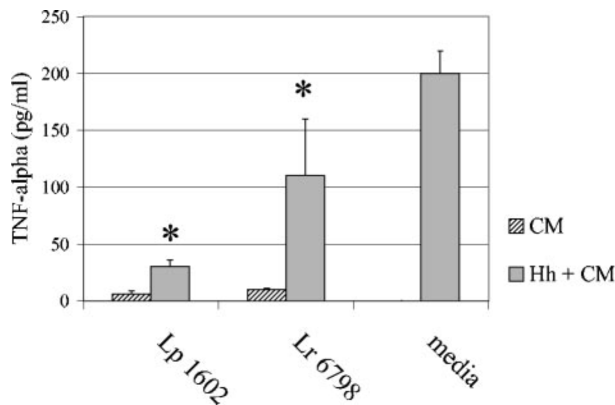

FIG. 2. In vitro selection of probiotic lactobacilli for studies in IL-10-deficient mice. *L. paracasei* 1602 (Lp1602) and *L. reuteri* 6798 (Lr6798) blocked TNF-α production by murine RAW 264.7 macrophages activated with bacterium-free stationary-phase culture medium of *H. hepaticus* (Hh) 3B1. Briefly, cells were coincubated with *H. hepaticus*-conditioned medium and *Lactobacillus*-conditioned medium (CM) derived from cultures of Lp1602 or Lr6798. TNF-α production was measured in picograms per milliliter by quantitative enzyme-linked immunosorbent assay. *, *Lactobacillus*-CM significantly decreased TNF-α output by Hh-stimulated macrophages ($P < 0.05$).

phages. *Lactobacillus* colonization was followed by infection with the mouse pathogen *H. hepaticus*. Sham-dosed mice and mice colonized with a combination of *L. paracasei* 1602 and *L. reuteri* 6798 in the absence of *H. hepaticus* were studied as controls. After 10 weeks, we semiquantitatively graded the cecocolic junction for inflammation, hyperplasia, and dysplasia by histopathologic examination. Median histologic scores of uninfected sham-dosed controls and *Lactobacillus*-colonized animals were similar (Fig. 3a). In contrast, mice infected with *H. hepaticus* developed moderately severe typhlocolitis. Animals treated with a combination of *L. paracasei* 1602 and *L. reuteri* 6798 prior to infection with *H. hepaticus* did not exhibit significant diminution of IBD-like lesions.

When animals were stratified by gender, we observed significant reductions in cecocolic lesion scores (inflammation, hyperplasia, and dysplasia; $P = 0.032$, 0.032, and 0.016, respectively) in female mice cocolonized with *Lactobacillus* and *H. hepaticus* versus animals that were monoinfected with *H. hepaticus* (Fig. 3b). In contrast, male mice cocolonized with murine lactobacilli displayed only a slight and statistically nonsignificant reduction in intestinal lesion grades. Real-time PCR confirmed that intestinal *Lactobacillus* DNA quantities were significantly elevated only in *Lactobacillus*-treated animals and were maintained for the duration of the study (data not shown). Because female mice appeared to derive the greatest benefit from probiotic therapy, we performed a follow-up study with female mice only ($n = 36$), using the same cocolonization and infection protocol. A summary of histologic scores for the follow-up study is provided (Table 1).

As in the pilot study, uninfected animals exhibited normal cecocolic histology (Fig. 4a), while those monoinfected with *H. hepaticus* (Fig. 4b) developed moderate to severe typhlocolitis characterized by infiltration of lymphocytes and macrophages, with fewer granulocytes, in the mucosa and submucosa. Reactive epithelial changes included hyperplasia with crypt elonga-

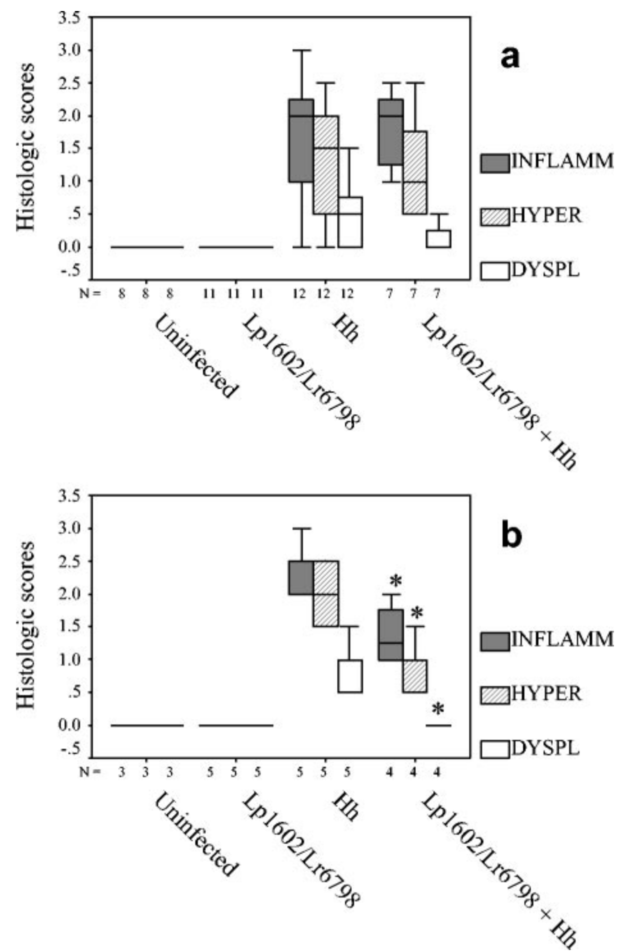

FIG. 3. *Lactobacillus* decreases typhlocolitis in female mice but not in male mice. The cecocolic region of mouse intestines was evaluated by hematoxylin-and-eosin staining and scored blindly by a board-certified veterinary pathologist. Independent scores (scale, 0 to 4) were obtained for degree of mucosal inflammation (INFLAMM), hyperplasia (HYPER), and dysplasia (DYSPL). (a) In a pilot study utilizing both genders, prophylaxis with lactobacilli failed to diminish visible disease by histologic examination. (b) When only female mice were analyzed, lactobacilli exerted significant protective effects. Mice were uninfected (not colonized or infected with exogenous bacteria), cocolonized with *L. paracasei* 1602/*L. reuteri* 6798 (Lp1602/Lr6798), infected with *H. hepaticus* (Hh), or cocolonized with *L. paracasei* 1602/*L. reuteri* 6798 and *H. hepaticus* (Lp1602/Lr6798 + Hh). Data are presented as box plots with median (line inside box), interquartile range (shaded box), and range (error bars). *, pretreatment with lactobacilli prior to *H. hepaticus* challenge (Lp1602/Lr6798 + Hh) significantly decreased all lesion types compared to results for animals receiving *H. hepaticus* alone (Hh) ($P < 0.05$).

tion as well as goblet cell loss and mild dysplasia with distortion of glandular architecture, cell crowding (piling), and early aberrant crypt foci including slit and back-to-back forms. Lesions were partially eliminated in mice treated with *L. paracasei* and *L. reuteri* (Fig. 4c). Subsequent molecular data presented in this study utilized samples from the follow-up population only.

***Lactobacillus*-mediated probiosis functions independently of quantities of intestinal *H. hepaticus*.** In order to correlate intestinal inflammation with the presence of *H. hepaticus*, cecal DNA was extracted and analyzed by real-time quantitative

TABLE 1. Summary of histologic scores of cecocolic region[a]

Group (n^b)	Infection status[c]	Inflammation	Hyperplasia	Dysplasia
1 (8)	Uninfected	0.5 (0.875)	0.5 (0.875)	0.25 (0.5)
2 (8)	Lp 1602/Lr 6798	0.5 (0.375)	0.5 (0.875)	0 (0)
3 (10)	Hh	2 (0.625)	2.25 (0.75)	0.75 (0.625)
4 (10)	Lp 1602/Lr 6798 + Hh	1.25 (1.25)	1.0 (1.125)	0.25 (0.5)

[a] Data are presented according to infection status. Median scores for inflammation, hyperplasia, and dysplasia are provided with interquartile ranges in parentheses.

[b] *n*, = no. of mice in group.

[c] Mice were uninfected (not colonized with probiotic lactobacilli), colonized with *L. paracasei* 1602/*L. reuteri* 6798 (Lp1602/Lr6798), infected with *H. hepaticus* (Hh), or precolonized with *L. paracasei* 1602/*L. reuteri* 6798 prior to *H. hepaticus* infection (Lp1602/Lr6798 + Hh).

PCR. As expected, *H. hepaticus* DNA was detectable only in animals deliberately infected with the pathogen. Unexpectedly, we identified no correlation between *H. hepaticus* colonization levels and lower bowel median lesion scores (inflammation [$\rho = 0.253$; $P = 0.082$] or hyperplasia [$\rho = 0.225$; $P = 0.108$]).

Animals receiving the *L. paracasei* 1602, *L. reuteri* 6798 combination (*L. paracasei* 1602/*L. reuteri* 6798) yielded quantities of *H. hepaticus* ($P = 1.0$) comparable to those for *H. hepaticus*-monoinfected controls (Fig. 5). *L. paracasei* 1602/*L. reuteri* 6798 provided partial protection against *H. hepaticus*-induced IBD-like lesions (Fig. 4c).

Reductions in proinflammatory cytokines correlate with probiosis. Since cytokine transcript levels have correlated with disease activity in IBD patients (41), we measured mRNA levels of selected cytokines in the ceca of mice (Fig. 6). Cecal levels of IL-12 (p40) and TNF-α were significantly elevated in *H. hepaticus*-infected mice compared to levels in uninfected controls ($P = 0.048$ and $P = 0.005$, respectively), while IFN-γ was not significantly altered. IL-4 levels were similar between uninfected and *H. hepaticus*-infected mice. In contrast, introduction of *L. paracasei* 1602/*L. reuteri* 6798 in the absence of *H. hepaticus* did not alter IL-12, TNF-α, IFN-γ, or IL-4 transcript levels compared to those for uninfected controls ($P < 0.05$ for all).

A significant reduction in mucosal IL-12p40 mRNA levels ($P = 0.016$) was observed when animals were cocolonized with *L. paracasei* 1602/*L. reuteri* 6798 prior to *H. hepaticus* challenge, while transcript levels for IFN-γ, IL-4, and TNF-α were not significantly altered. This difference may be biologically important considering the partial anti-inflammatory effect in the intestine without concomitant reductions in *H. hepaticus* levels. The data trends indicate reductions in overall proinflammatory cytokine profiles with the *L. paracasei* 1602/*L. reuteri* 6798 probiotic combination (Fig. 7).

We determined whether the presence of anti-inflammatory *Lactobacillus* clones rendered mucosal explants tolerant to *E. coli* LPS challenge with TNF-α as a biomarker. TNF-α production by LPS-stimulated colonic explants was blunted in mucosal tissue from mice cocolonized with *L. paracasei* 1602/*L. reuteri* 6798 and *H. hepaticus* compared to results for uninfected animals or animals infected only with *H. hepaticus*. Explants from mice treated with the *L. paracasei* 1602/*L. reuteri* 6798 combination yielded a trend toward reduced amounts of intestinal TNF-α when challenged with *E. coli* LPS (Fig. 8).

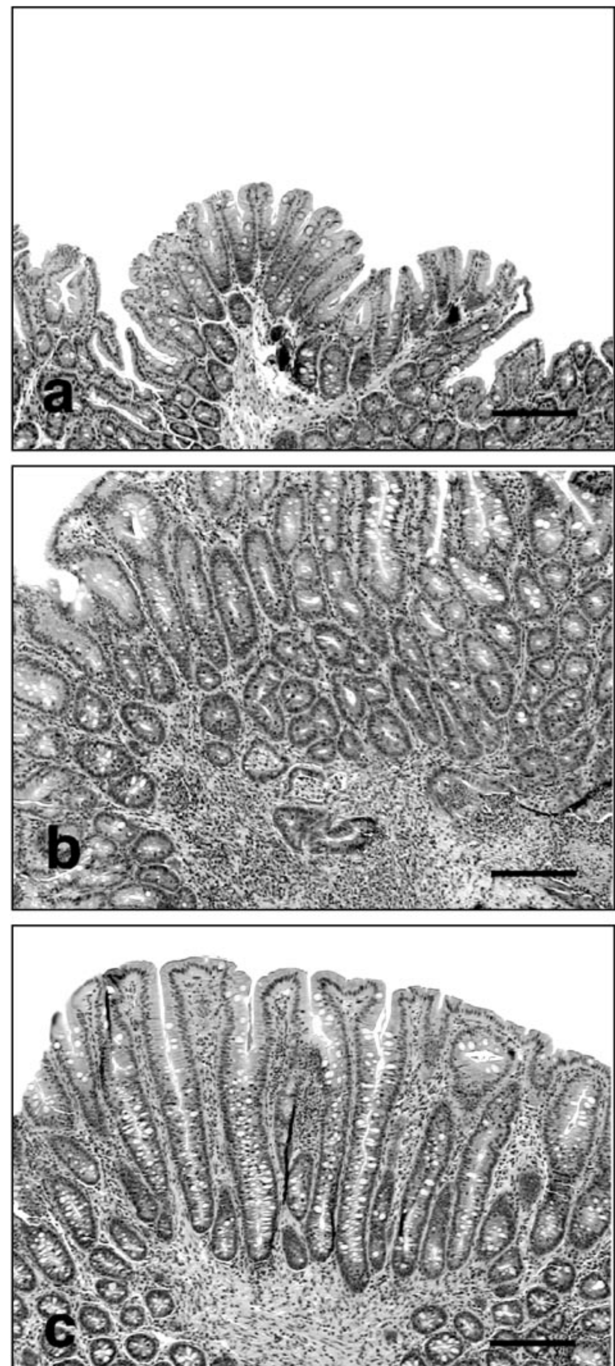

FIG. 4. *Lactobacillus* ameliorates typhlocolitis in *H. hepaticus*-infected IL-10-deficient mice. (a) Histopathology of cecocolic junction from an uninfected mouse demonstrates normal mucosal architecture. (b) Tissue from a mouse monoinfected with *H. hepaticus* exhibits moderately severe inflammation with reactive epithelial changes including goblet cell loss and crypt hyperplasia and dysplasia. (c) Tissue from a mouse administered a combination of *L. paracasei* 1602/*L. reuteri* 6798 prior to *H. hepaticus* infection exhibits a moderate reduction in intestinal inflammation and hyperplasia. Bar = 200 μm.

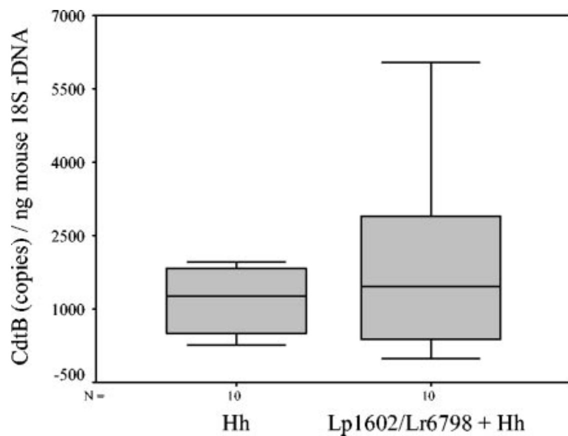

FIG. 5. Levels of *H. hepaticus* colonization in the ceca of IL-10-deficient mice. Cecal colonization by *H. hepaticus* (Hh) was evaluated by TaqMan probe-based real-time quantitative PCR, using the cytolethal distending toxin B-subunit gene (*cdtB*) as the species-specific DNA target. CdtB gene copy numbers were normalized by mouse DNA content, which was quantified by using murine 18S rRNA gene-based primers and probe. Quantitative results are expressed as copies of Hh *cdtB* per ng mouse 18S rRNA gene. Mice were infected with *H. hepaticus* (Hh) or precolonized with *L. paracasei* 1602/*L. reuteri* 6798 prior to *H. hepaticus* infection (Lp1602/Lr6798 + Hh). Data are presented as box plots with median (line inside box), interquartile range (shaded box), and range (error bars).

DISCUSSION

The IL-10$^{-/-}$ mouse is a well-established model for the study of colitis and IBD, including *Helicobacter*-induced disease (20). IL-10-deficient mice receiving lactic acid bacteria expressing recombinant murine IL-10 were protected from colitis (42), demonstrating the singular importance of this cytokine in regulating intestinal inflammation. Intestinal colonization with probiotic *Lactobacillus* clones, selected on the basis of TNF-α-inhibitory activity, diminished inflammation in IL-10-deficient mice infected with *H. hepaticus*. In the *L. paracasei*/*L. reuteri*-treated group, *Lactobacillus*-mediated protection was due to direct immunomodulatory activity and not reductions in the levels of *H. hepaticus*. The combination of *L. paracasei* and *L. reuteri* was associated with reductions in mucosal proinflammatory cytokines despite similar quantities of intestinal *H. hepaticus*. Interestingly, the anti-inflammatory effect was restricted to female mice in this model.

H. hepaticus induces IBD-like typhlocolitis in specific-pathogen-free IL-10-deficient mouse models (20). Chronic intestinal inflammation in this model has been presumably driven by a Th-1-predominant immune response. Elevated levels of mucosal TNF-α and nitric oxide are present in the intestines of these animals, and the colitis is associated with increased levels of IL-12 and IFN-γ (19, 20). In the present study, intestinal inflammation was partially eliminated by a combination of *L. paracasei* and *L. reuteri*. *L. paracasei*/*L. reuteri*-treated animals demonstrated a partial elimination of intestinal inflammation and significantly reduced mucosal IL-12 mRNA levels. The present findings support the role of intestinal IL-12 in mediating mucosal inflammation in this mouse colitis model independently of reductions of *H. hepaticus* quantities. Colitis in *H. hepaticus*-treated IL-10-deficient mice is dependent on mucosal IL-12 (20), and reduced intestinal inflammation was correlated with decreased mucosal IL-12 levels in *L. plantarum*-treated IL-10-deficient mice (38). Possibly, lactobacilli inhibit NF-κB activation in the intestinal mucosa, resulting in diminished expression of IL-12. The lack of an effect on intestinal IFN-γ, despite reductions in mucosal IL-12 mRNA, may be due to the absence of IL-10 in the intestine. IL-10 directly suppressed T- and NK cell-derived IFN-γ independently of TNF-α, IL-12, or IL-18 (37).

In this study, we selected *Lactobacillus* strains on the basis of macrophage TNF-α-inhibitory activity in vitro. Animal studies support the primary role of TNF-α in the pathogenesis of chronic colitis. Intraperitoneal injection of rat anti-mouse TNF-α monoclonal antibodies markedly reduced morbidity in IL-10-deficient mice with colitis (13). Murine macrophages represent important sources of mucosal TNF-α in murine colitis models. In vivo depletion of mucosal and lymphoid follicle-associated macrophages in mice deficient in IL-10 diminished colitis in these animals, suggesting that macrophages are important mediators of intestinal inflammation (44). Data obtained in this study indicate that the *L. paracasei*/*L. reuteri* combination may have reduced the mucosal TNF-α response to bacterial LPS. Although cell populations from the intestinal mucosa were not separated, both enterocytes and macrophages were likely affected in these explant studies. Colons from mice precolonized with *L. paracasei* 1602/*L. reuteri* 6798 yielded diminished responses to LPS stimulation. *L. reuteri* and *L. paracasei* may contribute to the development of LPS tolerance in intestinal epithelial cells or macrophages. LPS tolerance in macrophages includes hallmark molecular features, such as the up-regulation of NF-κB subunits p50 and p52 (22). Although induction of tolerance is a possibility, we have no evidence to support such a mechanism in this model.

Probiosis ultimately has a clonal basis and depends on the functional characterization of specific bacterial strains. Contrasting results have been obtained because investigators use a variety of different strains, cross species barriers, and work with different primary and cultured cell types. Some investigators have described the up-regulation of proinflammatory cytokine responses and NF-κB activation by intact lactobacilli (29–31). Cytokine-modulatory effects are strain dependent (3, 34) and vary with growth phase and cell preparation (29–31, 34). Recent data indicate that viable *L. reuteri* organisms were required for anti-inflammatory effects (25). *Lactobacillus* species differed in their abilities to modulate proinflammatory cytokine production in bone marrow-derived dendritic cells, and cytokine-inhibitory *L. reuteri* strains antagonized effects of cytokine-inducing *L. casei* clones (3). Interactions between cells in the intestinal mucosa are important, since leukocyte-sensitized intestinal epithelial cells (IECs) were differentially modulated by *Lactobacillus* clones, in contrast to human IECs cultured in the absence of leukocytes (15). The proinflammatory cytokines TNF-α and IL-1β were up-regulated in human IECs in the presence of *Lactobacillus sakei* but were not affected by *Lactobacillus johnsonii*. Furthermore, only *L. johnsonii* up-regulated the anti-inflammatory cytokine transforming growth factor β1 (15). In our experimental studies, anti-inflammatory effects in mouse models were obtained only with mouse-derived lactobacilli and raise the concern of using commensal bacteria derived from different species (including hu-

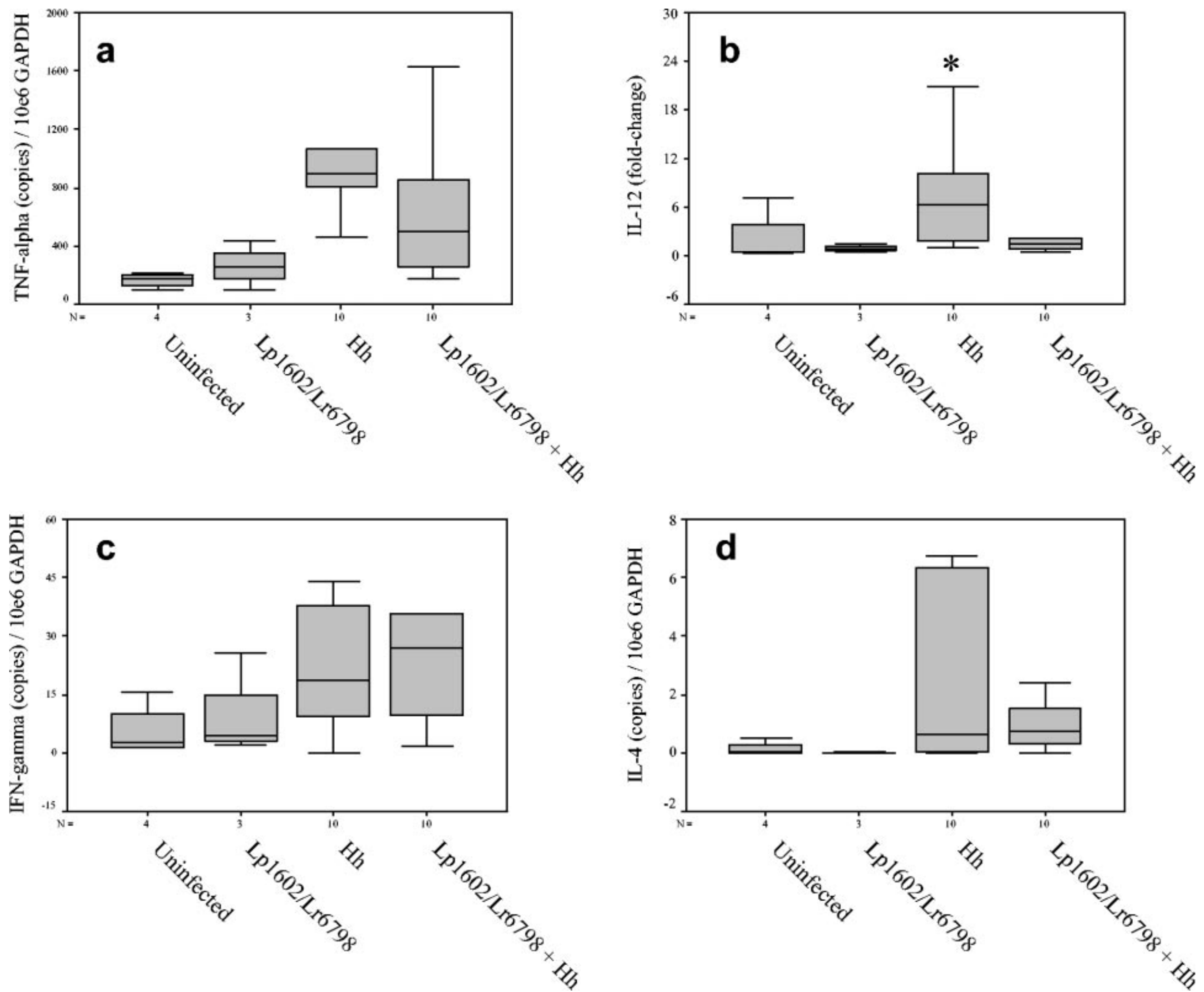

FIG. 6. Colonization with probiotic *Lactobacillus* affects levels of cytokine mRNA transcripts in the intestine. The mid-ceca of mice were homogenized, and total RNA was extracted. mRNA levels for (A) TNF-α, (B) IL-12 p40 subunit, (C) IFN-γ, and (D) IL-4 were quantitated by TaqMan probe-based real-time RT-PCR and normalized by mouse glyceraldehyde-3-phosphate dehydrogenase coamplification, using commercially available primer and probe sets for each target mRNA. IL-12 is presented as *n*-fold change from levels for uninfected control animals due to absence of a quantitation standard. Mice were uninfected (not colonized with probiotic lactobacilli), colonized with *L. paracasei* 1602/*L. reuteri* 6798 (Lp1602/Lr6798), infected with *H. hepaticus* (Hh), or precolonized with *L. paracasei* 1602/*L. reuteri* 6798 prior to *H. hepaticus* infection (Lp1602/Lr6798 + Hh). Data are presented as box plots with median (line inside box), interquartile range (shaded box), and range (error bars). *, mice monoinfected with *H. hepaticus* have IL-12 levels significantly greater than those for mice given *Lactobacillus* prior to *H. hepaticus* challenge ($P < 0.05$).

mans) in animal models. In our studies, IL-10-deficient mice precolonized with human-derived LGG developed more severe colitis after challenge with *H. hepaticus* (data not shown). In support of the potential importance of animal species origin for probiotic strategies, rat-derived *L. reuteri* was more effective than LGG at ameliorating intestinal inflammation in an acetic acid-induced rat colitis model (16).

Only female mice benefited significantly from the anti-inflammatory activity of probiotic *Lactobacillus* therapy in our model. Histologically, male and female IL-10-deficient mice challenged with *H. hepaticus* exhibited similar patterns of colitis (20), indicating that development of *H. hepaticus*-triggered colitis is not influenced by gender in C57BL mice. As expected, our present study shows that male and female *H. hepaticus*-infected C57BL mice yielded similar disease phenotypes in the absence of *Lactobacillus*. However, responsiveness to anti-inflammatory effects of probiotic bacteria appears to be affected by gender status in the present model. In contrast, A/JCr mice with an intact IL-10 gene demonstrated increased severity of colitis in females when challenged with *H. hepaticus* (23). Perhaps probiotic *Lactobacillus* strains modulate intestinal inflammation by an estrogen-dependent mechanism potentiated in an IL-10-deficient animal. Gender differences in immunologic responses have been well documented for humans and rodents (23, 43). Recent molecular studies of gender-specific responses point towards the role of sex hormones as possible determinants of immune responses and disease (23, 43). Specifically, estrogen has been implicated in T-cell-mediated autoimmune diseases (5). Of particular interest is that murine myeloid cells (dendritic cells or macrophages) lacking the estrogen receptor

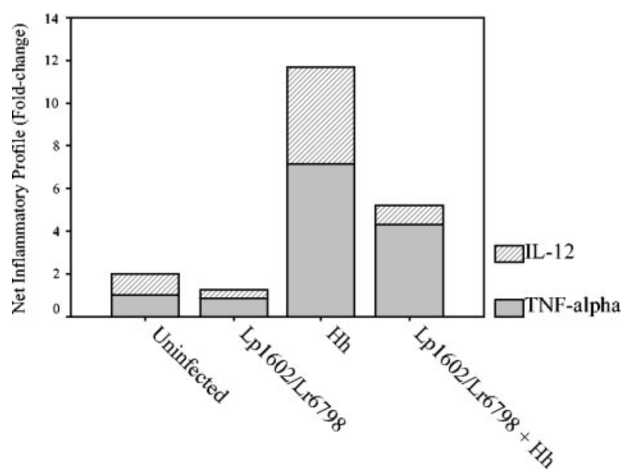

FIG. 7. Proinflammatory cytokine responses are modulated by probiotic lactobacilli in IL-10-deficient mice. Proinflammatory cytokine profiles were generated by using cecal levels of IL-12 and TNF-α mRNA transcripts. Mean quantities of IL-12 and TNF-α for all groups receiving exogenous bacteria were divided by means for uninfected mice. Bars represent *n*-fold change relative to results with uninfected sham-dosed controls. Note that the magnitudes of inflammatory profiles generated are proportional to histologic findings. Mice were uninfected (not colonized with probiotic lactobacilli), colonized with *L. paracasei* 1602/*L. reuteri* 6798 (Lp1602/Lr6798), infected with *H. hepaticus* (Hh), or precolonized with *L. paracasei* 1602/*L. reuteri* 6798 prior to *H. hepaticus* infection (Lp1602/Lr6798 + Hh).

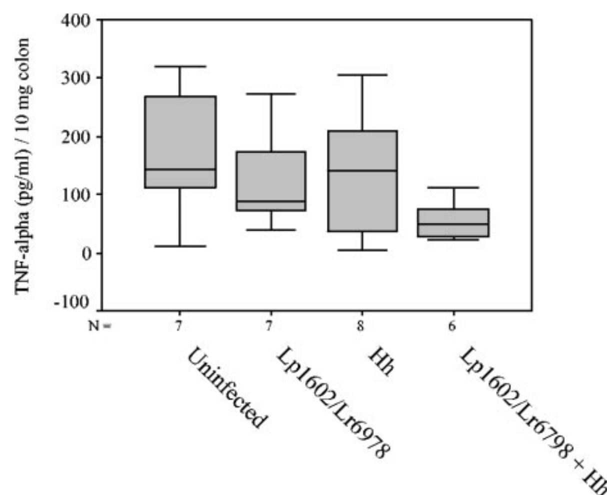

FIG. 8. Reduction of LPS-induced TNF-α production by colonic explants from mice treated with *Lactobacillus*. TNF-α output was corrected by the wet weight (measured at necropsy, prior to culture) and total DNA content of each explant. DNA content was determined by extraction of total explant DNA after culture and subsequent amplification of murine 18S rRNA gene by real-time quantitative PCR. TNF-α output in response to LPS stimulation is decreased in mice treated with *L. paracasei* 1602 and *L. reuteri* 6798 (Lp1602/Lr6798), indicating that Lp1602/Lr6798 may have direct anti-inflammatory effects. Mice were uninfected (not colonized with probiotic lactobacilli), colonized with *L. paracasei* 1602/*L. reuteri* 6798 (Lp1602/Lr6798), infected with *H. hepaticus* (Hh), or precolonized with *L. paracasei* 1602/*L. reuteri* 6798 prior to *H. hepaticus* infection (Lp1602/Lr6798 + Hh). Data corrected by weight are shown in this figure; data corrected by DNA (data not shown) were similar. Data are presented as box plots with median (line inside box), interquartile range (shaded box), and range (error bars).

alpha produce elevated TNF-α after challenge with *E. coli* LPS or *Mycobacterium avium* (21). Other studies revealed that estrogen receptor agonists suppressed inducible nitric oxide synthase and TNF-α production in LPS-stimulated macrophages (18, 45). Estrogen down-modulates monocyte chemoattractant protein-1 production by murine macrophages (7). The estrogen, 17-β estradiol, protects mice from experimental autoimmune encephalomyelitis in an estrogen receptor-dependent fashion (35). Differences in estrogen receptor subunit levels may partially account for the inability of *Lactobacillus*-derived immunomodulins to protect male mice from *H. hepaticus*-induced IBD. In data not shown, we also found that conditioned medium from probiotic lactobacilli decreased TNF-α production in LPS-stimulated primary peritoneal macrophages from female mice but not from male mice. Since probiosis appears to be a balancing act, with anti-inflammatory effectors (i.e., IL-10) competing with proinflammatory mediators (e.g., TNF-α and LPS), relative deficiencies of estrogen receptor alpha and circulating estrogens in male mice may tip the balance towards an inflammatory phenotype.

Probiosis may function by interference with enteric pathogens or proinflammatory components of the intestinal microbiota. *L. paracasei* 1602/*L. reuteri* 6798 may diminish inflammation by inhibition of *H. hepaticus* virulence gene expression or virulence factor activities without necessarily affecting quantities of intestinal *H. hepaticus*. *Lactobacillus gasseri* prevents gastritis by suppression of *Helicobacter pylori* type IV secretion system-dependent virulence factors, leading to decreased production of IL-8 by gastric epithelial cells (Y. Koga, personal communication). Commensal organisms may also enhance the production of antibacterial factors in the intestine. The commensal bacterium *Bacteroides thetaiotaomicron* induces the expression of bactericidal angiogenins (17) and prodefensin-processing matrilysin (24) by intestinal Paneth cells in the mouse. In our model, *L. paracasei* 1602/*L. reuteri* 6798 reduced mucosal IL-12 expression and LPS-stimulated colonic TNF-α production, possibly facilitating *H. hepaticus* colonization. Probiotics likely regulate inflammatory responses by direct interactions with the intestinal mucosa or antagonism of pathogenic or proinflammatory bacteria.

Data from the present study and prior studies suggest that probiotics exert their beneficial effects by one or a combination of mechanisms, including the following: (i) direct modulation of immune function (1, 2, 28, 34), (ii) competition with or exclusion of pathogen(s) (4), (iii) interference with virulence of enteric pathogens (36), or (iv) increasing epithelial/mucosal barrier function (26). The optimal application of probiotics for prevention or treatment of IBD and enteric infections may ultimately require the characterization of bacterial clones with specific probiotic properties. Individuals with enteric infections may benefit from probiotic strains with antagonistic properties towards a specific infectious agent. Patients with IBD may require probiotic strains with specific anti-inflammatory features that complement the immunomodulatory activities of other drugs or dietary changes in multicomponent treatment regimens. Additionally, the functional importance of host- or patient-specific characteristics (e.g., gender) should also be considered when designing clinical probiotic trials. The studies presented in this report stress the importance of rational se-

lection of probiotic bacterial clones for therapy of intestinal inflammatory diseases, including IBD.

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