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Preinoculation With the Probiotic *Lactobacillus acidophilus* Early in Life Effectively Inhibits Murine *Citrobacter rodentium* Colitis

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ABSTRACT

Enteropathogenic *Escherichia coli* (EPEC) is a common pathogen in infantile diarrhea, causing a characteristic histopathologic attaching and effacing (A/E) lesion in the intestinal mucosa. The mouse pathogen *Citrobacter rodentium* causes a similar A/E lesion in the murine intestine. Like EPEC, *C. rodentium* infection results in colonic crypt hyperplasia, goblet cell depletion, epithelial proliferation, and mucosal disruption. Using this murine model, we tested the hypothesis that preinoculation of murine gut with *Lactobacillus acidophilus* early in life can enhance host defense against enteric bacterial infection and attenuate bacteria-mediated colitis. Two-week old BALB/c mice were inoculated with *L. acidophilus* twice per week for 4 weeks before *C. rodentium* infection or concomitantly with the exposure to *C. rodentium* at 6–8 weeks of age. The probiotics were administered twice weekly thereafter. We observed that *L. acidophilus* inoculation in mice inhibits *C. rodentium*–induced colitis, which is associated with a decrease in *C. rodentium* colonization and translocation, an increase in its clearance, and a suppression of colonic myeloperoxidase (MPO) activity. Probiotic treatment also stimulates regulatory cytokine expression in the colon [transforming growth factor β (TGF-β), interleukin (IL)-10]. Preinoculation with *L. acidophilus* is more effective than concomitant use of probiotics in the induction of intestinal IgA secretion and in the downregulation of proinflammatory cytokine expression [tumor necrosis factor α (TNF-α), IL-6, and IL-12]. These observations suggest that inoculation with probiotics can effectively prevent bacteria-induced colitis by limiting enteric bacteria infection and promoting mucosal protective regulatory immune responses. This study may have ramifications for prevention of infectious diarrhea in human infants and children, particularly in developing countries.(***Pediatr Res* 58: 1185–1191, 2005**)

Abbreviations

MLN, mesenteric lymph node
MPO, myeloperoxidase
TGF-β, transforming growth factor β
TNF-α, tumor necrosis factor α

Infections caused by bacterial pathogens, such as enteropathogenic and enterohemorrhagic *E. coli* (EPEC and EHEC), represent a significant percentage of severe infantile diarrheas (1). EPEC and EHEC colonize the gastrointestinal mucosa by subverting intestinal epithelial cell function to produce a characteristic histopathological A/E lesion (2). The pathogenic features of EPEC or EHEC involve a disruption of the epithelial barrier and stimulation of a mucosal immune-mediated extensive inflammatory response. Similarly, the mouse enteric bacterial pathogen, *C. rodentium*, causes diarrhea, transmissible colonic hyperplasia, and colitis in mice as a consequence of its ability to colonize murine large intestinal enterocytes using the A/E lesion formation (3–5). *C. rodentium* is the only known murine A/E producing pathogen and therefore can serve as a small animal model for EPEC and EHEC infections.

Probiotics are live microorganisms that are ingested to promote beneficial effects on health by altering indigenous microflora. Probiotics have been used to prevent some intestinal pathogenic infections, such as *Shigella*, *Salmonella*, and enterohemorrhagic *E. coli* in murine models and enteropathogenic *E. coli* in a piglet model (6–9). It was also shown that probiotic treatment reduced *C. rodentium*–induced disease in adult mice (10). However, limited information is available regarding the effect of a probiotic inoculated during the preweaning stage on the subsequent host response to *C. rodentium* infection, which induces the intestinal pathologic alter-

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ations that are similar to those seen in many mouse models of colitis. Therefore, this model is an ideal model to study host-bacterial pathogen interactions *in vivo* (11) and can also be used to investigate the effect of probiotic inoculation on bacterial-induced intestinal diseases and on immune regulation of host responses to enteric infections.

The genus *Lactobacillus*, including at least 18 different species, has defined phenotypic and genotypic features in mice and humans (12–14). Probiotics have been considered a potentially important strategy to modulate inflammatory responses in the host gastrointestinal tract as they can enhance the host immune response and positively affect indigenous microflora in the host (15). *L. acidophilus* NCFM is a probiotic strain commercially available in the United States since the mid-1970s and supplemented in some conventional foods such as milk, yogurt, and toddler formula. This strain survives gastrointestinal tract transit in both healthy and diseased populations and is associated with the reduction in severity of pediatric diarrhea (16). We hypothesized that 1) preinoculation with *L. acidophilus* early in life (before weaning) in mice will establish a better protective defense against *C. rodentium* infection than that of concurrent administration and 2) that inoculation with *L. acidophilus* would be beneficial in the intestinal microbial ecosystem and in the establishment of host intestinal epithelial and mucosal immune responses, contributing to an enhanced protection and an attenuation of *Citrobacter*-mediated intestinal injury.

MATERIALS AND METHODS

Mice and probiotic bacteria inoculation. Six- to 8-week-old female and male BALB/c ByJ mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and bred in an animal facility at Massachusetts General Hospital. Neonatal mice were born to pregnant female Balb/c ByJ mice. Some mice were orally inoculated with *L. acidophilus* (Rhodia, Madison, WI) at 2 weeks of age for 4 weeks. All animal experiments were approved by the Institutional Animal Care and Use Committee. *L. acidophilus* were inoculated into deMan, Rogosa, and Sharpe broth (MRS; Difco, Detroit, MI) and grown at 37°C for 20 hours and then resuspended in PBS before being given orally to inoculate the mice (approximately 1×10^9 CFU).

C. rodentium infection and antigen preparation. Mice were orally inoculated with *C. rodentium* (strain DBS 100, from ATCC) at 6–8 weeks of age. Bacteria were grown overnight in Luria broth (LB) and resuspended in PBS before infecting the mice (approximately 5×10^8 CFU). *C. rodentium* antigen was prepared by collecting an overnight culture of *C. rodentium* in LB. The bacterial culture was washed in PBS and sonicated on ice. The homogenate was then centrifuged (14,000 rpm) at 4°C for 30 minutes. Supernatants were collected and the protein concentration was determined.

Experimental design. Randomly selected neonatal mice born to pregnant female Balb/c ByJ mice were fed with probiotic bacteria beginning at 2 weeks of age. Group A was preinoculated with *L. acidophilus* from 2 weeks on. Group B was preinoculated with *L. acidophilus* from 2 weeks of age and infected with *C. rodentium* at 6–8 weeks. Group C was concurrently inoculated with *L. acidophilus* and infected with *C. rodentium* on the same day at 6–8 weeks of age. Group D was infected with *C. rodentium* at 6–8 weeks of age without probiotic inoculation. After *C. rodentium* infection, *L. acidophilus* was administrated to groups A, B, and C twice per week throughout the experiment period. Group E consisted of normal controls. All mice were killed 7 or 14 d after *C. rodentium* infection. To assess the systemic effect of *C. rodentium* infection and probiotic inoculation, body weight and survival were measured throughout the experimental period.

Quantitation of C. rodentium and Lactobacillus acidophilus. To assess the clearance of *C. rodentium*, fecal pellets were collected from each mouse weekly. Fecal pellets were weighed, homogenized, serially diluted, and plated on selective MacConkey agar. To determine bacteria translocation, mice were killed at 7 and 14 d after *C. rodentium* infection. The MLN, spleen, and total segment of the colon were removed and homogenized in sterilized 1% Triton 100. An aliquot of the homogenate was cultured on MacConkey agar plates. Bacteria colonies were counted after overnight incubation. To assess the inoculating effect of the probiotic, fecal samples were also plated on Rogosa SL agar plates (Difco). After 72 hours of anaerobic incubation at 37°C, bacteria colonies were identified and quantitated (17,18).

Lymphocyte isolation. Mice were killed 2 weeks after *C. rodentium* infection. Lymphocyte suspensions were prepared from MLN and spleen as described previously (19). Cells (5×10^6 cells/mL) were cultured on 24-well plates in the presence or absence of *C. rodentium* antigen (50 μg/mL) or plate bound anti-CD3 MAb (10 μg/mL). Culture supernatants were collected 48 hours later and kept at −20°C for future measurement of cytokine production.

Measurement of IFN-γ and IL-10 production of MLN by ELISA. ELISA capture antibodies (R4-6A2, IFN-γ; JESS-2A5, IL-10) and biotinylated secondary antibodies (XMG1.2, IFN-γ; SXC-1, IL-10) were purchased from PharMingen (San Diego, CA). The biotinylated secondary antibodies were used as a second layer and reactions were visualized with *O*-phenylenediamine at 492 nm (OPD; Zymed Labs.). Standard curves were obtained using recombinant murine IFN-γ (Genzyme) and IL-10 (R&D Systems). OD values were converted to pg/mL for each cytokine by linear regression with Delta Soft II (Biometallics, Princeton, NJ).

Fecal IgA antibody assays. Fecal pellets were collected into a protease inhibitor cocktail and were then weighed, homogenized, and incubated at 4ºC for 1 hour. Supernatants were collected and stored at −80ºC. Immuno II plates were coated with goat anti-mouse IgA (2 μg/mL; Southern Biotechnology Associates, Birmingham, AL) or *C. rodentium* antigen (50 μg/mL) and incubated overnight at 4ºC. Two rows in each plate used was coated with a goat anti-mouse IgA (Southern Biotechnology Associates) and used to generate a standard curve by using a standard mouse IgA (Southern Biotechnology Associates). Total IgA and antigen-specific IgA were detected using HRP-conjugated anti-IgA as above. The reaction was developed with OPD (Zymed Labs) and read at 492 nm.

Histopathological examinations. At necropsy, colonic tissues were isolated and small fragments were then frozen in tissue Tek OCT compound (Miles Inc. Elkhart, IN) and stored at −80ºC. Some colonic fragments were snap frozen in liquid nitrogen and then stored at −80ºC for detection of cytokine expression and MPO activity. Five micrometer sections were cut on a 2800 Frigocut cryostat (Reichert-Jung, Germany) and stained with hematoxylin and eosin. Sections were analyzed without prior knowledge of treatment. Colonic pathology was scored according to a modified histologic scoring system previously published (20,21). The scoring system consists of two parts: Part 1 was the determination of inflammatory cell infiltration in the colon, scored from 0 to 4 (0: normal cells pattern; 1: scattered inflammatory cells in the lamina propria; 2: increased numbers of inflammatory cells in the lamina propria; 3: confluence of inflammatory cells extending into the submucosa; and 4: transmural extension of the infiltrative inflammatory cells). Part 2 was the evaluation of colon tissue damage which also ranged from 0 to 4 (0: normal tissue pattern; 1: discrete lymphoepithelial lesions; 2: mild colonic crypt hyperplasia ± focal invasion of epithelium; 3: obvious colonic crypt hyperplasia, invasion of the epithelium and goblet cells depletion; 4: excessive mucosal damage extending through deeper structures of the bowel wall). The total colon pathology score was the summation of the inflammatory cell score and the tissue damage score.

Detection of colonic cytokine expression. Cytokine mRNA expression in colonic tissues was evaluated by real-time polymerase chain reaction (PCR). Total cellular RNA was isolated from frozen colonic tissue (distal part of the colon) using TRIzol (GIBCO Life Technologies) according to the manufacturer's instructions. cDNA was synthesized and subjected to real-time PCR. Mouse IL-6, TNF-α, IL-12, IFN-γ, IL-10, TGF-β, and GAPDH real-time PCR probes, and primer pairs were purchased from Biosource International, Inc. (Camarillo, CA). Amplification of GAPDH was included for each experimental sample as an endogenous control. All experimental samples were amplified in duplicate.

MPO assay. MPO level was measured using a modified kinetic assay as described (22). Colonic segments were homogenized in 1% hexadecyl trimethylammonium in phosphate buffer to negate pseudoperoxidase activity. MPO activity was measured in supernatants following three cycles of sonication, freezing, thawing, and centrifuging at 13 200 rpm at 4°C for 15 minutes. The supernatant was mixed with potassium phosphate buffer containing 0.167 mg/mL *O*-dianiside dihydrochloride (Sigma Chemical Co.) and 0.0005% hydrogen peroxide. Activity was measured at 450 nm.

Statistic analysis. All the results are expressed as the mean ± SEM. N refers to the number of mice used. Statistical differences were determined using the two-tailed *t* test or one-way analysis of variance test with SPSS software (SigmaStat; SPSS, Chicago, IL); $p < 0.05$ was considered significant.

RESULTS

C. rodentium–induced colonic pathology in mice with and without probiotic inoculation. As expected, mice exposed to *C. rodentium* alone showed signs of disease early in the infection such as soft stools, a hunched posture, disturbed body hair, and body weight loss. In contrast, mice pretreated with *L. acidophilus* started at 2 weeks of age or given concurrently with *C. rodentium* developed less severe disease resulting in decreased body weight loss during the course of the experiment (data not shown) compared with mice infected with *C. rodentium*. Histologic examination of the distal part of the colon in mice infected with *C. rodentium* alone showed the typical pathologic changes associated with this bacterial infection including thickening of the wall of the colon, colonic crypt hyperplasia, an extensive inflammatory cellular infiltration and disruption of the epithelial surface (Fig. 1*D*). Histologic analysis of colonic tissue from mice treated concurrently (Fig. 1*C*) or preinoculated (Fig. 1*B*) showed a less severe pathology compared with *C. rodentium*–infected mice alone (Fig. 1*D*), including a mild colonic crypt elongation, less cellular infiltration of the colonic lamina propria, a mild uneven epithelial surface *versus* the control (Fig. 1*E*). Furthermore, the inflammation and intestinal damage scores (20,21) were significantly lower in preinoculated mice than those with *C. rodentium* infection alone (Fig. 1*G*) *versus* controls. In addition, a comparable colonic inflammation was also observed in the BLAB/c mice when preinoculated with a commensal *E. coli* twice weekly and then infected with *C. rodentium* compared with the mice infected with *C. rodentium* alone (Fig. 1*F*).

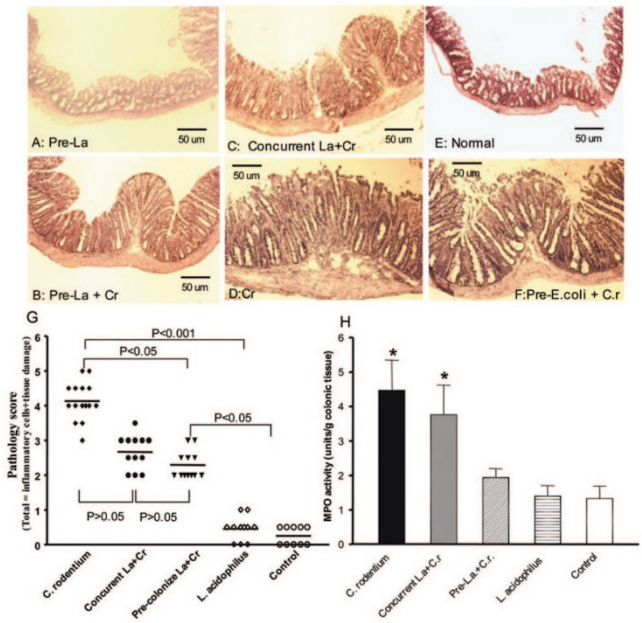

Figure 1. Representative histology of the distal colon 2 weeks after a *C. rodentium* infection. Colon tissue was prepared from the mice inoculated with *L. acidophilus* NCFM only (*A*) or preinoculated with *L. acidophilus* NCFM and then infected with *C. rodentium* (*B*, PreLa+Cr), concurrently administered with *L. acidophilus* NCFM and *C. rodentium* (*C*, concurrent La+Cr), *C. rodentium*–infected (*D*), untreated control (*E*), and *E. coli*-preinoculated and *C. rodentium*–infected (*E*) and stained with hematoxylin and eosin (magnification ×100). (*G*) The colonic pathology score of different groups of mice at 2 weeks after *C. rodentium* infection. The scores were assessed by determination of inflammation and tissue damage. The figures shown are measurements of individual mice pooled from three independent experiments. The horizontal line represents the mean score of different groups. Data from the colonic pathology scores was analyzed using one-way analysis of variance (nonparametric). (*H*) MPO activity was measured in the supernatants of homogenated colonic tissue. The data shown are the level of MPO activity per gram of colonic tissue at 2 weeks after *C. rodentium* infection. The data are represented of the mean ± SEM and statistical significant differences compared with healthy control group: $^*p < 0.05$ ($n = 10$–15).

MPO activity in the intestine can be used to quantitate inflammation (22,23). In this study, the impact of pre- and concurrent inoculation with probiotics on colonic inflammation was examined by measuring MPO activity of colonic tissues. Our results showed that mice infected with *C. rodentium* (pooled $n = 15$) and mice concurrently inoculated with probiotic ($n = 18$) had significantly higher MPO levels compared with healthy control mice ($n = 10$), suggesting that *C. rodentium* infection induces the infiltration of inflammatory cells into the colon and that concurrent inoculation with the probiotics at the adult stage has little or no effect on colonic MPO activity. In contrast, mice that were preinoculated with *L. acidophilus* early in life showed significantly decreased levels of MPO in the colon, suggesting an effect of probiotic in attenuating the bacteria-mediated inflammatory response in the colon. (Fig. 1*H*). Taken together, our results indicated that at an adult stage, a concurrent inoculation of probiotics and *C. rodentium* was unable to reduce MPO activity in the colon, whereas probiotic inoculation at an early age resulted in a reduced MPO activity in the intestine, contributing to the attenuation of the bacteria-induced intestinal inflammation.

Probiotics inhibit colonization, proliferation, and translocation of C. rodentium. We then determined whether *L. acidophilus* pre- and concurrent inoculation affected colonization, proliferation, and clearance of *C. rodentium*. At 1 and 2 weeks after *C. rodentium* infection, fecal pellets were collected, homogenized, and plated onto selective MacConkey agar plates. Our results showed that bacterial counts were lower in *L. acidophilus*–preinoculated mice (Fig. 2*A*) at an early stage of infection (1 week post-infection). A significantly lower level of bacteria shed in feces was also detected in *L. acidophilus*–preinoculated mice by the second week after infection (Fig. 2*B*), suggesting that the probiotic treatment reduced proliferation of *C. rodentium*. The intermediate levels of bacteria recovery from mice that were concurrently inoculated with *L. acidophilus* and *C. rodentium* at both time points (Fig. 2*A*, *B*) indicated that concurrent inoculation with the probiotic was less effective in altering establishment and persistence of *C. rodentium* infection in mice than preinoculation of *L. acidophilus*. An effective colonization by *L. acidophilus* was maintained by twice- weekly inoculation and confirmed by a series of stable bacterial counts on Rogosa SL agar plates (data not shown).

We next determined whether probiotic pre- and concurrent inoculation would have a beneficial effect on epithelial barrier function by examining *C. rodentium* mucosal transmigration in probiotic-treated infected and control mice. One and 2 weeks following *C. rodentium* infection, at the peak of inflammation, we killed mice and collected MLN, spleen, and total colonic segments from each mouse to assess bacterial load. We found

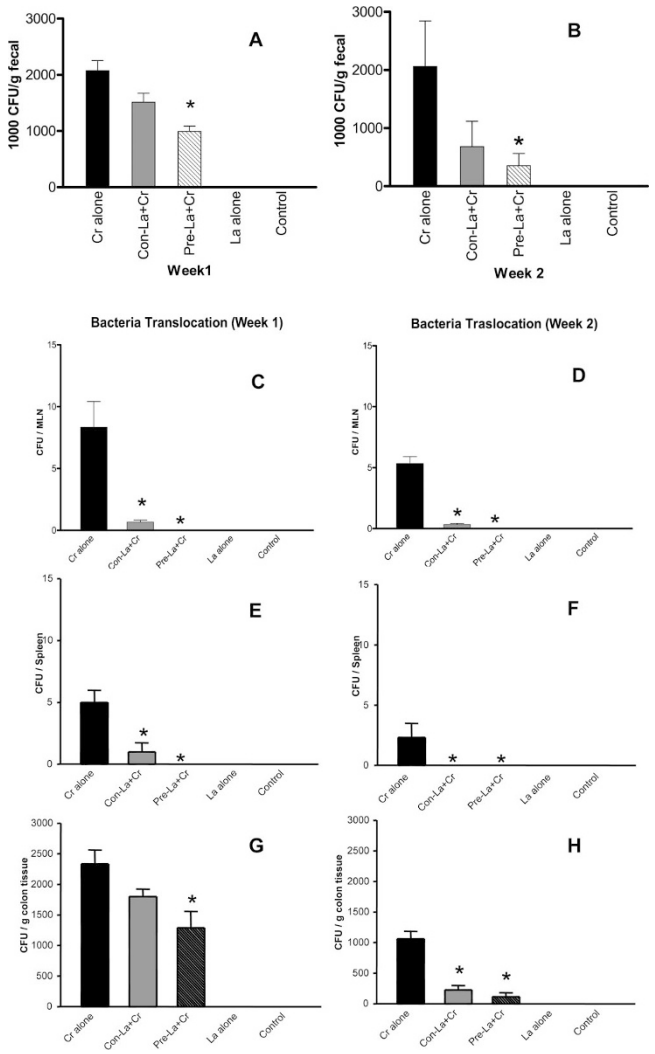

Figure 2. Analysis of fecal *C. rodentium* counts as determined by a selective MacConkey agar plate. The data shown are the number of *C. rodentium* recovered per gram of fecal samples from mice of different groups at 1 week (*A*) and 2 weeks (*B*) after *C. rodentium* infection. The data are represented as the mean ± SEM (n = 10–16 at each time point). Viable *C. rodentium* recovered in the MLN (*C*, *D*) spleen (*E*, *F*) and colonic tissue (*G*, *H*) at 1 and 2 weeks after *C. rodentium* infection. At necropsy, the MLN, spleen, and colonic tissue were removed and homogenized. An aliquot of diluted homogenized tissue was plated on selective MacConkey agar plates and incubated overnight. Viable bacteria were counted. The data are represented as the mean ± SEM (n = 5–10 at each time point) and statistical significant differences compared with the *C. rodentium*–alone group: $^*p < 0.05$.

that there were significantly fewer viable *C. rodentium* bacteria recovered from the MLN (Fig. 2*C*) and spleen (Fig. 2*E*) of both *L. acidophilus* pre- and concurrently inoculated mice 1 week after infection compared with mice infected with *C. rodentium* alone. At 2 weeks after *C. rodentium* infection, the number of *C. rodentium* bacteria recovered from MLN (Fig. 2*D*) and spleen (Fig. 2*F*) of mice infected with *C. rodentium* alone was similar to that detected the first week, whereas *L. acidophilus* inoculated mice (both pre- and concurrently) had significantly lower (or no) counts. These results suggest that pre- and concomitant treatment with probiotics may enhance colonic epithelial barrier function, resulting in a decrease in the entry of luminal bacteria across the colonic mucosa. There were also fewer viable *C. rodentium* bacteria recovered from the total colonic segments of mice both pre- and concomitantly treated with *Lactobacillus* 1 week (Fig. 2*G*) and 2 weeks (Fig. 2*H*) after infection compared with those of mice infected with *C. rodentium* alone. This suggests that probiotic treatment in mice inhibits *C. rodentium* colonization and promotes the clearance of *C. rodentium* from mouse intestine.

Probiotic pre- but not concurrent inoculation enhances IgA secretion in the intestinal lumen Our examination of the impact of probiotics on the host's intestinal IgA response showed that mice with *L. acidophilus* preinoculation alone (60 ± 2.0 μg/mL) and *L. acidophilus* preinoculation plus *C. rodentium* infection (63 ± 3.5 μg/mL) had higher total IgA levels compared with the other three groups (range, 20–41 μg/mL). Mice preinoculated with *L. acidophilus* early in life had significantly higher levels (8.2 ± 0.2 μg/mL, $p < 0.05$) of anti-*C. rodentium* specific IgA than mice with *C. rodentium* infection alone (4.1 ± 0.1 μg/mL) or *C. rodentium* plus concomitant probiotic administration (4.7 ± 0.2 μg/mL).

Probiotic pre- and concurrent inoculation alters cytokine responses in the intestinal mucosa. To examine the influence of *L. acidophilus* treatment on the pathogen-induced cytokine response in intestinal mucosa, we examined antigen-specific cytokine production in MLN. We found that both pre- and concomitant inoculation with *Lactobacillus* enhanced the *C. rodentium* antigen-specific IFN-γ (Fig. 3*A*) and IL-10 (Fig. 3*B*) responses and that preinoculation showed a more significant impact than concurrent treatment on the response of these cytokines.

To further examine the contribution of probiotic inoculation on the modulation of the local cytokine response in the intestine, we examined the expression of both proinflammatory and immune regulatory cytokines in the colon by real-time PCR. Our results show that *C. rodentium* infection induces proinflammatory cytokine (TNF-α and IL-6) expression (Fig. 4*A,B*), and that administration of *L. acidophilus* either alone or concurrently with *C. rodentium* results in a downregulation of the colonic TNF-α and IL-6 response (Fig. 4*A,B*). We have observed that preinoculation with *L. acidophilus* results in an enhanced colonic IFN-γ expression (Fig. 4*C*) and that concurrent inoculation with *L. acidophilus* reduces IFN-γ expression in colonic tissue. Our results also showed a significant increase in colonic IL-12 expression in *C. rodentium*–infected mice and

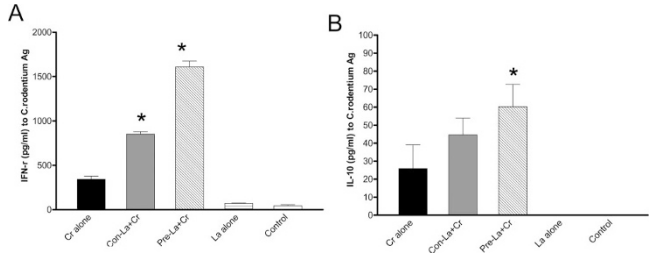

Figure 3. IFN- γ (*A*) and IL-10 (*B*) production by MLN cells from *C. rodentium*–infected and probiotic treated groups at 2 weeks after *C. rodentium* infection. MLN cells were collected and restimulated with *C. rodentium* antigen (50 μg /mL) for 48 hours. Cytokine secreted into culture supernatants was measured by ELISA. The results are expressed as the mean ± SEM and are representative of three independent experiments. $^*p < 0.05$, n = 5 mice.

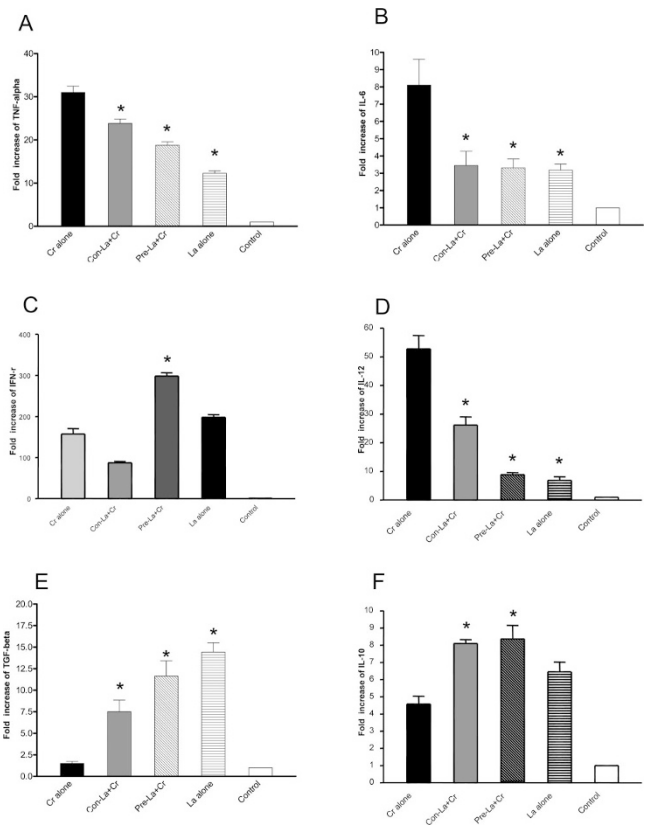

Figure 4. Cytokine mRNA expression in colonic tissue was measured by real-time PCR at 2 weeks after *C. rodentium* infection. Values are the mean fold increase compared with baseline obtained from control animals. (*A*) TNF-α, (*B*) IL-6, (*C*) IFN-γ, (*D*). IL-12, (*E*) TGF-β, and (*F*) IL-10. The data shown are from one of two experiments performed, showing similar results. Statistical significant differences compared with the *C. rodentium*–alone group: $*p < 0.05$, $n = 5$ mice per group.

that probiotic pre- and concomitant inoculation significantly reduced the *C. rodentium*–associated colonic IL-12 response (Fig. 4*D*).

Immune regulatory cytokines, such as IL-10 and TGF-β, are known to play a role in suppression of intestinal inflammation. To determine whether pre- and concurrent inoculation with probiotics could alter regulatory immune responses in the intestinal mucosa, we compared mRNA expression levels of TGF-β1 and IL-10 among different groups. The results presented in Fig. 4*E* demonstrate that probiotic treatment (both pre- and concurrent) significantly upregulates colonic TGF-β1 expression, which is absent in the colon of the mice infected with *C. rodentium* alone. Both probiotic pre- and concomitant inoculated mice showed an enhanced colonic IL-10 expression (Fig. 4*F*) compared with mice infected with *C. rodentium* alone. Taken together, our results suggest a role for probiotics in immune regulation during enteric bacterial infection.

DISCUSSION

Previous studies have demonstrated inhibitory effects of probiotic use on pathogenic bacteria in the intestinal tract. Most of these studies have shown that short-term probiotic administration at the time of infection can shorten and/or reduce the severity of the resultant disease course. Although it was also reported previously that administration of *L. acidophilus* attenuated *C. rodentium*–induced disease in adult mice (10), few studies have addressed the potential preventive effect of probiotics used during the preweaning stage on subsequent exposure to intestinal pathogens. In this study, we tested the hypothesis that preinoculation of murine gut with *L. acidophilus* early in life (*i.e.* during the time of immaturity of the intestinal immune system) will have profound consequences in host defense against subsequent enteric bacterial infection later in life.

Probiotics have been shown to be effective in inhibiting intestinal bacterial infection (6–9) and intestinal inflammation, such as dextran sulfate sodium- and trinitrobenzene sulfonic acid–induced colitis (24–27) in animal models. Multiple mechanisms by which probiotics enhance protection against pathogens have been suggested including stimulation of pathogen-specific immunity, competition for limited nutrients, inhibition of pathogen mucosal adherence, and invasion and production of antimicrobial substances (28). Our data suggested that inoculation with *L. acidophilus* NCFM could effectively inhibit *C. rodentium*–associated colitis. The protection activated by probiotic use in *C. rodentium*–infected mice correlates with reduced bacterial loads in the gut, enhanced mucosal immune responses evidenced by an increase in mucosal IFN-γ production and IgA secretion, and a downregulation of the proinflammatory cytokine response. Moreover, our results provide evidence suggesting that probiotic colonization induces an immune regulatory response, which may also be involved in suppression of bacteria-induced tissue injury. In an animal study in which a fermented mixture containing *L. acidophilus* (10^5 cells/mL) was fed to mice with *E. coli*–induced diarrhea, this measure helped to decrease the diarrhea (29). In a bovine model in which probiotic-treated calves were either given *E. coli* O157:H7 or *E. coli* O111: NM, fecal shedding was reduced compared with that for untreated infected calves (30). Shu *et al.* (31) also found that in a piglet model dietary treatment using probiotics could reduce the severity of weaning diarrhea associated with rotavirus and *E. coli* and was thought to be due to an enhanced immune-mediated protection.

The major finding of our study was that preinoculation with *L. acidophilus* NCFM significantly altered the dynamics of *C. rodentium* infection in the colon. In general, *C. rodentium* resides primarily in an extracellular location on the epithelial surface (32) and in the lamina propria or edematous submucosa (33). Our results show that inoculation with *L. acidophilus*, particularly preinoculation, in mice reduced *C. rodentium* infection, inhibited its proliferation, and facilitated its clearance. Such an impact of probiotics on *C. rodentium* infection is found to be more pronounced in mice with preinoculation of *L. acidophilus*, indicating a better protection than concurrent administration of the probiotic. Moreover, these preinoculated mice also prevented local or systemic spread of infection as evidenced by a decrease in bacterial translocation (*e.g.* no bacteria found in the MLN and spleen). This agrees with a previous study showing that feeding live *Lactobacillus plantarum* or *Lactococcus lactis* inhibited translocation of an administered bacteria in healthy as well as in TNBS-treated mice (26). Thus, our study and those discussed above demonstrate

that probiotics can enhance host defense by decreasing pathogenic bacterial loads, which acts to further protect the mucosal from invasion.

The inoculation with probiotics has been shown to increase the antibody response to gut pathogens (34,35). Our data show that mice inoculated with *L. acidophilus* produced significantly more total and antigen-specific IgA, which was accompanied by a less severe *C. rodentium*–induced colitis. This observation is in line with previous studies showing a significantly higher level of intestinal antibacterial IgA responses (36) among probiotic-fed mice and enhanced specific IgA to bacterial toxin in mice fed with yogurt-containing probiotics compared with controls (37). In an animal study, mice fed bifidobacteria for 12 d showed significantly higher levels of fecal total IgA compared with that of the control group (38). The above results suggest that probiotics can enhance local production of IgA in the intestine. However, the role of antibody production in host defense against *C. rodentium* infection is still inconclusive. Using various types of knockout mice, a recent study evaluated the importance of secreted antibodies (IgA, IgM, and IgG) and B cells in host defense against *C. rodentium* infection and found that IgG, but not IgA or IgM, antibodies are important in host protection against *C. rodentium* infection (39).

In this study we observed that *L. acidophilus* inoculation can activate colonic regulatory cytokine responses. The capacity of *L. acidophilus* to regulate intestinal cytokine responses is demonstrated by the results from the real-time PCR experiments showing that probiotic inoculation induces an upregulation of regulatory/anti-inflammatory cytokines and a downregulation of proinflammatory cytokine expression in the colon. These results may suggest the contribution of probiotic-induced immune regulatory responses in the reduction of *C. rodentium*–mediated colitis. IL-10 and TGF-β are regulatory cytokines produced by specialized subsets of T helper cells, which have anti-inflammatory activities. IL-10 might also have an indirect anti-inflammatory effect because it also stimulates together with TGF-β IgA synthesis (40). The probiotic *Lactobacillus paracasei* has been shown to maintain IL-10 and induce TGF-β secretion by CD4+ T cells (41). An *in vitro* study has demonstrated that the incubation of the probiotic *L. plantarum* with mononuclear cells isolated from actively inflamed colon results in a significant increase of IL-10 (42). Our results were further supported by a recent study that showed that probiotic treatment was able to ameliorate the severity of TNBS-induced colitis by inducing an immune-regulatory response involving TGF-β regulatory cells (43). In this study we also observed that infection with *C. rodentium* resulted in a reduction in a *L. acidophilus*–induced TGF-β response. Although the mechanism by which *C. rodentium* modifies host cytokine responses is still unknown, it may be possible that *C. rodentium* or its products have the capacity to inhibit certain host cytokine responses as suggested by a previous study (43).

Our results also show that *L. acidophilus* inoculation leads to a downregulation of the proinflammatory cytokine TNF-α and IL-6 mRNA expression as well as IL-12 mRNA levels in the colon. In a previous study, TNF-α levels and expression of their mRNA were decreased in mice treated with a *Bifidobacterium longum* HY8001 culture supernatant (44). It has been reported that *Lactobacillus* species can generate soluble molecules that inhibit TNF-α production in activated macrophages (45). Dendritic cells were unresponsive to *L. plantarum* and *Bifidobacterium adolescentis* but produced large amounts of IL-12p70, TNF-α, and IL-6 in response to *E. coli* (46). Our results also show the complexity of intestinal cytokine regulation by probiotics as evident by the opposite trend in the colonic IFN-γ response with an upregulation in pretreated and downregulation in concurrently inoculated mice. Although the Th1 response has been suggested to play a protective role in *C. rodentium* infection (47), the implications and mechanisms for the differentially altered colonic IFN-γ response observed in a current study are unclear and need to be further studied. Taken together, our results suggest that the probiotic *L. acidophilus* may have a role in controlling local inflammation by modulating the cytokine environment in the infected intestine.

In summary, our investigation of a prophylactic approach for bacteria-induced colitis in mice involving inoculation with *L. acidophilus* has shown that probiotic inoculation results in an attenuation of *C. rodentium*–mediated colonic pathology. Our results also showed that long-term preinoculation early in life had a better protective effect than concurrent administration of probiotics as evidenced by a more pronounced inhibitory effect on pathogenic bacterial proliferation and proinflammatory cytokine expression and by a more effective stimulatory effect on protective and regulatory immune responses to the enteric bacterial pathogen *C. rodentium*. These results may have medical implications in the prophylaxis and management of bacteria-induced diarrhea and intestinal inflammation in children and even adults at risk.

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