The Probiotic Lactobacillus Prevents Citrobacter rodentium-Induced Murine Colitis in a TLR2-Dependent Manner

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Introduction

Citrobacter rodentium, formerly Citrobacter freundii biotype 4280, is a gram-negative naturally occurring murine pathogen that causes colitis and transmissible colonic epithelial cell hyperplasia, and disrupts the mucosa in the descending colon [30]. C. rodentium infection in most adult mice is self-limiting. In contrast, young mice infected by C. rodentium develop diarrhea, retarded growth, and rectal prolapse, and in extreme cases, they exhibit significant mortality [1, 19]. Infection of young mice with C. rodentium induces a T-cell response similar to that seen in inflammatory bowel disease (IBD), a large infiltrate of CD4+ cells into the colonic lamina propria, and a highly polarized Th1 response. C. rodentium infection promotes the Th1 immune response, which is characterized by increased tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), and interleukin-12 (IL-12) mRNA expression in colonic tissue of young mice [12, 27].

C. rodentium is the only known murine model of an attaching and effacing enteropathogen that mimics changes observed in humans with colitis caused by Crohn’s disease [14]. Murine models are also used to study enteropathogenic Escherichia coli (EPEC) and enterohemorrhagic E. coli (EHEC), which pose an important threat to animal and human health worldwide [4, 11, 24]. Both EPEC and EHEC are poorly pathogenic in mice, whereas C. rodentium colonizes the colonic mucosa by attaching and effacing lesion formation [23].

Probiotic therapies have been clinically evaluated and used to treat human gastrointestinal disease, IBD, and their complications, including pouchitis [29, 33]. IBD symptoms are treated successfully by specific strains of probiotics, particularly Lactobacillus rhamnosus GG (LGG) [8, 21]. LGG...
is a short, gram-positive facultative anaerobic rod that often appears in chains. There have been preliminary studies of its probiotic use in the prevention of C. rodentium-induced colitis. In the C. rodentium-induced colitis murine model, LGG, or other Lactobacillus species, improved enteric lesions and decreased endotoxemia, weight loss, and mortality [14, 27].

Toll-like receptors (TLRs) play a central role in the initiation of the innate immune response. TLRs are activated by specific microbial ligands. For example, TLR2 recognizes a variety of microbial components, such as the gram-positive bacterial wall, whereas TLR4 recognizes the gram-negative bacterial wall. Activated TLRs lead to an association with Toll-interleukin-1-related domain-containing myeloid differentiation factor 88, mediating a signaling cascade that activates the NF-κB transcription factor [9, 31]. This activation results in the increase of pro-inflammatory cytokines and chemokines, responses that are required to protect against many types of bacterial pathogens. Defects in these innate immune responses can prevent clearance of the invading bacteria and activation of pathogenic T cells, and cause tissue damage, as observed during IBD and C. rodentium-induced colitis [9, 28]. TLR activation by commensal bacteria also plays an essential role in intestinal epithelial homeostasis. Recent studies have shown that TLR2, TLR4, and gut microbial flora play important roles in the process of ulcerative colitis [6, 25]. Probiotic administration modulates the inflammatory cytokine profile through TLR stimulation, mostly in cells involved in the innate immune response. For example, Lactobacillus probiotic administration to mice increases the expression of TLR2, TLR4, and TLR9 [5]. TLR2 and TLR4 monoclonal antibodies (mAb) can suppress the development of dextran sodium sulfate-induced colitis. In addition, Lactobacillus protects the intestinal epithelium in a TLR2/cyclo-oxygenase-2-dependent manner [2, 6].

The mechanism by which pretreatment with LGG results in a decreased response to C. rodentium colitis in mice is still unclear. We investigated the role of TLR2, TLR4, and pretreatment with LGG in C. rodentium-induced murine colitis in this study. In addition, we examined the effects of TLR2 and TLR4 deficiency on the protective effect of LGG against C. rodentium-induced colitis.

Materials and Methods

Animals

Procedures were approved by the Institutional Animal Care and Use Committee (KU15070). All mice were 4 weeks old at the time of infection. Wild-type C57BL/6 (B6) mice were obtained from the Korea Research Institute of Bioscience and Biotechnology (Korea). Isogenic TLR2 KO and TLR4 KO mice were purchased from Oriental Bio-Service Inc. (Japan). The genotypes of TLR2 KO and TLR4 KO mice were confirmed by polymerase chain reaction (PCR) amplification of tail DNA.

Experimental Design and Bacterial Strains

Lactobacillus rhamnosus GG (LGG, KCTC 5033) was first cultivated on de Man, Rogosa, and Sharpe (MRS) agar (Difco) at 37°C for 48 h, inoculated in MRS broth, and grown for 24 h with shaking. Mice were pretreated with 0.2 ml of PBS containing bacteria at a density of $2 \times 10^{9}$ colony forming units (CFU) by orogastric gavage. C. rodentium (strain DBS 100; ATCC 51459) was grown on Luria-Bertani (LB) agar plates for 24 h at 37°C, cultured in LB broth for 24 h at 37°C, and spun at 1,200 ×g for 10 min. Pelleted bacteria were resuspended in fresh LB broth and mice were infected by oral gavage using 0.2 ml of LB broth containing $2.5 \times 10^{9}$ CFU.

At 21 days of age, 63 female TLR2 KO, TLR4 KO, and B6 mice were divided into three groups per each strain. Each group had an uninfected control group ($n = 8$), C. rodentium-infected group ($n = 8$), and LGG-pretreated C. rodentium-infected group ($n = 8$). The mice injected only with PBS by orogastric gavage were used as uninfected controls for each strain. The C. rodentium-infected group was pretreated with sterile PBS for 7 days and infected with C. rodentium by orogastric gavage. The LGG-pretreated C. rodentium-infected group was pretreated with $2 \times 10^{8}$ CFU LGG for 7 days and infected with C. rodentium by orogastric gavage. Body weight and survival were monitored daily in a consecutive order. Mice were sacrificed 10 days after the infection and the severity of the disease was assessed.

Gross Examination

The spleen was removed and weighed. The entire large intestine from cecum to anus was excised and the length of the colon was measured from the ileocaecal junction to the anus.

Histopathological Evaluation of Colitis

Mid and distal colon tissues were immersed in 10% buffered formalin and embedded in paraffin. The paraffin blocks were sectioned into 4-µm-thick slices and hematoxylin and eosin (H&E) staining was performed. Grading of intestinal inflammation was determined as previously described [26], with some modifications (Table 1). Selected sections were scored independently by two blinded investigators.

Real-Time PCR (RT-PCR)

Total RNA was prepared from frozen spleen tissue using TRizol reagent (Ambion) according to the manufacturer’s instructions. The TaqMan fluorogenic probes and PCR primers for TNF-α, monocyte chemotactic protein 1 (MCP-1), IFN-γ, β-actin, and GAPDH were designed by Metabion (Martinsried, The Netherlands). Primers
Table 1. Histological scoring to quantify the degree of colitis.

<table>
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<tr>
<th>Criterion</th>
<th>Score</th>
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<tbody>
<tr>
<td>Loss of goblet cells</td>
<td>None</td>
<td>Mild/focal</td>
<td>Moderate</td>
<td>Severe</td>
</tr>
<tr>
<td>Mucosa thickening</td>
<td>None</td>
<td>Mild</td>
<td>Moderate</td>
<td>Severe</td>
</tr>
<tr>
<td>Inflammatory cells in mucosa</td>
<td>None</td>
<td>Mild/focal</td>
<td>Moderate</td>
<td>Severe</td>
</tr>
<tr>
<td>Inflammatory cells in submucosa</td>
<td>None</td>
<td>A few</td>
<td>Moderate</td>
<td>Severe</td>
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Table 2. PCR primers used in this study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequences</th>
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<tr>
<td>GAPDH</td>
<td>5'-TCC AAG GAG TAA GAA ACC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GGA AAT TGT GAG GGA GAT-3'</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5'-AGC CCT CCT TCT TGG GTA-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CAC TIT CGG TGC ACG ATG GA-3'</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5'-CCA TCC TTG TCC CAG TCT CT-3'</td>
</tr>
<tr>
<td></td>
<td>5'-ATG AAC GCT ACA CAC TGC AT</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5'-GTC TCA GCC TCT TCT GAT-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GCC ATT TGG GAA CTT CTC AT</td>
</tr>
<tr>
<td>MCP-1</td>
<td>5'-CAA CAA CTT CCT CTC CTG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-AAG GGC TTC AAT ATT GTG TTG-3'</td>
</tr>
</tbody>
</table>

used in this study are listed in Table 2. The relative changes in TNF-α, MCP-1, and IFN-γ mRNA levels were normalized to GAPDH and β-actin in the same samples.

Statistical Analysis

Data were analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA).

A two-tailed Student’s t-test was applied for comparison of two individual data. Survival analysis was determined by log-rank test. Data are expressed as the mean ± standard deviation (SD), and p < 0.05 was considered to be statistically significant.

Results

Pretreatment with LGG Ameliorates Growth Rate in C. rodentium-Infected B6 Mice

Body weight gain was significantly lower in C. rodentium-infected B6 mice than in uninfected or LGG-pretreated B6 mice. Pretreatment with LGG is effective in preventing both weight loss and death in B6 mice.

Murine colitis was induced by C. rodentium through orogastric administration as described in Materials and Methods. (A) The change in body weight of each group was determined by comparing the daily weight before and after the C. rodentium infection (n = 5 or 8 per group). *p < 0.05, **p < 0.01 vs. Control, †p < 0.05, ††p < 0.01 vs. C. rodentium-infected mice. (B) Mice survival was monitored daily for 10 days.
mice. Furthermore, pretreatment with LGG ameliorated the growth rate in *C. rodentium*-infected B6 mice. No body weight loss was recorded during infection in LGG-pretreated B6 mice, whereas weight recovery in *C. rodentium*-infected B6 mice did not start until the third day post infection. Body weight gain was significantly lower in *C. rodentium*-infected TLR2 KO and TLR4 KO mice compared with uninfected controls. Pretreatment with LGG did not contribute to growth rate recovery in either TLR2 KO or TLR4 KO mice. There were no significant differences in weight changes of LGG-pretreated TLR2 KO and TLR4 KO mice compared with non-pretreated animals (Fig. 1A).

**Pretreatment with LGG Increases Survival Rate in *C. rodentium*-Infected B6 Mice**

LGG-pretreated B6 mice displayed no mortality over the course of 10 days of infection with *C. rodentium* (*p* < 0.0291, log-rank test). In contrast, 10%, 20%, and 40% of infected non-pretreated B6 mice died after 2, 7, and 9 days, respectively (Fig. 1B). There were no significant differences between the survival rates of *C. rodentium*-infected TLR2 KO and TLR4 KO mice, regardless of pretreatment with LGG. Mortality correlated with loss of body weight in infected animals.

**Pretreatment with LGG Ameliorates Injury in the Colon during *C. rodentium* Infection in B6 Mice**

The length of the colon was measured because *C. rodentium*-induced colitis typically causes colon shortening in mice [6]. As shown in Fig. 2A, the colon in *C. rodentium*-infected mice decreased in length and two mice even exhibited bleeding ulcers. In contrast, the colon length of LGG-pretreated B6 mice was significantly longer than that of *C. rodentium*-infected B6 mice. A decrease of colon length (*p* < 0.05) was observed in *C. rodentium*-infected TLR2 KO and TLR4 KO mice, regardless of LGG pretreatment (Fig. 2B).

**Pretreatment with LGG Reduces the Increase in Spleen Weight during *C. rodentium* Infection in B6 and TLR4 KO Mice**

The spleen-to-body-weight ratio was significantly increased after *C. rodentium* infection in B6, TLR2 KO, and TLR4 KO mice compared with uninfected control mice. However, the ratio was significantly lower in LGG-pretreated B6 and TLR4 KO mice compared with LGG-non-pretreated *C. rodentium*-infected mice. An increase of the spleen-to-body-weight ratio was observed in infected TLR2 KO mice, regardless of LGG pretreatment (Fig. 2C).

**Reduction in Epithelial Damage Induced by *C. rodentium* Infection after Pretreatment with LGG in B6 and TLR4 KO Mice**

*C. rodentium* infection disrupted cellular morphological characteristics, such as loss of goblet cells, mucosal hyperplasia, and dramatic infiltration of inflammatory cells.
Fig. 3. Effect of LGG pretreatment on colonic histology in mice with *C. rodentium*-induced colitis.

(A) Representative histopathological examples of the distal colon of each group. (B) Analysis of total microscopic scores was performed based on mucosa thickening, loss of goblet cells, and the degree of infiltration of inflammatory cells into the mucosa and submucosa. Scores are expressed as the mean ± SD (*p < 0.05, **p < 0.01).
into the mucosa and submucosa [19]. LGG pretreatment decreased the histopathological scoring in the distal colon of B6 mice. Significantly higher histopathological scoring was observed in infected TLR2 KO and TLR4 KO mice, regardless of LGG pretreatment, compared with control animals. However, a decrease in distal colonic mucosal hyperplasia was observed in LGG-pretreated TLR4 KO mice compared with C. rodentium-infected controls. In contrast, severe mucosal hyperplasia was observed in TLR2 KO mice, regardless of LGG pretreatment (Figs. 3A and 3B).

As shown in Fig. 3B, LGG pretreatment decreased the histopathological scoring in the mid colon of B6 mice. Increased histopathological scoring in the mid colon was observed in C. rodentium-infected TLR4 KO mice compared with control mice. However, a decrease in mid colonic mucosal hyperplasia and goblet cell and inflammatory cell infiltration was observed in LGG-pretreated TLR4 KO mice compared with non-pretreated controls. Increased histopathological scoring was observed in the mid colon of TLR2 KO mice, regardless of LGG pretreatment (Fig. 3B).

The mRNA Levels of Inflammatory Cytokines Were Reduced in LGG-Pretreated B6 and TLR4 KO Mice

Cytokines play a fundamental role in modulating inflammation, phagocytosis, and tissue injury [31]. Total RNA was isolated and subjected to quantitative RT-PCR. TNF-α, MCP-1, and IFN-γ mRNA expression was lower in the spleen of LGG-pretreated B6 and TLR4 KO mice compared with non-pretreated B6 and TLR4 KO mice. In contrast, TNF-α, MCP-1, and IFN-γ mRNA expression was significantly higher in C. rodentium-infected TLR2 KO mice, regardless of LGG pretreatment (Fig. 4).

Discussion

Colitis refers to an inflammation of the colon and may become an acute or chronic digestive disease. Enteric pathogens such as EHEC and EPEC attach to and colonize the host gastrointestinal tract and cause diarrhea, transient colitis, and crypt hyperplasia [32]. The hallmark of EHEC and EPEC adhesion to host cells is the formation of distinctive attaching and effacing lesions. During infection, intimin, a bacterial outer membrane protein, interacts with Tir, an effector secreted via the type III secretion system into epithelial cells. This allows the bacterium to tightly attach to the epithelial surface by forming an actin pedestal [10, 17]. C. rodentium infection of mice represents the best murine model for the study of host defenses against attaching and effacing enteropathogens [21]. C. rodentium infection induces CD3+ and CD4+ T cells to infiltrate the colonic lamina propria, resulting in a Th1 response, characterized by elevated levels of IFN-γ and TNF-α [11].

Probiotics are viable nonpathogenic microorganisms that confer health benefits to the host by balancing the microflora [18]. The benefits of probiotic bacteria occur through various mechanisms, such as decreasing colonization, lowering of intestinal pH, and invasion by pathogenic organisms, and modifying the host immune response [34]. For example, Lactobacillus plantarum and Lactobacillus reuteri attenuate virulence by competitively excluding binding of pathogenic
organisms to the host epithelium and by acidifying the luminal environment [13, 22]. To explore the broad application of probiotics against diseases such as colitis, understanding the mechanism of probiotic action is a first step in the design of appropriate clinical trials. It should be noted that further mechanistic evidence in support of the effects of LGG on cellular responses is needed [36]. This study focused on the effect of LGG in C. rodentium colitis and its mechanisms of action.

The present study is a first attempt to investigate whether LGG exerts a preventive effect on C. rodentium-induced colitis in different mice mutants. It has been reported that, in comparison with the present study, TLR2 KO mice are more susceptible and TLR4 KO mice would be less susceptible to infection induced by a lower number (2.5 × 10⁶ CFU) of C. rodentium [9, 16]. In this study, the massive dose (2.5 × 10¹⁰ CFU) of C. rodentium failed to elicit any significant difference in body weight change in B6 and TLR4 KO mice, and the body weight change induced by C. rodentium infection was lower in TLR2KO mice than in B6 mice. TNF-α is capable of recruiting inflammatory cells to the site of infection, either directly or via the upregulation of adhesion molecules. In addition, it also stimulates the release of chemokines, which act as chemotactic cytokines for inflammatory cells [15]. MCP-1 is produced by monocytes, macrophages, T cells, and dendritic cells at sites of infection [35]. IFN-γ, a cytokine secreted by activated NK cells and T cells, has an immunomodulatory effect on several cell types. IFN-γ, the primary promoter of the Th1 immune response, is a major cytokine responsible for the activation of macrophages [3, 7]. A previous study reported that C. rodentium-induced increases in gene expression of IFN-γ and TNF-α could be prevented by pretreatment with LGG in colonic tissues [27]. In this study, TNF-α, MCP-1, and IFN-γ mRNA levels were lower in the spleen of LGG-pretreated B6 and TLR4 KO mice compared with isogenic controls. Instead, TNF-α, MCP-1, and IFN-γ mRNA levels were significantly higher in infected TLR2 KO mice, irrespective of LGG pretreatment.

We demonstrate that LGG pretreatment reduces injury derived from C. rodentium-induced colitis in B6 and TLR4 KO mice. For instance, mucosal hyperplasia is a hallmark of transmissible murine colonic hyperplasia induced by C. rodentium infection [20], and the thickness of mid and distal colon mucosa of LGG-pretreated B6 and TLR4 KO mice was decreased compared with C. rodentium-infected B6 and TLR4 KO mice. However, protection by LGG pretreatment was not observed in TLR2 KO mice. These findings suggest that LGG pretreatment protects mice from C. rodentium-induced colitis in a TLR2-dependent manner.

Acknowledgments

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References


