**Lactobacillus johnsonii N6.2 Stimulates the Innate Immune Response through Toll-Like Receptor 9 in Caco-2 Cells and Increases Intestinal Crypt Paneth Cell Number in BioBreeding Diabetes-Prone Rats**

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**Abstract**

*Lactobacillus johnsonii* (Ljo) N6.2 has been shown to mitigate the development of type 1 diabetes when administered to diabetes-prone rats. The specific mechanisms underlying this observed response remain under investigation. The objective of this study was to assess the effect of Ljo N6.2 on mucosal inflammatory response using differentiated Caco-2 monolayers. The mRNA expression levels of CCL20, CXCL8, and CXCL10 chemokines were determined by qRT-PCR. Ljo at 10^11 CFU/L induced a strong response in all chemokines examined. To assess the specific host-signaling pathways involved, we performed RT-PCR amplification of Toll-like receptors (TLR) and nucleotide-binding oligomerization domain-like receptors. TLR7 and TLR9 expression levels were induced 4.2- and 9-fold, respectively, whereas other TLR and nucleotide-binding oligomerization domain receptors were not modified. A similar effect was observed in Caco-2 monolayers treated with Ljo cell-free extract or purified nucleic acids (NA). Increased levels of IFN type 1 and IFN regulators Stat1 and IRF7 followed the upregulation of TLR9. Activation of TLR9 was also evidenced by increased Frizzled 5 expression in Ljo-treated Caco-2 cells and an increase in the number of Paneth cells in Ljo–fed, diabetes-prone rats. These results are in agreement with the polarizing-tolerizing mechanism recently described in which the apical stimulation of TLR9 in intestinal epithelial cells leads to a higher state of immunologic alertness. Furthermore, these results suggest that live probiotics could be, in the future, replaced with select cellular components. J. Nutr. 141: 1023–1028, 2011.

**Introduction**

The intestinal mucosal surface is a primary component of the host defense against potentially harmful microorganisms. Simultaneously, this surface is also the location where the intestinal microbiota and the host coexist in a tightly regulated balance. This symbiotic relationship is maintained through microbial recognition and tolerance by host cells. Intestinal epithelial cells (IEC)6 play an active role in the modulation of the innate immune system. IEC release chemokines that aid in the development of gut-associated lymphoid tissues. IEC can sense microorganisms or their components through membrane-bound pattern recognition receptors [or Toll-like receptors (TLR)] and/or cytoplasmic nucleotide-binding oligomerization domain (NOD)-like receptors. TLR expressed in epithelial cells recognize a wide variety of microbial compounds. NOD1 and NOD2 receptors sense peptidoglycan constituents and can elicit different protective or inflammatory pathways in the gut-associated lymphoid tissues. Host identification of microbial components is vital for the development of innate and adaptive immune responses to gastrointestinal infections. Furthermore, both innate receptors and microbes have been identified as important players in the balance between the induction and prevention of autoimmunity in the host (1).

In a recent study, we determined that the administration of *Lactobacillus johnsonii* (Ljo) N6.2 to BioBreeding diabetes-prone rats (BBDP) reduced the incidence of type 1 diabetes (2). The feeding of this microorganism postweaning was followed by changes in the native microbiota, host mucosal proteins, and oxidative stress response. Lower levels of the proinflammatory cytokines, IFNγ and TNFα, were also observed in the Ljo-fed...
Materials and Methods

Bacterial preparations. Ljo N6.2 isolated from BioBreeding diabetes-resistant rats (Biomedical Research Models) was cultured as described in Lai et al. (4). Cells grown for 24 h were centrifuged at 7400 × g for 20 min at 4°C, washed twice with sterile PBS, and resuspended in sterile PBS buffer. Aliquots containing 1 × 10^{11} to 4 × 10^{12} CFU/L were stored at −80°C until use. Chromosomal DNA extraction of Ljo DNA was performed using a DNeasy Blood and Tissue kit (Qiagen).

Ljo cell-free extracts were prepared by collecting cultures by centrifugation at 7400 × g for 10 min. Cells were washed with ultrapure water 3 times. Cells were resuspended in ultrapure water to an OD600 = 1.2 and incubated at 37°C for 4 h. The supernatant was collected after centrifugation at 7400 × g for 20 min at 4°C and stored at −80°C.

Caco-2 cell culture and stimulation. Caco-2 cells were obtained from American Type Culture Collection and grown in a humidified incubator frozen at −80°C for determination of the concentrations of IL-8 and TNF-α. When required, 10^{-8} g/L of TNF-α for Assessment and Accreditation of Laboratory Animal Care.

Statistical analysis. Values presented are means ± SD. Two-way ANOVA was used to compare effects of Ljo treatment with and without TNFα stimulation. Significant values from 2-way ANOVA were subject to Bonferroni-Dunn post hoc testing. For all other comparisons, significance was assessed using 1-way ANOVA. Tukey honest significance difference tests were conducted when significant differences resulted from 1-way ANOVA. Differences were considered significant at P < 0.05.

Ethics statement. The Institutional Review Board of the University of Florida approved all methods and procedures performed in the rat studies. Rat care was based on guidelines established by the Association for Assessment and Accreditation of Laboratory Animal Care.

Results

Chemokine expression as a result of Ljo stimulation. A 10^{11}-CFU/L dose of Ljo induced a strong response in the mRNA levels of CCL20 (MIP3α), CXCL8 (IL-8), and CXCL10 (IP10) by 20-, 90-, and 4-fold, respectively (Fig. 1). TNFα stimulation of untreated Caco-2 cells resulted in a 2-fold increase in the CXCL8 chemokine mRNA levels (Fig. 1A). The addition of TNFα to 1 × 10^{10} CFU/L Ljo-treated Caco-2 monolayers amplified CXCL8 expression 2-fold (P < 0.001), whereas incubation with 1 × 10^{11} CFU/L did not exacerbate the inflammatory response. These results were confirmed by measuring IL-8...
concentrations. As observed with the mRNA levels, the IL-8 concentration in Caco-2 cells without TNFα stimulation showed a significant increase that correlated with the amount of Ljo administered (Fig. 1D). A similar trend in the induction of mRNA levels was observed for CCL20 (P, 0.001) (Fig. 1C). CXCL10 mRNA levels also increased (P, 0.001) upon incubation with 1 × 10¹¹ CFU/L Ljo. The addition of TNFα induced the expression of CXCL10 in untreated Caco-2 cells or at low concentrations of Ljo (P < 0.05), whereas no significant effect was observed at higher Ljo concentrations (1 × 10¹¹ CFU/L; P > 0.1). These results indicate that Ljo may prime the innate immune system when in contact with the intestinal epithelium.

Modulation of host-signaling pathways. To identify the signaling pathway(s) stimulated by Ljo, the expression of TLR or NOD-like receptors was evaluated. It was determined that the expression levels of NOD1 and NOD2 were not affected by stimulation of Caco-2 cells for up to 24 h with 1 × 10¹¹ CFU/L Ljo (data not shown). The expression levels of TLR2, TLR5, TLR7, and TLR9 were also analyzed. As expected, TLR5 expression was not modified by Ljo as it senses flagellin (18). The TLR2 receptor and its mediator, Tollip, showed variable results. At the highest Ljo concentration, TLR7 and TLR9 expression was induced 4.2- and 10-fold, respectively (Fig. 2A).

Because TLR7 and TLR9 are involved in nucleic acid (NA) sensing (18,19), the effects of cell-free extracts and pure nucleic acid preparations of Ljo were tested. The mRNA levels of TLR7 and TLR9 were induced in Caco-2 cells stimulated with Ljo cell-free extracts containing nucleic acid concentrations of 3.2–4.8 mg/L (P < 0.05). Similarly, the treatment of Caco-2 cells with a pure NA extraction of Ljo (1 mg/L) showed a 4.5 ± 0.7 and 6.5 ± 0.9 fold increase in the expression of TLR7 and TLR9, respectively (Fig. 2B). These results indicate that IEC sensing of DNA and/or RNA from Ljo may be involved in the observed increases in select chemokines.

The effect of inhibitors of the TLR7-TLR9 (chloroquine), PI3K (LY2940021), and p38 (SB203580) signaling pathways was assessed (Fig. 3). In nonstimulated cells, chloroquine and SB203580 repressed the expression of TLR7 5-fold. Similarly, the stimulation of TLR7 that resulted from addition of Ljo NA was reduced in the presence of these inhibitors. On the contrary, none of the inhibitors affected the upregulation of TLR7 or TLR9 upon incubation with Ljo (Fig. 3A,B). The TLR9 induction obtained with NA was inhibited in the presence of LY2940021 and SB203580 (P < 0.01) (Fig. 3B).

Ljo has no effect on epithelial barrier functionality in Caco-2 cells. The effect of Ljo on epithelial barrier functionality was determined using TER. As expected, TNFα treatment caused an 8% decrease in TER through the monolayers; however, treatment with Ljo did not induce a significant change in TER in TNFα-treated or untreated cells (data not shown). The expression levels of claudin-1, claudin-2, occludin, and zonula occludens were determined after incubation with 10¹⁰ and 10¹¹ CFU/L Ljo. A 3-fold increase in the expression level of zonula occludens was observed when treating cells with Ljo 10¹¹ cells/L, whereas no significant differences were observed for claudin-1, claudin-2, or occludin. These results are in agreement with the lack of induction of TLR2, a known inducer of tight junction proteins (20,21).

FIGURE 1 Changes in expression of CXCL8 (A), CXCL10 (B), and CCL20 (C) mRNA and IL-8 concentration (D) in Caco-2 cells stimulated with Ljo N6.2 and TNFα. Ljo was administered at 10¹⁰ CFU/L (Ljo e10) or 10¹¹ CFU/L (Ljo e11) followed by 10 μg/L TNFα where indicated. Values are means ± SD, n = 3. Means without a common letter differ, P < 0.05. *Different from TNFα, P < 0.05.

FIGURE 2 Expression of TLR receptor mRNA after stimulation of Caco-2 cells with Ljo (A) or Ljo cell-free extracts containing varying concentrations of NA or purified NA (B). Values are means ± SD, n = 3. Means without a common letter differ, P < 0.05.
**FIGURE 3** Effects of signaling inhibitors on Caco-2 TLR7 cells (A) and TLR9 (B) mRNA expression after stimulation with 10^11 CFU/L Ljo e11 or its pure NA. Values are means ± SD, n = 3. Means without a common letter differ, P < 0.05.

TLR9 induced by Ljo N6.2 upregulates expression of Fzd5. The consequences of Ljo-induced TLR9 stimulation were studied on the expression of Frizzled 5 receptors (fzd5). These receptors bind signaling proteins in the Wnt pathway, which induces Paneth cell maturation (22). We found that the expression of fzd5 was induced 3-fold upon stimulation with 1 × 10^11 CFU/L Ljo N6.2 after 24 h, whereas its target gene, human β-defensin-2 (hbd2), was upregulated 123-fold (P < 0.0001). Similarly, the stimulation of Caco-2 cells with NA showed a 44-fold increase (P < 0.05) in hbd2 expression (Fig. 4).

To establish a correlation between the upregulation of fzd5 and Paneth cell maturation, we analyzed slices of distal intestine of BBDP rats administered Ljo (2). The number of Paneth cells per crypt in the Ljo-fed group (4.3 ± 0.13) was higher (P < 0.0001) than those in the control group (3.6 ± 0.09) (Fig. 5). Granule concentration and mRNA expression levels of defensin 6 did not differ between the 2 groups.

**FIGURE 4** Modulation of fzd5 and human β-defensin 2 genes as a result of Ljo or NA stimulation. Caco-2 was stimulated with Ljo e11 or its pure NA. Values are means ± SD, n = 3. Means without a common letter differ, P < 0.05.

**FIGURE 5** Modulation of mRNA expression of CCL20 (MIP3α), CXCL8 (IL-8), and CXCL10 (IP10). A gradual response to the dose concentration of Ljo was observed. CXCL8 and CCL20 had a similar pattern of induction, whereas CXCL10 was most efficiently induced by 10^11 CFU/L. At this bacterial concentration, the proinflammatory response obtained was not exacerbated by further stimulation with TNFα. These results suggest that Ljo may promote an early activation of the immune system. This activation could improve the alertness of the host defensive system, thus preventing a subsequent strong inflammatory response. In the related species L. acidophilus, a lower proinflammatory response was induced compared with Escherichia coli. However, the downstream effects were not analyzed (7).

It is also possible that Ljo induces the expression of CXCL10 as a defensin-like molecule, although the canonical role of CXCL10 is to attract activated Th1 cells and NK cells (24). Cole et al. (25) reported CXCL10 defensin-like antibacterial activity. Among the inducers of CXCL10 are microbial components such as peptidoglycan and lipopolysaccharide. These cellular fractions also induce high levels of CXCL8 (26).

The innate immune system initiates a response to microorganisms or their components via pattern recognition receptors such as TLR and NOD receptors. The activation of these receptors influences the nature of the subsequent adaptive immune response. Although many components within the signaling pathway are shared by the TLR, important consequences in gene expression are observed upon stimulation of an individual TLR (27). The current literature describes 2 major localizations of epithelial TLR: TLR that recognize NA localized at the

**Discussion**

In the BioBreeding rat model of type 1 diabetes, we determined that continuous administration of a native strain of Ljo isolated from BioBreeding diabetes-resistant rats reduces disease development in BBDP rats (2). In this study, an in vitro approach was used to study the initial effects of Ljo N6.2 on human IEC. We determined that Ljo activates the innate immune response as evidenced by the increase in expression of CCL20 (MIP3α),
endosome (TLR3, TLR7, TLR8, TLR9) and TLR localized at the apical side of IEC that recognize other bacterial components (28). To assess the signaling pathways induced by Ljo, we determined the expression levels of TLR2 (lipoproteins, peptidoglycan, and lipoteichoic acid), TLR7 (RNA), and TLR9 (nonmethylated CpG motifs in DNA) (20,28). The expression of NOD1 and NOD2 were also determined (29). Interestingly, in our study, Ljo caused only the upregulation of TLR7 and TLR9 mRNA levels. The data obtained using cell-free extracts of Ljo, as well as Ljo pure NA, suggest that DNA/RNA is one of the main components of Ljo-mediated signaling. The fact that TLR2 and NOD2 receptors were not upregulated under any condition analyzed indicates that peptidoglycan is not a mediator of the observed effects.

In a previous study, Rachmilewitz et al. (30) found that TLR9 signaling mediated the antiinflammatory effects of a probiotic mix (VSL3) in a murine model of experimental colitis. Similarly, Lee et al. (31) have linked DNA stimulation to higher expression of NfκB and c-Jun N-terminal kinases in response to administration of probiotic DNA to mice. Our experiments with pathway inhibitors and pure Ljo NA indicate that the MAPK signaling pathways are involved. However, stimulation with whole Ljo cells was not interrupted by any chemical tested. Chloroquine has been described as an inhibitor of TLR9 and TLR7 signaling pathways when localized in the endosome (32,33). Pretreatment of Caco-2 cells with chloroquine prior to exposure to pure Ljo NA or Ljo cells did not affect TLR7 or TLR9 induction (Fig. 3). The “polarizing-tolerizing” mechanism described by Lee et al. (34) showed that the apical localization of TLR9 triggers a tolerogenic response conducive to the maintenance of colonic homeostasis and prevention of further inflammation. Similarly, a neutral effect of chloroquine was reported (32). Consistent with this model, we also observed upregulation of ifnar and ifnb were observed. The IFN regulatory proteins IRF7 and STAT1 were also induced in response to Ljo cells or purified NA. Production of type 1 IFN leads to the activation of the JAK/STAT signaling pathway and serves as a first line of defense against infections via immune modulator effects on dendritic cells, macrophages, T cells, and B cells. Administration of type 1 IFN has been proposed as an immunotherapeutic strategy for the treatment of autoimmune diseases such as multiple sclerosis (36). However, the role of IFNα in diabetes type 1 development is not fully understood. A high level of IFNα can be used as a marker for viral infection, and positive correlations between patients with type 1 diabetes and high IFNα levels were reported (35). Interestingly, the protective role of viral infections in T1D development is variable and seems to be linked to immune system maturity (37,38). It is tempting to speculate that TLR9-mediated responses induced by Ljo could mimic a subtle viral infection and promote a protective effect. This hypothesis needs to be tested in a rodent experimental model.

Based on the present results, it is likely that Ljo mediates induction of type 1 IFN through cell-signaling pathways requiring TLR9. As a consequence, a tolerizing effect of the IEC and a higher state of immunologic activation would be achieved. Furthermore, our results indicate that NA are one of the active components mediating these responses and suggest that live probiotics could be, in the future, replaced with cellular components.

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Literature Cited


**FIGURE 5** Numbers of Paneth cells per crypt in control and Ljo-fed (10⁸ CFU daily) BBDP rats. (A) Values are means ± SD, n = 10. *Different from control, P < 0.0001. Hematoxylin and eosin-stained slides with arrows indicating representative Paneth cells are shown in Ljo-fed (B) and control (C) rats.

**FIGURE 6** Expression of IFNa, IFN8, and their modulators, Stat1 and IRF7, in Caco-2 cells stimulated with 10¹¹ CFU/L Ljo e11 or its pure NA. Values are means ± SD, n = 3. Means without a common letter differ, P < 0.05.


