The effect of probiotic Lactobacillus rhamnosus colonization on the Toll-like receptor expression in immunodeficient mice

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Abstract

Probiotics are non pathogenic bacteria with beneficial effects on mammal's health. Little is known about effects of probiotic bacteria on the development of innate immunity, especially on the activation of the Pattern Recognition Receptors, such as Toll-like receptors (TLRs). The aim of the study was to evaluate the influence of probiotic Lactobacillus rhamnosus strains on the expression of TLRs and the number of Paneth’s cells in the gut of severe combined immunodeficient (SCID) mice. Two-month old SCID mice reared in gnotobiotic conditions were either maintained germ-free (GF) or associated with L. rhamnosus strains. SCID mice associated with commensal intestinal microflora reared in conventional conditions (CONV-SCID) were used as controls. TLR-2, and -4 were determined by immunohistochemical methods. Paneth’s cells were enumerated in hematoxylin and eosin stained intestinal sections. The number of Paneth’s cells in GF animals was significantly lower in comparison with CONV mice. L. rhamnosus colonization increased the number of Paneth’s cells, and induced TLR expression pattern observed in CONV animals. GF-SCID mice showed high expression of TLR-2 in brush border of small intestine as well as in goblet cells of the colon, whereas TLR-4 was observed only in the colon. Commensal intestinal microflora influenced the expression pattern of TLRs in the colon. In GF animals TLR-4 was mainly expressed in colonic crypts and at the surface of epithelial cells, whereas in CONV mice this receptor was found only in crypts and in lower amounts in intracellular compartment of mature epithelial cells. Our study showed that L. rhamnosus bacteria influenced the development of innate immunity in SCID mice by increasing the number of Paneth’s cells and induction of TLR expression similar to that found in mice associated with commensal microflora.

Key words: innate immunity, Lactobacillus, Paneth’s cells, probiotics, TLR
Introduction

The human intestinal tract contains a thriving microbial flora, which functions are essential to maintaining digestive processes, gut motility and protection against pathogen colonization. Recently, the role of intestine microflora in the development of immune system reactions has been stressed, however still little is known about their influence on the development of gut innate immunity [16]. The innate immune system of gut comprises a large number of cell populations present in mucosa and in mucosa associated organized lymphoid tissue [5]. The cells of innate immunity produce cytokines essential for inflammatory reactions as well as factors critical for the subsequent initiation of specific immunity. The first layer of innate defense is the intestinal epithelial cells that line the luminal surface of the gastrointestinal tract. The single layer of epithelial cells, especially in the colon, forms an impermeable barrier to luminal bacteria. This is critical given that this organ contains approximately $10^{11}$-$10^{12}$ bacteria per gram of stool [16], and innate immunity responses are induced upon recognition of the conserved molecules produced by both commensal and pathogenic microorganisms [3]. These molecules, know as microbial pathogen –associated molecular patterns (PAMPs) or commensal-associated molecular patterns are recognized by specific host Pattern Recognition Receptors (PRRs) including the Toll – like receptors (TLRs) and nucleotide binding oligomerization domain (NOD) proteins, NOD1 and NOD2 [2]. PRRs receptors initiate signals for anti-microbial peptide expression, barrier fortification, proliferation of epithelial cells, production of pro-inflammatory cytokines [1, 7]. Anti-microbial peptides (defensins, cathelicidins, crydtins, angiogenins, bactericidal/permeability increasing protein, chemokine CCL20) are produced by specialized gram-negative bacteria such as Lactobacillus rhamnosus Pen, L. rhamnosus E/N, L. rhamnosus Oxy (available as Lakcid®, Biomed, Lublin, Poland) in dose $10^9$/mouse were reared in sterile plastic isolators. Conventional mice (CONV) kept in CONV animal facility were fed the same but not sterile food. The animals were kept in a room with 12h light-dark cycle at 22°C. Mice were either maintained GF or associated with mentioned Lactobacillus rhamnosus strains. Offspring from the second generation of colonized mice were reared in the incubator for next 2 months, and such naturally L. rhamnosus colonized SCID mice were used for our experiments. Samples of small intestine and colon descendens were collected from GF, CONV and L. rhamnosus-associated mice.

Material and methods

Animal model

Two- month old severe combined immunodeficient mice, background Balb/cJHanHsd-SCID (SCID) were used for our experiments. Germ-free (GF) and associated with three genetically identified Lactobacillus rhamnosus strains: L. rhamnosus Pen, L. rhamnosus E/N, L. rhamnosus Oxy (available as Lakcid®, Biomed, Lublin, Poland) in dose $10^9$/mouse were reared in sterile plastic isolators. Conventional mice (CONV) kept in CONV animal facility were fed the same but not sterile food. The animals were kept in a room with 12h light-dark cycle at 22°C. Mice were either maintained GF or associated with mentioned Lactobacillus rhamnosus strains. Offspring from the second generation of colonized mice were reared in the incubator for next 2 months, and such naturally L. rhamnosus colonized SCID mice were used for our experiments. Samples of small intestine and colon descendens were collected from GF, CONV and L. rhamnosus-associated mice.

Enumeration of Paneth’s cells

Paneth cells were analyzed in hematoxylin and eosin stained tissue sections of ileum. The results are expressed as arithmetic mean ± standard deviation of the number of Paneth’s cells per one crypt. Paneth’s cells were enumerated at least in 20 crypts of the each animal.

Immunohistochemistry of TLR

The expression of TLR was analyzed on paraffin sections of ileum and colon. Four µm sections were de-paraffined and rehydrated using a graded alcohol series. Antigen retrieval was done using 1,5 M citrate buffer pH 6,0. Slides were boiling for 30 min. and washed in phosphate buffered saline (PBS) pH 7,4. Endogenous peroxidase was blocked by treatment with 3% hydrogen peroxide solution for 10 min. After incubation for 30 min. with 10% normal rabbit serum, primary goat anti-TLR-4 or anti-TLR-2 antibodies (Santa Cruz Biotechnology, INC., USA) in final dilution 1:50 were applied for overnight in 4°C. Then slides were washed in PBS and secondary antibody rabbit anti-goat peroxidase labeled (Jackson ImmunoResearch) in final dilution 1:250 was used. Reactions were developed using APC – chromogen (Dakocytomation). Finally, slides were counterstained with haematoxylin. Negative controls were also prepared by replacing the primary antibody with PBS.

Results

The influence of L. rhamnosus applications on the number of Paneth’s cells

Paneth’s cells were found at the bottom of the small intestine crypts in all groups of mice (Fig. 1). In GF mice their number was significantly lower compared to CONV mice (Table 1). Association of GF mice with L. rhamnosus strains induced an increase of amounts of Paneth’s cells in crypts, but their number was lower than that found in
both in GF and CONV animals (Fig. 2). In contrast to TLR-2, TLR-4 was not detected in epithelial cells of small intestine in GF as well as in CONV animals. Animals associated with L. rhamnosus showed similar expression pattern of those receptors in small intestine as that found in GF and CONV mice.

In contrast to the ileum in which TLR-4 was not expressed, in colon both receptors were observed, but they presented different expression pattern. TLR-2 was found mainly in goblet cells, whereas TLR-4 expression was confined to crypt cells (Fig. 3 and 4). Some positive cells bearing TLR-2 and -4 were also found in lamina propria of ileum and colon.

Intestinal microflora of CONV animals affected mainly expression pattern of TLR-4. In GF animals the highest amounts of TLR-4 was detected in cytoplasm of colonic crypts, and intracellular expression was not found in mature epithelial cells (Fig. 3). Interestingly, in GF animals this receptor was also observed at the luminal surface of all epithelial cells. In CONV animals TLR-4 was also found mainly in crypt cells (Fig. 4). The amounts of TLR-4 decreased when epithelial cells matured and moved up towards the gut lumen, and in contrast to GF animals small amounts of this

**Table 1**

The number of Paneth’s cells in small intestine of mice

<table>
<thead>
<tr>
<th>Group of mice</th>
<th>Numbers of Paneth’s cells/crypt</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCID-GF</td>
<td>3.6 ±1.3</td>
</tr>
<tr>
<td>SCID associated with L. rhamnosus</td>
<td>4.3 ± 1.0</td>
</tr>
<tr>
<td>SCID-CONV</td>
<td>6.9 ±1.1</td>
</tr>
</tbody>
</table>

Paneth’s cells were enumerated in ileum (n=5 mice/group). The number of Paneth cells were calculated from at least twenty crypts and expressed as arithmetical means±SD per one crypt. Statistical analyses with Tukey-Kramer Multiple Comparison Test showed significant differences between following groups of mice: SCID-GF versus SCID-CONV (p<0.001), SCID associated with L. rhamnosus versus SCID-CONV (p<0.01).

SCID-CONV mice and did not achieve statistical significance.

**Expression of TLRs in the gut**

TLR-2 and -4 were detected in the gut of GF and CONV mice. TLR-2 was found mainly in the brush border of ileum both in GF and CONV animals (Fig. 2). In contrast to TLR-2, TLR-4 was not detected in epithelial cells of small intestine in GF as well as in CONV animals. Animals associated with L. rhamnosus showed similar expression pattern of those receptors in small intestine as that found in GF and CONV mice.

In contrast to the ileum in which TLR-4 was not expressed, in colon both receptors were observed, but they presented different expression pattern. TLR-2 was found mainly in goblet cells, whereas TLR-4 expression was confined to crypt cells (Fig. 3 and 4). Some positive cells bearing TLR-2 and -4 were also found in lamina propria of ileum and colon.

Intestinal microflora of CONV animals affected mainly expression pattern of TLR-4. In GF animals the highest amounts of TLR-4 was detected in cytoplasm of colonic crypts, and intracellular expression was not found in mature epithelial cells (Fig. 3). Interestingly, in GF animals this receptor was also observed at the luminal surface of all epithelial cells. In CONV animals TLR-4 was also found mainly in crypt cells (Fig. 4). The amounts of TLR-4 decreased when epithelial cells matured and moved up towards the gut lumen, and in contrast to GF animals small amounts of this
receptor was found in intracellular compartment of mature epithelial cells. However, we did not observe TLR-4 expression at the surface of epithelium. Colonization of mice with *L. rhamnosus* strains induced TLR-4 expression similar to that found in CONV animals. Conventional microflora did not influence the expression pattern of TLR-2, which in CONV animals was present mainly in goblet cells (Fig. 4). Similar expression was observed in *Lactobacillus* colonized animals.

**Discussion**

It is known fact that TLRs recognise pathogen associated molecular patterns and trigger the activation of inflammatory responses against pathogens [2, 3]. On the other hand, TLRs sense commensal associated molecular patterns, and the recent study stressed that TLR signalling mediated by commensal bacteria is essential for intestinal barrier function and repair [15]. MyD88 (TLR signaling adaptor molecule)-, TLR2- and TLR4-deficient mice, i.e. mice markedly impaired in their ability to respond to commensal microflora, exhibited severe mortality following dextran sulphate sodium induced intestinal injury. Our study showed that TLR-2 and -4 are highly expressed in the gut of animals avoided of intestinal microflora and reared in germ-free conditions, and whereas TLR-2 was found both in small intestine and colon, TLR-4 was detected only in the colon. Interestingly, commensal bacteria affected mainly expression pattern of TLR-4. In GF animals this receptor was found in immature epithelial cells of colonic crypts as well as at the surface of epithelium. In mice colonized with physiological intestinal non-pathogenic bacteria TLR-4 was confined to the crypt epithelial cells, but this receptor did not occur at the luminal surface. In contrast to GF animals, mature epithelial cells of mice associated with commensal microflora showed intracellular expression of small amounts of TLR-4. In contrast to our study Naik and coauthors [14] showed absence of TLR-4, but at mRNA level, in intestinal epithelium. They supposed that the lack of TLR-4 expression in epithelium is associated with endotoxin hyporesponsiveness of intestinal epithelium. However, Furrie et al [8] using immunohistochemistry analyses showed expression of TLR-4 in human colonic tissue both in the epithelium and in the lamina propria. Similar to our study, this receptor was mainly expressed by colonic crypts and was absent at the luminal surface of epithelium. Simultaneously, they observed very strong expression of TLR-4 in large mononuclear cells.
of the lamina propria, being particularly localized near the crypts. In our model TLR-4 was expressed by a few cells occurring in lamina propria, but we analyzed SCID mice, i.e. mice devoided T and B lymphocytes infiltrating lamina propria of healthy animals.

Our results confirm the hypothesis that physiological microflora control epithelial TLR activation and play a role in regulation of unintended stimulation and mucosal inflammation. We believe that the contact of TLR-4, expressed primary at the luminal surface of colon, with intestinal microflora induces down-regulation of the immune response against various bacterial components including lipopolysaccharide (LPS), and finally affects the expression pattern of TLR-4 in such way which avoids direct recognition of bacteria colonizing the intestine. That is why, in mice associated with commensal microflora, TLR-4 was expressed in crypt cells and in significantly lower amounts in intracellular compartments of mature epithelial cells. Recent studies performed by Lotz et al [13] present mechanisms that prevent inappropriate stimulation of TLRs by the colonizing microflora [13]. They showed that although TLR-4 was present in fetal, neonatal, and adult intestinal epithelial cells, LPS-induced nuclear factor NF-kappa B activation was only detected in fetal cells. Importantly, the spontaneous epithelial cell activation occurred only in vaginally born mice but not in neonates delivered by Cesarean section or in TLR4-deficient mice. Thus, epithelial cells acquire TLR tolerance immediately after birth by exposure to exogenous endotoxin to facilitate microbial colonization and the development of a stable intestinal host–microbe homeostasis [12]. As colonization of mice with L. rhamnosus strains induced TLR-4 expression similar to that found in CONV animals, we supposed that those probiotic bacteria could play an important role in regulation of TLRs.

In addition, our study showed that L. rhamnosus strains influenced Paneth’s cells. We observed the presence of Paneth’s cells in GF mice, but their number was significantly lower in comparison with CONV mice. Colonization of mice with probiotic bacteria increased the number of Paneth’s cells. In contrast to our results Bry et al [6] showed that an increase of Paneth’s cell number per crypt occurs during postnatal days 14-28, when crypt proliferate by fission. However, this process was not dependent upon interactions with microflora. Recently, it has been demonstrated that probiotic bacteria (Escherichia coli Nissle 1917) stimulate the upregulation of inducible antimicrobial peptides such as human beta-defensin-2 in Caco-2 intestinal epithelial cells [17]. Our study shows that probiotic L. rhamnosus may stimulate the intestinal innate defense through activation of Paneth’s cell proliferation.

In conclusion, our data provide the evidence that probiotic Lactobacillus rhamnosus strains may exert their beneficial effects through stimulation of TLR expression and induction of increased number of Paneth’s cells in intestinal epithelium. Both stimulation of Paneth’s cells and induction of TLRs by probiotics may contribute to an enhanced mucosal barrier to the luminal bacteria, and to the use of such probiotic bacteria for treatment and/or protection of intestinal injury during chronic inflammation, such as inflammatory bowel disease.

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