Lactobacillus acidophilus Induces Cytokine and Chemokine Production via NF-κB and p38 Mitogen-Activated Protein Kinase Signaling Pathways in Intestinal Epithelial Cells

Yujun Jiang,a,b Xuena Lü,a Chaoxin Man,b Linlin Han,a Yi Shan,b Xingguang Qu,a Ying Liu,a Shi Qin Yang,a Yuqing Xue,a and Yinghua Zhanga

Key Lab of Dairy Science, Ministry of Education, College of Food Science and Engineering, Northeast Agricultural University, Harbin, People's Republic of China,a and National Research Center of Dairy Engineering and Technology, Northeast Agricultural University, Harbin, People's Republic of Chinab

Intestinal epithelial cells can respond to certain bacteria by producing an array of cytokines and chemokines which are associated with host immune responses. Lactobacillus acidophilus NCFM is a characterized probiotic, originally isolated from human feces. This study aimed to test the ability of L. acidophilus NCFM to stimulate cytokine and chemokine production in intestinal epithelial cells and to elucidate the mechanisms involved in their upregulation. In experiments using intestinal epithelial cell lines and mouse models, we observed that L. acidophilus NCFM could rapidly but transiently upregulate a number of effector genes encoding cytokines and chemokines such as interleukin 1α (IL-1α), IL-1β, CCL2, and CCL20 and that cytokines showed lower expression levels with L. acidophilus NCFM treatment than chemokines. Moreover, L. acidophilus NCFM could activate a pathogen-associated molecular pattern receptor, Toll-like receptor 2 (TLR2), in intestinal epithelial cell lines. The phosphorylation of NF-κB p65 and p38 mitogen-activated protein kinase (MAPK) in intestinal epithelial cell lines was also enhanced by L. acidophilus NCFM. Furthermore, inhibitors of NF-κB (pyrrolidine dithiocarbamate [PDTC]) and p38 MAPK (SB203580) significantly reduced cytokine and chemokine production in the intestinal epithelial cell lines stimulated by L. acidophilus NCFM, suggesting that both NF-κB and p38 MAPK signaling pathways were important for the production of cytokines and chemokines induced by L. acidophilus NCFM.

The human gastrointestinal (GI) tract, which is populated by a complex mixture of more than 10^{14} microorganisms, is lined by a single monolayer of intestinal epithelial cells (IEC) (7). IEC are recognized as immunological sentinels of the GI tract and play a key regulatory role in maintaining host innate and adaptive mucosal immunity (16, 40). IEC act as the first line of host defense against a pathogenic bacterial invasion or inflammatory stimuli by secreting an array of cytokines and chemokines, which affect the immune cells scattered in the GI tract and recruit immune cells to the GI tract, respectively (13, 19, 24, 36). Because IEC are continually exposed to the GI tract microbiota, it is clear that commensal bacteria should not elicit as intense an inflammatory response as pathogenic bacteria (31). In addition, some investigators showed that IEC remain hyporesponsive to nonpathogenic commensal bacteria (23, 29). However, it has also been reported that IEC, exposed to some commensal bacteria, such as Bacillus subtilis, Bacteroides ovatus, Escherichia coli, Lactobacillus rhamnosus, Bifidobacterium lactis, Lactobacillus casei, or Lactobacillus acidophilus, could produce inflammatory cytokines (e.g., interleukin 1 [IL-1], IL-8, and tumor necrosis factor alpha [TNF-α]) or chemokines (e.g., CCL2 and CCL20) (6, 12, 21, 33, 40).

Probiotics exert beneficial effects on the health of the host through establishing mutualistic relationships with the IEC (22). Some strains have been shown to enhance the host immune responses by regulating cytokine and chemokine production (12, 18, 21, 33, 39, 40). Of these, the strain Lactobacillus acidophilus NCFM is a well-characterized probiotic bacterium, with several reports showing beneficial effects on the host (1, 20, 32, 35). These studies have shown that L. acidophilus NCFM is able to modulate the production of inflammatory mediators, such as TNF-α, IL-1β, CCL2, and IL-6, in dendritic cells (DC) and IEC (32, 40). However, little is known about the basic molecular mechanism of L. acidophilus NCFM regulation of the host immune responses.

IEC sense bacteria through expression of conserved pattern recognition receptors (PRRs), such as the Toll-like receptors (TLRs) (21, 26). Some studies have shown that TLR2 and TLR4 were constitutively expressed both in IEC lines and in primary IEC isolated from intestinal tissue (3, 21). These receptors activated nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK), the immune-related transcriptional factors that induced the synthesis of cytokines and chemokines (25). It has been reported that B. lactis, the dominant microbial population group in the human GI tract, induced inflammatory cytokine IL-6 production through NF-κB and p38 MAPK signaling pathways in IEC (33). L. casei could activate these signaling pathways in production of innate cytokines, such as TNF-α and IL-12, in spleen cells (18). Miettinen et al. also showed that L. rhamnosus GG can initiate NF-κB, STAT1, and STAT3 DNA-binding activity in human macrophages (27). Therefore, it is likely that the activation of these transcriptional factors of host cells by L. acidophilus plays important roles in the generation of immune-related cytokines and chemokines that function to benefit the host.

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Address correspondence to Yujun Jiang, yujunjiang163.com. Y.J. and X.L. contributed equally to this study.
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In this study, we examined the ability of L. acidophilus NCFM to stimulate cytokine and chemokine production in native IEC and IEC lines, and elucidated the mechanisms involved. We found that L. acidophilus NCFM could rapidly but transiently induce cytokine and chemokine production, and cytokines showed lower expression levels than chemokines. Furthermore, our research suggested that the activation of TLR2-mediated NF-
B and p38 MAPK signaling pathways played a key role in the production of cytokines and chemokines in IEC.

MATERIALS AND METHODS

Bacterial strain and culture conditions. L. acidophilus NCFM was obtained from American Type Culture Collection (ATCC) (Rockville, MD). For stimulation experiments, the bacteria were anaerobically grown at 37°C in de Man, Rogosa, and Sharp broth (MRS broth) (Difco, Detroit, MI) overnight prior to use. The bacterial cells were harvested by centrifugation (4,000 x g, 10 min) at stationary phase, washed twice with sterile phosphate-buffered saline (PBS), and then diluted with Dulbecco’s modified Eagle’s minimal essential medium (DMEM) (GIBCO-BRL, Grand Island, NY) and sterile 10% skimmed milk for in vitro and in vivo experiments, respectively. The number of bacterial cells was determined by the plate counting agar method.

Cell culture. Human colorectal adenocarcinoma cell line Caco-2 cells were purchased from ATCC and maintained in an incubator at 37°C, 5% CO2, in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) (NQB, Australia), 1% nonessential amino acids, 10 U/ml penicillin, and 10 µg/ml streptomycin. The Caco-2 cells (3 x 10⁴ cells/well), which were used for stimulation experiments, were allowed to attach and grow in plastic six-well culture plates (Costar; Corning). Culture medium was changed every second day for approximately 17 days until the cells reached full differentiation and polarization (38). Subsequently, the Caco-2 cells were used in experimental investigations as specified below.

Stimulation experiment. Prior to stimulation, polarized epithelial cell monolayers were washed twice with prewarmed PBS, and then the cells were incubated with the bacteria suspensions at a multiplicity of infection (MOI) (ratio of bacteria number to epithelial cell number) of 10, which did not affect the composition of the culture medium and IEC viability (21), for various times at 37°C and 5% CO2. Culture medium was used as a negative control. Where indicated, the experiments were terminated by thoroughly washing the cells with cold PBS.

Animal studies. BALB/c mice, 10 to 12 weeks old, weighing from 20 g to 24 g, were used to study the in vivo kinetics of how L. acidophilus NCFM induced cytokine and chemokine expression. The mice were housed in plastic cages kept at a constant room temperature of 22 ± 2°C and relative humidity of 55% ± 5% and exposed to a 12-h light/dark cycle. They had free access to a conventional balanced diet and distilled water. The experimental group was administered intragastrically with L. acidophilus NCFM for 2 h at an MOI of 10. The experiments were terminated by thoroughly washing the cells with cold PBS, and then total RNA was prepared for real-time reverse transcription-PCR (RT-PCR).

RNA isolation and real-time RT-PCR. RNA from cell lines or cecum and colon was extracted using TRIzol (Invitrogen, Carlsbad) by repetitive pipetting (17). The purity and integrity of RNA were evaluated by spectrophotometry and electrophoresis on 1% agarose gels. cDNA was synthesized using the cDNA RT reagent kit (Takara, Dalian, China) according to the manufacturer’s protocol. Real-time RT-PCRs were carried out using the ABI Prism 7500 system using SYBR green buffer according to the manufacturer’s instructions (Applied Biosystems), subjected to 30-s denaturation at 95°C, followed by 40 cycles of 5 s at 95°C and 34 s at 60°C. The sequences of specific primers used in the PCR are shown in Table 1. The data were analyzed by using ABI Prism 7500 system software. All gene quantifications were performed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal standard, and the relative quantitative gene expression was analyzed by using the standard formula \( 2^{-\Delta\Delta CT} \), where \( \Delta\Delta CT \) is the cycle number where the amplified target reaches the defined threshold; \( E_t \) is the \( C_T \) of the experimental gene in treated samples, \( R_t \) is the \( C_T \) of GAPDH in treated samples, \( R_c \) is the \( C_T \) of the experimental gene in control samples, and \( R_c \) is the \( C_T \) of GAPDH in control samples (28). Application plot and dissociation curves were used for the examination of the amplified products.

Western blot analysis. Caco-2 cells, which were treated with L. acidophilus NCFM or DMEM, were lysed in lysis buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% NP-40 and sodium deoxycholate, 10 mg/ml aprotinin and leupeptin, 100 mg/ml phenylmethylsulfonyl fluoride [PMSF], 400 µM Na3VO4, and 5 Mm NaF), incubated at 4°C for 30 min, and centrifuged at 13,000 x g for 10 min at 4°C. The supernatants were transferred to fresh tubes and stored at −70°C until required. The protein concentration in the supernatants was determined by Bradford’s method. Approximately 20 µg protein per lane was loaded on a sodium dodecyl sulfate–12% polyacrylamide gel and then transferred to polyvinylidene fluoride membranes (Millipore; Bedford, MA) in 25 mM Tris base, 190 mM glycine, and 20% methanol using a wet blotter. Subsequently, the membranes were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) supplemented with 0.1% Tween 20 for 1 h and washed with TBS supplemented with 0.1% Tween 20 for 5 min three times. Afterwards, the membranes were incubated at 4°C over-

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**TABLE 1 Primer sequences for cytokines and chemokines for real-time RT-PCR**

| Gene product | Primer sequence (5' → 3')a | Fragment size (bp) |
|--------------|---------------------------|-------------------|
| **Human**    |                           |                   |
| CCL2 (MCP-1) | F, CTGACGCGATGAGATCAATG 129 |
|              | R, AGATCGACAGTCTTTGGGACACC |
| CCL20 (MCP-3α) | F, TGGACTGCTGTCCTGGATACAC 150 |
|              | R, TCTGTGTTGATTGCGGCA |
| IL-1β        | F, GTTGGCAGAGGATGACCTGTTC 130 |
|              | R, TTGTGTTGACTTGCCGAAGGGA |
| IL-1α        | F, AGAAAGACAGTCTCCCATTTGA 136 |
|              | R, CTTGAGTTGATTAGGTTGTTT |
| GAPDH        | F, ACGGATTTGCGTGTATTG 214   |
|              | R, GCTCCTTGAAAGATGTTGAT |
| **Mouse**    |                           |                   |
| CCL2         | F, AGCTGGTGGGGCTGGCAAGA 136 |
|              | R, ACTTACGCTTTCCTTTGGGACACC |
| CCL20        | F, TACTGTCGCTGCTACCTC 112   |
|              | R, ATCTGTCGTGCACAAAAA |
| IL-1β        | F, AAGTTCGAGCAACCA 126      |
|              | R, GTGATACGTCCTGCCGTA |
| IL-1α        | F, TCTGCCATTCGATCATCTC 183  |
|              | R, AATCTCCGTTGCGTTG |
| GAPDH        | F, GCCTGGAGAACCTGCC3' 200    |
|              | R, ATACCCAAGGATAGCTGACCA |

a F, forward; R, reverse.
night with rabbit anti-Ser(p)-NF-κB p65 (phosphor-specific Ser536), anti-Th(p)-p38 MAPK (phosphor-specific Thr180/Tyr182) (Cell Signaling Technology, Inc., Beverly, MA), anti-TLR2, and anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA). After incubation with horseradish peroxidase (HRP)-conjugated anti-rabbit antibody, the membranes were incubated with ECL chemiluminescence reagent (TransGen Biotech, Beijing, China), and the film was then exposed to the membranes.

**Statistical analysis.** Data were expressed as means ± standard deviation (SD) of triplicates. The statistical significance of the difference between the two means was evaluated by using Student’s t test. P values of <0.05 were considered significant.

**RESULTS**

**Kinetics of cytokine and chemokine expression in Caco-2 cells stimulated with L. acidophilus NCFM.** In order to assess the effect of *L. acidophilus* NCFM on cytokine and chemokine production in IEC, Caco-2 cells were incubated with bacteria at an MOI of 10 for 0, 2, 4, 8, and 12 h, and cytokines and chemokines, including IL-1α, IL-1β, CCL2, and CCL20, associated with the host immunity, were measured. As shown in Fig. 1, *L. acidophilus* NCFM induced cytokine and chemokine expression with the same kinetics, and the expression of these genes was significantly upregulated (*P* < 0.05) at 2 h after bacterial stimulation, except for that encoding IL-1β, which was significantly upregulated (*P* < 0.05) at 4 h. All gene expression peaked at 4 h after stimulation and then gradually declined. The chemokine mRNA expression represents a higher fold change than the cytokines.

**Kinetics of cytokine and chemokine expression in mice administered L. acidophilus NCFM intragastrically.** In order to further investigate whether *L. acidophilus* NCFM can induce cytokine and chemokine expression *in vivo*, BALB/c mice were administered bacteria intragastrically for 0, 1, 3, 5, and 7 days. Figure 2 shows that *L. acidophilus* NCFM could induce cytokine and chemokine production, and the trends of gene expression levels were comparative *in vivo* and *in vitro*. Expression of both cytokines and chemokines was highest on day 5 after the initial bacterial association in mice. However, the expression of these genes was significantly upregulated (*P* < 0.05) on day 5 except for that encoding IL-1α, the expression of which was not significant (*P* > 0.05) compared to that for the control group during the bacterial association. Similar to the *in vitro* data, the expression level of cytokines was lower than that observed for the chemokines. The above results indicated that *L. acidophilus* NCFM had the ability to regulate transient cytokine and chemokine expression both *in vitro* and *in vivo*.

**Induction of TLR2 in Caco-2 cells by L. acidophilus NCFM.** Expression of the pattern recognition receptors, TLRs, plays an essential role in activation of the host immune responses, and TLR2 has been shown to be activated by Gram-positive bacteria (6, 18). Therefore, we investigated whether *L. acidophilus* NCFM could induce TLR2 expression in IEC. Caco-2 cells were treated with bacteria at an MOI of 10 for 0, 0.5, 1, 2, and 4 h. As shown in Fig. 3, TLR2 was induced after stimulation with *L. acidophilus* NCFM, and the activation was started as early as 0.5 h after treatment. The data suggested that probiotic *L. acidophilus* NCFM could upregulate the expression of the pattern recognition receptor molecule TLR2 in Caco-2 cells.

**Activation of NF-κB and p38 MAPK signaling pathways in Caco-2 cells stimulated with L. acidophilus NCFM.** TLRs have been shown to lead to the activation of the NF-κB and p38 MAPK signaling pathways, which were important in the production of many immune-related factors, including cytokines and chemokines (25). Therefore, in order to examine whether the NF-κB and p38 MAPK signaling pathways have been activated, the activation state of these two signaling pathways was studied when Caco-2 cells were stimulated with *L. acidophilus* NCFM at an MOI of 10 for 0 to 4 h. As shown in Fig. 4A, *L. acidophilus* NCFM could activate the p38 MAPK signaling pathway in IEC. The levels of p38 MAPK phosphorylation increased until 2 h and then slowly de-
creased, despite persistent bacterial stimulation. To verify the activation of the NF-κB signaling pathway, cell lysates were analyzed for levels of phosphorylated NF-κB p65, since phosphorylation of the NF-κB p65 subunit was associated with the activation of the NF-κB signaling pathway (11). *L. acidophilus* NCFM could rapidly activate the NF-κB signaling pathway with a kinetics similar to that for the p38 MAPK signaling pathway (Fig. 4B). These results demonstrated that the NF-κB and p38 MAPK signaling pathways were transiently activated when *L. acidophilus* NCFM stimulated Caco-2 cells and that this activation occurred before a significant increase in cytokine and chemokine expression (Fig. 1).

To further test whether the NF-κB and p38 MAPK signaling pathways are necessary for cytokine and chemokine production, the Caco-2 cells were stimulated with or without the existence of PDTC, a specific inhibitor for NF-κB, or SB203580, a specific inhibitor for p38 MAPK. The Caco-2 cells were preincubated with PDTC (40 μM) or SB203580 (20 μM) for 30 min then treated with *L. acidophilus* NCFM for 2 h. Inhibition of the NF-κB or p38 MAPK signaling pathway resulted in a partial yet significant decline (P < 0.05) in cytokine and chemokine expression compared to results for the uninhibited groups treated with bacteria only (Fig. 5). The above results suggested that *L. acidophilus* NCFM could rapidly induce IL-1α, IL-1β, CCL2, and CCL20 production through NF-κB and p38 MAPK signaling pathways in Caco-2 cells.

**DISCUSSION**

It is well known that cytokines and chemokines, which affect the immune cells scattered in the GI tract and recruit immune cells to the GI tract, respectively, play a major role in mediating immune and intestinal inflammatory responses (13, 19, 24, 36). Recently, it was been reported that commensal bacteria, such as *L. rhamnosus*, *L. acidophilus*, and *E. coli*, could upregulate the production of many members of the cytokine and chemokine family, such as IL-1, CCL2, and CCL20 (3, 11, 21), although some studies have shown that the intestine appeared to be tolerant toward commensal bacteria (23, 29).

In line with these studies, our data also showed that *L. acidophilus* NCFM induced the production of some cytokines (IL-1α and IL-1β) and chemokines (CCL2 and CCL20) which were of crucial importance in the control of normal homeostasis and host gut immunity. IEC showed a rapid but transient upregulation of cytokines and chemokines (Fig. 3) despite the persistence of bacte-
recognition molecules, such as TLRs, which are thought to recognize the signature molecules of microorganisms during the early period of innate immune responses (21, 26). It has been reported that IEC could induce TLR2 and TLR4 when responding to Gram-positive bacteria, but TLR2 was mainly involved in response to Gram-positive bacteria (6, 18, 21, 26). Commensal bacteria, such as L. casei, L. rhamnosus, L. plantarum and B. lactis, all activated TLR2 in many cells, including IEC and macrophages (6, 18, 33). In this study, we found that the expression of TLR2 was upregulated in a rapid manner in IEC after treatment with L. acidophilus NCFM compared to results for the unstimulated controls (Fig. 3), which is in line with a study that showed that L. acidophilus NCFM could activate TLR2 in HEK293 cells (20), while others indicated that the mouse fetal epithelial cells were nonresponsive to the expression of TLR2 after L. acidophilus NCFM stimulation (40). It is likely that different cells respond differently even to the same bacteria (5).

The consequences of signaling through TLRs have been reported to trigger both NF-κB and p38 MAPK activation. These play important roles in the production of cytokines and chemokines involved in regulating immune responses (25). Y. G. Kim and colleagues have shown that the p38 MAPK signaling pathway was important for the production of cytokines in L. casei-treated mouse spleen cells, whereas NF-κB P65 also contributed but to a lesser extent (18). B. lactis has also been shown to induce cytokine IL-6 gene expression in IEC through the NF-κB and p38 MAPK signaling pathways (33). In this study, phosphorylation of NF-κB p65 and p38 MAPK in Caco-2 cells was shown to be rapidly but transiently enhanced in the L. acidophilus NCFM-treated groups (Fig. 4), indicating that both signaling pathways could be activated by L. acidophilus NCFM. Consistent with our findings, previous studies also showed that the direct contact of L. acidophilus NCFM with IEC was able to activate the NF-κB pathway (32). Inhibition of the NF-κB or p38 MAPK signaling pathway, using the specific inhibitor PDTC or SB203580, respectively, significantly reduced cytokine (IL-1α and IL-1β) and chemokine (CCL2 and CCL20) production in Caco-2 cells after stimulation by L. acidophilus NCFM (Fig. 5). These results suggested that activation of both NF-κB and p38 MAPK could play an important role in augmenting the production of cytokines and chemokines by L. acidophilus NCFM. It has been reported that p38 MAPK has numerous direct and indirect interactions with NF-κB (4, 34), so it is necessary to further examine the role of the interactions of the p38 MAPK and NF-κB signaling pathways in cytokine and chemokine production.

Interestingly, both in vivo and in vitro data demonstrated that the cytokines (IL-1α and IL-1β) showed a lower expression level with L. acidophilus NCFM treatment than chemokines (CCL2 and CCL20) (Fig. 1 and 2). Some studies also showed that expression of proinflammatory cytokines secreted by IEC stimulated with an agonist or bacteria was generally much lower than that observed for the chemokines (8, 15). IL-1α and IL-1β are proinflammatory mediators which have been shown to induce chemokine responses. IL-1α can upregulate CCL20 mRNA expression and protein production in IEC lines, including Caco-2 cells and HT-29 cells (14). IL-1β has been shown to significantly induce CCL2 and CCL20 expression in Caco-2 cells or macrophages (10, 39). Fichorova et al. and Perkins also demonstrated that IL-1 would induce the secretion of chemokines such as CCL2 via the NF-κB signaling pathway (9, 30). In line with these reports, the NF-κB signaling pathway was reported to be important for IL-1β-stimulated CCL2 production in rat astrocytes, and the MAPK signaling pathway also contributed (37). In addition, IL-1β was able to induce phosphorylation of p38 MAPK in IEC-6 cells (2). Taken together, the synergism between cytokines, chemokines, and L. acidophilus NCFM may be explained as follows. L. acidophilus NCFM induced an early-phase response with subsequent cytokine (IL-1α and IL-1β) and chemokine (CCL2 and CCL20) production through the TLR2-mediated NF-κB and p38 MAPK signaling pathways in Caco-2 cells. Then, the secreted cytokines (IL-1α and IL-1β) might have further stimulated the cells through the NF-κB and p38 MAPK signaling pathways, which initiated a late-phase response to express the chemokines (CCL2 and CCL20). However, further studies would be required to determine whether IL-1α and IL-1β have important roles as chemokine-inducing factors in L. acidophilus NCFM-stimulated Caco-2 cells.

In this study, our data demonstrated that the commensal bacterium L. acidophilus NCFM can induce cytokine and chemokine production in IEC, with the cytokines showing a lower expression level with bacterial treatment than chemokines. This may help to provide important insights to elaborate the host immune responses triggered by probiotic bacteria. Moreover, L. acidophilus NCFM could induce TLR2 signaling to trigger cytokine and chemokine expression in IEC through the NF-κB and p38 MAPK signaling pathways, and the activation in IEC after L. acidophilus NCFM stimulation is rapid but transient. Although we examined the signaling pathways involved, the study does not fully reveal the mechanisms, and further research is needed. Together, this study allows for a better understanding of how L. acidophilus NCFM contributes to the immune responses of the host, and it will be important in establishing the basis for further studies on the molecular mechanisms of interactions between commensal bacteria and the host.

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