

***Lactobacillus rhamnosus* GG and *Lactobacillus casei* Suppress *Escherichia coli*-Induced Chemokine Expression in Intestinal Epithelial Cells**

Shinji Toki^{a,b} Shinji Kagaya^a Miwa Shinohara^{a,c} Hiroshi Wakiguchi^c
Takashi Matsumoto^b Yoshihisa Takahata^b Fumiki Morimatsu^b Hirohisa Saito^a
Kenji Matsumoto^a

^aDepartment of Allergy and Immunology, National Research Institute for Child Health and Development, Tokyo,

^bR&D Center, Nippon Meat Packers, Inc., Tsukuba, and ^cDepartment of Pediatrics, Kochi Medical School, Kochi, Japan

Key Words

Lactobacillus • Chemokine • Intestinal epithelial cells • Intestinal microbiota

Abstract

Background: Recently, some strains of lactic acid bacteria (LAB) have been reported to prevent the development of atopic dermatitis and to improve allergic symptoms, especially in young children. However, the mechanisms involved in these effects are not fully understood. Intestinal microbiota play critical roles in the development of host immune development and are recognized and regulated by the host through intestinal epithelial cells (IECs). We thus hypothesized that LAB influence the host immune system through the activation of IECs. To begin testing this hypothesis, chemokine expression in IECs exposed to intestinal bacteria was investigated. **Methods:** Caco-2 cell monolayers were stimulated with different concentrations of various live or heat-killed intestinal bacteria or bacterial components for up to 3 h. Changes in the gene expressions of various chemokines were measured using quantitative real-time PCR. **Results:** The expressions of CCL20, CXCL8, CXCL10 and CX3CL1 were strongly induced by nonpathogenic *Escherichia coli* in a

dose-dependent manner and were partially induced by some commensal LAB. In contrast, *Lactobacillus rhamnosus* GG (LGG) and *Lactobacillus casei* did not induce these chemokine expressions. In addition, LGG significantly suppressed the expressions of CCL20 and CXCL10 induced by *E. coli*, peptidoglycan or flagellin when cultured simultaneously. **Conclusions:** LGG and *L. casei* markedly suppressed *E. coli*-induced chemokine expression, presumably through the suppression of the Toll-like receptor-mediated signal transduction pathway, at least in part. The clinical importance of this suppressive effect and the mechanisms involved require further investigation; however, such effects can be used as a marker to identify clinically useful LAB.

Copyright © 2008 S. Karger AG, Basel

Introduction

The onset of allergic diseases is thought to be regulated by an interaction between genetic and environmental factors [1, 2]. After the first report by Strachan [3] in 1989, several environmental factors, including exposure to viral and bacterial microorganisms during early childhood, have been shown to play important roles in the on-

set of allergic diseases [4–6]; these associations are now referred to as the ‘hygiene hypothesis’. On the other hand, intestinal microbiota, an internal environment, might also be involved in the onset of allergic diseases [7].

Intestinal microbiota consist of approximately 10^{14} bacteria that can be classified into more than 400 species, almost half of which remain unclassified to date. Intestinal microbiota are thought to play an important role in the development of the immune system in the host [8] because mucosal immunity is impaired in germ-free mice [9]. Intestinal microbiota have also been reported to play a critical role in the induction and maintenance of oral tolerance [10].

Not just the presence or absence of intestinal microbiota, but the type of bacteria is also a key issue. Children with allergies reportedly have a lower frequency of colonization with lactobacilli, compared with nonallergic children, whereas children with allergies tend to harbor higher counts of aerobic microorganisms [11]. This observation has been further confirmed by a couple of prospective studies [12, 13]. In addition, the colonization of *Bifidobacterium*-like bacteria and *Lactobacillus*-like bacteria in infants born by cesarean delivery was delayed, compared with infants born by vaginal delivery [14], and the prevalence of adulthood asthma is significantly higher among subjects born by cesarean delivery [15]. These epidemiological facts indicate that some mechanisms for recognizing types of bacteria in the gut must exist during early infancy, with subsequent consequences for host immune development.

Indeed, the administration of lactic acid bacteria (LAB) prenatally to pregnant woman at risk and postnatally to newborn infants significantly reduced the prevalence of atopic dermatitis in early childhood [16, 17]. In addition, the administration of LAB also improved the symptoms or severity of young children with atopic dermatitis or food allergy [18–20].

However, the administration of LAB produced no clinical benefits in some interventional trials [21, 22]. This finding can be explained by the heterogeneity of allergic diseases and of patients in terms of age, phenotype, severity or environmental factors [23]. However, an additional possibility is that different strains of LAB may have been used. As a matter of fact, several different strains of LAB were used, including *Lactobacillus rhamnosus* GG (LGG) and mixtures containing LGG [16, 19, 24–26], *Bifidobacterium lactis* (Bb-12) [27, 28], *Lactobacillus paracasei* [29] and other strains [30, 31]. The effects of each LAB strain differ, with LGG generally providing a better outcome than the other strains, with a few excep-

tions, suggesting that the host immune system can distinguish beneficial strains of LAB from others or from normal commensal bacteria in the gut microbiota. However, the mechanisms by which some, but not all, LAB exert beneficial effects remain unclear.

So far, several in vitro attempts have been made to clarify the different signals provided by different strains of intestinal bacteria using peripheral blood mononuclear cells [32–34], mouse splenocytes [29] or dendritic cells [35]; these studies have produced valuable information. Of note, commensal bacteria have no opportunity to come into direct contact with peripheral blood mononuclear cells or splenocytes in vivo. In other words, these in vitro models are thought to mimic bacteremia or bacterial invasion in vivo; thus, the production of huge amounts of inflammatory cytokines, such as tumor necrosis factor- α or interleukin (IL)-6, was also observed.

Intestinal epithelial cells (IECs) normally function as a physical barrier and are constantly exposed to intestinal bacteria without inducing inflammatory reactions or the production of inflammatory cytokines [36]. IECs are capable of distinguishing pathogenic from nonpathogenic bacteria [37, 38]; thus, the responses of IECs participate in the formation and function of gut-associated lymphoid tissues (GALT). In particular, chemokines released from IECs play important roles in the selective recruitment of leukocytes to the GALT [39].

We hypothesize that intestinal bacteria influence the host immune system through the induction of chemokines in IECs, which in turn recruit specific types of leukocytes to the GALT to regulate subsequent immune responses, either promoting or suppressing the development of allergies. To begin testing this hypothesis, the chemokine gene expressions in IECs exposed to intestinal bacteria were investigated.

Materials and Methods

Bacteria Strains and Preparation of Heat-Killed Bacteria

Two LAB strains, LGG (ATCC 53103) and *Pediococcus pentosaceus* (ATCC 33316), as well as *E. coli* (ATCC 29425) were purchased from American Type Culture Collection (ATCC; Rockville, Md., USA). The other 5 LAB strains, *L. casei*, *Lactobacillus acidophilus*, *Streptococcus thermophilus*, *Lactococcus lactis* and *B. infantis*, were provided by Nippon Luna Inc. (Kyoto, Japan). All LAB strains and *E. coli* were cultured aerobically at 37°C for 20 h in MRS broth (Difco Laboratories, Detroit, Mich., USA) or nutrient broth (Difco Laboratories), respectively. After counting the viable bacteria, they were killed by heating at 70°C for 20 min. Heat-killed bacteria were centrifuged at 8,000 rpm for 10 min,

Table 1. Primers for RT-PCR and real-time RT-PCR

Target gene	Genbank accession No.	Forward sequence (5'–3')	Reverse sequence (5'–3')	Amplicon size, bp
TLR1	NM_003263	CTATACACCAAGTGTGAGC	GTCTCCAACTCAGTAAGGTG	220
TLR2	NM_003264	GCCAAAGTCTTGATTGATTGG	TTGAAGTTCTCCAGCTCCTG	347
TLR3	NM_003265	GCTGGAAAATCTCCAAGAGC	CCGAATGCTTGTGTTTGCTA	247
TLR4	NM_138554	AGTTGAACGAATGGAATGTGCA	CTGAGGACCGACACACCAATG	111
TLR5	NM_003268	AGATGGCTGGTGCCTTGAA	CCAACCACCACCATGATGA	87
TLR6	NM_006068	CCTCCCAGGATCAAGGTACTTG	ATCAGGCCAGCCCTCTAACAC	327
TLR7	NM_016562	TCTACCTGGGCCAAAAGTGT	GGCACATGCTGAAGAGAGTTA	388
TLR8	NM_138636	CCGACTTGGAAGTTCTAGATC	AATGCTTCATTTGGGATGTGCT	316
TLR9	NM_017442	GCTGCGTCTCCGTGACAATTA	AGCTGACATCCAGCCTCCG	158
TLR10	NM_030956	GGCAAACCTTCGAGCTGCTATT	TCCCAACAGTGTATGTGGTCCC	179
CARD4	NM_006092	CCTGCAGCAGAACACGTCTCT	GGCAGTCCCCTTAGCTGTGAT	154
CARD15	NM_022162	GGCTCTGTATTTGCGCGATAA	ACAGCCGTCAGTCAATTTGTTG	124
CCL20	NM_002990	GGCCAATGAAGGCTGTGACAT	TGGATTTGCGCACACAGACA	73
CXCL8	NM_000584	GTCTGCTAGCCAGGATCCACAA	GAGAAACCAAGGCACAGTGGAA	51
CXCL10	NM_001565	GCCAATTTTGTCCACGTGTTG	AGCCTCTGTGTGGTCCATCCT	193
CX3CL1	NM_002996	TGCTGGCTGGTTAGAGGTTT	TGTGTGAGATGGGAGGACTG	123
CCL22	NM_002990	TACTCTGATGACCGTGGCCTTG	AGAGAGTTGGCACAGGCTTCTG	100
CCL28	NM_148672	CTGATGGGGATTGTGACTTG	TGTGCCCTGTTACTGTTCTCTC	181
GAPDH	NM_002046	GAAGGTGAA GGTCGGAGTC	GAAGATGGTGATGGGATTTC	226

washed with PBS and were resuspended at each concentration and stored at -70°C until use.

Intestinal Epithelial Cells

The human colon adenocarcinoma cell line Caco-2 was purchased from Dainippon Sumitomo Pharma Co., Ltd. (Tokyo, Japan) and cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM; Gibco, Gaithersburg, Md., USA) supplemented with 10% FBS (Equitech-Bio, Ingram, Tex., USA), 1% nonessential amino acid solution (Gibco), 0.1% penicillin and streptomycin (Gibco) at 37°C in an atmosphere of 5% CO_2 , 95% air. The culture medium was renewed every other day. To obtain a monolayer, Caco-2 cells were seeded on a 12-well cell culture insert (0.4- μm microporous filters; Falcon, BD Biosciences, Franklin Lakes, N.J., USA). Two weeks later, this resulted in a tight polarized monolayer displaying a transepithelial electrical resistance of at least $150\ \Omega/\text{cm}^2$.

Stimulation of IECs

The apical surfaces of the Caco-2 cell monolayers were stimulated with 1×10^6 CFU/ml of live *E. coli*, LGG or *L. casei* for 1 h or with up to 5×10^{11} CFU/ml of heat-killed *E. coli* or an LAB strain for 3 h at 37°C , 5% CO_2 . The Caco-2 cells were then washed with PBS to remove excess bacteria and lysed in RLT buffer for the following RNA extraction (RNeasy Kit, Qiagen, Valencia, Calif., USA). In some experiments, Caco-2 cells were stimulated with 1×10^{10} CFU/ml of *E. coli* with various concentrations of LAB or 10, 20 and 40 mM of butyrate (Wako Pure Chemicals, Osaka, Japan) for 3 h. In other experiments, the Caco-2 cells were stimulated with 10 $\mu\text{g}/\text{ml}$ of peptidoglycan (PGN; Invivogen, San Diego, Calif., USA), 10 $\mu\text{g}/\text{ml}$ of double-stranded RNA (poly I:C;

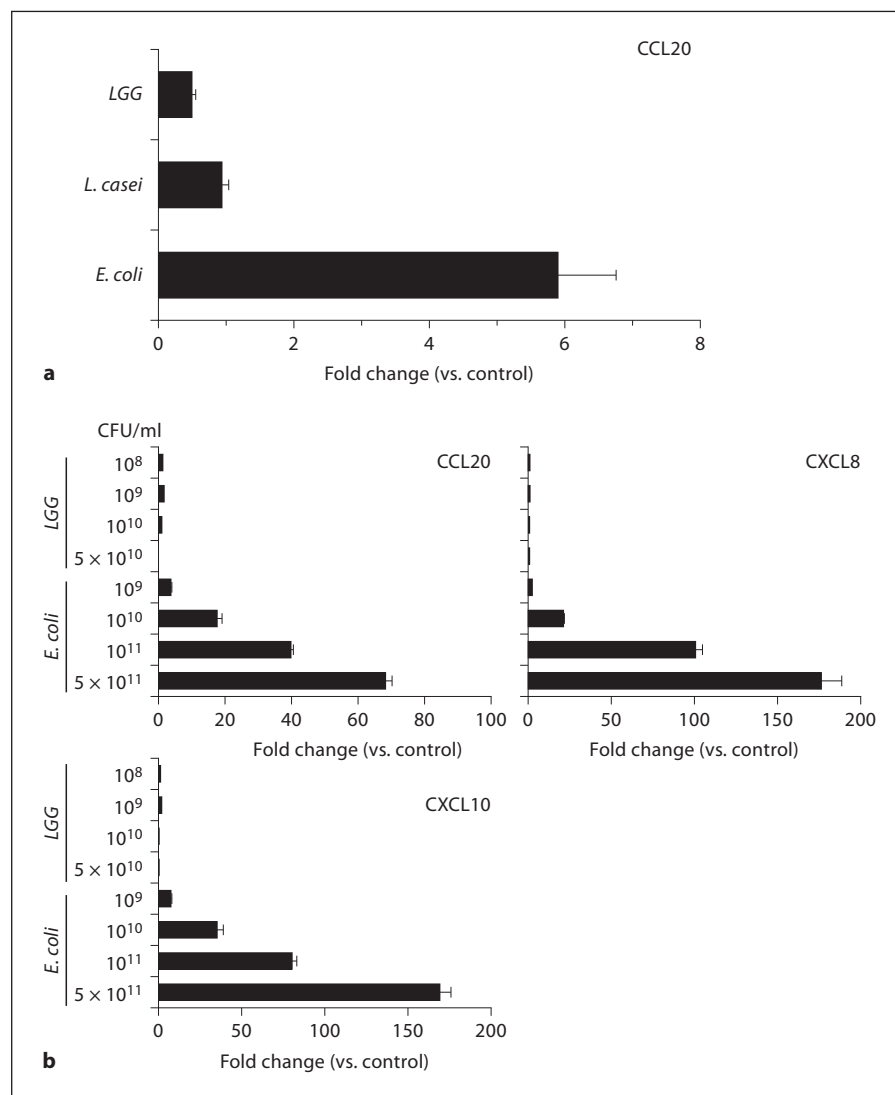
Invivogen), 1 $\mu\text{g}/\text{ml}$ of lipopolysaccharide (LPS; Sigma, St. Louis, Mo., USA), 1 $\mu\text{g}/\text{ml}$ of flagellin (Alexis Biochemicals, San Diego, Calif., USA), or 5 $\mu\text{g}/\text{ml}$ of CpG oligonucleotides (CpG 2006, 5'-tcgtcggtttgtcgtttgtcgtt-3', and CpG 2216, 5'-ggGGGACGATC-GTCgggggG-3'; Operon Biotechnology, Tokyo, Japan) [40] for 6 h. The cell culture supernatants were collected from the basolateral compartments and frozen at -20°C until further measurement.

Real-Time PCR and RT-PCR

Total RNA was extracted from Caco-2 cells using the RNeasy Kit (Qiagen) and was digested using RNase-free DNase I (Qiagen) following the manufacturer's instructions. For RT-PCR, an aliquot of 1 μg total RNA was reverse transcribed using iScript (Bio-Rad, Hercules, Calif., USA). cDNA generated from 50 ng of total RNA was amplified using Platinum Taq PCR SuperMix (Invitrogen Inc., Carlsbad, Calif., USA).

Quantitative real-time PCR analyses were performed using the ABI Prism 7,700 Sequence Detection System (Applied Biosystems, Foster City, Calif., USA) and SYBR Green PCR reagents (Bio-Rad), as previously reported [41]. To determine the exact copy numbers of the target genes, the quantified concentrations of subcloned PCR fragments of CCL20 (LARC), CXCL8 (IL-8), CXCL10 (IP-10), CCL22 (MDC), CCL28 (MEC), CX3CL1 (fractalkine), heat shock protein A1 (HSPA1) and HSPA6 were serially diluted and used as standards in each experiment. Aliquots of cDNA equivalent to 5 ng of total RNA samples were used for each real-time PCR. Data were normalized using the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels in each sample. All primer sets used for the real-time PCR and RT-PCR were synthesized by Fasmac Co., Ltd. (Kanagawa, Japan) and are shown in table 1.

Fig. 1. Chemokine mRNA expression in Caco-2 cells after stimulation with *LGG*, *L. casei* or *E. coli*. **a** Caco-2 cell monolayers were stimulated with 10^6 CFU/ml of heat-killed *LGG*, *L. casei* or *E. coli* for 1 h. mRNA expression of CCL20 was examined using real-time PCR. **b** Caco-2 cell monolayers were stimulated with various concentrations of heat-killed *LGG* or *E. coli* for 3 h. mRNA expression of CCL20, CXCL8 and CXCL10 was examined using real-time PCR. Each bar shows the fold change in mRNA expression from the unstimulated control mRNA expression level. Values represent the mean $\pm$ SEM of 3 separate experiments.



Chemokines in the Culture Supernatant

The chemokine concentrations in the culture supernatant from the basolateral compartments of the Caco-2 monolayers were measured using ELISA kits that specifically recognized CCL20, CXCL8 or CXCL10 (R&D Systems, Minneapolis, Minn., USA). The minimal detection limits of these ELISA kits were 7.8, 31.2 and 7.8 pg/ml for CCL20, CXCL8 and CXCL10, respectively.

Statistical Analysis

All data are presented as the mean $\pm$ SEM, unless otherwise noted. Differences between groups were analyzed using the paired Student t test and were considered significant if $p < 0.05$.

Results

Chemokine Expression in Caco-2 Cells after Stimulation with *LGG* or *E. coli*

After forming a tight monolayer, Caco-2 cells were stimulated with live or heat-killed *LGG* or *E. coli* for up to 3 h, and the expression of mRNA for 3 chemokines, CCL20, CXCL8 and CXCL10, was examined using quantitative real-time PCR with specific primer sets. The expression of CCL20 was significantly induced by live commensal, nonpathogenic *E. coli* but not by live *LGG* or live *L. casei* at a concentration of 1×10^6 CFU/ml after 1 h of incubation (fig. 1a). The expressions of CCL20, CXCL8 and CXCL10 were also strongly induced by heat-killed *E. coli* in a dose-dependent manner (fig. 1b). At the highest

concentration of 5×10^{11} CFU/ml, heat-killed *E. coli* induced a 65- to 160-fold increase in mRNA for all 3 chemokines. In contrast, almost no induction of these 3 chemokines was observed in Caco-2 cells stimulated with heat-killed *LGG*, even at the highest concentration of 5×10^{10} CFU/ml.

Chemokine Expression in Caco-2 Cells after Stimulation with Various LAB

Caco-2 cells were stimulated with various heat-killed LAB or *E. coli* for 3 h, and the expression of mRNA for 6 chemokines, CCL20, CXCL8, CXCL10, CX3CL1, CCL22 and CCL28, was examined using real-time PCR with specific primer sets. Heat-killed *E. coli* significantly induced the expression of all the chemokines except for CCL28 in a dose-dependent manner (fig. 2). In sharp contrast, heat-killed *LGG* and *L. casei* showed almost no induction of these 6 chemokines, even at the highest concentrations used. Other LAB strains, *L. acidophilus*, *S. thermophilus*, *L. lactis*, *P. pentosaceus* and *B. infantis*, induced a certain amount of mRNA expression for these chemokines, mostly in a dose-dependent manner. The expression of CCL28, a homeostatic chemokine, was unchanged in Caco-2 cells after stimulation with any of these LAB strains or *E. coli* (fig. 2).

Chemokine Concentration in the Culture Supernatant from the Basolateral Compartment of the Caco-2 Monolayer

Caco-2 cells were stimulated with various heat-killed LAB or *E. coli* for 24 h at a concentration of 10^{10} CFU/ml or with *S. thermophilus* at a concentration of 10^9 CFU/ml. Chemokine concentrations in the culture supernatant from the basolateral compartment were measured with ELISA kits. Heat-killed *E. coli* induced 54.7, 8.9 and 18.3 pg/ml of CCL20, CXCL8 and CXCL10, respectively. In contrast, all LAB strains induced a trace amount of these chemokines, which were close to or below the detection limit of each ELISA kit.

Effect of LGG or Butyrate on the E. Coli-Induced Chemokine Expression in Caco-2 Cells

Caco-2 cells were stimulated with 10^{10} CFU/ml of heat-killed *E. coli* and various concentrations of heat-killed *LGG*, *L. casei* or butyrate for 3 h, and the expression of mRNA for 3 chemokines, CCL20, CXCL8 and CXCL10, was examined using real-time PCR. In the absence of LAB, *E. coli* significantly induced the expression of all 3 chemokines. The addition of *LGG* and *L. casei* significantly suppressed the *E. coli*-induced expression of all 3 chemokines

in Caco-2 cells and reversed the chemokine expressions to the control levels (fig. 3, upper and middle panels). The addition of butyrate suppressed the expression of *E. coli*-induced CXCL8 and CXCL10 in a dose-dependent manner; however, 10 and 20 mM of butyrate significantly enhanced the expression of CCL20 (fig. 3, bottom panels).

Expression and Function of Toll-Like Receptors in Caco-2 Cells

The mRNA expressions of Toll-like receptors (TLRs) in Caco-2 cells were evaluated using conventional RT-PCR and primer sets specific to each TLR (table 1). As shown in figure 4a, the Caco-2 cells expressed TLR2, TLR3, TLR5, TLR6, TLR7, TLR9 and CARD4 (NOD1), but not TLR4, TLR8 or TLR10. The faint expressions of TLR1 and CARD15 (NOD2) were also detected. This result confirms the findings of a previous study [42]. Next, Caco-2 cells were stimulated with 10 µg/ml of PGN, 10 µg/ml of poly I:C, 1 µg/ml of LPS, 1 µg/ml of flagellin or 5 µg/ml of CpG 2006 for 6 h, and the chemokine expression was examined as described above. As a result, PGN and flagellin significantly induced the expression of CCL20, CXCL8 and CXCL10 (fig. 4b), whereas poly I:C and LPS showed only marginal effects. In addition, CpG 2006 (B-oligo) and CpG 2216 (A-oligo) had no effects on chemokine expression (data not shown).

Effect of LAB on Pathogen-Associated Molecular Patterns Induced Chemokine Expression in Caco-2 Cells

As shown in figure 4, the Caco-2 cells expressed functional TLR2 and TLR5. Thus, Caco-2 cells were cultured with various LAB in the presence of 10 µg/ml of PGN or 1 µg/ml of flagellin for 3 h, and the expressions of CCL20, CXCL8 and CXCL10 were evaluated in the manner described above. As a result, *LGG* significantly suppressed the expression of CCL20 and CXCL10 in Caco-2 cells after stimulation with PGN and flagellin. *LGG* also marginally suppressed the expression of CXCL8 in Caco-2 cells after stimulation with PGN, but did not alter the expression of CXCL8 when Caco-2 cells were stimulated with flagellin (fig. 5).

HSP Expression in Caco-2 Cells after Stimulation with Various LAB and Effect of HSP on E. coli-Induced Chemokine Expression

The expressions of HSPA1 and HSPA6 were significantly induced by a high concentration of heat-killed *LGG*. In contrast, the other strains of heat-killed LAB or *E. coli* failed to induce HSPA1 or HSPA6, even at the high-

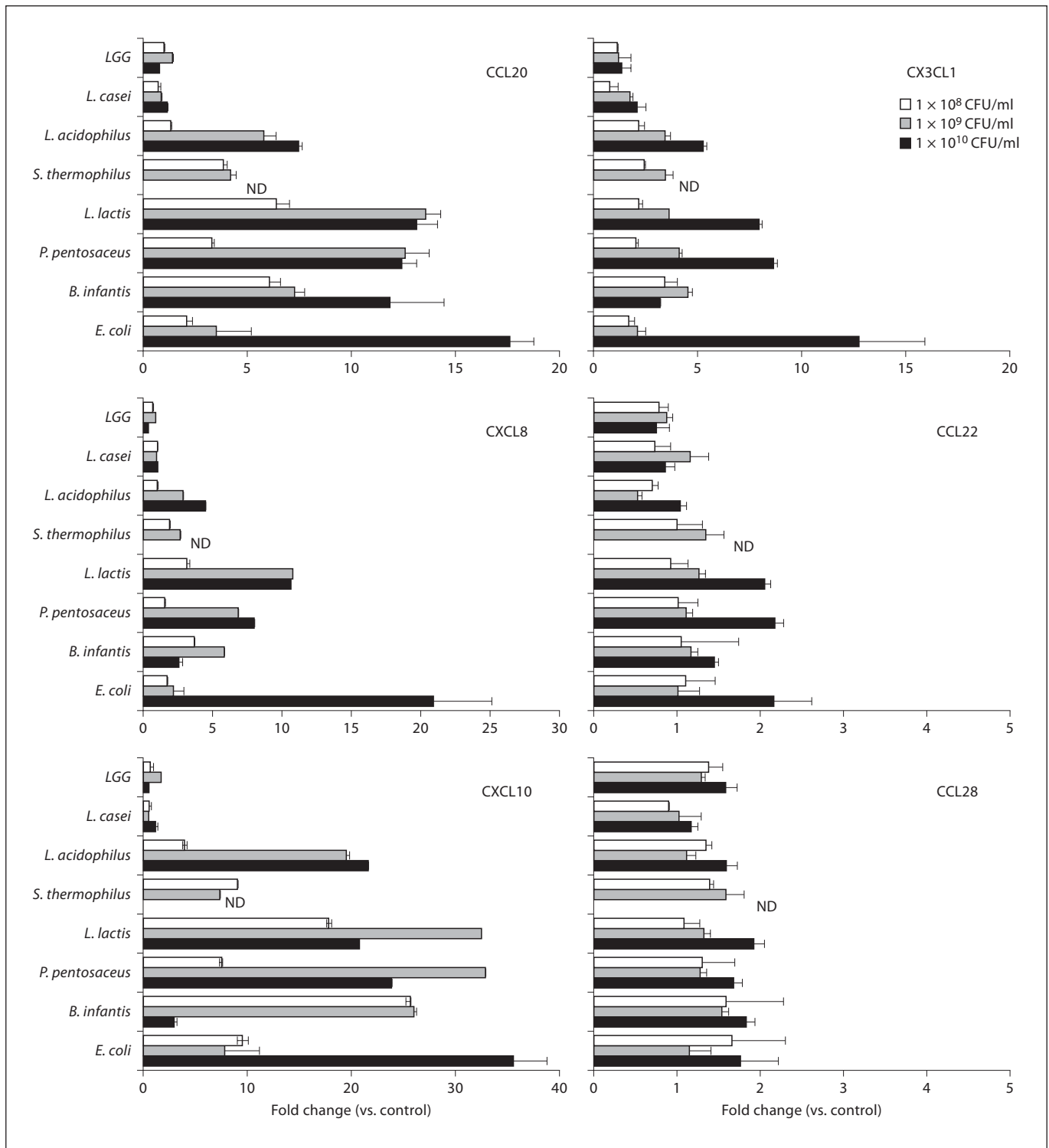


Fig. 2. Chemokine mRNA expression in Caco-2 cells after stimulation with various LAB. Caco-2 cell monolayers were stimulated with various concentrations of different LAB for 3 h. mRNA expression of CCL20, CXCL8, CX3CL1, CCL22 and CCL28 was examined using real-time PCR. Each bar shows the fold change in mRNA expression from the unstimulated control mRNA expression level. Values represent the mean $\pm$ SEM of 3 separate experiments. ND = Not done, because a concentration of 1×10^{10} CFU/ml of *S. thermophilus* could not be prepared using conventional centrifugation.

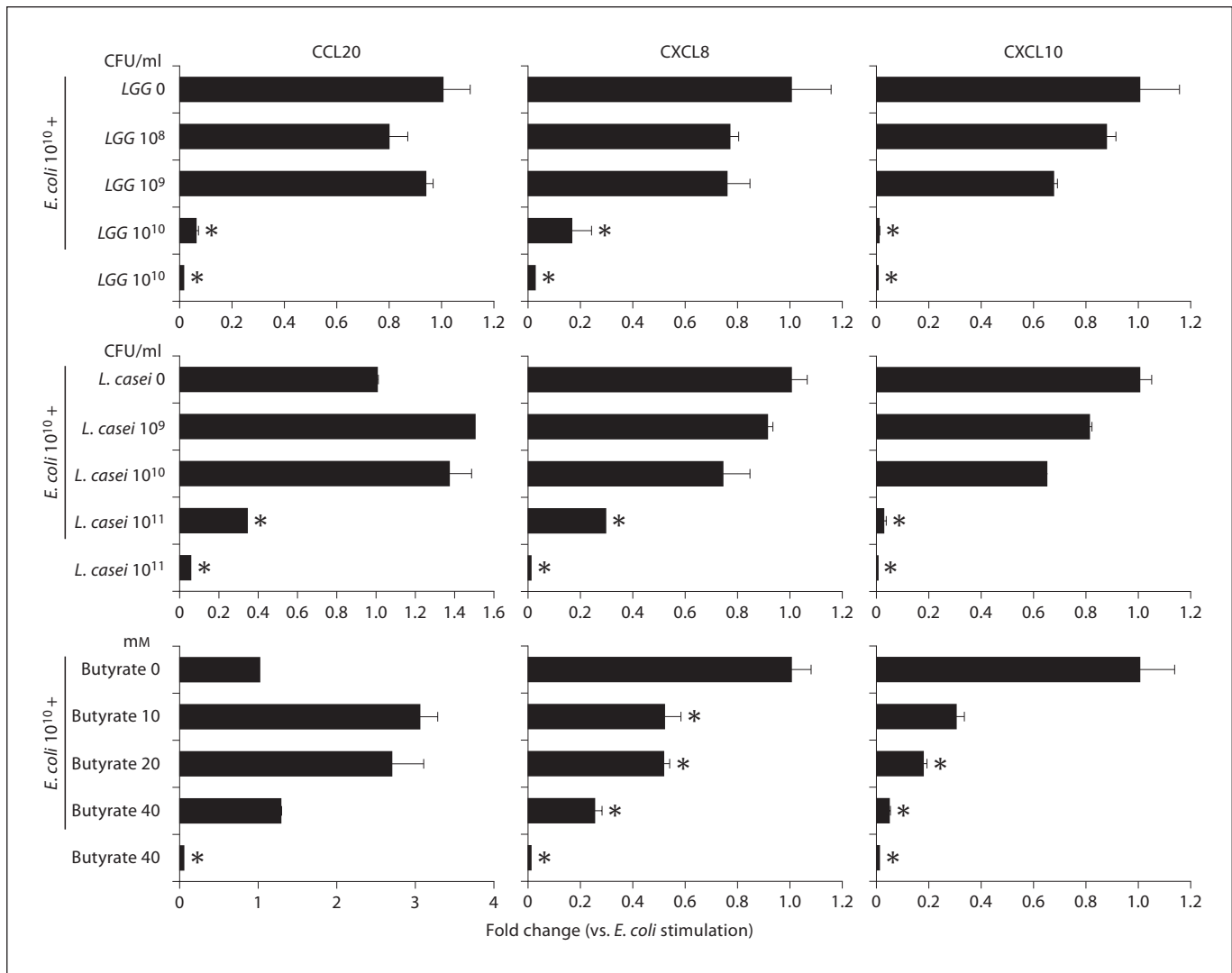


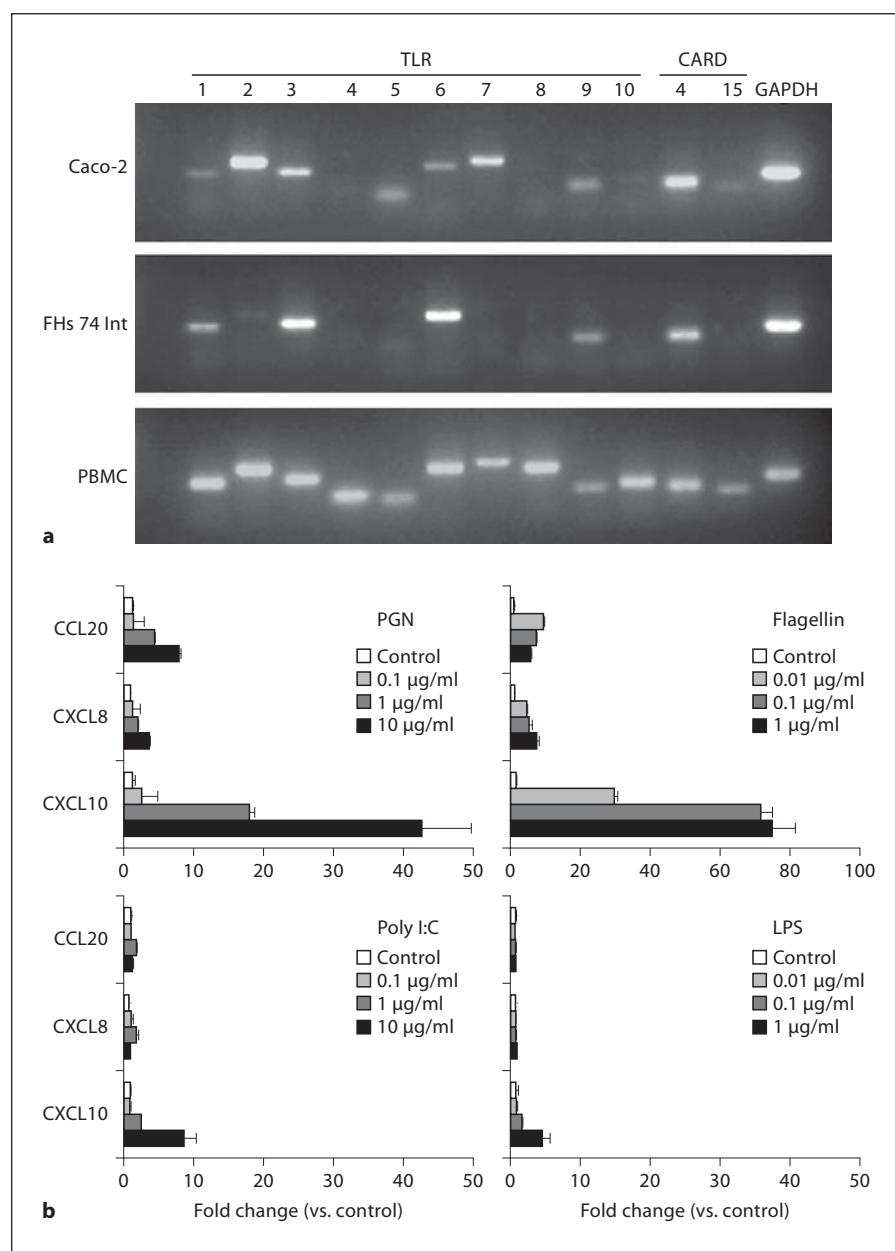
Fig. 3. Effect of LGG on *E. coli*-induced chemokine expression in Caco-2 cells. Caco-2 cell monolayers were stimulated with 1×10^{10} CFU/ml of *E. coli* and various concentrations of LGG or butyrate for 3 h. mRNA expression of CCL20, CXCL8 and CXCL10 was examined using real-time PCR. Each bar shows the fold change in mRNA expression from the unstimulated control mRNA expression level. Values represent the mean $\pm$ SEM of 3 separate experiments. * $p < 0.05$ compared with the mRNA expression level induced by *E. coli* alone.

est concentrations tested (fig. 6a). A high concentration (10^{10} CFU/ml) of heat-killed LGG and butyrate significantly induced both HSPA1 and HSPA6 expression in Caco-2 cells (fig. 6b). In contrast, 10^{10} CFU/ml of *E. coli* did not induce HSPA1 or HSPA6. When the Caco-2 cells were stimulated with *E. coli* and the highest concentrations of LGG or butyrate, a significant expression of both HSPA1 and HSPA6 was observed; however, the expression levels were lower than those found in cells simulated with LGG or butyrate alone.

Discussion

Intestinal microbiota, especially during early childhood, are associated with the onset and symptoms of allergic diseases, including atopic dermatitis. However, the mechanisms by which intestinal microbiota affect host immune responses are not fully understood. We hypothesize that in response to the microbiota, IECs produce and release chemokines that participate in the formation of GALT, because IECs are constantly exposed to micro-

Fig. 4. Expression and function of TLRs in Caco-2 cells. **a** mRNA expression patterns of TLR1–TLR10, CARD4 and CARD15 in Caco-2 and human fetal intestinal epithelial cells (FHs 74 Int, ATCC CCL-241) were examined using conventional RT-PCR. Peripheral blood mononuclear cells (PBMC) were used as a positive control. **b** Quantitative real-time PCR analysis of mRNA expression of CCL20, CXCL8 and CXCL10 in Caco-2 cells after stimulation with PAMPs. Caco-2 cell monolayers were stimulated with various concentrations of PAMPs for 6 h. Each bar shows the fold change in mRNA expression from the unstimulated control mRNA expression level. The values represent the mean $\pm$ SEM of 3 separate experiments.



biota and are reportedly capable of distinguishing commensal bacteria from pathogenic strains [37, 38]. To begin testing this hypothesis, we performed in vitro experiments in which IECs were exposed to intestinal bacteria, and the levels of chemokines in the IECs were measured.

In our first series of experiments, we compared chemokine expression in Caco-2 cells after stimulation with live commensal bacteria or LAB at a relatively low concentration (1×10^6 CFU/ml). We found that nonpatho-

genic *E. coli* significantly induced CCL20 expression in Caco-2 cells, whereas live LGG or *L. casei* did not. Next, we tried to confirm this phenomenon using different doses of live *E. coli* and LGG, since the size and shape of these bacteria vary and such differences may affect the distinct induction of chemokine expression shown in figure 1a. However, we found that Caco-2 cells were seriously damaged when they were treated with live bacteria at concentrations of more than 10^8 CFU/ml. Therefore, we used different doses of heat-killed bacteria to prove

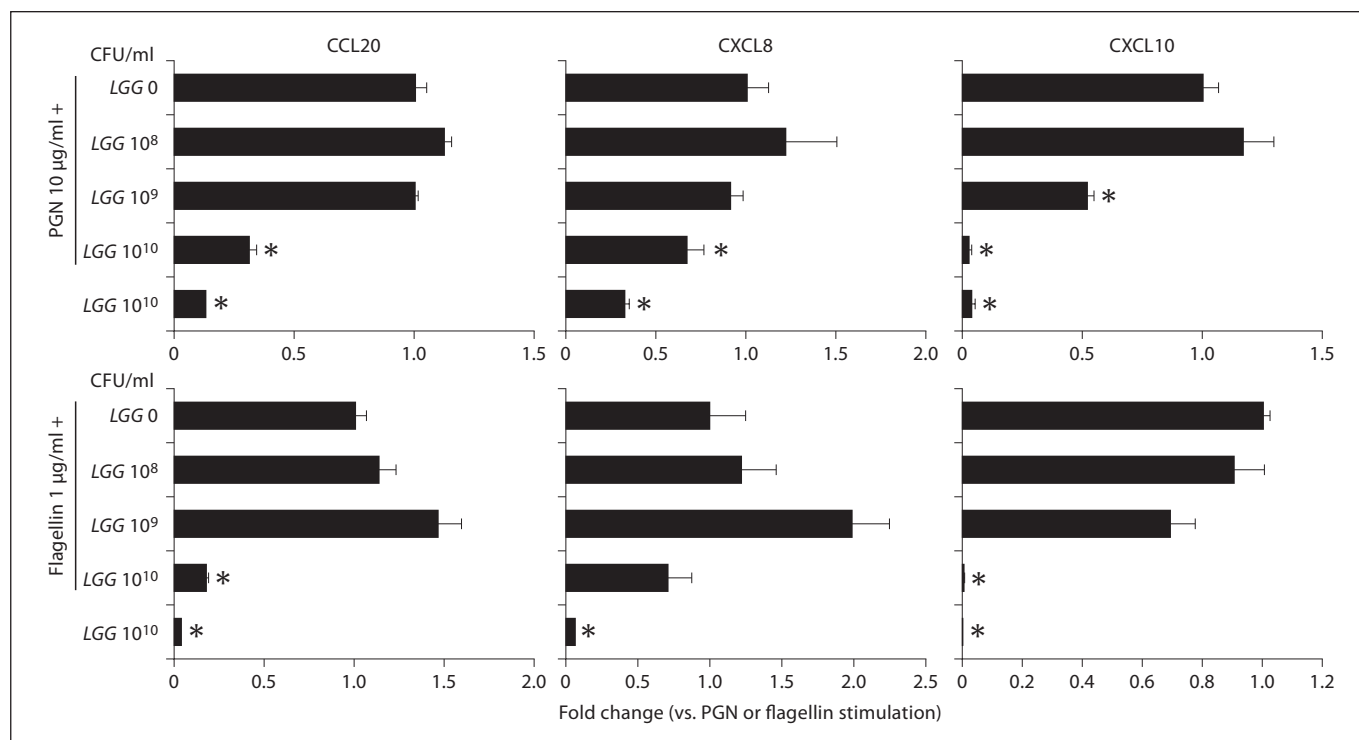


Fig. 5. Effect of *LGG* on PGN- and flagellin-induced chemokine expression in Caco-2 cells. Caco-2 cell monolayers were stimulated with 10 µg/ml of PGN (upper panels) or 1 µg/ml of flagellin (lower panels) and various concentrations of *LGG* for 3 h. mRNA expression of CCL20, CXCL8 and CXCL10 was examined using real-time PCR. Each bar shows the fold change in mRNA expression from the unstimulated control mRNA expression level. Values represent the mean $\pm$ SEM of 3 separate experiments. * $p < 0.05$ compared with the mRNA expression level induced by PGN or flagellin alone.

the essential differences in the induction of chemokines in Caco-2 cells between *E. coli* and *LGG*.

We found that heat-killed *E. coli* significantly induced chemokine expression in IECs in a dose-dependent manner (fig. 1b), which confirmed the findings of a previous study [43]. In contrast, *LGG* did not significantly induce any of these 3 chemokines even at the highest concentration that was used. In addition, after 3 h of incubation, the viability was virtually unchanged when assessed using the trypan blue dye exclusion test and the amount of mRNA extracted from the cells; cell damage was minimal unless live bacteria were used. This fact clearly indicates that regardless of the size or shape of the bacterial body or its concentration, *LGG* and *E. coli* exerted fundamental differences in their induction of chemokines in IECs.

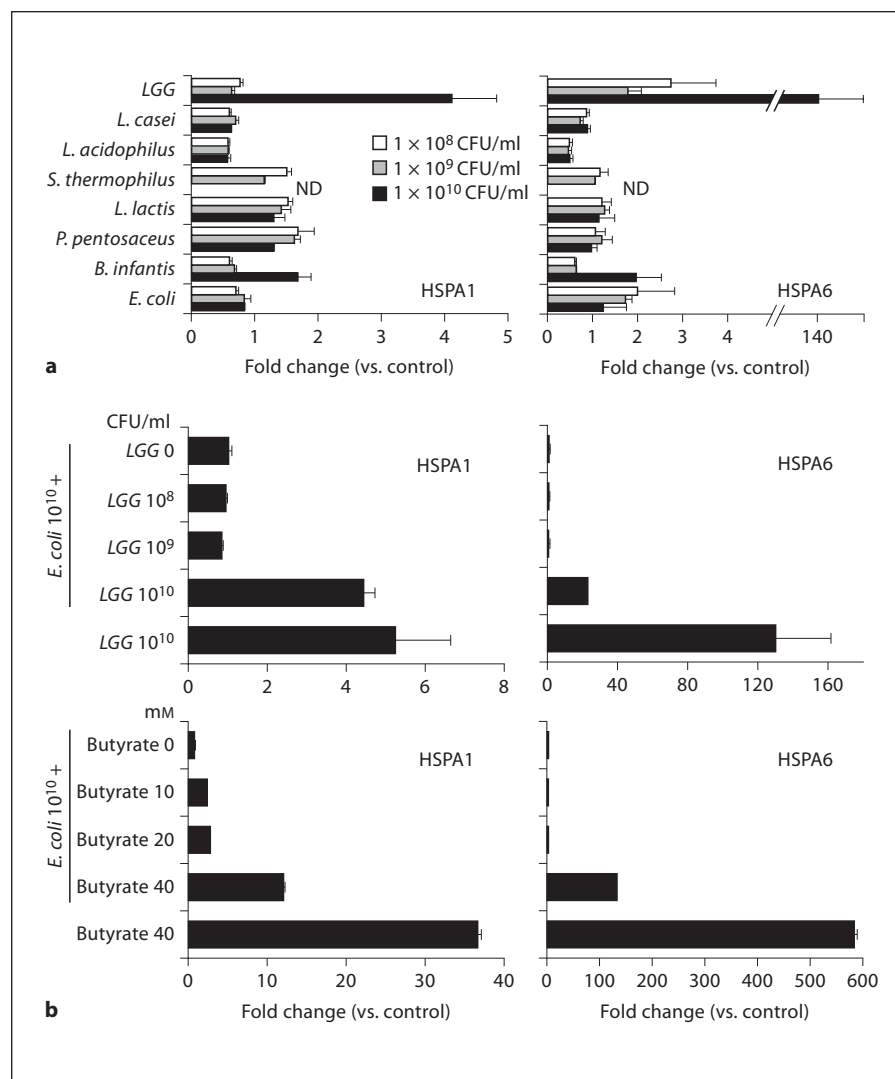
We next compared chemokine expression using different strains of LAB and found that heat-killed *LGG* and *L. casei* produced almost no induction of any of the 6 chemokines that were tested, whereas the other strains of

LAB induced some amounts of mRNA for these chemokines (fig. 2). The induction of these chemokines was not thought to be due to the carryover of LAB-cultured broth with a low pH, because virtually all the LAB-cultured broths had a similar pH. Therefore, some strain-specific signals may exist in IECs when cultured with intestinal bacteria.

To confirm that the chemokines were indeed produced by IECs, we measured the chemokine concentrations in the culture supernatant from the basolateral compartment. Heat-killed *E. coli* induced the release of some amounts of all 3 chemokines from IECs in the supernatant. In contrast, all heat-killed LAB induced almost no release of these chemokines. Thus, the mRNA expression of these chemokines in IECs seems to reflect protein synthesis.

In the following series of experiments, heat-killed *E. coli* and LAB were simultaneously added to wells, and the chemokine expression was investigated, since intestinal microbiota consist of hundreds of different types of

Fig. 6. HSP mRNA expression in Caco-2 cells after stimulation with various LAB or butyrate. **a** Caco-2 cell monolayers were stimulated with various concentrations of different LAB or *E. coli* for 3 h. mRNA expression of HSPA1 and HSPA6 was examined using real-time PCR. Each bar shows the fold change in mRNA expression from the unstimulated control mRNA expression level. Values represent the mean $\pm$ SEM of 3 separate experiments. ND = Not done, because a concentration of 1×10^{10} CFU/ml of *S. thermophilus* could not be prepared using conventional centrifugation. **b** Caco-2 cell monolayers were stimulated with 10×10^{10} CFU/ml of *E. coli* with various concentrations of LGG or butyrate for 3 h. mRNA expression of HSPA1 and HSPA6 was examined using real-time PCR. Each bar shows the fold change in mRNA expression from the unstimulated control mRNA expression level.



Gram-positive and -negative bacteria, and interactions between different strains may exist. As a result, *LGG* significantly suppressed the *E. coli*-induced expression of all 3 chemokines tested in a dose-dependent manner (fig. 3). A suppressive tendency on the expressions of CXCL8 and CXCL10 was clearly found in experiments with *LGG* and *L. casei*, whereas the suppression of CCL20 was only found at a bacterial concentration higher than 10^{10} CFU/ml. These findings suggested that some strain-specific induction and suppression mechanisms may exist and that the effect of LAB differs depending on the strain.

To clarify whether the suppressive effect of some LAB on *E. coli*-induced chemokine expression is mediated by a simple occupation of the surface area or by an inhibi-

tion of some intracellular signaling pathways, we tested whether LAB influenced chemokine expression by pathogen-associated molecular patterns (PAMPs) or not. Before stimulation with PAMPs, the expression of TLR in Caco-2 cells was examined using conventional RT-PCR, and the function of TLR was assessed by measuring PAMPs-induced chemokine expression using quantitative real-time PCR. The functional expression of TLR2, TLR6 and TLR5 in resting Caco-2 cells was observed (fig. 4), confirming the results of a previous report [43]. Therefore, we stimulated IECs with PGN or flagellin, a ligand for TLR2 and TLR5, respectively, in the presence of *LGG*. As a result, *LGG* significantly suppressed PAMPs-induced chemokine expression in Caco-2 cells (fig. 5). This fact clearly indicates that *LGG* are

capable of suppressing the TLR signal transduction pathway. We believe that this may, at least in part, explain how *LGG* suppress *E. coli*-induced chemokine expression in IECs.

Chemokines play critical roles in the formation and function of GALT [39]. CCL20, a ligand for CCR6, predominantly recruits dendritic cells [38]. CXCL8, a ligand for CXCR1 and CXCR2, predominantly recruits neutrophils. CXCL10, a ligand for CXCR3, predominantly recruits Th1-type T cells and natural killer cells [44], and in contrast, CCL22 predominantly recruits Th2-type T cells. The precise roles of these chemokines released from IECs in the onset of allergic disease mostly remain unknown. In addition, whether the suppression of these chemokines directly or indirectly influences the risk of allergic disease also remains uncertain. Very recently, Th17 cells have been reported to express CCR6, a receptor for CCL20 [45, 46]. Annunziato et al. [47] demonstrated that Th17 cells play critical roles in the pathogenesis of Crohn's disease. The oral administration of LAB has been reported to improve the clinical symptoms of Crohn's disease [48], suggesting that the suppression of CCL20 by LAB found in this study may reflect the mechanisms underlying this clinical observation, at least in part.

However, we would like to emphasize that the in vitro effects of LAB are not the same. Epidemiological [49] and intervention studies [18, 19, 29] also suggest that the in vivo effects of LAB are not the same. Some LAB reportedly suppress pathogenic bacteria-induced chemokine expression [50, 51]. Our study revealed that *LGG* and *L. casei* also suppressed nonpathogenic bacteria-induced chemokine expression in IECs [37, 38], whereas other LAB failed to suppress *E. coli*-induced chemokine expression.

Since *LGG* and *L. casei* are associated with better clinical outcomes, the lack of chemokine induction by IECs or the suppression of signaling pathways may mediate clinical benefits. Our findings shed new light on the potential mechanism for the prevention of or the improvement in allergic diseases. We hypothesized that beneficial LAB strains provide favorable clinical effects through the induction of some beneficial chemokine expression, thereby preventing the development of allergic diseases; however, our results could not prove this hypothesis. The lack of induction of chemokines by LAB themselves or the suppression of *E. coli*-induced chemokines may be a marker to identify beneficial LAB strains. Interestingly, cytokine production from splenocytes showed that most of the substrains of *LGG* and *L. casei* produce more IL-12

and less IL-4, whereas other LAB strains produce less IL-12 and more IL-4 [29].

Recently, apical, but not basolateral, stimulation of TLR9 in IECs was reported to suppress the activation of a transcription factor, nuclear factor (NF)- κ B, and to contribute to the maintenance of colonic homeostasis [52]. However, in our preliminary study, the DNA fraction extracted from *LGG* failed to suppress *E. coli*-induced chemokine expression in Caco-2 cells (data not shown). In addition, DNA from *E. coli* also contains CpG motifs. Thus, whether signaling through TLR9 by bacterial DNA really mediates the suppression induced by *LGG* and *L. casei* remains unproven. Recently, the induction of CCL20, CXCL8 and CXCL10 through the activation of NF- κ B via TLRs has been reported (CXCL10 is strongly induced by an interferon-dependent pathway; however, an NF- κ B-dependent pathway also plays a role) [53]. Thus, we speculate that *LGG* consequently suppresses activation of NF- κ B through unknown pathway(s). On the other hand, lipoteichoic acid from LAB has been reported to inhibit LPS-induced cytokine production from IECs [54]. However, whether or not the differences in lipoteichoic acid are responsible for the differences in their functions that we observed remains uncertain [55, 56].

Lastly, the induction of Hsp70 by *L. casei* was reported to be critical for the inhibition of *Salmonella*-induced CXCL8 induction [57]. Actually, the mRNA expression of 2 major components of Hsp70, HSPA1 and HSPA6, in Caco-2 cells was induced by 10^{10} CFU/ml or higher concentrations of heat-killed *LGG*. However, in our experiments, *L. casei* failed to induce both HSPA1 and HSPA6 (fig. 6a), even at a high concentration sufficient to suppress *E. coli*-induced chemokine expression (fig. 3). In addition, as previously reported [58, 59], HSPA1 and HSPA6 are induced by cellular stress, such as exposure to a high concentration of butyrate (fig. 6). However, lower concentrations (10 and 20 mM) of butyrate resulted in the marked suppression of *E. coli*-induced CXCL8 and CXCL10 expression (fig. 3, bottom panels), though such low concentrations of butyrate did not induce either HSPA1 or HSPA6 expression. Moreover, lower concentrations (10 and 20 mM) of butyrate actually suppressed CXCL8 and CXCL10 expression, whereas they enhanced CCL20 expression (fig. 3, bottom panels). Thus, as the induction of HSPA1 and HSPA6 is not correlated with the suppression of *E. coli*-induced chemokine expression, other factors are likely involved in the suppression of *E. coli*-induced chemokine expression by *LGG* and *L. casei*. Most LAB do not produce butyric acid; however, some bacterium strains in the intestinal microbiota are known

to utilize lactate and to produce butyrate [60]. In a clinical setting, fecal butyrate levels are reportedly increased by yogurt consumption [61]. In addition, butyrate plays an important role in intestinal barrier function [62]. Thus, we used butyrate as a positive control for the suppression of *E. coli*-induced chemokine expression and HSP induction.

Taken together, these findings suggest that further study is warranted to clarify the mechanisms by which some LAB strains significantly suppress chemokine production, while others do not. If these precise mechanisms can be clarified, this knowledge may help to establish new preventative strategies for allergic diseases.

In the present study, we used both live and heat-killed bacteria. Since Caco-2 cells were seriously damaged by live bacteria at high bacterial concentrations of more than 1×10^8 CFU/ml, we were unable to examine the chemokine expressions of live bacteria in Caco-2 cells. However, the effect of live bacteria should be examined further [63]. Because the bacteria that we used in most experiments were heat-killed, the adhesion of the bacteria is unlikely to be involved in the induction or suppression of chemokine expression [64, 65].

In conclusion, we found that the induction of chemokines by LAB differs depending on the strain of LAB and that induction was inversely associated with the inhibi-

tion of commensal *E. coli*-induced chemokine expression in IECs. The mechanisms by which different LAB produce different results in vitro requires further investigation; however, *LGG* and *L. casei* markedly suppressed *E. coli*-induced chemokine expression, presumably through the suppression of the TLR-mediated signal transduction pathway. The significance of this inhibition to the onset of allergic diseases requires further investigation; however, the induction of chemokines in Caco-2 cells by intestinal bacteria may be useful as a marker for predicting the effect of LAB in vitro. In a very recent review published in the *Journal of Allergy and Clinical Immunology*, Prescott et al. [66] suggested that the varied clinical effects of LAB administration may be explained by differences in the LAB strains that were used in the trials. Our findings may add a new method for selecting potentially effective strains of LAB.

Acknowledgments

The authors would like to thank Ms. Noriko Hashimoto (National Research Institute for Child Health and Development) for her skillful technical assistance. This work was supported in part by a grant (ID05-24) from the National Institute of Biomedical Innovation.

References

- Holgate ST: Genetic and environmental interaction in allergy and asthma. *J Allergy Clin Immunol* 1999;104:1139–1146.
- Sengler C, Lau S, Wahn U, Nickel R: Interactions between genes and environmental factors in asthma and atopy: new developments. *Respir Res* 2002;3:7.
- Strachan DP: Hay fever, hygiene, and household size. *BMJ* 1989;299:1259–1260.
- von Mutius E: Infection: friend or foe in the development of atopy and asthma? The epidemiological evidence. *Eur Respir J* 2001;18: 872–881.
- Gehring U, Bolte G, Borte M, Bischof W, Fahlbusch B, Wichmann HE, Heinrich J: Exposure to endotoxin decreases the risk of atopic eczema in infancy: a cohort study. *J Allergy Clin Immunol* 2001;108:847–854.
- Matricardi PM, Ronchetti R: Are infections protecting from atopy? *Curr Opin Allergy Clin Immunol* 2001;1:413–419.
- Wold AE: The hygiene hypothesis revised: is the rising frequency of allergy due to changes in the intestinal flora? *Allergy* 1998;53: 20–25.
- Macpherson AJ, Harris NL: Interactions between commensal intestinal bacteria and the immune system. *Nat Rev Immunol* 2004;4: 478–485.
- Rhee KJ, Sethupathi P, Driks A, Lanning DK, Knight KL: Role of commensal bacteria in development of gut-associated lymphoid tissues and preimmune antibody repertoire. *J Immunol* 2004;172:1118–1124.
- Moreau MC, Corthier G: Effect of the gastrointestinal microflora on induction and maintenance of oral tolerance to ovalbumin in C3H/HeJ mice. *Infect Immun* 1988;56: 2766–2768.
- Bjorksten B, Naaber P, Sepp E, Mikelsaar M: The intestinal microflora in allergic Estonian and Swedish 2-year-old children. *Clin Exp Allergy* 1999;29:342–346.
- Bjorksten B, Sepp E, Julge K, Voor T, Mikelsaar M: Allergy development and the intestinal microflora during the first year of life. *J Allergy Clin Immunol* 2001;108:516–520.
- Kalliomaki M, Kirjavainen P, Eerola E, Kero P, Salminen S, Isolauri E: Distinct patterns of neonatal gut microflora in infants in whom atopy was and was not developing. *J Allergy Clin Immunol* 2001;107:129–134.
- Gronlund MM, Lehtonen OP, Eerola E, Kero P: Fecal microflora in healthy infants born by different methods of delivery: permanent changes in intestinal flora after cesarean delivery. *J Pediatr Gastroenterol Nutr* 1999;28: 19–25.
- Xu B, Pekkanen J, Hartikainen AL, Jarvelin MR: Caesarean section and risk of asthma and allergy in adulthood. *J Allergy Clin Immunol* 2001;107:732–733.
- Kalliomaki M, Salminen S, Arvilommi H, Kero P, Koskinen P, Isolauri E: Probiotics in primary prevention of atopic disease: a randomized placebo-controlled trial. *Lancet* 2001;357:1076–1079.
- Kukkonen K, Savilahti E, Haahtela T, Jun-tunen-Backman K, Korpela R, Poussa T, Tuure T, Kuitunen M: Probiotics and prebiotic galacto-oligosaccharides in the prevention of allergic diseases: a randomized, double-blind, placebo-controlled trial. *J Allergy Clin Immunol* 2007;119:192–198.
- Kirjavainen PV, Salminen SJ, Isolauri E: Probiotic bacteria in the management of atopic disease: underscoring the importance of viability. *J Pediatr Gastroenterol Nutr* 2003;36: 223–227.

- 19 Rosenfeldt V, Benfeldt E, Nielsen SD, Michaelsen KF, Jeppesen DL, Valerius NH, Paerregaard A: Effect of probiotic *Lactobacillus* strains in children with atopic dermatitis. *J Allergy Clin Immunol* 2003;111:389–395.
- 20 Prescott SL, Dunstan JA, Hale J, Breckler L, Lehmann H, Weston S, Richmond P: Clinical effects of probiotics are associated with increased interferon-gamma responses in very young children with atopic dermatitis. *Clin Exp Allergy* 2005;35:1557–1564.
- 21 Taylor AL, Dunstan JA, Prescott SL: Probiotic supplementation for the first 6 months of life fails to reduce the risk of atopic dermatitis and increases the risk of allergen sensitization in high-risk children: a randomized controlled trial. *J Allergy Clin Immunol* 2007;119:184–191.
- 22 Brouwer ML, Wolt-Plompen SA, Dubois AE, van der Heide S, Jansen DF, Hoijer MA, Kauffman HF, Duiverman EJ: No effects of probiotics on atopic dermatitis in infancy: a randomized placebo-controlled trial. *Clin Exp Allergy* 2006;36:899–906.
- 23 Saavedra JM: Clinical applications of probiotic agents. *Am J Clin Nutr* 2001;73:1147S–1151S.
- 24 Pohjavuori E, Viljanen M, Korpela R, Kuitunen M, Tiittanen M, Vaarala O, Savilahti E: *Lactobacillus* GG effect in increasing IFN-gamma production in infants with cow's milk allergy. *J Allergy Clin Immunol* 2004;114:131–136.
- 25 Viljanen M, Kuitunen M, Haahtela T, Juntenen-Backman K, Korpela R, Savilahti E: Probiotic effects on faecal inflammatory markers and on faecal IgA in food allergic atopic eczema/dermatitis syndrome infants. *Pediatr Allergy Immunol* 2005;16:65–71.
- 26 Viljanen M, Savilahti E, Haahtela T, Juntenen-Backman K, Korpela R, Poussa T, Ture T, Kuitunen M: Probiotics in the treatment of atopic eczema/dermatitis syndrome in infants: a double-blind placebo-controlled trial. *Allergy* 2005;60:494–500.
- 27 Weizman Z, Asli G, Alsheikh A: Effect of a probiotic infant formula on infections in child care centers: comparison of two probiotic agents. *Pediatrics* 2005;115:5–9.
- 28 Isolauri E, Arvola T, Sutas Y, Moilanen E, Salminen S: Probiotics in the management of atopic eczema. *Clin Exp Allergy* 2000;30:1604–1610.
- 29 Fujiwara D, Inoue S, Wakabayashi H, Fujii T: The anti-allergic effects of lactic acid bacteria are strain dependent and mediated by effects on both Th1/Th2 cytokine expression and balance. *Int Arch Allergy Immunol* 2004;135:205–215.
- 30 Rosenfeldt V, Benfeldt E, Valerius NH, Paerregaard A, Michaelsen KF: Effect of probiotics on gastrointestinal symptoms and small intestinal permeability in children with atopic dermatitis. *J Pediatr* 2004;145:612–616.
- 31 Helin T, Haahtela S, Haahtela T: No effect of oral treatment with an intestinal bacterial strain, *Lactobacillus rhamnosus* (ATCC 53103), on birch-pollen allergy: a placebo-controlled double-blind study. *Allergy* 2002;57:243–246.
- 32 Karlsson H, Larsson P, Wold AE, Rudin A: Pattern of cytokine responses to gram-positive and gram-negative commensal bacteria is profoundly changed when monocytes differentiate into dendritic cells. *Infect Immun* 2004;72:2671–2678.
- 33 Pochard P, Gosset P, Grangette C, Andre C, Tonnel AB, Pestel J, Mercenier A: Lactic acid bacteria inhibit TH2 cytokine production by mononuclear cells from allergic patients. *J Allergy Clin Immunol* 2002;110:617–623.
- 34 Niers LE, Timmerman HM, Rijkers GT, van Bleek GM, van Uden NO, Knol EF, Kapsenberg ML, Kimpfen JL, Hoekstra MO: Identification of strong interleukin-10 inducing lactic acid bacteria which down-regulate T helper type 2 cytokines. *Clin Exp Allergy* 2005;35:1481–1489.
- 35 Hart AL, Lammers K, Brigidi P, Vitali B, Rizzello F, Gionchetti P, Campieri M, Kamm MA, Knight SC, Stagg AJ: Modulation of human dendritic cell phenotype and function by probiotic bacteria. *Gut* 2004;53:1602–1609.
- 36 Smith DW, Nagler-Anderson C: Preventing intolerance: the induction of nonresponsiveness to dietary and microbial antigens in the intestinal mucosa. *J Immunol* 2005;174:3851–3857.
- 37 Lan JG, Cruickshank SM, Singh JC, Farrar M, Lodge JP, Felsburg PJ, Carding SR: Different cytokine response of primary colonic epithelial cells to commensal bacteria. *World J Gastroenterol* 2005;11:3375–3384.
- 38 Sierro F, Dubois B, Coste A, Kaiserlian D, Kraehenbuhl JP, Sirard JC: Flagellin stimulation of intestinal epithelial cells triggers CCL20-mediated migration of dendritic cells. *Proc Natl Acad Sci USA* 2001;98:13722–13727.
- 39 Kunkel EJ, Campbell DJ, Butcher EC: Chemokines in lymphocyte trafficking and intestinal immunity. *Microcirculation* 2003;10:313–323.
- 40 Kato A, Ogasawara T, Homma T, Batchelor J, Imai S, Wakiguchi H, Saito H, Matsumoto K: CpG oligodeoxynucleotides directly induce CXCR3 chemokines in human B cells. *Biochem Biophys Res Commun* 2004;320:1139–1147.
- 41 Kato A, Homma T, Batchelor J, Hashimoto N, Imai S, Wakiguchi H, Saito H, Matsumoto K: Interferon-alpha/beta receptor-mediated selective induction of a gene cluster by CpG oligodeoxynucleotide 2006. *BMC Immunol* 2003;4:8.
- 42 Furrie E, Macfarlane S, Thomson G, Macfarlane GT: Toll-like receptors-2, -3 and -4 expression patterns on human colon and their regulation by mucosal-associated bacteria. *Immunology* 2005;115:565–574.
- 43 Bambou JC, Giraud A, Menard S, Begue B, Rakotobe S, Heyman M, Taddei F, Cerf-Bennussan N, Gaboriau-Routhiau V: In vitro and ex vivo activation of the TLR5 signaling pathway in intestinal epithelial cells by a commensal *Escherichia coli* strain. *J Biol Chem* 2004;279:42984–42992.
- 44 Ono SJ, Nakamura T, Miyazaki D, Ohbayashi M, Dawson M, Toda M: Chemokines: roles in leukocyte development, trafficking, and effector function. *J Allergy Clin Immunol* 2003;111:1185–1199.
- 45 Lim HW, Lee J, Hillsamer P, Kim CH: Human Th17 cells share major trafficking receptors with both polarized effector T cells and FOXP3+ regulatory T cells. *J Immunol* 2008;180:122–129.
- 46 Hirota K, Yoshitomi H, Hashimoto M, Maeda S, Terada S, Sugimoto N, Yamaguchi T, Nomura T, Ito H, Nakamura T, Sakaguchi N, Sakaguchi S: Preferential recruitment of CCR6-expressing Th17 cells to inflamed joints via CCL20 in rheumatoid arthritis and its animal model. *J Exp Med* 2007;204:2803–2812.
- 47 Annunziato F, Cosmi L, Santarlasci V, Maggi L, Liotta F, Mazzinghi B, Parente E, Fili L, Ferri S, Frosali F, Giudizi F, Romagnani P, Parronchi P, Tonelli F, Maggi E, Romagnani S: Phenotypic and functional features of human Th17 cells. *J Exp Med* 2007;204:1849–1861.
- 48 Suzuki S, Shimojo N, Tajiri Y, Kumemura M, Kohno Y: Differences in the composition of intestinal *Bifidobacterium* species and the development of allergic diseases in infants in rural Japan. *Clin Exp Allergy* 2007;37:506–511.
- 49 Fujimori S, Tatsuguchi A, Gudis K, Kishida T, Mitsui K, Ehara A, Kobayashi T, Sekita Y, Seo T, Sakamoto C: High dose probiotic and prebiotic cotherapy for remission induction of active Crohn's disease. *J Gastroenterol Hepatol* 2007;22:1199–1204.
- 50 Fajdiga S, Koninkx JF, Tooten PC, Marinsek-Logar R: Interference of *Salmonella enteritidis* and *Lactobacillus* spp. with IL-8 levels and transepithelial electrical resistance of enterocyte-like Caco-2 cells. *Folia Microbiol (Praha)* 2006;51:268–272.
- 51 Roselli M, Finamore A, Britti MS, Mengheri E: Probiotic bacteria *Bifidobacterium animalis* MB5 and *Lactobacillus rhamnosus* GG protect intestinal Caco-2 cells from the inflammation-associated response induced by enterotoxigenic *Escherichia coli* K88. *Br J Nutr* 2006;95:1177–1184.
- 52 Lee J, Mo JH, Katakura K, Alkalay I, Rucker AN, Liu YT, Lee HK, Shen C, Cojocaru G, Shenouda S, Kagnoff M, Eckmann L, Ben-Neriah Y, Raz E: Maintenance of colonic homeostasis by distinctive apical TLR9 signaling in intestinal epithelial cells. *Nat Cell Biol* 2006;8:1327–1336.

- 53 Kobayashi K, Inohara N, Hernandez LD, Galan JE, Nunez G, Janeway CA, Medzhitov R, Flavell RA: RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. *Nature* 2002;416: 194–199.
- 54 Vidal K, Donnet-Hughes A, Granato D: Lipoteichoic acids from *Lactobacillus johnsonii* strain La1 and *Lactobacillus acidophilus* strain La10 antagonize the responsiveness of human intestinal epithelial HT29 cells to lipopolysaccharide and gram-negative bacteria. *Infect Immun* 2002;70:2057–2064.
- 55 Gusils C, Cuozzo S, Sesma F, Gonzalez S: Examination of adhesive determinants in three species of *Lactobacillus* isolated from chicken. *Can J Microbiol* 2002;48:34–42.
- 56 Velez MP, Verhoeven TL, Draing C, Von Aulock S, Pfitzenmaier M, Geyer A, Lambrichts I, Pot B, Vanderleyden J, De Keersmaecker SC: Functional analysis of D-alanylation of lipoteichoic acid in the probiotic strain *Lactobacillus rhamnosus* GG. *Appl Environ Microbiol* 2007;103:666–674.
- 57 Nemeth E, Fajdiga S, Malago J, Koninkx J, Tooten P, van Dijk J: Inhibition of *Salmonella*-induced IL-8 synthesis and expression of Hsp70 in enterocyte-like Caco-2 cells after exposure to non-starter lactobacilli. *Int J Food Microbiol* 2006;112:266–274.
- 58 Malago JJ, Koninkx JF, Tooten PC, van Liere EA, van Dijk JE: Anti-inflammatory properties of heat shock protein 70 and butyrate on *Salmonella*-induced interleukin-8 secretion in enterocyte-like Caco-2 cells. *Clin Exp Immunol* 2005;141:62–71.
- 59 Petrof EO, Kojima K, Ropeleski MJ, Musch MW, Tao Y, De Simone C, Chang EB: Probiotics inhibit nuclear factor-kappaB and induce heat shock proteins in colonic epithelial cells through proteasome inhibition. *Gastroenterology* 2004;127:1474–1487.
- 60 Duncan SH, Louis P, Flint HJ: Lactate-utilizing bacteria, isolated from human feces, that produce butyrate as a major fermentation product. *Appl Environ Microbiol* 2004;70: 5810–5817.
- 61 Matsumoto M, Aranami A, Ishige A, Watanabe K, Benno Y: LKM512 yogurt consumption improves the intestinal environment and induces the T-helper type 1 cytokine in adult patients with intractable atopic dermatitis. *Clin Exp Allergy* 2007;37:358–370.
- 62 Hamer HM, Jonkers D, Venema K, Vanhoutvin S, Troost FJ, Brummer RJ: Review article: the role of butyrate on colonic function. *Aliment Pharmacol Ther* 2008;27:104–119.
- 63 Zhang L, Li N, Caicedo R, Neu J: Alive and dead *Lactobacillus rhamnosus* GG decrease tumor necrosis factor-alpha-induced interleukin-8 production in Caco-2 cells. *J Nutr* 2005;135:1752–1756.
- 64 Tuomola EM, Salminen SJ: Adhesion of some probiotic and dairy *Lactobacillus* strains to Caco-2 cell cultures. *Int J Food Microbiol* 1998;41:45–51.
- 65 He F, Ouwehand AC, Isolauri E, Hashimoto H, Benno Y, Salminen S: Comparison of mucosal adhesion and species identification of bifidobacteria isolated from healthy and allergic infants. *FEMS Immunol Med Microbiol* 2001;30:43–47.
- 66 Prescott SL, Bjorksten B: Probiotics for the prevention or treatment of allergic diseases. *J Allergy Clin Immunol* 2007;120:255–262.