Inhibitory effects of *Lactobacillus casei* subsp. *rhannosus* on *Salmonella* lipopolysaccharide-induced inflammation and epithelial barrier dysfunction in a co-culture model using Caco-2/peripheral blood mononuclear cells

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In this study, we investigated the anti-inflammatory and reinforcing barrier effects of *Lactobacillus casei* subsp. *rhannosus* (Lcr35) on Caco-2 intestinal epithelial cells already exposed to *Salmonella* LPS. Using the Transwell co-culture model, *Salmonella* LPS was apically added to polarized Caco-2 cells co-cultured with peripheral blood mononuclear cells (PBMCs) in the basolateral compartment. LPS-stimulated Caco-2 cells were incubated with Lcr35 for 1, 6, 24 or 48 h. Apical inoculation of Lcr35 after 48 h significantly inhibited the basolateral secretion of interleukin-8 (IL-8) in the Caco-2/PBMC co-culture. The PCR analysis showed that Lcr35 significantly downregulated mRNA expression of monocyte chemoattractant protein 1 (MCP-1) ($P<0.05$) and had a trend of decreasing mRNA expression of IL-8 ($P=0.05$), but did not alter mRNA expression of transforming growth factor- β 1 in LPS-stimulated Caco-2 cells at 48 h after addition of Lcr35. Compared to non-LPS-pretreated controls, transepithelial electrical resistance (TEER) of the polarized Caco-2 cell monolayers pretreated with LPS for 48 h was decreased by 9.9 % ($P<0.05$). Additionally, compared to those cells only treated with LPS, apical co-incubation with Lcr35 showed biphasic TEER levels increased by 12.1 % ($P<0.001$), 5.7 % ($P<0.05$) and 86.8 % ($P<0.001$) in the Caco-2 cell monolayers compared to those without Lcr35 treatment after 1, 6 and 48 h, respectively. In conclusion, Lcr35 can exert anti-inflammatory effects and ameliorate barrier dysfunction in the *Salmonella* LPS-pretreated inflamed intestinal epithelium *in vitro*.

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Abbreviations: IEC, intestinal epithelial cell; IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein 1; NF- κ B, nuclear factor-kappa B; PBMC, peripheral blood mononuclear cell; TEER, transepithelial electrical resistance; TGF- β 1, transforming growth factor- β 1; TNF- α , tumour necrosis factor alpha.

INTRODUCTION

The intestinal epithelium maintains a physical barrier to separate the luminal environment from the host milieu, and acts as a guard of the intestinal tract resisting bacteria, pathogens and other antigens (Sierro *et al.*, 2001). Such intestinal barrier integrity can stabilize the entire intestinal

ecosystem. In the maintenance of barrier integrity, tight junction complexes play a decisive role (Mitic *et al.*, 2000). Intestinal infections by microbial pathogens, such as *Salmonella* spp., often impair or subvert host tight junction barrier function and can subsequently result in acute diarrhoea. Moreover, certain enteric viruses and bacteria can cause deterioration in the structure and function of intestinal tight junctions that may lead to chronic intestinal disorders (O'Hara & Buret, 2008).

In addition to behaving as a physical barrier, the intestinal epithelium also plays a key role in the host immune system. Various lymphocyte populations interspersed among the epithelial cells or in the underlying intestinal lamina propria constitute the gut-associated lymphoid tissue to provide innate and adaptive immune responses against pathogens (Ramiro-Puig *et al.*, 2008). Accordingly, an *in vitro* co-culture model with human intestinal epithelial cells (IECs) and human blood leukocytes has been developed to assess ion transport and barrier function (McKay *et al.*, 1996), or to investigate whether reactivity of enterocytes to bacterial signals depends on their interactions with immunocompetent cells (Haller *et al.*, 2000). In addition, both human peripheral blood mononuclear cells (PBMCs) and lamina propria mononuclear cells are involved in the mucosal defence against bacterial translocation and systemic inflammation (Guzy *et al.*, 2009). Therefore, PBMCs in the basolateral compartment of Transwell plates can be *in vitro* co-cultured with apical IECs to evaluate the effect of bacteria on immunological interactions between these two main cell populations in the gut.

In the past decade, the clinical implications and immunomodulatory effects of probiotic bacteria, such as lactobacilli, have been increasingly investigated in both clinical trials and experimental models. In clinical practice, commercial probiotics have been used as therapeutic agents to ameliorate diarrhoea. Treatment with *Lactobacillus rhamnosus* strains can shorten the duration of diarrhoea up to 5 days in patients with rotavirus infections (Szymanski *et al.*, 2006). On the other hand, some *in vitro* studies showed that lactobacilli can not only blunt the pro-inflammatory immune response induced by *Salmonella enterica* serotype Typhimurium (*S.* Typhimurium) in HT-29 human IECs (O'Hara *et al.*, 2006), but also inhibit the synthesis of interleukin-8 (IL-8) in *Salmonella*-stimulated Caco-2 cells (Nemeth *et al.*, 2006). However, long-duration kinetic impacts of probiotic lactobacilli on pro-inflammatory cytokine responses and integrity of intestinal barrier function after pathogenic stimulation in an enterocyte-leukocyte co-culture model has rarely been discussed. Thus, we conducted this study to assess the inhibitory effects of the commercial probiotic *Lactobacillus casei* subsp. *rhamnosus* (Lcr35), which has been used as a probiotic therapeutic agent (Fang *et al.*, 2009), upon *Salmonella* LPS-induced inflammation and epithelial barrier dysfunction in IECs over a 48 h period *in vitro*.

METHODS

Bacterial strain and cultivation. The commercial strain *Lactobacillus casei* subsp. *rhamnosus*, Lcr35 (Antibiophilus), was used in this study. Lcr35 was cultured in Difco Lactobacilli MRS (de Man–Rogosa–Sharpe) broth (Becton Dickinson) at 37 °C with 5% CO₂ and used in the early stationary phase.

Cell culture. Enterocyte-like Caco-2 cells with the structural and functional characteristics of intestinal cells were developed into differentiated intestinal cells (Koninkx *et al.*, 1996). The human Caco-2 cells (passage 33–35) were purchased from the Cell Bank at the Bioresource Collection and Research Centre (Hsinchu, Taiwan), seeded at a density of 1×10^5 cells cm⁻² and grown on 0.9 cm² cell culture inserts in the Transwell system (0.4 µm nucleopore size BD Falcon; Milian). The inserts were pre-coated with collagen (Sigma) and placed into 12-well tissue culture plates before cell seeding. Cells were maintained in Dulbecco's modified Eagle medium (DMEM, 4500 mg glucose l⁻¹; Amimed) with 20% (v/v) fetal bovine serum (FBS) and 1% (v/v) non-essential amino acids (Sigma). Cell culture medium was changed every 2–3 days until 12–14 days later when full polarization of the Caco-2 cell monolayer was achieved.

Isolation of PBMCs. The study protocol and consent documents were approved by the Institutional Review Board of Mackay Memorial Hospital. PBMCs were obtained from healthy donors and provided as buffy coats (Blood Transfusion Centre, Taipei, Taiwan). Leukocytes were freshly purified by Ficoll–Hypaque (Pharmacia) centrifugation (400 g, 20 min) and were collected from the interface. The final PBMCs were stored in RPMI 1640 medium (Sigma), supplemented with 1.0 mM HEPES, 1.0 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol, 10% (v/v) FBS and adjusted to 3×10^6 cells ml⁻¹.

LPS stimulation of Caco-2/PBMCs in the co-culture model.

Confluent monolayers of polarized Caco-2 cells were developed in tissue culture inserts and transferred to 12-well tissue culture plates. In the Transwell cell culture system, 1 ml fresh purified PBMCs and 1 ml DMEM, respectively, were added to the basal and apical compartments of the culture plates. The apical surface of Caco-2 cell monolayers was challenged with *S. Typhimurium* LPS (Sigma), at the optimal concentration of 10 ng ml⁻¹, to activate maximal cytokine tumour necrosis factor alpha (TNF-α) secretion (Ménard *et al.*, 2004). In order to trigger an inflammatory response in the whole co-culture system, the Caco-2 cell monolayers were incubated with LPS at 37 °C with 5% CO₂ for 48 h (Matsumoto & Benno, 2006). After then, the Caco-2 cells and PBMCs were collected and washed once with cold PBS (Fig. 1). After centrifugation (300 g, 5 min), the washed PBMCs were resuspended in the supplemented RPMI 1640 medium, and then were added back to the original basal compartments in the Transwell plates for further experiments (Fig. 1).

Probiotic anti-inflammatory effects on intestinal cells *in vitro*.

To investigate the probiotic anti-inflammatory effects on Caco-2 cells, an inoculum of 2×10^7 c.f.u. Lcr35 ml⁻¹ was added onto the apical side of enterocytes after removal of LPS (Fig. 1). The cell survival was assessed using a 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Matsumoto & Benno, 2006). Compared to the untreated controls, the relative survival rate of polarized Caco-2 cells treated with Lcr35 was still maintained (>95%) after 48 h. Caco-2/PBMCs co-cultures were incubated for 1, 6, 24 or 48 h. The immune responses of Caco-2 cells and PBMCs upon stimulation with Lcr35 were monitored by analysis of cytokine gene transcription including IL-8, monocyte chemoattractant protein 1 (MCP-1) and transforming growth factor-β1 (TGF-β1) using RT-PCR, and by determination of IL-8 secretion in cell culture supernatants using ELISA. In all co-culture experiments, IL-8 protein levels were measured in both the

Fig. 1. Schematic illustration of the co-culture model showing experimental arrangements in the Transwell system for assessment of Caco-2/PBMCs interactions after various kinds of stimulation: inflammation (A), Caco-2 cells were challenged with LPS; anti-inflammation (B), LPS was removed and then Caco-2 cells were challenged with Lcr35 with the same population of PBMCs washed, spun, resuspended in complemented RPMI medium, and reloaded into the lower chamber.

apical and basal media, which were collected by centrifugation at 2000 r.p.m. for 3 min and frozen in aliquots at -80°C . IL-8 protein levels were quantified by ELISA using the DuoSet kit (R&D Systems) according to the manufacturer's instructions. Absorbance was measured in a microplate reader (SpectraMax 190; Molecular Devices) at 450 nm.

RNA extraction and PCR. Total RNA from Caco-2 cells was isolated using the RNA extraction miniprep system (Total RNA Mini kit; Viogene) according to the manufacturer's instructions for animal cells. Template cDNA was synthesized from RNA using reverse transcription with Oligo dT₁₂₋₁₈ primers (Protech) and amplified in a total volume of 50 μl reaction using *Taq* DNA polymerase (Genet Bio) in the GeneAmp PCR System 9700 (Applied Biosystems) with 40 cycles of denaturation at 94°C for 30 s, annealing at 56°C (actin and MCP-1) or 63°C (IL-8 and TGF- β 1) for 30 s, and extension at 72°C for 30 s. Subsequently, the PCR products were subjected to gel electrophoresis on 2% agarose gels with $1\times$ Tris borate EDTA and visualized by staining with ethidium bromide. Pairs of oligonucleotide primers specific to IL-8 (forward 5'-TGGCTCTCTTGGCAGCC-TTC-3', reverse 3'-TGCACCCAGTTTTCCTTGGG-5'; product length 238 bp), MCP-1 (forward 5'-CTCAGCCAGATGCAATC-AAT-3', reverse 3'-AGATTCTTGGGTTGTGGAGT-5'; 259 bp), and TGF- β 1 (forward 5'-AGGACTGCGGATCTCTGTGT-3', reverse 3'-AGTGCCCAAGGTGCTCAATA-5'; 196 bp) were used as described by Saegusa *et al.* (2007) and purchased from Invitrogen. Primer sequence of the housekeeping gene actin (forward 5'-GTCACCAACT-GGGACGACAT-3', reverse 3'-GATAGCAGCCTGGATAGCAA-5'; 197 bp) was designed by the primer 5 program. The intensity of the specific bands was measured using the Multi Gauge v2.1 image software.

Electrical resistance measurements. Transepithelial electrical resistance (TEER) of Caco-2 cells was monitored using an EVOM TEER meter (World Precision Instruments). TEER increased until day 12–14 when a steady state of higher than $450\ \Omega\ \text{cm}^2$ was reached, indicating development of functional polarity and an intact monolayer, which is referred to as completed tight junction formation and maximized integrity of barrier function, and is similar to that described by Klingberg *et al.* (2005), Mine & Zhang (2003) and Satsu *et al.* (2003). LPS stimulation of Caco-2/PBMCs in the co-culture model lasted for 48 h until addition of Lcr35. After removal of LPS by washing the Caco-2 monolayer with PBS, TEER readings were

recorded at five time points (0, 1, 6, 24 and 48 h) during the subsequent co-incubation of Caco-2 and Lcr35 (2×10^7 c.f.u. ml^{-1}). An m.o.i. of 25 was calculated when Lcr35 was inoculated.

Statistics. The results were obtained using PBMCs from at least three blood donors in each independent experiment. The quantitative data were expressed as mean $\pm$ SEM of triplicate measurements. Statistical analyses were performed with one-way ANOVA or Student's *t*-test using SPSS 12.0. Statistical significance was defined as a *P* value of <0.05 .

RESULTS AND DISCUSSION

Salmonella LPS-induced inflammation

Our pilot study demonstrated that IL-8 levels in the apical and basal compartment of Caco-2/PBMCs co-culture system were, respectively, $838\ \text{pg}\ \text{ml}^{-1}$ ($P>0.05$) and $7046562\ \text{pg}\ \text{ml}^{-1}$ ($P<0.001$) after *Salmonella* LPS challenge for 48 h, compared to 703 and $6729\ \text{pg}\ \text{ml}^{-1}$, respectively, in non-LPS controls. In the present study, *Salmonella* LPS caused a significant inflammatory response, which was prolonged as late as 6–24 h after removal of the LPS, in polarized Caco-2 cells by triggering the mRNA expression of three cytokine genes, including IL-8 (0 vs 1, 6 and 24 h, Fig. 2). Both IL-8 gene and its post-transcriptional protein expression validate that *Salmonella* LPS can induce an inflammation in our Caco-2/PBMCs co-culture system. Previous reports demonstrated that *Salmonella* (Bolton *et al.*, 2000), or its cell wall components (Sierro *et al.*, 2001), may participate in initiating the mucosal inflammation of Caco-2 cells. LPS interacts with the intestinal epithelium and indirectly induces the inflammatory responses of the lymphoid system. Moreover, LPS has been reported to moderately enhance IL-8 secretion in Caco-2 cells (Chen *et al.*, 2006; Weglarz *et al.*, 2007). Such an LPS challenge to Caco-2 cells can simulate *Salmonella* enteric infections *in vivo* because increased IL-8 secretion triggers recruitment of neutrophils to intestinal epithelium, which is the histopathological hallmark of salmonellosis (McCormick *et al.*, 1993).

IL-8 secretion in the presence of Lcr35

Our preliminary studies indicated that the IL-8 secretion in the apical compartment was lower than that in the basal compartment in the Caco-2/PBMCs co-cultures, and also was negligible in the Caco-2 cells without co-cultured PBMCs (data not shown). In a similar Transwell system, the relevant cytokines were only detectable in the basolateral component (Haller *et al.*, 2000). Therefore, the IL-8 concentration was exclusively measured from the medium in the basal compartment. Our data showed that the inhibition of IL-8 levels by Lcr35 was initially apparent at 24 h, and the trend reached its maximum at 48 h (Fig. 3). The IL-8 levels in the medium from Lcr35-treated cells were lower than those from non-Lcr35-treated cells at 48 h ($P<0.05$).

Fig. 2. mRNA expression of various cytokines (MCP-1, IL-8 and TGF- β 1) in Caco-2 cells after treatment with Lcr35 for 1, 6, 24 and 48 h. Caco-2/PBMCs were pre-incubated with *Salmonella* LPS for 48 h. (a) Representative PCR showing identified bands accompanied by 100 bp DNA ladder markers. (b) Quantification of mRNA expression in MCP-1, IL-8 and TGF- β 1, converted into relative intensity of individual PCR bands, was compared after normalization using the housekeeping gene actin. *, $P < 0.05$ indicates a significant difference between the Lcr35-treated group and the control group (Student's t -test, $n = 3$); #, $P = 0.05$. Grey bars, Non-Lcr35; white bars, Lcr35.

A putative mechanism of the aforementioned phenomenon is that probiotic bacteria could either bind to cell surface receptors on enterocytes, or stimulate the translocation of dendritic cells or secrete mediators diffusing into the cytosols of enterocytes triggering the related signal transduction pathways (Koninkx *et al.*, 1996). IL-8

Fig. 3. Lcr35 inhibited IL-8 secretion in the Caco-2/PBMCs co-culture model after LPS-induced inflammatory responses for 48 h. Secretion of IL-8 into the basolateral compartment was determined at various time points (1, 6, 24 and 48 h). Culture medium from non-Lcr35-treated cells was used as a control. The data are expressed as means $\pm$ SEM (error bars) of measurements in triplicate. *, $P < 0.05$ indicates a significant difference between the Lcr35-treated group and the control group (one-way ANOVA, $n = 3$). ■, Non-Lcr35; ▲, Lcr35.

secretion can be induced through the nuclear factor-kappa B (NF- κ B) pathway after challenge of TNF- α or pathogen-associated molecular patterns (PAMPs), such as LPS and flagellin. The signalling pathway of NF- κ B activation after *Shigella flexneri* infection involves the inhibitory protein, I- κ B α , which can be upregulated by *L. casei* to block NF- κ B activation in Caco-2 cells (Tien *et al.*, 2006). Probiotic *Escherichia coli* strain Nissle 1917 suppresses TNF- α -induced IL-8 production in human colonic epithelial cells (Kamada *et al.*, 2008). The secretion of IL-8 in the apical compartment was low in both Lcr35-treated and non-Lcr35-treated cells in our study. After Lcr35 treatment, IL-8 secretion was obviously reduced in the basolateral compartment with time compared to the controls. The immune response in Caco-2 cells could be further enhanced by presence of PBMCs after adequate interaction. Furthermore, the inhibition of IL-8 secretion demonstrated that Lcr35 is able to suppress the inflammatory response *in vitro*.

mRNA expression in Caco-2/PBMCs co-cultures with Lcr35

Our preliminary experiment confirmed that IL-8 mRNA expression was strongly increased by LPS stimulation for 48 h, accompanied by corresponding increased IL-8 protein secretion (data not shown). In this study, the LPS-stimulated polarized Caco-2 monolayers, treated with or without Lcr35 for different periods of time, showed that the bands of PCR-amplified IL-8, TGF- β 1 and MCP-1 were similarly maintained in their density before 24 h, but only MCP-1 obviously decreased after 48 h after inoculation of Lcr35 (Fig. 2). First, mRNA expression of MCP-1 in the

presence of Lcr35 was significantly lower than non-Lcr35 controls at the 48 h time point ($P<0.05$, Fig. 2). Second, incubation of Caco-2 cells with Lcr35 for 48 h showed a decreasing trend of IL-8 mRNA expression ($P=0.05$). Third, there was no significant difference in mRNA expression of TGF- β 1 between Lcr35-treated cells and non-Lcr35 controls.

In this study, we used a simplified epithelial-immune cell co-culture system to investigate the anti-inflammatory effects of probiotic Lcr35 by analysis of gene expression in the inflamed IECs. The results demonstrated that IL-8, a chemoattractant for neutrophils (Huber *et al.*, 1991), was significantly downregulated by Lcr35 in the inflamed Caco-2 cells after 48 h duration, but not within 24 h. Similarly, *Lactobacillus rhamnosus* GG can decrease IL-8 production in TNF- α stimulated Caco-2 cells (Zhang *et al.*, 2005). In addition, TGF- β 1 is an important inhibitor of the synthesis of pro-inflammatory cytokines (Parlesak *et al.*, 2004). In the present study, Lcr35 did not significantly affect TGF- β 1 gene expression in the inflamed Caco-2 cells over 48 h. Hence, TGF- β 1 might not play the key role of inhibiting IL-8 secretion of Caco-2/PBMCs after *Salmonella* LPS challenge. Interestingly, a previous study revealed that *Lactobacillus johnsonii* stimulated TGF- β 1 mRNA expression but *Lactobacillus sakei* did not in the Caco-2/PBMCs co-culture model (Haller *et al.*, 2000). Thus, various strains of lactobacilli may exert different regulation upon TGF- β 1 gene expression. Moreover, inflammatory stimuli can induce MCP-1, which is a potent chemoattractant for monocytes, memory T lymphocytes and natural killer cells (Gu *et al.*, 2000). Probiotic *E. coli* Nissle 1917 specifically upregulated MCP-1 mRNA expression in Caco-2 cells within 6 h (Ukena *et al.*, 2005); this is similar to our finding of increasing band density of PCR-amplified MCP-1 mRNA within 1 h (Fig. 2). In contrast, *L. sakei* and non-pathogenic *E. coli* upregulated MCP-1 gene expression in Caco-2/PBMCs co-cultures at 16 h (Haller *et al.*, 2000). These data suggested that Lcr35 might initially stimulate host defence mechanisms via the expression of MCP-1 at an earlier stage than other probiotic strains. Strain specificity of lactobacilli or co-existence of PBMCs might explain the varied dynamics in upregulation of MCP-1 gene expression. All of these studies indicated that cytokines or chemokines might be secreted from IECs. Therefore, alteration in gene expression of various cytokines or chemokines in Caco-2 cells could result in subsequent variation of individual protein expression and potentially interact with PBMCs to induce appropriate host immune responses.

Effect of Lcr35 on integrity of Caco-2 monolayer

In the treatment of intestinal inflammatory disorders it is fundamental to restore and stabilize physical barrier function. Probiotics have been reported to strengthen gut barrier function (Klingberg *et al.*, 2005). During the first 48 h period in our Caco-2/PBMCs co-cultures, TEER

decreased from 420.7 to 414.0 $\Omega \text{ cm}^2$ in LPS-treated Caco-2 cells but increased from 420.7 to 455.0 $\Omega \text{ cm}^2$ in non-LPS-treated cells ($P<0.05$). To determine the effect of Lcr35 on LPS-induced epithelial barrier dysfunction of Caco-2 cells, TEER was monitored for 48 h and this showed a significant reinforcement by Lcr35 of the TEER of the Caco-2 cell monolayers with time. Compared to the controls, the TEER of the Lcr35-treated cells increased by 12.1 % (598.8 vs 534.2 $\Omega \text{ cm}^2$, $P<0.001$) after 1 h and by 5.7 % (517.7 vs 489.7 $\Omega \text{ cm}^2$, $P<0.05$) after 6 h. The TEER levels of the Lcr35-treated Caco-2 cells were 86.7 % higher than those of the non-Lcr35-treated cells (874 vs 468 $\Omega \text{ cm}^2$, $P<0.001$) at 48 h (Fig. 4). Such a biphasic booster effect of Lcr35 on the TEER of the Caco-2 monolayers appeared firstly at 1 h with an increase of 64.6 $\Omega \text{ cm}^2$, followed by another second surge of 406 $\Omega \text{ cm}^2$ at 48 h when compared to non-Lcr35-treated cells (Fig. 4). In this time-dependent phenomenon, withdrawal of LPS and replacement of the medium might account for the first peak; competitive consumption of nutrients in the medium by apical Lcr35 and basal PBMCs might temporarily reduce TEER between 1 and 24 h, but accumulation of released probiotic mediators or their influences upon Caco-2/PBMCs co-cultures taking longer (than 24 h) might exceed the above transitory decrease in TEER to form the second higher peak at 48 h. TEER of polarized Caco-2 monolayers exposed to *Lactobacillus salivarius* DC5 and *Lactobacillus plantarum* MF1298 also showed a similar trend (Klingberg *et al.*, 2005). Unlike employing probiotic lactobacilli as a post-infectious therapeutic agent in our experiment, in a different study another probiotic mixture, VSL#3, was applied to prevent a pathogen-induced decrease in TEER (Otte & Podolsky, 2004). To sum up, Lcr35 can retain or even enhance the TEER of Caco-2 cell monolayers, which validates its

Fig. 4. Lcr35 increased TEER levels of the Caco-2 cell monolayers. The cell monolayers were pre-stimulated with *Salmonella* LPS for 48 h, followed by treatment with Lcr35 that reinforced LPS-suppressed transepithelial resistance. TEER values were measured at various time points (1, 6, 24 and 48 h) and the data are expressed as means $\pm$ SEM (error bars) of measurements in triplicate. *, $P<0.05$ indicates significant difference between the Lcr35-treated group and the control group (one-way ANOVA, $n=3$); #, $P<0.001$. ■, Non-Lcr35; ▲, Lcr35.

beneficial role as a post-infectious therapeutic agent in the maintenance and reinforcement of intestinal barrier integrity. This finding is supported by the fact that several probiotics and commensal bacteria, including *L. rhamnosus* GG and *Bifidobacterium infantis*, have been shown to stabilize tight junction-associated protein, zonula occludens-1, or increased zonula occludens-1 expression (Ewaschuk *et al.*, 2008; Johnson-Henry *et al.*, 2008) so as to strengthen epithelial barrier function. Thus, judging from the overall results of inhibition in IL-8 secretion, altered gene expression of MCP-1 and enhancement of TEER in the Caco-2 cell monolayers in our study, Lcr35 could post-infectiously inhibit inflammation of enterocytes caused by enteric pathogens through a putative mechanism involving the integrity of the tight junctions between enterocytes. In other words, Lcr35 functions as a barrier-sustaining agent, or barrier-enhancing agent, to protect the breakdown of intestinal epithelium from enteric pathogens.

In conclusion, our study using the Caco-2/PBMCs co-culture model clearly demonstrated the pivotal role of Lcr35 in inhibition of the intestinal anti-inflammatory responses and its beneficial effect upon re-establishment of the intestinal epithelial barrier function after inflammation. Moreover, Lcr35 induced the Caco-2 cells to express various pro-inflammatory cytokines at the gene level. Whether Lcr35 could further signal the PBMCs after such gene regulation to mediate the subsequent responses needs further studies for elucidation. Upon the inhibitory effect of probiotic Lcr35, the secretion of IL-8, a major pathogen-elicited epithelial chemoattractant of the inflammatory responses in salmonellosis, was significantly reduced in the basolateral compartment of a co-culture model where PBMCs predominantly resided and mimicked lymphocytes in the lamina propria. PBMCs have been used elsewhere to study the effect of immunocompetent cells on human IECs (Haller *et al.*, 2000; McKay *et al.*, 1996). Hence, our proposed Caco-2/PBMCs co-culture model is not only a useful *in vitro* system for investigating the modulation of probiotics on immune responses and barrier integrity of the gut after enteric infection or inflammation, but also a feasible platform for screening probiotic therapeutic agents for further animal studies and clinical trials.

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