Lactobacillus rhamnosus GG attenuates interferon- γ and tumour necrosis factor- α -induced barrier dysfunction and pro-inflammatory signalling

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The intestinal epithelium forms a protective barrier against luminal contents and the external environment, mediated via intercellular tight junctions (TJs). The TJ can be disrupted via cell signalling induced by either enteric pathogens or pro-inflammatory cytokines, thereby contributing to various intestinal disorders ranging from acute infectious diarrhoea to chronic inflammatory bowel diseases. Probiotics, such as *Lactobacillus rhamnosus* GG (LGG), are reported to confer beneficial effects on epithelial cells, including antagonizing infections and reducing overt pro-inflammatory responses, but the underlying mechanisms of these observed effects require further characterization. We hypothesized that probiotics preserve barrier function by interfering with pro-inflammatory cytokine signalling. Caco-2bbe cells were seeded into Transwells to attain polarized monolayers with intercellular TJs. Monolayers were inoculated apically with the probiotic LGG 3 h prior to the addition of IFN- γ (100 ng ml⁻¹) to the basolateral medium overnight. The monolayers were then placed in fresh basal medium $\pm$ TNF- α (10 ng ml⁻¹) and transepithelial electrical resistance (TER) measurements were taken over the time-course of TNF- α stimulation. To complement the TER findings, cells were processed for zona occludens-1 (ZO-1) immunofluorescence staining. As a measure of TNF- α downstream signalling, cells were immunofluorescently stained for NF- κ B p65 subunit and CXCL-8 mRNA was quantified by qRT-PCR. Basal cell culture medium was collected after overnight TNF- α stimulation to measure secreted chemokines, including CXCL-8 (interleukin-8) and CCL-11 (eotaxin). Following LGG inoculation, IFN- γ priming and 24 h TNF- α stimulation, epithelial cells maintained TER and ZO-1 distribution. LGG diminished the nuclear translocation of p65, demonstrated by both immunofluorescence and CXCL-8 mRNA expression. CXCL-8 and CCL-11 protein levels were decreased in LGG-inoculated, cytokine-challenged cells. These findings indicate that LGG alleviates the effects of pro-inflammatory cytokines on epithelial barrier integrity and inflammation, mediated, at least in part, through inhibition of NF- κ B signalling.

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INTRODUCTION

The intestinal epithelium forms a protective barrier against luminal contents, such as microbes and dietary food antigens, present in the external environment. Barrier function is mediated through apical junction complexes, which consist of paracellular proteins integrated into tight

junctions (TJs) (Van Itallie & Anderson, 2006). Abnormalities of intercellular TJs contribute to a variety of intestinal disorders, including acute diarrhoeal illness, gluten-sensitive enteropathy (celiac disease) and chronic inflammatory bowel disease (McGuckin *et al.*, 2009). The challenging of intact polarized epithelia with either pro-inflammatory cytokines or pathogenic bacteria dismantles the TJ protein structure and thereby disrupts barrier function (Donato *et al.*, 2008; Wang *et al.*, 2005, 2006; Zareie *et al.*, 2005).

We have shown that probiotic lactobacilli antagonize infection by pathogenic *Escherichia coli* (Johnson-Henry *et al.*, 2005, 2007) and other groups have shown that

Abbreviations: ERK-1/2, extracellular signal-related kinase; IFN- γ , interferon- γ ; LGG, *Lactobacillus rhamnosus* GG; MAPK, mitogen-activated protein kinase; MEK-1/2, mitogen-activated protein kinase kinase-1/2 (activator of ERK-1/2); TER, transepithelial electrical resistance; TJ, tight junction; TNBS, trinitrobenzenesulfonic acid; TNF- α , tumour necrosis factor- α ; ZO-1, zona occludens-1.

probiotic effects also extend to the modulation of downstream pro-inflammatory cytokine signalling in epithelial cells, which serves to maintain epithelial barrier function and regulate chemokine secretion (Resta-Lenert & Barrett, 2006). We have demonstrated that the probiotic *Lactobacillus rhamnosus* strain GG (LGG) blocks the deleterious effects of *E. coli* O157:H7, a bacterium which directly disrupts epithelial cell architecture and intercellular TJs (Johnson-Henry *et al.*, 2008). LGG also prevents pro-inflammatory-cytokine-induced apoptosis (Yan *et al.*, 2007), and ameliorates barrier dysfunction due to oxidative insults (Seth *et al.*, 2008). Recent research has elucidated the role of functional mitogen-activated protein kinase (MAPK) signalling, especially extracellular signal-related kinase (ERK-1/2) in the maintenance of epithelial barrier integrity by LGG and other probiotic strains (Ewaschuk *et al.*, 2008; Seth *et al.*, 2008; Tao *et al.*, 2006). The mechanism by which LGG engages in a 'microbial-epithelial crosstalk' in a model of proinflammatory cytokine-induced barrier disruption remains unclear. Therefore, the aim of the research described in this report was to determine if LGG inhibits epithelial barrier dysfunction mediated by IFN- γ and TNF- α co-stimulation, and modulates downstream pro-inflammatory signalling and chemokine secretion, and also to assess if there is a role for ERK-1/2.

METHODS

Bacterial cultures. *Lactobacillus rhamnosus* strain GG (ATCC 53103; LGG) was grown in 10 ml of deMan–Rogosa–Sharpe (MRS) broth (BD) at 37 °C until the culture reached a concentration of $\sim 10^8$ c.f.u. ml $^{-1}$. Related bacteria, *Lactobacillus farciminis* and *Lactobacillus plantarum* strain RO403 (Institut Rosell-Lallemand, Montreal, Quebec, Canada) were grown in MRS broth and used for comparative purposes. Bacteria were pelleted, rinsed once in PBS, and resuspended to the original culture volume in antibiotic-free eukaryotic cell culture medium. Bacterial cultures were diluted to obtain a working concentration of 5×10^5 – 5×10^6 c.f.u. ml $^{-1}$. For some experiments, bacteria were heat-killed for 45 min at 100 °C. To inhibit *de novo* bacterial protein synthesis while maintaining cellular architecture, chloramphenicol (20 μ g ml $^{-1}$) was added to the cell culture medium.

Epithelial cell culture. Caco-2bbe cells (ATCC CRL-2102) are human colonic adenocarcinoma cells that form confluent, polarized epithelial monolayers with well-differentiated intercellular TJ structures, and a pattern of brush border protein expression that is comparable to that of primary human enterocytes (Peterson & Mooseker, 1992). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum, 0.01 mg human transferrin ml $^{-1}$, 1 mM sodium pyruvate, 200 U penicillin ml $^{-1}$ and 200 μ g streptomycin ml $^{-1}$ (all reagents from GIBCO). Cell culture medium was changed to antibiotic-free culture medium prior to experimental trials.

Measurements of transepithelial electrical resistance (TER). Caco-2bbe monolayers were grown on Transwell (Costar; Corning) semi-permeable filter supports (seeded at approximately 10^6 cells cm $^{-2}$; 6.5 mm diameter wells, polystyrene membranes with 0.4 μ m pores) and grown until a TER indicative of established TJ formation ($>500 \Omega$ cm $^{-2}$) was achieved. TER was measured in each monolayer before adding 200 μ l of culture medium containing 10^5 – 10^6 c.f.u. of lactobacilli onto the apical surface for 3 h prior to treatment of the

basolateral medium with IFN- γ (100 ng ml $^{-1}$; R&D Systems) overnight at 37 °C. It was determined empirically that 10^5 c.f.u. of LGG or 10^6 c.f.u. of *L. farciminis*/*L. plantarum* did not cause bacterial overgrowth and deleterious effects on epithelial cells over the time-course of the experiments. The media of the basal compartment wells were changed and some of the wells were supplemented with TNF- α (10 ng ml $^{-1}$; Sigma). TER measurements were taken over the time-course of TNF- α stimulation and the resulting data presented as a percentage of the TER at the beginning of TNF- α stimulation.

To determine a role for ERK-1/2, the MEK-1/2 extracellular inhibitor PD-98059 (50 μ M, ALEXIS Biochemicals) was added at the time of LGG inoculation and levels of the inhibitor (or vehicle, 0.1%, v/v, DMSO) were maintained throughout the course of the experiment. The inhibitor was serially diluted in some instances to establish a dose–response curve (vehicle concentration was maintained for all dilutions).

Immunofluorescence for the integral TJ-associated protein zona occludens-1 (ZO-1). As a complementary approach to study TJ structure–function relationships, immediately following TER measurements, cell monolayers were processed for indirect immunofluorescence microscopy to detect ZO-1. Monolayers were fixed in 100% methanol for 10 min at -20 °C. After rinsing in PBS, the monolayers were blocked with 5% normal goat serum and then incubated with rabbit anti-ZO-1 antibody (Zymed) overnight at 4 °C. The monolayers were rinsed in PBS before the addition of DyLight 488-conjugated anti-rabbit IgG (Jackson ImmunoResearch; 1 h, room temperature) and 300 nM DAPI dilactate (Invitrogen) for nuclear counterstaining. Images were captured with a Leica DMI6000B fluorescence microscope and companion DFC 360FX camera (Leica Microsystems). The images were then deconvolved, using software provided by the manufacturer.

Immunofluorescence to detect NF- κ B translocation to the nucleus. Caco-2bbe cells were grown on Permanox four-well chamber slides (MP Biomedicals) overnight at 37 °C in 5% CO $_2$, prior to inoculation with LGG and overnight incubation in IFN- γ (100 ng ml $^{-1}$). The cell culture medium was replaced and some wells were supplemented with TNF- α (10 ng ml $^{-1}$) for 0.5 h at 37 °C, rinsed in PBS, and fixed in 3.7% (v/v) formaldehyde for 0.5 h at room temperature. The cells were rinsed with 5% (w/v) BSA prior to permeabilization with 0.1% (v/v) Triton X-100 (5 min, room temperature) and blocking in 1% (w/v) BSA for 30 min at room temperature. Cells were then incubated in rabbit polyclonal anti-NF- κ B p65 subunit IgG antibody (C-20; Santa Cruz Biotechnology) with 5% (w/v) BSA for 1 h at room temperature. Unbound primary antibody was rinsed away, and cells were incubated with secondary DyLight 488-conjugated anti-rabbit IgG for 1 h at room temperature; 300 nM DAPI dilactate was used as a nuclear counterstain. Images were obtained as described above for ZO-1 immunofluorescence staining. Counts of 100 random cells were performed for each treatment group, using non-adjacent fields of view under $\times 630$ magnification.

mRNA isolation and quantitative reverse transcriptase PCR (qRT-PCR). Transwell-grown cell monolayers were treated with IFN- γ and LGG overnight, and TNF- α was then added for 6 h. The monolayers were then rinsed twice in cold PBS and scraped into 800 μ l TRIZOL reagent (Invitrogen) for RNA isolation, according to the manufacturer's instructions. Briefly, approximately 1 μ g RNA was treated with a DNase I kit (Invitrogen) prior to conversion of RNA into cDNA using the iScript cDNA synthesis kit system (Bio-Rad). cDNA (1:10 dilution) was subjected to q-PCR with iQ SYBR Green supermix and 500 nM of a primer pair, according to the manufacturer's instructions. Primer sequences (5'–3') were: CXCL-8 – forward, ACT GAG AGT GAT TGA GAG TGG AC; reverse, AAC

CCT CTG CAC CCA GTT TTC; β -actin – forward, TGC GTG ACA TTA AGG AGA AG; reverse, AGG AAG GAA GGC TGG AAG AG. A CFX1000 thermocycler and CFX96 q-PCR detection system was used (Bio-Rad) with 60 °C annealing temperature. Relative CXCL-8 mRNA expression was quantified by using $2^{-\Delta\Delta C_t}$ values for comparison against β -actin mRNA expression.

Basal medium chemokine assay. Samples of cell culture media from basal wells were collected following TER measurements after overnight stimulation with TNF- α . Aliquots of the basolateral culture medium were then placed into a human 7-plex chemokine electro-immunosorbent plate kit to measure levels of CXCL-8 (interleukin-8), CCL-11 (eotaxin-1), monocyte chemotactic proteins-1 and -4 (MCP-1, -4), macrophage inflammatory protein-1 β (MIP-1 β), thymus- and activation-related chemokine (TARC), and interferon-inducible protein-10 (IP-10) (Meso Scale Discovery), following incubation procedures according to the manufacturer's instructions, and detection using a proprietary SECTOR imager (Meso Scale Discovery).

RESULTS

Viable LGG preserves epithelial barrier function against the effects of the pro-inflammatory cytokines IFN- γ and TNF- α

As shown in Fig. 1(A), untreated Caco-2bbe monolayers maintained TER levels over the time-course of the experiments,

whereas IFN- γ -primed monolayers demonstrated significantly decreased TER after 24 h of TNF- α stimulation ($61.8 \pm 3.1\%$ of pre-TNF- α stimulation values; $P < 0.01$ compared to untreated; $n = 5$ separate trials) and this effect was sustained at 32 h post-stimulation. These effects were prevented when the monolayers were pre-treated with LGG ($89.6 \pm 6.0\%$; $P = \text{ns}$ compared to untreated).

The protective effect of LGG required live, metabolically active bacteria, as heat-killing or the addition of chloramphenicol to the culture medium both abolished the response, even if 10-fold more LGG were added (Fig. 1B).

The probiotic effect was strain specific, because two other *Lactobacillus* species, *L. farciminis* (TER $65.6 \pm 3.5\%$ versus $63.8 \pm 5.1\%$ in cytokine-challenged epithelia in the absence of any bacteria) and *L. plantarum* (TER $71.2 \pm 3.6\%$) did not provide the same barrier-protective effect, even if the inoculum of these species was increased 10-fold (Fig. 2).

Probiotic strain specificity in the preservation of TJ morphology

In comparison to untreated monolayers (Fig. 3A) ZO-1 immunostaining showed that IFN- γ - and TNF- α -challenged cells had disrupted TJ architecture, with strand

Fig. 1. LGG lessens the decrease in TER induced by pro-inflammatory cytokine challenge. (A) TER levels in Caco-2bbe cells. Measurements were taken immediately prior to and throughout the time-course of TNF- α stimulation. (B) TER levels (24 h post TNF- α stimulation) in the presence of either heat-killed LGG or LGG in the presence of chloramphenicol (20 $\mu\text{g ml}^{-1}$). The data are from four or five separate experimental trials, using duplicate treatments per trial. Values are reported as mean $\pm$ SEM. ANOVA with Tukey post test, ** $P < 0.01$.

Fig. 2. The effect of probiotics on the amelioration of pro-inflammatory-cytokine-induced barrier dysfunction is strain dependent. TER was monitored after apical probiotic inoculation (3 h), basal IFN- γ stimulation overnight (100 ng ml⁻¹) and basal addition of TNF- α (10 ng ml⁻¹). Measurements of TER were taken immediately prior to and after 4, 8 and 24 h of TNF- α stimulation. Results are reported as a percentage of the pre-TNF- α values. The data are from four or five separate experimental trials, using duplicate treatments per trial. Trials using 10⁵ c.f.u. *L. farciminis* and *L. plantarum* were repeated twice. Values are reported as mean $\pm$ SEM. ANOVA with Tukey post test, ** P <0.01.

Fig. 3. Prevention of TJ disruption in cytokine-challenged Caco-2bbe monolayers is strain specific. Representative (of three separate trials) deconvolved immunofluorescence microscopy photomicrographs of monolayers immunostained for ZO-1 (green) are shown (A) Untreated epithelial monolayers with well-circumscribed TJ bands. (B) Cytokine-challenged cells (24 h post TNF- α stimulation) displaying multiple areas of junctional disruption and punctate staining (arrows). (C) *L. rhamnosus* GG (LGG) inoculation of monolayers largely prevented disruption of ZO-1 due to cytokine stimulation. (D) *L. farciminis*-inoculated and cytokine-stimulated monolayers also demonstrated disruptions of ZO-1 TJ protein staining (arrows), while those inoculated with *L. plantarum* RO403 (E) demonstrated disruptions of ZO-1, albeit to a lesser extent. Scale bars, 10 μ m.

breaks and diffuse, punctate areas of protein expression (Fig. 3B). By contrast, LGG largely prevented TJ ZO-1 perturbations in response to the cytokine challenges (Fig. 3C). *L. farciminis* did not prevent these disruptive effects (Fig. 3D), and cells challenged with cytokines after *L. plantarum* exposure had relatively less TJ disruption (Fig. 3E).

LGG suppresses TNF- α -induced nuclear translocation of NF- κ B

NF- κ B p65 subunit translocated to the nucleus in IFN- γ -primed Caco-2bbe cells after 0.5 h of TNF- α stimulation (Fig. 4C, D), whereas unstimulated epithelial cells demonstrated a predominantly cytoplasmic localization of NF- κ B (Fig. 4A, B). LGG inoculation reduced the nuclear translocation of NF- κ B in response to TNF- α (Fig. 4E, F). Counts of 100 random cells per sample (Fig. 4G) demonstrated that LGG-inoculated, cytokine-stimulated cells had less nuclear p65 expression (18.5 ± 5.8 % cells with nuclear localization) compared to cells treated with cytokines in the absence of probiotic (43.3 ± 1.3 %; ANOVA $P < 0.01$, $n = 3$).

Complementing these findings, CXCL-8 mRNA was isolated 6 h after TNF- α stimulation and quantified using qRT-PCR. CXCL-8 mRNA levels increased over 200-fold in cytokine-stimulated cells versus untreated cells (215.32 ± 45.69 -fold greater, $n = 5$). However, this upregulation was reduced by 50 % when the cytokine-stimulated cells were inoculated with LGG (79.90 ± 23.52 -fold greater than untreated cells, $n = 4$; unpaired Student's t -test $P < 0.05$, compared to uninoculated, cytokine-treated cells; Fig. 4H).

LGG suppresses pro-inflammatory-cytokine-induced epithelial secretion of CXCL-8 and CCL-11

As shown in Fig. 5(A) (for CXCL-8) and Fig. 5(B) (for CCL-11), basal tissue culture medium from IFN- γ primed, TNF- α -treated cells contained elevated levels of the chemokines CXCL-8 and CCL-11, compared to samples obtained from untreated monolayers. Neither *L. farciminis* nor *L. plantarum* reduced CXCL-8 and CCL-11 secretion induced by IFN- γ and TNF-stimulation (ANOVA, $P > 0.05$;

Fig. 4. LGG suppresses NF- κ B translocation from the cytoplasm to the nucleus. Representative deconvolved immunofluorescent micrographs of NF- κ B p65 subunit (green; panels A, C and E) and DAPI nuclear counterstain (blue; panels B, D and F) are shown. (A) Untreated cells show cytoplasmic p65 localization whereas IFN- γ -primed and TNF- α -stimulated cells (C) show nuclear p65 localization within 30 min of TNF- α addition (arrows). In contrast, few of the cells treated with LGG 3 h prior to cytokine stimulation (E) expressed nuclear p65. Scale bars, 10 μ m. (G) Percentage of p65-positive-staining nuclei in 100 random cells counted per treatment (mean $\pm$ SEM for three separate experimental trials; ANOVA with Tukey post test, $P < 0.01$ **compared to untreated, *compared to cells treated with cytokines alone). (H) CXCL-8 mRNA quantification by qRT-PCR (datum from untreated group set to 1.0). LGG inoculation reduced the increased expression of CXCL-8 mRNA in response to cytokine stimuli (overnight with 100 ng ml^{-1} IFN- γ followed by 10 ng ml^{-1} TNF- α for 6 h; unpaired Student's t -test, $*P < 0.05$; data from four or five separate experimental trials).

Fig. 5. Selected probiotics reduce CXCL-8 and CCL-11 secretion from cytokine-stimulated epithelial monolayers. (A) CXCL-8 (interleukin-8) and (B) CCL-11 (eotaxin-1) concentrations in basal tissue culture medium samples recovered from monolayers after IFN- γ priming (100 ng ml⁻¹) and 24 h of TNF- α (10 ng ml⁻¹) stimulation. The number of experimental trials, each run in duplicate, is indicated in each bar. Values are reported as mean $\pm$ SEM. ANOVA with Tukey post test, ** P <0.01, * P <0.05.

$n=4$). By contrast, LGG significantly reduced levels of CXCL-8 (286.8 ± 17.2 pg ml⁻¹ in cytokine-challenged cells compared to 189.2 ± 16.4 pg ml⁻¹ in LGG-pretreated, cytokine-challenged cells; $n=7$, ANOVA, P <0.01). LGG also reduced CCL-11 secretion following the cytokine stimulation (27.3 ± 1.8 pg ml⁻¹ in cytokine-challenged cells compared with 17.1 ± 2.8 pg ml⁻¹ in LGG-pretreated, cytokine-challenged cells; $n=7$, ANOVA, P <0.05). Live, metabolically active bacteria were required, because neither heat-killed LGG nor chloramphenicol treatment of the culture medium caused the suppression of CXCL-8 and CCL-11 secretion (258.0 and 342.1 pg ml⁻¹, respectively, for CXCL-8; and 23.7 and 28.9 pg ml⁻¹, respectively, for CCL-11; $n=2$).

The other chemokines tested also were secreted to a significantly greater extent in cytokine-challenged cells (except for MIP-1 α , which remained at undetectable levels in all treatments), but none of the three lactic-acid-producing bacterial strains tested diminished chemokine secretion due to the cytokine challenges (data not shown).

MEK-ERK-1/2 signalling is required for LGG-mediated maintenance of TER

As shown in Fig. 6, addition of the inhibitor targeting MEK-1/2 (PD-98059; 50 μ M) was not detrimental to polarized cell monolayers, since TER levels were maintained throughout the experiment. The presence of the inhibitor did not hinder the ability of LGG to maintain epithelial resistance during overnight IFN- γ incubation; however, TER was decreased following TNF- α stimulation.

DISCUSSION

The principal finding of this study is that inoculation of epithelial cells with the live, active probiotic *Lactobacillus rhamnosus* strain GG (LGG) prevents deleterious effects on TJ structure–function induced by pro-inflammatory cytokines. The findings also demonstrate strain specificity, because two other *Lactobacillus* species tested did not provide comparable protection. Our findings also show that LGG modulates host-epithelial cell signalling in this model, at least in part, by dampening TNF- α -induced NF- κ B activation and barrier protection responses requiring intact ERK-1/2 signalling.

The use of probiotics represents an opportunity for alleviating the pathobiology of a variety of acute and chronic digestive diseases, largely by regulating the cell signalling that modulates the inflammatory responses mediated both by the intestinal epithelium and by associated immune cells present in the underlying lamina propria (Round & Mazmanian, 2009). The findings in the present study build on recent work that has focused on altered signalling as a result of probiotic inoculation of epithelial cells. Increasing evidence shows that probiotics suppress the deleterious effects of pro-inflammatory cytokines, and that they also modulate the signalling of the stress-pathway-related MAPKs for cell survival and maintenance of an intact epithelial barrier (Vanderpool *et al.*, 2008).

Exposure to pro-inflammatory cytokines IFN- γ or TNF- α , individually, and over long time periods (36–48 h), decreases barrier function in model polarized epithelial monolayers (Ko *et al.*, 2007; Ma *et al.*, 2004; Utech *et al.*, 2005). However, the two cytokines also act synergistically to impair epithelial barrier function (Wang *et al.*, 2006). IFN- γ sensitizes epithelial cells to TNF- α stimulation, which further potentiates the disruption of intercellular TJs (Fish *et al.*, 1999; Wang *et al.*, 2006).

The transcription factor NF- κ B activated in response to TNF- α also plays a role in the disruption of the epithelial barrier (Ma *et al.*, 2004). Previous studies have investigated the role of proinflammatory-cytokine-induced apoptosis (stimulated by TNF- α) and found that programmed cell death may be a mediator of barrier dysfunction (Bruewer *et al.*, 2003). However, the concentration of cytokines used in our model was much less than what has been reported to induce apoptosis (10 versus 100 ng ml⁻¹). Furthermore,

Fig. 6. ERK-1/2 sustains barrier function during co-incubation of LGG with epithelial cells challenged with pro-inflammatory cytokines. Monolayers were inoculated with probiotic and pro-inflammatory cytokines, and measurements of TER were taken immediately prior to and at 24 h after TNF- α stimulation. The inhibitor PD-98059, which is specific for MEK-1/2, the upstream kinase that activates ERK-1/2, was added (50 μ M) at the time of bacterial inoculation and remained in the culture medium for the duration of the experiment. All treatments contained vehicle (DMSO) at 0.1 % (v/v). The number of separate experimental trials, each run in duplicate, is indicated in each bar. Values are reported as mean $\pm$ SEM. ANOVA with Tukey post test, ** P < 0.01, * P < 0.05.

other groups have demonstrated that the proapoptotic effects of these cytokines are separable from their ability to induce barrier permeability (Bruewer *et al.*, 2003; Marano *et al.*, 1998; Yan & Polk, 2002), suggesting that apoptosis is not the major factor mediating barrier dysfunction in this model system.

In the present study, we demonstrated that LGG prevented TNF- α -induced activation of NF- κ B, since the probiotic reduced the cytoplasm-to-nucleus translocation of the p65 subunit. The suppressive effects of LGG on TNF- α signalling were probably not due to the bacterium either interfering with TNF receptors directly or causing breakdown of the cytokine, because the probiotic and cytokines were added to separate compartments of the polarized epithelial (Transwell) model (Resta-Lenert & Barrett, 2006).

Recent experimental evidence indicates that the beneficial effects of probiotics are species and strain specific (Yan & Polk, 2010). For instance, *L. farciminis* has anti-nociceptive effects and preserves TJs in a rat model of irritable bowel syndrome involving stress-induced visceral hypersensitivity (Ait-Belgnaoui *et al.*, 2006) and trinitrobenzenesulfonic acid (TNBS)-induced colitis, through its capacity to spontaneously release nitric oxide as an anti-inflammatory mediator (Lamine *et al.*, 2004). Various strains of *L. plantarum* have been shown, by using epithelial model cell lines, to protect TJs against enteroinvasive *E. coli* (Qin *et al.*, 2009) and to dampen the effects of TNF- α -induced CXCL-8 secretion by Caco-2 cells (Ko *et al.*, 2007). *L. plantarum* inhibits the TNF-receptor, MyD-88-dependent, and MyD-88-independent induction of the NF- κ B pathway in young adult mouse colon (YAMC) cells, RAW macrophages, and murine dendritic cells (Petrof *et al.*, 2009). Despite positive findings in these other models, both *L. farciminis* and *L. plantarum* were ineffective in protecting epithelial barrier function and suppressing pro-inflammatory cytokine signalling in our studies. Further work should

aim to determine if various strains of *L. rhamnosus*, in addition to LGG, impart the same protective effects on polarized epithelia.

LGG secretes factors that prevent YAMC and HT-29 cell apoptosis in response to TNF- α exposure, a finding that was not replicated when either *Lactobacillus casei* or *Lactobacillus acidophilus* were used as the inoculate strain (Yan *et al.*, 2007). The same secreted factors protect Caco-2 epithelial barrier function against damage induced by exposure to hydrogen peroxide (Seth *et al.*, 2008), whereas only whole LGG provided protective effects in our studies. Varying experimental conditions could account for this discrepancy. For instance, previous studies did not employ LGG for relatively long-term *in vitro* studies in the context of extended stimulation of human colonic epithelial cells with both IFN- γ and TNF- α .

The findings of the present study also demonstrate that the dampened levels of NF- κ B signalling have functional significance. LGG decreased secretion of the NF- κ B-regulated chemokines CXCL-8 and CCL-11, two chemokines that attract neutrophils and eosinophils, respectively, to sites of injury in a variety of human intestinal conditions characterized by mucosal inflammation (Ahrens *et al.*, 2008; Blanchard & Rothenberg, 2009; Chen *et al.*, 2001; Stadnyk, 2002). LGG has previously been shown to suppress CXCL-8 secretion in Caco-2 cells using IL-1, TNF- α or bacterial flagellin as stimuli (Choi *et al.*, 2008; Lopez *et al.*, 2008; Zhang *et al.*, 2005). *Bifidobacterium infantis* decreases production of CCL-11 in a dextran-sodium-sulfate-induced murine colitis model (Tanabe *et al.*, 2008). The finding in the present study that LGG dampens secretion of CCL-11 is novel and may explain the mechanisms of efficacy behind a recent study in infants which employed LGG to supplement extensively hydrolysed casein formula for the alleviation of cow's milk protein-induced allergic colitis (Baldassarre *et al.*, 2010).

It is important to note that LGG does not completely inhibit NF- κ B activation in response to the TNF- α stimulus and that dampening of this signalling cascade, rather than complete blockade, is probably beneficial for the epithelium. Using a variety of inhibitors and varying concentrations of NF- κ B pathway second messengers, Wang *et al.* (2005) demonstrated that complete inhibition NF- κ B signalling serves to exacerbate IFN- γ - and TNF- α -induced barrier disruption. NF- κ B also mediates both protective and disruptive functions in the intestine *in vivo* (Spehlmann & Eckmann, 2009). For instance, deletion of IKK- γ /NEMO in a mouse model induces spontaneous colitis and ablation of epithelial barrier function, and allows translocation of luminal bacteria (Nenci *et al.*, 2007). In the context of chemically (TNBS) or bacterially induced colitis, NF- κ B inhibition with p65 subunit-interfering oligonucleotides or pharmacological inhibitors (BAY 11-7085) attenuates inflammation (Karrasch *et al.*, 2007; Neurath *et al.*, 1996).

We observed that addition of PD-98059 also abolished the protective effects of LGG. This finding suggests that epithelial cells require a working ERK-1/2 response to transduce the LGG-stimulated effects on the host to maintain epithelial barrier function. ERK-1/2 has a role in the modulation of Caco-2 TJs in a hydrogen peroxide stress model (Basuroy *et al.*, 2006). Epidermal growth factor prevents TJ disruption by activating ERK-1/2, which then colocalizes and regulates occludin to maintain its location at the apical interface (Basuroy *et al.*, 2006). This protective effect is also abolished with pharmacological inhibition of the ERK-1/2 pathway (Basuroy *et al.*, 2006). The finding that MAPKs like ERK-1/2 mediate a protective barrier response has been reported in previous studies involving probiotics, including strain LGG. For instance, the use of the specific MEK-1/2 inhibitor U0126 abolished the protective effects of isolated LGG proteins (p40 and p75) against hydrogen peroxide-mediated damage (Seth *et al.*, 2008). Secreted factors from *B. infantis* also confer barrier-enhancing properties in T84 human colonic cells and protection against either IFN- γ or TNF- α stimulation that requires ERK-1/2 activity (Ewaschuk *et al.*, 2008). The anti-inflammatory properties of *L. acidophilus* strain ATCC 4356 against either IFN- γ or TNF- α stimulation are also abolished with the use of MAPK inhibitors (Resta-Lenert & Barrett, 2006). LGG also mediates ERK-1/2 activation and subsequent upregulation of cytoprotective heat-shock proteins involved in epithelial cell survival and barrier maintenance (Tao *et al.*, 2006).

Taken together, this report further elucidates the mechanisms of action of some probiotics in the context of chronic inflammatory models. The signalling interactions between LGG and a model epithelium appear twofold. Firstly, LGG suppresses TNF- α -induced NF- κ B activation, as evidenced by maintenance of epithelial barrier integrity against pro-inflammatory cytokine stimuli and by a reduction in the secretion of specific chemokines induced by activation of this signal transduction pathway. Secondly,

the effects of this probiotic are mediated, at least in part, by an ERK-1/2 response, which when suppressed abolishes the protective effect of LGG on the integrity of a polarized epithelial monolayer. These findings provide insight for new directions in the design of novel interventions that could be used in the prevention of both acute and chronic inflammatory bowel diseases.

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