Functional modulation of human intestinal epithelial cell responses by *Bifidobacterium infantis* and *Lactobacillus salivarius*

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Summary

Intestinal epithelial cells (IECs) and dendritic cells (DCs) play a pivotal role in antigen sampling and the maintenance of gut homeostasis. However, the interaction of commensal bacteria with the intestinal surface remains incompletely understood. Here we investigated immune cell responses to commensal and pathogenic bacteria. HT-29 human IECs were incubated with *Bifidobacterium infantis* 35624, *Lactobacillus salivarius* UCC118 or *Salmonella typhimurium* UK1 for varying times, or were pretreated with a probiotic for 2 hr prior to stimulation with *S. typhimurium* or flagellin. Gene arrays were used to examine inflammatory gene expression. Nuclear factor (NF)-κB activation, interleukin (IL)-8 secretion, pathogen adherence to IECs, and *mucin*-3 (MUC3) and *E-cadherin* gene expression were assayed by TransAM assay, enzyme-linked immunosorbent assay (ELISA), fluorescence, and real-time reverse transcriptase–polymerase chain reaction (RT-PCR), respectively. IL-10 and tumour necrosis factor (TNF)-α secretion by bacteria-treated peripheral blood-derived DCs were measured using ELISA. *S. typhimurium* increased expression of 36 of the 847 immune-related genes assayed, including *NF-κB* and *IL-8*. The commensal bacteria did not alter expression levels of any of the 847 genes. However, *B. infantis* and *L. salivarius* attenuated both IL-8 secretion at baseline and *S. typhimurium*-induced pro-inflammatory responses. *B. infantis* also limited flagellin-induced IL-8 protein secretion. The commensal bacteria did not increase *MUC3* or *E-cadherin* expression, or interfere with pathogen binding to HT-29 cells, but they did stimulate IL-10 and TNF-α secretion by DCs. The data demonstrate that, although the intestinal epithelium is immunologically quiescent when it encounters *B. infantis* or *L. salivarius*, these commensal bacteria exert immunomodulatory effects on intestinal immune cells that mediate host responses to flagellin and enteric pathogens.

Keywords: commensal bacteria; flagellin; interleukin-8; intestinal epithelium; mucins

Introduction

The human gastrointestinal tract is divergently challenged in that it must be able to tolerate dietary antigens and endogenous microflora, and simultaneously recognize and signal the presence of pathogens. The epithelium plays a crucial role in the maintenance of intestinal homeostasis[1] and actively samples resident bacteria, pathogens and other antigens.[2,3] The epithelium is covered by mucus that protects the mucosal surface by limiting pathogen

Abbreviations: CFU, colony-forming units; ct, crossing threshold; DCs, dendritic cells; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; IBD, inflammatory bowel disease; IECs, intestinal epithelial cells; IL, interleukin; MRS, de Man Rogosa Sharpe; NF, nuclear factor; PBMCs, peripheral blood mononuclear cells; RT, reverse transcriptase; TLR, Toll-like receptor; TNF, tumour necrosis factor; TSA, tryptic soy agar; TSB, tryptic soy broth.

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access.[4] Mucus is mainly composed of complex glycoproteins called mucins which are encoded by various *mucin* (*MUC*) genes.[4,5] Intestinal epithelial cells (IECs) secrete many mediators involved in immune responses to potentially pathogenic organisms, including antibacterial peptides such as defensins,[6] mucins including MUC3,[4] and chemokines and cytokines such as interleukin (IL)-8.[7]

IL-8, a C-X-C chemokine that is transcriptionally regulated by nuclear factor (NF)-κB,[8] shows potent chemotactic activity for neutrophils.[9] IL-8 is secreted by IECs in response to various pathogenic bacteria[10] and pro-inflammatory cytokines such as tumour necrosis factor (TNF)-α. Furthermore, it has been reported that certain non-pathogenic commensal bacteria, such as *Escherichia coli* Nissle 1917,[11,12] but not *Lactobacillus reuteri*, *Lactobacillus rhamnosus* GG or the probiotic cocktail VSL#3,[11–13] induce the secretion of IL-8 by IECs.

Antigen-presenting dendritic cells (DCs) survey and sample commensal and pathogenic bacteria at mucosal interfaces.[14,15] Moreover, intestinal DCs can directly sample the contents of the gut lumen by extending dendrites between IECs.[16] Host pattern recognition receptors, including Toll-like receptors (TLRs), play a fundamental role in immune cell activation in response to specific microbial-associated molecular patterns that are associated with a variety of organisms including bacteria, fungi and viruses.[17] IECs and DCs constitutively express several TLRs, including TLR5 which responds to the monomeric flagellin subunits of bacterial flagella.[18–20] In the epithelium, TLR5 is a key mediator of pro-inflammatory responses to flagellin from pathogenic and commensal bacteria.[21–23] Flagellin also stimulates the maturation of responsive DCs.[18] Together with DCs and IECs, TLRs thus represent integral components of the mucosal innate immune system.

Probiotics, commensal organisms that can be harnessed for health benefits, have demonstrated therapeutic effects in murine models of colitis[24,25] and in patients with inflammatory bowel diseases (IBDs),[26–28] diarrhoea[29,30] and, most recently, irritable bowel syndrome.[31] Despite increased recognition of the importance of luminal flora in the development of colitis[32,33] and the almost paradoxical benefits conferred by certain probiotics in these conditions, therapeutic approaches that modulate the bacterial load are hampered by a limited understanding of host flora interactions at the epithelial interface. In this study, we examined immune cell responses to *Salmonella typhimurium*, flagellin, and *Bifidobacterium infantis* and *Lactobacillus salivarius*, two commensal strains demonstrated to have probiotic properties.[24,31,34] We demonstrate that, although the epithelium is immunologically unresponsive to *B. infantis and L. salivarius*, these commensal bacteria induce the secretion of regulatory cytokines by DCs. Furthermore, the data show that *B. infantis* and *L. salivarius* functionally modulate the epithelium by attenuating *S. typhimurium*- and flagellin-induced pro-inflammatory responses.

Materials and methods

Bacteria and growth conditions

Salmonella typhimurium UK1 (kindly provided by R. Curtiss III, Washington University in St Louis, MO), *Bifidobacterium infantis* 35624 and *Lactobacillus salivarius* subspecies *salivarius* UCC118[34,35] were stored in 50% glycerol at −70°. Prior to use in experiments, *S. typhimurium* was cultured at 37° in tryptic soy broth (TSB) (Merck, Darmstadt, Germany) for 18 hr under aerobic conditions, *B. infantis* was cultured anaerobically at 37° in de Man Rogosa Sharpe (MRS) (Merck) broth supplemented with 0·05% cysteine (Sigma-Aldrich, St Louis, MO) for 48 hr, and *L. salivarius* was cultured anaerobically at 37° in MRS broth for 18 hr. The stationary-phase bacteria were centrifuged, resuspended in sterile phosphate-buffered saline (PBS), and Gram-stained to confirm purity. Bacterial number was estimated by measuring the absorbance at 600 nm, and relating the absorbance value to a standard curve of colony-forming units (CFU) on MRS agar or tryptic soy agar (TSA) (Merck).

Epithelial cell culture

In this study, the HT-29 human colonic epithelial cell line (American Type Culture Collection, Manassas, VA) was chosen as this IEC model has been extensively used by many groups investigating epithelial responses to bacteria.[11,13,36,37] HT-29 cells were cultured in modified McCoy's 5A medium (Gibco-BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Sigma-Aldrich) in the presence or absence of 100 U/ml penicillin G and 100 μg/ml streptomycin (Gibco-BRL). The HeLa (human cervix epithelial-like) cell line (ATCC) was cultured in minimal essential medium (Gibco-BRL) supplemented with 0·1 mM non-essential amino acids (Gibco-BRL), 1·5 mM L-glutamine, 10% heat-inactivated FCS, 100 U/ml penicillin G and 100 μg/ml streptomycin. The cells were routinely propagated in 75-cm^2 tissue culture flasks at 37° in a humidified, 5% CO_2 incubator until they approached 80–90% confluency. Subsequently, the cells were trypsinized and used in experimental investigations as specified below.

HT-29 cell treatments

For all assays, cell viability was determined by trypan blue exclusion, and a known number of HT-29 cells were seeded into 25-cm^2 culture flasks, 9·6-cm^2 six-well plates, or 3·8-cm^2 12-well plates. In some experiments, after 24 hr HT-29 cells were incubated for varying times with *B. infan-*

tis, *L. salivarius* or *S. typhimurium* at a bacterial to epithelial cell ratio of 10 : 1, a dose used previously by others.[13] In other investigations, HT-29 cells were grown to confluence, and confluent monolayers were treated with 1×10^5, 1×10^6 or 1×10^7 CFU/ml *B. infantis* or *L. salivarius.* Dose–response studies were performed to determine the optimal concentrations of flagellin and TNF-α to use for stimulation of IECs and, subsequently, HT-29 cells were treated with 0·5 μg/ml purified *S. typhimurium* flagellin (InvivoGen Corp., San Diego, CA) or 5 ng/ml TNF-α (R & D Systems, Minneapolis, MN). Bacterial survival following 1, 2, 6 or 11 hr of incubation with HT-29 cells was assessed by plating serial dilutions of cell culture supernatants on MRS agar or TSA. Following a 24-hr incubation (for *S. typhimurium* and *L. salivarius*) or a 48-hr incubation (for *B. infantis*), the CFU were quantified. In some experiments, HT-29 cells were pretreated for 2 hr with a known dose of commensal bacteria and subsequently were infected with an equivalent dose of *S. typhimurium* or treated with 0·5 μg/ml flagellin or 5 ng/ml TNF-α for varying times.

Cell viability assay

IEC viability was assessed using propidium iodide. Briefly, cell culture supernatants were removed from untreated and bacteria-treated HT-29 cells. The cells were scraped, washed in PBS, and resuspended in 50 μg/ml propidium iodide (Sigma-Aldrich) and 5 Kunitz units/ml ribonuclease A in PBS (Sigma-Aldrich). The stained cells were analysed using an Epics Elite flow cytometer (Beckman Coulter, Inc., Fullerton, CA), and propidium iodide fluorescence was detected at 675 nm. The pH of the corresponding cell culture supernatants was measured using a PHM61 Laboratory pH Meter (Radiometer A/S, Copenhagen, Denmark).

Gene arrays

Human Cytokine Expression Arrays (R & D Systems) were used to examine inflammatory gene expression in bacteria-treated HT-29 cells. Each array membrane comprised 847 cloned cDNAs representing immune-related genes including various cytokines, chemokines, and other immunoregulatory factors, printed as polymerase chain reaction (PCR) products on a positively charged nylon membrane. Briefly, HT-29 cells were seeded in six-well plates in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) supplemented with 10% heat-inactivated FCS and 50 μg/ml gentamycin (Gibco-BRL) and were grown to confluence. HT-29 monolayers were treated with approximately 1×10^7–10^8 cells/well stationary-phase *B. infantis*, *L. salivarius* or *S. typhimurium* for 0·5, 2 or 6 hr. Untreated cell monolayers served as controls. Following treatment, the cells were scraped and centrifuged at 400 ***g*** for 10 min. PolyA mRNA was extracted from the cell pellets using the Dynalbeads® mRNA Direct™ kit (Dynal, Oslo, Norway) according to the manufacturer's recommendations, and was treated with RNase-free DNase (Ambion, Cambridgeshire, UK). mRNA integrity was assessed by 2% agarose gel electrophoresis and ethidium bromide staining. Subsequently, human cytokine-specific primers (R & D Systems) were annealed to 600 ng of mRNA in 11 μl of diethyl pyrocarbonate (DEPC)-treated water. The mRNA was reverse-transcribed using a cDNA labelling and hybridization kit (R & D Systems) according to the manufacturer's instructions. The reaction comprised 20 μCi [α-^{33}P]dCTP (Amersham Pharmacia, Buckinghamshire, UK), dNTP mix (333 μM dATP, dTTP and dGTP, and 1·67 μM dCTP) (R & D Systems), 30 Units of RNase inhibitor (Ambion), and 50 Units of avian myeloblastosis virus reverse transcriptase (RT). Unincorporated [α-^{33}P]dCTP was removed from the cDNA using a Sephadex® G-25 Spin column (Sigma-Aldrich).

Hybridization of the [α-^{33}P]dCTP-labelled cDNA probe to the Human Cytokine Expression Array membrane was performed according to the manufacturer's protocol. Following hybridization, array membranes were exposed to high-intensity phosphor screens at room temperature overnight. Screens were examined using Molecular Dynamics Storm Phosphorimager (Amersham Biosciences, Piscataway, NJ), and arrays were analysed using PHORETIX™ Array 2 (Nonlinear Dynamics, Newcastle-upon-Tyne, UK) and FOCUS© (Steve Cole, Los Angeles, CA) software. Each array was performed in duplicate in each of two independent experiments.

Real-time RT-polymerase chain reaction (RT-PCR) analysis of IL-8 mRNA expression

The 2-hr mRNA samples from the gene array experiments were selected for RT-PCR analysis of IL-8 mRNA. mRNA (250 ng) was reverse-transcribed to yield cDNA using 40 U/μl RNase inhibitor (Ambion) and the Expand Reverse Transcriptase system (Roche Diagnostics Ltd, East Sussex, UK) according to the manufacturer's protocol. Real-time RT-PCR for human IL-8 was performed in a LightCycler 1·5 (Roche) using a previously described IL-8 primer set[38] (MWG-Biotech Ltd, Ebersberg, Germany) and a Fast Start DNA Master SyBr Green I kit as specified by the manufacturer (Roche). Calculations were performed using *GAPDH* as a relative standard, and the *GAPDH* primers (forward, 5′-ACCACAGTCCATGCCATCAC-3′; reverse, 5′-TCCAC CACCCTGTTGCTGTA-3′) were synthesized by Clontech (BD Biosciences, San Jose, CA).

Analysis of MUC3A, MUC3B *and E-cadherin mRNA expression*

In humans, MUC3 is among the predominant ileocolonic mucins[4] and E-cadherin is a cell surface protein involved

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in cell adhesion.[39] We used real-time RT-PCR to examine the expression of *E-cadherin* and two *MUC3* genes, *MUC3A* and *MUC3B*,[40] in commensal-treated HT-29 cells. In the absence of antibiotics, confluent HT-29 monolayers were treated with 1×10^7 cells/ml *B. infantis* or *L. salivarius* for 1 or 2 hr. The cells were harvested by trypsinization, and total RNA was isolated using the Absolutely RNA® Miniprep kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Samples were treated twice with RNase-free DNase I. Total RNA (1 μg) was reversed-transcribed from random primers (Roche) using ImProm-II RT (Promega, Madison, WI) and RNasin Plus RNase inhibitor (Promega). Real-time RT-PCR analysis was performed on 5 μl of the resulting cDNA using LightCycler TaqMan Master (Roche), and LightCycler Uracil-DNA glycosylase (Roche) as specified by the manufacturer, together with Universal ProbeLibrary probes (Exiqon, Vedbaek, Denmark) in a final reaction volume of 20 μl. Primers designed using the Universal ProbeLibrary Assay design centre (http://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp) were synthesized by MWG Biotech Ltd. PCR amplification was performed using a LightCycler 1·5 instrument (Roche). Thermal cycling conditions comprised 2 min at 40° and 10 min at 95°, followed by 45 amplification cycles at 95° for 10 seconds, 60° for 30 seconds, and 72° for 1 second, and a 40° cooling cycle for 30 seconds. Calculations were performed using *GAPDH* as the endogenous control reference gene. Fold difference in gene expression was calculated according to the standard formula $2^{(Ec - Rn) - (Et - Rt)}$, where *Ec* is the crossing threshold (ct) of the experimental gene in untreated control samples, *Rn* is the ct of *GAPDH* in untreated samples, *Et* is the ct of the experimental gene in treated samples, and *Rt* is the ct of *GAPDH* in treated samples. Data are presented as the mean ± standard error (SE) of three independent experiments.

Immunohistochemical staining for intracellular NF-κB

HeLa cells were selected as an epithelial cell model to visualize intracellular NF-κB localization as they have an optimal nucleus-to-cytoplasm ratio and are epithelial cells.[13] Intracellular NF-κB activation in the model was assessed using immunohistochemistry as described previously.[13] Briefly, HeLa cells (1×10^7 cells/ml) were grown on coverslips in six-well plates for 3 days, and incubated with 1×10^7 *B. infantis* or 20 ng/ml TNF-α for 30 min. The coverslips were washed, fixed, blocked, and incubated with 6 μg of mouse anti-NF-κB antibody (BD Pharmingen, Mississauga, Ontario, Canada), followed by fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (IgG) (1 : 64) (Sigma-Aldrich) as described previously.[13] The coverslips were mounted in glycerol-PBS, and slides were viewed using a LSM510 (Carl Zeiss, Jena, Germany) confocal microscope.

NF-κB p65 transcription factor assay

HT-29 cells (3×10^6) were seeded in 25-cm^2 tissue culture flasks in 5 ml of antibiotic-free medium. After 24 hr of incubation, the medium was replaced with antibiotic- and serum-free medium, and the cells were treated with *S. typhimurium*, flagellin or TNF-α in the presence or absence of probiotic pretreatment for varying times. Nuclear proteins were extracted using the Active Motif Nuclear Extract kit (Active Motif Europe, Rixensart, Belgium) according to the manufacturer's instructions, and the total protein concentration of the lysates was determined by Bradford assay (Bio-Rad, Hercules, CA). Activation of the NF-κB p65 subunit in 5 μg of HT-29 nuclear extracts was determined using an NF-κB p65 enzyme-linked immunosorbent assay (ELISA)-based transcription factor assay kit (TransAM assay) (Active Motif Europe) according to the manufacturer's protocol. The NF-κB detecting antibody recognizes an epitope on p65 that is accessible only when NF-κB is activated. The positive control Jurkat nuclear extract provided with the kit was used to assess assay specificity.

IL-8 ELISA

Subconfluent or confluent HT-29 cells grown in antibiotic- and serum-supplemented media were treated with known doses of probiotic bacteria or pro-inflammatory stimuli for 6 or 24 hr. Following treatment, immunoreactive IL-8 protein levels in cell-culture supernatants were quantified using an ELISA DuoSet kit (R & D Systems) according to the manufacturer's protocol. In the gene array assays, the IL-8 DuoSet kit was used also to assess extracellular IL-8 protein levels in cell-culture supernatants and intracellular IL-8 in these cells following lysis with ice-cold water.

Bacterial interference assays

Probiotic interference with pathogen association with HT-29 cells was assessed using two independent techniques, the plate dilution method and biofluorescence. For the plate dilution method, HT-29 cells (1×10^6) seeded in six-well plates in antibiotic-free medium were incubated for 24 hr. Subsequently, the medium was replenished, and the cells were pretreated with or without 10 : 1 *B. infantis* or *L. salivarius* for 2 hr prior to infection with 10 : 1 *S typhimurium* for a further 2 hr. After infection, the medium was discarded and the cells washed five times with 2 ml of warm medium to remove non-adherent bacteria. Cells with associated bacteria were lysed using sterile distilled water supplemented with 0·1% bovine serum albumin for 30 min at 4° with agitation as described previously.[41] IEC-associated *S. typhimurium* were defined as adherent plus intracellular bacteria and

were quantified by CFU counts of diluted cell lysates on TSA.

Independently, *S. typhimurium* grown overnight in TSB were pelleted by centrifugation and washed with sterile 0·85% NaCl. *S. typhimurium* (2×10^8) were labelled using 1·5 μl of SYTO 9® (Molecular Probes, Inc., Eugene, OR), a green-fluorescent nucleic acid stain, as specified by the manufacturer. HT-29 cells were then infected with 10 : 1 SYTO 9-labelled *S. typhimurium* for 2 hr in the presence or absence of probiotic pretreatment. Subsequently, the cells were washed five times with warm 0·85% NaCl and cells with associated bacteria were lysed as outlined above. Aliquots of lysate (200 μl) were transferred to wells of flat-bottom 96-well plates, and fluorescence was quantified by *in vivo* imaging using the IVIS™ 100 Imaging System and Living Image Software (Xenogen, Alameda, CA). The numbers of IEC-associated bacteria were determined from a standard curve of serial dilutions of SYTO 9-labelled *S. typhimurium* relative to fluorescence intensity. For both techniques, probiotic interference was evaluated by comparing *S. typhimurium* association with HT-29 cells in the presence or absence of probiotic pretreatment.

Bacterial treatment of DCs isolated from human peripheral blood mononuclear cells (PBMCs)

In accordance with a protocol approved by the Ethics Committee of Cork University Hospital, Cork, Ireland, peripheral blood from healthy volunteers ($n = 3$) was collected by venepuncture into sterile ethylenediamine-tetraacetic acid (EDTA) vacutainer tubes. PBMCs were purified from buffy coats in histopaque tubes (Greiner Bio-one, Inc., Longwood, FL) using Ficoll-Hypaque (Pharmacia, Dübendorf, Switzerland) gradient centrifugation (400 ***g*** for 30 min). PBMCs were taken from the interface and washed four times with Ca^{2+}- and Mg^{2+}-free PBS, and were finally resuspended in Ca^{2+}- and Mg^{2+}-free degassed column buffer (PBS, pH 7·2, supplemented with 0·5% BSA and 2 mM EDTA). Subsequently, monocytes were isolated from mononuclear cells by negative selection using the Monocyte Isolation Kit II (Miltenyi Biotec) with MACS Column and MACS Separator (Miltenyi Biotec) according to the manufacturer's instructions. To generate DCs, monocytes were incubated for 5 days in DMEM containing 10% FCS, 100 000 IU/ml granulocyte–monocyte colony-stimulating factor, and 40 000 IU/ml IL-4 (BD Biosciences). Cell purity was assessed by cell surface staining and flow cytometry using antibodies obtained from BD Biosciences, and viability was determined by trypan blue exclusion. Cell purity as determined by HLA-DR-positive and CD3/CD14/CD16/CD19/CD20/CD56-negative cells was consistently > 85%, and cell viability was consistently > 98%. Subsequently, PBMC-derived DCs (1×10^6 cells/ml) were seeded in 1·9-cm^2 24-well plates in DMEM supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 2·5 μg/ml fungizone (Gibco-BRL). Cells were treated with medium alone (negative untreated controls), or with 10 : 1 *B. infantis* or *L. salivarius* for 48 hr. Subsequently, IL-10 and TNF-α protein levels in cell-culture supernatants were quantified using commercially available ELISA kits (R & D Systems) according to the manufacturer's protocols. In order to determine the levels of endotoxin in the commensal bacterial preparations we used a *Limulus* amebocyte lysate (LAL) gel-clot assay with a sensitivity of 0·03 Endotoxin units/ml (Charles River Laboratories, Inc., Charleston, SC). The assay was used according to the manufacturer's instructions and *Escherichia coli* control standard endotoxin (Charles River Laboratories, Inc.) was used to confirm LAL-reagent sensitivity. Tap water was used as a positive control and LAL-reagent water (Charles River Laboratories, Inc.) as a negative control.

Statistics

All data are expressed as mean ± SE. Statistical analyses were performed using unpaired two-tailed Student's *t*-tests or analysis of variance (ANOVA). *P*-values < 0·05 were considered to be statistically significant, and *n* represents the number of independent experiments performed.

Results

IEC viability following bacterial treatment

To determine whether the bacteria used in this study had any immediate direct effect on the cell line model, HT-29 cells were exposed to *B. infantis*, *L. salivarius* or *S. typhimurium* in the presence of antibiotics for varying times. Compared with untreated controls, neither the two probiotic strains nor *S. typhimurium* exerted a toxic effect on HT-29 cells over a 24-hr time-period (Fig. 1a). Parenthetically, compared with untreated cells, there was a marginal improvement in survival 24 hr after HT-29 cells were exposed to bacteria, although the biological significance of this is uncertain. Moreover, co-incubation of HT-29 cells with each individual bacterial strain did not adversely affect the pH of the growth media (Fig. 1b); no bacterial overgrowth was observed, and after 6 hr < 0·02% of the bacteria remained viable (Fig. 1c). Therefore, under the assay conditions, IEC integrity was not compromised during co-incubation with *B. infantis*, *L. salivarius* or *S. typhimurium.*

Inflammatory gene expression following bacteria incubation

Human Cytokine Expression Arrays were used to examine inflammatory gene expression in bacteria-treated HT-29

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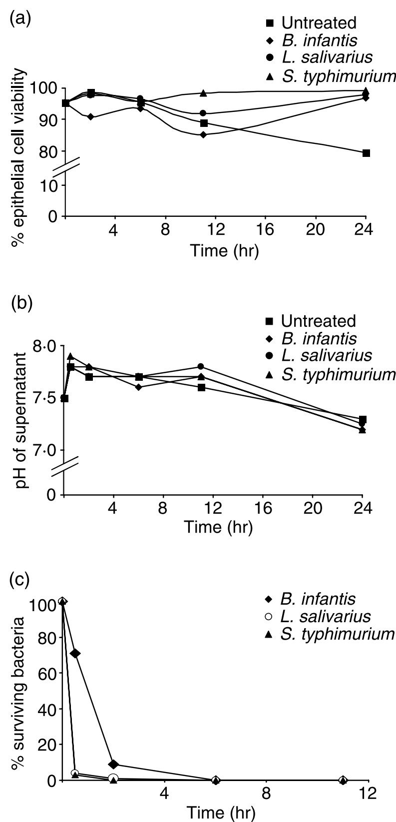

Figure 1. Effects of bacterial treatment on the HT-29 cell line model. HT-29 cells in antibiotic-supplemented media were untreated, or were exposed to *Bifidobacterium infantis*, *Lactobacillus salivarius,* or *Salmonella typhimurium* at a bacterial to epithelial cell ratio of 10 : 1 for up to 24 hr. (a) Epithelial cell viability was measured at the indicated time-points and results are expressed as per cent viable HT-29 cells detected at each time-point. (b) The pH of the corresponding cell culture supernatants recorded at each indicated time-point is shown. (c) Bacterial survival at various times post treatment was determined by quantifying colony-forming units and results are expressed as per cent surviving bacteria. The graphs are representative of $n = 3$ independent experiments.

cells. Treatment with *B. infantis* or *L. salivarius* for 0·5, 2 or 6 hr did not augment the expression of any of the 847 immune-related genes assayed, whereas infection with *S. typhimurium* increased expression of 36 genes associated with pro-inflammatory responses (Table 1). These included a repertoire of signal transducers, immunoreceptors, chemokines, and transcription factors, among them *NF-κB* and the chemokine *IL-8*.

NF-κB activation following treatment of epithelial cells

In order to confirm the gene array results, we used HeLa cervical epithelial cells as an independent epithelial model to assess NF-κB activation by immunohistochemistry. Untreated HeLa cells expressed NF-κB p65 constitutively in the cytoplasm (Fig. 2a), and treatment with 20 ng/ml TNF-α caused a rapid cytoplasmic to nuclear translocation of NF-κB, and increased levels of NF-κB p65 were detected in the nucleus within 30 min. In contrast, treatment with *B. infantis* for the same time did not induce nuclear translocation and NF-κB remained localized to the cytoplasm (Fig. 2a). Similarly, in HT-29 cells, treatment with *B. infantis* or *L. salivarius* for 30 min or 1 hr did not stimulate the DNA-binding activity of NF-κB p65 compared with untreated cells, whereas infection with *S. typhimurium* or treatment with TNF-α significantly increased NF-κB DNA-binding activity at 1 hr or 30 min, respectively (Fig. 2b). NF-κB DNA-binding activity was detected in the positive control Jurkat nuclear extract, and the specificity of NF-κB binding in the assay was confirmed by competition with free wild-type NF-κB consensus oligonucleotide or mutated NF-κB oligonucleotide (Fig. 2b). The data demonstrate that epithelial cells respond differently to various antigens, and, in contrast to *S. typhimurium* and TNF-α, the commensal bacteria used in this study did not induce NF-κB nuclear translocation or DNA-binding activity.

S. typhimurium, but not *B. infantis* or *L. salivarius*, induces IL-8 expression

In subconfluent HT-29 cells treated with *B. infantis* or *L. salivarius*, the levels of *IL-8* mRNA or protein detected after 2 or 24 hr, respectively, were similar to those found at baseline in untreated cells (Figs 3a and b). Conversely, infection with *S. typhimurium* stimulated a significant increase in *IL-8* mRNA expression after 2 hr (13·3-fold) and IL-8 protein secretion after 24 hr (7-fold) compared with untreated cells. As shown in Fig. 3(c), *S. typhimurium* quickly induced *IL-8* mRNA and maximum levels were detected within 2 hr of infection. Infection also resulted in a time-dependent accumulation of extracellular IL-8 that was associated with a corresponding decrease in the amount of detectable intracellular IL-8 protein (Fig. 3c).

B. infantis and *L. salivarius* inhibit IL-8 secretion at baseline

We next examined whether treatment with increasing doses of *B. infantis* or *L. salivarius* (1×10^5, 1×10^6, or 1×10^7 CFU/ml) affected IL-8 secretion by confluent HT-29 cell monolayers. IL-8 protein levels were measured after 6 hr. A dose-dependent inhibition of baseline IL-8 secretion by confluent HT-29 monolayers was observed (Fig. 4). At a concentration of 1×10^7 CFU/ml, which under the experimental conditions was equivalent to approximately 10 bacteria per epithelial cell, both *B. infantis* and *L. salivarius* significantly inhibited basal IL-8 secretion by 38 and 44%, respectively. This suggests that these commensal bacteria exert anti-inflammatory

Table 1. Immunoregulatory genes stimulated by *Salmonella typhimurium*

Gene (official nomenclature)	Function	Maximum induction		
		$n = 1$*	$n = 2$*	Time (hr)
CXCL8, interleukin-8 (*IL-8*)	CXC chemokine	17	20	2
CXCL1, growth related oncogen protein-alpha (*Gro-α*)	CXC chemokine	17	21	2
CXCL2, growth related oncogene protein-beta (*Gro-β*)	CXC chemokine	15	20	2
CXCL3I, growth related oncogene protein-gamma (*Gro-γ*)	CXC chemokine	15	21	2
CCL20, macrophage inflammatory protein 3-alpha (*MIP-3-α*)	CC chemokine	5	7	2
CXCL5, epithelial neutrophil activating peptide-78 (*ENA-78*)	CXC chemokine	7	4	6
Tumour necrosis factor-alpha, (*TNF-α*)	Pro-inflammatory cytokine	7	4	2
Interleukin-1-beta (*IL-1β*)	Pro-inflammatory cytokine	5	6	2
CSF2, Colony stimulating factor 2 (*GM-CSF*)	Pro-inflammatory cytokine	3	3	2
Bone morphogenetic protein 4 (*BMP-4*)	Growth and differentiation factor	1·5	1·7	0·5
Amphiregulin (*AREG*)	Growth factor and mitogen	2	3	2
Cyclin-dependent kinase inhibitor 1A (p21) (*CDKN1A* (p21))	Kinase inhibitor	4	4	2
v-myc myelocytomatosis viral oncogene homolog (avain) *MYC* (*c-Myc*)	Transcription factor activity	3	2	6
Signal recoginition particle 72 kDa (*SRP72*)	Signal transduction	1·6	2	0·5
Myeloid differentiation primary response gene (88) (*MYD88*)	Signal transduction	2	1·7	0·5
Tumour necrosis factor receptor-associated factor-2 (*TRAF2*)	Signal transduction	2·75	3·5	2
Nuclear factor of kappa light polypeptide gene enhancer in B-cells (p105) (*NF-κB-1* (p50))	Transcription factor	1·8	2·6	2
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100) (*NF-κB-2* (p100))	Transcription factor	2·6	2·8	2
Interferon regulatory factor 1 (*IRF-1*)	Transcription activator	2·8	2·6	2
B-cell CLL/lymphoma 3 (*BCL3*)	Transcription coactivator	2·7	4	2
Interleukin 6 signal transducer (*IL-6ST* (gp130))	Signal transduction	2·1	2·2	0·5
Toll-like receptor 3 (*TLR3*)	Receptor activity	2	2·3	0·5
Interferon gamma receptor 1 (*IFNGR1*)	Receptor activity	3	2	6
G protein-coupled receptor 19 (*GPR19*)	Receptor activity	8	10	2
Interleukin 18 receptor 1 (*IL-18R1*)	Receptor activity	2	2	6
Interleukin 10 receptor, beta (*IL-10RB*)	Receptor activity	2·4	2·5	6
Intercellular adhesion molecule type 1 (*ICAM-1*)	Receptor activity/adhesion	6	5·75	2
Integrin-alpha-4 (*ITGA4*)	Receptor activity	4	8	2
CD3G antigen, gamma polypeptide (TTT3 complex) (*CD3G*)	T-cell receptor binding	2·4	1·5	0·5
CD40 antigen (TNF receptor superfamily member 5) (*CD40*)	Receptor activity	3	2	6
Lymphocyte adhesion molecule 1 (*SELL* (L-selectin))	Receptor activity	3·5	2·7	6
Expressed in non-metastatic cells 6, protein (nucleoside diphosphate kinase) *NME6* (*NM23-H6*)	Kinase activity	1·7	1·7	0·5
Ephrin receptor A2 (*EPHA2*)	Enzymatic and receptor activity	1·9	1·9	2
Prostaglandin-endoperoxide synthase 2 (*Cox-2*)	Enzymatic activity	1·7	2·8	2
Matrix metallopeptidase 7 (*MMP-7*)	Enzymatic activity	2·8	2·5	6
Matrix metallopeptidase 8 (*MMP-8*)	Enzymatic activity	2·3	2·2	6

Inflammatory gene expression was evaluated using Human Cytokine Expression Arrays.

*Each array was performed in duplicate, and the mean result of each of two independent experiments expressed as maximum fold induction compared with baseline gene expression in untreated HT-29 cells is indicated. The time postinfection at which maximum gene induction occurred is also shown. See Supplementary Table S1 for a complete list of the genes examined in the cDNA array.

effects on the epithelium by down-regulating the secretion of IL-8.

Probiotic bacteria attenuate *S. typhimurium*-induced pro-inflammatory responses

IL-8 mRNA expression is under the regulatory control of NF-κB,[8] and our data indicated that *B. infantis* or *L. salivarius* do not activate NF-κB or elicit IL-8 production by HT-29 cells. Considering this and the finding that the probiotic bacteria affected IL-8 secretion at baseline, we explored whether *B. infantis* or *L. salivarius* might inhibit NF-κB activation or IL-8 secretion in response to *S. typhimurium*. Subconfluent HT-29 cells were pretreated with 10 : 1 *B. infantis* or *L. salivarius* for 2 hr prior to stimulation with *S. typhimurium* or TNF-α. The levels of

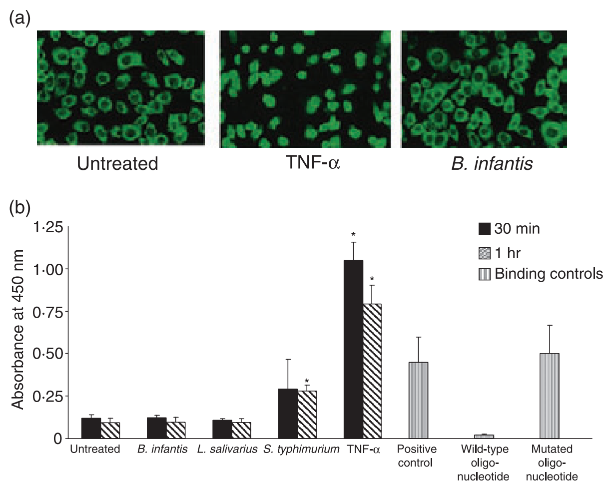

Figure 2. *Bifidobacterium infantis* or *Lactobacillus salivarius* do not activate nuclear factor (NF)-κB in epithelial cells. (a) Immunohistochemical staining for NF-κB p65 demonstrated a constitutive expression of NF-κB p65 in the cytoplasm of untreated HeLa cells. Incubation with tumour necrosis factor (TNF)-α (20 ng/ml) for 30 min caused p65 nuclear translocation, whereas *B. infantis* did not induce translocation of p65. (b) HT-29 cells were untreated, or were treated with 10 : 1 *B. infantis, L. salivarius, Salmonella typhimurium,* or 5 ng/ml TNF-α for 30 min or 1 hr. The DNA-binding activity of NF-κB p65 in HT-29 nuclear extracts was determined using an enzyme-linked immunosorbent assay (ELISA)-based transcription factor assay. The positive control Jurkat nuclear extract provided with the kit was used to verify assay specificity in competition assays with wild-type or mutated NF-κB oligonucleotides. The data represent mean absorbance readings ± standard errors of five separate experiments. *$P < 0·05$ relative to untreated HT-29 cells.

activated NF-κB in nuclear extracts were determined after 0·5, 1 and 2 hr, and IL-8 protein was measured after 24 hr. Prior exposure to either *B. infantis* or *L. salivarius* significantly reduced *S. typhimurium*-induced NF-κB binding activity following 1 or 2 hr of infection (Fig. 5a). Within 30 min, TNF-α stimulated a 8·9-fold increase in activated NF-κB compared with untreated cells, and, although pretreatment with *L. salivarius* or *B. infantis* did not affect NF-κB activation by TNF-α at 30 min, a significant reduction (16% decrease) was observed in *B. infantis*-treated cells 1 hr post TNF-α-stimulation (data not shown).

As shown in Fig. 5(b), *S. typhimurium* induced 1897 (± 292) pg/ml IL-8, and pretreatment with *B. infantis* or *L. salivarius* significantly attenuated *S. typhimurium*-induced IL-8 secretion by 23·5 or 31%, respectively. Although TNF-α potently induced 50 550 (± 9933) pg/ml IL-8, probiotic pretreatment had no significant effect on TNF-α-induced IL-8 secretion (data not shown). Collectively, these data show that *B. infantis* and *L. salivarius* significantly attenuate *S. typhimurium*-induced pro-inflammatory responses.

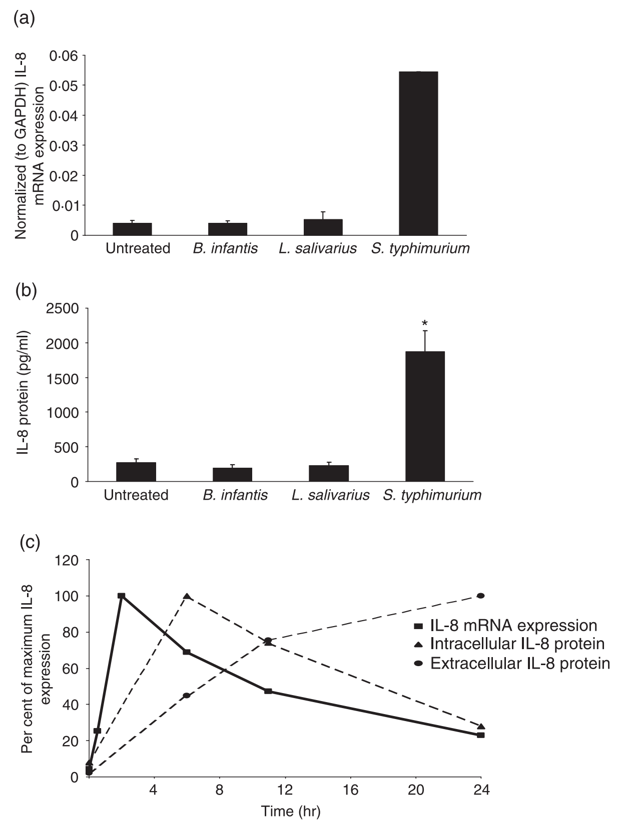

Figure 3. *Salmonella typhimurium*-induced interleukin (IL)-8 expression. Subconfluent HT-29 cells were treated with 10 : 1 *Bifidobacterium infantis*, *Lactobacillus salivarius* or *S. typhimurium* for varying times. Untreated cells served as controls. (a) *IL-8* mRNA expression was determined after 2 hr by real-time reverse transcriptase–polymerase chain reaction (RT-PCR). The data are presented as normalized (to *GAPDH*) *IL-8* mRNA expression levels and are mean ± standard error (SE) ($n = 3$). *$P < 0·05$ relative to untreated cells. (b) IL-8 protein levels (pg/ml) in cell culture supernatants were measured after 24 hr by enzyme-linked immunosorbent assay (ELISA). The data represent mean ± SE ($n = 3$). *$P < 0·05$ relative to untreated cells. (c) Time–course of *S. typhimurium*-induced IL-8 mRNA expression (normalized to *GAPDH*), intracellular *IL-8* protein depletion, and extracellular IL-8 protein accumulation in HT-29 cells. The data are expressed as percentage of maximum IL-8 mRNA/protein expression and are representative of two independent experiments.

S. typhimurium association with HT-29 cells is not blocked by the probiotic bacteria

We next determined whether the modulation of *S. typhimurium*-induced pro-inflammatory responses by the probiotic bacteria was attributable to interference with pathogen binding to IECs. Binding of *S. typhimurium* to HT-29 cells was evaluated in the presence or absence of probiotic pretreatment using biofluorescence and the plate dilution method. The two methods yielded complementary data and, as shown in Table 2, pretreatment with neither *B. infantis* nor *L. salivarius* affected the number of

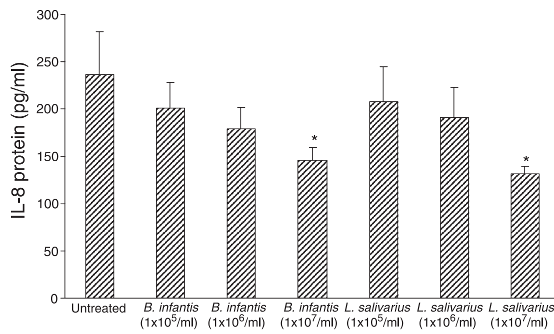

Figure 4. Probiotic pretreatment down-regulates interleukin (IL)-8 secretion at baseline. Confluent HT-29 cells were treated with *Bifidobacterium infantis* or *Lactobacillus salivarius* at doses of 1×10^5, 1×10^6, or 1×10^7 colony-forming units (CFU)/ml, and IL-8 protein levels were measured after 6 hr. *B. infantis* and *L. salivarius* caused a dose-dependent inhibition of baseline IL-8 secretion by confluent HT-29 monolayers ($*P < 0{\cdot}05$ compared with untreated monolayers). The data are expressed as pg/ml IL-8 and represent the mean ± standard error ($n = 3$ independent experiments).

IEC-associated *S. typhimurium* detected 2 hr after infection. These data indicate that the immunoregulatory effects of the probiotic bacteria observed in this study are not attributable to competitive inhibition of *S. typhimurium* association with HT-29 cells.

B. infantis inhibits flagellin-induced IL-8 secretion

Flagellin is a key activator of pro-inflammatory responses to *Salmonella* in IECs.[22,42] Therefore, we explored whether the probiotic bacteria could inhibit IEC responses to *Salmonella* flagellin. Firstly, in dose–response studies, we incubated various doses of flagellin from *S. typhimurium* (0·1, 0·5 and 1·0 µg/ml) with confluent HT-29 monolayers, and found that, at a concentration of 0·5 µg/ml, flagellin significantly induced NF-κB activation and IL-8 secretion. Treatment with 0·5 µg/ml flagellin for 1 hr resulted in a 3·15 (± 0·3)-fold increase in NF-κB binding activity compared with untreated cells, and 1876 (± 262) pg/ml IL-8 was detected after 6 hr ($P < 0{\cdot}05$) (data not shown). HT-29 monolayers were treated for 2 hr with either *B. infantis* or *L. salivarius* (1×10^6 or 1×10^7 CFU/ml), followed by 0·5 µg/ml flagellin for 6 hr. As shown in Fig. 6, pretreatment with *L. salivarius* did not affect flagellin-induced IL-8 at either dose tested. However, flagellin-induced IL-8 was significantly inhibited in cells that were pretreated with 1×10^7 CFU/ml *B. infantis.* The results suggest strain-specific antagonistic effects on inducible IL-8 expression in IECs.

MUC3A, *MUC3B* and *E-cadherin* expression following probiotic treatment

It has been proposed that some strains of probiotic bacteria exert beneficial effects on the intestinal epithelium

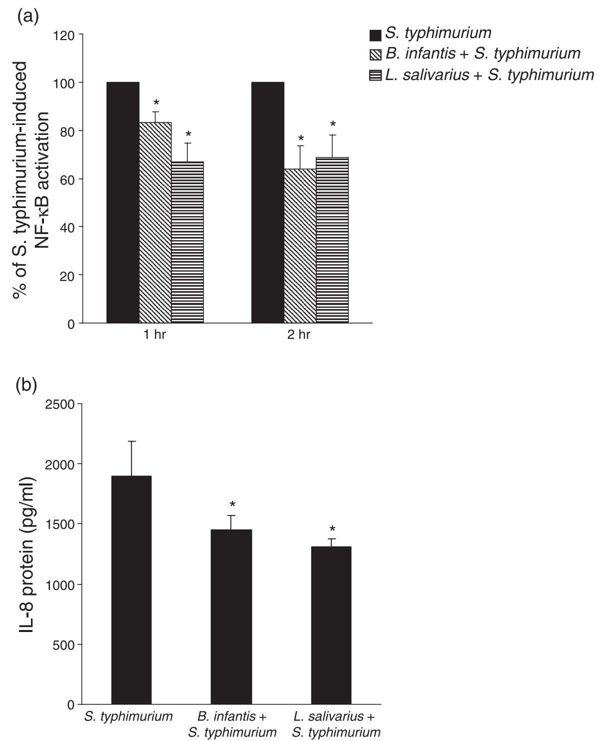

Figure 5. Probiotic pretreatment attenuates *Salmonella typhimurium*-induced pro-inflammatory responses. Subconfluent HT-29 cells were pretreated with 10 : 1 *Bifidobacterium infantis* or *Lactobacillus salivarius* for 2 hr prior to infection with 10 : 1 *S. typhimurium*. The levels of activated nuclear factor (NF)-κB in nuclear extracts were determined after 1 and 2 hr, and interleukin (IL)-8 protein levels were measured after 24 hr. (a) Pretreatment with *B. infantis* or *L. salivarius* reduced NF-κB activation by *S. typhimurium* after 1 and 2 hr ($*P < 0{\cdot}05$ compared with *S. typhimurium*-infected HT-29 cells). The data are expressed as per cent of *S. typhimurium*-induced NF-κB activation, and represent the mean ± standard error (SE) ($n = 5$ independent experiments). (b) Pretreatment with *B. infantis* or *L. salivarius* inhibited *S. typhimurium*-induced IL-8 secretion ($*P < 0{\cdot}05$ relative to *S. typhimurium*-infected HT-29 cells). The data are expressed as pg/ml IL-8 and represent the mean ± SE ($n = 6$ independent experiments).

by up-regulating the expression of mucin genes.[36,43] We used real-time RT-PCR to determine the expression of *MUC3A* and *MUC3B* mRNA in confluent HT-29 cells treated for 1 or 2 hr with 1×10^7 cells/ml probiotic bacteria. *MUC3A* and *MUC3B* mRNA was detected in untreated HT-29 cells, and, as shown in Fig. 7, treatment with *B. infantis* or *L. salivarius* had no significant effect on the expression of these genes. Similarly, no difference was observed in *E-cadherin* mRNA expression following treatment with the commensal bacteria (data not shown).

DC cytokine responses following probiotic incubation

We examined the effect of the commensal bacteria used in this study on cytokine secretion by myeloid DCs. DCs

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Table 2. Probiotic bacteria do not interfere with *Salmonella typhimurium*–HT-29 cell association

Sample	Plate dilution method	Biofluorescence method
	Cell-associated *S. typhimurium* (log CFU/ml)*	% of cell-associated *S. typhimurium*†
S. typhimurium	3·24E + 05 ± 1·25E + 05	100
B. infantis + *S. typhimurium*	3·25E + 05 ± 8·85E + 04	90·02 ± 13·75
L. salivarius + *S. typhimurium*	3·61E + 05 ± 7·23E + 04	112·04 ± 10·85

Probiotic interference with pathogen binding was evaluated by quantifying the numbers of *S. typhimurium* associated with HT-29 cells in the presence or absence of probiotic pretreatment. Data represent the mean ± standard error of three independent experiments.

*Intestinal epithelial cell (IEC)-associated *S. typhimurium* were quantified by colony-forming units (CFU) counts of diluted cell lysates on tryptic soy agar (TSA) and results are expressed as log CFU/ml.

†IEC-associated *S. typhimurium* were quantified by *in vivo* imaging fluorescence using a standard curve of serial dilutions of SYTO 9-labelled *S. typhimurium* relative to fluorescent intensity. Results are expressed as per cent HT-29 cell-associated *S. typhimurium*.

B. infantis, *Bifidobacterium infantis*; *L. salivarius*, *Lactobacillus salivarius*.

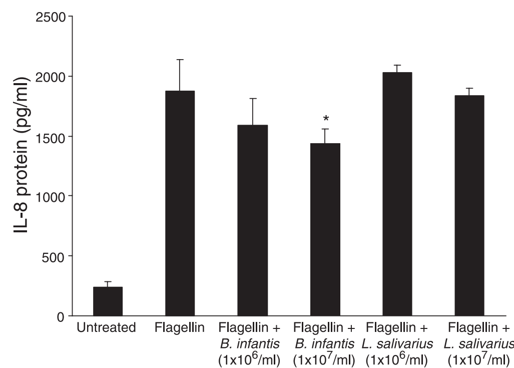

Figure 6. *Bifidobacterium infantis* attenuates flagellin-induced interleukin (IL)-8 secretion. Confluent HT-29 cells were pretreated for 2 hr with *B. infantis* or *Lactobacillus salivarius* at doses of 1×10^6 or 1×10^7 colony-forming units (CFU)/ml prior to stimulation with 0·5 μg/ml flagellin for 6 hr. Pretreatment with 1×10^7 CFU/ml *B. infantis*, but not *L. salivarius*, significantly inhibited flagellin-induced IL-8 secretion (*$P < 0{\cdot}05$ relative to flagellin-treated HT-29 monolayers). The data are expressed as pg/ml IL-8 and represent the mean ± standard error of three independent experiments.

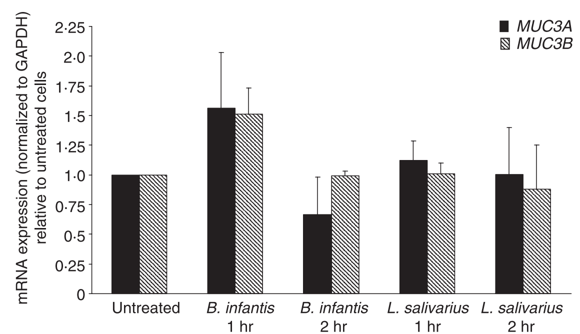

Figure 7. *MUC3* mRNA expression is unaltered by probiotic treatment. Confluent HT-29 monolayers were treated with 1×10^7/ml *Bifidobacterium infantis* or *Lactobacillus salivarius* for 1 or 2 hr. Real-time reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of *MUC3A* and *MUC3B* mRNA expression was performed and the fold difference in gene expression compared with that of untreated samples was calculated using *GAPDH* as the reference gene. Data are presented as mean ± standard error of three separate experiments.

were treated with *B. infantis* or *L. salivarius* for 48 hr, and IL-10 and TNF-α protein levels were quantified. Both bacteria stimulated a significant increase in IL-10 and TNF-α secretion by PBMC-derived DCs compared with untreated DCs (Fig. 8). These effects were not a result of endotoxin contamination of the bacterial suspensions as < 0·03 Endotoxin units/ml, the detection limit of the LAL assay, were detected in the medium controls and bacterial preparations.

Discussion

In the present study, we have shown that IECs respond differentially to various bacterial species. In contrast to *S. typhimurium*, *B. infantis* or *L. salivarius* did not induce pro-inflammatory responses by IECs. However, *B. infantis* and *L. salivarius* functionally modulated the epithelium by inhibiting the constitutive secretion of IL-8 and attenuating *S. typhimurium*-induced NF-κB activation and IL-8 secretion. Moreover, *B. infantis* inhibited flagellin-induced IL-8 protein, and both commensal strains stimulated the secretion of IL-10 and TNF-α by myeloid DCs. Taken together, these data demonstrate that *B. infantis* and *L. salivarius* exert immunomodulatory effects that mediate intestinal immune cell responses to enteric pathogens and their antigenic components.

Commensal bacteria, their ligands and commensal-derived symbiosis factors have been shown to be essential for normal development of the mucosal immune system

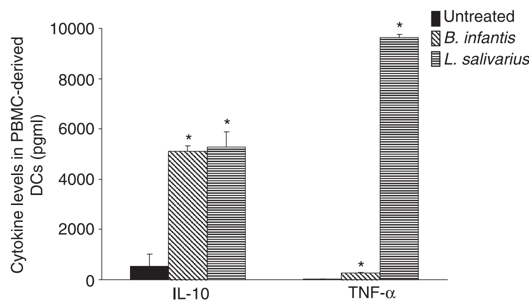

Figure 8. Commensal bacteria stimulate interleukin (IL)-10 and tumour necrosis factor (TNF)-α secretion by myeloid dendritic cells (DCs). DCs isolated from peripheral blood mononuclear cells (PBMCs) of healthy volunteers ($n = 3$) were exposed to *Bifidobacterium infantis* or *Lactobacillus salivarius* for 48 hr, and IL-10 and TNF-α levels were determined. *B. infantis* and *L. salivarius* stimulated IL-10 and TNF-α secretion (*$P < 0{\cdot}05$ relative to untreated PBMC-derived DCs). The data are expressed as mean pg/ml ± standard error of three independent experiments.

and the maintenance of gut homeostasis,[2,44,45] but their role in intestinal physiology is incompletely understood. Here we have demonstrated that the intestinal epithelium mounts a robust pro-inflammatory response to *S. typhimurium*, but not to *B. infantis* or *L. salivarius.* In particular, *B. infantis and L. salivarius* did not stimulate NF-κB activation or IL-8 secretion. Similarly, other commensal bacteria, including *L. rhamnosus* GG, *L. reuteri*, *Lactobacillus plantarum*, and the VSL#3 cocktail, do not activate NF-κB or IL-8.[11–13,46] The current study considerably extends these observations, and the gene array analysis demonstrated that, in addition to NF-κB and IL-8, *B. infantis* or *L. salivarius* did not augment the expression of any of the other 845 immune-related genes assayed. However, immune cells were not universally unresponsive to these commensal bacteria and they induced the secretion of regulatory cytokines by myeloid DCs. This supports previous studies that showed that probiotic bacteria modulate DC function and promote tolerance-inducing DCs.[47,48] Furthermore, certain non-pathogenic bacteria can activate gut inflammatory processes and induce IL-8 secretion by IECs.[11–13,49] The non-pathogenic commensal strain *Bacteroides vulgatus* has been shown to activate NF-κB and pro-inflammatory gene expression in IECs.[49] However, these effects were inhibited by the presence of immune cells[37] and immune–IEC cross-talk differentially affects pro-inflammatory gene expression in response to commensal bacteria.[50]

Our data indicate that both *B. infantis* and *L. salivarius* limited the constitutive secretion of IL-8. This effect was only observed in confluent HT-29 monolayers, demonstrating the importance of an intact barrier for normal epithelial immune function. Moreover, pretreatment with *B. infantis* and *L. salivarius* inhibited *S. typhimurium*-, but not TNF-α-induced IL-8 secretion, although *B. infantis* delayed TNF-α-induced NF-κB activation. This suggests the involvement of different intracellular signalling pathways between commensal-mediated affects on *S. typhimurium*- and TNF-α-mediated pro-inflammatory responses. Considering the crucial role of IL-8 in inflammatory processes and the implication of enteric bacteria in the pathogenesis of IBD,[51] our finding that *B. infantis* and *L. salivarius* attenuated IL-8 secretion at baseline and in response to *S. typhimurium* may be of particular physiologic relevance in IBD and other inflammatory conditions. Other commensal strains, including *L. reuteri*, *Bacteroides thetaiotaomicron*, *Salmonella pullorum* and VSL#3, have exhibited similar effects on pathogen-induced IL-8 secretion in IECs,[11,13,52,53] and *L. reuteri* was shown to inhibit the constitutive synthesis, but not secretion, of IL-8.[13]

It has been established that flagellin is a prerequisite for *Salmonella*-induced TLR5-mediated pro-inflammatory responses in IECs.[22,42] In our study *B. infantis*, but not *L. salivarius*, attenuated flagellin-induced IL-8, suggesting that different modes of action mediate strain-specific antagonistic effects on inducible IL-8 expression. To our knowledge, only one other commensal strain, *B. thetaiotaomicron*, has been reported to restrict flagellin-mediated signalling.[52] Although the responsible mechanism(s) remains unknown, it is possible that commensal surface structures act through host cell receptors to modulate inflammatory responses,[54,55] but our data do not implicate the TNF receptor in this process. It has been proposed that receptor systems antagonistic to TLR action, analogous to SIGIRR (single Ig IL-1R-related molecule),[56] are involved in mediating protective effects of the commensal flora.[57] Moreover, the composition of cell surface structures such as lipotechoic acid of *Lactobacillus plantarum* has been shown to differentially impact on the host immune system.[58] The ability of the gut to tolerate large numbers of potentially pro-inflammatory commensal bacteria could, in part, be attributed to the anti-inflammatory effects of bacteria such as *B. thetaiotaomicron* and *B. infantis* that limit the signalling induced by flagellin and *Salmonella.* The possibility that *B. infantis* exerts similar effects on other flagellated pathogens is being investigated.

The data suggest that the inhibitory effects of *B. infantis* and *L. salivarius* were mediated, at least in part, through the NF-κB pathway. *B. infantis* and *L. salivarius* inhibited *S. typhimurium*-induced NF-κB activation, and *B. infantis* did not stimulate nuclear translocation of p65 in HeLa cells. Several distinct modes of action by which commensal bacteria impinge on NF-κB signalling to limit inflammation have been elucidated. These include inhibition of IκB-α ubiquitination or epithelial proteasome function[53,59] or the nuclear export of transcriptionally active p65.[52] Mitogen-activated protein kinase and protein kinase B pathways have also been implicated in the protective effects mediated by various commensal bacteria.[11,60] In addition,

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several host homeostatic mechanisms that limit inflammatory responses in the gut have been described.[1,57,61]

We queried whether the inhibitory effects of *B. infantis* and *L. salivarius* on *S. typhimurium*-induced pro-inflammatory responses were attributable to competitive exclusion at the epithelial surface. Such a defensive layer could comprise the commensal bacteria themselves, or the induction of mucin genes. The data indicate that these commensal bacteria did not hinder the association of *S. typhimurium* with HT-29 cells. Consistent with this observation, *B. infantis* and *L. salivarius* did not induce the expression of *MUC3A* or *MUC3B*. VSL#3 and other *Lactobacillus* strains have been reported to induce mucin expression,[11,36,43,62] thereby preventing adhesion of pathogens to the epithelial surface.[36,43] However, commensal-induced mucin up-regulation is strain-specific, and *Lactobacillus acidophilus* does not stimulate *MUC3* expression.[36] Of note, mucin expression is affected by the growth medium, and in other studies HT-29 cells were progressively transferred to glucose-free medium to preferentially increase *MUC3* mRNA expression.[36,43] Although commensal bacteria have been shown to modify pathogen-induced alterations in cytoskeletal and tight junction proteins such as zonula occludens, occludin and actinin,[11,41] we have shown that *B. infantis* or *L. salivarius* do not induce alterations in *E-cadherin* mRNA expression in HT-29 cells.

In conclusion, we have demonstrated that IECs display immunological quiescence when exposed to *B. infantis* or *L. salivarius*, but these bacteria can modulate epithelial responses to limit inflammatory signals. Functional capabilities appear to be strain-specific, and the precise mechanisms by which *B. infantis* and *L. salivarius* limit inflammatory responses are being explored. Collectively, the data demonstrate that *B. infantis* and *L. salivarius* exert immunomodulatory effects on IECs that mediate host responses to flagellin and enteric pathogens.

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Supplementary material

The following supplementary material is available for this article online:
Table S1. The 847 cloned cDNAs representing immune-related genes on the Human Cytokine Expression Arrays used to examine inflammatory gene expression in bacteria-treated HT29 cells.
This material is available as part of the online article from http://www.blackwell-synergy.com