

Regulation of the Polymeric Immunoglobulin Receptor in Intestinal Epithelial Cells by *Enterobacteriaceae*: Implications for Mucosal Homeostasis

Maria E. C. Bruno,¹ Eric W. Rogier,¹ Aubrey L. Frantz, Andrew T. Stefka, Stephanie N. Thompson, and Charlotte S. Kaetzel

Department of Microbiology, Immunology & Molecular Genetics, University of Kentucky, Lexington, Kentucky 40536, USA

The commensal microbiota of the human colon profoundly impacts host gene expression and mucosal homeostasis. Secretory IgA antibodies, which influence the composition of the intestinal microbiota and provide immunity against pathogens, are transported across intestinal epithelial cells (IEC) by the polymeric immunoglobulin receptor (pIgR). To compare the effects of different colonic bacteria on pIgR expression, the human IEC line HT-29 was stimulated with various species representing the 4 major phyla of colonic bacteria. Only bacteria from the family *Enterobacteriaceae* (phylum *Proteobacteria*) induced expression of pIgR and other target genes of bacterial pattern recognition receptors. HT-29 cells responded to purified ligands for Toll-like receptor (TLR)4 but not TLR2. Expression of pIgR and transport of IgA were significantly reduced in colons of mice deficient in the TLR adaptor MyD88, consistent with a role for TLR signaling in the regulation of pIgR by colonic bacteria. Induction of pIgR expression in HT-29 cells required NF- κ B signaling but not MAPK signaling, in contrast to the requirement for both NF- κ B and MAPK signaling for induction of pro-inflammatory genes. These results suggest that commensal *Enterobacteriaceae* may promote intestinal homeostasis by enhancing pIgR expression in IEC.

¹Authors Bruno and Rogier contributed equally to this work.

Address correspondence to Charlotte S. Kaetzel, Ph.D., Department of Microbiology, Immunology & Molecular Genetics, University of Kentucky, 124D Combs Cancer Research Building, 800 Rose Street, Lexington, KY 40536-0096; E-mail: csaet@uky.edu

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INTRODUCTION

Secretory IgA (SIgA) antibodies play a critical role in shaping the composition of the gut microbiota and in maintenance of intestinal homeostasis (Woof et al., 2005, 2006; Brandtzaeg et al., 2007; Macpherson et al., 2008). The polymeric immunoglobulin receptor (pIgR) transports polymeric IgA antibodies from the basolateral to the apical surface of intestinal epithelial cells (IEC), where proteolytic cleavage of the extracellular domain of pIgR (also known as secretory component, SC) releases SIgA into the intestinal lumen (Norderhaug et al., 1999; Kaetzel, 2005; Kaetzel et al., 2007). SC, either free or complexed to SIgA, participates in a variety of innate defense mechanisms, including prevention of bacterial adherence to the intestinal mucous layer and neutralization of potential pro-inflammatory factors (Phalipon et al., 2003; Corthésy, 2007). The significance of pIgR in intestinal homeostasis is highlighted by observations that *Pigr*^{-/-} mice are more susceptible than are wild-type mice to infection with *Salmonella typhimurium* (Wijburg et al., 2006) and to development of chemically-induced colitis (Murthy et al., 2006).

Colonic bacteria promote intestinal homeostasis by enhancing the physical integrity of the epithelium and preventing bacteria from translocating into the lamina propria and draining lymphoid tissues (Artis, 2008; Sartor, 2008). The 100 trillion bacteria that comprise the microbiota of the human colon represent upwards of 40,000 species that encode millions of unique gene products (Backhed et al., 2005; Frank et al., 2008). A recent analysis of combined data from 3 independent metagenomic studies indicated that the majority of colonic bacteria in healthy individuals belonged to one of 4 phyla: *Firmicutes* (about 65% of total species); *Bacteroidetes* (about 23%); *Proteobacteria* (about 8%); and *Actinobacteria* (about 3%) (Frank et al., 2008). Alterations in the composition of the colonic microbiota, characterized by relative increases in *Proteobacteria* and *Actinobacteria* and relative decreases in *Firmicutes* and *Bacteroidetes*, have been associated with increased risk for inflammatory bowel disease (IBD) (Frank et al., 2007). Probiotic strains of colonic bacteria have shown promise in the prevention and treatment of IBD, but further clinical and fundamental studies are needed to delineate the mechanisms by which different probiotics exert their beneficial effects (Sartor, 2005; Damaskos et al., 2008; Seksik et al., 2008).

Cross-talk between commensal bacteria and IEC is mediated by several families of pattern recognition receptors (PRRs), including the Toll-like (TLR) and NOD-like receptor families (Hargreaves et al., 2005; Strober et al., 2006; Takeuchi et al., 2007). In previous studies, we demonstrated that signaling through TLR3 and TLR4 up-regulates pIgR expression in the human IEC line

HT-29 (Schneeman et al., 2005). We recently reported that pIgR expression was significantly higher in the colon than in the small intestine of normal human subjects, suggesting that colonic bacteria may regulate pIgR expression in IEC (Arsenescu et al., 2008). In that study we also found that expression of pIgR was lower in the colonic mucosa of patients with Crohn's disease (CD) than that of normal controls. It is not known whether changes in the composition of the colonic microbiota affect pIgR expression in IEC, since the effect of different species of colonic bacteria on pIgR regulation has not been compared systematically. The aim of the present work was to determine whether bacterial species representing the four major phyla of the colonic microbiota differentially regulate the expression of pIgR and other epithelial genes involved in intestinal immunity. We found that pIgR expression was selectively up-regulated in HT-29 cells in response to bacteria of the family *Enterobacteriaceae* of the phylum *Proteobacteria*, including the probiotic *E. coli* strain Nissle 1917 (*EcN*). We also found that expression of pIgR and transport of IgA was reduced in colonic mucosa from mice deficient in the TLR adaptor protein MyD88 compared to wild-type mice. Our findings suggest that bacteria of the family *Enterobacteriaceae* regulate transport of SIgA antibodies into intestinal secretions by inducing pIgR expression in IEC, and propose a novel mechanism through which *EcN* may exert its probiotic effects and ameliorate the symptoms of IBD.

MATERIALS AND METHODS

Bacterial Cell Culture. The phylogeny of bacterial species used in this study is described in Table I. *Escherichia coli* Nissle 1917 was the generous gift of Dr. Ulrich Sonnenborn, Ardeypharm GmbH, Herdecke, Germany. *Salmonella typhimurium* SL1344 was the generous gift of Dr. Sarah D'Orazio, University of Kentucky. All other bacteria were obtained from the American Type Culture Collection (Manassas, VA). Prior to incubation with eukaryotic cells, bacteria were grown to mid-log phase in Schaedler broth (Oxoid, LTD, Basingstoke, UK) under anaerobic conditions at 37°C. For quality control, the identity of each bacterial species was confirmed by PCR amplification of the 16S rRNA gene with species-specific primers. Heat-killed bacteria were prepared by diluting liquid cultures to approximately 10⁸ colony-forming units (CFU)/ml in eukaryotic tissue culture medium and heating at 65°C for 30 min. Aliquots of bacterial cultures prior to heat treatment were plated on Schaedler agar to quantify CFU. For experiments with live bacteria, aliquots of bacterial suspensions before and after co-culture with eukaryotic cells were plated on Schaedler agar to quantify CFU.

Eukaryotic Cell Culture. The HT-29v20 subclone of the human colon adenocarcinoma cell-line HT-29 (Blanch et al., 1999) and the human acute myeloid leukemia cell line THP-1 (Tsuchiya et al., 1980) (a generous gift of Dr. Sarah

D'Orazio, University of Kentucky) were cultured in DMEM Ham's F-12 (1:1) media supplemented with 5% fetal bovine serum and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml fungizone) at 37°C and 5% CO₂. All cell culture reagents were from Lonza Walkersville (Walkersville, MD) or Invitrogen Life Technologies (Carlsbad, CA). Preliminary dose-response experiments (data not shown) demonstrated that maximal induction of pIgR and IL-8 mRNA was achieved at a ratio of 10-20 bacteria to HT-29 cells, and this ratio was used in all subsequent experiments. HT-29 cells were plated in 24-well dishes and grown for 24-48 h to a density of approximately 10⁶ cells/well prior to initiation of experiments.

THP-1 cells were maintained in suspension culture, and were diluted to a density of 1 × 10⁶ cells/well in 24-well dishes immediately before exposure to bacteria. Heat-killed or live bacteria were diluted to a final concentration of approximately 10⁷ cells/well. Where indicated, HT-29 and THP-1 cells were treated with the following TLR ligands at a final concentration of 1 µg/ml: N-palmitoyl-S-[2,3-bis(palmitoyloxy)-propyl]-Cys-Ser-Lys trihydrochloride (Pam3CSK4) (InvivoGen, San Diego, CA), a synthetic tripalmitoylated lipopeptide that acts as a ligand for TLR2; or highly purified lipopolysaccharide (LPS) from *E. coli* O26:B6 (Sigma-Aldrich, St. Louis, MO), which acts as ligand for TLR4. Where indicated, cells were treated 30 min prior to TLR stimulation with the nuclear factor-kappa B (NF-κB) inhibitor Bay11-7082 (Calbiochem, San Diego, CA) at a final concentration of 10 µM, or with mitogen-activated protein kinase (MAPK) inhibitors (PD98059, extracellular signal-related kinase (ERK) inhibitor; SB20358, p38 inhibitor; SP600125, Jun N-terminal kinase (JNK) inhibitor) (Sigma-Aldrich), individually or combined, at a final concentration of 10 µM each.

Bacterial Invasion Assay. The ability of live *E. coli* Nissle or *S. typhimurium* SL1344 to invade monolayers of HT-29 cells was analyzed as described (Criss et al., 2001). Approximately 10⁷ bacteria (see Fig. 3B for enumeration of bacterial CFU) were added to 24-well dishes containing approximately 10⁶ HT-29 cells. After 1 h, triplicate cultures of HT-29 cells were washed extensively with Hank's Balanced Salt Solution (HBSS), lysed with 1% Triton X-100, and plated on Schaedler agar to enumerate total cell-associated CFU. Separate triplicate cultures of HT-29 cells were treated with gentamicin (480 µg/ml in HBSS) for 90 min at 37°C to kill bacteria bound to the external cell surface, washed extensively, lysed, and plated on Schaedler agar to enumerate internalized CFU. Membrane-associated bacteria were calculated by subtracting internalized CFU from total cell-associated CFU.

Mice. Female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). *Myd88*^{-/-} mice were obtained from Dr. Douglas Golenbock (Division of Infectious Diseases and Immunology, University of Massachusetts). The *Myd88*^{-/-} mice were originally engineered in the laboratory of Dr. Shizuo Akira (Hyogo College of Medicine, Hyogo, Japan) and backcrossed onto the

C56BL/6 genetic background for 6 generations (Adachi et al., 1998). *Myd88*^{-/-} mice were bred in the animal facility at the University of Kentucky College of Medicine. All mice were kept in microisolator cages with sterile bedding and were fed sterile food and water. Mice were maintained and used in accordance with the policies and guidelines set forth by the University of Kentucky Institutional Animal Care and Use Committee.

mRNA Analysis. Total cellular RNA was extracted from HT-29 and THP-1 cells using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). For extraction of tissue RNA, whole colons and small intestines were washed thoroughly in cold PBS and stored in a stabilizing solution (RNA later™, Qiagen) at -20°C. Total RNA was extracted using the Qiagen RNeasy Protect Midi kit. The quality of purified RNA was analyzed by agarose gel electrophoresis and by measurement of the ratio of absorbance at 260 and 280 nm. cDNA was synthesized using 200–300 ng of total cellular RNA and the TaqMan Gold RT-PCR kit with random hexamers (Applied Biosystems, Foster City, CA). Specific mRNA levels were quantified by real-time reverse transcriptase PCR (qRT-PCR), using the ABI Prism® 7700 Sequence Detection System (Applied Biosystems). The sequences of primers and fluorescent probes for the human genes pIgR, interleukin (IL)-8, tumor necrosis factor (TNF) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) have previously been reported (Bruno et al., 2005). The proprietary software PrimerExpress™ (Applied Biosystems) was used to design the primers and probe for mouse pIgR as follows (5'-3'): forward primer: CTATTGGTGTCTTACCAATGGTGACT; reverse primer: CTGTTGCGTTCTGTGGCGT; TaqMan probe: ACAATAGAACTCCAGGTTGCCGAAGCTACAAGG. Pre-designed primers and probes for A20, MAPK phosphatase (MKP)-1, TLR2, TLR4, myeloid differentiation primary response gene (MyD)88, single immunoglobulin and toll-interleukin 1 receptor (SIGIRR), Toll interacting protein (TOLLIP) and β 2-microglobulin (β 2-m) were purchased from Applied Biosystems.

PCR was performed using 40 ng reverse-transcribed RNA and TaqMan Universal PCR Master Mix, no UNG (Applied Biosystems). Amplification of the cDNA template was measured at every cycle by fluorescence resonance energy transfer (ΔR_n). The threshold cycle (C_T), which is inversely proportional to the level of a given mRNA transcript, is defined as the cycle at which the ΔR_n exceeds the threshold of fluorescence detection. To determine the relative level of target mRNA in individual samples, the C_T values for each message were normalized to the C_T for the control genes GAPDH (HT-29 and THP-1 cells) or β 2-m (mouse tissues), by the formula $(2^{-C_{T, \text{target}} - C_{T, \text{control}}}) \times 100\%$.

Measurement of Mucosal IgA Levels. Immediately following euthanasia, colonic mucosal secretions were collected with Polywick filters (Polyfiltronics Group, Inc., Rockland, MA) as described (Haneberg et al., 1994). Briefly, 2 mm \times 25 mm filters were introduced through a glass tube inserted into the rectum, then pushed into the colon such that the wick was in direct contact with the

mucosal surface of the distal 3 cm of colon. The section of the colon containing the wick was excised, and the wick was removed by inverting the tissue. Captured mucus was extracted in PBS containing BSA (1% w/v), pepstatin A (1 μ g/ml), leupeptin (1 μ g/ml), PMSF (1 mM), aprotinin (1 μ g/ml) and benzamidine (150 μ M). Mucosal IgA levels were measured using the mouse IgA ELISA quantitation kit (Bethyl Laboratories, Montgomery, TX) according to the manufacturer's protocol.

Statistical Analyses. Statistical differences among treatment groups were determined by ANOVA and Fisher's protected least significant difference test. Student's paired *t* test was used to compare levels of gene expression in paired samples of small and large intestine from individual mice.

RESULTS

Regulation of Gene Expression in Human Intestinal Epithelial Cells by Commensal Bacteria Representing the Four Major Phyla of the Colonic Microbiota
We used the human colon carcinoma cell-line HT-29 as an *in vitro* model to examine the responses of IEC to colonic bacteria. HT-29 cells were utilized for the first studies demonstrating that pIgR mediates epithelial transport of IgA and IgM, thus illuminating the pathway through which secretory immunoglobulins are formed in human mucosal epithelia (Crago et al., 1978; Brandtzaeg et al., 1984). We and others have demonstrated that pIgR expression is regulated in HT-29 cells by cytokines known to modulate intestinal immunity in humans, including IFN- γ , TNF, IL-1 and IL-4 (Sollid et al., 1987; Kvale et al., 1988a, 1988b, 1995b; Phillips et al., 1990; Krajci et al., 1993; Piskurich et al., 1993; Youngman et al., 1994; Denning, 1996; Hayashi et al., 1997; Sarkar et al., 1998; Ackermann et al., 1999; Blanch et al., 1999; Nilsen et al., 1999; Takenouchi-Ohkubo et al., 2004; Bruno et al., 2005). The physiological relevance of these findings was supported by our demonstration that stimulation of HT-29 cells with supernatants from freshly isolated human intestinal lamina propria mononuclear cells dramatically up-regulated pIgR expression, and that this effect was blocked by neutralizing IFN- γ activity (Youngman et al., 1994).

The HT-29 cell-line has subsequently been used as a model system to identify basal and cytokine-inducible regulatory elements in the human *PIGR* gene (Piskurich et al., 1997; Johansen et al., 1998; Takenouchi-Ohkubo et al., 2000; Schjerven et al., 2001, 2003, 2004; Solorzano-Vargas et al., 2002; Ackermann et al., 2004; Bruno et al., 2004). A potential role for commensal bacteria in pIgR regulation was first suggested by the finding that butyrate, a by-product of bacterial fermentation, up-regulated pIgR expression in HT-29 cells (Kvale et al., 1995a). We subsequently demonstrated that bacterial LPS up-regulated pIgR expression in HT-29 cells, suggesting that bacterial-epithelial cross-talk via TLR signaling may regulate transport of secretory immunoglobulins (Schneeman et al., 2005).

To compare the ability of different species of colonic bacteria to regulate pIgR expression, we analyzed pIgR mRNA levels in HT-29 cells stimulated with heat-killed bacteria of representative species from the four major bacterial phyla of the human colonic microbiota (Fig. 1A). Preliminary studies with selected species demonstrated that induction of pIgR expression in response to heat-killed or live bacteria were similar (data not shown). Table 1 describes the phylogeny of the bacteria used in this study, all of which were environmental strains originally isolated from human fecal material. Individual species were chosen based on their relevance to the pathogenesis or prevention of

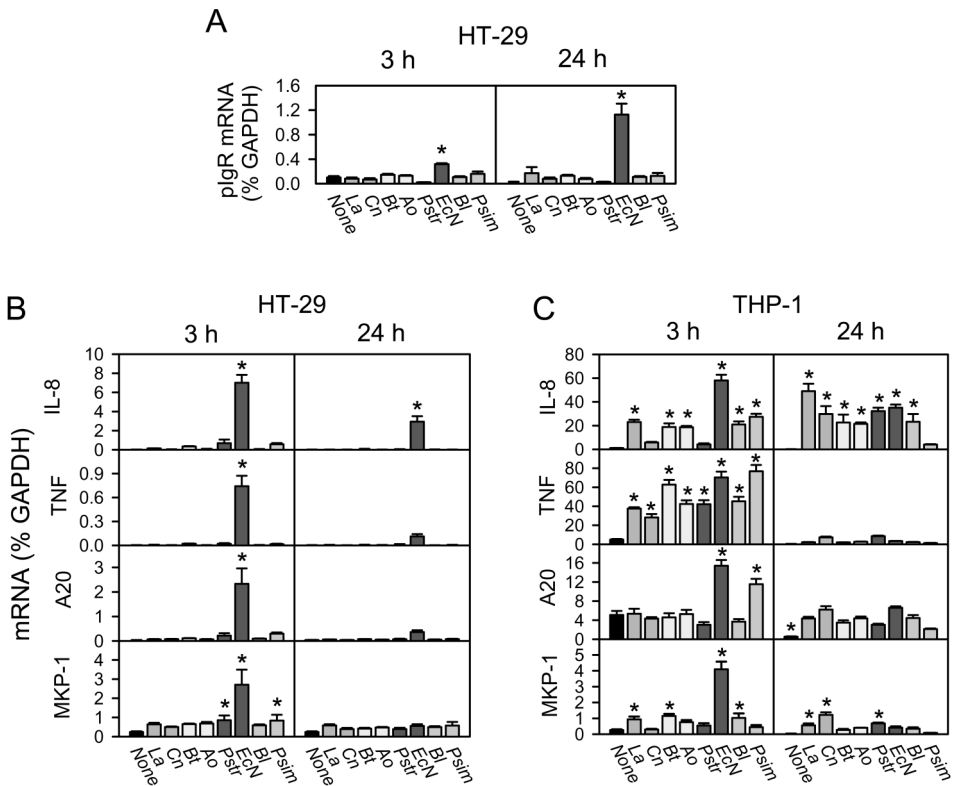


Figure 1: Regulation of gene expression by commensal bacteria representing the four major phyla of the human colonic microbiota. See Table 1 for a description of bacterial phylogeny. Abbreviations: La, *L. acidophilus*; Cn, *C. nexile*; Bt, *B. thetaiotaomicron*; Ao, *A. onderdonkii*; Pstr, *P. straminea*; EcN, *E. coli* Nissle; Bl, *B. longum*; Psim, *P. simplex*. A. HT-29 cells were cultured for 3 or 24 h with heat-killed bacteria at a ratio of 10-20 bacterial cells per eukaryotic cell. pIgR mRNA levels were quantified by qRT-PCR and normalized to GAPDH mRNA. Data from 3 independent experiments were combined and expressed as mean \pm SEM (n = 8). Asterisks indicate that the mean is significantly different from the mean for untreated HT-29 cells at 3 h (p < 0.05). B,C. Analysis of mRNA for pro-inflammatory factors and negative regulators in HT-29 and THP-1 cells treated as described for panel A. Data from 3 independent experiments were combined and expressed as mean \pm SEM (HT-29, n = 8; THP-1, n = 9). Asterisks indicate that the mean is significantly different from the mean for untreated cells from the same cell-line at 3 h (p < 0.05).

Table 1: Phylogeny of bacterial species used in this study.

Phylum	Class	Order	Family	Genus	Species/strain	Gram stain
Commensals:						
Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	acidophilus	Positive
Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	nexile	Positive
Bacteroidetes	Bacteroidetes	Bacteroidales	Bacteroidaceae	Bacteroides	thetaiotaomicron	Negative
Bacteroidetes	Bacteroidetes	Bacteroidales	Rikenellaceae	Alistipes	onderdonkii ^a	Negative
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	sframinea	Negative
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Escherichia	coli Nissle 1917	Negative
Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium	longum	Positive
Actinobacteria	Actinobacteria	Actinomycetales	Nocardoidaceae	Pimelobacter	simplex	Positive
Pathogenic:						
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Salmonella	typhimurium SL1344	Negative

^aPhylogenetic names are according to the National Center for Biotechnology Information Taxonomy database (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=taxonomy>).

^bAlternate name: *Alistipes* sp. WAL 8169.

^cAlternate name: *Nocardioides simplex*.

IBD in humans. A recent study reported that relative numbers of *Clostridium nexile* (*Cn*), *Bacteroides thetaiotaomicron* (*Bt*) and *Alistipes onderdonkii* (*Ao*) were reduced in the colonic microbiota of patients with IBD, whereas relative numbers of *Pseudomonas straminea* (*Pstr*), *Escherichia coli* (*Ec*) and *Pimelobacter simplex* (*Psim*) were increased (Frank et al., 2007).

In contrast to potentially pathogenic strains of *E. coli* isolated from IBD patients (La Ferla et al., 2004), the avirulent *E. coli* strain Nissle 1917 (*EcN*) has been shown to have probiotic properties and to ameliorate inflammation in IBD patients (Schultz, 2008). The probiotic species *Lactobacillus acidophilus* (*La*) and *Bifidobacterium longum* (*Bl*) were included in this experiment because of their widespread use in fermented milk products and other probiotic formulations (Damaskos et al., 2008; Douglas et al., 2008). Interestingly, *EcN* was unique among these 8 commensal species in its ability to induce expression of pIgR. Levels of pIgR mRNA increased slowly in response to *EcN* stimulation, with a small but significant increase at 3 h and a much larger increase at 24 h. In subsequent experiments, no significant change in pIgR expression was observed at 3 h (see Figs. 3 and 4). The delayed increase in pIgR expression in response to *EcN* is consistent with our previous observations with LPS and other TLR ligands (Schneeman et al., 2005; Bruno et al., 2007).

We and others have observed delayed responses of pIgR to cytokine stimulation, due to a requirement for *de novo* synthesis of one or more transcription factors (Krajci et al., 1993; Piskurich et al., 1993, 1997; Schjerven et al., 2000, 2001, 2003, 2004; Schneeman et al., 2005). The delayed response of pIgR to bacterial stimulation suggests that there may be a similar requirement for a newly synthesized transcription factor. In our previous studies involving stimulation of HT-29 cells with cytokines and TLR ligands, we found that levels of membrane and secreted pIgR protein were directly correlated with levels of pIgR mRNA (Piskurich et al., 1993; Schneeman et al., 2005). Although we did not measure pIgR protein levels in the present study, it is reasonable to conclude that increased production of pIgR mRNA resulted in increased expression of pIgR protein.

In contrast to the slow increase in pIgR mRNA, we found that *EcN* caused a rapid induction in mRNA for the pro-inflammatory factors IL-8 and TNF, which declined significantly by 24 h (Fig. 1B). The observed down-regulation of IL-8 and TNF could be attributed in part to the induction of A20, a ubiquitin-editing enzyme that down-regulates NF- κ B signaling (Heyninck et al., 2005), and mitogen-activated protein kinase phosphatase (MKP)-1, a negative regulator of MAP kinase signaling (Chi et al., 2006) (Fig. 1B).

As a control to assess the potential activity of bacteria that did not induce gene expression in HT-29 cells, the THP-1 human monocyte cell-line was stimulated with heat-killed bacteria of the same species (Fig. 1C). Levels of pIgR mRNA were negligible in THP-1 cells even after bacterial stimulation (data not shown), consistent with the epithelial-specific expression of pIgR

(Kaetzel, 2005; Kaetzel et al., 2007). Every commensal species induced expression of IL-8 and TNF in THP-1 cells to levels 100-fold higher than those seen in HT-29 cells. Only selected species, including *EcN*, induced expression of the negative regulators A20 and MKP-1 in THP-1 cells. These results suggest that the potential to induce a pro-inflammatory response in innate immune cells is widespread among commensal bacteria, but that IEC may respond only to a subset of bacteria.

Regulation of pIgR Expression by TLR4 but not TLR2 Signaling. We previously reported that LPS from bacteria of the family *Enterobacteriaceae* (*E. coli* or *Salmonella typhimurium*) induces expression of *pIgR* in HT-29 cells via TLR4 signaling (Schneeman et al., 2005; Bruno et al., 2007). The hyporesponsiveness of HT-29 cells to many commensal bacteria suggests that they may not respond to those bacterial cell wall constituents, common to both Gram-positive and Gram-negative bacteria, that act as ligands for TLR2. Consistent with this notion, we found that *pIgR* expression was up-regulated in HT-29 cells following stimulation with highly purified LPS from *E. coli* O26:B6 (TLR4 ligand), but not Pam3CSK4 (TLR2 ligand) (Fig. 2A).

Expression of IL-8 was also induced by LPS but not Pam3CSK in HT-29 cells (Fig. 2B), indicating that the lack of response to TLR2 stimulation was not gene-specific. By contrast, stimulation of THP-1 cells with either Pam3CSK4 or LPS induced high levels of IL-8 mRNA. The steady-state level of TLR2 mRNA in HT-29 cells was about 2000-fold lower than in THP-1 cells, which likely contributed to the lack of response to Pam3CSK4. Despite the much higher IL-8 response to LPS in THP-1 cells than in HT-29 cells, expression of TLR4 was only marginally higher. Expression of MyD88, a cytoplasmic adaptor protein associated with both TLR2 and TLR4 signaling, was also similar in HT-29 and THP-1 cells. To examine whether the hyporesponsiveness of HT-29 cells to LPS was due to inhibition of TLR4 signaling, expression of two negative regulators of TLR signaling was analyzed. Single Ig IL-1-related receptor (SIGIRR; also known as Toll-IL-1 receptor 8) is a member of the TLR family that acts as a decoy to inhibit ligand binding and/or intracellular signaling (Mantovani et al., 2004). Toll-interacting protein (TOLLIP) is a cytoplasmic factor that binds to the intracellular domain of TLRs and inhibits phosphorylation of the downstream kinase IRAK (Cario et al., 2005).

Basal expression of SIGIRR was about 3-fold lower in THP-1 cells than in HT-29 cells, and was down-regulated by TLR2 or TLR4 ligation in THP-1 but not HT-29 cells. Basal expression of TOLLIP was lower in HT-29 cells than in THP-1 cells, but was modestly up-regulated by TLR2 or TLR4 ligation. Neither HT-29 nor THP-1 cells expressed NOD2, and did not respond to the NOD2 ligand muramyl dipeptide (MDP), either alone or in combination with the TLR2 ligand Pam3CSK4 (data not shown). In conclusion, these results suggest that the relative hyporesponsiveness of HT-29 cells to most species of commensal

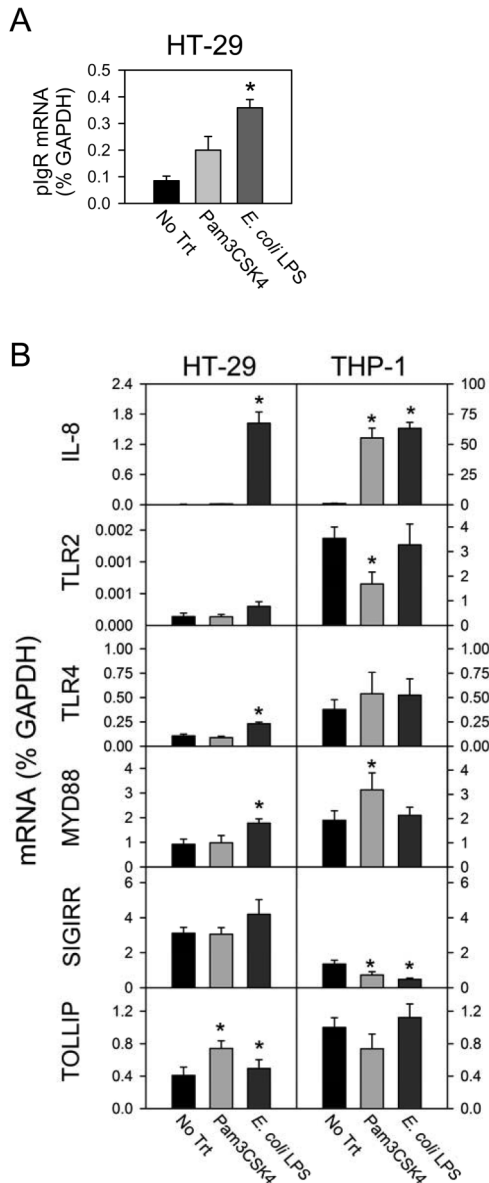


Figure 2: Regulation of gene expression by purified ligands for TLR2 and TLR4. A. HT-29 cells were cultured for 24 h in the absence or presence of Pam3CSK4 (TLR2 ligand) or highly purified LPS from *E. coli* O26:B6 (TLR4 ligand), each at a concentration of 1 μ g/ml. mRNA levels were quantified by qRT-PCR and normalized to GAPDH mRNA. Data from 2 independent experiments were combined and expressed as mean \pm SEM (n = 8). Asterisks indicate that the mean is significantly different from the corresponding mean for untreated HT-29 cells (p < 0.05). B. HT-29 and THP-1 cells were cultured for 3 h in the absence or presence of Pam3CSK4 or LPS, and mRNA levels were analyzed as described for panel A. Data from 2 independent experiments were combined and expressed as mean \pm SEM (n = 8). Asterisks indicate that the mean is significantly different from the corresponding mean for untreated cells from the same cell-line (p < 0.05).

bacteria may be due to a combination of low TLR expression (especially TLR2) and high expression of the decoy receptor SIGIRR and other factors that may dampen downstream signaling pathways.

Regulation of pIgR expression in HT-29 Cells by Commensal and Pathogenic Bacteria of the Family Enterobacteriaceae. We found that *EcN*, a commensal bacterium of the family *Enterobacteriaceae* (phylum *Proteobacteria*) was unique in its ability to induce gene expression in HT-29 cells (Fig. 1). The failure of other commensal species to induce gene expression could be due to a lack of recognition of those species by cellular PRRs or active inhibition of PRR signaling. If the latter mechanism were operative, the presence of “inhibitory” bacteria might down-regulate the response to *EcN*. To test this hypothesis, HT-29 cells were exposed to heat-killed *EcN* in the presence or absence of equivalent doses of *Lactobacillus acidophilus* (*La*, phylum *Firmicutes*), *Bacteroides thetaiotaomicron* (*Bt*, phylum *Bacteroidetes*) or *Bifidobacterium longum* (*Bl*, phylum *Actinobacteria*) (Fig. 3). As expected, *EcN* stimulation resulted in early induction of IL-8 mRNA and delayed induction of pIgR mRNA. None of the bacteria from other phyla, alone or in combination with *EcN*, significantly affected expression of pIgR or IL-8. These findings suggest that concurrent exposure to other types of commensal bacteria does not significantly dampen the response of intestinal epithelial cells to *EcN*. However, the possibility remains that a complex mixture of commensal bacteria could modify the response to *EcN* or other bacteria of the family *Enterobacteriaceae*.

To examine the ability of IEC to discriminate between closely related commensal and pathogenic species of the family *Enterobacteriaceae*, HT-29 cells

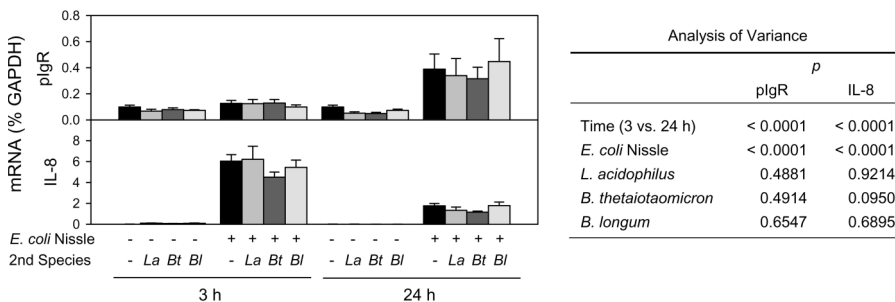


Figure 3: Effect of addition of bacteria from different phyla on the response of HT-29 cells to *E. coli* Nissle. HT-29 cells were cultured for 3 or 24 h with heat-killed *E. coli* Nissle, *L. acidophilus* (*La*), *B. thetaiotaomicron* (*Bt*), or *B. longum* (*Bl*), as indicated. Each bacterial species was added at a ratio of 10-20 bacterial cells per eukaryotic cell. mRNA levels were quantified by qRT-PCR and normalized to GAPDH mRNA. Data from 2 independent experiments were combined and expressed as mean \pm SEM ($n = 7$). Statistical comparisons of the overall effects of time and bacterial stimulation on pIgR and IL-8 mRNA are noted in the analysis of variance table.

were co-cultured with *EcN* or *Salmonella typhimurium* (*St*) strain SL1344. Following 1 h of culture, relative numbers of *EcN* or *St* associated with the plasma membrane of HT-29 cells were found to be similar, whereas only *St* was capable of cellular invasion (Fig. 4A). This result is consistent with reports demonstrating higher invasiveness of *St* in HT-29 cells compared to several nonpathogenic *E. coli* strains, including *EcN* (Wells et al., 1999; La Ferla et al., 2004). To compare the responses to living vs. dead bacteria, HT-29 cells were exposed to live or heat-killed *EcN* or *St* for 3 h (Fig. 4B). Interestingly, early induction of IL-8 expression was greater in response to live *EcN* than live *St*, whereas no differences were observed in the responses to heat-killed *EcN* vs. *St*. Expression of pIgR was not induced following 3 h of stimulation with either live or dead bacteria.

Comparison of the responses at 24 h to *EcN* and *St* was complicated by the fact that the viability of HT-29 cells declined after exposure to live bacteria for more than 3 h (data not shown). To determine whether changes in gene expression persisted after removal of bacterial stimuli, HT-29 cells were treated for 3 h with live or heat-killed *EcN* or *St*, washed with antibiotic-containing culture medium, and cultured for an additional 21 h (24 h harvest). Under these conditions, pIgR mRNA levels were not significantly different from those in untreated HT-29 cells, suggesting that continuous exposure to bacterial components is required to induce pIgR expression (compare Fig. 4B to Fig. 1A and Fig. 3). The lack of significant IL-8 expression at 24 h was likely due to a combination of withdrawal of bacterial stimulation and induction of negative regulatory pathways.

Induction of pIgR by EcN is enhanced by NF- κ B signaling but not MAPK signaling. To examine the contribution of the NF- κ B and MAPK signaling pathways to induction of pIgR expression, HT-29 cells were stimulated with *EcN* in the presence or absence of a chemical inhibitor of NF- κ B activation (Bay 11-7082) (Fig. 5A) or MAPK inhibitors (PD98059, ERK inhibitor; SB20358, p38 inhibitor; SP600125, JNK inhibitor) (Fig. 5B). Inhibition of NF- κ B signaling completely blocked the induction of both pIgR and IL-8, consistent with a central role for this signaling pathway in the response to *EcN*. Inhibition of p38, but not JNK or ERK, significantly reduced the induction of IL-8 by *EcN*, but not to as great an extent as did inhibition of NF- κ B signaling. By contrast, none of the MAPK inhibitors affected the induction of pIgR by *EcN*.

These findings suggest that activation of p38 by *EcN* may synergize with NF- κ B to promote the early pro-inflammatory response. Concurrent induction of the NF- κ B regulator A20 and the MAPK regulator MKP-1 by *EcN* may contribute to the down-regulation of early response genes such as IL-8 and TNF (Fig. 1B). The delayed rise in pIgR mRNA in response to pro-inflammatory stimuli has been attributed to a requirement for *de novo* synthesis of one or more transcription factors (reviewed in Kaetzel, 2005). Our new results suggest

A

Species	<i>E. coli</i> Nissle	<i>S. typhimurium</i>
	(CFU)	(CFU)
Initial concentration	6.1×10^6	9.8×10^6
Final concentration	6.3×10^7	1.6×10^7
Membrane-associated ^a	$1.6 \pm 0.28 \times 10^4$	$2.8 \pm 0.32 \times 10^4$
Internalized	0	$3.2 \pm 0.31 \times 10^2$

B

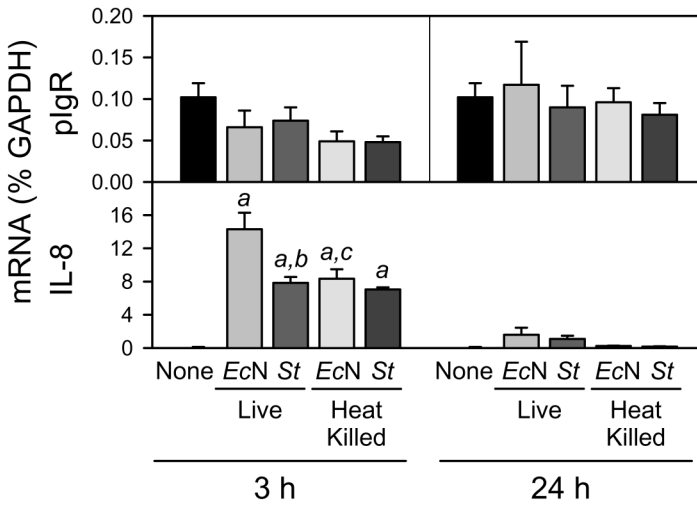


Figure 4: Induction of gene expression in HT-29 cells by live or heat-killed bacteria of the family *Enterobacteriaceae*. A. Bacterial invasion assay. Six replicate cultures of HT-29 cells (approximately 10^6 cells/culture) were incubated for 1 h with the indicated concentration of *E. coli* Nissle or *S. typhimurium*. After removal of culture supernatants to obtain final bacterial concentrations, triplicate cultures of HT-29 cells were analyzed for either membrane-associated or internalized bacteria as described in Materials and Methods. Statistical comparison: a, numbers of membrane-associated *S. typhimurium* were significantly greater than numbers of membrane-associated *E. coli* Nissle ($p < 0.05$). B. HT-29 cells were cultured for 3 h with live or heat-killed bacteria of the commensal *E. coli* Nissle (EcN) or the pathogen *Salmonella typhimurium* (St) at a ratio of 10 bacterial cells per eukaryotic cell. HT-29 cells were either harvested immediately (3 h harvest), or washed and cultured in antibiotic-containing medium for an additional 21 h (24 h harvest). mRNA levels were quantified by qRT-PCR and normalized to GAPDH mRNA. Data from 3 independent experiments were combined and expressed as mean \pm SEM ($n = 10$). Statistical comparisons: a, mean is significantly different from the corresponding mean for untreated HT-29 cells ($p < 0.05$); b, mean for HT-29 cells treated with St is significantly different from the corresponding mean for cells treated with EcN ($p < 0.05$); c, mean for HT-29 cells treated with heat-killed bacteria is significantly different from the corresponding mean for cells treated with live bacteria ($p < 0.05$).

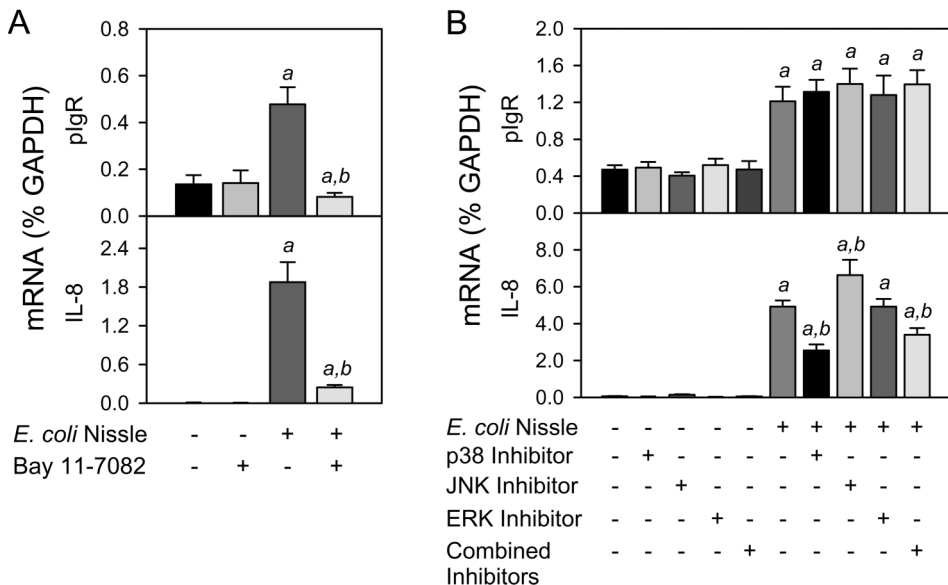


Figure 5: Effect of NF- κ B or MAPK inhibition on the response of HT-29 cells to *E. coli* Nissle. **A.** HT-29 cells were stimulated for 3 or 24 h with heat-killed *EcN*, at a ratio of 10 bacterial cells per eukaryotic cell, in the presence or absence of an inhibitor of NF- κ B (10 μ M Bay 11 7082). mRNA levels were quantified by qRT PCR and normalized to GAPDH mRNA. Data from 3 independent experiments were combined and expressed as mean \pm SEM ($n = 9$): *a*, mean for cells stimulated with *EcN* is significantly different from the corresponding mean for unstimulated cells; *b*, mean for cells treated with Bay 11-7082 is significantly different from the corresponding mean for untreated cells. **B.** HT-29 cells were stimulated with *EcN* for 3 or 24 h in the presence or absence of MAPK inhibitors (PD98059, ERK inhibitor; SB20358, p38 inhibitor; SP600125, JNK inhibitor), individually or combined, each at a concentration of 10 μ M. Gene expression was analyzed as described for panel A. Data from 2 independent experiments were combined and expressed as mean \pm SEM ($n = 8$): *a*, mean for cells stimulated with *EcN* is significantly different from the corresponding mean for unstimulated cells; *b*, mean for cells treated with MAPK inhibitors is significantly different from the corresponding mean for untreated cells.

that induction of these factor(s) may require NF- κ B but not MAPK signaling. Another non-mutually exclusive possibility is that continued stimulation by *EcN* may lead to a second wave of NF- κ B activation and nuclear translocation, which could enhance *PIGR* gene transcription by binding to the NF- κ B element in intron 1 of the *PIGR* gene. In this context it is significant that we found that induction of *PIGR* transcriptional activity by LPS in HT-29 cells was blocked by mutation of the intron 1 NF- κ B site (Schneeman et al., 2005).

Colonic Bacteria Regulate Gene Expression in Vivo Through MyD88-dependent Signaling Our *in vitro* experiments suggested that colonic epithelial cells have the inherent ability to regulate gene expression in response to commensal bacteria through TLR signaling. Consistent with this conclusion, we found that the steady-state level of pIgR mRNA was significantly higher in colonic than small intestinal mucosa of wild-type C57BL/6 mice with a

normal colonic microbiota (Fig. 6A). These results are similar to our previous finding that the steady-state level of *pIgR* mRNA was significantly higher in colonic than in small intestinal mucosa of healthy humans (Arsenescu et al., 2008).

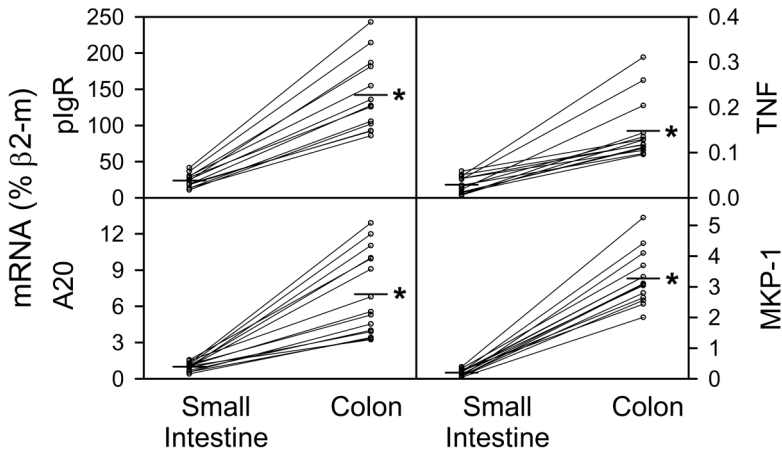
Steady-state levels of TNF, A20 and MKP-1 were also higher in the colon than in the small intestine of wild-type mice, presumably due to stimulation by greater numbers of bacteria in the large bowel. It should be noted that the relative expression level of *pIgR*, an anti-inflammatory factor, was 1000-fold higher than the relative expression of TNF, a pro-inflammatory factor, suggesting that colonic bacteria may promote intestinal homeostasis by selectively inducing or repressing individual target genes. The relatively high levels of A20 and MKP-1 in the colon suggest that steady-state expression of these negative regulators may suppress inappropriate pro-inflammatory responses to colonic bacteria.

The cytoplasmic adaptor protein MyD88 is utilized by multiple TLRs that respond to bacterial products, including TLRs 1, 2, 4, 5, 6 and 9. We hypothesized that *pIgR* expression would be compromised in *Myd88*-deficient mice due to reduced responses of IEC to colonic bacteria. Consistent with this hypothesis, we found that the steady-state level of *pIgR* mRNA was significantly reduced in colonic mucosa of *Myd88*^{-/-} mice as compared to wild-type mice (Fig. 6B). Numbers of culturable fecal bacteria did not differ significantly between wild-type and *Myd88*^{-/-} mice ($8.8 \pm 2.7 \times 10^9$ vs. $4.4 \pm 1.1 \times 10^9$ CFU/g feces), suggesting that reduced signaling rather than differences in bacterial load was responsible for the observed differences in *pIgR* expression. Importantly, levels of IgA in colonic mucus were also significantly lower in *Myd88*^{-/-} mice than in wild-type mice. The reduction in mucosal IgA could be due to reduced production of IgA by mucosal plasma cells or decreased epithelial transport of polymeric IgA, or both. The finding that steady-state levels of TNF, A20 and MKP-1 mRNA were lower in colonic mucosa of *Myd88*^{-/-} mice as compared to wild-type mice (Fig. 6C) is consistent with the hypothesis that commensal bacteria maintain the balance of gene expression in the colonic mucosa through TLR signaling.

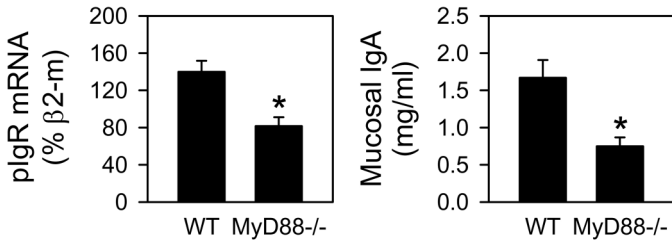
DISCUSSION

The single layer of epithelial cells lining the intestinal tract provides both a physical barrier and the first line of communication between the host and its microbial community, setting the stage for a mutually beneficial relationship. The polymeric immunoglobulin receptor promotes the homeostatic functions of IEC by participating in innate defense and by transporting IgA antibodies across the epithelial layer (Kaetzel, 2005; Kaetzel et al., 2005, 2007; Corthésy, 2007; Phalipon et al., 2007). It has been estimated that up to 3 g of SIgA are transported daily into the intestinal secretions of the normal human adult

A



B



C

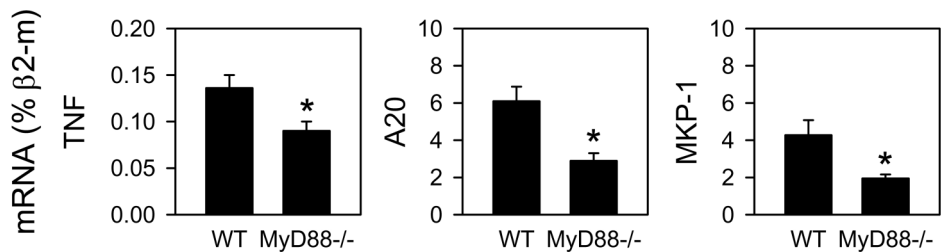


Figure 6: Regulation of gene expression and mucosal IgA levels by colonic bacteria *in vivo*. **A.** RNA was isolated from whole small intestines and colons of 12 to 15 week-old C57BL/6 mice. mRNA levels were quantified by qRT-PCR and normalized to β 2-microglobulin mRNA. Each data point represents an individual mouse, and lines connect the value for small intestine and colon. Horizontal bars indicate the mean mRNA level for each tissue. Asterisks indicate that the mean is significantly higher in the colon than in the small intestine ($p < 0.002$). **B.** RNA was isolated from whole colons of 12 to 15 week-old C57BL/6 wild-type and *Myd88*^{-/-} mice. plgR mRNA levels were quantified by qRT-PCR and normalized to β 2 microglobulin mRNA. Data are expressed as mean \pm SEM ($n = 20$). Mucosal secretions were collected from the colons of 12 week-old wild-type ($n = 16$) and *Myd88*^{-/-} ($n = 11$) mice using absorbent wicks, and IgA levels were determined by ELISA. Data are expressed as mean \pm SEM. Asterisks indicate that the mean for *Myd88*^{-/-} mice is significantly less than the corresponding mean for wild-type mice ($p < 0.05$). **C.** mRNA levels for TNF, A20 and MKP-1 were analyzed as described for panel B.

(Mestecky et al., 1986; Conley et al., 1987), necessitating very high levels of pIgR expression by IEC. At the mucosal surface, secretory IgA antibodies carry out the dual functions of neutralizing potentially invasive pathogens and shaping the composition of the commensal microbiota (Woof et al., 2005, 2006; Brandtzaeg et al., 2007; Macpherson et al., 2008).

Alterations in the composition of the commensal microbiota, including emergence of aggressive strains of typically avirulent bacteria, are thought to contribute to the pathogenesis of IBD (Strober et al., 2007; Frank et al., 2007; Sokol et al., 2008; Tannock, 2008a, 2008b; Sartor, 2008). Prominent among the aggressive bacteria associated with the ileal and colonic mucosa of IBD patients are adherent-invasive *E. coli* (AIEC) (Darfeuille-Michaud et al., 2004; Martin et al., 2004; Kotlowski et al., 2007; Sasaki et al., 2007).

Probiotic treatments for intestinal inflammation are receiving increasing attention, but questions remain regarding the choice of bacteria and their mechanisms of action (Sartor, 2005; Damaskos et al., 2008; Seksik et al., 2008). Therapy with probiotic *E. coli* strains may offer the potential to neutralize the damaging effects of mucosa-associated AIEC that are a prominent feature of IBD. The best characterized probiotic *E. coli* strain is Nissle 1917 (*EcN*), first used by German physician and scientist Alfred Nissle during World War I as a treatment for infectious colitis in soldiers (Nissle, 1918). *EcN* has been tested as a treatment for IBD in several clinical trials, and has been shown to be as effective as mesalazine in maintaining remission in ulcerative colitis (Schultz, 2008; Kruis, 2004).

Experiments in cultured IEC lines have revealed a number of mechanisms through which *EcN* may exert beneficial effects on the intestinal epithelium, including inhibition of invasion by aggressive bacteria such as AIEC and *Salmonella* (Boudeau et al., 2003; Altenhoefer et al., 2004; Otte et al., 2004), inhibition of signaling by the pro-inflammatory cytokine TNF (Kamada et al., 2008), stimulation of tight junction formation and barrier function (Ukena et al., 2007; Zyrek et al., 2007), induction of chemokine synthesis (Lammers et al., 2002; Otte et al., 2004; Ukena et al., 2005; Zoumpopoulou et al., 2009), and induction of antimicrobial β -defensins (Wehkamp et al., 2004; Schlee et al., 2007; Mondel et al., 2009). A recent study demonstrated that treatment of healthy volunteers with probiotic *E. coli* caused an increase in fecal excretion of human β -defensin-2, suggesting that the observed *in vitro* mechanisms of *EcN* may also be operative *in vivo* (Mondel et al., 2009).

We found *EcN* to be unique among representative species from the 4 major phyla of human colonic bacteria in its ability to up-regulate expression of pIgR in the human IEC line HT-29 (Fig. 1A). Enhanced expression of pIgR and transport of SIgA could represent a novel mechanism through which *EcN* mediates its probiotic effects. We previously reported that a subset of Crohn's disease patients with chronic symptoms and poor responses to therapy had decreased expression of pIgR in the colonic mucosa (Arsenescu et al., 2008). It will be

interesting to determine if probiotic therapy with *EcN* can restore normal expression of pIgR and improve clinical outcomes in these patients.

We previously reported that signaling through TLR3 and TLR4 up-regulates pIgR expression in the HT-29 IEC line (Schneeman et al., 2005; Kaetzel, 2005; Bruno et al., 2007; Kaetzel et al., 2007). Consistent with a role for TLR signaling in maintenance of pIgR expression *in vivo*, we now report that expression of pIgR and secretion of IgA were significantly reduced in the colonic mucosa of *MyD88*^{-/-} mice compared to wild-type mice (Fig. 6B). We have recently observed that siRNA-mediated knockdown of MyD88 expression in HT-29 cells inhibits the up-regulation of pIgR by *EcN*, consistent with a direct role for MyD88 signaling in IEC (Frantz et al., manuscript in preparation).

Other investigators have reported that TLR signaling in IEC enhances B cell recruitment and IgA class switching in the intestinal lamina propria by inducing IECs to secrete the cytokine APRIL (He et al., 2007; Shang et al., 2008). Thus *EcN* offers the potential to enhance both IgA production and IgA transport by stimulating TLR signaling in IEC. Although we did not measure serum IgA levels in *MyD88*^{-/-} mice, it is possible that inhibition of IgA transport in the intestine could result in increased levels of IgA in the circulation, as has been reported for pIgR^{-/-} mice (Johansen et al., 1999; Shimada et al., 1999). In this regard it is significant that we observed a correlation between reduced pIgR expression in the colonic epithelium and increased serum IgA levels in Crohn's Disease patients (Arsenescu et al., 2008).

Here we found that the IEC line HT-29 was unresponsive to TLR2 stimulation (Fig. 2), which may explain the lack of response to Gram-positive bacteria. Although TLR2 has been detected at low levels in IEC *in vivo* and in some IEC lines, functional studies have suggested that TLR2-mediated pro-inflammatory responses by IEC are suppressed by a variety of mechanisms (Melmed et al., 2003; Cario, 2008). TLR4 is normally expressed at low levels in adult IEC, but unlike TLR2, its expression can be up-regulated by pro-inflammatory stimuli (Fukata et al., 2007).

We found that HT-29 cells responded to purified *E. coli* LPS and to whole *EcN*, although not as robustly as the THP-1 monocyte cell-line (Figs. 1 and 2). Interestingly, HT-29 cells did not respond to other Gram-negative bacteria, including *Pseudomonas straminea*, which like *EcN* is a member of the phylum *Proteobacteria*, and two members of the phylum *Bacteroidetes*, *Bacteroides thetaiotaomicron* and *Alistipes onderdonkii*. Analyses of the structural requirements for TLR4-mediated signaling have suggested that the ability to act as a ligand for TLR4 is restricted to LPS molecules from a limited group of Gram-negative bacteria, in particular those of the family *Enterobacteriaceae* (Backhed et al., 2003; Munford et al., 2006). LPS molecules from many other Gram-negative commensals, including *Pseudomonas aeruginosa* (phylum *Proteobacteria*) and *Bacteroides fragilis* (phylum *Bacteroidetes*), have been shown to signal through TLR2 rather than TLR4 (Erridge et al., 2004).

Our data suggest that the capacity of *EcN* to stimulate TLR4 signaling in IEC may contribute to its unique probiotic properties. *EcN* may also promote intestinal homeostasis by signaling through other TLRs in addition to TLR4. For example, purified flagellin from *EcN*, a ligand for TLR5, has been shown to induce expression of β -defensin 2 in the human IEC line Caco-2 (Schlee et al., 2007). It remains to be determined whether TLR5 signaling enhances expression of pIgR and/or transport of IgA in IEC.

In order to maintain intestinal homeostasis in the face of continuous interactions with gut bacteria, IEC face the unique challenge of promoting epithelial barrier function and transport of IgA antibodies while preventing excess production of pro-inflammatory factors. Significantly, we found that steady-state levels of pIgR mRNA were 1000-fold higher than steady-state levels of TNF mRNA in colonic mucosa from normal mice (Fig. 6A) and humans (Arsenescu et al., 2008). One mechanism by which IEC may differentially regulate gene expression in response to bacterial stimulation may be through gene-specific effects of downstream signaling pathways.

We found that induction of the pro-inflammatory chemokine IL-8 by *EcN* in HT-29 cells required both NF- κ B and p38 MAPK signaling (Fig. 5). The increase in IL-8 production was rapid and transient, returning to near baseline levels following continuous stimulation with *EcN*. By contrast, the slow and sustained induction of pIgR by *EcN* required NF- κ B signaling but not MAPK signaling. In this context it is significant that TNF-induced MAPK signaling has been reported to inhibit induction of pIgR in HT-29 cells (Takenouchi-Ohkubo et al., 2008). Importantly, we found that *EcN* induced expression of MKP-1, a negative regulator of MAPK signaling (Fig. 1B).

Consistent with a role for colonic bacteria in maintaining expression of MKP-1 in IEC, we found that steady-state levels of MKP-1 mRNA were 16-fold higher in the colon than in the small intestine of normal mice (Fig. 6A). Furthermore, MKP-1 mRNA levels were reduced by more than 50% in colons of MyD88^{-/-} mice compared to wild-type mice (Fig. 6C). Taken together, these results suggest that activation of TLR signaling by gut bacteria may be important for maintaining expression of MKP-1 and preventing excess activation of MAPK signaling pathways in IEC.

In conclusion, we have demonstrated several novel mechanisms by which bacteria of the family *Enterobacteriaceae* may promote homeostasis through direct interactions with the intestinal epithelium. These mechanisms may explain some of the beneficial effects of *EcN* treatment that are unique from those of probiotic bacteria from other phyla, such as *Lactobacilli* and *Bifidobacteria*. Stimulation of pIgR expression by *EcN* would enhance pIgR-mediated transport of protective IgA antibodies as well as innate immune functions mediated by SC. Transient induction of moderate levels of IL-8 would promote influx of neutrophils in sufficient numbers to enhance innate immunity without causing excess inflammation. Finally, induction of negative regulatory molecules

such as A20 and MKP-1 by *EcN* would prevent excess activation of NF- κ B and MAPK signaling pathways.

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