

The bacterial signal indole increases epithelial-cell tight-junction resistance and attenuates indicators of inflammation

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Interkingdom signaling is established in the gastrointestinal tract in that human hormones trigger responses in bacteria; here, we show that the corollary is true, that a specific bacterial signal, indole, is recognized as a beneficial signal in intestinal epithelial cells. Our prior work has shown that indole, secreted by commensal *Escherichia coli* and detected in human feces, reduces pathogenic *E. coli* chemotaxis, motility, and attachment to epithelial cells. However, the effect of indole on intestinal epithelial cells is not known. Because intestinal epithelial cells are likely to be exposed continuously to indole, we hypothesized that indole may be beneficial for these cells, and investigated changes in gene expression with the human enterocyte cell line HCT-8 upon exposure to indole. Exposure to physiologically relevant amounts of indole increased expression of genes involved in strengthening the mucosal barrier and mucin production, which were consistent with an increase in the transepithelial resistance of HCT-8 cells. Indole also decreased TNF- α -mediated activation of NF- κ B, expression of the proinflammatory chemokine IL-8, and the attachment of pathogenic *E. coli* to HCT-8 cells, as well as increased expression of the antiinflammatory cytokine IL-10. The changes in transepithelial resistance and NF- κ B activation were specific to indole: other indole-like molecules did not elicit a similar response. Our results are similar to those observed with probiotic strains and suggest that indole could be important in the intestinal epithelial cells response to gastrointestinal tract pathogens.

host-pathogen interactions | interkingdom signaling | probiotics

The human gastrointestinal (GI) tract is rich in a diverse range of signaling molecules. A wide range of bacterial signals (e.g., autoinducer-2, autoinducer-3, and indole) (1) are produced by the $\sim 10^{14}$ nonpathogenic commensal bacteria that coexist with host cells in the GI tract, and neuroendocrine hormones (e.g., norepinephrine and dopamine) are also synthesized in situ in the GI tract via the enteric nervous system. The close proximity of bacteria and the host cells in the GI tract, as well as the high local concentrations of the signals they secrete, has led to a signal-centric paradigm wherein GI tract signals are considered to be important mediators of homeostasis and infections through intrakingdom (i.e., recognition of bacterial signals by other bacteria) and/or interkingdom (i.e., recognition of host signals by bacteria and vice versa) signaling and communication.

Interkingdom recognition of hormones by pathogens was first demonstrated by Lyte et al. (2). Subsequently, Sperandio et al. (3) showed that enterohemorrhagic *Escherichia coli* (EHEC) virulence is increased upon exposure to epinephrine and norepinephrine, and that epinephrine binds and signals through the QseC receptor (4). Work from our group (5) has also shown that epinephrine and norepinephrine increase EHEC chemotaxis, motility, adherence to epithelial cells, and virulence gene expression. Although these studies provide evidence for the recognition of human hormones by pathogens, the converse (i.e., recognition of bacterial specific signaling molecules by intestinal

epithelial cells and their role in GI tract function) has not been fully investigated. Although several studies have shown that culture supernatants from probiotic strains attenuate pathogen infection (6, 7), modulate inflammation in dendritic cells (8) and Caco-2 cells (9), and increase intestinal epithelial cell barrier function (10), the specific molecules contributing to these responses and the mechanisms involved are not known. In fact, most individual bacterial signals have been shown to be deleterious to host cells; for example, the *Pseudomonas aeruginosa* quorum-sensing signaling molecule *N*-(3-oxododecanoyl)-L-homoserine lactone disrupts epithelial barrier integrity in Caco-2 cells (11), increases the expression of proinflammatory cytokines in murine fibroblasts and human lung epithelial cells (12), and disrupts nuclear factor kappa B (NF- κ B) signaling to promote persistence of a local *P. aeruginosa* infection (13).

Indole is produced in *E. coli* from L-tryptophan via tryptophanase (encoded by *tnaA*) (14). Prior work from our laboratory has shown that indole is an extracellular signal, represses *E. coli* K-12 biofilm formation through the sensor of quorum sensing signals, SdiA (14), is more effective in repressing *E. coli* K-12 biofilm at low temperatures (i.e., 25°C and 30°C) (15), and exerts the opposite effect as epinephrine and norepinephrine on EHEC chemotaxis, motility, adherence to epithelial cells, and virulence gene expression, at 37°C (5).

Because commensal *E. coli* produce as much as 600 μ M indole in suspension cultures (16) and indole has been detected in human feces at comparable concentrations (~ 250 – $1,100$ μ M) (17, 18), it is likely that intestinal epithelial cells are continually exposed to high concentrations of indole. Therefore, we hypothesized that indole is recognized as an interkingdom signal in intestinal epithelial cells. Measurements of changes in gene expression in HCT-8 intestinal epithelial cells and phenotypic measurements of transepithelial resistance, NF- κ B activation, IL-8 and IL-10 secretion, and EHEC attachment, show that indole modulates expression of proinflammatory genes, increases expression of antiinflammatory genes, strengthens epithelial cell barrier properties, and decreases pathogen colonization. Our data strongly suggest that indole is a beneficial interkingdom signal for intestinal epithelial cells.

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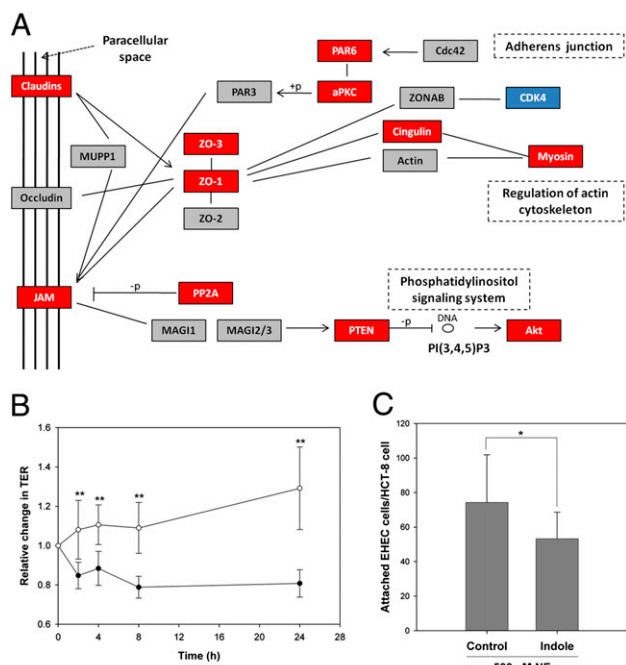


Fig. 1. Changes in tight junction proteins and TER. (A) Pathways containing differentially expressed genes involved in tight junction formation were adapted from Pathway Express using KEGG classifications. Red, up-regulation; blue, down-regulation; gray, no change in expression; arrow, molecular interaction leading to activation; blunt line without an arrowhead, molecular interaction leading to inhibition. The pathway scheme shown is based on microarray data and does not include posttranscriptional regulation. (B) Changes in the TER of polarized HCT-8 cells exposed to either solvent (filled circles) or indole (open circles) for 24 h. Data represent mean \pm SD from seven measurements at each time point and two independent experiments. (C) Effect of indole pretreatment on adherence of EHEC to HCT-8 cells in the presence of 500 μ M norepinephrine. Data represent mean \pm SD from three wells per experiment and three independent experiments. *, statistical significance, using Student's *t* test, at $P < 0.05$; **, significance at $P < 0.005$.

Downstream mediators of cytokine signaling (i.e., signaling kinases) such as the mitogen-activated protein kinase (MAPK) pathway, which is central to the inflammatory signaling, were differentially expressed. Specifically, the expression of dual-specificity phosphatases (DUSPs)—Dusp1, Dusp3, Dusp10, Dusp16, Dusp18, and Dusp22, which function as inhibitors of MAPK signaling (27)—was increased upon indole exposure. However, because several MAPK genes were also up-regulated, further analysis of the phosphorylation state of different pathway components is required to fully understand the changes in MAPK signaling upon indole exposure. Indole also induced the expression of genes in the Jak/STAT signaling pathway, which is activated by proinflammatory molecules such as IFN- γ leading to increased microbial killing and cytokine production (28). Jak-1, -2, and -3 were induced along with STAT-1, -2, -3, -5, and -6 (Table S2). The IFN-stimulated transcription factor 3 γ (ISGF3G), which is activated by STAT1 to regulate IFN- γ -stimulated genes, was also induced in expression upon exposure to indole, along with SOCS, a repressor of Jak-STAT signaling. Therefore, in addition to inducing changes in pro- and antiinflammatory mediators, exposure to indole also induced coordinated changes in the downstream signaling pathways used by these cytokines and chemokines.

We also monitored the activation of the prototypical proinflammatory transcription factor NF- κ B, which has been shown to mediate the inflammatory response both *in vitro* and *in vivo* (29, 30), using an HCT-8 GFP reporter cell line for NF- κ B (31). Our data show that indole reduced the TNF- α -mediated increase in

NF- κ B activation in HCT-8 intestinal epithelial cells by 3-fold over 18 h (Fig. 3A and B) and clearly indicate the ability of indole to attenuate TNF- α -mediated inflammation in HCT-8 cells.

Specificity of Indole in Attenuating NF- κ B Activation and Promoting Epithelial Cell Barrier Properties. We investigated the effect of five indole-like molecules (Fig. S2)—¹H-indole-2,3-dione (isatin), 7-hydroxyindole (7-HI), 5-hydroxyindole (5-HI), 2-hydroxyindole (2-HI), and indole-3-acetic acid (I3AA)—for establishing the specificity of indole in attenuating NF- κ B activation and promoting TER. Fig. 4A shows that only 5-HI significantly repressed NF- κ B activity in a manner similar to that observed with indole (Fig. 3), whereas the effectiveness of the other molecules varied from having no effect or marginal (7-HI, I3AA, and 2-HI) to actually increasing NF- κ B activation (isatin). However, 5-HI did not cause a significant change in the TER of HCT-8 cells after 24 h (Fig. 4B), and among the molecules tested, only 7-HI increased the TER to levels comparable with indole (Fig. 1B). Thus, although indole-like molecules were able to either attenuate NF- κ B activation or increase TER at the concentrations tested, changes in both phenotypes were observed only with indole.

To further establish that the beneficial effects of indole were not caused by an increase in HCT-8 cell number or viability, we determined the change in HCT-8 cell density after exposure to indole for 24 h. Cells exposed to indole showed >99% viability after 24 h (live-cell density in control and indole-treated cells was $3.1 \times 10^6 \pm 0.6 \times 10^6$ and $3.0 \times 10^6 \pm 0.7 \times 10^6$ cells per mL, respectively), with a similar percentage of live cells for the solvent control. These data suggest that the beneficial effects of indole are not metabolic (i.e., because of the lack of an increase in cell numbers).

The whole-transcriptome data were substantiated with qRT-PCR where genes from inflammatory cytokines and signaling, and epithelial barrier function were profiled. ISGF3G was induced 4.1-fold in the microarrays and 5.1 ± 0.1 -fold in qRT-PCR. Similar results were seen for CXCL5 (−5.2-fold in the arrays, -13.6 ± 0.6 -fold in qRT-PCR), pre-IL-1 (5.6-fold in the arrays, 5.9 ± 0.2 -fold in qRT-PCR), IL-8 (−1.8-fold in the arrays, -1.4 ± 0.1 -fold in qRT-PCR), cldn3 (3.5-fold in the arrays, 4.9 ± 0.2 -fold in qRT-PCR), TJP3 (5.7-fold in the arrays, 5.5 ± 0.2 -fold in qRT-PCR), and muc13 (4.4-fold in the arrays, 2.3 ± 0.2 -fold in qRT-PCR). Thus, the expression results from whole-transcriptome analysis were in good agreement with the qRT-PCR results.

Discussion

The recognition of host cell signaling molecules by pathogens through interkingdom communication and its role in pathogenesis has been recently documented (3, 5). However, little is known about recognition of bacterial signals by host cells. To date, specific prokaryotic signals such as the *P. aeruginosa* quorum-sensing molecules have been shown to be deleterious to host cells as they help pathogen infections. Here, we show that the bacterial stationary-phase signal indole is recognized by intestinal epithelial cells and used to strengthen host cell-barrier properties and maintain controlled inflammation. This is supported by multiple lines of evidence showing that indole (i) increases the expression of genes involved in the formation of tight junctions and actin cytoskeleton, and increases the transepithelial resistance of polarized HCT-8 intestinal epithelial cells; (ii) attenuates IL-8 secretion and TNF- α -mediated NF- κ B activation in HCT-8 cells while increasing IL-10 secretion; (iii) increases the resistance of HCT-8 cells to norepinephrine-mediated increase in EHEC colonization; and (iv) reduces the expression of proinflammatory cytokines and chemokines while inducing the expression of antiinflammatory cytokines. Therefore, we propose that indole can be a beneficial interkingdom signal that helps improve intestinal epithelial-cell function, maintain controlled inflammation, and increase resistance to pathogen colonization.

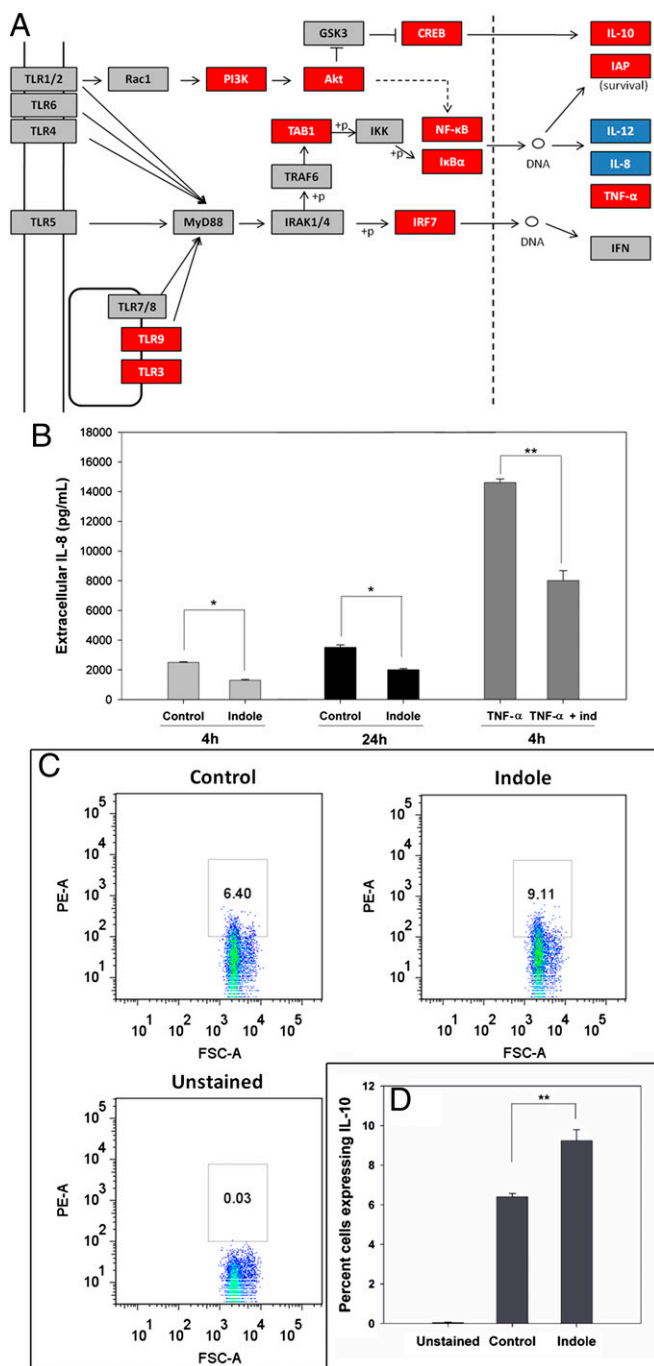


Fig. 2. Changes in Toll-like receptor, IL-8, and IL-10 signaling. (A) Pathways containing differentially expressed genes involved in tight junction formation were adapted from Pathway Express using KEGG classifications. Colors and symbols as in Fig. 1. The pathway scheme shown is based on microarray data and does not include posttranscriptional regulation. (B) Secretion of IL-8 in HCT-8 cells exposed to indole for 4 h or 24 h, in the presence or absence of 40 ng/mL TNF- α . Data represent mean \pm SD from three independent experiments. (C) Intracellular staining and flow cytometry of IL-10 in HCT-8 cells exposed to indole for 24 h. Two-color dot plots of number of PE-stained cells and forward scatter for solvent control, indole-treated, and unstained cells are shown. Percentages in the boxed region indicate the proportion of HCT-8 cells producing IL-10 in one representative experiment. (D) Average increase in IL-10 expression data from three independent experiments. *, statistical significance, using Student's *t* test, at $P < 0.05$; **, significance at $P < 0.005$.

Several studies have shown that cell-free supernatants of probiotic bacterial cultures increase host epithelial-cell barrier properties and resistance to pathogen colonization. However, the identity of specific molecule(s) that contribute to this protective effect and the mechanisms involved are not known, because culture supernatants are complex mixtures comprised of media components, metabolites, and signals secreted by different bacterial species. The data presented here show that indole elicits a response similar to probiotic strains by strengthening host cell-barrier properties. Because indole is produced by several commensal bacteria (e.g., *E. coli*, *Bacteroides ovatus*, and *Clostridium bifementans*) that colonize the human GI tract (32), it is intriguing to speculate that the beneficial effects of some probiotic strains are mediated, in part, through indole.

Although 4,102 genes (or 8% of the transcriptome) were differentially regulated by indole, it is worth noting that only 83 genes were regulated by 4.0-fold or higher. A moderate response to indole is to be expected, especially because indole is abundant in the human GI tract ($\sim 100 \mu\text{M}$ in human feces) (17), and is less likely to perturb the epithelial cell transcriptome like a pathogen. In this regard, our data are similar to that reported by Hasegawa et al. (27), who showed that two different species of commensal bacteria, *Streptococcus gordonii* and *Fusobacterium nucleatum*, perturbed the gingival epithelial cell transcriptome to a lesser extent than the pathogens *Poryphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*, which elicited more widespread organism-specific changes in the transcriptome. Striking similarities are also evident between our data and changes in gene expression elicited by *S. gordonii* and *F. nucleatum* in gingival epithelial cells. For example, gene families that were differentially expressed in intestinal epithelial cells upon exposure to indole—tight junction proteins, actin cytoskeleton, cell cycle regulators, phosphatidylinositol signaling, MAP kinase signaling, TLR signaling, and Wnt signaling—were all altered in gingival epithelial cells as well when exposed to commensal bacteria.

In addition to the similarities with commensal response in gingival epithelial cells, significant differences are also observed between the indole response in intestinal epithelial cells and pathogen-mediated changes in expression in gingival epithelial cells. One such example is the activation of caspase-1 by pathogenic bacteria (e.g., lipopolysaccharide, *Salmonella typhimurium* type III secretion), which leads to increased secretion of proinflammatory cytokines such as IL-1 β and the induction of apoptosis. Hasegawa et al. (27) reported that commensal bacteria did not activate caspase-1, thus indicating physiological balance between the host and the organism. Similarly, no caspase-1 gene expression was observed in HCT-8 intestinal epithelial cells exposed to indole, which also suggests favorable recognition of indole by intestinal epithelial cells. Furthermore, the lack of activation of TLR signaling by indole, similar to that seen with oral commensal bacteria (27) and opposite to that of oral pathogens (30), also strongly suggests that indole is recognized as a beneficial signal and not detected as a pathogenic signal by intestinal epithelial cells.

IL-8 is an alarm chemokine that is highly induced by pathogens in a wide variety of cells, including human colon adenocarcinoma cells. For example, a 4- to 30-fold increase in IL-8 and ENA-78 (CXCL5) has been observed in HT-29 and Caco-2 intestinal epithelial cells upon exposure to *Salmonella dublin* (33). However, in our study, exposure to indole decreased the expression of ENA-78 and IL-8, which suggests that indole is not recognized as a pathogen signal by intestinal epithelial cells and strongly proposes an antiinflammatory role for indole in GI tract inflammation.

It has been proposed that bacteria (or molecules secreted by them) induce the expression of pro- and antiinflammatory cytokines (34), which function coordinately (i.e., as a network) to regulate the host response to commensal bacteria (34). Disruption of this regulated cytokine response leads to sustained inflammation, as has been shown with IL-10 knockout mice developing chronic enterocolitis even in the absence of pathogen

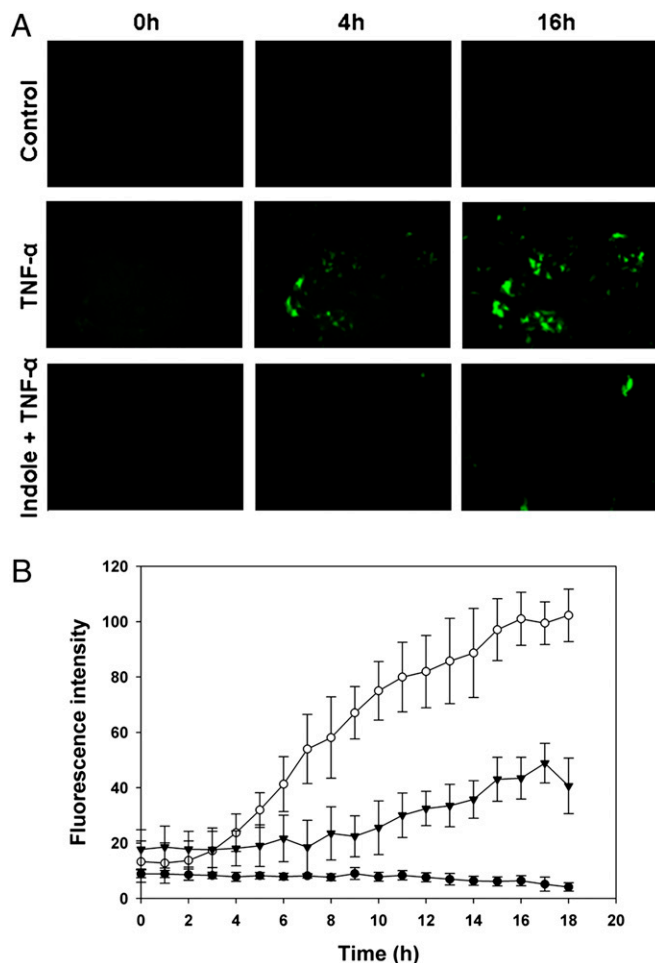


Fig. 3. Modulation of NF- κ B activity by indole. (A) Effect of indole on NF- κ B activation by TNF- α in HCT-8 NF- κ B-GFP reporter cells and the resultant GFP expression. (B) Quantification of NF- κ B activity in control cells (filled circles), cells treated with TNF- α to induce NF- κ B activation (open circles), and cells treated with indole and TNF- α (filled inverted triangles). Data shown are mean \pm SD from images obtained at four locations in three independent experiments.

colonization (35). In addition, this heightened level of alertness also enables host cells to respond rapidly to the presence of pathogens. Our data showing that indole up-regulates the expression of some proinflammatory cytokines, in addition to attenuating other indicators of inflammation, are consistent with this hypothesis, and suggest that indole-mediated communication is involved in the balance between pro- and antiinflammatory cytokine signaling in intestinal epithelial cells.

The intestinal epithelial cell inflammatory response to pathogens is orchestrated, in part, through activation of NF- κ B (29, 30), which is well established. Our data showing that indole reduced TNF- α -mediated NF- κ B activation in intestinal epithelial cells support the antiinflammatory role for indole. Because commensal bacteria reduce the *S. typhimurium*-induced NF- κ B activity (29) and inflammation in mice, it is intriguing to speculate that indole is a signal through which probiotics reduce intestinal inflammation.

The effect of indole on HCT-8 cells also extends beyond alterations in gene expression to changes in prototypical intestinal epithelial cell functions such as barrier integrity. First, the increase in tight junction protein expression and TER is significant, because an increase in tight junction resistance leads to decreased paracellular permeability and reduces the ability of pathogens to cross the epithelial cell barrier. Second, the

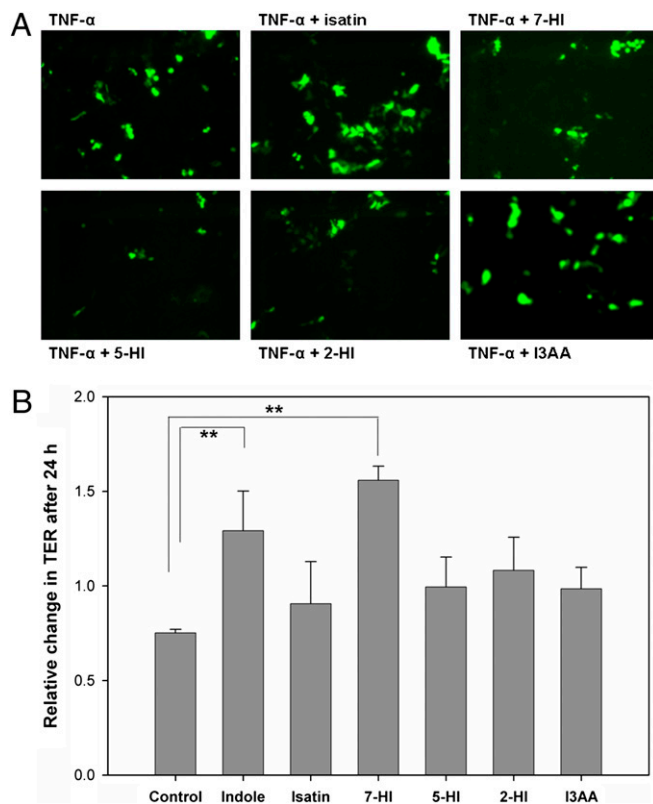


Fig. 4. Changes in HCT-8 cell phenotypes with aromatic bicyclic molecules. (A) NF- κ B activation in HCT-8 NF- κ B-GFP reporter cells and the resultant GFP expression. Images shown are after 18 h of exposure to TNF- α and are representative of data from three independent experiments. (B) Changes in TER after exposure to indole-like molecules for 24 h. Data represent mean \pm SD from three independent experiments. **, statistical significance, using Student's *t* test, at $P < 0.005$.

induction of 50 cytoskeleton genes suggests increased resistance to pathogen colonization because pathogens like *P. aeruginosa*, *Shigella flexneri*, *Listeria monocytogenes*, and enteropathogenic *E. coli* disrupt epithelial cell morphology during infection by interfering with the actin cytoskeleton (36–38). Third, the increase in the expression of several mucin genes is also likely to decrease pathogen colonization: Kim et al. (21) recently reported that lactobacilli increased the production of mucin 2 (*muc2*) to inhibit EHEC attachment to epithelial cells in vitro. These three lines of evidence strongly support the hypothesis that indole elicits beneficial changes in intestinal epithelial cells.

The alteration of TER and the regulation of inflammation by indole has significant implications in the treatment of chronic intestinal inflammatory diseases such as Crohn's disease, which is an autoimmune disease characterized by sustained inflammation and deterioration of epithelial cell barrier function. Currently no drug-based or surgical cure for this disease exists. Based on the data showing that indole elicits antiinflammatory changes in expression and increases epithelial cell barrier properties, we propose that indole could represent a new, yet safe (as it is naturally present in the human GI tract), modality for regulating intestinal inflammation and promoting epithelial cell function.

In summary, our in vitro studies strongly indicate the importance of indole in favorable interkingdom signaling interactions between the intestinal epithelial cells and commensal bacteria. Our results show coordinated control of inflammation in the presence of indole through the repression of several inflammatory cytokines and coordinated regulation of signaling pathways,

along with fortification of cell structure through up-regulation of actin cytoskeleton and tight junction proteins. The recognition of indole as an interkingdom signal by human cells is also consistent with the recognition of the bacterial product indole-3-acetic acid, and provides an initial basis for understanding symbiotic interkingdom interactions at the molecular level, as well as insights into some GI tract diseases and pathogenic infections.

Materials and Methods

Details for all methods can be found in *SI Materials and Methods*.

HCT-8 Cell Culture. The human colon-cancer cell line HCT-8 (ATCC), derived from enterocytes at the junction of the large and small bowel, is a polarizable cell line that has been used for investigating mechanisms underlying host-cell response to pathogens and inflammation (39, 40). Cells were maintained according to standard ATCC protocols.

RNA Isolation and Microarrays. HCT-8 cells were exposed to 1 mM indole or solvent for 4 h or 24 h. RNA isolation, reverse transcription, cDNA labeling, hybridization of arrays, array scanning, and identification of genes exhibiting a statistically significant change in expression were performed as described in *SI Materials and Methods*.

NF- κ B Activity Measurements. HCT-8 cells transduced with a NF- κ B-GFP reporter lentivirus (31) were exposed to nontoxic concentrations of indole or indole-like molecules for 4 h, followed by addition of 40 ng/mL TNF- α . Methods used for fluorescence microscopy and image analysis are described in *SI Materials and Methods*.

Flow Cytometry and Intracellular Cytokine Staining. IL-10 expression in HCT-8 cells was determined by intracellular cytokine staining using a phycoerythrin-conjugated anti-human IL-10 antibody (eBioscience). Data (8,000 events per sample) were acquired on a BD FACSAria II cell sorter system (BD Biosciences) and analyzed by using FlowJo software (Tree Star).

In Vitro Colonization, Cell Viability Assay, Extracellular Indole Assay, Transepithelial Resistance, qRT-PCR, and IL-8 ELISA. Assays were performed according to standard protocols, the details of which are described in *SI Materials and Methods*.

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