

- theoretical fit than other least squares regressions (37). Therefore, we conducted regression analyses on the basis of both the OLS($y|x$) and OLS_{BIS} methods.
20. From the data of Peters [appendix IIIb in (2)], we estimated the hypothetical energy use OLS_{BIS} slope of aquatic invertebrates to be 0.79 (median value; $n = 17$). Thus, abundance would have to scale with mass to the -0.8 power for energy equivalence to hold if we assume the slope of OLS_{BIS} of log body mass against log metabolic rate to be 0.8.
 21. ANCOVA comparing regression lines gives statistically insignificant differences ($P > 0.5$) for both intercepts and slopes in the two contrasts performed (Mynach versus Seebach).
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Prokaryotic Regulation of Epithelial Responses by Inhibition of I κ B- α Ubiquitination

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Epithelia of the vertebrate intestinal tract characteristically maintain an inflammatory hyporesponsiveness toward the luminal prokaryotic microflora. We report the identification of enteric organisms (nonvirulent *Salmonella* strains) whose direct interaction with model human epithelia attenuate synthesis of inflammatory effector molecules elicited by diverse proinflammatory stimuli. This immunosuppressive effect involves inhibition of the inhibitor κ B/nuclear factor κ B (I κ B/NF- κ B) pathway by blockade of I κ B- α degradation, which prevents subsequent nuclear translocation of active NF- κ B dimer. Although phosphorylation of I κ B- α occurs, subsequent polyubiquitination necessary for regulated I κ B- α degradation is completely abrogated. These data suggest that prokaryotic determinants could be responsible for the unique tolerance of the gastrointestinal mucosa to proinflammatory stimuli.

In humans, the mucosal lining of the intestine coexists in intimate contact with a diverse prokaryotic microflora. The intestinal epithelial cells have necessarily evolved mechanisms to prevent or limit activation of cellular immuno-inflammatory stress responses in this microbe- and antigen-rich environment (1). Immune and inflammatory responses in the gut and other immuno-competent tissues often involves the transcription factor NF- κ B. This DNA binding protein is the transcriptional effector of an evolutionarily conserved regulatory pathway that is activated by a myriad of proinflammatory stimuli and is required for the de novo synthesis of numerous proinflammatory cytokines, chemokines, adhesion proteins, and other molecules critical for

normal immuno-inflammatory function (2). Proinflammatory stimuli activate NF- κ B through tightly regulated phosphorylation, ubiquitination, and proteolysis of a physically associated class of inhibitor molecules, I κ B's (3). I κ B is inducibly phosphorylated by a multisubunit kinase complex (I κ B kinase) and is subsequently ubiquitinated by a second multiprotein complex, recently isolated by several groups and designated E3-SCF $^{\beta}$ -TrCP, with the β -TrCP subunit functioning as the I κ B-specific ubiquitin ligase (E3) (4–7). Polyubiquitinated I κ B is thus targeted for degradation by the 26S proteasome (8), allowing NF- κ B to translocate to the nucleus, bind to its sequence recognition motif on target promoters, and activate transcription of effector genes, an example of which is the neutrophil chemokine interleukin-8 (IL-8). A variety of endogenous anti-inflammatory mediators, as well as clinically effective anti-inflammatory drugs, are known to exert their effects, at least in part, by blockade

of various aspects of the NF- κ B signal transduction pathway (9); however, there has been no description of interference with the ubiquitination step.

Although the vast majority of enteric organisms do not elicit intestinal inflammation, it is becoming increasingly recognized that enteropathogens that cause acute inflammatory colitis do activate the NF- κ B pathway, resulting in secretion of chemokines including IL-8 (10–12). We hypothesized that if proinflammatory enteric pathogens activate NF- κ B and subsequent events, nonpathogenic microorganisms may be able to selectively attenuate this pathway as a mechanism of intestinal immune tolerance. We observed that colonization of the apical aspect of polarized T84 model epithelia with nonpathogenic *Salmonella* strains, both laboratory-derived and naturally occurring (*S. typhimurium* PhoP^c and *Salmonella pullorum*), attenuates basolateral IL-8 secretion characteristically elicited by apical infection with proinflammatory strains (wild-type *S. typhimurium* and Hil A mutant) (Fig. 1A) (13–15). Colonization with *S. typhimurium* PhoP^c and *S. pullorum* was also capable of attenuating the IL-8 secretion elicited by a spectrum of proinflammatory stimuli [tumor necrosis factor- α (TNF- α), the calcium mobilizing agent carbachol, and the phorbol ester PMA (phorbol 12-myristate 13-acetate)] (Fig. 1B). This anti-inflammatory effect could be eliminated by nondenaturing heat killing of the organisms and was not present in bacterial lysates or conditioned media (Fig. 1C), suggesting the phenomenon is mediated through direct interactions of the epithelium with viable bacteria. The anti-inflammatory effects were maximal after a precolonization period of 30 min, though detectable suppressive effects were observed with colonization 10 min after addition of proinflammatory agonist (16). Colonization of T84 model epithelia with the anti-inflammatory organisms showed no strain-specific effects on transepithelial resistance or short-circuit currents elicited with forskolin. These indices of normal

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REPORTS

signaling and barrier function indicated cytotoxicity was not responsible for the anti-inflammatory effect (17).

To determine if the observed effects involved changes in IL-8 transcription or RNA turnover, Northern blotting was performed (Fig. 1D, top row). As expected, TNF- α treatment resulted in de novo appearance of IL-8 mRNA within 30 min, with levels increasing up to 2 hours (18). In contrast, colonization with *S. pullorum* resulted only in trace appearance of IL-8 mRNA at 30 min, with no detectable transcript at 1 or 2 hours. In epithelia colonized with *S. pullorum* and subsequently exposed to TNF- α , levels of IL-8 transcript were markedly reduced at all time points compared to cells treated with TNF- α alone, consistent with data obtained with secreted IL-8. Transcript levels of I κ B- α , itself a rapidly inducible NF- κ B-dependent gene (2), behaved in a very similar manner (Fig. 1D, middle row), consistent with a generalized inhibition of NF- κ B dependent genes. Hybridization of a glyceraldehyde phosphate dehydrogenase (GAPDH) probe was unchanged in all experimental conditions, indicating that the repressive effect was not secondary to a global increase in mRNA turnover (Fig. 1D, bottom row).

Because IL-8 and I κ B- α are NF- κ B-dependent genes, we studied the effects of the anti-inflammatory organisms on the NF- κ B activation pathway. Anti-inflammatory salmonellae, but not wild-type *S. typhimurium*, inhibited activation of reporter constructs controlled both by the native IL-8 promoter and synthetic NF- κ B response elements (Fig. 2A). Immunolocalization of the p65 subunit of NF- κ B in HeLa epithelial cells revealed that colonization with the anti-inflammatory organisms prevented nuclear translocation of p65 in response to TNF- α (Fig. 2B). Furthermore, the anti-inflammatory organisms caused the p65 to be redistributed in a granular, perinuclear localization even in the absence of proinflammatory stimulation. To determine if the observed blockade of NF- κ B activation was occurring at the level of I κ B- α degradation, Western blot analysis of whole-cell lysates was performed (Fig. 2, C through E). Colonization of T84 model epithelia with anti-inflammatory organisms prior to TNF- α or proinflammatory wild-type *S. typhimurium* challenge resulted in stabilization of I κ B- α , in contrast to the nearly complete I κ B- α degradation induced by TNF- α or wild-type *S. typhimurium* alone (Fig. 2, C and D). Identical results were obtained with two other human epithelial cell lines, HT-29 and HeLa, but not with monocytic U293 cells or human umbilical vein endothelial cells, suggesting that the anti-inflammatory effect may be a property of epithelial cells in general (16). The anti-inflammatory effects on I κ B- α degradation required a minimal multiplicity of

infection (MOI) of 10 organisms/cell and, consistent with the IL-8 secretion data, could not be mediated by heat-killed organisms or bacterial culture supernates (Fig. 2E). The anti-inflammatory effect on I κ B- α degradation was seen maximally after 30 min of colonization and could not be inhibited with cycloheximide (20 μ g/ml, added 30 min before experimental infection) (16).

Surprisingly, colonization of T84 model epithelia with anti-inflammatory organisms induced the phosphorylation of I κ B- α and did so to an even greater extent than did wild-type *S. typhimurium*, *InvA*, or TNF- α (Fig. 3A). The presence of continuously phosphorylated I κ B- α

in the absence of degradation and subsequent NF- κ B translocation is distinct from the reported mechanism of NF- κ B blockade mediated by the enteroinvasive pathogen *Yersinia* sp. (19, 20). These organisms prevent I κ B phosphorylation via the activity of the translocated prokaryotic effector molecule YopJ, which inhibits mitogen-activated protein kinase (MAPKK) and subsequent I κ B kinase activation. In contrast, all *Salmonella* strains studied, including the anti-inflammatory strains, activated the MAPKK/JNK pathway, which is the only other signaling pathway necessary for IL-8 transcription in response to *Salmonella* (Fig. 3B) (11).

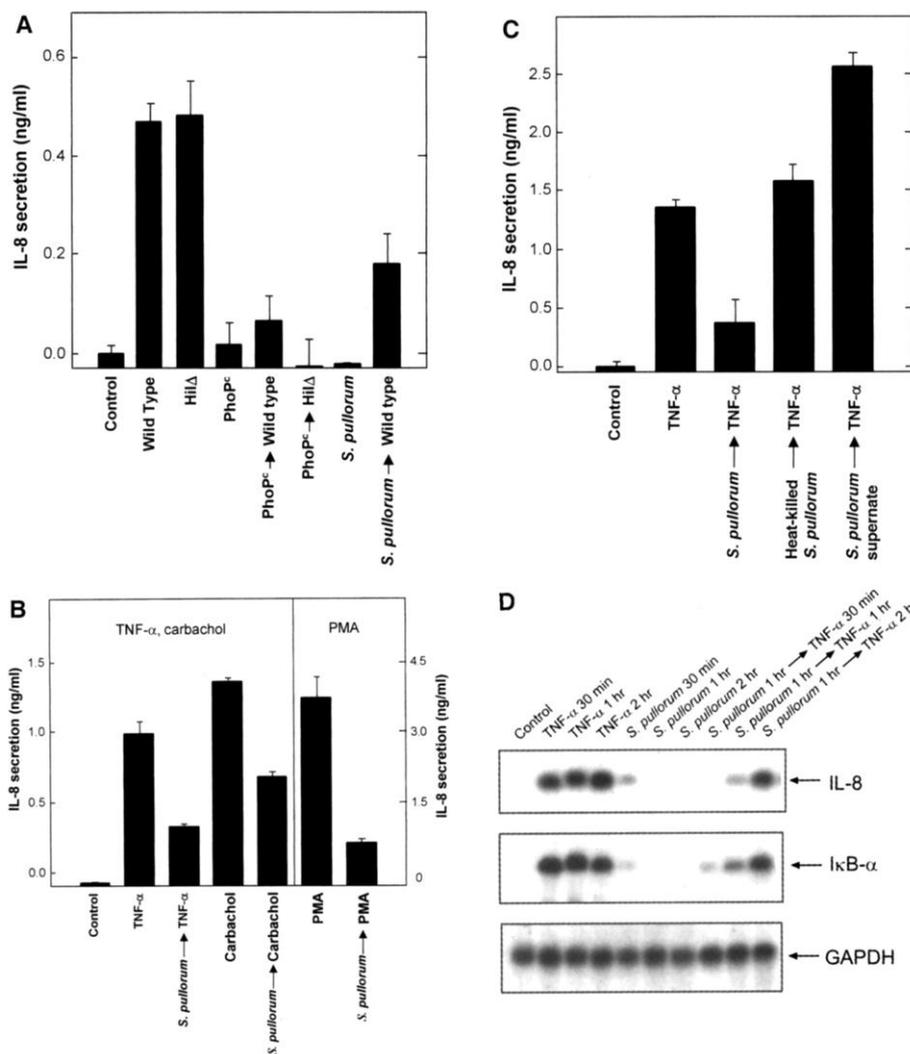


Fig. 1. Colonization with anti-inflammatory *Salmonella* inhibits induced epithelial responses. [(A) through (C)] Enzyme-linked immunosorbent assay (ELISA) of secreted IL-8 from model epithelia (13, 14). Data are the means \pm SEM of three experiments. (A) Cells were colonized apically with the indicated bacteria for 1 hour and washed, after which the second strain of bacteria was added for an additional hour. (B) Cells were colonized apically as in (A), washed, and subsequently challenged with basolateral TNF- α (10 ng/ml), carchanol (100 μ M), and PMA (1 μ g/ml) for 30 min as indicated. (C) Cells were treated apically with live and heat-killed (80°C for 10 min) cultures and with conditioned media (full-density cultures equivalent to a MOI of 30 were incubated alone at 37°C for 1 hour and sterilized through a 0.2- μ m filter) for 1 hour, washed and challenged with basolateral TNF- α . (D) Total RNA was analyzed by Northern hybridization as described (17). Blots were sequentially hybridized with ³²P-labeled cDNA probes encoding human IL-8, I κ B- α , and GAPDH.

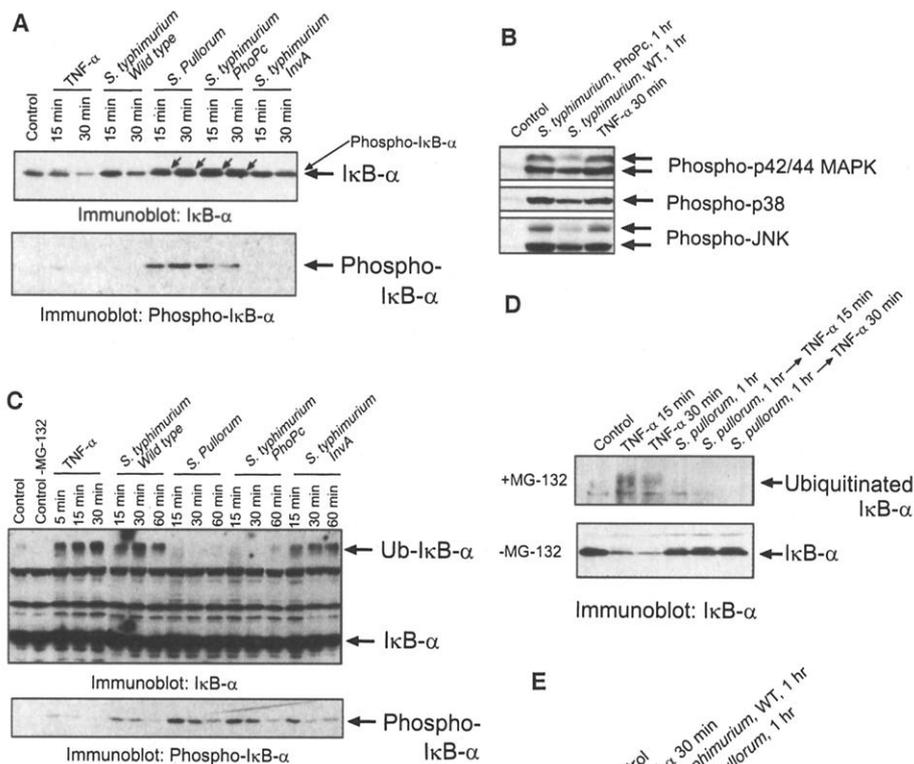
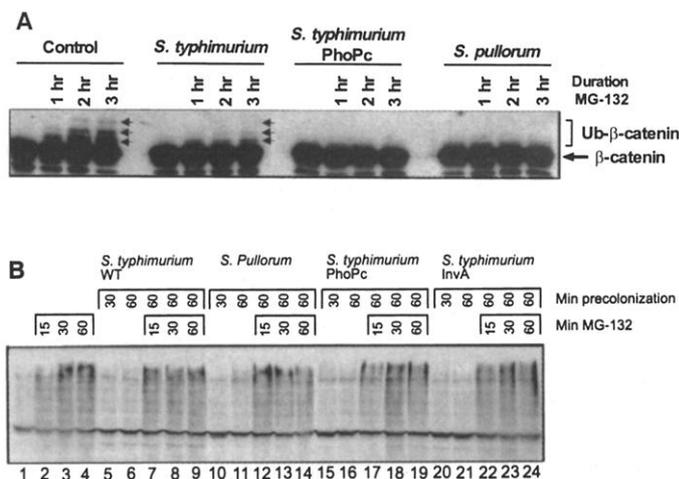


Fig. 3. Colonization with anti-inflammatory *Salmonella* induces IκB-α phosphorylation but inhibits ubiquitination. (A) Whole-cell extracts from cells colonized apically with the indicated organisms. Immunoblots with anti-IκB-α (upper panel, high molecular weight phospho IκB-α species are flagged) and anti-phospho IκB-α antibodies (lower panel) (12). (B) Immunoblots with anti-phospho-specific antibodies (New England Biolabs). Whole-cell extracts were prepared from cells treated as indicated. (C and D) Cells were pretreated with 50 μM MG-132 for 30 min and activated/colonized as indicated. Immunoblots with anti-IκB-α antibodies [(C), upper panel and (D)] and anti-phospho IκB-α antibodies [(C), lower panel]. (E) Immunoprecipitation with IκB-α followed by immunoblot with anti-ubiquitin conjugate antibody (22). HT-29 cells were pretreated with 50 mM MG-132 for 1 hour and activated/colonized as indicated.

Fig. 4. (A) Immunoblots with anti-β-catenin antibodies (Transduction Laboratories). Model epithelia were colonized with the indicated strains for 30 min before addition of 50 μM MG-132. (B) Immunoblot with anti-ubiquitin conjugate antibodies (Affiniti Research). Model epithelia were treated as indicated with 50 μM MG-132, with test bacteria applied apically, or with test bacteria followed by wash and subsequent addition of MG-132.



interactions may induce local inhibition of acute inflammatory responses, utilizing either host- or bacteria-derived factors. Our

observations suggest that the anti-inflammatory bacterial/epithelial interaction may provide a factor that either reduces activity

of the IκB E3-SCF complex or increases a specific de-ubiquitinating activity. Such a hypothetical factor could be translocated from the bacterium into the host cell via a type III secretion apparatus (25), or alternatively, represents a eukaryotic-derived anti-inflammatory compound elicited by cellular interactions with a specific bacterial factor (26). At equilibrium, attenuation of acute inflammatory responses by certain prokaryotes could contribute to the characteristic tolerance of the mucosal immune system, whereas disruption of this equilibrium could contribute to the pathogenesis of enteric infections as well as idiopathic inflammatory bowel disease. In relation to this hypothesis, it is relevant that the use of nonpathogenic enteric organisms, termed “probiotics,” are now being explored as therapeutic agents in inflammatory bowel disease. Although the mechanism of probiotic action is not understood, the beneficial effects are consistent with an anti-inflammatory state conferred by nonpathogenic organisms. It appears that the luminal microflora can send negative as well as positive signals to mucosal lining cells as a means of productive, symbiotic interactions with the host.

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22. Model epithelia were treated with 50 μM MG-132 for 30 min before apical infection of test organisms or basolateral addition of TNF-α. Cells were lysed in 200 μl of RIPA. We added 20 μl of agarose-conjugated antibodies to IκB-α (anti-IκB-α) (Santa Cruz) to 70 μl of clarified lysate and allowed it to bind for 4 hours. Beads were washed three times in 500 μl of lysis buffer, and bound proteins were released by boiling in denaturing loading buffer. Samples were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting with anti-ubiquitin conjugate (Affiniti).
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