Inflammation-Induced Acid Tolerance Genes *gadAB* in Luminal Commensal *Escherichia coli* Attenuate Experimental Colitis

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Dysregulated immune responses to commensal intestinal bacteria, including *Escherichia coli*, contribute to the development of inflammatory bowel diseases (IBDs) and experimental colitis. Reciprocally, *E. coli* responds to chronic intestinal inflammation by upregulating expression of stress response genes, including *gadA* and *gadB*. GadAB encode glutamate decarboxylase and protect *E. coli* from the toxic effects of low pH and fermentation acids, factors present in the intestinal lumen in patients with active IBDs. We hypothesized that *E. coli* upregulates *gadAB* during inflammation to enhance its survival and virulence. Using real-time PCR, we determined *gadAB* expression in luminal *E. coli* from ex-germfree wild-type (WT) and interleukin-10 (IL-10) knockout (KO) (IL-10$^{-/-}$) mice selectively colonized with a commensal *E. coli* isolate (NC101) that causes colitis in KO mice in isolation or in combination with 7 other commensal intestinal bacterial strains. *E. coli* survival and host inflammatory responses were measured in WT and KO mice colonized with NC101 or a mutant lacking the *gadAB* genes (NC101Δ*gadAB*). The susceptibility of NC101 and NC101Δ*gadAB* to killing by host antimicrobial peptides and their translocation across intestinal epithelial cells were evaluated using bacterial killing assays and transwell experiments, respectively. We show that expression of *gadAB* in luminal *E. coli* increases proportionately with intestinal inflammation in KO mice and enhances the susceptibility of NC101 to killing by the host antimicrobial peptide cryptdin-4 but decreases bacterial transmigration across intestinal epithelial cells, colonic inflammation, and mucosal immune responses. Chronic intestinal inflammation upregulates acid tolerance pathways in commensal *E. coli* isolates, which, contrary to our original hypothesis, limits their survival and colitogenic potential. Further investigation of microbial adaptation to immune-mediated inflammation may provide novel insights into the pathogenesis and treatment of IBDs.

T he human gastrointestinal tract is home to a complex community of microbes whose number exceeds the number of host cells by over an order of magnitude (1). In the healthy state, protective host mechanisms, including bacterium-induced regulatory T cells and rapid microbial adaptation, result in a dynamic equilibrium between the intestinal immune system and this large number of microbial symbionts (2, 3). However, mounting evidence suggests that members of the intestinal microbiota, including adherent-invasive strains of *Escherichia coli*, also induce maladaptive immune responses in patients with inflammatory bowel diseases (IBDs) (4). Currently, the prevailing hypothesis is that a subset of commensal enteric bacteria provides antigenic stimulation that continuously activates pathogenic T cells to cause chronic intestinal injury (5). Similar to human IBDs, the importance of the intestinal microbiota in the pathogenesis of chronic, immune-mediated experimental colitis is evident in gnotobiotic studies using animals with immunoregulatory defects, including the interleukin-10 (IL-10)-deficient (IL-10$^{-/-}$) mouse. In this model, development of chronic intestinal inflammation requires the presence of commensal microbes (6, 7). Moreover, selective colonization (monoassociation) of IL-10$^{-/-}$ mice with defined commensal bacteria, including adherent-invasive *E. coli*, causes colitis (7–10).

While the influence of the microbiota on host immune responses has been extensively studied, little is known about the effects of inflamed intestine on bacterial properties that may perpetuate the cycle of mucosal inflammation. Host inflammatory responses enhance the virulence of certain enteric pathogens (11–13), and we recently reported that colitis in *E. coli*-monoassociated IL-10$^{-/-}$ mice over a long period induces upregulation of stress-response genes, including *gadA* and *gadB*, in commensal luminal *E. coli* (14).

Although the *gadA* and *gadB* genes are not located in the same operon, their function appears to be redundant. *gadA* and *gadB* (*gadAB*) encode glutamate decarboxylase proteins that are part of the glutamate-dependent acid resistance system 2 (AR2) in *E. coli*, which protects the bacteria from acid conditions (pH 2.5 to 4.5), including those encountered during passage through the stomach into the intestine (15, 16). While the roles of *E. coli* GadAB in bacterial survival during transit through the normal gastrointestinal tract have been described (15, 17), little is known about the

effect of these proteins on *E. coli* viability in the inflamed gut and how they affect host immune responses.

Since active IBDs are variably associated with decreased luminal pH (18) and increased colonic levels of some fermentation acids (19, 20) and because *gadAB* protect *E. coli* from the toxic effects of low pH and fermentation acids (16, 21), we hypothesized that *E. coli* upregulates *gadAB* in the inflamed colon in order to survive and perpetuate colitis. In the present study, we show that, contrary to our hypothesis, expression of *gadAB* in luminal commensal *E. coli* is associated with reduced bacterial survival and attenuated colitis, which may be due in part to increased susceptibility to bacterial killing by host antimicrobial peptides (AMPs) and decreased translocation across intestinal epithelial cells. These results suggest that inflammation-associated changes in commensal bacterial gene expression may paradoxically decrease luminal bacterial survival and attenuate the host inflammatory response.

MATERIALS AND METHODS

Bacterial strains, lysates, and growth curves. The nonpathogenic murine *E. coli* strain NC101 was isolated as described previously (8). The *Enterococcus faecalis* human oral isolate (strain OG1RF) was provided by Mark Huycke. Mutant strain NC101Δ*gadAB* was constructed using the bacteriophage λ-*red* recombinase method (22). Mutant strains NC101Δ*gadAB* attTn7::*gadBA* and NC101Δ*gadAB*(pgadB) were generated using standard molecular biology techniques (see the methods in the supplemental material). Bacterial lysates were prepared and bacterial growth curves were generated as described previously (8, 14). In separate experiments, germfree mice were colonized by oral gavage with a mixture of *Enterococcus faecalis* OG1RF, *Bacteroides thetaiotaomicron* VPI-5482, *Bacteroides vulgatus* ATCC 8482, *Bifidobacterium longum* subsp. *infantis* ATCC 15697, *Lactobacillus rhamnosus* GG, *Ruminococcus gnavus* ATCC 29149, *Faecalibacterium prausnitzii* A2-165, and either *E. coli* NC101 or *E. coli* NC101Δ*gadAB* that had been cultured anaerobically overnight.

Antimicrobial peptide bacterial killing assays. Bacteria were grown to mid-exponential phase and washed in 10 mM PIPES buffer, pH 7.3, and the optical density at 600 nm was adjusted to ~1.0 using PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] containing 1% LB. Bacterial suspensions were incubated with either medium control (PIPES plus 1% LB) or recombinant murine cryptdin-4 (a gift from Andrew Oulette) for 1 h, after which viable bacteria were enumerated by plating serial dilutions.

Mice. Germfree (GF) IL-10$^{-/-}$ and wild-type (WT) controls (both on the 129S6/SvEV background) were originally derived under sterile conditions by hysterectomy at the Gnotobiotic Laboratory (University of Wisconsin, Madison). Mice maintained under GF conditions at the National Gnotobiotic Rodent Resource Center at the University of North Carolina (UNC)—Chapel Hill were monoassociated with bacteria by swabbing the mouth and anus with an overnight bacterial culture of *E. coli* NC101 or NC101Δ*gadAB*. The absence of isolator contamination was confirmed by Gram stain and culture of cecal contents on brain heart infusion (BHI) agar plates under aerobic and anaerobic conditions. All animal protocols were approved by the UNC—Chapel Hill Institutional Animal Care and Use Committee.

RNA isolation from cecal contents. Freshly harvested cecal or midcolonic contents (100 to 300 mg) were snap-frozen in liquid N$_2$ and stored at −80°C. Frozen samples were thawed into 1 ml of Qiagen RNAprotect bacterial reagent while vortexing and incubated at 25°C for 5 min, and bacterial RNA was isolated as described previously (23).

Extraction of DNA from colon tissue. Bacterial DNA was isolated from snap-frozen cecal tissue using phenol-chloroform extraction, physical disruption, and DNA purification on Qiagen DNeasy blood and tissue columns (see the methods in the supplemental material).

Real-time PCR. Real-time oligonucleotide primers were designed using either Primer 3 or Applied Biosystems Primer Express (version 3.0) software based on *E. coli* NC101 genomic DNA sequences published in GenBank (see Table S1 in the supplemental material). First-strand cDNA was synthesized, and quantitative real-time PCRs were performed as described previously (14), except that Bio-Rad 2× iTaq SYBR green PCR Supermix was used. To quantify the number of *E. coli* 16S rRNA gene copies in cecal tissue, 50 ng of tissue DNA was added to each real-time PCR mixture and the results were normalized to those for murine GAPDH (glyceraldehyde-3-phosphate dehydrogenase) using the ΔC_T (where C_T is the threshold cycle) method.

Quantification of luminal bacteria. Luminal *E. coli* concentrations in monoassociated mice were measured by dilution plating on BHI agar as described previously (14). Cecal *E. coli* concentrations in animals with bacterial octoassociation (animals colonized with the mixture of the seven commensal strains representative of the normal gut microbiota indicated above plus an *E. coli* strain) were measured by quantitative PCR of the *E. coli* 16S rRNA gene as described above, but using bacterial genomic DNA isolated from cecal contents rather than cecal tissue. Absolute quantification of *E. coli* genome copies per ng of total DNA was performed using a standard curve generated from purified *E. coli* genomic DNA, assuming that the *E. coli* NC101 genome is 5.02 Mb and contains 7 copies of the 16S rRNA gene.

Histological scoring. Composite histological inflammation scores of colon tissue were determined as described previously (14).

Immunofluorescence staining. To detect *E. coli* in intestinal tissue, paraffin-embedded intestinal sections were rehydrated in a xylene-alcohol gradient, permeabilized with phosphate-buffered saline (PBS)–0.1% Triton X-100 for 10 min at 25°C, blocked with PBS–5% bovine serum albumin–1% Triton X-100, and incubated with a 1:50 dilution of biotinylated rabbit anti-*E. coli* polyclonal antibody (Fitzgerald Industries) for 1 h at 25°C, followed by a 1:400 dilution of streptavidin-phycoerythrin (BD Biosciences) for 1 h. Three images per tissue section were acquired from randomly chosen areas using an Olympus IX71 fluorescence microscope. In a blinded fashion, apical epithelial cell-associated bacteria per ×20-magnification field were enumerated in each image, and the numbers from six individual intestinal sections (ileum; 2 sections of the cecum; and the proximal, mid-, and distal colon) were summed for each animal.

Colonic tissue fragment and MLN cultures. Colonic tissue fragment cultures and IL-12/23p40 enzyme-linked immunosorbent assays (ELISAs) were performed as described previously (7, 14). Multiplex immunoassays for gamma interferon (IFN-γ), IL-1β, IL-6, IL-13, IL-17, monocyte chemoattractant protein 1, and tumor necrosis factor alpha (TNF-α) were performed using a Milliplex system (EMD Millipore) on undiluted colon tissue fragment culture supernatants according to the manufacturer's instructions. For mesenteric lymph node (MLN) cultures, single-cell suspensions of MLNs were stimulated with medium or bacterial lysates, after which the IFN-γ in the culture supernatants was measured by ELISA as described previously (14).

T84 cell cultures and transwell experiments. The human T84 colorectal cancer cell line was provided by Scott Plevy. T84 cells ($2.5 × 10^5$) in high-glucose Dulbecco modified Eagle medium containing 10% fetal bovine serum were cultured in the upper chamber of 12-well transwell plates with a 3-μm pore size (Corning Costar) for 10 to 14 days, at which point tight junctions formed and transepithelial electrical resistance was ~3,500 Ω for 1 day, as measured with an EVOM2 ohmmeter and an Endohm-12 device. Bacteria ($8 × 10^7$) were added in the upper chamber and incubated at either 37°C or 4°C. At the indicated times, viable bacteria in the lower chamber were quantified by plating on LB agar. To enumerate epithelial cell-associated bacteria, monolayers were washed 5 times with PBS and lysed with 1% Triton X-100 for 10 min, and dilutions were plated on LB agar. For confocal imaging, monolayers infected for 5 h were fixed in 4% paraformaldehyde, washed, stained with anti-*E. coli* antibody as described above but without permeabilization, counterstained with DAPI (4′,6-diamidino-2-phenylindole), and visualized on a Zeiss LSM710 confocal microscope.

Gentamicin protection assays. T84 cells were cultured and infected with *E. coli* as described above. Monolayers were washed 3 times with PBS, treated with gentamicin (100 μg/ml) at 37°C for 15 min, followed by 4°C for 2 h, and washed 3 times with PBS. Epithelial cells were lysed as described above, and intracellular bacteria were enumerated by plating dilutions on LB agar.

Statistical analysis. All data are presented as the mean ± standard error of the mean (SEM). *P* values were calculated using the unpaired, 2-tailed Student's *t* test.

RESULTS

Expression of *gadA* and *gadB* is upregulated in luminal commensal *E. coli* during intestinal inflammation. We have previously shown that *E. coli* stress response genes, including *gadAB*, are upregulated in cecal bacteria from IL-10$^{-/-}$ mice with colitis monoassociated with *E. coli* for 7 weeks compared to their expression in healthy WT controls (14). However, whether increased *E. coli gadAB* expression is the direct result of IL-10 deficiency or colitis is unknown, nor are the kinetics of *gadAB* expression during the development of colitis. To test this, we monoassociated germfree (GF) WT and IL-10$^{-/-}$ mice for 5 days to 10 weeks with a commensal murine strain of *E. coli* (NC101) that has previously been shown to induce colitis in IL-10$^{-/-}$ mice (8). Consistent with previous results, we observed a time-dependent increase in histological colonic inflammation in IL-10$^{-/-}$ but not WT mice (Fig. 1A). Similar to the colitis scores, *gadAB* expression in cecal NC101 bacteria from IL-10$^{-/-}$ mice increased over time and was significantly higher than that in cecal NC101 bacteria from WT mice at later time points, when colitis was the most severe. Surprisingly, we observed higher expression of *gadAB* in cecal NC101 bacteria from healthy WT mice than IL-10$^{-/-}$ mice at 5 and 14 days (Fig. 1B and C). These data suggest that multiple factors govern *gadAB* expression in luminal *E. coli* in a time-dependent fashion. During early phases of colonization, non-inflammation-dependent factors predominate, whereas in later phases, inflammation-related factors are paramount.

Expression of *gadAB* enhances susceptibility of *E. coli* to killing by the host α-defensin cryptdin-4 *in vitro* and is associated with decreased bacterial survival *in vivo*. Based on our hypothesis that *E. coli* upregulates *gadAB* as a survival strategy, we predicted that a mutant strain of NC101 that lacks *gadAB* (NC101Δ*gadAB*) would be less viable in the inflamed intestine. In contrast, we observed significantly higher concentrations of NC101Δ*gadAB* bacteria than NC101 bacteria in cecal and colonic luminal contents from both WT and IL-10$^{-/-}$ monoassociated mice (Fig. 2A; see Fig. S1 in the supplemental material). The differences in bacterial viability in the cecum and colon are likely due to the unique environment of these locations, since the growth of NC101Δ*gadAB* was virtually identical to that of NC101 *in vitro* (Fig. 2B). The absence of *gadAB* expression in cecal bacteria from mice monoassociated with NC101Δ*gadAB* was confirmed by real-time PCR (see Fig. S2 in the supplemental material). These data demonstrate that, contrary to our original hypothesis, expression of *gadAB* in *E. coli* impairs luminal bacterial growth *in vivo* but not *in vitro*.

Host antimicrobial peptides (AMPs), including α-defensins, are secreted into the intestinal lumen, where they limit the growth of certain pathogenic and commensal bacteria. The antimicrobial activity of some AMPs depends in part on a normal proton motive force across the bacterial inner membrane (24–26). Since GadAB help maintain the normal proton gradient in acidic environments
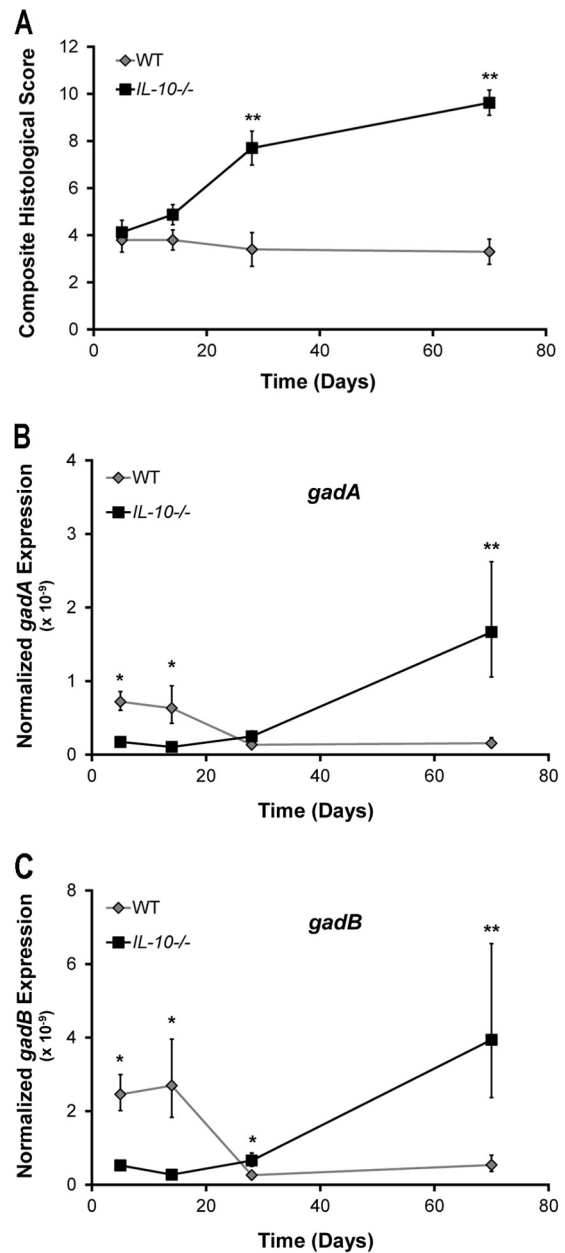

FIG 1 Expression of *gadAB* is upregulated in luminal *E. coli* during inflammation. WT and IL-10$^{-/-}$ mice were monoassociated with *E. coli* NC101 for 5, 14, 35, and 70 days, after which colonic histological inflammation (A) and expression of *gadA* (B) and *gadB* (C) normalized to that of the 16S rRNA gene in cecal NC101 were determined. Data are presented as the mean ± SEM (*n* = 4 to 8 mice per group). *, *P* < 0.05 relative to WT; **, *P* < 0.01 relative to WT.

(15, 16), we reasoned that expression of *gadAB* increases the susceptibility of NC101 to killing by AMPs. To test this hypothesis, we treated mid-log-phase NC101 and NC101Δ*gadAB* with increasing concentrations of the recombinant murine α-defensin cryptdin-4 and found that NC101Δ*gadAB* was more resistant to cryptdin-4-mediated killing than NC101 at all concentrations of cryptdin-4 tested (Fig. 2C). Complementation of NC101Δ*gadAB* with chromosomal copies of *gadAB* partially reversed the phenotype (Fig. 2C). These results indicate that expression of *gadAB* enhances the susceptibility of NC101 to killing by cryptdin-4 *in vitro*, but not
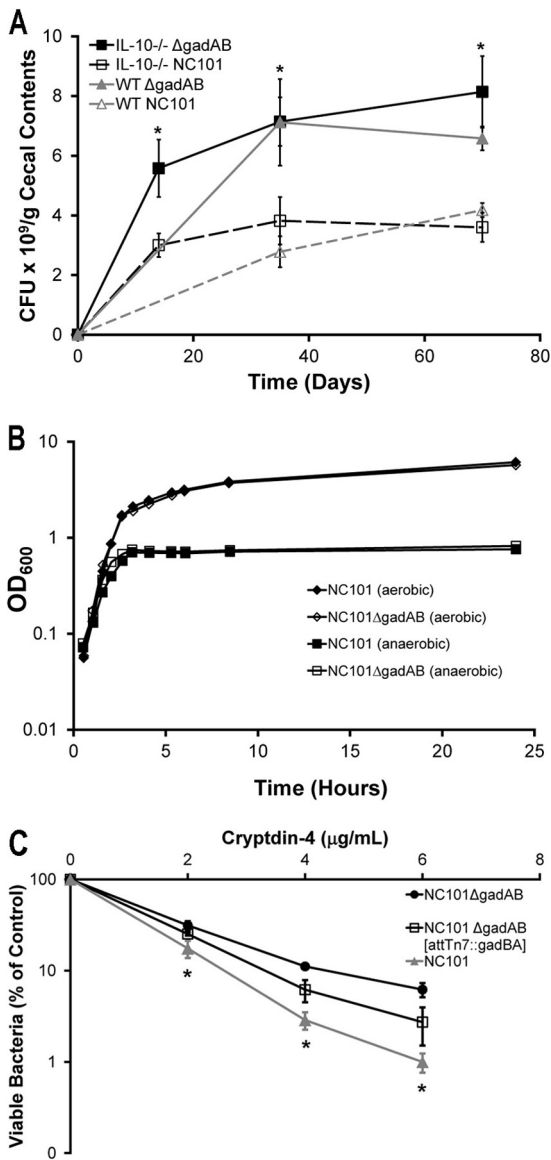
FIG 2 GadAB inhibits luminal *E. coli* survival and enhances cryptdin-4-mediated killing *in vitro*. (A) Cecal bacterial concentrations in monoassociated WT and IL-10$^{-/-}$ mice. Data are presented as mean ± SEM (n = 5 mice per group). *, P < 0.05 relative to mice with the same genetic background monoassociated with NC101. (B) NC101 and NC101Δ*gadAB* growth curves under aerobic or anaerobic conditions. Data are presented as the mean ± SEM (n = 2 separate cultures/strain). OD$_{600}$, optical density at 600 nm. (C) Killing curves of mid-log-phase NC101, NC101Δ*gadAB*, and complemented NC101Δ*gadAB* (NC101Δ*gadAB attTn7::gadBA*) bacteria treated with recombinant mouse cryptdin-4. Data show the percentage of surviving bacteria relative to the numbers of untreated controls, presented as the mean ± SD of three separate experiments. *, P < 0.05 relative to NC101Δ*gadAB*.

necessarily *in vivo*. However, they suggest the possibility that the decreased luminal bacterial concentrations in NC101- versus NC101Δ*gadAB*-monoassociated mice might be due to differences in AMP susceptibility. Transcription of the cryptdin-4 gene (*defa4*) and those of other AMPs in ileal tissue from NC101- and NC101Δ*gadAB*-monoassociated mice was similar, indicating that differences in luminal bacterial concentrations in the two groups of mice are not due to differences in AMP gene expression (see Fig. S3 in the supplemental material).

GadAB in luminal *E. coli* attenuate the development of experimental colitis. To determine whether the decreased luminal bacterial concentrations of *gadAB*-expressing NC101 correlate with decreased colitis, we monoassociated WT and IL-10$^{-/-}$ mice with *E. coli* NC101 and NC101Δ*gadAB* for 2, 5, and 10 weeks and quantified colonic inflammation using a validated blinded histological scoring system described previously (8). As expected, IL-10$^{-/-}$ mice colonized with NC101 exhibited increased histological inflammation compared to WT mice at 5 and 10 weeks (Fig. 3A and B). Consistent with the increased luminal concentrations of NC101Δ*gadAB* compared to those of NC101, histological inflammation scores in NC101Δ*gadAB* monoassociated IL-10$^{-/-}$ and WT mice were higher than those in NC101-monoassociated mice (Fig. 3A and B).

The activation of innate immune pathways, including IL-12 and IL-23 secretion, is associated with experimental colitis and human IBDs (8, 27). We therefore tested whether the expression of *gadAB* in luminal *E. coli* isolates of monoassociated mice affects the secretion of IL-12/23p40 by colon explants. In accordance with decreased colonic histological inflammation, spontaneous secretion of IL-12/23p40 was lower in colon explants from IL-10$^{-/-}$ mice monoassociated with NC101 for 2 and 5 weeks and WT mice monoassociated with NC101 for 5 and 10 weeks than the corresponding NC101Δ*gadAB*-monoassociated mice (Fig. 3C). Using multiplex immunoassays, we also detected increased levels of the proinflammatory cytokines IFN-γ, IL-6, IL-17, and TNF-α in cultures of colon explants from IL-10$^{-/-}$ mice monoassociated with NC101Δ*gadAB* for 5 weeks compared to those in cultures of colon explants from NC101-monoassociated IL-10$^{-/-}$ mice (Fig. 3D).

Because IL-12 and IL-23 promote the clonal expansion of IFN-γ-producing Th1 and IL-17-producing Th17 cells, respectively, both of which play a crucial role in the development of chronic-immune mediated colitis (28), we next aimed to determine whether the increased innate immune responses observed in mice monoassociated with NC101Δ*gadAB* were associated with enhanced antigen-specific adaptive immune responses. Therefore, we measured IFN-γ secretion by NC101 lysate-stimulated MLN cells isolated from NC101- or NC101Δ*gadAB*-monoassociated WT and IL-10$^{-/-}$ mice. Consistent with the increased levels of proinflammatory cytokines in cultures of colon explants from NC101Δ*gadAB*-monoassociated mice, stimulated MLN cells from both WT and IL-10$^{-/-}$ mice colonized with NC101Δ*gadAB* produced significantly more IFN-γ than MLN cells from mice monoassociated with NC101 (Fig. 3E). MLN cells isolated from NC101- and NC101Δ*gadAB*-monoassociated mice and stimulated with lysate from the unrelated bacterium *Enterococcus faecalis* secreted similar and very small amounts of IFN-γ (data not shown).

To confirm that the increased inflammatory responses in NC101Δ*gadAB*-monoassociated IL-10$^{-/-}$ mice were due to the absence of glutamate decarboxylase function and not an artifact of creating the deletion mutant, we repeated monoassociation studies using a strain of NC101Δ*gadAB* in which *gadB* was expressed from a stable episome [NC101Δ*gadAB*(pgadB)]. Luminal bacterial concentrations, histological scores, and spontaneous IL-12/23p40 secretion by colon explants from IL-10$^{-/-}$ mice monoassociated with NC101Δ*gadAB*(pgadB) for 2 and 5 weeks were not different from those for NC101-monoassociated IL-10$^{-/-}$ mice (Fig. 4), confirming that glutamate decarboxylase activity in NC101 is responsible for the observed attenuated colitis pheno-
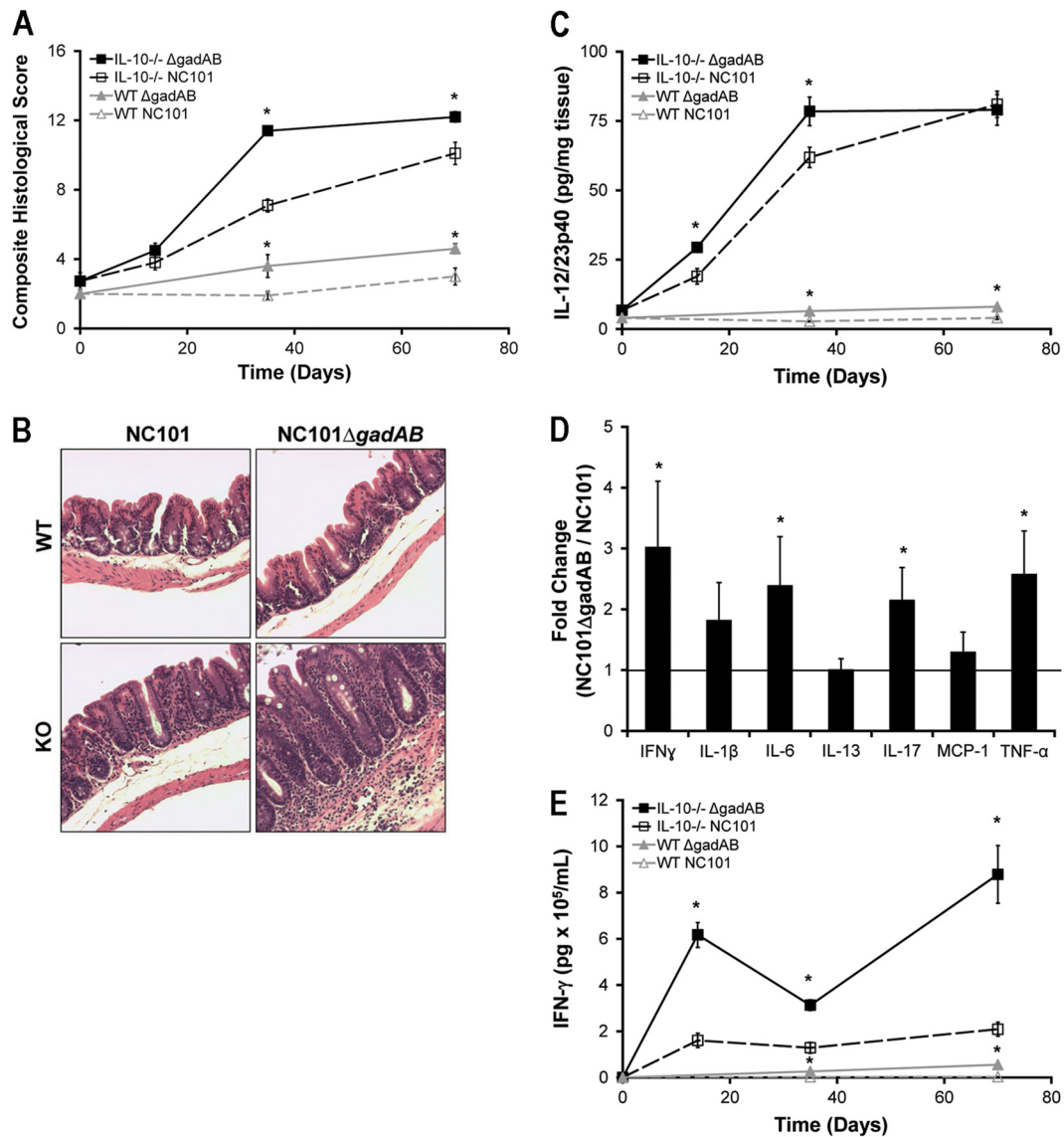
FIG 3 The presence of *E. coli gadAB* is associated with attenuated host proinflammatory immune responses in NC101-monoassociated IL-10$^{-/-}$ and WT mice. (A) Composite blinded histological inflammation scores of colonic segments from IL-10$^{-/-}$ or WT mice monoassociated for the indicated times. (B) Representative photomicrographs of cecum from WT and IL-10$^{-/-}$ mice monoassociated for 5 weeks, as described in panel A. Magnifications, ×20. KO, knockout. (C) Spontaneous secretion of IL-12/23p40 in culture supernatants from proximal colon explants measured by ELISA. (D) Proinflammatory cytokine levels in colon explant cultures from IL-10$^{-/-}$ mice monoassociated with NC101Δ*gadAB* for 5 weeks relative to those in mice monoassociated with NC101 measured by multiplex immunoassays. MCP-1, monocyte chemoattractant protein 1. (E) IFN-γ secretion by unfractionated MLN cells stimulated *ex vivo* with *E. coli* NC101 lysate. Data are presented as the mean ± SEM ($n = 5$ to 6 mice per group for all panels except panel D, for which 4 mice were used per group). *, $P < 0.05$ relative to NC101-monoassociated mice of the same genetic background.

type. Overall, these data indicate that expression of *gadAB* in luminal *E. coli* isolates is associated with reduced colonic inflammation and decreased mucosal innate and adaptive immune responses in monoassociated IL-10$^{-/-}$ mice.

Since results obtained using monoassociated mice may not accurately represent those observed in mice with a complex intestinal microbiota, we colonized WT and IL-10$^{-/-}$ mice with a mixture of seven commensal strains representative of the normal gut microbiota, together with either *E. coli* NC101 or NC101Δ*gadAB*, for 5 and 10 weeks (octoassociation). We found significantly increased expression of *gadA* and *gadB* in luminal *E. coli* isolates from octoassociated IL-10$^{-/-}$ mice at 10 weeks (Fig. 5A and B).

Similar to results obtained in monoassociated IL-10$^{-/-}$ mice, luminal *E. coli* densities, histological inflammation scores, and spontaneous IL-12/23p40 secretion by colon explants from IL-10$^{-/-}$ mice colonized with a complex bacterial mixture that included NC101Δ*gadAB* were significantly increased compared to those for mice colonized with a complex bacterial mixture that included NC101 at 5 weeks (Fig. 5C to E). These results suggest that expression of *gadAB* in luminal *E. coli* NC101 attenuates colitis even in the presence of a complex microbiota.

Expression of *gadAB* decreases translocation of *E. coli* across intestinal epithelial cells. Since our initial observations indicated that *gadAB* expression is associated with reduced colonic inflam-
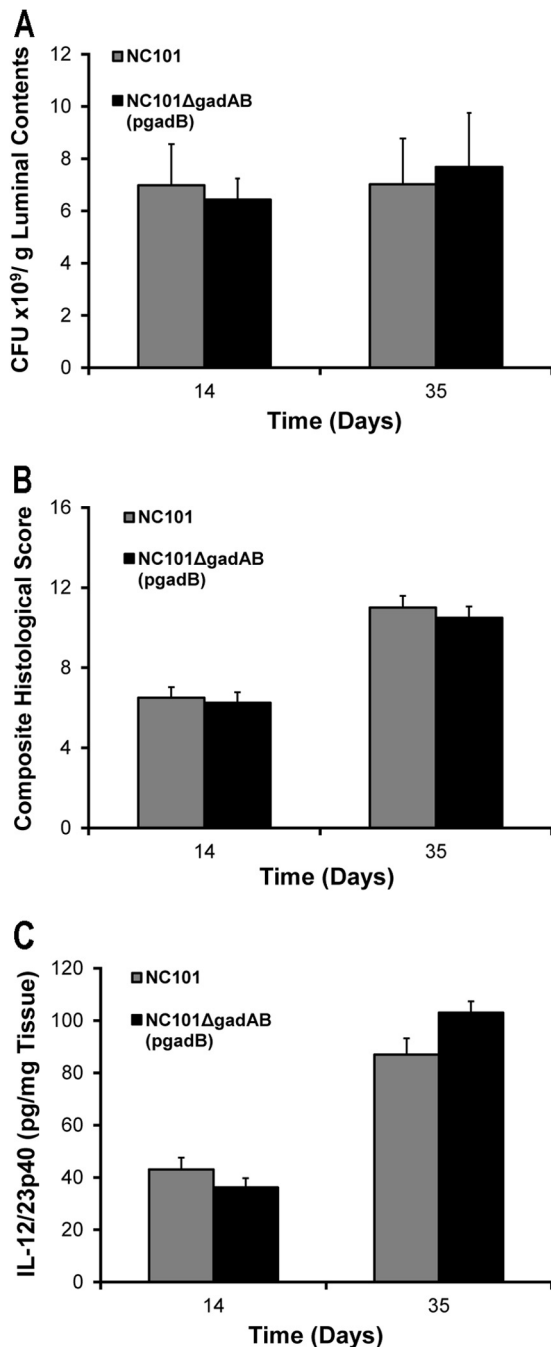
FIG 4 Complementation of NC101Δ*gadAB* restores the *E. coli* NC101 phenotype in IL-10$^{-/-}$ monoassociated mice. (A) Luminal bacterial concentrations in the cecum; (B) composite blinded histological inflammation scores of colonic segments; (C) spontaneous secretion of IL-12/23p40 in culture supernatants from proximal colon explants of IL-10$^{-/-}$ mice monoassociated with NC101 or complemented NC101Δ*gadAB* [NC101Δ*gadAB*(pgadB)] for the indicated times. Data are presented as the mean ± SEM ($n = 6$ mice per group).

mation and decreased mucosal immune responses to NC101 and because access to lamina propria antigen-presenting cells is necessary for bacteria to stimulate antigen-specific immune responses, we examined whether decreased colitis in NC101-monoassociated IL-10$^{-/-}$ mice corresponds to a reduced ability of *E. coli* NC101 to adhere to the intestinal epithelium compared with

that for NC101Δ*gadAB*. We determined the densities of epithelial cell-associated *E. coli* in intestinal fragments from IL-10$^{-/-}$ mice monoassociated with NC101 and NC101Δ*gadAB* for 2, 5, and 10 weeks using fluorescence microscopy. Semiquantitative analysis of anti-*E. coli* antibody-stained intestinal sections revealed significantly increased numbers of luminal bacteria adjacent to the intestinal epithelium in NC101-monoassociated IL-10$^{-/-}$ mice compared to NC101Δ*gadAB*-monoassociated IL-10$^{-/-}$ mice at 2 weeks but a less pronounced increase at 5 weeks ($P = 0.06$) (Fig. 6A). To confirm these results, we measured the relative levels of the *E. coli* 16S rRNA gene copies on extensively washed cecal tissue from monoassociated IL-10$^{-/-}$ mice using quantitative PCR. We detected significantly more *E. coli* 16S rRNA gene copies on cecal tissue from IL-10$^{-/-}$ mice monoassociated with NC101 than IL-10$^{-/-}$ mice monoassociated with NC101Δ*gadAB* for 2 and 5 weeks (Fig. 6B). Though the difference was not statistically significant, numerically more *E. coli* 16S rRNA gene copies and antibody-stained *E. coli* cells were detected on intestinal tissue from NC101-monoassociated mice than NC101Δ*gadAB*-monoassociated mice at 10 weeks (Fig. 6A and B). These results indicate that expression of *gadAB* in NC101 is associated with increased numbers of colonic epithelial cell surface-associated *E. coli* soon after colonization and are counter to our hypothesis that the attenuated colitis observed in NC101-monoassociated mice compared to that observed in NC101Δ*gadAB*-monoassociated mice correlates with decreased numbers of epithelial cell-associated bacteria.

Based on these unexpected results, we postulated that NC101 cells accumulate at the epithelial surface because of decreased bacterial translocation to the lamina propria and therefore cause less colitis than NC101Δ*gadAB* cells. To test this hypothesis, we infected polarized T84 intestinal epithelial cell monolayers in transwells with NC101 and NC101Δ*gadAB* under translocation-permissive (37°C) and -nonpermissive (4°C) conditions and determined the numbers of epithelial cell-associated and translocated *E. coli* cells using culture plating. Similar to the *in vivo* results, we detected significantly more NC101 bacteria than NC101Δ*gadAB* bacteria associated with T84 cells after infection at the translocation-permissive temperature of 37°C (Fig. 7A). However, there was no significant difference in the numbers of epithelial cell-associated bacteria between the two strains after infection at the translocation-nonpermissive temperature of 4°C (Fig. 7A). In addition, confocal imaging of infected polarized T84 monolayers in transwells at 37°C also showed higher densities of NC101 bacteria than NC101Δ*gadAB* bacteria on the epithelial surface (Fig. 7B). These data indicate that NC101Δ*gadAB* may translocate across epithelial cell monolayers more efficiently than NC101.

To confirm that expression of *gadAB* in NC101 impairs *E. coli* translocation across epithelial cells *in vitro*, we directly measured the numbers of NC101 and NC101Δ*gadAB* bacteria that migrated to the basolateral compartment of polarized T84 cell monolayers at 37°C and found significantly fewer NC101 than NC101Δ*gadAB* bacteria at all time points investigated (Fig. 7C). Complementation of NC101Δ*gadAB* with *gadAB* partially restored the translocation phenotype to that observed for NC101 at 8 and 10 h (see Fig. S4 in the supplemental material). In addition, we detected fewer intracellular bacteria in polarized T84 cells infected with NC101 at 37°C than cells infected with NC101Δ*gadAB* (Fig. 7D). These data suggest that *gadAB* expression reduces the ability of NC101 to enter, survive within, and/or exit intestinal epithelial
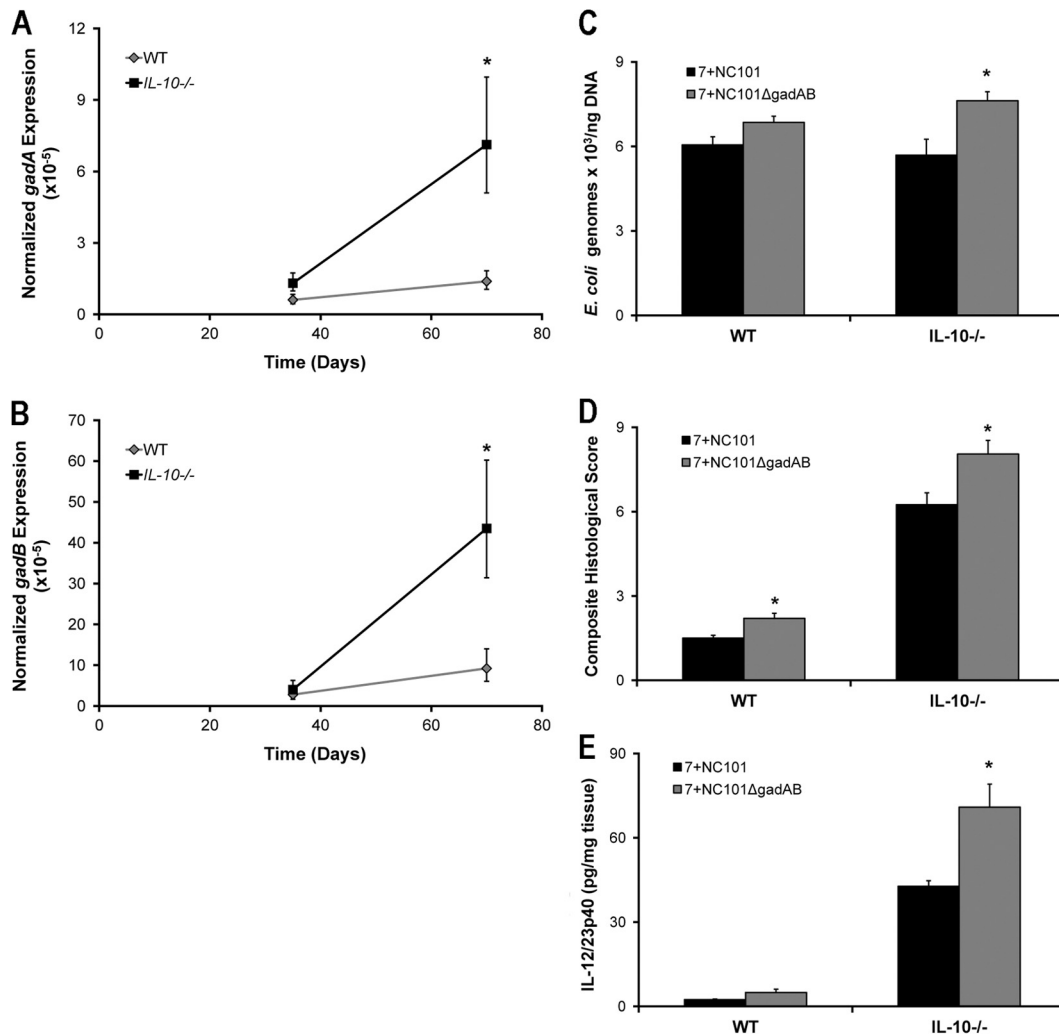
FIG 5 Presence of *E. coli gadAB* is associated with attenuated host proinflammatory immune responses in octoassociated IL-10$^{-/-}$ and WT mice. Expression of *gadA* (A) and *gadB* (B) in cecal *E. coli* from octoassociated mice. (C) Number of *E. coli* genomes per ng cecal content DNA. (D and E) Composite blinded histological inflammation scores of colonic segments (D) and spontaneous secretion of IL-12/23p40 in culture supernatants from proximal colon explants (E) of WT and IL-10$^{-/-}$ mice octoassociated for 5 weeks with 7 bacteria and either NC101 or NC101Δ*gadAB*. Data are presented as the mean ± SEM ($n = 5$ mice per group). *, $P < 0.05$ relative to WT mice (A and B) or relative to octoassociated and NC101-monoassociated mice of the same genetic background (C to E).

cells *in vitro*. Together, the results of these *in vitro* experiments indicate that *E. coli gadAB* expression inhibits translocation across intestinal epithelial cells, resulting in increased numbers of apical epithelial cell-associated bacteria and may explain how these inflammation-induced bacterial genes attenuate colitis *in vivo*.

DISCUSSION

While the importance of the commensal enteric microbiota, including *E. coli*, in the development of IBDs and experimental colitis has previously been established, the mechanisms by which the host inflammatory responses influence the intestinal bacteria are still poorly understood. We reported in a previous study that *E. coli* bacterial stress response genes, including *gadAB*, are highly upregulated in IL-10$^{-/-}$ mice with a long-term bacterial mono-association (14). We show here that the increased expression of *gadAB* in luminal *E. coli* NC101 from IL-10$^{-/-}$ mice coincides with advanced histological intestinal inflammation at 10 weeks.

Unexpectedly however, *gadAB* expression is also elevated in *E. coli* isolates from WT mice at early time points, suggesting that factors unrelated to histological inflammation also upregulate *gadAB*. Although the mechanisms involved in the early upregulation of *gadAB* in WT mice remain to be determined, innate immune responses might be suspected due to the early timing. Alternatively, IL-10 produced by intestinal tissue during early phases of bacterial colonization could directly upregulate *E. coli gadAB*. However, addition of recombinant IL-10 to *E. coli* cultures *in vitro* had no effect on *gadAB* expression (data not shown). Whatever the cause, it is tempting to speculate that early expression of *gadAB* in luminal *E. coli* isolates in WT mice may facilitate intestinal homeostasis, since WT mice monoassociated with NC101Δ*gadAB* had increased proinflammatory immune responses at later time points (Fig. 3).

The mechanisms responsible for later inflammation-associated expression of *gadAB* are equally enigmatic. While stationary-phase growth, osmotic shock, norepinephrine, indole, and sodium have all been implicated as potential regulators of *gadAB*
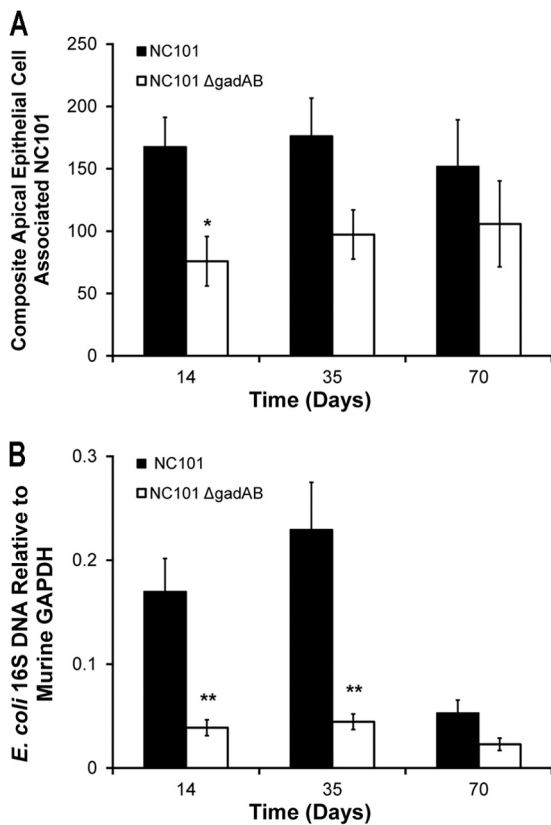
FIG 6 The presence of *E. coli gadAB* is associated with increased numbers of bacteria at the apical surface of intestinal epithelial cells *in vivo*. (A) Numbers of apical epithelial cell-associated bacteria in 18 ×20-magnification fields of intestinal tissue per IL-10$^{-/-}$ mouse monoassociated with NC101 or NC101Δ*gadAB*. (B) Relative numbers of *E. coli* 16S DNA copies in washed cecal tissue from the same IL-10$^{-/-}$ mice used for the experiment whose results are shown in panel A. Results are normalized to the level of tissue GAPDH expression. Data are presented as the mean ± SEM (*n* = 5 mice per group). *, *P* < 0.05 relative to NC101Δ*gadAB*-monoassociated mice; **, *P* < 0.01 relative to NC101Δ*gadAB*-monoassociated mice.

expression (17, 29–31), none of these factors have been reported to be increased in the lumen during colitis. Most of the published data support a role for *gadAB* in acid tolerance (16). While others have reported decreased luminal pH during colitis (18), we found no significant differences in pH or glutamine or glutamate levels in cecal contents from IL-10$^{-/-}$ mice with colitis versus WT controls (data not shown). Therefore, factors other than low pH and glutamate/glutamine levels are likely stimulating expression of *E. coli gadAB* in this model system. Interestingly, others have demonstrated that *gadB* expression is regulated by *oxyS*, a small regulatory RNA which is important in *E. coli* stress responses to oxidative environments, such as the oxidative environment found in the inflamed intestine, and which is also upregulated in luminal *E. coli* isolates from colitic mice (14, 32, 33). Therefore, we speculate that inflammation-associated upregulation of *E. coli gadAB* may be caused by oxidative stress rather than acidic pH.

Contrary to our initial hypothesis, *gadAB* expression decreases survival of *E. coli* NC101 in the inflamed intestine and is associated with reduced innate and adaptive immune responses and attenuated colitis. We provide *in vitro* evidence that one reason for the decreased luminal concentrations of *gadAB*-expressing *E. coli* may

be an increased susceptibility to killing by the mouse AMP cryptdin-4. How *gadAB* expression enhances the susceptibility of *E. coli* to AMP killing and whether this phenomenon occurs *in vivo* remain to be determined. However, since *gadAB* helps maintain a normal proton motive force across the bacterial inner membrane, which may be necessary for cryptdin-4-mediated killing (24–26), we propose that the proton-exporting function of the GadAB system mediates this effect.

Due to the intestinal epithelial barrier that separates potentially proinflammatory microbes from the mucosal immune system, luminal bacterial concentrations do not necessarily correlate with colitis. Translocation of the commensal microbiota or microbial products across the epithelium is required for host immune activation. We show that *E. coli* GadAB inhibits bacterial translocation in polarized colonic epithelial cell cultures, but the mechanism is unclear. Others have reported that activation of the PhoB regulon is associated with decreased adherence of pathogenic *E. coli* to intestinal epithelial cells (34). Since PhoB also regulates acid stress responses (35), it is conceivable that GadAB-mediated inhibition of *E. coli* translocation is also PhoB dependent. However, regulation of *gadAB* by PhoB has not yet been reported.

We therefore propose a model in which *gadAB* expression in luminal *E. coli* increases susceptibility to killing by antimicrobial peptides and decreases migration across the epithelium, which reduces the density of priming microbes in the lamina propria and diminishes host inflammatory responses.

The reason why *E. coli* would upregulate a gene that ultimately proves to be detrimental to its survival in the colon is still unclear. However, we and others have observed similar findings with different stress response genes (14, 36). It might be more important for commensal *E. coli* to dampen inflammation triggered by any gut microbe rather than to develop mechanisms to overcome host immune responses. Indeed, others have shown that enterohemorrhagic *E. coli* suppresses host inflammatory responses to its own toxin (37). In addition, the paradox could be explained by limitations of our experimental system, since selective colonization does not accurately reflect the natural gut environment, where many other commensal microbes are present. These commensals may not only contribute to the inflammatory responses but also potentially interact with *E. coli* and affect its gene expression. However, we demonstrate that *gadAB* expression has a detrimental effect on *E. coli* survival and its colitogenic potential even in the presence of a more complex microbiota (Fig. 5). Moreover, others have also reported that *E. coli* lacking *gadX* and *gadW* genes that regulate expression of *gadAB* outcompete parental *E. coli* MG1655 in the streptomycin-treated mouse intestine, which harbors a complex microbiota (38). Interestingly, colitis is not associated with significant upregulation of *gadB* in cecal *E. coli* strain LF82 from IL-10$^{-/-}$ mice colonized with a complex defined microbiota relative to its expression in WT controls (data not shown), suggesting that *E. coli* transcriptional responses to inflammation are not shared by all *E. coli* strains (data not shown). Lastly, it is possible that mucosa-associated bacteria that are closer to epithelial and immune cells exhibit gene expression profiles that are different from those of most luminal bacteria. Further studies that dissect the effects of different host cells on bacterial transcription will extend the biological relevance of our findings.

In summary, we have shown that expression of the bacterial stress response genes *gadAB* is differentially regulated in commensal luminal *E. coli* isolates from mice with colitis than healthy
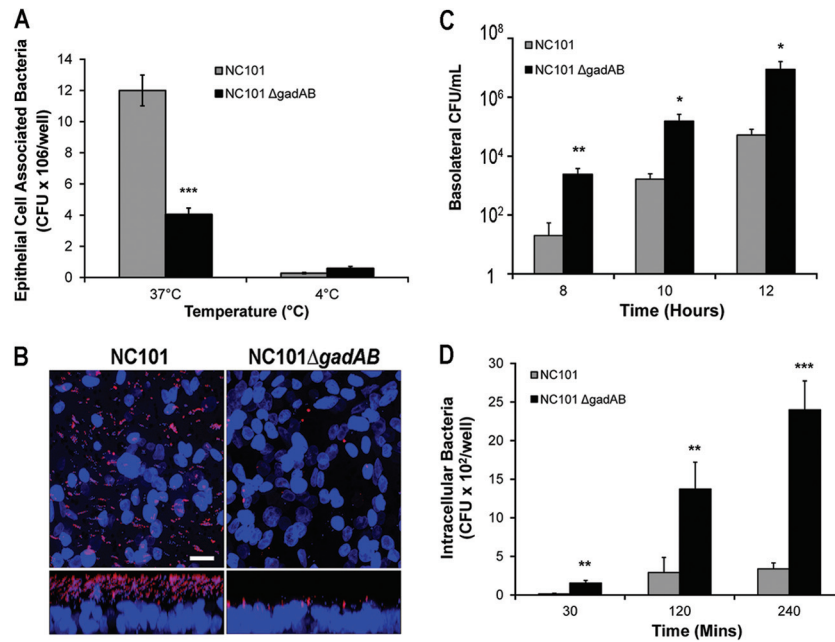
FIG 7 GadAB inhibits translocation of NC101 across intestinal epithelial cells *in vitro*. (A) Numbers of epithelial cell-associated bacteria in T84 cell monolayers infected at the indicated temperatures. (B) Representative confocal images of T84 intestinal epithelial cells infected for 5 h at 37°C and stained with anti-*E. coli* antibody (red) and DAPI (blue). (Top) *x-y* axis; (bottom) *z* axis. Bar = 20 μm. (C) Numbers of bacteria in the basolateral chamber of monolayers at the indicated times postinfection at 37°C. (D) Intracellular numbers of NC101 and NC101Δ*gadAB* bacteria in infected intestinal epithelial cell monolayers at 37°C. Data are presented as the mean ± SEM ($n = 6$ wells per group). *, $P < 0.05$ relative to NC101; **, $P < 0.01$ relative to NC101; ***, $P < 0.001$ relative to NC101.

controls. Furthermore, *gadAB* expression is associated with decreased luminal bacterial survival and reduced host inflammatory responses *in vivo* as well as increased sensitivity to the α-defensin cryptdin-4 and an impaired ability to migrate across intestinal epithelial cells. These studies provide evidence that host intestinal inflammation affects commensal microbial function in a manner that reduces inflammation. An enhanced understanding of the mechanisms by which inflammation controls bacterial gene expression and how inflammation-induced bacterial stress responses impact chronic immune-mediated colitis will improve our knowledge of the pathogenesis of IBDs and could lead to the development of novel therapies that target the host-microbe dialogue.

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