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Murine Guanylate Cyclase C Regulates Colonic Injury and Inflammation

Kris A. Steinbrecher,*† Eleana Harmel-Laws,‡ Monica P. Garin-Laflam,*†1 Elizabeth A. Mann,‡ Lucas D. Bezerra,‡ Simon P. Hogan,*† and Mitchell B. Cohen*†

Guanylate cyclase C (GUCY2C or GC-C) and its ligands, guanylin (GUCA2A or Gn) and uroguanylin (GUCA2B or Ugn), are expressed in intestinal epithelial cells and regulate ion secretion, intestinal barrier function, and epithelial monolayer homeostasis via cGMP-dependent signaling pathways. The aim of this study was to determine whether GC-C and its ligands direct the course of intestinal inflammation. In this article, we show that dextran sodium sulfate (DSS)-induced clinical disease and histological damage to the colonic mucosa were significantly less severe in GC-C−/− mice and moderately reduced in Gn−/− mice. Relative to wild-type controls, GC-C−/− and Gn−/− mice had reduced apoptosis and increased proliferation of intestinal epithelial cells during DSS colitis. Basal and DSS-induced production of resistin-like molecule β (RELMβ) was substantially diminished in GC-C−/− mice. RELMβ is thought to stimulate cytokine production in macrophages in this disease model and, consistent with this, TNF-α and IFN-γ production was minimal in GC-C−/− animals. RELMβ and cytokine levels were similar to wild-type in Gn−/− mice, however. Colonic instillation of recombinant RELMβ by enema into GC-C−/− mice restores sensitivity to DSS-mediated mucosal injury. These findings demonstrate a novel role for GC-C signaling in facilitating mucosal wounding and inflammation, and further suggest that this may be mediated, in part, through control of RELMβ production. The Journal of Immunology, 2011, 186: 7205–7214.

Infection with enterotoxigenic strains of Escherichia coli (ETEC) causes secretory diarrhea. Pathogens such as ETEC that elaborate heat-stable (ST) peptide toxins are among the most important causes of acute diarrhea in infants and travelers (1). In developing countries, young children experience two to three episodes of diarrhea each year caused by infections with ETEC; this represents >25% of all diarrheal illness and results in significant morbidity. In addition to their tremendous burden of acute diarrhea, ETEC infections are associated with growth failure and persistent diarrhea. That diarrhea caused by bacterial ST results from molecular mimicry was shown by the identification of guanylin (Gn) and uroguanylin (Ugn), ST-like peptides present in the mammalian intestine (2, 3). Both peptides are produced and secreted from intestinal epithelial cells (IECs), with Ugn expressed predominantly in the small bowel and Gn in the colon. All three ligands (ST, Gn, and Ugn) bind the transmembrane receptor guanylate cyclase C (GC-C), which in the intestine is expressed only on IECs but can be found at low levels in extra-intestinal tissues such as the brain and kidney (4). Ligand-induced activation of GC-C increases intracellular cGMP and, via cGMP-dependent protein kinase II, leads to opening of cystic fibrosis transmembrane conductance regulator (CFTR) and inhibition of Na+/H+ exchanger 3 (NHE3, SLC9a3) (5–7). Overproduction of cGMP by ST stimulation results in the hypersecretion of electrolytes and water (8). Gn and Ugn, however, are less potent activators of GC-C than is ST, and their presence does not result in secretory diarrhea (2, 3). GC-C and its ligands may be important in systemic salt balance, hydration of the intestinal lumen, regulation of cell cycle, and small-bowel barrier function (9–11). However, it remains unclear as to what critical physiological function is supplied by GC-C that counterbalances the well-defined role this receptor plays in susceptibility to infectious diarrheal disease.

Although the mechanism is poorly understood, it is apparent that transmembrane guanylate cyclase (GC) receptors and their peptide ligands regulate inflammation. For example, although GC-A signaling plays an important role in fluid regulation and coronary heart disease, it is also critical for controlling inflammation. Deletion of GC-A results in cardiac hypertrophy and is associated with increased proinflammatory cytokine expression (12). Treatment with atrial natriuretic peptide, a GC-A activating ligand, diminishes TNF-α, IL-1β, and inducible nitric oxide synthase activity in hepatocytes and macrophages (13–16). Conversely, within the specific context of ischemia/reperfusion injury, the presence of GC-A seems to drive tissue damage and inflammation (17). It is evident that the role of GC/cGMP signaling in regulation of inflammation and tissue injury is not fully understood.

There is intriguing evidence that expression of GC-C and its ligands may impact the pathogenesis of intestinal inflammation. Microarray analysis shows that Gn and Ugn are downregulated in inflammatory bowel disease (18). In the Citrobacter rodentium
murine model of infectious colitis, Gn and Ugn expression is depressed early during the course of infection (19). Notably, recent work indicates that CFTR and NHE3, end points in the GC-C/GMP signaling cascade, are critically important in regulation of mucosal innate immunity (20–24). CFTR activity is required for suppression of cytokine-stimulated proinflammatory signaling cascades in vitro (25, 26). Mice that lack CFTR or NHE3 overproduce proinflammatory cytokines and chemokines in the colon and are prone to intestinal inflammation. Despite evidence to suggest the loss of GC-C ligands in inflammatory disease and the clear impact that intestinal electrolyte movement has on mucosal immunity, there has been no direct analysis of whether epithelial receptor GC/cGMP signaling is important for the pathogenesis or progression of intestinal inflammation. Our previous work indicates that genetic deletion of GC-C or its ligands in mice results in a significant decrease in steady-state cGMP levels in IECs, making these animals ideal models with which to address this question (9, 27, 28). In this article, we show that mice deficient in GC-C or Gn have a striking resistance to chemically induced colonic inflammation and demonstrate that epithelial GC signaling regulates mucosal immune homeostasis in the intestine.

Materials and Methods

Mice

All animal studies were approved by the Cincinnati Children’s Hospital Medical Center Institutional Animal Care and Use Committee. Mice with gene-ablated GC-C (Gucya2; GC 2c; Gene ID: 14917) or Gn (Gucya2a, GC activator 2a; Gene ID: 14915) have been described previously (27, 28). GC-C+/− and Gn−/− mice were bred into the C57BL/6J background for >10 generations and were housed under specific pathogen-free conditions.

Analysis of dextran sodium sulfate-induced colonic injury

The dextran sodium sulfate (DSS) model of colonic wounding was performed as previously detailed (29, 30). In brief, 3% DSS (m.w. 36,000–50,000; MP Biomedical) was provided to 8–12 wk-old male mice for 5 d in studies termed “acute,” whereas 3% DSS for 5 d followed by water for 6 d constituted the “recovery” protocol. Scoring of histological damage was performed with the observer blind to sample genotype, as previously described (29, 30). Immediately on sacrifice of each animal, distal colons were placed in “swiss roll” fashion in cassettes for paraffin embedding, or subdivided and flash frozen in OCT material for immunofluorescence (IF) staining. In some studies, colonic tissue was frozen for protein extraction or biopsies were taken for cytokine ELISA analysis. Disease activity index included a summation of three components: weight loss (0 = 0%, 1 = 1–5%, 2 = 6–10%, 3 = 11–15%, 4 = 16–20%, 5 = >20%), diarrhea (0 = normal stool, 1 = soft stool and minimal wet anal fur/tail, 2 = diarrhea and moderate-to-severe wet anal fur/tail), and frank rectal bleeding (0 = absent, 1 = present but minimal, 2 = moderate/severe).

Histology, immunohistochemistry, and IF

H&E and Alcian blue staining, IHC, and IF were all performed as previously described (29–31). IHC and IF were performed on formalin-fixed, 5-μm paraffin sections or OCT frozen sections, respectively. Animals injected with BrdU (Invitrogen) before sacrifice were used solely for IEC proliferation analysis. Abs were supplied by the following: cleaved caspase 3 (CC3; #9661; Cell Signaling Technology), resistin-like molecule β (Relmβ; #500-P215; PeproTech), and β-tubulin (Ab4; Thermo Fisher Scientific). As previously described, analysis of distal colon IEC proliferation and apoptosis in acute or recovery DSS studies was performed either by counting positive epithelial cells within 8–12 micrograph fields (original magnification ×200) per mouse or by counting positive epithelial cells per well-oriented crypt (28–30).

Real-time RT-PCR and immunoblotting

Total RNA extraction, DNase treatment, cDNA preparation, and real-time RT-PCR analysis were performed as described previously (29, 30). Primer sequences are available upon request. GC-C and Gn Abs were produced as indicated previously (27, 32). Relmβ and β-tubulin Abs were provided by PeproTech and Santa Cruz, respectively.

ELISA of organ culture supernatant

Quantification of cytokines in organ culture supernatant was performed as described with minor modifications (29, 30). Multiple biopsy punches (3 mm) were taken from distal colon of untreated or DSS-treated animals and cultured separately overnight in 400 μl organ culture media (DMEM 10% FBS, penicillin/streptomycin [#15140-122; Invitrogen], and Primocin (50 mg/ml; #ant-pm; Invivogen)). Supernatant for each animal was pooled, aliquoted, and snap frozen with liquid nitrogen until analysis. ELISA was performed according to the manufacturers’ recommendations (eBioscience, R&D Systems, and PeproTech).

Rectal RELMβ instillation

Once-daily enemas were used to supplement RELMβ levels in wild-type (WT) and GC-C−/− mice during DSS-induced colitis. Using an approach modified from previous reports (33, 34), acute DSS studies were performed as described earlier (3% DSS for 5 d) except that daily enemas were performed on study days 1–4 using recombinant RELMβ (PeproTech; 400 ng RELMβ in 200 μl saline per mouse). Study groups included those receiving active or heat-inactivated (90°C for 10 min) RELMβ. Enemas were performed with a 25-gauge catheter such that liquid was placed 2.5 cm proximal to the anal verge. Mice were anesthetized with ketamine/xylazine during the procedure.

Statistics

Unless otherwise stated, data were presented as mean with SEM and were considered significant at p ≤ 0.05. Statistical analysis was performed using the Mann–Whitney U test.

Results

Mice that lack GC-C, or its ligand Gn, are resistant to DSS-induced colonic injury

Wounding of the distal colon by DSS is initiated by direct IEC monolayer ulceration and entry of luminal Ags into the mucosa. Mice that lack GC-C were provided DSS in drinking water in studies termed acute (3% DSS for 5 d) or recovery (3% DSS for 5 d followed by 6 d of water). Although this dose of DSS caused only minimal weight loss in all mice in the 5-d acute protocol, weight loss was more profound during the recovery phase. GC-C+/− mice lost significantly less weight than WT controls (Fig. 1A). During these studies, GC-C−/− mice also had a substantially diminished disease activity index (weight change, rectal bleeding, stool consistency; Fig. 1B). Colonic atrophy is an expected response to wounding by DSS and was noted in WT animals, but it occurred to a much lesser degree in GC-C−/− mice in both acute and recovery studies (Fig. 1C). Improved clinical disease parameters suggested that loss of GC-C may provide resistance to this model of intestinal wound-induced inflammation.

Analysis of histology in both acute and recovery studies confirmed that GC-C facilitates DSS-induced mucosal injury. After 5 d of DSS, WT mice had apparent mucosal damage characterized by loss of crypt epithelia, robust inflammatory cell infiltrate, and ulceration of the IEC monolayer, all parameters that affected GC-C−/− mice to a limited extent (Fig. 2A). Histopathology scoring confirmed that DSS-mediated acute injury is strongly attenuated in the absence of GC-C (Fig. 2B). In recovery studies, WT mice responded with widespread epithelial hypertrophy and continued to have a significant submucosal inflammatory cell presence. GC-C−/− mice remained highly resistant to DSS-induced inflammation, possibly as a result of milder initial injury and/or enhanced epithelial restitution (Fig. 2C, 2D).

Gn is the primary colonic ligand that mediates GC-C–dependent cGMP production in IECs (28). Acute DSS studies were performed with Gn−/− mice to determine whether ligand-induced activation of GC-C mediates DSS injury. Although acute exposure to DSS caused similar shortening of the colon in mice lacking Gn as compared with WT (K. Steinbrecher and E. Harmel-Laws, unpublished observations), histological damage was significantly reduced in Gn−/− mice.
compared with control mice.

Dependent on epithelial monolayer ulceration for pathogenesis. Inflammatory disease in experimental colitis models that are demonstrated to be because of the presence of low levels of Ugn in the colon that may manifest from an IEC monolayer prone to resist cell death. Although numerous CC3+ epithelial cells were evident in WT animals after 5 d of DSS drinking water, GC-C−/− mice had obviously fewer apoptotic cells (Fig. 3A). Quantification of CC3+ epithelial cells per high-power microscope field in untreated mice indicated that there was no significant difference in basal levels of cell death between the genotypes (Fig. 3B). As expected, the number of CC3+ IECs per field was greatly enhanced in WT mice after 5 d of DSS and, to a lesser extent, after an additional 6 d of recovery (Fig. 3B). In contrast, however, GC-C−/− mice were highly resistant to epithelial cell death. Although the increase in CC3 staining in GC-C−/− mice was significant relative to basal conditions, the level of IEC apoptosis was substantially less than that seen in DSS-treated WT controls (Fig. 3B) and is consistent with diminished histopathology in these mice.

To measure corresponding changes in proliferation in distal colon of WT and GC-C null mice, we used IHC to stain cells that had incorporated BrdU. Tissue staining clearly indicated that GC-C−/− mice had multiple BrdU-labeled cells within each crypt, but that WT mice had noticeably fewer (Fig. 3C). Quantification of BrdU-stained cells in distal colon revealed that deletion of GC-C had no effect on basal IEC proliferation relative to WT (Fig. 3D). However, in response to acute exposure to DSS, a time point when GC-C−/− mice display less IEC death, significantly more cell division was present in the GC-C−/− IEC monolayer as compared with DSS-treated WT (Fig. 3D). Recovery from DSS-induced wounding resulted in the expected hyperplastic response in WT animals, but because there was initially less DSS-induced injury, proliferation in GC-C−/− mice was significantly less than in recovering WT animals and had returned to levels similar to that of untreated GC-C−/− mice (Fig. 3D). These data suggest a strong resistance to injury in the distal colon of mice that lack GC-C that may manifest from an IEC monolayer prone to resist cell death and maintain proliferative self-renewal.

We next determined the impact of DSS injury on IEC proliferative and apoptotic homeostasis in Gn−/− mice. During acute exposure to DSS, staining for CC3 indicated significantly reduced IEC apoptosis in Gn−/− colon (Fig. 3E, 3F). We used Ki-67 staining to identify proliferating cells and found that Gn−/− mice retained highly proliferative IECs during acute DSS injury (Fig. 3G, 3H). As in GC-C−/− mice, reduced IEC death and sustained cell division in Gn−/− mice in the presence of acute DSS inflammation is consistent with the strong resistance to epithelial monolayer damage and loss of crypt IECs noted during histological analysis of these mice (Fig. 2E, 2F).

Robust production of RELMβ in colonic goblet cells requires GC-C activity

Multiple factors mediate the sensitivity of the colon to DSS-induced injury. Of proven importance is the colonic goblet cell

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**FIGURE 1.** Deletion of GC-C reduced clinical disease parameters during colonic injury. A, Mice were given 3% DSS for 5 d and water for an additional 6 d. Weight loss was significantly less in GC-C−/− mice as compared with WT animals. $n = 28–32$ mice/group; *$p < 0.05$). B, Disease activity index (DAI), defined as weight loss, rectal bleeding, and stool consistency, was increased to a lesser degree in GC-C−/− animals as compared with control mice. $n = 9–11$ mice/group. *$p < 0.05$. C, Colon length was measured after both acute (5 d 3% DSS) and recovery (5 d 3% DSS and 6 d water) studies, and showed that mice that lacked GC-C experienced much less tissue atrophy as compared with WT mice. $n = 5–9$ mice/group. *$p < 0.03$.GC-C and Gn facilitate apoptosis and suppress proliferation during DSS-induced colonic injury

Clinical and histological measurements indicated that GC-C was instrumental in facilitating IEC monolayer ulceration and crypt cell loss during DSS treatment. We and others have reported that GC-C and its ligands are important for IEC proliferative/apoptotic homeostasis and susceptibility to some forms of damage-induced cell death (10, 37). Because the degree of epithelial cell apoptosis and cell division is a critical determinant of the severity of DSS-induced monolayer wounding and recovery, we next determined the response of the epithelia to DSS exposure in GC-C WT and null mice. Immunofluorescent staining of CC3 was used as a marker of apoptosis and indicated that, in the distal colon of WT and GC-C−/− mice, there were obvious and striking differences in cell death. Although numerous CC3+ epithelial cells were evident in WT animals after 5 d of DSS drinking water, GC-C−/− mice had obviously fewer apoptotic cells (Fig. 3A). Quantification of CC3+ epithelial cells per high-power microscope field in untreated mice indicated that there was no significant difference in basal levels of cell death between the genotypes (Fig. 3B). As expected, the number of CC3+ IECs per field was greatly enhanced in WT mice after 5 d of DSS and, to a lesser extent, after an additional 6 d of recovery (Fig. 3B). In contrast, however, GC-C−/− mice were highly resistant to epithelial cell death. Although the increase in CC3 staining in GC-C−/− mice was significant relative to basal conditions, the level of IEC apoptosis was substantially less than that seen in DSS-treated WT controls (Fig. 3B) and is consistent with diminished histopathology in these mice.
lineage, which produces a number of secreted proteins that influence initial injury, as well as mucosal healing, in this model of intestinal inflammation. For example, genetic deletion of the goblet cell proteins Muc2 or TFF3 result in highly increased sensitivity to DSS-induced injury and inflammation (38–40). Of note, mice with a significant reduction in intestinal goblet cells produce only slightly lower levels of mucin but are strongly protected from DSS injury (41). This may be mediated by a decrease in the goblet cell protein RELMβ. Similar to Gn and Ugn, RELMβ is predominantly expressed in goblet cells and secreted into the intestinal lumen (32, 33, 42). During DSS-induced inflammation, RELMβ−/− mice have diminished clinical and histological signs of disease, reduced TNF-α expression, and diminished inflammatory cell infiltrate in the colon (33, 43). Based on the phenotypic overlap between mice lacking GC-C or Gn and those deficient in RELMβ, we next determined whether RELMβ production was altered in these mice. Real-time RT-PCR analysis indicated that basal RELMβ expression, although highly variable, was diminished in the distal colon of GC-C−/− mice relative to WT controls (GC-C+/+ 2.2 ± 1.1 versus GC-C−/− 0.5 ± 0.1; p = 0.07; n = 7–8/group). RELMβ is highly induced during intestinal inflammation, such as that caused by DSS (33, 44). Immunoblot analysis readily identified RELMβ in WT animals after an acute 5-d DSS treatment, but GC-C−/− mice produced very little (Fig. 4A). Quantification of multiple blots indicated that RELMβ production is diminished in the GC-C−/− colon by ~75% (Fig. 4B). Similarly, IHC of distal colon from DSS-treated WT and GC-C−/− mice indicated very little RELMβ production in the absence of

**FIGURE 2.** GC-C−/− and Gn−/− mice were resistant to DSS-mediated colonic wounding. A and B, H&E staining of distal colon from mice treated acutely for 5 d with 3% DSS indicated that GC-C−/− mice have less severe histopathology. Disease scoring showed that GC-C deficiency provided significant protection from acute DSS exposure, especially with respect to edema and ulceration of the IEC monolayer. n = 14–15 mice/group. *p < 0.009. C and D, Histological analysis suggested that on day 11 of recovery from DSS injury, extensive epithelial hyperplasia and inflammatory cell infiltrate was still present in WT but not GC-C−/− mice. Quantification of disease parameters indicated that deletion of GC-C resulted in less persistent injury as compared with WT mice. n = 24–28 mice/group. *p < 0.003. E and F, Retention of crypt-surface epithelial structure is moderately better in Gn−/− mice versus WT animals after acute DSS-mediated injury. Acute DSS colitis in Gn−/− mice is characterized by reduced edema, ulceration, and crypt loss. n = 12–14 mice/group. *p < 0.05. Scale bars, 100 μm.
GC-C (Fig. 4C). These studies indicate that the robust increase in RELMβ that occurs during intestinal injury-induced inflammation requires GC-C.

To determine whether the primary colonic ligand for GC-C, Gn, provided sufficient GC-C activity for efficient RELMβ production, we assessed RELMβ levels in distal colon of Gn−/− mice. Acute DSS injury resulted in highly variable induction of RELMβ in Gn−/− mice as measured by immunoblot analysis, and quantification of multiple blots suggested that, although levels trended lower, there was no significant decrease in RELMβ in these animals (Fig. 4D, 4E). Similarly, by IHC it was evident that RELMβ levels were only slightly blunted (Fig. 4F) and showed a stark
contrast from the profound reduction noted in GC-C\(^{-/-}\) mice. This suggested that partial activity of GC-C is retained in the distal colon of Gn\(^{-/-}\) mice such that RELM\(\beta\) production is nearly that of WT mice, and that multiple pathways likely influence the resistance of GC-C\(^{-/-}\) and Gn\(^{-/-}\) mice to DSS-mediated inflammation.

IHC of RELM\(\beta\) suggested that the drastic reduction of RELM\(\beta\) in GC-C\(^{-/-}\) mice was not due to a profound loss of goblet cells. To confirm this, we chose to quantitate goblet cells on a per crypt basis to determine whether GC-C in the distal colon affects differentiation of this cell type. Alcian blue-stained goblet cells were quantitated per well-oriented crypt of the distal colon and found to be similar in number in WT and GC-C\(^{-/-}\) mice under resting conditions (Fig. 5A,5B). Furthermore, goblet cells were decreased during acute DSS injury in a manner that was not genotype dependent (Fig. 5A, 5B). Although the histopathology in GC-C\(^{-/-}\) mice is not as severe as that of control mice, the inflammation that does occur in these animals is evidently enough to reduce the number of goblet cells produced per crypt to a level similar to WT. Collectively, these studies indicated that the phenotypic overlap between GC-C\(^{-/-}\) and RELM\(\beta\)^{-/-} mice in response to DSS-induced injury may be, in part, caused by the GC-C–dependent nature of RELM\(\beta\) expression.

**GC-C regulates mucosal TNF-α and IFN-γ production during colonic injury**

Efficient mucosal restitution is highly dependent on the local cytokine profile, as this directly affects the rate of epithelial homeostasis and migration, as well as the number and composition of infiltrating immune cell types. The minimal production of RELM\(\beta\) in mice that lack GC-C suggested that, as is the case in RELM\(\beta\)^{-/-} animals, proinflammatory cytokine expression may be diminished (33, 43). Accordingly, we next used organ culture and ELISA to measure the production of several cytokines and chemokines that are key to pathogenesis in the DSS injury model. After acute exposure to DSS, mice that lack GC-C produced far less TNF-α (Fig. 6A) and IFN-γ (Fig. 6B) than did WT animals. Analysis of IL-12/23p40 also indicated that GC-C\(^{-/-}\) mice produced significantly less than controls (WT 42.4 ± 9.2 versus GC-C\(^{-/-}\) 14.2 ± 4.0 pg/FIGURE 4. RELM\(\beta\) was diminished in GC-C\(^{-/-}\), but not Gn\(^{-/-}\), mice after acute colonic injury by DSS. A. After 5 d of 3% DSS, RELM\(\beta\) protein production in GC-C\(^{-/-}\) distal colon is depressed relative to WT mice. B. Quantification of multiple Western blots by densitometry indicated that RELM\(\beta\) is produced in the GC-C\(^{-/-}\) colon at ∼25% that of WT animals after DSS injury. n = 12 mice/group. *p = 0.0035. C, IHC of mice treated acutely with DSS clearly shows the minimal amount of RELM\(\beta\) staining present in GC-C\(^{-/-}\) distal colon, whereas abundant staining is present in WT mice. D. Acute DSS colitis induced RELM\(\beta\) expression in Gn\(^{-/-}\) mice to a degree similar to WT controls. E. Quantification of multiple Western blots indicated that no significant difference in RELM\(\beta\) levels in the distal colon of WT and Gn\(^{-/-}\) mice. n = 12–16 mice/group. F, IHC of Gn\(^{-/-}\) distal colon showed that RELM\(\beta\) staining, although mildly reduced, was still present. Scale bars, 100 μm.

efficiency during DSS induction (34, 35).
Deletion of GC-C resulted in a differential cytokine response during acute DSS-induced injury. A and B, ELISA of organ culture supernatants indicated that GC-C<sup>−/−</sup> mice produced significantly less TNF-α and IFN-γ after acute DSS injury as compared with WT animals. Basal: n = 3–4 mice/group; acute DSS: n = 10–11 mice/group. *p < 0.05 versus basal GC-C<sup>+/+</sup>. C–G, A survey of cytokines and chemokines relevant to DSS-induced disease showed similar levels in WT and GC-C<sup>−/−</sup> mice during mucosal injury. Basal: n = 3–4 mice/group; acute DSS: n = 14–17 mice/group. *p < 0.05 versus basal GC-C<sup>+/+</sup>; †p < 0.05 versus basal GC-C<sup>−/−</sup>.

Unlike GC-C<sup>−/−</sup> mice, the absence of Gn provided only partial protection from DSS colitis. Accordingly, we speculated that mucosal cytokine levels would not be substantially different during acute DSS challenge in Gn<sup>−/−</sup> animals versus WT controls. ELISA-based analysis of organ culture supernatant indicated that there were no significant changes in any measured cytokine/chemokine (Fig. 7A–D). This is consistent with both the retention of RELMβ inducibility and substantial inflammatory cell infiltrate in GN<sup>−/−</sup> mice at levels similar to that noted in WT controls (Figs. 2F, 4D). These data underscore the likelihood that GC-C signaling regulates several independent mechanisms that provide protection from mucosal damage.

Having established that complete loss of GC-C activity (GC-C<sup>−/−</sup> mice) provided resistance to chemical-induced colonic injury that was associated with minimal production of RELMβ, we next determined whether the introduction of recombinant RELMβ into the colon of GC-C<sup>−/−</sup> mice would sensitize these mice to injury. Acute DSS studies were performed in which WT and GC-C<sup>−/−</sup> mice were given once-daily saline enemas that contained either active or heat-inactivated RELMβ protein. After 5 d of 3% DSS, we found the expected colonic shortening in WT mice that received inactive RELMβ, as well as less atrophy in similarly treated GC-C<sup>−/−</sup> mice (Fig. 8A). Colon length was further decreased in WT animals that received active RELMβ, suggesting that RELMβ supplementation enhanced disease in these animals. Importantly, enemas with active RELMβ in GC-C<sup>−/−</sup> mice resulted in colon shortening similar to that seen in control mice (Fig. 8A). Histological analysis revealed that GC-C<sup>−/−</sup> mice that received enemas with active RELMβ had more mucosal damage and inflammatory cell infiltrate than GC-C<sup>−/−</sup> mice that were dosed with inactive peptide (Fig. 8B). Composite histopathology disease scores indicated that, although GC-C<sup>−/−</sup> mice given enemas with inactive RELMβ had significantly lower disease scores as compared with WT mice, the presence of active RELMβ partially removed the resistance of these mice to DSS-induced injury (Fig. 8C). It was notable, however, that some level of protection was still present in GC-C<sup>−/−</sup> mice in that mucosal damage was less than that seen in WT mice given active RELMβ. These observations indicated that the resistance to DSS-induced
intestinal inflammation in GC-C−/− mice was due, in part, to poor induction of RELMβ.

Discussion
Transmembrane receptor GCs and cGMP signaling are understood to directly regulate tissue injury and inflammation in the cardiovascular, pulmonary, and renal systems (48). This report extends our understanding of GC/cGMP signaling to include a role in regulation of colonic wounding and mucosal immunity, and indicates that this is achieved through cGMP-regulated signaling pathways specific to the epithelial cell monolayer. We show that deletion of GC-C, and to a lesser degree Gn, has a dramatic impact on the course of injury-induced inflammation in the colon. Significantly less IEC apoptosis coupled with sustained proliferation in GC-C−/− and Gn−/− distal colon relative to WT animals may be an important aspect of disease resistance in these mice. Production of RELMβ, a goblet cell protein that is critical for inducing TNF-α expression in macrophages during DSS injury (33), is dependent on the presence of GC-C but is unaffected by deletion of Gn. Consistent with this, reduced RELMβ levels are coincident with diminished elaboration of TNF-α in the colonic mucosa of GC-C−/− mice. Restoration of RELMβ in the GC-C−/− distal colon lumen partially abolishes resistance to DSS injury. Collectively, this work establishes GC-C signaling in the IEC monolayer as an important regulator of the mucosal injury response and further suggests that the intracellular pathway(s) that affect this process may be sensitive to differential levels of GC-C activity.

Mice that lack Gn are only moderately protected from DSS-induced injury and inflammation. Similar to GC-C−/− mice, Gn−/− animals responded to the acute DSS protocol with significantly less IEC apoptosis and increased epithelial cell proliferation. This was evident in histology scoring, which indicated a strong retention of crypts and surface epithelia in Gn−/− mice. However, our analysis indicated that in Gn−/− mice, RELMβ levels, the degree of inflammatory infiltrate, and mucosal cytokine production were similar to control animals. Our previous work suggests that the overlapping proximal-to-distal expression pattern of GC-C ligands has important physiological implications (9, 28). Although Gn is the primary colonic GC-C ligand, Ugn is present in the colon at low levels. Deletion of GC-C diminishes colonic mucosal cGMP levels to a greater degree than loss of either ligand and suggests that each ligand can only partially compensate for loss of the other. Ugn produced in the colon of Gn−/− mice may activate GC-C at a minimal level and drive moderate resistance to DSS colitis. However, at this time, we are not able to completely exclude another mechanism of GC-C activation (i.e., an unidentified peptide ligand or ligand-independent GC-C functions).

Unfortunately, we are unable to directly address this by breeding Gn/Ugn double null mice because of the close proximity of these genes on mouse chromosome 4 (49).

Work in several experimental colitis models indicates that RELMβ is an important modulator of intestinal inflammation. In addition to inducing TNF-α production in the context of DSS-mediated injury, RELMβ is required for TNF-α and IFN-γ production during parasite-associated intestinal inflammation, and its levels increase substantially as inflammation occurs in the SAMP1/YitFc model of murine ileitis (33, 43, 50, 51). RELMβ is reduced in GC-C−/− colon under basal conditions, and these animals fail to increase RELMβ production during DSS-induced injury. As in RELMβ−/− mice, DSS colitis elicits minimal TNF-α production and reduced inflammatory infiltrate in GC-C−/− animals. Gn−/− mice, however, expressed similar levels of RELMβ during colonic injury and did not present with reduced cytokine expression, suggesting RELMβ-dependent and -independent mechanisms through which the GC-C signaling cascade regulates mucosal damage. Expression of some cytokines and neutrophil/macrophage chemokines in GC-C−/− colonic mucosa is similar to that of WT animals. Differential cytokine/chemokine expression such as this is not without precedent. For example, reduced disease in DSS-treated fibroblast mutant mice is shaped by minimal expression of IL-6 and IL-1β, but not TNF-α and IFN-γ (29). Importantly, IL-6, as well as chemokine-mediated recruitment of prorestitution neutrophils and macrophages, may be an important aspect of apoptosis resistance in IECs and effective mucosal wound healing (52–56). Further work is necessary to determine the importance of RELMβ-directed cytokine production in intestinal inflammation in GC-C−/− mice, as well as the manner in which GC-C/cGMP control RELMβ production.

This study indicates that cGMP-dependent pathways in the IEC monolayer sensitize the colon to chemical-induced damage and ulceration. This work also shows that regulation of the goblet cell protein RELMβ by GC/cGMP may be instrumental in inflammatory cell infiltration and cytokine expression. However, although RELMβ−/− mice are resistant to the innate immune cell-driven disease of the DSS model, loss of RELMβ increases...
susceptibility to hapten-induced T cell colitis (43). In addition, the severity of T cell colitis is reduced by treatment of WT mice with recombinant RELMβ (34). This suggests that GC-C+ mice may also have a differential sensitivity to DSS versus T cell-mediated colitis models. Consistent with this notion are murine studies suggesting that small-bowel barrier defects similar to that which we have found in GC-C−/− mice can exacerbate spontaneous inflammation in the large intestine (11, 57). Therefore, be it through an RELMβ-dependent mechanism or through preservation of small-intestinal barrier function, we suggest that epithelial GC/GM signaling may have differential effects on the progression of intestinal inflammation that are dependent on disease model. We are currently focused on studies that will address the role of GC-C in spontaneous T cell colitis with the expectation that these studies will demonstrate that epithelial cGMP signaling is necessary for suppression of this form of intestinal inflammation. This is especially timely because clinical trials to evaluate the therapeutic activation of the GC-C signaling pathway are under way (58, 59). Efforts to clarify the function of GC-C in intestinal injury and inflammation are critical to the development of the GC-C signaling pathway as a pharmacological target.

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