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Laila D. McVay, … , Angela Haczku, Gary D. Wu

Research Article   **Immunology**

Although inflammatory bowel disease (IBD) is the result of a dysregulated immune response to commensal gut bacteria in genetically predisposed individuals, the mechanism(s) by which bacteria lead to the development of IBD are unknown. Interestingly, deletion of intestinal goblet cells protects against intestinal injury, suggesting that this epithelial cell lineage may produce molecules that exacerbate IBD. We previously reported that resistin-like molecule β (RELMβ; also known as FIZZ2) is an intestinal goblet cell–specific protein that is induced upon bacterial colonization whereupon it is expressed in the ileum and colon, regions of the gut most often involved in IBD. Herein, we show that disruption of this gene reduces the severity of colitis in the dextran sodium sulfate (DSS) model of murine colonic injury. Although RELMβ does not alter colonic epithelial proliferation or barrier function, we show that recombinant protein activates macrophages to produce TNF-α both in vitro and in vivo. RELMβ expression is also strongly induced in the terminal ileum of the SAMP1/Fc model of IBD. These results suggest a model whereby the loss of epithelial barrier function by DSS results in the activation of the innate mucosal response by RELMβ located in the lumen, supporting the hypothesis that this protein is a link among goblet cells, commensal bacteria, and the pathogenesis of IBD.

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Absence of bacterially induced RELMβ reduces injury in the dextran sodium sulfate model of colitis

Laila D. McVay,[1] Sue A. Keilbaugh,[1] Tracie M.H. Wong,[2] Sonja Kierstein,[3] Marcus E. Shin,[1] Michael Lehrke,[4] Martina I. Lefterova,[4] D. Edward Shifflett,[5] Sean L. Barnes,[6] Fabio Cominelli,[6] Steven M. Cohn,[6] Gail Hecht,[5] Mitchell A. Lazar,[4] Angela Haczku,[3] and Gary D. Wu[1]

[1]Division of Gastroenterology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA. [2]Division of Gastroenterology, Hepatology and Nutrition, The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania, USA. [3]Division of Pulmonary Allergy and Critical Care and [4]Division of Endocrinology, Diabetes, and Metabolism, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA. [5]Department of Medicine, Section of Digestive Diseases and Nutrition, University of Illinois at Chicago, Chicago, Illinois, USA. [6]Digestive Health Center of Excellence, University of Virginia Health Sciences Center, Charlottesville, Virginia, USA.

Although inflammatory bowel disease (IBD) is the result of a dysregulated immune response to commensal gut bacteria in genetically predisposed individuals, the mechanism(s) by which bacteria lead to the development of IBD are unknown. Interestingly, deletion of intestinal goblet cells protects against intestinal injury, suggesting that this epithelial cell lineage may produce molecules that exacerbate IBD. We previously reported that resistin-like molecule β (RELMβ; also known as FIZZ2) is an intestinal goblet cell–specific protein that is induced upon bacterial colonization whereupon it is expressed in the ileum and colon, regions of the gut most often involved in IBD. Herein, we show that disruption of this gene reduces the severity of colitis in the dextran sodium sulfate (DSS) model of murine colonic injury. Although RELMβ does not alter colonic epithelial proliferation or barrier function, we show that recombinant protein activates macrophages to produce TNF-α both in vitro and in vivo. RELMβ expression is also strongly induced in the terminal ileum of the SAMP1/Fc model of IBD. These results suggest a model whereby the loss of epithelial barrier function by DSS results in the activation of the innate mucosal response by RELMβ located in the lumen, supporting the hypothesis that this protein is a link among goblet cells, commensal bacteria, and the pathogenesis of IBD.

Introduction

Members of the RELM/FIZZ protein family, which includes resistin, and RELMα, -β, and -γ, are characterized by a signature carboxyl terminus sequence containing 10 completely conserved cysteine residues with invariant spacing (1). Each family member has a distinct pattern of tissue expression. Although they are best characterized as regulators of glucose homeostasis (2), more recent studies have suggested that one member of this protein family, resistin, may have proinflammatory properties. Recombinant resistin can activate proinflammatory cytokine expression in peripheral blood mononuclear cells (3), activate macrophages in vitro (4), and induce arthritis when injected into mice (3). Furthermore, serum levels of resistin are elevated in patients with both ulcerative colitis and Crohn disease (5). We have shown that RELMβ is produced specifically by intestinal goblet cells and is secreted apically into the intestinal lumen, where it exists intact in the stool of both mice and humans (6), and likely plays a role in the defense against parasitic nematode infections of the intestine (7). RELMβ expression is essentially undetectable in the colon of both germ-free immunocompetent and SCID mice but can be induced within 48 hours upon transfer to a conventional environment, demonstrating that the expression of this gene is dependent upon bacterial colonization of the gut (6). The other 2 RELM isoforms, α and γ, as well as

resistin, are not induced in the colon by colonization of the intestine with commensal bacteria (8). In contrast to the constitutive expression of RELMβ in the colon of conventionally housed mice and low but detectable expression in the terminal ileum, expression of this gene is absent in the rest of the gastrointestinal tract, namely the small intestine and stomach (1).

There is compelling evidence for the role of commensal gut bacteria as a central factor in the pathogenesis of IBD. First, intestinal disease activity in animal models of IBD is ameliorated when they are housed in a germ-free environment (9, 10). Second, IBD afflicts regions of the intestinal tract most heavily colonized with gut bacteria (11, 12), and antibiotics have efficacy in treating Crohn disease (11, 13). Finally, the identification of NOD2/CARD15 as an IBD susceptibility gene provides a genetic link among innate immunity, bacteria, and the pathogenesis of IBD (14, 15). As the interface between the bacteria-laden intestinal lumen and the mucosal immune system, the intestinal epithelium has been hypothesized to play a critical role in the development of IBD. Indeed, the ablation of goblet cells in the colon reduces the development of colitis in mice (16), suggesting that this cell lineage may be of particular relevance to the pathogenesis of IBD.

The observations that RELMβ protein expression is goblet cell specific and is expressed in those regions of the gut with the highest concentrations of commensal bacteria (12) and the highest frequency of involvement in patients with IBD (11) led us to determine whether RELMβ plays a role in the pathogenesis of intestinal inflammation in murine models of IBD. Herein, we show that RELMβ is an activator of the innate immune response and
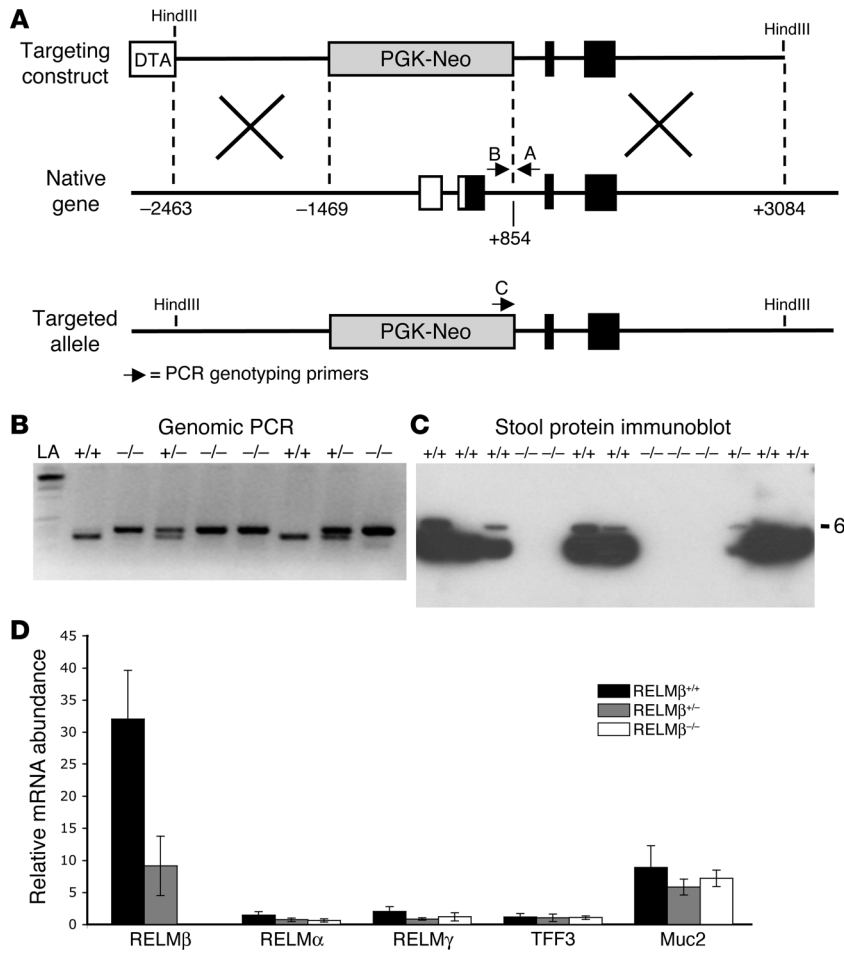
Figure 1
Characterization of the RELMβ knockout. (**A**) Schematic diagram of the knockout strategy. The phosphoglycerate kinase–Neo (PGK-Neo) cassette was used to disrupt 2323 bp of the RELMβ gene locus, replacing the promoter region and the first 2 exons. (**B**) Genomic PCR using primers shown in **A**. Amplification of a 295-bp product by primers A and B indicate the wild-type allele (lower band), whereas a 413-bp product amplified by primers A and C indicates the null allele (upper band). LA, ladder. (**C**) Immunoblot of RELMβ using protein isolated from the stool of wild-type and RELMβ-knockout mice. (**D**) Quantification of mRNA expression for RELM isoforms and goblet cell–specific genes using RNA isolated from the proximal colon of RELMβ$^{+/+}$, RELMβ $^{+/-}$, and RELMβ$^{-/-}$ mice ($n = 3$; mean ± SD).

that disruption of this gene protects against dextran sodium sulfate–induced (DSS-induced) colitis in mice. These results provide a potential explanation for the results described by Itoh et al. (16) showing that mice with a reduction in colonic goblet cells are protected from DSS-induced colitis.

Results

Development and initial characterization of the RELMβ knockout. In order to determine the function of RELMβ in vivo, we utilized gene targeting to disrupt the locus for this gene and develop a RELMβ$^{-/-}$ mouse (Figure 1, A and B). The RELMβ-null allele was inherited in the appropriate Mendelian pattern. RELMβ$^{-/-}$ mice did not express detectable levels of either mRNA or protein for this gene, whereas RELMβ$^{+/-}$ mice exhibited a gene dosage–dependent decrease in RELMβ mRNA expression (Figure 1, C and D). RELMβ$^{-/-}$ mice developed normally and bred well, without obvious gross phenotype. Furthermore, there was no alteration in the mRNA expression of the other RELM isoforms or goblet cell–specific genes such as *mucin 2* (*Muc2*) and *trefoil factor 3* (*Tff3*) in the proximal colon of RELMβ$^{-/-}$ mice (Figure 1D). Similar findings were observed in both the cecum and distal colon (data not shown).

RELMβ$^{-/-}$ mice are protected from DSS-induced colitis. In light of the central role that commensal bacteria play in the development of IBD in both mice and humans, together with the innate immune-dependent activation of RELMβ protein expression in the intestine, we sought to determine whether intestinal inflammation

would be altered in RELMβ$^{-/-}$ mice by inducing colitis using DSS (17). Four-percent DSS was delivered in drinking water for 8 days to age- and sex-matched RELMβ$^{-/-}$ mice and their wild-type littermates. To quantify clinical parameters of disease activity, we documented, on a daily basis, throughout the course of this study, all 3 components of a previously described disease activity index (DAI) (18): weight loss, intestinal bleeding, and stool consistency. The results, presented in Figure 2, are representative of 3 independent experiments. RELMβ$^{-/-}$ mice lost considerably less weight than the wild-type controls commencing on day 4. The greater body weight of RELMβ$^{-/-}$ mice relative to controls was sustained throughout duration of the study (Figure 2A). In addition, the onset of intestinal bleeding and the development of loose stool were also significantly delayed in RELMβ$^{-/-}$ mice compared with their wild-type littermates (Figure 2B). At day 7 of this study, the overall DAI was significantly less for RELMβ$^{-/-}$ mice compared with wild-type mice (3.29 ± 0.12 versus 3.63 ± 0.09; $P < 0.05$). Importantly, survival after DSS administration was dramatically enhanced in RELMβ$^{-/-}$ mice (Figure 2C). Consistent with the evidence for reduced injury, the percentage of the colonic mucosa containing ulcerations (Figure 2D) and the mRNA expression of an important mediator of inflammation in both patients with IBD and mice with DSS-induced colitis, TNF-α (19, 20), were significantly reduced in RELMβ$^{-/-}$ mice (Figure 2E). Taken together, these results provide compelling clinical evidence that RELMβ$^{-/-}$ mice are significantly less susceptible to DSS-induced colitis.
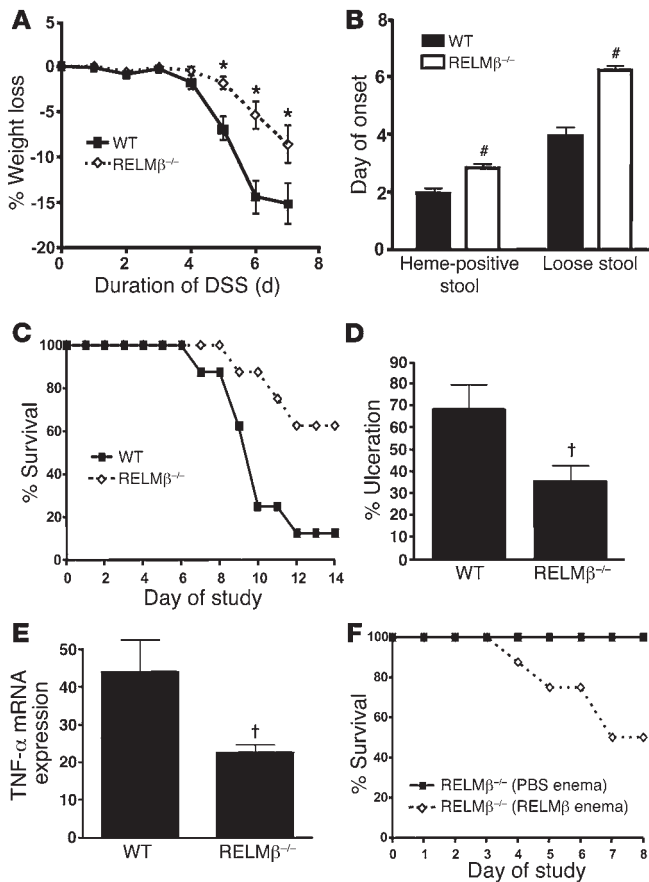
Figure 2

Clinical evidence that RELMβ exacerbates DSS-induced colitis. (**A**) Daily percent weight loss of wild-type and RELMβ$^{-/-}$ mice treated with 4% DSS ($n = 8$ per group; mean ± SEM; *$P < 0.01$). (**B**) Mean time (days) to onset of intestinal bleeding (hemoccult positivity) and loose stools in wild-type and RELMβ$^{-/-}$ mice treated with 4% DSS ($n = 8$ per group; mean ± SEM; #$P < 0.05$). (**C**) Percent survival of wild-type and RELMβ$^{-/-}$ mice treated with 4% DSS for 7 days ($n = 8$ per group). (**D**) Percentage of the colonic mucosa ulcerated in DSS-induced colitis in wild-type and RELMβ$^{-/-}$ mice treated with 4% DSS for 7 days (†$P < 0.05$). (**E**) Quantification of TNF-α mRNA expression in the colon of wild-type and RELMβ$^{-/-}$ mice treated with 4% DSS for 7 days assessed by Northern blot normalized to 18S RNA (†$P < 0.05$). (**F**) Percent survival of RELMβ$^{-/-}$ mice treated with 4% DSS that received enemas twice daily with either vehicle (PBS) or recombinant murine RELMβ (2 μg/ml) ($n = 8$ per group).

and B). By contrast, upon treatment with DSS, analysis of colonic tissue obtained from wild-type mice revealed severe inflammation of the colon, with near complete loss of the colonic crypts and epithelium (Figure 3, C and E). By contrast, analysis of colonic tissue obtained from DSS-treated RELMβ$^{-/-}$ mice revealed the preservation of colonic crypt structures containing isolated goblet cells despite the presence of an intense inflammatory infiltrate (Figure 3, D and F). The dilated crypt structures lined with isolated goblet cells suggest that, in the absence of RELMβ, the colonic epithelium and its associated crypt structures may either be better able to survive destruction or may be more rapidly reconstituted in the setting of intestinal inflammation. Although recent evidence that RELMβ acts as a mitogenic factor in lung epithelium via a PI3K-dependent mechanism might be consistent with this notion (21), there was no difference in epithelial apoptosis, which was very rare, as determined by TUNEL staining, in both wild-type and RELMβ$^{-/-}$ mice that received DSS (data not shown). Alternatively, the RELMβ-null mice may be more resistant to the initiation of disease induced by DSS.

In order to determine the specificity with which RELMβ exerts its proinflammatory effects in DSS-induced colitis, we examined the effect of RELMβ, administered by enema twice daily, on the development of DSS-induced colitis in RELMβ$^{-/-}$ mice. Reintroduction of RELMβ into the knockout mice resulted in a 50% mortality rate within 1 week of DSS administration, whereas all RELMβ$^{-/-}$ mice receiving the vehicle as an enema survived (Figure 2F). Because the administration of enemas altered stool consistency and may have contributed to the development of Hemoccult-positive (Beckman Coulter) stool, we were unable to determine DAI in these mice. However, even with the exclusion of the RELMβ-treated mice that died early in the study, there was a trend toward an increase in percent body weight loss observed in the RELMβ-treated mice compared with controls at the termination of the study (14.34% ± 2.35% versus 12.78% ± 1.46%).

Histologically, there was no difference in morphology of the colonic mucosa, including the number of goblet cells or the size of their granules, in wild-type versus RELMβ$^{-/-}$ mice (Figure 3, A

Figure 3

Preservation of colonic crypt cell proliferation, architecture, and goblet cells in RELMβ$^{-/-}$ mice treated with DSS. RELMβ immunostaining in the colon of wild-type (**A**) and RELMβ$^{-/-}$ mice (**B**) (magnification, ×200). (**C**–**F**) Representative photomicrographs of "Swiss-rolled" colonic tissue obtained after 7 days of 4% DSS administration. H&E-stained sections of wild-type (**C**) and RELMβ$^{-/-}$ colons (**D**) (magnification, ×100). Alcian blue–stained sections of wild-type (**E**) and RELMβ$^{-/-}$ colons (**F**) (magnification, ×100).
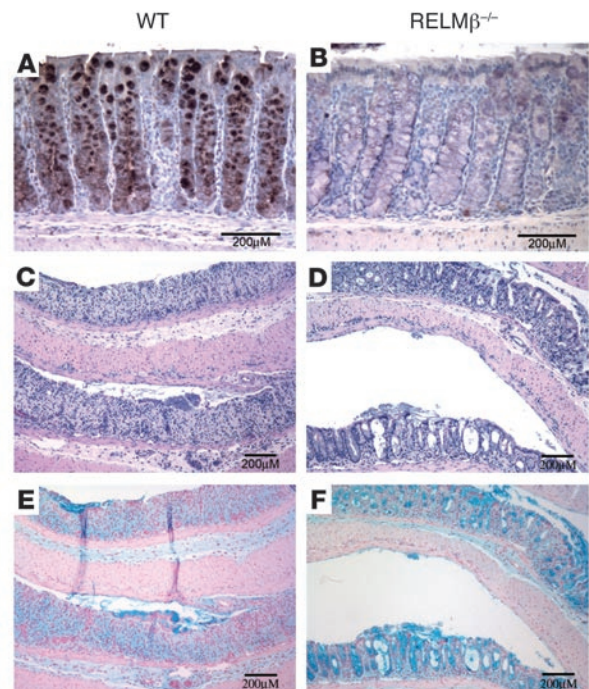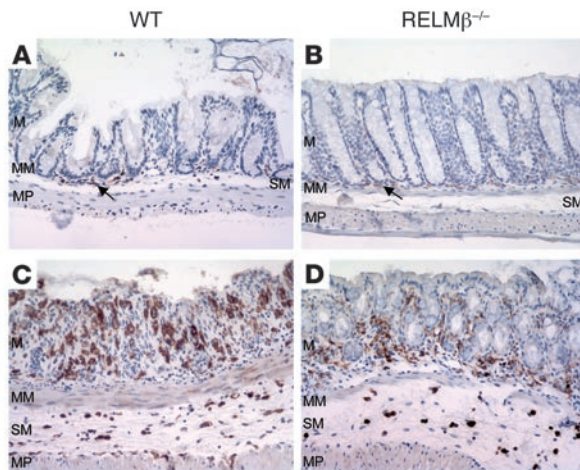
Figure 4
Analysis of the inflammatory cell infiltrate in DSS-induced colitis of wild-type and RELMβ$^{-/-}$ mice. F4/80 staining in the colon wild-type mice (**A**) or RELMβ$^{-/-}$ mice (**B**) treated with 4% DSS for 2 days (magnification, ×200). Arrows indicate F4/80-positive macrophages at the based of colonic crypts. F4/80 staining in the colon of wild-type mice (**C**) or RELMβ$^{-/-}$ mice (**D**) treated with 4% DSS for 7 days (magnification, ×200). M, mucosa; MM, muscularis mucosa; SM, submucosa; MP, muscularis propria.

In order to more thoroughly characterize the inflammatory process in the wild-type and RELMβ$^{-/-}$ mice treated with DSS, the leukocyte components of the inflammatory infiltrate were determined histologically in a time-course study. After 2 days of DSS administration, the histologic appearance of both the wild-type and RELMβ$^{-/-}$ mice appeared normal, with only occasional F4/80-positive macrophages identified adjacent to the base of the colonic crypts (Figure 4, A and B). This was similar to the histologic appearance observed in untreated wild-type controls (data not shown). By contrast, the distal colons of both the wild-type and RELMβ$^{-/-}$ mice demonstrated an inflammatory infiltrate after 7 days of DSS administration that was significantly less pronounced in the RELMβ$^{-/-}$ than the wild-type mice (Figure 4, C and D, and Table 1), consistent with the evidence of less disease (Figure 2) and the preservation of colonic crypt structures in the RELMβ$^{-/-}$ mice (Figure 3). Importantly, a significant proportion of the leukocytic infiltrate in both the wild-type and RELMβ$^{-/-}$ mice (approximately 30%) consisted of F4/80-positive macrophages (Figure 4, C and D, and Table 1). By contrast, granulocytes were responsible for only a small component of the inflammatory infiltrate in both the wild-type and RELMβ$^{-/-}$ mice (Table 1). In total, these results are consistent with previous notions that DSS-induced colitis is primarily an acute innate immune model in which macrophages may play a predominant role (22). Since the total inflammatory infiltrate was reduced in the RELMβ$^{-/-}$ mice without any alteration in the proportion of either macrophages or granulocytes, these results are consistent with a role for RELMβ as a more general upstream modifier of disease activity rather than a chemoattractant for a specific innate immune cell type.

Epithelial function in response to DSS is not altered in RELMβ$^{-/-}$ mice. In order to determine whether RELMβ exacerbates colitis by modifying early events associated with the initiation of DSS-induced colitis, we examined 2 parameters of colonic epithelial function in the RELMβ$^{-/-}$ mice, cell proliferation and barrier function. Colonic crypt cell proliferation was determined by immunohistochemical detection of Ki-67 in colonic tissue sections from both wild-type and RELMβ$^{-/-}$ given 4% DSS for 2 days. Although the histologic appearance of the colonic mucosa was normal in both groups of mice, crypt cell proliferation was virtually absent in the distal colon of both wild-type (data not shown) and RELMβ$^{-/-}$ mice (Figure 5A). Importantly, Ki-67 staining of the proximal colon and cecum of both wild-type and RELMβ$^{-/-}$ mice, where colitis does not develop after longer-term treatment with DSS, was not different from that seen in these gut segments obtained from untreated mice. In order to determine whether RELMβ expression alters colonic epithelial function in response to DSS treatment, prior to the onset of inflammation, we assessed epithelial barrier function in the distal colon of both wild-type and RELMβ$^{-/-}$ mice treated with 4% DSS for 2 days. Alterations in intestinal epithelial barrier function have been hypothesized to play an important role in the pathogenesis of IBD (23). Barrier function was equally reduced in the distal colon of both wild-type and RELMβ$^{-/-}$ mice, as demonstrated by a decrease in transepithelial resistance (TER) (Figure 5B) and a corresponding increase in short-circuit current (data not shown). To determine whether RELMβ can directly activate a proinflammatory response in the intestinal epithelium, we examined the effect of recombinant RELMβ on the inflammatory response of an intestinal cell line, LS174T, in vitro. A dose-ranging study of RELMβ from 0.1 to 1.0 μg/ml failed to induce the expression of IL-8 mRNA expression or activate the IL-8 promoter, an NF-κB–dependent gene, in reporter gene studies (data not shown). Together, these results demonstrate that RELMβ does not have a primary effect on the colonic epithelium associated with the initiation of DSS-induced colitis.

Table 1
Quantification of leukocyte populations in DSS-induced colitis

	Wild-type (DSS, 2 d)	Wild-type (DSS, 7 d)	RELMβ$^{-/-}$ (DSS, 2 d)	RELMβ$^{-/-}$ (DSS, 7 d)
Total leukocytes/HPF	145 ± 14.4	458 ± 60.0	128 ± 13.5	250 ± 31.0[A]
Macrophages/HPF	25 ± 2.0 (17%)	136 ± 18.1 (30%)	32 ± 2.4 (25%)	74 ± 11.3[A] (29%)
Granulocytes/HPF	4 ± 0 (3%)	16 ± 2.6 (4%)	6 ± 2.1 (5%)	8 ± 1.5[A] (3%)

Cells counted in H&E- and F4/80-stained distal colonic tissue sections using 3 random ×600-magnification fields for each of 4 mice of each genotype treated with 4% DSvS for the times periods indicated (mean ± SEM; numbers in parentheses indicate percentage of total leukocyte count; [A]P < 0.03 relative to wild-type). HPF, high-power field.
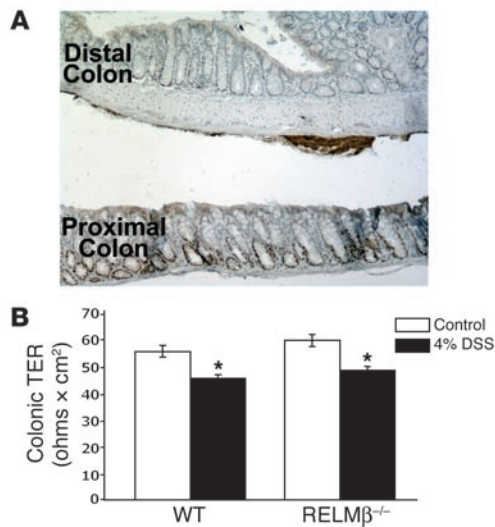
Figure 5
Short-term DSS treatment inhibits colonic crypt cell proliferation and reduces barrier function in RELMβ$^{-/-}$ mice in the absence of gross inflammation. (**A**) Ki-67 staining of a Swiss-rolled colon isolated from a RELMβ$^{-/-}$ mouse after treatment with 4% DSS for 2 days (magnification, ×100). (**B**) Assessment of epithelial barrier function through the measurement of transepithelial resistance (TER) in wild-type and RELMβ$^{-/-}$ mice with and without 4% DSS for 2 days. *$P \leq 0.01$.

RELMβ activates innate immune responses both in vitro and in vivo. Upon disruption of colonic epithelial barrier function, it is possible that RELMβ located in the lumen may augment the inflammatory response by activating leukocytes that normally reside in the lamina propria of the intestinal mucosa. Macrophages have been suggested to be a primary innate immune cell type that is activated upon the loss of epithelial barrier function in DSS-induced colitis (22). Indeed, immunohistochemical staining for F4/80 revealed that macrophages are a predominant cell type associated with DSS-induced colitis in both wild-type and RELMβ$^{-/-}$ mice (Figure 4 and Table 1). To determine whether RELMβ could activate macrophages in vitro, a multiplex assessment of protein secretion induced by the treatment of thioglycolate-isolated macrophages with recombinant RELMβ revealed the specific induction of only 2 proteins, TNF-α and IL-15 (Figure 6A), out of 22 cytokines and chemokines assayed (IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p70, IL-13, IL-17, G-CSF, GM-CSF, IFN-γ, IP-10, KC, MCP-1, MIP-1α, and RANTES). Interestingly, both TNF-α and IL-15 have been previously shown to play an important role in DSS-induced colitis (20, 24). Although TNF-α levels were reduced in RELMβ$^{-/-}$ compared with wild-type mice treated with DSS (Figure 2E), there was no difference in the mRNA expression of IL-15 (data not shown). A dose-ranging study revealed that isolated macrophages secrete high levels of TNF-α in response to recombinant RELMβ at a low microgram per milliliter concentration (Figure 6B). The maximal secretion of TNF-α occurred rapidly, within 3 hours of stimulation (data not shown).

To determine the effect of RELMβ on the immune system in vivo, recombinant RELMβ was injected i.p. into mice, and peritoneal cells were collected for gene expression studies as well as assessment of the cellular profile. Quan-

titative assessment of the innate immune cellular profile in the peritoneal lavage fluid by cytospin analysis showed that RELMβ induced a significant, time-dependent influx of neutrophils (Figure 7A). The peak neutrophil influx occurred 24 hours after the injection of RELMβ ($P = 0.005$ compared with vehicle-treated controls). There was also an increase in the level of eosinophils, but this change did not reach statistical significance when compared with that in mice treated with vehicle control (data not shown). By contrast, the abundance of mast cells, which in the peritoneum was approximately 5% of that of macrophages, did not change with RELMβ administration. While injection of the vehicle, which consisted of PBS spiked with trace amounts of LPS equivalent to that measured in the recombinant RELMβ, also induced an increase in the number of infiltrating neutrophils (Figure 7A), this increase was not significantly different from that obtained by injection of PBS alone (data not shown). While the underlying mechanism for the peritoneal neutrophil influx is unclear, we found that 1 hour after RELMβ injection, there was a significant increase in mRNA expression of TNF-α, a cytokine responsible for initiation of proinflammatory changes, as measured by quantitative RT-PCR (Figure 7B). Interestingly, RELMβ injection also caused morphologic changes characteristic of macrophage activation, including enlargement and foamy transformation of the cytoplasm, which was most apparent after 48 hours (Figure 7C). FACS analysis of the F4/80-positive cell population revealed that the proportion of these cells did not change significantly, upon injection of RELMβ, relative to the total cell population (Figure 7D). There was, however, a striking increase in the amount of TNF-α–producing cells within this macrophage population (28% of TNF-α–positive cells in the F4/80-positive population) when compared with that in vehicle-treated mice (7%; $P = 0.011$) (Figure 7, E and F). These data together with the morphological changes suggest that RELMβ is an immune activator of macrophages in vivo. In contrast, no activation was apparent in the other mononuclear cell populations following RELMβ treatment, as we observed no increase in the proportion of B220-positive B cells, CD3-positive T cells, the CD4- or CD8-positive T cell subclasses or their expression of the IL-2 receptor, CD25 (Table 2). Thus, recombinant RELMβ activated the production of the proinflammatory cytokine TNF-α by macrophages and induced inflammatory cell infiltration when injected locally. The ability of RELMβ to activate the expression of TNF-α, an upstream mediator of the general inflammatory response, is
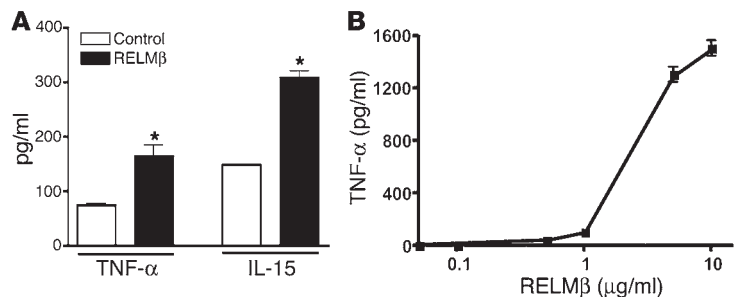

Figure 6
Recombinant RELMβ is an activator of macrophages in vitro. (**A**) Recombinant RELMβ activates TNF-α and IL-15 protein expression in thioglycolate-isolated macrophages (*$P < 0.01$). (**B**) Dose-dependent induction of TNF-α protein expression in thioglycolate-isolated macrophages treated with recombinant murine RELMβ.
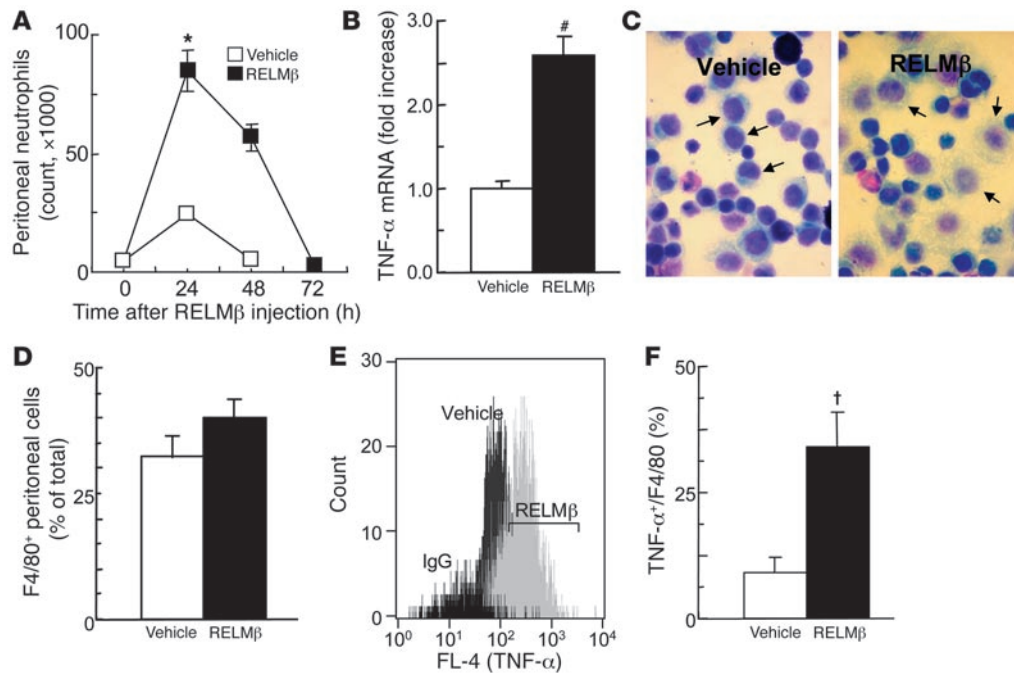
Figure 7

Intraperitoneal injection of RELMβ induces a proinflammatory response. (**A**) Time course of neutrophil influx (mean ± SEM of n = 4–8 mice; *P = 0.005) in isolated peritoneal cells after vehicle or RELMβ injection. (**B**) Quantification of TNF-α mRNA expression in isolated peritoneal cells by RT-PCR 1 hour after vehicle or RELMβ injection (n = 4; mean ± SEM; #P = 0.041). (**C**) Peritoneal macrophages (arrows) show enlargement and foamy transformation of the cytoplasm 48 hours after i.p. RELMβ injection (magnification, ×1,000). (**D**) Quantification of F4/80-positive macrophages by FACS (n = 4; mean ± SEM). (**E**) Representative overlay histograms of TNF-α expression in F4/80-positive cells from vehicle or RELMβ-treated mice. (**F**) Quantification of intracellular TNF-α expression in F4/80-positive macrophages 48 hours after i.p. RELMβ injection (mean ± SEM of n = 5 mice; †P = 0.011).

consistent with the previously defined role of macrophages and TNF-α in DSS-induced colitis (20, 22). This ability also correlates well with the general decrease in the inflammatory infiltrate observed in RELMβ$^{-/-}$ mice treated with DSS for 7 days compared with similarly treated wild-type mice (Figure 4 and Table 1).

RELMβ expression is strongly induced in the terminal ileum of the SAMP1/Fc model of Crohn disease. Our results in DSS-induced colitis suggest that RELMβ may play a role in the exacerbation of intestinal inflammation in other more spontaneous, chronic, and immunologically based models of IBD such as that observed in SAMP1/Fc mice. Although the AKR background strain of the SAMP1/Fc mice exhibits low levels of RELMβ expression in the terminal ileum (1) (Figure 8B), mRNA and protein expression was strongly induced in the ileum of SAMP1/Fc mice concurrent with the onset of inflammation and the development of goblet cell hyperplasia (Figure 8, A and C). Interestingly, IL-4, a Th2 cytokine, has recently been implicated as a critical mediator in the perpetuation of disease in SAMP1/Fc mice (25). We have also shown that Th2-mediated inflammatory responses, induced by intestinal parasites in an IL-4 receptor–dependent fashion, strongly activate the expression of RELMβ in the intestinal tract (7).

Discussion

Our results demonstrate that RELMβ$^{-/-}$ mice are protected from DSS-induced colitis. As an acute model of intestinal injury, the DSS-induced colitis model is ideal for assessing the role of RELMβ in the intestinal inflammatory response for several reasons. First, DSS-induced colitis can be ameliorated by treatment with antibiotics used clinically in patients with IBD (11), which have also been shown to reduce RELMβ expression (26), demonstrating that commensal bacteria play a role in the development of inflammation in this model system (27). Second, as with the induction of RELMβ by bacterial colonization, DSS-induced colitis also develops in SCID mice, eliminating the role of adaptive immunity as a factor in this model (28). Finally, the ablation of goblet cells in the colon reduces mucosal damage in DSS-induced colitis (16).

In order to ascertain the mechanism by which RELMβ$^{-/-}$ mice are protected from DSS-induced colitis, we first focused on the effect of this protein on epithelial cell proliferation and barrier function. The initiating event by which DSS induces colitis appears to be a reduction in colonic crypt epithelial proliferation leading to the

Table 2

FACS analysis of peritoneal lymphocyte subpopulations 48 hours after vehicle or RELMβ injection

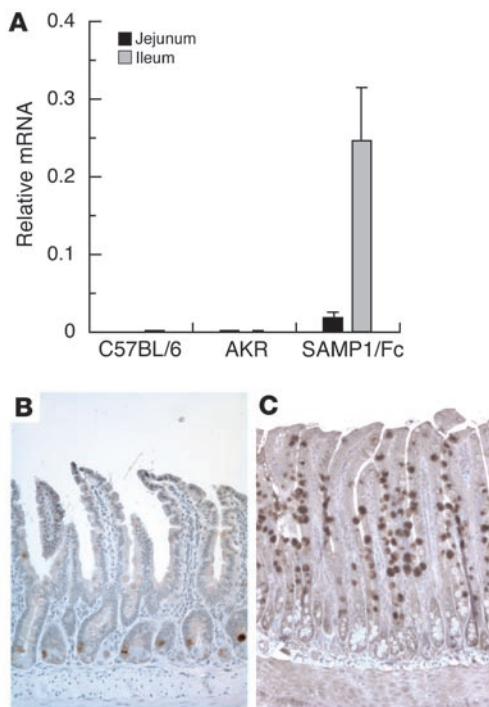| Marker	Vehicle	RELMβ	P value
B220$^+$/total (%)	85.5 ± 9.0	87.0 ± 9.1	NS
CD3$^+$/total (%)	23.0 ± 2.9	26.2 ± 2.5	NS
CD4$^+$/CD3$^+$ (%)	73.7 ± 1.1	71.4 ± 2.6	NS
CD8$^+$/CD3$^+$ (%)	23.7 ± 0.9	22.8 ± 2.1	NS
CD25$^+$/CD4$^+$ (%)	21.9 ± 3.8	14.4 ± 2.8	NS
CD25$^+$/CD8$^+$ (%)	1.7 ± 0.6	1.8 ± 0.7	NS

Mean ± SEM of n = 4.

Figure 8
RELMβ expression is strongly activated in the terminal ileum of the SAMP1/Fc mice. (**A**) RELMβ mRNA levels were determined by quantitative real-time PCR in the jejunum and ileum of 40-week-old C57BL/6, AKR, and SAMP1/Fc mice. 18S rRNA was used as an endogenous control. RELMβ mRNA levels are expressed as mean ± SEM relative to 18S rRNA ($\times 10^{-2}$) ($n = 5$ per group). Immunohistochemical detection of RELMβ in the terminal ileum of AKR (**B**) and SAMP1/Fc mice (**C**) (magnification, ×200).

loss of this progenitor cell compartment (29). Subsequent reduction of epithelial barrier function (30) may then allow antigenic lumenal contents to activate resident innate immune cell populations in the lamina propria, such as macrophages, resulting in colonic inflammation (22). Although RELMβ does not have any epithelial cell autonomous effects in the setting of DSS-induced colitis, our data suggest that RELMβ derived from the lumen, delivered to the lamina propria through a disrupted epithelial barrier, may activate immune cells to produce inflammatory mediators such as TNF-α.

In mammals, TNF-α is secreted by macrophages, monocytes, neutrophils, NK cells, and T cells in response to proinflammatory changes in the local environment (31). In Figure 2E, we show that TNF-α expression is significantly lower in the DSS-induced colitis in RELMβ$^{-/-}$ mice compared with that induced in wild-type mice. Macrophages, which appear to be a prominent inflammatory cell type in DSS-induced colitis in both wild-type and RELMβ$^{-/-}$ mice (Figure 4 and Table 1), are therefore likely to be the principal source of TNF-α. This suggests that RELMβ may regulate the expression of TNF-α in macrophages. Indeed, we show that in vitro treatment of thioglycolate-induced peritoneal macrophages with recombinant RELMβ stimulated the release of TNF-α protein (Figure 6) and that, in vivo, i.p. injection of RELMβ rapidly induced de novo TNF-α protein synthesis in F4/80-positive macrophages isolated from the peritoneum (Figure 7, E and F).

Together with the induction of morphologic changes in peritoneal macrophages consistent with an activated phenotype (Figure 7C), the induction of TNF-α expression in macrophages demonstrates that RELMβ is capable of directly activating macrophages in vivo. Indeed, this is consistent with the ability of resistin, a closely related protein, to induce the expression of proinflammatory cytokines such as TNF-α in peripheral blood mononuclear cells (3) as well as macrophage cell lines (4).

Although our data implicate macrophages as the predominant immune cell type that is activated by RELMβ, it is certainly possible that this protein may influence the biology of other immune and inflammatory cells present in the setting of DSS-induced colitis (Figure 4 and Table 1; and ref. 18). In this regard, we show in Figure 7A that i.p. injection of RELMβ leads to the influx of neutrophils into the peritoneum, a process that possibly influences the inflammatory changes induced by DSS, particularly during the initiation of colitis in this model.

Although the physiologic function of RELMβ has yet to be determined, like resistin (32), RELMβ seems to induce insulin resistance in vivo when either administered systemically or ectopically expressed (33, 34). There is growing evidence that insulin resistance, the hallmark of type 2 diabetes mellitus, is due in part to a state of chronic inflammation perpetuated by circulating inflammatory mediators such as TNF-α (35). Indeed, previous studies have reported that TNF-α inhibits insulin signaling by serine phosphorylation of IRS1 (36) and that the deletion of this gene or its receptors in mice prevents diet-induced insulin resistance (37). Most of these immune mediators are associated with an innate immune response in which macrophages may play an important role (35). In this regard, our results showing that RELMβ is an activator of macrophages leading to TNF-α production are consistent with the role that this molecule may play in host metabolism.

In Figure 6B, we provide results of a dose-response study of RELMβ on the induction of TNF-α protein expression in isolated peritoneal macrophages. The induction of TNF-α in this model occurs at higher levels of RELMβ protein than we have previously reported in the stool (7). It is possible that levels of RELMβ concentrated in the granules of goblet cells and released in proximity to inflammatory cells located in the lamina propria may be considerably greater than the steady-state levels of RELMβ in the stool. Furthermore, it must be noted that RELMβ, like resistin, which is the prototypical member of this family, are highly cysteine-rich proteins (1). It is currently unknown whether the recombinant protein folds correctly, a process that may have an effect on the potency of its bioactivity. This is why we have utilized the RELMβ$^{-/-}$ mouse to focus on the role of the endogenous RELMβ protein on the development of colitis in the DSS model.

The presence of resistin and RELMβ has been shown to correlate with the severity of inflammatory changes in patients with rheumatoid arthritis (3) and with intestinal bacterial overgrowth observed in CFTR-null mice (26), respectively. In fact, intraarticular injection of resistin into mice results in inflammatory infiltrate and pannus formation in the synovium (3). Taken together, our data and the results of these previously published studies support a proinflammatory function for RELMβ. We speculate that this molecule may act by the rapid induction of TNF-α synthesis and release by macrophages followed by the subsequent recruitment of other inflammatory cells such as neutrophils (Figure 7A).

RELMβ is also associated with inflammation in a more chronic and immunologically mediated model of IBD, SAMP1/Fc mice

(Figure 8). The pattern of inflammation in these mice is remarkably similar to that of Crohn disease in humans, with the spontaneous development of disease located primarily in the terminal ileum, and is characterized by discontinuous transmural inflammation with the formation of granulomas, the development of perirectal fistulas, and the amelioration of disease by the administration of TNF-α–neutralizing monoclonal antibodies (25, 38). As occurs in DSS-induced colitis, and particularly relevant to patterns of RELMβ expression, the inflammatory response in these SAMP1/Fc mice is accelerated by gut bacteria (39). The strong induction of RELMβ in the terminal ileum of these mice concurrent with disease activity (Figure 8) together with the reduction in the severity of DSS-induced colitis in RELMβ−/− mice (Figure 2) and the ability of exogenous RELMβ to activate the innate immune response both in vitro and in vivo (Figures 6 and 7) provide compelling evidence for a role of RELMβ in modulating intestinal inflammation. The ability of RELMβ to activate the expression of TNF-α may be particularly relevant to human IBD given the therapeutic efficacy of anti–TNF-α biologics (19). Although our present studies clearly demonstrate a role for RELMβ in acute intestinal inflammation, breeding the RELMβ−/− genotype onto the SAMP1/Fc background will help to ascertain the role of RELMβ within the context of a more chronic disease process.

It is generally accepted that the goblet cell lineage plays an important role in maintaining mucosal integrity in the gut through the production of mucin and trefoil factors (40, 41). However, recent studies have questioned this because disruption of goblet cells reduces susceptibility of mice to colonic injury (16). Our results reconcile these seemingly disparate observations by showing that the absence of goblet cell RELMβ dramatically reduces susceptibility to DSS-induced colitis. In this regard, the characteristic depletion of goblet cell granules in the presence of active IBD (42) may actually be a protective compensatory mechanism. The ability of RELMβ to activate the innate immune response is consistent with the recently described crystal structure of both RELMβ and resistin, which reveals a multimeric complex with similarity to the C1q complement protein and the TNF family of immune regulatory proteins (43, 44). The identification of RELMβ as a mediator of the mucosal inflammatory response emphasizes the importance of the goblet cell lineage in the innate response to commensal bacteria in the gut and the pathogenesis of IBD.

Methods

Generation of RELMβ-knockout mice. The targeting strategy for the disruption of the endogenous RELMβ gene locus is depicted in Figure 1A. A 129SvEv BAC library was screened by PCR, and a 5547-bp *Hind*III fragment of the RELMβ gene locus was cloned into pKS− (Promega). The targeting vector, which contains phosphoglycerate kinase–Neo^r (PGK-Neo^r) and diphtheria toxin A (DTA) cassettes, was introduced into mouse ES stem cells (inGenious Targeting Laboratory Inc.) by electroporation, and the cells were subjected to selection under G418. Homologous recombinants, identified by genomic PCR and confirmed by Southern blotting, were then injected into blastocysts, and the resulting male chimeras were mated with female C57BL/6 mice. After germline transmission of the null allele was confirmed by genomic PCR and Southern blotting, F$_1$ mice were then interbred to obtain mice on a mixed 129SVEV/C57BL6 background. Genomic PCR to genotype the resulting mice and identify wild-type, RELMβ+/−, and RELMβ−/− was performed using the following primers: A: 5′-TAGTGCAGGAGATCGTCTTA-3′; B: 5′-TCTGAT-GCCCTCTTCTGGCT-3′; C: 5′-TGGATGTGGAATGTGTGCGA-3′. A representative example of a genotyping PCR results is shown in Figure 1B.

SAMP1/Fc mice. These mice were maintained under specific pathogen–free conditions at the Animal Facility of the University of Virginia. AKR mice used as controls were purchased from The Jackson Laboratory. All experiments were approved by the Animal Care and Use Committee at the University of Virginia.

Immunoblots, immunohistochemistry, TUNEL staining, Northern blots, and quantitative RT-PCR. The methods used for the isolation of stool protein and immunoblot detection using a polyclonal rabbit anti-mouse antibody to mRELMβ and F4/80 (AbD Serotec) have been previously described (6). Sections for TUNEL staining were preincubated with Proteinase K Solution (Roche Diagnostics) for 15 minutes at room temperature followed by the use of the Apoptosis Detection Kit (Chemicon International) per the instructions of the manufacturer. Positive controls for TUNEL staining were generated by treating sections of normal mouse colon with RNase-free DNase I (Roche Diagnostics).

Colonic tissues from both wild-type and RELMβ−/− mice were divided lengthwise with one-half of the colon used for RNA isolation and the other one-half "Swiss rolled" with the distal colon in the center and fixed in 10% neutral buffered formalin for paraffin embedding. The method for isolation of RNA; primer sequences for RELMβ, RELMα, RELMγ, Muc2, and TFF3; as well as the conditions for quantitative RT-PCR have been previously described (6–8). The primer sequences utilized to quantify TNF-α expression in peritoneal lavage cells by RT-PCR were: forward, 5′-CAGACCCTCACACTCAGATCATCTT-3′, and reverse, 5′-CCACTTGGT-GGTTTGCTACGA-3′. Alcian blue, which stains mucopolysaccharides, was used to identify goblet cells. These sections were counterstained with nuclear fast red as previously described (45).

IL-8 expression was quantified using RNA isolated from LS174T cells treated for 24 hours with 0.1, 0.5, or 1.0 μg/ml hRELMβ. Quantification of mRNA expression of IL-8, normalized to GAPDH levels, was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using SYBR Green as the fluorescent probe. The oligonucleotide primer sequences used for human IL-8 were: forward, 5′-GTGCTAAAGAACTTAGATGT-CAGTGCATA-3′, and reverse, 5′-TGGTCCACTTCTCAATCACTCTCA-3′.

For the TNF-α and IL-15 quantification by Northern blotting, total RNA (10 μg) for each sample was electrophoretically separated, transferred to a nylon membrane, and UV cross-linked. After imaging the membrane using a Bio-Rad ChemiDoc system, the blot was hybridized to a PCR fragment of either murine TNF-α (forward, 5′-AGGGGCCACCACGCTCTTCTGT-3′; reverse, 5′-GCAAATCGGCTGACGGTGTGG-3′) or murine IL-15 (forward, 5′-TCTCGTGCTACTTGTGTTTCCTTC-3′; reverse, 5′-GTTTTCTCCTC-CAGCTCCTCACAT-3′). The PCR fragments were isolated from agarose gels, radioactively labeled using Megaprime DNA Labeling Systems (Amersham Biosciences), and isolated from unincorporated nucleotides by NICK columns (Amersham Biosciences). Signal quantification was performed using a Storm 840 PhosphorImager (Amersham Biosciences). The bands were normalized to the intensity of the 18S band for each sample as measured by Quantity One 4.3.1 software on a Bio-Rad ChemiDoc (Bio-Rad).

IL-8 promoter reporter gene studies. The luciferase reporter gene constructs containing 135 bp of the IL-8 5′ flank has been previously described (46). Transient transfections of LS174T cells were performed using Lipofectamine 2000 (Invitrogen) and SV40 β-galactosidase as a transfection control. hRELMβ (1 μg/ml; PeproTech) was added to the culture medium 24 hours after transfection. Forty-eight hours later, the cells were lysed and assayed for luciferase (Luciferase Assay System; Promega) and β-galactosidase. Each sample was assayed in triplicate, with the results expressed as mean light units per unit of β-galactosidase activity.

DSS-induced colitis. Eight male wild-type and RELMβ−/− littermate mice of 10 weeks age and weighing 23–28 g were fed 4% DSS for 7 days (MP Biomedicals Inc.; molecular mass 36–50 kDa, dissolved in drinking water)

as previously described (17). The mice had access to standard mice chow pellets. Weights, intestinal bleeding (hemoccults), and stool character were assessed on a daily basis by an investigator blinded to the genotype of the mice. All statistical analyses were performed using 2-tailed Student's *t* test. *P* values less than 0.05 were considered significant. The DSS was discontinued in the drinking water on day 8 of the study, and the survival of the mice was determined over the course of an additional 7 days. In a second experiment, wild-type and RELMβ$^{-/-}$ mice treated in an identical fashion were euthanized on day 7 for harvesting of colonic tissue to be analyzed by immunohistochemistry as described above.

The RELMβ enema add-back experiment utilized 16 male RELMβ$^{-/-}$ mice, which were 20 weeks of age and weighed 24–33 g. Enemas were given twice daily at 12-hour intervals using an 18 French angiocatheter inserted 2 cm proximal to the anal verge in a volume of 200 μl. Mice were held upside down for 30 seconds after enema administration. Eight mice were given murine recombinant RELMβ (PeproTech) at a concentration of 2 μg/ml in PBS, and 8 mice were given enemas containing only 1× PBS. After the first day of enema administration, all mice were fed 4% DSS dissolved in drinking water as described above.

Electrophysiology measurements of epithelial barrier function. Mice were euthanized via CO_2 asphyxiation after administration of 4% DSS for 2.5 days. Colonic tissue was then resected and mounted in an Üssing chamber for measurements of TER, an indicator of barrier function, and short-circuit current (Isc). Full-thickness, unstripped mucosa was bathed on both mucosal and serosal surfaces with 5 ml of oxygenated (95% O_2/5% CO_2) Ringer solution containing (in mM): NaCl 109.8, KCl 5.3, $CaCl_2$ 1.2, $MgCl_2$ 1.2, $NaHCO_3$ 25, Na_2HPO_4 2.4, NaH_2PO_4 0.4. Additionally, the serosal bathing solution contained 10 mM glucose, which was osmotically balanced by 10 mM mannitol on the mucosal side. Bathing solutions were circulated in water-jacketed reservoirs and maintained at 37°C.

Stimulation of thioglycolate-isolated macrophages with mRELMβ. Peritoneal macrophages were harvested 3 days after injection of 10% Brewer thioglycolate (BD) by peritoneal lavage with PBS and plated in 24-well plates using 10% FBS/DMEM. After 4 hours, adherent cells were washed twice with DMEM and thereafter kept in macrophage serum-free medium (Invitrogen) for further treatment. Supernatants were analyzed for the expression of 22 different cytokines after a 48-hour treatment interval with either recombinant murine RELMβ or human resistin protein (1 μg/ml; PeproTech) using a Luminex multiplex protein assay system (catalog MCYTO-70K; Linco). Each measurement was performed in triplicate, with the results normalized to cellular protein content and expressed as picograms per milliliter.

Intraperitoneal administration of RELMβ, cytospins, cell counts, and FACS analysis. Murine recombinant RELMβ (2 μg in 100 μl PBS; PeproTech) was injected i.p. into BALB/c mice, followed by peritoneal lavage after 1, 24, 48, and 72 hours as described previously (47). The vehicle control was spiked with 5 pg of LPS from *E. coli* (Sigma-Aldrich) to approximate the amount of endotoxin in the recombinant RELMβ, less than 2.5 pg/μg, measured by limulus assay. Total RNA was isolated from cells of the peritoneal lavage 1 hour after injection of RELMβ (0.5 μg in 100 μl PBS), and TNF-α mRNA expression was determined by quantitative RT-PCR analysis (as described above). The morphological changes in macrophages, 24 hours after RELMβ administration, were assessed on cytospin preparations stained by Shandon Kwik-Diff (Thermo Electron Corp.), and total and differential cell

counts were determined as described previously (47). Two hundred cells were counted for each of the cytospin slides under immersion oil, using a Nikon light microscope (at ×1,000 magnification).

Peritoneal cells for analysis were harvested, 1 hour after injection of RELMβ (0.5 μg in 100 μl PBS), by lavage with cold, sterile PBS and resuspended in staining buffer (0.1% Na azide, 2.5% FBS in PBS) containing a predetermined optimal concentration of fluorochrome-conjugated antibodies or the corresponding isotype controls. The following antibodies were used for cell-surface staining: PE-conjugated anti-mouse F4/80, FITC-conjugated anti-mouse CD3e, APC-conjugated anti-mouse CD8a, PE-Cy5.5–conjugated anti-mouse/human CD45R (B220) (eBioscience), APC-conjugated anti-mouse CD4, PE-conjugated anti-mouse CD25 (BD Biosciences — Pharmingen).

For intracellular protein expression of TNF-α in macrophages, cells were washed and resuspended in Macrophage-SFM Medium (Invitrogen) containing L-glutamine, 10% FBS, penicillin, and streptomycin. Cells were cultured ex vivo in the presence of GolgiPlug (BD Biosciences — Pharmingen) protein transport inhibitor for 5 hours. Test samples were cultured without further stimulation. A pool of 5% of the cells from each mouse was stimulated with 0.5 μg LPS and used as positive control. For analysis by flow cytometry, approximately 1×10^6 cells were incubated for 15 minutes with 1 μg Fc Block (BD Biosciences — Pharmingen) followed by a 30-minute incubation with PE-conjugated anti-mouse F4/80 antibody at 4°C. Cells were then fixed and permeabilized using a Cytofix/Cytoperm kit (BD Biosciences —Pharmingen) and stained with APC-conjugated rat anti-mouse TNF-α antibody (BD Biosciences — Pharmingen). Background fluorescence was less than 0.5% after incubation with Ig isotype controls. Cells were analyzed on a FACSCalibur flow cytometer, and data were analyzed using CellQuest software version 5.2.1 (BD).

Address correspondence to: Gary D. Wu, Suite 600 Clinical Research Building, 415 Curie Blvd., University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104, USA. Phone: (215) 898-0158; Fax: (215) 573-2024; E-mail: gdwu@mail.med.upenn.edu.

Laila D. McVay and Sue A. Keilbaugh contributed equally to this work.

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