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Resistin-like Molecule α Promotes Pathogenic Th17 Cell Responses and Bacterial-Induced Intestinal Inflammation

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Resistin-like molecule (RELMα) belongs to a family of secreted mammalian proteins that have putative immunomodulatory functions. Recent studies have identified a pathogenic role for RELMα in chemically induced colitis through effects on innate cell populations. However, whether RELMα regulates intestinal adaptive immunity to enteric pathogens is unknown. In this study, we employed Citrobacter rodentium as a physiologic model of pathogenic Escherichia coli–induced diarrheal disease, colitis, and Th17 cell responses. In response to Citrobacter, RELMα expression was induced in intestinal epithelial cells, infiltrating macrophages, and eosinophils of the infected colons. Citrobacter-infected RELMα−/− mice exhibited reduced infection-induced intestinal inflammation, characterized by decreased leukocyte recruitment to the colons and reduced immune cell activation compared with wild-type (WT) mice. Interestingly, Citrobacter colonization and clearance were unaffected in RELMα−/− mice, suggesting that the immune stimulatory effects of RELMα following Citrobacter infection were pathologic rather than host-protective. Furthermore, infected RELMα−/− mice exhibited decreased CD4 T cell expression of the proinflammatory cytokine IL-17A. To directly test whether RELMα promoted Citrobacter-induced intestinal inflammation via IL-17A, infected WT and IL-17A−/− mice were treated with rRELMα. RELMα treatment of Citrobacter-infected WT mice exacerbated intestinal inflammation and IL-17A expression whereas IL-17A−/− mice were protected from RELMα-induced intestinal inflammation. Finally, infected RELMα−/− mice exhibited reduced levels of serum IL-23p19 compared with WT mice, and RELMα−/− peritoneal macrophages showed deficient IL-23p19 induction. Taken together, these data identify a proinflammatory role for RELMα in bacterial-induced colitis and suggest that the IL-23/Th17 axis is a critical mediator of RELMα-induced inflammation. The Journal of Immunology, 2013, 190: 2292–2300.
responses, and we provide data that suggest RELMα is an upstream regulator of the proinflammatory cytokine IL-17A. Following exposure to DSS, RELMα−/− mice were protected from excessive intestinal inflammation, and ameliorated disease severity was associated with reduced CD4+ T cell–derived IL-17A.

To test whether the immune stimulatory effects of RELMα in the colon may be beneficial for host adaptive immunity to enteric pathogens, we employed the natural gastrointestinal pathogen of mice C. rodentium. Citrobacter belongs to the group of attaching and effacing bacteria, including enteropathogenic Escherichia coli (EPEC) and enterohemorrhagic Escherichia coli (EHEC), which are major causative agents of diarrheal diseases (14). Diarrheal and effacing bacteria, including enteropathogenic infections, factors, including IL-17A (17–21). In addition to defining the pendent on innate and adaptive immunity and several immune factors, including IL-17A (15, 16). Immunity to Citrobacter is dependent on innate and adaptive immunity and several immune factors, including IL-17A (17–21). In addition to defining the critical factors that are important for resistance to enteric bacterial infections, Citrobacter infection has been used as a model for inflammatory bowel disease, as it induces colonic inflammation characterized by crypt hyperplasia, thickening of the mucosa, and inflammatory cell infiltrate in wild-type (WT) mice (22).

Following Citrobacter infection, RELMα expression was upregulated early and expressed at the site of infection by epithelial cells, infiltrating macrophages, and eosinophils. Employing RELMα−/− mice and through administration of rRELMα, we demonstrate that RELMα promotes APC activation, Citrobacter-specific Th17 cell responses, and intestinal inflammation. Although Th17 cells are necessary for optimal immunity to Citrobacter (18), RELMα−/− mice did not exhibit significant differences in Citrobacter clearance compared with WT mice. Critically, Citrobacter-specific Th1 cell responses in RELMα−/− mice were not impaired, and mice could successfully eradicate Citrobacter, suggesting that targeting RELMα to prevent intestinal inflammation may not significantly compromise host intestinal immunity to bacterial pathogens. Furthermore, RELMα-mediated intestinal inflammation was abrogated in IL-17A−/− mice. These data place RELMα upstream of IL-17A and suggest that RELMα-directed inflammation requires IL-17A expression. Finally, infected RELMα−/− mice exhibited reduced serum levels of Th17-associated cytokine IL-23p19 compared with infected WT mice, and peritoneal macrophages isolated from naïve RELMα−/− mice showed impaired LPS-induced IL-23p19 expression, suggesting that RELMα promotes Th17 cell responses through stimulatory effects on macrophages. In conclusion, using two models of intestinal inflammation, we present data that identify a previously unrecognized pathway where RELMα exacerbates colitis through the IL-23/IL-17A immune axis.

Materials and Methods
Mice
WT C57BL/6 mice were purchased from The Jackson Laboratory or bred in-house, RELMα−/− mice were generated as previously described (10), IL-17A−/− mice were provided by Y. Iwakura and bred at the University of Pennsylvania. Mice were maintained in a specific pathogen-free facility. All experiments were carried out under the guidelines of the Institutional Animal Care and Use Committee at the University of Pennsylvania.

Dextran sodium sulfate
DSS (MP Biomedicals, Solon, OH) was added to drinking water at 5% w/v throughout the course of the experiment. Mice were monitored daily for morbidity (piloerection, lethargy), weight loss, and rectal bleeding. Severity of colitis (1–4) was scored as an average of the following parameters: feces: normal, 0; pasty, semiformalized, 1; sticky, 2; sticky with some blood, 3; completely liquid, bloody, or unable to defecate after 10 min, 4; rectal bleeding: no blood, 0; visible blood in rectum, 1–2; visible blood on fur, 3–4; general appearance: normal, 0; piloerection, 1; lethargic and piloerection, 2; lethargic and hunched, 3; motionless, sickly, 4.

C. rodentium infection model
Mice were infected by oral gavage with 0.2 ml overnight culture in Luria broth containing ∼5 × 10^8 CFU wild-type C. rodentium as previously described (19). Where indicated, control PBS or rRELMα (10 μg; PeproTech) was injected i.p. in 100 μl volumes. For bacterial counts, fecal pellets were collected, weighed, homogenized in PBS, and serial dilutions were plated on MacConkey agar (Sigma-Aldrich) and incubated overnight at 37°C. Bacterial colonies were counted the following day.

Histological staining
At necropsy, a 1-cm section of the distal colon was removed and flushed with PBS following by fixing in 4% paraformaldehyde and wax-embedded or frozen in OCT for cryosections. Five-micrometer sections were prepared and stained for H&E or with alcan blue–periodic acid-Schiff (PAS) reagent. Blinded clinical scoring of Citrobacter-infected mice was performed according to the following criteria: crypt hyperplasia (1–5) and mural inflammation/edema (1–5). For immunofluorescence and immunohistochemistry (IH), sections were dewaxed and stained with rabbit polyclonal anti-RELMα (PeproTech).

For immunofluorescence, dewaxed sections were stained with anti-RELMα and biotinylated anti-Siglec-F (R&D Systems), followed by secondary staining with Cy2-conjugated anti-rabbit secondary Ab and Cy3-conjugated streptavidin (Jackson ImmunoResearch Laboratories) and counterstaining with DAPI (Molecular Probes). For IH, the diaminobenzidine peroxidase substrate kit (Vector Laboratories) was used according to the manufacturer’s instructions.

Isolation of immune cells for analysis
At necropsy, mesenteric lymph nodes (mLNs) and spleens were harvested and single-cell suspensions were prepared. For lamina propria cell isolation, the colon was harvested, flushed with PBS, and cut into 1-cm pieces. To strip epithelial cells, colonic tissue was incubated with shaking in 5% FBS, 1 mM EDTA, and 1 mM DTT in PBS at 37°C for 20 min. Intestinal epithelial leukocytes were further isolated by shaking for 20 min in 1 mM EDTA/PBS. To obtain lamina propria lymphocytes, the remaining tissue was digested in a shaker with collagenase/dispase (0.5 mg/ml; Roche) and DNase type IV (30 μg/ml; Sigma-Aldrich) for 30 min followed by isolation of live cells by Percoll gradient. Recovered cells were stained with Aqua Live/Dead stain (Molecular Probes) followed by standard surface staining for flow cytometric analysis with fluorochrome-conjugated Abs (eBioscience, Becton-Dickinson Bioscience). For RELMα intracellular staining, cells were fixed and permeabilized using a fixation/permeabilization kit (eBioscience) and biotinylated anti-RELMα (1 μg/ml; PeproTech) followed by fluorochrome-conjugated streptavidin.

For peritoneal macrophage cultures, peritoneal lavage cells from naïve mice were recovered by thorough washing of the peritoneal cavity with 10 ml PBS. Peritoneal cells were plated in 96-well flat-bottom plates at 1 × 10^5 cells/well for 2–1 h, washed in warm media to enrich for adherent peritoneal macrophages, and stimulated with LPS (25 ng/ml; Sigma-Aldrich) and IFN-γ (20 ng/ml). At time points indicated, supernatants were recovered for analysis by ELISA, and cells were resuspended in RLT buffer (Qiagen) for RNA isolation.

Cytokine analysis
To examine CD4+ T cell activation, single-cell suspensions from spleens and/or mLNs were stimulated for 4 h ex vivo with PMA (50 ng/ml), ionomycin (500 ng/ml), and brefeldin A (10 μg/ml) (all from Sigma-Aldrich) or cultured for 48–72 h in medium alone, freeze-thawed Citrobacter Ag (30 μg/ml), or anti-CD3/anti-CD28 (1 μg/ml; eBioscience) followed by a brief (4 h) PMA/ionomycin stimulation in the presence of brefeldin A. Cells were surface and intracellular stained with the combination of fluorochrome Abs as indicated (obtained from eBioscience and Becton-Dickinson Biosciences) using the Cytofix/Cytoperm kit (Becton-Dickinson Biosciences) according to the manufacturer’s instructions.

Stained cells were acquired on a BD LSRII flow cytometer (Becton-Dickinson Biosciences) and analyzed using FlowJo software (Tree Star). To confirm analysis of CD4+ T cells, cells were also examined for CD3 and/or TCRRP surface expression. For restimulation cultures, cell-free supernatants were recovered and cytokine production was measured by sandwich ELISA. RELMα ELISAs were performed on serum recovered by cardiac puncture at necropsy, or on 1-cm distal colonic tissue mechanically homogenized in PBS. For RELMα ELISA, anti-RELMα capture Ab and biotinylated anti-RELMα detection Ab (both from PeproTech) were used.
Real-time RT PCR
Colonic tissue RNA was isolated by TRizol (Invitrogen) and peritoneal macrophage RNA by the RNeasy kit (Qiagen) in accordance with the manufacturer’s instructions. cDNA was generated and analyzed by real-time PCR using SYBR Green technology (Applied Biosystems) with customized primers (Qiagen). Reactions were run on the GeneAmp 7500 sequence detection system (Applied Biosystems). Results were standardized to the housekeeping gene β-actin.

Statistical analysis
Results represent the mean ± SEM of individual animals or replicate wells. Statistical significance was determined by the two-tailed Student t test, one-way ANOVA, or two-way ANOVA using Prism GraphPad software (version 4). Results were considered significant when p < 0.05.

Results
RELMα promotes DSS-induced intestinal inflammation and Th17 cell responses
Previous studies reported that RELMα was proinflammatory in response to DSS, where it promoted innate immune cell activation and proinflammatory chemokine and cytokine expression in DSS-exposed mice (2, 3). Because DSS-induced intestinal inflammation is mediated both by innate and adaptive immune cells (23), and given recent findings that RELMα regulates CD4+ T cell responses (10), we first examined whether, in addition to regulation of innate immune cell activation, RELMα also regulated CD4+ T cell responses in this model.

Following 5% DSS treatment in the drinking water as a model for acute DSS colitis, WT C57BL/6 mice exhibited increased expression of Retnla (the gene encoding RELMα) in the colon (Supplemental Fig. 1) as well as recruitment of RELMα+ cells to the lamina propria (Supplemental Fig. 1B). Consistent with previous studies showing that RELMα expression promoted intestinal inflammation, RELMα−/− mice exhibited less severe DSS-induced weight loss (Supplemental Fig. 1C) and reduced disease severity at day 7, as measured by fecal consistency, rectal bleeding, and general appearance (Supplemental Fig. 1D). Histological examination of colonic tissue sections from day 7 DSS-treated mice revealed that RELMα−/− animals were protected from DSS-induced colonic lesions and demonstrated normal crypt architecture, lack of ulceration, and less severe inflammatory cell infiltration than did WT controls (Supplemental Fig. 1E).

Intestinal inflammation resulting from 5% DSS treatment is associated with CD4+ Th1 and Th17 cell activation (24, 25). To test whether RELMα regulated these helper T cell subsets, mLN cells from DSS-treated WT or RELMα−/− mice were polyclonally stimulated and IFN-γ and IL-17A production were examined by ELISA. In comparison with DSS-treated WT mice, mLN cells from DSS-treated RELMα−/− mice exhibited equivalent IFN-γ production but significantly reduced IL-17A production (Supplemental Fig. 1F). Furthermore, intracellular flow cytometric analysis revealed significantly reduced CD4+ T cell-derived IL-17A in the absence of RELMα (Supplemental Fig. 1G). Associated with reduced Th17 cell responses in RELMα−/− mice, real-time PCR analysis of the colons of DSS-treated WT and RELMα−/− mice revealed reduced expression of factors associated with Th17 cell polarization, including Rorc, Il23a, and Il17a (Supplemental Fig. 1H). Collectively, this provides the first demonstration that RELMα contributes to CD4+ Th17-mediated inflammation and implicates RELMα in exacerbating intestinal inflammation following DSS exposure.

C. rodentium infection induces local and systemic RELMα expression
Although Th17 cell activation and IL-17A production are associated with multiple inflammatory diseases, including colitis, arthritis, and asthma, IL-17A expression is necessary for host immunity to several fungal and bacterial pathogens (18, 26–29). To test the hypothesis that RELMα-mediated IL-17A expression is host-protective in the context of infection-induced inflammation, we employed C. rodentium, a natural bacterial pathogen of mice that colonizes the colon, induces intestinal inflammation (22), and requires IL-17A for optimal clearance (18, 20).

Quantification of Citrobacter loads in infected WT C57BL/6 mice revealed detectable Citrobacter colonization at day 3 postinfection, maximal bacterial burden at day 5 postinfection, and clearance by day 21 postinfection (Fig. 1A). Colonies from naïve and Citrobacter-infected mice were recovered for Retnla mRNA and RELMα protein quantification. Compared to naïve mice, RELMα expression in the colon was upregulated at days 3 and 9 postinfection, correlating with maximal fecal Citrobacter burdens (Fig. 1B, 1C). Examination of RELMα protein expression by IF of colon tissue sections revealed Citrobacter-induced RELMα expression in the intestinal crypts and lamina propria (Fig. 1D). High-power magnification of anti-RELMα immunofluorescent-stained sections (Fig. 1E, green) confirmed staining by intestinal epithelial cells in the crypts and by macrophages and Siglec-F+ eosinophils in the lamina propria. In addition to expression of RELMα locally at mucosal sites, previous reports have shown that RELMα can be detected systemically (2). Quantification of RELMα serum levels by ELISA revealed that Citrobacter infection induced increased and maintained systemic RELMα production (Fig. 1F).

To quantify the cell populations in the colon that express RELMα following Citrobacter infection, cells isolated from the colonic tissue of naïve or day 10 Citrobacter-infected mice were analyzed by intracellular RELMα staining. To control for background staining and inherent autofluorescence of colonic cell preparations, colon cells from infected RELMα−/− mice were also examined. Viable cells were gated, and intestinal epithelial cells were determined according to epithelial cell adhesion molecule surface expression (Fig. 2A). Colonic epithelial cells from naïve mice (Fig. 2A, dashed histogram) did not exhibit significant positive RELMα staining in comparison with control RELMα−/− epithelial cells (gray histogram). Following Citrobacter infection, there was a noticeable shift in RELMα+ cells with 64% of epithelial cells staining positive for RELMα (black histogram). Additionally, colonic F4/80Ly6G+ macrophages (Fig. 2B) and F4/80+ Siglec-F+ eosinophils (Fig. 2C) exhibited infection-induced RELMα expression. Given previous studies showing dendritic cell (DC) expression of RELMα (5, 30), CD11c+F4/80− colon cells were examined but no RELMα+ staining above background was observed (data not shown). To determine the relative contribution of RELMα by epithelial cells, macrophages, and eosinophils, the frequency of RELMα+ cells as a percentage of live cells was quantified (Fig. 2D). Although significant infection-induced increases in RELMα expression by epithelial cells and macrophages were observed, there was no obvious contribution of RELMα by eosinophils due to the low frequency of this population in the infected colons. Taken together, these data employ two separate methods to identify epithelial cells and macrophages as dominant cellular sources of RELMα in the colon following exposure to Citrobacter.

RELMα−/− mice are resistant to Citrobacter-induced colitis
To examine whether the upregulation of RELMα played a functional role in enteric bacterial infection, WT or RELMα−/− mice were infected with C. rodentium and sacrificed at day 10 postinfection, a time point that correlates with maximal bacterial burden, intestinal inflammation, and RELMα expression. Macroscopic examination of the colons of naïve and infected mice revealed that
FIGURE 1. C. rodentium infection induces RELMα expression. C57BL/6 mice were infected with Citrobacter. (A) Bacterial burdens in the feces were measured. (B) Retnla mRNA in colon tissue was quantified by real-time PCR as fold induction over naive. (C) RELMα protein was quantified in naive and Citrobacter-infected colon tissue homogenate by ELISA. (D and E) Localization of RELMα expression in the colon was examined by IH (D, RELMα, brown) and by immunofluorescent staining (E, RELMα, green; Siglec-F, red; DAPI, blue) at day 6 post-infection. C, crypt; LP, lamina propria. Scale bar, 20 μm. (F) RELMα serum levels. *p < 0.05. Data are representative of three experiments with two to four mice per group.

Infected WT mice exhibited characteristic signs of Citrobacter-induced colonic inflammation, including loose stools and significant colon shortening (Fig. 3A). In contrast, infected RELMα−/− mice exhibited minimal signs of infection-induced inflammation with fecal pellet consistency and colon lengths that were comparable to naive mice. Examination of PAS/alcan blue–stained colonic tissue sections did not reveal histological differences between naive WT and RELMα−/− colons at steady-state (Fig. 3B, left panels). Citrobacter infection induced severe colitis in WT mice characterized by leukocyte infiltration in the lamina propria and submucosa, reduced mucin production, crypt hyperplasia, and thickening of the muscularis externa. In contrast, colons from RELMα−/− mice were minimally affected and exhibited little to no intestinal inflammation (Fig. 3B, right panels). Blind clinical scoring of colon sections from infected WT and RELMα−/− mice confirmed that there was significantly reduced Citrobacter-induced intestinal inflammation in the absence of RELMα (Fig. 3C).

Taken together, these data reveal a proinflammatory function for RELMα in enteric bacterial infection.

Ameliorated Citrobacter-induced intestinal inflammation in RELMα−/− mice is associated with reduced immune cell activation and lower Th17 cell responses

Previous studies demonstrated an immunomodulatory role for RELMα on APCs and T cells (2, 10). We therefore examined whether the ameliorated intestinal inflammation observed in RELMα−/− mice correlated with reduced intestinal DC, macrophage, and CD4+ T cell activation. First, no significant differences in the frequency of DCs, monocytes, or macrophage populations were detected between Citrobacter-infected WT and RELMα−/− mice (Supplemental Fig. 2A, 2C). Additionally, there were no obvious differences in surface MHC class II expression in macrophages or DCs isolated from the colons of naive WT or RELMα−/− mice (Supplemental Fig. 2D, 2E). However, following Citrobacter infection, colonic DCs from WT mice exhibited increased surface MHC class II expression (Fig. 4A, 4B, black histogram) compared with DCs isolated from RELMα−/− mice (Fig. 4B, gray histogram). Quantification of infection-induced MHC class II expression

FIGURE 2. C. rodentium infection induces RELMα expression in epithelial cells, macrophages, and eosinophils. Colons of naive (N, dashed) or day 10 infected (Inf, solid line) WT mice were recovered for flow cytometric analysis of intracellular RELMα. Plots shown were previously gated on live cells. (A) Epithelial cells (EC) were gated as epithelial cell adhesion molecule (Epcam)+F4/80− cells. (B) Macrophages (Mac) were gated as F4/80+Ly6G− cells. (C) Eosinophils (Eos) were gated as Ly6G+F4/80−Siglec-F+ cells. Numbers indicated represent frequency of positive cells in infected WT mice. For control RELMα staining (filled gray graph), gated epithelial cells (A), macrophages (B), or eosinophils (C) from day 10–infected RELMα−/− mice were stained with anti-RELMα. (D) RELMα+ cell populations in the colon as a proportion of live cells was measured. *p < 0.05. Data from three naive or infected mice per group are representative of three separate experiments.
Given the reduced proliferative capacity of the RELMα−/− mice, we hypothesized that the reduced Citrobacter-specific immune cell response in RELMα−/− mice may result in increased Citrobacter burden. Although there was a modest delay in Citrobacter elimination in RELMα−/− mice at day 14 postinfection (Fig. 4I), we observed no significant differences in the kinetics of Citrobacter colonization and clearance between WT or RELMα−/− mice, suggesting that in the context of enteric bacterial infection, the immunostimulatory effects of RELMα contribute to inflammatory pathology rather than a critical host-protective function.

**FIGURE 3.** RELMα−/− mice are resistant to Citrobacter-induced colitis. WT or RELMα−/− mice were infected with Citrobacter for 10 d. (A) Infection-induced colon shortening was determined and quantified as percentage of uninfected controls. Scale bars, 1 cm. (B and C) Histologic examination of infection-induced inflammation in PAS/alcan blue stained sections of distal colonic tissue was performed and scored. Scale bars, 50 μm. Data are representative of four experiments with three to four infected mice per group. *p < 0.05, **p < 0.01.

**RELMA-induced intestinal inflammation following Citrobacter infection is dependent on IL-17A.**

Employing RELMα−/− mice in two models of intestinal inflammation, these data have revealed a previously unrecognized function for RELMα in influencing Th17 cell responses. However, Citrobacter-infected RELMα−/− mice also exhibited reduced macrophage activation and CD4+ T cell proliferation, suggesting that RELMα may promote intestinal inflammation via mechanisms other than IL-17A production. To test this hypothesis, Citrobacter-infected WT and IL-17A−/− mice were treated with rRELMα and examined at day 10 postinfection for intestinal inflammation and T cell activation. In WT mice, Citrobacter infection induced characteristic colonic lesions consisting of leukocyte infiltration, submucosal edema, and crypt hyperplasia, and treatment of WT mice with RELMα−/− mice also exhibited reduced colon inflammation and T cell activation. In contrast, infected IL-17A−/− mice exhibited less severe intestinal inflammation, edema, and crypt hyperplasia, consistent with the known proinflammatory function of IL-17A (Fig. 6A, right panels). Strikingly, unlike WT mice, RELMα treatment of IL-17A−/− mice did not exacerbate Citrobacter-associated intestinal inflammation, suggesting that IL-17A is a necessary mediator of RELMα-directed inflammation. Blind pathology scoring confirmed that RELMα treatment significantly increased the severity of Citrobacter-induced inflammation in WT mice but not IL-17A−/− mice (Fig. 6B). To examine the effect of RELMα treatment on CD4+ T cell activation, CD4+ T cells were stimulated ex vivo with PMA/ ionomycin and stained for intracellular cytokines. Compared to naive control WT mice, there was an increase in the frequency of CD4+ T cell–derived IL-17A following Citrobacter infection, differentially express IFN-γ. Collectively, these data identify an immunostimulatory role for RELMα in promoting bacterial infection-induced intestinal macrophage and Th17 cell activation. Because immunity to Citrobacter is dependent on macrophage activation and on Th17 cells (20, 31), we hypothesized that the reduced Citrobacter-specific immune cell response in RELMα−/− mice may result in increased Citrobacter burden. Although there was a modest delay in Citrobacter elimination in RELMα−/− mice at day 14 postinfection (Fig. 4I), we observed no significant differences in the kinetics of Citrobacter colonization and clearance between WT or RELMα−/− mice, suggesting that in the context of enteric bacterial infection, the immunostimulatory effects of RELMα contribute to inflammatory pathology rather than a critical host-protective function.

**rRELMα treatment induces Citrobacter-induced colitis**

To determine whether the dampened intestinal inflammation seen in RELMα−/− mice could be restored by exogenous administration of rRELMα, Citrobacter-infected RELMα−/− mice were injected i.p. with PBS or rRELMα throughout infection. Despite high concentrations of administered rRELMα, rRELMα-treated mice had much lower RELMα levels than did WT mice (Fig. 5A, compare with Fig. 1F). However, histologic examination of H&E-stained colon tissue sections from PBS and RELMα-treated mice revealed exacerbated Citrobacter-induced intestinal lesions following RELMα treatment characterized by increased crypt hyperplasia, submucosal edema (Fig. 5B, arrow), and leukocyte infiltration (Fig. 5B, box) relative to PBS-treated mice. Finally, treatment of Citrobacter-infected RELMα−/− mice with rRELMα was sufficient to induce significantly increased intestinal inflammation compared with PBS-treated mice (Fig. 5C). Collectively, these data suggest that RELMα directly contributes to intestinal inflammation during Citrobacter infection.
which was enhanced with RELMα treatment (Fig. 6C). To examine CD4+ T cell activation in infected IL-17A−/− mice, CD4+ T cell–derived IFN-γ and TNF-α were quantified (Fig. 6D, 6E). Whereas RELMα treatment of infected WT mice resulted in the increased frequency (Fig. 6D, top panels) and total number (Fig. 6E) of IFN-γ+TNF-α+ coproducers, RELMα treatment had no effect on CD4+ T cells from infected IL-17A−/− mice (Fig. 6D, bottom panels, 6E). Taken together, these data suggest that RELMα-induced intestinal inflammation following Citrobacter infection is dependent on IL-17A.

FIGURE 4. Ameliorated Citrobacter–induced intestinal inflammation in RELMα−/− mice is associated with reduced immune cell activation and Th17 cell responses. WT and RELMα−/− mice were infected with Citrobacter for 10 d followed by leukocyte preparations of the colonic tissue. (A) Gating strategy of live, CD45+Ly6G−Ly6C+ for CD11c+ DCs and F4/80+ macrophages (Mac). Number denotes frequency of gated cells. (B and C) DCs (B) and Macs (C) were analyzed for MHC class II expression. The change in mean fluorescence intensity (∆MFI) was calculated as the difference between naive and infected MHC class II MFI. (D–F) CD4+CD3+ T cells from the mLNs were analyzed ex vivo for expression of Ki67 (D, E) and CD44 (F). (G–I) mLNs from Citrobacter-infected WT and RELMα−/− mice were stimulated with Citrobacter Ag for 48 h followed by intracellular cytokine staining for CD4+ T cell–derived IL-17A (G, H) IL-17F, IL-22, and IFN-γ (I). (J) Citrobacter burdens from fecal pellets harvested from WT and RELMα−/− mice throughout the course of infection. *p < 0.05, **p < 0.01. Data are from three to four naive or infected mice per group are representative of three experiments.

FIGURE 5. rRELMα induces Citrobacter–induced colitis. Citrobacter–infected RELMα−/− mice were treated with PBS, rRELMα (10 µg) at days 0, 3, 6, and 9 postinfection, and sacrificed at day 10. (A) RELMα serum levels. (B) H&E-stained sections of colonic tissue from PBS- or rRELMα-treated Citrobacter–infected mice. (C) Pathology score based on histological sections shown in (B). Scale bars, 50 µm. Arrow points to submucosal inflammation. Box, crypt inflammation; LOD, limit of detection. *p < 0.05. Data are representative of two experiments of three to five mice per group.
Macrophages from RELMα−/− mice exhibit impaired production of IL-23p19

Given the selective impairment in Citrobacter-induced Th17 cell responses in the absence of RELMα, we hypothesized that RELMα−/− mice may exhibit impaired expression of IL-23, a critical cytokine for the development and maintenance of CD4+ Th17 cells. Consistent with this, IL-23p19 levels in the serum of Citrobacter-infected RELMα−/− mice were significantly reduced compared with infected WT mice (Fig. 7A). Taken together, these data suggest that the immunostimulatory effects of RELMα act through promoting the IL-23/Th17 immune axis; however, whether RELMα was required for CD4+ Th17 cell differentiation or for activation of APCs such as macrophages was unknown. In vitro Th17 cell polarized splenocyte cultures from WT or RELMα−/− mice revealed no defect in IL-17A production by RELMα−/− CD4+ T cells (data not shown).

Because cytokine-mediated Th17 polarization was not affected in RELMα−/− CD4+ T cells in vitro, and we had observed impaired macrophage activation in vivo following Citrobacter infection, we tested the hypothesis that intrinsic defects in RELMα−/− macrophages could explain the impaired infection-induced Th17 cell response. Peritoneal macrophages from naive WT or RELMα−/− mice were treated ex vivo with the bacterial ligand LPS and immunostimulatory IFN-γ and assayed for expression of inflammatory cytokines IL-12p40, TNF-α, and IL-23p19. Although there was no significant difference in LPS/IFN-γ-induced TNF-α and IL-12p40 by RELMα−/− peritoneal macrophages (data not shown), there was a selective impairment in the ability of RELMα−/− macrophages to upregulate expression of IL-23p19 RNA transcripts (Fig. 7B) and protein (Fig. 7C). Collectively, the data presented in this study demonstrate that macrophages in RELMα−/− mice exhibit intrinsic defects in IL-23p19 expression, which may have important consequences for CD4+ Th17 development and intestinal inflammation.

Discussion

RELMα is a constitutively expressed protein that has been associated with multiple infectious and inflammatory responses. Previous work has demonstrated dysregulated RELMα expression in insulin resistance, helminth infection, type 2–associated lung inflammation, and chemically induced colitis (2, 3, 10, 30, 32). These studies have identified that in these various settings, RELMα plays a critical role in glucose homeostasis, can interact directly with innate immune responses following intestinal injury. However, the role of RELMα in coordinating a Th17 immune response and its potential function in response to bacterial infection were unknown. In this study, we report that following infection with Citrobacter, a murine model for EPEC/EHEC intestinal diseases in humans, RELMα exacerbates intestinal inflammation. Collectively, this study demonstrates several key findings that contribute to our understanding of this immunomodulatory molecule (Supplemental Fig. 3). First, we demonstrate that intestinal epithelial cells and macrophages are potent sources of RELMα in the colon of Citrobacter-infected mice. Second, using both RELMα−/− mice and treatment with exogenous RELMα, we show that RELMα pro-

FIGURE 6. RELMα-induced intestinal inflammation is dependent on IL-17A. Citrobacter-infected WT or IL-17A−/− mice were treated i.p. with PBS or 10 μg rRELMα at days 0, 3, 6, and 9 postinfection and sacrificed at day 10. (A) H&E-stained colonic tissue sections of naive or infected mice were examined. Scale bars, 100 μm. (B) Infected mice were scored for intestinal inflammation. (C–E) Splenocytes were stimulated ex vivo with PMA/ionomycin. (C) Expression of IFN-γ and TNF-α by CD4+CD44hi T cells expressing IL-17A. (D) Number of CD44hiCD4+ T cells expressing IL-17A. (E) Number of CD44hiCD4+ splenocytes that are IFN-γ+TNF-α+ coproducers. N, WT naive; n.d., not detected. *p < 0.05, **p < 0.01. Data are representative of two experiments (naive, n = 4; infected, n = 7).

FIGURE 7. Macrophages from RELMα−/− mice exhibit impaired production of IL-23p19. (A) IL-23p19 serum levels in Citrobacter-infected WT and RELMα−/− mice were measured. (B and C) Adherent peritoneal macrophages from naive WT or RELMα−/− mice were treated overnight with medium or activated with LPS/IFN-γ. (B) At time points indicated cells were recovered for IL23a real-time RT-PCR analysis. (C) Overnight supernatants were recovered for measurement of IL-23p19 by ELISA. *p < 0.05. Data are representative of two separate experiments with three to four replicate wells.
motes APC and CD4+ T cell activation at the site of infection, and that genetic deletion of RELMα limits infection-induced colitis. Third, we identify the IL-23/Th17 immune axis as a downstream effector pathway that mediates RELMα-induced intestinal inflammation. Specifically, this study identifies a new secreted factor that influences intestinal disease following enteric bacterial infection. Additionally, our findings suggest that targeting this protein, the cell types that express it, or the downstream effector pathways may offer new therapies to alleviate the symptoms of EPEC/EHEC intestinal diseases.

Although RELMα is a signature gene of alternatively activated macrophages and has important roles in helminth infection and allergy, its function in other inflammatory environments is less well characterized. To our knowledge, for the first time we have examined whether RELMα is involved in the immune response to a pathogenic bacterial infection and demonstrate a critical role for RELMα expression in promoting infection-induced inflammation. These findings are consistent with a previous report demonstrating that RELMα−/− mice were protected from DSS-induced colitis and extend our knowledge of how RELMα contributes to intestinal immunity and tissue inflammation. Importantly, our studies demonstrate that although RELMα−/− mice exhibited diminished Citrobacter-specific Th17 cell responses, they did not suffer from impaired immunity to Citrobacter. Thus, in this study we have effectively demonstrated that host-protective adaptive immunity can be uncoupled from tissue-damaging inflammation mediated by RELMα and Th17 cell responses in a model of infection-induced colitis.

Given the importance of IL-17A in clearance of Citrobacter infection (18, 20), we were surprised that RELMα−/− mice successfully cleared their bacteria. However, although the frequency is decreased compared with WT mice, infected RELMα−/− animals do generate a pool of Citrobacter-responsive CD4+ Th17 cells, as well as equivalent Citrobacter-specific Th1 cell responses (Fig. 4). Indeed, the protective role of Ag-specific CD4+ T cells that has been demonstrated, and mice lacking IFN-γ-producing CD4+ T cells demonstrated greater weight loss and fecal bacterial burden following Citrobacter infection (33). The combination of these responses may be sufficient for successful Citrobacter clearance in infected RELMα−/− mice. In addition to selective defects in IL-17A cytokine expression, CD4+ T cells from the colon and draining mLN of RELMα−/− mice exhibited striking defects in their activation and proliferation, as examined by CD44 and Ki67 staining. RELMα is highly mitogenic in certain lung inflammation models (34), and we have previously shown that RELMα can bind CD4+ T cells (10). We tested the hypothesis that intrinsic RELMα expression was necessary for Th17 differentiation and/or proliferation through in vitro polarization assays, and although we did not observe defects in RELMα−/− CD4+ T cells in this setting, it is possible that in vivo inflammatory conditions RELMα may affect local T cell activation and proliferation.

Because direct effects of RELMα deletion in CD4+ T cells were not the apparent cause of the diminished Citrobacter-specific Th17 response in RELMα−/− mice, we tested the influence of RELMα expression on innate immune cell populations that could ultimately influence the quality of the adaptive immune response.

We demonstrate in this study that Citrobacter infection induced upregulation of RELMα in colonic macrophages and eosinophils as well as nonhematopoietic intestinal epithelial cells in WT animals. Quantification of the contribution of RELMα-expressing innate immune cell populations demonstrated that following Citrobacter infection, macrophages were the primary source of hematopoietic-derived RELMα. Previous studies have shown increased RELMα expression in the lung in response to bacterial LPS (35), and we have previously proposed that RELMα may be induced directly in response to injury (36). The Citrobacter-induced expression of RELMα in the colon that we report in this study may therefore be triggered by Citrobacter LPS and/or as a consequence of the injury induced by pathogenic bacterial infection. Consistent with this hypothesis and previous reports, we show in this study that RELMα expression is also induced in the intestine in response to chemically induced injury with DSS.

To determine whether the infection-induced upregulation of RELMα in colonic macrophages had a functional role, we examined whether RELMα−/− macrophage activation or function was impaired in response to bacterial stimulation. Indeed, following Citrobacter infection, colonic RELMα−/− macrophages failed to upregulate MHC class II to the same extent as in WT mice. Additionally, RELMα−/− macrophages displayed selective defects in their ability to express the Th17-associated cytokine IL-23 following bacterial ligand stimulation. Previous studies have shown that RELMα treatment of macrophages in vitro induces JNK signaling and proinflammatory cytokine expression (3). Thus, these data suggest that RELMα promotes CD4+ T cell IL-17A expression via macrophage activation and polarization. Taken together with our previous studies demonstrating that RELMα plays a critical role in limiting type 2 inflammation, our present data provoke the hypothesis that RELMα may act as an immunological rheostat and play a role in tuning the type of immune response generated following infection. Importantly, our results suggest that targeting RELMα may be beneficial for ameliorating intestinal inflammation without compromising intestinal immunity to enteric bacteria.

Critically, RELMα-induced intestinal inflammation was abrogated in the absence of IL-17A, demonstrating that IL-17A is downstream of the proinflammatory function of RELMα. In contrast to most pathogens, where infection-induced T cell activation occurs 1–2 wk postinfection, recent studies reported that Citrobacter induces a significant population of CD4+ TCRβ+ IL-17A−producing T cells at the infection site as early as day 4 postinfection (20). The early induction of RELMα at the site of infection is consistent with the possibility that RELMα directly influences this early Th17 cell response to Citrobacter infection.

Collectively, the results presented in this study reveal a previously unrecognized role for RELMα in enteric bacterial infection, and they uncover a new pathway by which RELMα promotes intestinal inflammation via an IL-23/IL-17A-dependent inflammatory pathway. These findings suggest that immunotherapies targeting RELMα may provide a way to limit intestinal inflammation without significantly impairing mucosal Th17 immune responses.

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Disclosures

The authors have no financial conflicts of interest.

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