Resistin-like molecule α enhances myeloid cell activation and promotes colitis

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Background: Resistin-like molecule (Relm) α is a secreted protein and a hallmark signature gene for alternatively activated macrophages. Relm-α is highly induced by allergic inflammatory triggers and perceived to promote tissue repair. Yet the function of Relm-α remains unknown.

Objective: We sought to determine the role of Relm-α in dextran sodium sulfate (DSS)-induced colonic injury.

Methods: The cellular source of Relm-α was determined after oral DSS-induced colitis. Retnla<sup>-/-</sup> mice were generated, subjected to DSS treatment, and monitored for disease progression (clinical and histopathologic features). Cytokine production in the supernatants of ex vivo colon cultures, and of LPS-stimulated macrophages incubated with Retnla<sup>-/-</sup> was assessed. Relm-α was administered intraperitoneally, and the cellular recruitment to the peritoneum was assessed.

Results: After innate intestinal stimulation with DSS, Relm-α was highly expressed by eosinophils and epithelial cells. Retnla<sup>-/-</sup> gene–targeted mice were protected from DSS-induced colitis (eg, decreased diarrhea, rectal bleeding, colon shortening, disease score, and histopathologic changes). Relm-α coactivated IL-6 and TNF-α release and inhibited IL-10 release from LPS-activated bone marrow–derived macrophages. Consistent with these findings, colon cultures of DSS-treated Retnla<sup>-/-</sup> mice produced decreased IL-6 and increased IL-10<sup>ex vivo</sup>. Furthermore, Retnla<sup>-/-</sup> mice had substantially decreased c-Jun N-terminal kinase phosphorylation in vivo. In vivo administration of Relm-α initiated cellular recruitment to the peritoneum, and Relm-α was able to induce eosinophil chemotaxis in vitro.

Conclusions: These findings demonstrate a central proinflammatory role for Relm-α in colonic innate immune responses, identifying a novel pathway for regulation of macrophage activation. (J Allergy Clin Immunol 2008;122:1200-7.)

Key words: Resistin-like molecule α, colitis, macrophages, eosinophils, LPS, inflammation, inflammatory bowel disease

Immune-related diseases, such as inflammatory bowel disease (IBD), diabetes, and obesity, have become some of the fastest growing and most persistent public health problems in the Western world and are currently on the rise. These diseases have a shared component of inflammation involved in disease pathogenesis and complications. Substantial evidence suggests that macrophages have a key role in the pathogenesis of inflammatory diseases, such as IBD. After diverse stimuli, macrophages are capable of releasing a wide plethora of mediators, including superoxide anions, reactive nitrogen intermediates (eg, nitric oxide and peroxynitrite), bioactive lipids, and cytokines, that might lead to cell death and tissue injury. For example, LPS-stimulated macrophages produce TNF-α and IL-6, and increased levels of these cytokines and activated macrophages correlate with IBD severity.

Resistin-like molecule (Relm) α belongs to a newly defined family of secreted proteins (Resistin, Relm-α, Relm-β, and Relm-y) that contain highly conserved C-terminal cysteine residues, which support the assembly of disulfide-dependent multimeric units. Interestingly, and despite opposing physiologic effects on insulin resistance, the multimeric assembly of the resistin family is similar to that of adiponectin and suggests a functional role for Relm in the regulation of glucose. formerly known as “found in inflammatory zone 1,” is a hallmark signature gene of murine alternatively activated macrophages and is thought to be involved in processes such as tissue repair. Functional studies demonstrate that Relm-α stimulates α-smooth muscle actin and collagen I production by fibroblasts. Furthermore, other hallmark alternatively activated macrophage products, such as arginase-1, have been shown to contribute to tissue repair. Collectively, these data suggest an anti-inflammatory role for Relm-α. Nevertheless, the function of Relm-α in vivo and especially in intestinal inflammation is yet to be defined. Importantly, Relm-α and Relm-β are highly induced by allergic inflammatory triggers (eg, T<sub>H2</sub> cytokines, such as IL-13) yet have differential expression patterns and pleiotropic...
functions. The expression of Relm-β at baseline is restricted to the colonic epithelium and regulated by commensal bacteria through Caudal type homeobox transcription factor 2 (Cdx2), whereas Relm-α is mainly expressed by hematopoietic cells and adipose tissue and is more similar to the expression pattern of resistin.

In this study we aimed to determine the cellular source and role of Relm-α in vivo. Herein, we demonstrate that after innate intestinal stimulation with dextran sulfate sodium (DSS), Relm-α is highly expressed by eosinophils and colonic epithelial cells. Furthermore, Relm-α deficiency protects against experimental colitis, and recombining Relm-α had potent proinflammatory effects on macrophages, identifying a key role for Relm-α in colonic inflammation.

METHODS

Mice

Male and female, 8- to 12-week-old Retnla<sup>b−</sup> mice (backcrossed to c57Bl/6 or BALB/c background for at least 7 and 10 generations, respectively) were generated by using Velocigene technology (Regeneron Pharmaceuticals, Inc., Tarrytown, NY).<sup>10</sup> Mice were genotyped by means of PCR analysis with the following primers: Relm-α forward, GTCAAGAATCCCCGTTGCTA; Relm-α reverse, ACTTCTTACCCACCTTACC; and Lac-Z, GTCGTCGC TAGCTTCCACTG. This yielded an 800-bp band for wild-type mice and a 400-bp band for gene-targeted mice. Wild-type mice (4-5 weeks old) were obtained from Taconic Laboratories (Hudson, NY) and environmentally matched with the Retnla<sup>b−</sup> mice for 2 to 3 weeks. All mice were housed under specific pathogen-free conditions and treated according to institutional guidelines.

DSS-induced colonic injury and histopathologic examination

DSS (average molecular weight of 41 kd; ICM Biomedical, Inc, Costa Mesa, Calif) was administered in the drinking water as a 2.5% (wt/vol) solution for up to 10 days, and disease monitoring and histopathologic changes in the colon were scored as previously described.<sup>10</sup>

Cell activation

Bone marrow–derived macrophages (2 × 10<sup>7</sup> cells) were incubated for 24 hours with recombining Relm-α (kind gift of Peprotech, Rocky Hill, NJ) or LPS (Sigma-Aldrich, St Louis, Mo). Thereafter, the supernatant was collected and stored at −70°C until assessed for cytokines.

Immunofluorescence

Fixed frozen sections were treated with 100% acetone and blocked with 3% goat serum in PBS. Slides were incubated with isotype controls (Rat IgG1 and Rabbit IgG; Vector, Burlingame, Calif), anti–Mac-3 (BD PharMingen, San Jose, Calif), anti–major basic protein (anti-MBP; kindly provided by James Lee, Mayo Clinic, Rochester, Minn), anti–phospho c-Jun N-terminal kinase (anti-pJNK; Cell Signaling, Danvers, Mass), and anti–Relm-α and anti–Relm-β (kind gift of Peprotech, overnight at 4°C), followed by goat anti-rabbit Alexa 488 and donkey anti-rat Alexa 494 (Invitrogen, Carlsbad, Calif) and counterstaining with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI)/Supernount G solution (Fluoromount-G; Southern Biotech, Birmingham, Ala). Images were captured with a Zeiss microscope and Axiosview image analysis software (Deutschland; Carl Zeiss Corp, Jena, Germany).

Western blotting

Whole colon lysates were loaded to 4% to 12% Bis-Tris Gels and transferred to a nitrocellulose membrane (Invitrogen). Arginase and YM1 were detected by using rabbit anti–arginase-1 (Abcam, Cambridge, Mass) and anti–YM1 (StemCell Technologies, Vancouver, British Columbia, Canada) antibodies followed by anti-rabbit peroxidase-conjugated antibody (Cell Signaling) and ECL-plus detection reagents (GE Healthcare, Buckinghamshire, United Kingdom). Anti-actin (Santa Cruz Biotechnology, Santa Cruz, Calif) was used as loading control.

ELISA

Cytokine levels were measured in the supernatants of LPS-activated (Sigma-Aldrich) and Relm-α–activated (Peprotech) bone marrow–derived macrophages or supernatants of colon punch biopsy specimens with a commercially available ELISA Duo-Set kit, according to the manufacturer’s instructions (R&D Systems, Minneapolis, Minn). Lower detection limits for IL-6, TNF-α, and IL-10 were 15.6, 32.25, and 15.6 pg/mL, respectively. For detection of serum Relm-α, purified anti–Relm-α and biotinylated anti–Relm-α (Peprotech) were used according to a protocol provided by the manufacturer. The lower detection limit for Relm-α was 15.62 pg/mL.

Relm-α administration

Recombinant Relm-α (2 and 20 μg per mouse) was injected intraperitoneally in 200 μL of saline. Twenty-four and 48 hours after injection, the mice were killed, and peritoneal lavage and cell counts were performed.

Chemotaxis assay

Chemotaxis of eosinophils obtained from the spleens of CD2-IL-5Tg mice toward Relm-α was assessed as previously described.<sup>11</sup>

Statistical analysis

Data were analyzed by means of ANOVA, followed by the Tukey post-hoc test with GraphPad Prism 4 (San Diego, Calif). Data are presented as the mean ± SD. P values of less than .05 were considered statistically significant.

RESULTS

Relm-α expression in experimental colitis

We first aimed to determine the cellular source of Relm-α in the colon during a DSS-induced model of experimental colitis. This model directly damages the intestinal epithelial barrier, resulting in activation of innate immune cells (predominantly tissue macrophages) and recruitment of additional macrophages, neutrophils, and eosinophils.<sup>2</sup> After DSS treatment, CD68<sup>+</sup> myeloid cells accumulated in the colon (see Fig E1 in this article’s Online Repository at www.jacionline.org). Phenotyping of these cells by means of flow cytometry revealed a substantial increase from (4.82% ± 1.33% to 16.23% ± 2.88%) of F4/80<sup>+</sup>/CD11b<sup>+</sup>/FSC<sup>high</sup> cells in the colon (see Fig E1). Western blot analysis of whole colon lysates from control or DSS-treated mice showed a significant increase in expression of the alternatively activated macrophage markers arginase-1 and YM1 (see Fig E1).<sup>12</sup> Immunofluorescence staining of colon samples of DSS-treated mice revealed a marked increase in Relm-α<sup>−</sup> cells, whereas Relm-α was hardly expressed in the normal colon (Fig 1, A). Unexpectedly, analysis of the cellular source of Relm-α expression identified
that the majority of Relm-α− cells were not macrophages but rather eosinophils (MBP+ cells; Fig 1, B and C) and epithelial cell subsets (Fig 1, D). Supporting our immunofluorescence findings, quantitative PCR analysis of Relm-α expression failed to detect upregulation of Relm-α in purified DSS-treated colonic macrophages (data not shown). Further characterization of Relm-α expression in eosinophils showed that Relm-α was found to colocalize with MBP in eosinophil granules (Fig 1, A). In fact, isolated eosinophils expressed 0.25 pg of Relm-α per cell at baseline (data not shown). Although occasional Mac-3+/Relm-α− cells corresponding with macrophage morphology were observed (Fig 1, E), they were less than 5% of the macrophage population, and the majority of the macrophages were Relm-α− (Fig 1, F). Collectively, these data demonstrate that eosinophils are a chief source of Relm-α after DSS.

**Generation of Retnla−/− mice**

To define the role of Relm-α in vivo, we used the Velocigene technology to delete genomic Retnla by means of replacement with a LacZ neoexpression cassette (Fig 2, A). The specific genetic ablation of Retnla (Fig 2, B) was confirmed by assessing serum levels of Relm-α in wild-type, Retnla−/−, and Retnla−/− mice (Fig 2, C). Indeed, Relm-α was not detected in Retnla−/− mice, whereas it was readily detected in the sera of wild-type and Retnla−/− mice. Retnla−/− mice were fertile and produced offspring at predicted mendelian inheritance patterns, with no gross abnormalities observed. Analysis of peripheral blood cell counts did not show any differences in differential cell numbers (data not shown). Further validation of the gene deletion and anti–Relm-α antibody specificity was obtained by demonstrating that Retnla−/− mice exhibited minimal reactivity with the anti–Relm-α antibody in DSS-treated colon samples (Fig 2, D).

**Retnla−/− mice are protected from DSS-induced experimental colitis**

Because Relm-α expression was induced during DSS-triggered colitis, we aimed to determine the role of Relm-α in intestinal inflammation. Retnla−/− mice were markedly protected against DSS-induced colitis (Fig 3) in comparison with wild-type mice. Retnla−/− mice displayed delayed development of diarrhea (Fig 3, A), substantially less rectal bleeding (Fig 3, B), and weight loss (Fig 3, C). In addition, Retnla−/− mice displayed decreased colon shortening as a result of the DSS insult (Fig 3, D). Notably, Retnla−/− mice were protected from DSS-induced death (Fig 3, E). For example, by day 10, 80% of wild-type mice were dead, and 100% of Retnla−/− mice were still viable.

Analysis of the histopathology induced by DSS revealed that Retnla−/− mice had significantly less erosion and damage of the epithelial layer (Fig 4, A, right panel) and substantially decreased submucosal inflammation compared with that seen in wild-type mice (Fig 4, A, middle and right panels). Quantitation of the histology score revealed significant reduction in the histologic features of DSS-induced disease in Retnla−/− mice (Fig 4, B). Importantly, all of the aforementioned parameters were independent of mouse strain because both c57BL/6 and BALB/c mice...
It is notable that Retnlb−/− mice are also protected from DSS-induced colitis.10,13 Therefore we examined Relm-β expression in Retnla−/− mice. Notably, Retnla−/− mice expressed Relm-β; Relm-β was readily detected in colonic epithelial cells and upregulated in the colons of DSS-treated Retnla−/− mice similar to that seen in wild-type mice (Fig 4, C, and data not shown).

The proinflammatory role of Relm-α
We hypothesized that Relm-α can induce its effects by altering the balance of proinflammatory (ie, IL-6) and anti-inflammatory (ie, IL-10) cytokine production.2,3 Thus we subsequently examined the effects of recombinant Relm-α on macrophage activation in vitro. Activation of bone marrow–derived macrophages with Relm-α in combination with LPS significantly increased induction of IL-6 and TNF-α secretion compared with LPS alone, whereas Relm-α alone did not induce any cytokine production (Fig 5, A-C). Moreover, Relm-α markedly inhibited LPS-induced IL-10 secretion (Fig 5, C). These results highlight that Relm-α acts as a proinflammatory cofactor that regulates macrophage activation and consequent cytokine production.

We aimed to determine whether these mechanistic findings might be responsible for the in vivo phenotype observed in Retnla−/− mice. Accordingly, colon punch biopsy specimens were obtained from control and DSS-treated wild-type and Retnla−/− mice and assessed for IL-6 and IL-10 production ex vivo. Consistent with the observed proinflammatory role of Relm-α in vitro, colonic organ cultures obtained from DSS-treated Retnla−/− mice displayed marked reduction in the levels of IL-6 and a substantial increase in IL-10 levels (Fig 5, D and E).

Consistent with these findings, DSS-treated Retnla−/− mice displayed a marked reduction in JNK phosphorylation in vivo in comparison with that seen in wild-type mice (Fig 6, A and B). Of note, the proximal colons of DSS-treated Retnla−/− mice showed a marked protection, and pJNK+ cell levels were similar to those seen in control mice, whereas in the distal colon pJNK+ cells were observed, albeit in significantly lower numbers. Nevertheless, the expression pattern was similar between proximal and distal colon segments and correlated with the overall protection that was observed in the Retnla−/− mice. Interestingly, the cellular source for pJNK+ cells in the DSS-treated Retnla−/− mice was different than that of wild-type mice. In the wild-type mice pJNK+ cells were mainly localized to the lamina propria and corresponded with infiltrating cells, whereas in Retnla−/− mice most of the pJNK+ cells were epithelial cells similar to those seen in control mice, and infiltrating pJNK+ immunoreactive cells were
observed only in the distal colon (Fig 6, A and B, and data not shown).

In addition, intraperitoneal injection of Relm-α induced a local inflammatory response. Kinetic analysis of peritoneal cell populations after Relm-α administration revealed that Relm-α initiated neutrophil accumulation that culminated after 24 hours, and a significant increase in eosinophils and lymphocytes was observed after 48 hours (Fig 6, C). Indeed, in vitro chemotaxis assays revealed that Relm-α is capable of inducing up to a 3-fold increase in eosinophil chemotaxis (Fig 6, D).

**DISCUSSION**

Effector molecules involved in the regulation of gastrointestinal inflammation have been an area of intense research in the last decade. Of even greater interest are molecular pathways that might be shared between several immune-related diseases, such as asthma, obesity, and IBD.1,14-16 The role of Relm-α in colonic inflammation and a novel regulatory role for Relm-α in the promotion of inflammation (Fig 6, A and B, and data not shown). Furthermore, macrophages are a major source of TNF-α, which is mainly expressed by epithelial cells,10,17 might explain the non-redundant roles of the Relm family members. Although Relm-β alone was sufficient to induce TNF-α and IL-15 release from bone marrow–derived macrophages (of 22 measured cytokines),13 Relm-α did not induce cytokine secretion by itself but rather acted as a coactivator to amplify LPS-induced IL-6 and TNF-α secretion and regulate IL-10 levels. Thus Relm-α might regulate inflammatory processes by polarizing a proinflammatory rather than an anti-inflammatory response.

Recent findings demonstrate a proinflammatory role for eosinophils in DSS-induced colitis. In fact, eosinophil peroxidase has been shown to promote the pathogenesis of ulcerative colitis.18 Interestingly, eosinophil peroxidase can synergize with macrophage reactive oxygen species to kill tumor cells or potentially catalyze the oxidation of nitrite to generate additional cytotoxic radicals.19,20 Recent evidence suggests an active cross-talk between eosinophils and macrophages. Alternatively activated macrophages have a role in recruiting eosinophils after *Nippostrongylus brasiliensis* infection, and we have recently detected eotaxin in human macrophages from patients with ulcerative colitis and in murine intestinal macrophages after DSS treatment.21,22 Furthermore, macrophages are a major source of TNF-α that can induce eosinophil activation and survival.23 Thus it is likely that eosinophil recruitment by macrophages can initiate a self-perpetuating cross-talk in the inflamed tissue in which macrophages are activated by eosinophil-derived products and vice versa. However, similar to macrophages, eosinophils share the potential to promote tissue repair through multiple yet distinct pathways. For example, activated eosinophils are higher in the quiescent phase of ulcerative colitis than in the active phase, and eosinophils have been linked to activation of fibroblasts to explain the phenomenon of fibrosis and stricture formation in Crohn disease.24 Taken together, our results highlight a new pathway in which eosinophils can amplify macrophage activation and perpetuate colonic inflammation.18,26
Future studies are required to shed more light on the eosinophil and macrophage interaction.

Notably, Retnla\textsuperscript{−/−} mice are also protected against DSS-induced colitis.\textsuperscript{10,13} Nevertheless, examination of Relm-\(\beta\) expression in Retnla\textsuperscript{−/−} mice revealed that Relm-\(\beta\) levels were intact at baseline and after DSS treatment. These data demonstrate that Relm-\(\alpha\) and Relm-\(\beta\) independently and through distinct cell types contribute to eliciting intestinal inflammation.
In agreement with this hypothesis and supporting a proinflammatory role for resistin and the Relm protein family are recent findings showing that resistin increases the production of TNF, IL-1, IL-6, and IL-12 and induces the activation of p38, extracellular signal-regulated kinase, and phosphatidylinositol 3-kinase.27,28 Resistin and Relm-β have been attributed important roles in glucose metabolism. It has been recently reported that transgenic mice overexpressing resistin exhibit impaired insulin-mediated glucose transport.29 Indeed, mice lacking resistin exhibit low blood glucose levels because of reduced hepatic glucose production.30 Furthermore, lack of resistin diminishes the increases in glucose levels that are associated with increased weight, suggesting a role for resistin in mediating hyperglycemia associated with obesity.30 Moreover, infusion of Relm-β (or resistin) into rats decreased hepatic insulin sensitivity and was not attributed to peripheral insulin resistance.31 It is thus likely that Relm-α will possess similar activities as well.

In summary, our results define the involvement of Relm-α in colonic inflammation and highlight an eosinophil–Relm-α–macrophage axis. These results imply a similar role for Relm-α under other inflammatory conditions in which Relm-α and eosinophils are observed (eg, asthma). Furthermore, these results outline a novel innate immune pathway in which eosinophils have the potential to regulate immune polarization through regulating macrophage activation.

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**Key Messages**

- Relm-α is expressed by intestinal eosinophils and epithelial cells and has a critical role in DSS-induced colitis.
- Relm-α promotes macrophage activation by synergizing with LPS to induce proinflammatory cytokine release and inhibit anti-inflammatory cytokines.
REFERENCES


FIG E1. Macrophage accumulation in the colon and arginase-1 and YM1 expression after DSS treatment. Wild-type mice were treated with DSS for up to 8 days. At the indicated time points (days 0, 3, and 5), the colon was excised and either frozen for slide and protein lysate preparation (A, B, and D) or enzymatically digested for differential cell analysis (C). Representative microphotographs of anti-CD68–stained slides are shown (Fig E1, A). Anti-CD68<sup>+</sup> cells were quantified by using digital morphometric analysis (Fig E1, B). Single-cell suspensions were stained with anti-CD11b and anti-F4/80 and analyzed by means of FlowJo software (Tree Star, Inc, Ashland, Ore) for CD11b<sup>+</sup>/F4/80<sup>+</sup> cells in the colon (Fig E1, C). Total protein lysates of whole colon specimens were subjected to Western blot analysis and probed with anti-arginase, anti-YM1, and anti-actin as loading control. A representative Western blot is shown in which each lane represents the colon from 1 mouse (n = 4; Fig E1, D).