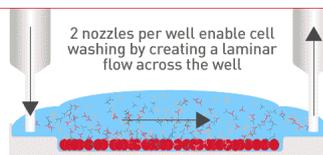


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## Resistin-Like Molecule $\alpha$ Decreases Glucose Tolerance during Intestinal Inflammation

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# Resistin-Like Molecule $\alpha$ Decreases Glucose Tolerance during Intestinal Inflammation<sup>1</sup>

Ariel Munitz, Luqman Seidu, Eric T. Cole, Richard Ahrens, Simon P. Hogan, and Marc E. Rothenberg<sup>2</sup>

Resistin-like molecule  $\alpha$  (Relm- $\alpha$ ) is a secreted cysteine-rich protein belonging to a newly defined family of proteins, including resistin, Relm- $\beta$ , and Relm- $\gamma$ . Resistin was initially defined based on its insulin resistance activity, but the family members are highly up-regulated in various inflammatory states, especially those involving intestinal inflammation. In this study, we report the role of Relm- $\alpha$  at baseline and following an experimental model of colitis. Relm- $\alpha$  was readily detected in the serum at baseline (4–5 ng/ml), and its level was regulated by energy uptake. *Retnla*<sup>-/-</sup> mice had decreased baseline circulating leptin levels, but displayed normal glucose, glucose clearance, and insulin levels. Following exposure to the oral innate trigger dextran sodium sulfate (DSS), a nonredundant proinflammatory role for Relm- $\alpha$  was uncovered as *Retnla*<sup>-/-</sup> mice were markedly protected from DSS-induced disease activity and histopathological features. Relm- $\alpha$  regulated eosinophil-directed cytokines (e.g., IL-5, CCL11/eotaxin-1, and CCL5/RANTES) and IL-17 *ex vivo*. Consistently, DSS-treated *Retnla*<sup>-/-</sup> mice displayed substantially decreased eosinophil accumulation and decreased phosphorylation of NF- $\kappa$ B, ERK1/2, and p38 in macrophages and eosinophils. Following DSS exposure, serum level of Relm- $\alpha$  was up-regulated, and DSS-treated *Retnla*<sup>-/-</sup> mice were markedly protected from hyperglycemia induced by glucose injection independent of changes in insulin levels. *Retnla*<sup>-/-</sup> mice were protected from increases in gut hormone serum levels of gastric inhibitory polypeptide and peptide YY that were induced following DSS treatment. These findings demonstrate a central proinflammatory role for Relm- $\alpha$  in the regulation of colonic inflammation and a novel link between colonic injury, glucose tolerance, and energy intake. *The Journal of Immunology*, 2009, 182: 2357–2363.

Resistin is a hormone that was originally identified in the adipose tissue with physiological roles in promoting insulin resistance and linked to obesity with insulin resistance (1, 2). Recently, a new family of resistin-like molecules (Relm),<sup>3</sup> including Relm- $\alpha$ , Relm- $\beta$ , and Relm- $\gamma$ , has been identified (3). This group of Relm proteins shares sequence homology with resistin and contains highly conserved C-terminal cysteine residues, which support the assembly of disulfide-dependent multimeric units (4). Interestingly, and despite opposing physiological effects on insulin resistance, the

multimeric assembly of the resistin family is similar to that of adiponectin and suggests a functional role for the Relm proteins in the regulation of glucose (4). Indeed, it is assumed that resistin and the Relm protein family may have a role in the metabolism and energy balance (5).

Relm- $\alpha$ , formerly known as found in inflammatory zone 1 (FIZZ1), has been implicated in various inflammatory conditions, including asthma and helminth infections (6, 7). Following Th2 stimuli, Relm- $\alpha$  is highly up-regulated in the lung and gastrointestinal tract and is thought to promote fibrosis via direct stimulation of fibroblasts (7, 8). Although Relm- $\alpha$  has not been identified in the human genome, the expression pattern of human resistin is more similar to that of Relm- $\alpha$  than to murine resistin (6). Thus, Relm- $\alpha$  may share functional roles similar to those of resistin.

Notably, various metabolic hormones, including resistin and Relm- $\beta$ , have been attributed proinflammatory roles in inflammatory bowel disease (IBD). Patients with IBD display increased serum levels of resistin, leptin, and adiponectin (9, 10). In addition, gut hormones such as gastric inhibitory polypeptide (GIP) and peptide YY (PYY) are elevated in patients with Crohn's disease (11, 12). Importantly, studies with *Retnlb*<sup>-/-</sup> mice revealed a significant role for Relm- $\beta$  in experimental models of intestinal inflammation (13, 14). Taken together, these data implicate that Relm family members may have a role in intestinal inflammation and metabolism. Nevertheless, the role of Relm- $\alpha$  in intestinal inflammation is not determined, and its contribution to glucose metabolism or energy uptake is currently unknown. In this study, we further characterize recently generated *Retnla*-deficient mice (15) and elucidate a nonredundant role for Relm- $\alpha$  in regulating innate colitis and inflammation-associated glucose tolerance.

Division of Allergy and Immunology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229

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<sup>2</sup> Address correspondence and reprint requests to Dr. Marc E. Rothenberg, Division of Allergy and Immunology, Cincinnati Children's Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, OH 45229. E-mail address: Rothenberg@cchmc.org

<sup>3</sup> Abbreviations used in this paper: Relm, resistin-like molecule; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; DSS, dextran sodium sulfate; GIP, gastric inhibitory polypeptide; GLP-1, glucagon-like peptide-1; IBD, inflammatory bowel disease; MBP, major basic protein; PYY, peptide YY.

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## Materials and Methods

### Mice

Male and female, 8- to 12-wk-old *Retnla*<sup>-/-</sup> mice (backcrossed to C57BL/6 or BALB/c background at least 7 and 10 generations, respectively) were generated using the Velocigene technology, as described (15). *IL6*<sup>-/-</sup> (C57BL/6 background) mice were obtained from The Jackson Laboratory. For all experiments, 4- to 5-wk-old wild-type mice were obtained from Taconic Farms and environmentally matched with the *Retnla*<sup>-/-</sup> mice for 2–3 wk. All mice were housed under specific pathogen-free conditions and treated according to institutional guidelines.

### High-fat diet

In some experiments, mice were fed a high-fat diet consisting of 58% fat, 25.6% carbohydrate, and 16.4% protein (total 23.4 kJ/g), whereas the normal diet contained 11.4% fat, 62.8% carbohydrate, and 25.8% protein (total 12.6 kJ/g).

### Dextran sodium sulfate (DSS)-induced colonic injury

DSS (ICN Biomedical; average molecular weight of 41 kDa) was supplied in the drinking water as a 2.5% (w/v, for C57BL/6 mice) and 5% (w/v, for BALB/c mice) solution for up to 8 days. The appearance of diarrhea was defined as mucus-fecal material adherent to anal fur. The presence or absence of diarrhea was scored as either 1 or 0, respectively. The presence or absence of diarrhea was confirmed by means of examination of the colon after completion of the experiment. Mice were killed, and the colon was excised from the animal. Diarrhea was defined by the absence of fecal pellet formation in the colon and the presence of continuous fluid fecal material in the colon. The appearance of rectal bleeding was defined as diarrhea containing visible blood, mucus, or both, or gross rectal bleeding, and scored as described for diarrhea. A change in body weight was calculated by the percentage change (gain/loss) from the initial weight. The disease activity index was derived by scoring three major clinical signs (weight loss, diarrhea, and rectal bleeding) (13).

### Intestinal histopathologic examination

Animals were killed on day 7, and the colon was excised. Tissue specimens were then fixed in 4% paraformaldehyde and stained with H&E using standard histologic techniques. The histological score was determined by calculating the percentage of colon length with mucosal ulceration, edema, lymphoid aggregates, and epithelial cell loss, by performing a blinded morphometric analysis of the colon with the ImageProPlus 4.5 software package (Media Cybernetics) (13, 16).

### 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 Laboratories) anti-F4/80 (BD Pharmingen), anti-major basic protein (MBP; provided by J. Lee, Mayo Clinic, Phoenix, AZ), anti-phospho-NF- $\kappa$ B, anti-phospho-ERK1/2, anti-phospho-p38 (Cell Signaling Technology), and anti-Relm- $\alpha$  (gift from PeproTech, Rocky Hill, NJ) (overnight, 4°C), followed by goat anti-rabbit Alexa 488 and donkey anti-rat Alexa 594 (Invitrogen), and counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI)/Supermount G solution (Fluoromount-G). Images were captured using a Zeiss microscope and Axioviewer image analysis software (Deutsland; Carl Zeiss). Quantification of eosinophil numbers in the tissue was performed by counting the number of immunoreactive cells (magnification  $\times$ 40) from at least three random sections/mouse. Values were expressed as eosinophils per high power field.

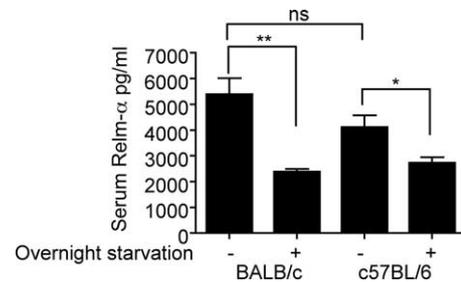
### Punch biopsies

The colons of control or DSS-treated mice were flushed with PBS and opened along a longitudinal axis. Thereafter, 3-mm<sup>2</sup> punch biopsies were obtained and incubated for 24 h in RPMI 1640 supplemented with 10% FCS and antibiotics. Supernatants were collected and kept in  $-20^{\circ}\text{C}$  until assessed for chemokines.

### ELISA and multiplex chemokine assays

Insulin and leptin levels were examined in the serum using a commercial enzyme immunoassay kit (Crystal Chem), according to the manufacturer's instructions. Lower detection limits for insulin and leptin were 0.2 and 0.1 ng/ml, respectively.

For detection of serum Relm- $\alpha$ , purified biotinylated anti-Relm- $\alpha$  and anti-Relm- $\alpha$  (PeproTech) were used, according to a protocol provided by the manufacturer. Lower detection limit for Relm- $\alpha$  was 125.62 pg/ml.



**FIGURE 1.** Serum expression of Relm- $\alpha$ . The expression of Relm- $\alpha$  in the serum of BALB/c or C57BL/6 mice was assessed at baseline or following an overnight fasting period;  $n = 22$ – $27$  mice; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

Chemokines and gut hormone levels were determined by a mouse multiplex kit (Millipore), according to the manufacturer's instructions. Lower detection limits for chemokines were 6.4 pg/ml. Lower detection limits for gut hormones were 41.5 pg/ml (amylin and gherlin), 8.7 pg/ml (glucagon-like peptide-1 (GLP-1) and PYY), and 2.7 pg/ml (GIP), respectively.

### Glucose tolerance and measurements

For glucose tolerance tests, D-glucose (2 mg/g body weight) was i.p. injected into overnight fasted mice, and glucose levels were monitored at 0, 15, 30, and 60 min after injection by retro-orbital bleeding using an Accu-Chek glucometer (Roche Diagnostics).

### Statistical analysis

Data were analyzed either by ANOVA, followed by Tukey post hoc test, or by unpaired, two-tailed Student's  $t$  test using GraphPad Prism 4. Data are presented as mean  $\pm$  SD; values of  $p < 0.05$  were considered statistically significant.

## Results

### Baseline serum Relm- $\alpha$ expression

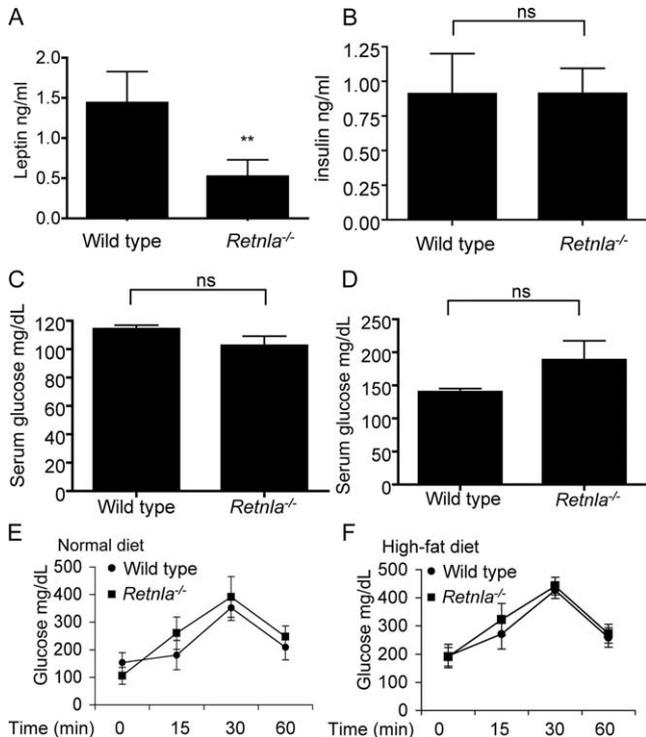
Because Relm- $\alpha$  is a secreted protein, we examined the baseline circulating levels of Relm- $\alpha$  in wild type BALB/c and C57BL/6 mice. Relm- $\alpha$  was detected at high levels at baseline in the serum, and no significant differences were observed between both mice strains (Fig. 1) and between male and female mice (data not shown). Interestingly, following overnight fasting, both BALB/c and C57BL/6 mice displayed a significant reduction in Relm- $\alpha$  expression (Fig. 1). To control for nonspecific binding of the anti-Relm- $\alpha$  Ab, serum from *Retnla*<sup>-/-</sup> was subjected to the ELISA and displayed no immunoreactivity (data not shown).

### Regulation of leptin and weight gain by Relm- $\alpha$

Next, we were interested to examine whether Relm- $\alpha$  may regulate metabolic features and/or affect the expression of other adipokine expression (17, 18). Interestingly, *Retnla*<sup>-/-</sup> mice displayed significantly lower levels of leptin at baseline, whereas no alterations in insulin levels were detected (Fig. 2, A and B). No baseline difference was observed in serum levels of TNF- $\alpha$  and IL-6. Furthermore, *Retnla*<sup>-/-</sup> mice exhibited similar weight to wild-type mice following normal food (data not shown) and gained weight similarly under high-fat diet conditions (data not shown).

### Baseline glucose metabolism in *Retnla*<sup>-/-</sup> mice

Given the association between insulin resistin and glucose metabolism (2), we aimed to examine the role of Relm- $\alpha$  in glucose metabolism and tolerance. Thus, we examined glucose levels in *Retnla*<sup>-/-</sup> mice at baseline and following normal or high-fat diet. *Retnla*<sup>-/-</sup> mice had comparable glucose levels to wild-type mice at baseline ( $114.3 \pm 4.5$  and  $102.5 \pm 13.3$  mg/dL in wild-type and *Retnla*<sup>-/-</sup> mice, respectively) (Fig. 2C). In addition, following a high-fat diet, serum glucose levels were comparable between



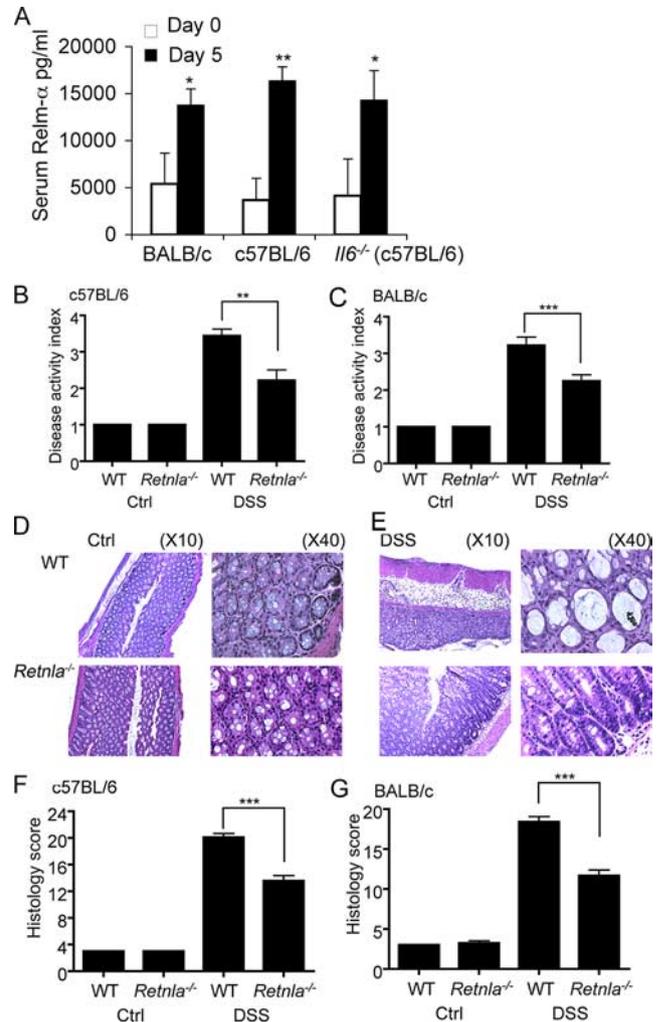
**FIGURE 2.** The effects of Relm- $\alpha$  on leptin and baseline glucose metabolism. Serum of 24-h starved wild-type and *Retnla*<sup>-/-</sup> mice, kept on a normal diet, was analyzed for leptin and insulin levels (A and B, respectively);  $n = 14$ ; \*\*,  $p < 0.01$ . Following normal (C and E) or high-fat diet (D and F), wild-type and *Retnla*<sup>-/-</sup> mice were starved for 24 h, and blood glucose levels were assessed (C and D). In addition, the mice were subjected to a glucose tolerance test (E and F). Glucose clearance was measured at the indicated time points;  $n = 3$  (4–8 mice per experimental group).

*Retnla*<sup>-/-</sup> and wild-type mice ( $147.3 \pm 1.8$  and  $183.4 \pm 28.57$  mg/dL in wild-type and *Retnla*<sup>-/-</sup> mice, respectively) (Fig. 2D).

Resistin has been shown to regulate blood glucose levels in association with increased weight gain (2). Therefore, we examined whether Relm- $\alpha$  regulates glucose clearance when mice were fed with a normal or high-fat diet. These sets of experiments revealed that *Retnla*<sup>-/-</sup> mice cleared glucose normally under regular diet, and displayed similar kinetics to wild-type mice (Fig. 2E). Furthermore, i.p. glucose challenge following a high-fat diet revealed no significant difference in glucose clearance between wild-type and *Retnla*<sup>-/-</sup> mice (Fig. 2F).

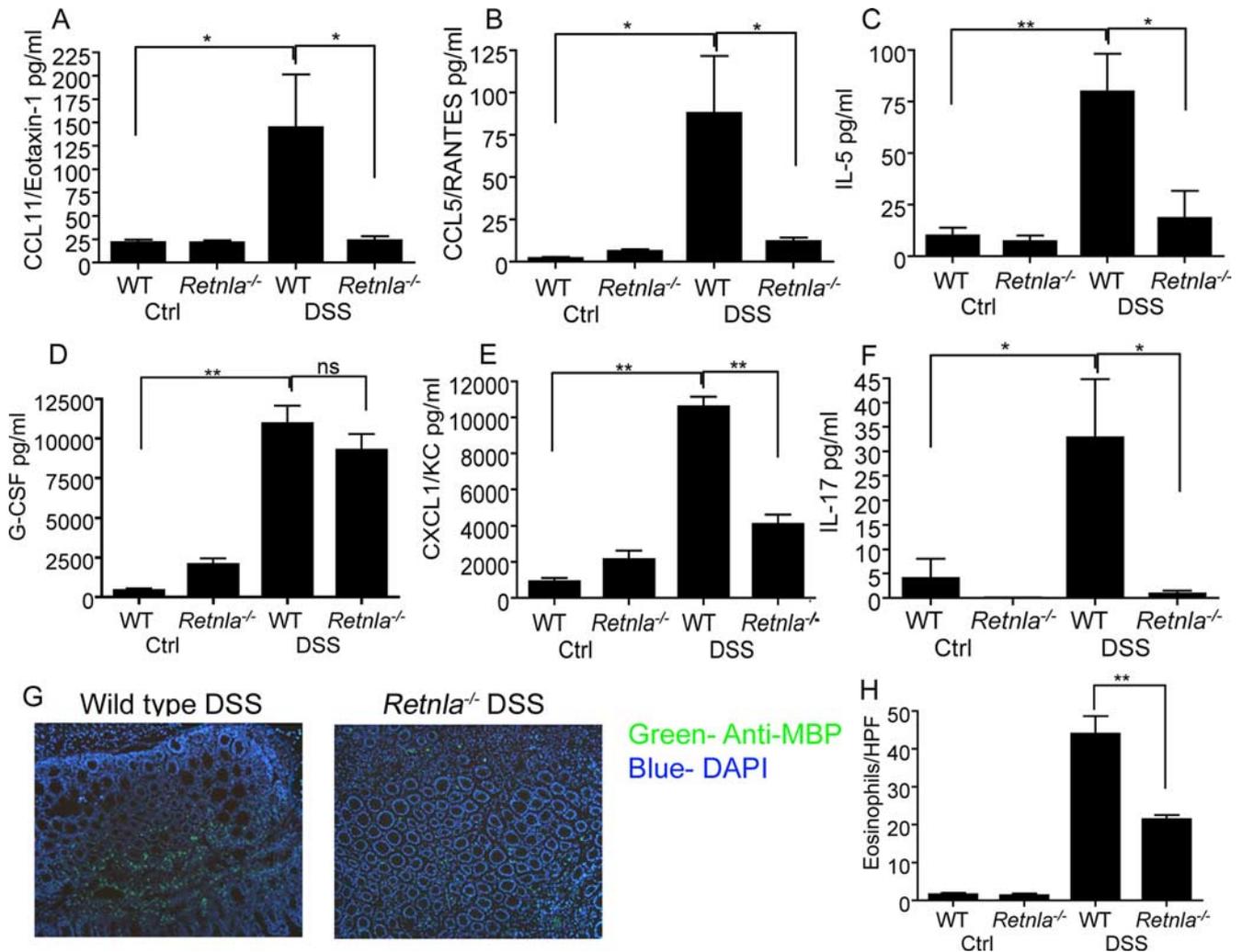
#### *Retnla*<sup>-/-</sup> mice are protected from DSS-induced colitis

Following DSS treatment, wild-type BALB/c and C57BL/6 mice displayed increased levels of circulating Relm- $\alpha$  (Fig. 3A). For example, in BALB/c mice, Relm- $\alpha$  was elevated in the serum after DSS treatment from  $5.4 \pm 3.2$  (baseline) to  $13.8 \pm 1.7$  ng/ml (DSS treated,  $p < 0.05$ ) (Fig. 3A); the ng/ml level of Relm- $\alpha$  in the serum was notably high. The increase in Relm- $\alpha$  levels was independent of IL-6, because *IL6*<sup>-/-</sup> mice, which have been previously shown to be protected from DSS-induced colitis (19), increased Relm- $\alpha$  similar to control (C57BL/6) mice (from  $4.1 \pm 4.3$  at baseline to  $14.1 \pm 3.9$  ng/ml following DSS treatment). To examine the role of Relm- $\alpha$  in experimental colitis, *Retnla*<sup>-/-</sup> mice were subjected to DSS in their drinking water and assessed for disease progression. *Retnla*<sup>-/-</sup> mice were protected from the major clinical features of DSS-induced colitis and displayed reduced rectal bleeding, diarrhea, and weight loss that was reflected



**FIGURE 3.** Relm- $\alpha$  expression and function in DSS-induced colonic injury. Wild-type BALB/c and C57BL/6 mice as well as *IL6*<sup>-/-</sup> mice were subjected to DSS (5% for BALB/c and 2.5% for C57BL/6 and *IL6*<sup>-/-</sup> mice) in their drinking water. Five days after disease induction, serum samples of untreated or DSS-treated mice were collected and Relm- $\alpha$  expression was examined using an ELISA (A). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ;  $n = 8$ –12 mice. Wild-type and *Retnla*<sup>-/-</sup> mice were subjected to the aforementioned experimental regime and analyzed for the clinical disease features, including diarrhea, rectal bleeding, and weight loss, and scored for the overall disease activity index (B and C) and histological score (F and G);  $n = 4$  (6–8 mice per experimental group); \*\*\*,  $p < 0.001$ . Seven days after DSS treatment, the colons were excised, fixed, and stained with H&E. Representative photomicrographs of low power magnification (D and E, left panels,  $\times 10$ , respectively) and high power (D and E, right panels,  $\times 40$ , respectively) magnification of control-treated mice (Ctrl, D) and DSS-treated mice (E) are shown. Digital morphometric analysis of the histological score of C57BL/6 and BALB/c mice is shown (F and G). Data represent  $n = 4$  (6–8 mice per experimental group); \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

by reduced disease activity index (Fig. 3, B and C). Importantly, the protection from DSS-induced damage was observed in both C57BL/6 and BALB/c mouse strains (Fig. 3, B and C). Furthermore, histological examination of colons obtained from *Retnla*<sup>-/-</sup> mice showed significant reduction in histopathological findings (both in C57BL/6 and BALB/c mice). Upon DSS treatment, wild-type mice displayed increased inflammation, whereas *Retnla*<sup>-/-</sup> displayed decreased edema formation, epithelial cell damage, and leukocyte infiltration (Fig. 3, D and E). Quantitation of the histological findings showed marked protection in *Retnla*<sup>-/-</sup> mice that was observed both in C57BL/6 and BALB/c mice (Fig. 3, F and G).



**FIGURE 4.** The effects of Relm- $\alpha$  on colon cytokine production and eosinophil levels. Punch biopsies were obtained from control and DSS-treated wild-type (WT) or *Retnla*<sup>-/-</sup> mice 6 days after the beginning of DSS treatment. Following a 24-h incubation period, the supernatants were obtained and assessed for chemokine and cytokine expression (A–F);  $n = 4$ ; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ . Six days following DSS treatment, the colons were excised, frozen sections were prepared, and slides were stained with anti-MBP (green) and DAPI (blue). A representative photomicrograph (magnification  $\times 10$ ) (G) and quantitative analysis (H) are shown. HPF, high power field. \*\*,  $p < 0.01$ .

#### Regulation of eosinophil-active cytokines by Relm- $\alpha$

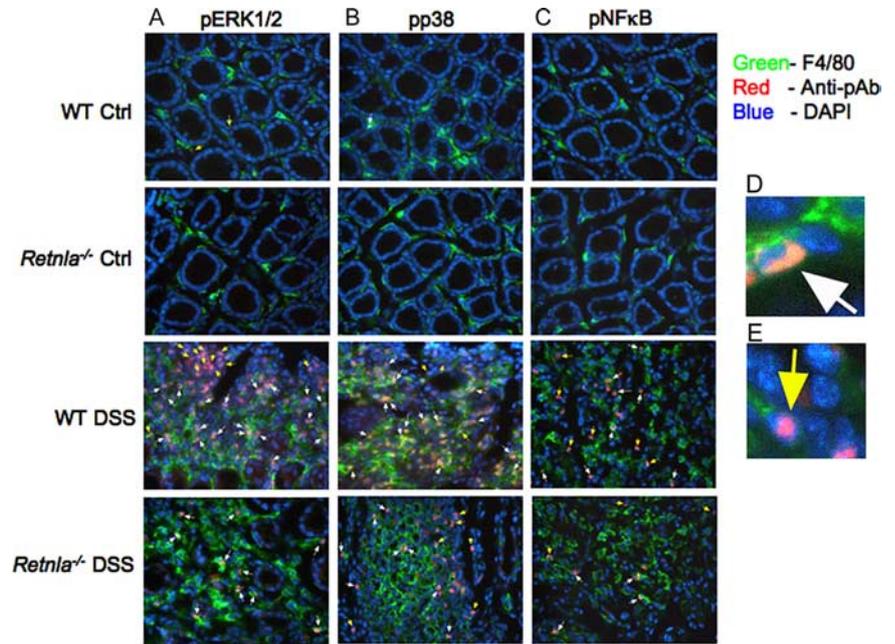
We have previously shown that Relm- $\alpha$  is capable of inducing cellular infiltration, including eosinophil accumulation into the peritoneal cavity (15). This suggested that the proinflammatory effect of Relm- $\alpha$  may involve regulation of chemokine expression. To determine whether *Retnla*<sup>-/-</sup> mice display an altered chemokine expression pattern following DSS treatment, punch biopsies from DSS-treated *Retnla*<sup>-/-</sup> were analyzed for chemokine production *ex vivo*. Consistent with our previous findings, DSS-treated *Retnla*<sup>-/-</sup> mice displayed an altered eosinophil-related chemokine response; CCL11/eotaxin-1 and CCL5/RANTES levels were substantially reduced *ex vivo* (Fig. 4, A and B). In addition, IL-5 levels were significantly reduced as well (Fig. 4C). These results were not attributed to a general inhibition because the levels of G-CSF, JE/CCL2, and IFN- $\gamma$ -inducible protein-10/CXCL10 were not significantly altered (Fig. 4D and data not shown). Notably, the DSS-induced CXCL1/KC was also Relm- $\alpha$  dependent (Fig. 4E). Consequently, we hypothesized that eosinophil recruitment into the colon, previously shown to be mediated by CCL11/eotaxin-1 (20), would be altered in DSS-treated *Retnla*<sup>-/-</sup> mice. Indeed, eosinophil accumulation in the colon of DSS-treated *Retnla*<sup>-/-</sup> mice was significantly attenuated (Fig. 4, G and H). Of

note, *Retnla*<sup>-/-</sup> mice also displayed a significant reduction in IL-17 production (Fig. 4F). Importantly, the aforementioned altered chemokine response is likely not due to the effects of Relm- $\alpha$  on macrophages because Relm- $\alpha$  was not capable of inducing or potentiating chemokine release from bone marrow macrophages directly or in combination with LPS, respectively (data not shown).

#### Activation of proinflammatory signaling cascades *in vivo*

Given our previous findings on the proinflammatory effects of Relm- $\alpha$  on macrophage activation (15) and the aforementioned data on eosinophil accumulation into the colon, we hypothesized that both cell types will display decreased activation *in vivo* in DSS-treated *Retnla*<sup>-/-</sup> mice. To explore this hypothesis, frozen sections of control and DSS-treated wild-type and *Retnla*<sup>-/-</sup> mice were stained with the F4/80 Ab and analyzed for activation of NF- $\kappa$ B, ERK1/2, and p38. In agreement with the proinflammatory role for Relm- $\alpha$ , DSS-treated *Retnla*<sup>-/-</sup> mice displayed substantially decreased NF- $\kappa$ B, ERK1/2, and p38 phosphorylation. These alterations were observed both in F4/80<sup>+</sup> cells corresponding with macrophage morphology (Fig. 5, A–C, white arrows, and D) and in

**FIGURE 5.** The effects of Relm- $\alpha$  on proinflammatory signaling cascades in vivo. Wild-type (WT) and *Retnla*<sup>-/-</sup> BALB/c mice were exposed to 5% DSS for 7 days. Thereafter, colon was excised, frozen sections were prepared, and slides were stained with F4/80 (green), anti-phospho-ERK1/2 (pERK1/2, A), anti-phospho-p38 (pp38, B), anti-phospho-NF- $\kappa$ B (pNF $\kappa$ B, C), and DAPI (blue). Representative photomicrographs are shown. White and yellow arrows indicate F4/80<sup>+</sup> and F4/80<sup>-</sup> phospho-Ab<sup>+</sup> (pAb) immunoreactive cells, respectively. A high power magnification of F4/80<sup>+</sup>/pAb<sup>+</sup> and F4/80<sup>-</sup>/pAb<sup>+</sup> cells is shown (D and E).



F4/80<sup>low/negative</sup> cells that corresponded with eosinophil morphology (Fig. 5, A–C, yellow arrows, and E). Importantly, we specifically analyzed microinflammatory foci where the distribution of F4/80<sup>+</sup> cells was homogeneous. This analysis revealed that the altered phosphorylation in *Retnla*<sup>-/-</sup> mice was most likely due to decreased phosphorylation and not due to overall less cellular recruitment (Fig. 5, A–C).

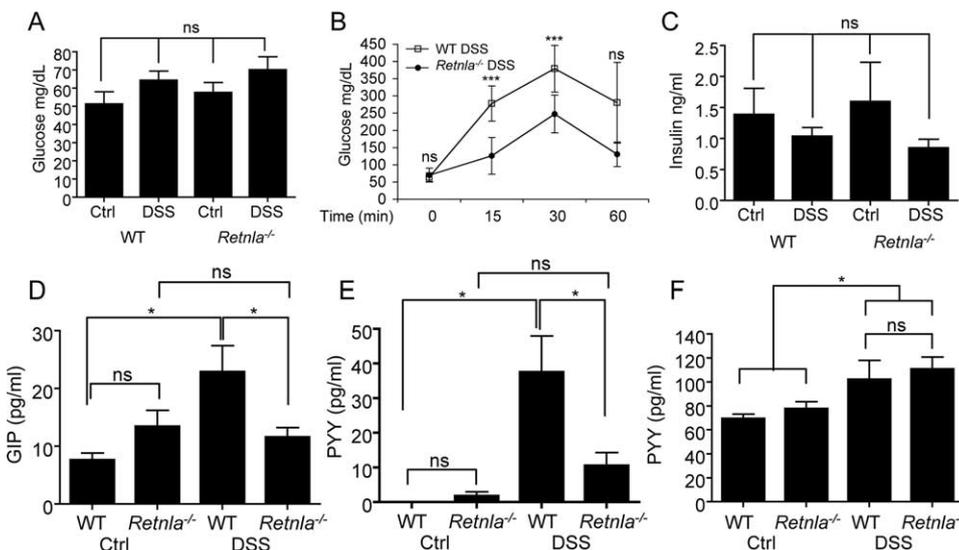
*Glucose tolerance and insulin assessment following colonic inflammation*

Given the substantial role for Relm- $\alpha$  during colonic inflammation (Figs. 3–5) and the role of resistin in glucose metabolism, we hypothesized that Relm- $\alpha$  may have a role in glucose metabolism specifically under inflammatory conditions. Assessment of glucose levels 6 days following DSS treatment demonstrated normal serum glucose levels in *Retnla*<sup>-/-</sup> mice (Fig. 6A). Because the levels of circulating Relm- $\alpha$  were highly induced following the DSS experimental regime, we hypothesized that under colonic inflammatory conditions, Relm- $\alpha$  may regulate glucose clearance. Although

baseline glucose levels were unaltered in *Retnla*<sup>-/-</sup> mice following DSS treatment (Fig. 6A), DSS-treated *Retnla*<sup>-/-</sup> mice were significantly protected from hyperglycemia induced by glucose challenge, whereas wild-type mice displayed markedly elevated levels of serum glucose (Fig. 6B). For example, whereas the levels of glucose in wild-type mice increased after 15 min to 278  $\pm$  84 mg/dL, glucose levels in *Retnla*<sup>-/-</sup> mice hardly increased ( $p < 0.001$ ). Even more striking was the difference observed at 30 min, in which glucose levels increased up to 362  $\pm$  48 mg/dL in wild-type mice, whereas in *Retnla*<sup>-/-</sup> mice it was increased only up to 223  $\pm$  74 mg/dL ( $p < 0.001$ ).

To determine whether the changes in glucose clearance may be due to a DSS-induced change in insulin levels in the *Retnla*<sup>-/-</sup> mice, serum insulin levels were assessed. Importantly, the metabolic effects of Relm- $\alpha$  were independent of changes in insulin, because insulin levels were similar at baseline and following DSS administration between wild-type and *Retnla*<sup>-/-</sup> mice (Fig. 6C).

Next, we hypothesized that gut hormone levels that have been linked to glucose metabolism and energy uptake (such as gherlin,



**FIGURE 6.** The effects of Relm- $\alpha$  on glucose metabolism and serum levels of gut-derived hormones during DSS-induced colon injury. Wild-type (WT) and *Retnla*<sup>-/-</sup> BALB/c mice were exposed to 5% DSS in their drinking water. Mice were starved overnight 7 days after disease induction, and serum glucose was assessed (A). Glucose clearance following i.p. glucose injection was measured at the indicated time points (B). \*\*\*,  $p < 0.05$ ;  $n = 15$  mice. Serum samples of untreated or DSS-treated mice were collected, and insulin (C), GIP (D), and PYY (E) levels were examined using ELISA or a multiplex array kit; \*,  $p < 0.05$ ;  $n = 12$ . Punch biopsy samples were analyzed for PYY expression (F); \*,  $p < 0.05$ ;  $n = 6$ .

amylin, GIP, GLP-1, and PYY) might be altered in response to DSS and modulated by Relm- $\alpha$ . Thus, we assessed the levels of active ghrelin, active amylin, total GLP-1, GIP, and PYY. Following DSS treatment, GIP and PYY levels were significantly increased in the serum of wild-type BALB/c mice, whereas *Retnla*<sup>-/-</sup> mice did not display enhanced gut hormone levels (Fig. 6, D and E). Ghrelin and amylin were not detected (data not shown). Although readily detected, no changes were observed in GLP-1 following DSS treatment (data not shown). To further elucidate whether the changes in PYY directly correlated with the decreased disease phenotype that was observed in *Retnla*<sup>-/-</sup> mice, we examined PYY levels in colon punch biopsies obtained from DSS-treated wild-type and *Retnla*<sup>-/-</sup> mice. Although PYY levels were significantly up-regulated in DSS-treated punch biopsies, no difference was observed in PYY levels between wild-type and *Retnla*<sup>-/-</sup> mice (Fig. 6F).

## Discussion

Immune-related diseases such as IBD, diabetes, obesity, and asthma have become some of the fastest growing and persistent public health problems in the western world, and are currently on the rise (21–23). These diseases share a component of inflammation that is involved in disease pathogenesis and complications (21–25). Thus, defining molecular pathways that may be shared between several immune-related diseases such as asthma, obesity, and IBD that often co-occur is of great interest and importance (21–24).

The Relm family of proteins draws much attention, because these proteins share sequence and structural homology to resistin and are highly up-regulated in various inflammatory states including asthma and IBD. Nevertheless, the role of Relm- $\alpha$  is still unclear (6, 26). In this study, we demonstrate several key findings. First, we demonstrate that Relm- $\alpha$  is consistently detectable in the serum, and its expression levels are regulated by food intake and colonic inflammation. Second, *Retnla*<sup>-/-</sup> mice are protected from DSS-induced colitis and, under these conditions, Relm- $\alpha$  has a role in hyperglycemia induced by glucose injection and in regulating gut-derived hormones such as GIP and PYY. Third, we provide substantial evidence that Relm- $\alpha$  regulates proinflammatory eosinophil-directed cytokines in vivo (e.g., CCL11/eotaxin-1 and IL-5) and activates intracellular proinflammatory signaling cascades. Our data support a model in which Relm- $\alpha$  contributes to glucose metabolism when it is induced during the setting of specific intestinal inflammatory conditions and the host is exposed to increased proinflammatory cytokines (e.g., IL-6 and TNF- $\alpha$ ) and high glucose intake.

Several molecules that are involved in energy intake were found to be dysregulated in *Retnla*<sup>-/-</sup> mice either at baseline (e.g., leptin) or following DSS treatment (e.g., GIP and PYY). In fact, GIP stimulates glucose-dependent insulin secretion and PYY regulates satiety via the hypothalamus (12, 27). Moreover, the findings that the levels of Relm- $\alpha$  are regulated by food intake strongly suggest that Relm- $\alpha$  has a metabolic role. Notably, leptin, an important protein regulating energy intake and expenditure, including appetite metabolism (28, 29), is up-regulated in IBD patients and has a proinflammatory role in experimental colitis (17, 18, 30). Furthermore, PYY and GIP are up-regulated in the serum of patients with Crohn's disease (11, 12). This suggests that under colonic-inflammatory conditions in which the body is in energy deficit, multiple pathways (either gut derived or from other endocrine sources) act in concert to increase food ingestion. These latter findings are of specific interest because resistin was initially described as a factor linking obesity and insulin resistance (2). In particular, PYY has been found to be up-regulated in the serum of humans and mice

with diet-induced obesity (31, 32). Hence, the ability of Relm- $\alpha$  to regulate glucose metabolism in DSS-induced colitis may be due to the overall energy deficit state and the alteration in energy-related hormonal status; these findings argue against the protection from hyperglycemia simply due to the protection from inflammation. Consistent with this hypothesis, we show that GIP and PYY are up-regulated in the serum (and PYY in the colon) following DSS treatment in wild-type, but not *Retnla*<sup>-/-</sup> mice. Interestingly, whereas circulating PYY levels were altered in the *Retnla*<sup>-/-</sup> mice, colonic generation of PYY was not dysregulated, indicating that a central pathway may be regulated by Relm- $\alpha$  during colonic inflammatory conditions. Furthermore, no difference is observed in glucose tolerance between control- and DSS-treated wild-type mice (data not shown), indicating that hyperglycemia involves cooperativity between DSS treatment and Relm- $\alpha$ .

Although the inflammatory state alone is not likely to be the main factor leading to glucose tolerance in *Retnla*<sup>-/-</sup> mice (given all the metabolic alterations), our findings cannot exclude this possibility. In fact, several observations link increased inflammation and glucose metabolism. For example, adipokines (leptin, resistin, and adiponectin) have been all shown to have important roles in inflammation and are elevated in the serum of IBD patients (9, 10, 30). Of note, and similar to Relm- $\alpha$ , the serum levels of leptin and resistin are also detected in the ng/ml range (9). Furthermore, high-fat diet induces increased serum endotoxin levels, and mice that are chronically perfused with low-dose LPS develop hepatic insulin resistance and increased IL-6 and TNF- $\alpha$  (33). In these settings, TLR 4-mediated MyD88 activation has a key role in promoting insulin resistance by diet-induced obesity (34). In addition, there are recent reports that overweight Crohn's disease patients (body mass index >24) develop more severe disease (as indicated by more frequent anoperineal complications, a marked year-by-year disease activity, and requirement of earlier surgical intervention) compared with lean patients (35, 36). In agreement with our data, a recent study by Al-Azzawi et al. (37) demonstrated that prolonged administration of i.p. Relm- $\alpha$  (but not resistin) significantly increased insulin resistance that is associated with decreased gallbladder tension. Thus, whereas Relm- $\alpha$  and resistin share similar structure and expression pattern, they may have distinct roles under different settings.

The ability of Relm- $\alpha$  to regulate leptin levels may also contribute to its overall proinflammatory role in vivo. Nevertheless, we have recently shown that Relm- $\alpha$  acts as a cofactor with LPS to induce IL-6 and TNF- $\alpha$  production (15), and we now demonstrate that Relm- $\alpha$  can regulate eosinophil-directed chemokines (e.g., CCL11/eotaxin-1) and cytokines (e.g., IL-5). This latter effect is relatively specific because G-CSF and other chemokines, which are significantly induced by DSS treatment, were not attenuated. These data argue for a specific effect and not a general inhibition of chemokine production due to decreased disease state, and further distinguishes the role of Relm- $\alpha$  and leptin. Our findings regarding the proinflammatory role of Relm- $\alpha$  suggest that Relm- $\alpha$  is a novel link between the innate and adaptive immune response. It is likely that Relm- $\alpha$  induces its responses via regulating various cell types. Supporting this hypothesis are our findings that Relm- $\alpha$  did not induce or potentiate chemokine release from macrophages. Thus, the effects of Relm- $\alpha$  on chemokine expression are possibly by other cells including epithelial cells and T cells. Of note, Relm- $\alpha$  was found to significantly regulate colonic expression of IL-17, a cytokine that has been shown to be critical in colitis (38). These findings suggest that Relm- $\alpha$  can either directly (via acting on T cells) or indirectly (via regulating macrophage IL-6 production (15)) regulate Th17 cell function. Although the receptor for Relm- $\alpha$  has yet to be identified, our data suggest

that Relm- $\alpha$  is capable of inducing intracellular MAPK and NF- $\kappa$ B activation.

In summary, we demonstrate a novel role for Relm- $\alpha$  in the orchestration of the colonic immune reaction in response to DSS by regulating colon-derived eosinophil-directed cytokines. Furthermore, our data establish a novel link between colonic inflammation, energy uptake, and glucose metabolism, and provide an important insight into the role of Relm- $\alpha$  in this process. Because the health of the modern world is under increasing threat of chronic co-occurring inflammatory diseases, defining the roles of shared components such as Relm- $\alpha$  in the pathophysiology of multiple diseases may provide new targets for future therapeutics.

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## Disclosures

A.M., E.T.C., L.S., R.A., and S.P.H. declare no conflict of interests. M.E.R. is a consultant for Merck, Ception Therapeutics, Novartis, and Nycomed.

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