Allergen-induced resistin-like molecule-α promotes esophageal epithelial cell hyperplasia in eosinophilic esophagitis

Parm Mavi,1 Rituraj Niranjan,2 Parmesh Dutt,1 Asifa Zaidi,1 Jai Shankar Shukla,1 Thomas Korfhagen,4 and Anil Mishra1

1Section of Pulmonary Diseases, Department of Medicine, Tulane Eosinophilic Disorder Center, Tulane University School of Medicine, New Orleans, Louisiana; 2University of Cincinnati College of Medicine, Cincinnati, Ohio; 3Allergy and Immunology, Cincinnati Children’s Medical Center, Cincinnati, Ohio; and 4Perinatal Institute, Cincinnati Children’s Medical Center, Cincinnati, Ohio

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Mavi P, Niranjan R, Dutt P, Zaidi A, Shukla JS, Korfhagen T, Mishra A. Allergen-induced resistin-like molecule-α promotes esophageal epithelial cell hyperplasia in eosinophilic esophagitis. Am J Physiol Gastrointest Liver Physiol 307: G499–G507, 2014. First published July 3, 2014; doi:10.1152/ajpgi.00141.2014.—Resistin-like molecule (Relm)-α is a secreted, cysteine-rich protein belonging to a newly defined family of proteins, including resistin, Relm-β, and Relm-γ. Although resistin was initially defined based on its insulin-resistance activity, the family members are highly induced in various inflammatory states. Earlier studies implicated Relm-α in insulin resistance, asthmatic responses, and intestinal inflammation; however, its function still remains an enigma. We now report that Relm-α is strongly induced in the esophagus in an allergen-challenged murine model of eosinophilic esophagitis (EoE). Furthermore, to understand the in vivo role of Relm-α, we generated Relm-α gene-inducible bitransgenic mice by using lung-specific CC-10 promoter (CC10-rtTA-Relm-α). We found Relm-α protein is significantly induced in the esophagus of CC10-rtTA-Relm-α bitransgenic mice exposed to doxycycline food. The most prominent effect observed by the induction of Relm-α is epithelial cell hyperplasia, basal layer thickness, accumulation of activated CD4+ and CD4− T cell subsets, and eosinophilic inflammation in the esophagus. The in vitro experiments further confirm that Relm-α promotes primary epithelial cell proliferation but has no chemotactic activity for eosinophils. Taken together, our studies report for the first time that Relm-α induction in the esophagus has a major role in promoting epithelial cell hyperplasia and basal layer thickness, and the accumulation of activated CD4+ and CD4− T cell subsets may be responsible for partial esophageal eosinophilia in the mouse models of EoE. Notably, the epithelial cell hyperplasia and basal layer thickness are the characteristic features commonly observed in human EoE.

eosinophils; esophagus; epithelial cells

RESEARCH IN THE FIELD OF EOSINOPHILIC ESOPHAGITIS (EoE) has largely focused on analysis of the cellular and molecular events induced by allergen exposure in sensitized mice and humans (6–8, 13, 17, 34). These studies have identified elevated production of IgE, mucus hypersecretion, eosinophilic inflammation, including eosinophilic microabscess in epithelial mucosa, and basal cell hyperplasia (6–8, 13, 17, 34). Clinical and experimental investigations have demonstrated a strong correlation between the presence of CD4+ T helper 2 lymphocytes, including γδNKT cells and disease severity, suggesting an integral role for these cells and cytokines in the pathophysiology of EoE (14, 34) through the secretion of an array of cytokines like IL-5, IL-13, and IL-15 that activate inflammatory and resident effector pathways (32, 38). Interestingly, this mechanistic pathway in the induction and progression of EoE is found to be closely associated with allergic asthma, which is one of the reasons that EoE is also termed eosinophilic asthma (2). Aiming to elucidate novel pathways involved in the pathogenesis of allergic EoE, earlier we studied a number of genes based on our microarray analysis that were induced or reduced in disease pathogenesis. One of the genes induced in the microarray analysis of the allergen murine model of EoE and asthma that did not get much attention is the resistin family of molecules (Relm).

The resistin family of cytokines consists of several conserved, ~12.5-kDa proteins with 10 or 11 cysteine residues that promote the formation of oligomeric molecular species (3, 30). The first family member, resistin, also called adipocyte-secreted factor or found in inflammatory zone (FIZZ) 3, is a novel hormone secreted by adipocytes and has been proposed to link obesity with insulin resistance and type II diabetes (5). Relm-α was originally found in inflammatory zones in a murine model of experimental asthma and was subsequently designated FIZZ1 (31). The Relm family of cytokines is expressed in a number of tissues such as heart, lung, tongue, intestine, and adipose tissue (4, 18, 37). Earlier, the highest levels of Relm-γ expression have been shown in hematopoietic tissues (11). Based on the relatively strong data emerging that links the Relm family of molecules with inflammatory processes that are an important cytokine involved in innate mucosal responses (16), we aimed to investigate the role of Relm-α in allergic esophageal inflammation. In this study, we demonstrate that Relm-α transcript is induced in the esophagus of a murine model of experimental EoE and CD4+/CD4− T cells; eosinophilic inflammation and epithelial cell proliferation are induced in the esophagus following 6 wk of doxycycline (DOX) food exposure in CC10-rtTA-Relm-α bitransgenic mice. Furthermore, in vitro experiments demonstrated that Relm-α had no eosinophil chemotactic activity but induces primary epithelial cell proliferation. In conclusion, we identified for the first time a novel in vivo and in vitro role of Relm-α molecule that is involved in basal layer thickening in the esophagus. Of note, basal layer thickening is found in both EoE and gastroesophageal reflux disease (GERD).

MATERIALS AND METHODS

Mice. Specific pathogen-free BALB/c mice were obtained from Jackson Laboratory (Bar Harbor, ME) and housed in a specific...
pathogen-free barrier facility. The Relm-α gene-deficient mice in Balb/c background were bred in the laboratory at Cincinnati Children’s Hospital Medical Center (Cincinnati, OH) along with the littermate controls as described earlier (27). All of the experiments were performed on age- and sex-matched mice (6–8 wk old) and handled according to National Institutes of Health guidelines. The institutional animal care and use committee approved all studies reported.

DOX-regulated lung-specific Relm-α bitransgenic mice. Relm-α bitransgenic mice used were generated as described previously (20). The single transgenic lines were the tetracycline reverse-transactivator transgenic mouse line CC10-rtTA (expressing rtTA cDNA under the regulation of the lung-specific rat 2.3-kb CC-10 promoter) and the responder transgenic mouse line tetO-Relm-α [containing germine Relm-α cDNA under the regulation of the tetracycline operator (tetO)]. These transgenic lines were crossed to generate tetracycline-induced CC10-rtTA-Relm-α bitransgenic mice for the experiments. The constructs for these transgenic mice are shown in Fig. 2A.

Allergen challenge in mice. Wild-type or Relm-α gene-deficient mice were sensitized by intraperitoneal injection with 100 mg of OVA or peanut allergen and 1 mg aluminum hydroxide in saline on days 0 and 14. On days 24 and 27, mice were lightly anesthetized with inhaled isoﬂurane and challenged intranasally with 100 μg OVA or peanut in 50 μl of saline or with saline, and mice were killed 18–20 h following challenge (40). Furthermore, as required, the wild-type or Relm-α gene-deficient mice were also challenged with nine doses of intranasal Aspergillus fumigatus antigen over the course of 3 wk (28). The allergen challenge was performed by applying 100 μg of A. fumigatus in 50 μl of saline or saline to the nares using a micropipette with the mouse held in a supine position. After instillation, the mice were held upright until alert. Mice were killed 18–20 h following allergen challenge.

Real-time PCR analysis. The RNA samples (300 ng) were subjected to reverse transcription analysis using Biotypec reverse transcriptase (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. Transcripts of IL-5, IL-13, eotaxin-1, eotaxin-2, and Relm-α were quantified by real-time PCR using the LightCycler instrument and LightCycler FastStart DNA master SYBR green I as a positive control. The Transwell unit was kept at 37°C for 5 h in a humidified 95% air-5% CO2 atmosphere. After 5 h, media from the lower chamber was centrifuged at 250 g, and cells were resuspended in 0.05 ml of PBS. The number of migrated cells in the lower chamber was counted with a hemocytometer. Each assay was set up in duplicate and repeated at least three times.

Isolation and purification of mouse primary epithelial cells. Mouse primary epithelial cells were isolated from the sterile esophagus removed from naïve mice. The esophagus was longitudinally cut, washed with sterile Hanks’ buffered salt solution (containing 100 μg/ml penicillin/streptomycin, 0.1 μg/ml hydrocortisone (Sigma), 25 ng/ml epidermal growth factor (Sigma), 10 mM HEPES, 10 μg/ml insulin (Sigma), and 1.25 μg/ml amphoterin B (Sigma)] and cultured in Falcon tissue culture flasks (Fischer Scientific, New Hampton, NH) at 37°C and 5% CO2.

Isolation and purification of mouse eosinophils. Mouse eosinophils were isolated from the spleen of CD2-IL-5 transgenic mice. The spleen was minced and a single-cell suspension was incubated with Miltenyi magnetic separation beads (Miltenyi Biotech), anti-Thy1.2, and anti-CD19 antibodies (BD Biosciences) for 20 min at 4°C. Mouse eosinophils were purified using a CS column (Miltenyi Biotech) following the manufacturer’s protocol.

Eosinophil migration assay. Transwell units (24 wells) of 5-μm porosity polycarbonate filters (Corning, Corning, NY) coated with 1% gelatin were used for monitoring in vitro cell migration assay of mouse eosinophils (1 × 106 cells/well). Mouse eosinophils in HBSS (Life Technologies, St. Paul, MN) pH 7.2 were placed in the upper chamber, and different concentrations of Relm-α (1, 10, 100, and 500 ng/ml) or eotaxin-2 (100 ng/ml) were added to the lower chamber. Eotaxin-2, a known chemoattractant for eosinophils, was used as a positive control. The Transwell unit was kept at 37°C for 5 h in a humidified 95% air-5% CO2 atmosphere. After 5 h, media from the lower chamber was centrifuged at 250 g, and cells were resuspended in 0.05 ml of PBS. The number of migrated cells in the lower chamber was counted with a hemocytometer. Each assay was set up in duplicate and repeated at least three times.

Eosinophil analysis in the esophagus. Esophageal, 5-μm paraffin sections were immunostained with antiserum against mouse eosinophil major basic protein (anti-MBP) as previously described (21, 24). In brief, endogenous peroxide in the tissue was quenched with 0.3% hydrogen peroxide in methanol followed by nonspecific protein blocking with normal goat serum. Tissue sections were then incubated with rat anti-MBP (1:2,000) overnight at 4°C, followed by incubation with 1:200 dilution of biotinylated anti-rat IgG secondary antibody and avidin-peroxidase complex (Vector Laboratories, Burlingame, CA) for 30 min each. These slides were further developed with nickel diaminobenzidine-coalt chloride solution to form a black precipitate and counterstained with hematoxylin. Negative controls included replacing the primary antibody with normal rat serum.

Enzyme-linked immunosorbent assay. Relm-α protein concentrations in the esophageal homogenates were quantified by established enzyme-linked immunosorbent assay (ELISA) analysis using anti-Relm-α-captured, biotinylated anti-Relm-α detection and purified Relm-α standard (Peprotech, Rocky Hill, NJ). In brief, Relm-α-specific monoclonal capture antibody was used to precoat a 96-well ELISA plate and blocked for nonspecific protein binding with fetal bovine serum, and then the samples were applied. The plate was incubated 2 h at room temperature and washed with 0.05% Tween 20 containing PBS, and biotinylated cytokine-specific monoclonal antibody was applied to each well followed by avidin-horseradish peroxidase conjugate reagent. Finally, TMB substrate solution (BD Biosciences Pharmingen, San Diego, CA) was added to each well, the color was developed in the dark at room temperature, and the optical density was immediately read at 450 nm. The cytokine concentration of each sample was calculated by using a standard curve. The lower detection limit for Relm-α was 125.62 pg/ml.

Quantification of eosinophils. Eosinophils were quantified by counting the MBP-positive cells in the epithelial mucosa and lamina propria of the esophagus. Eosinophil numbers and area of each esophageal tissue section were measured and calculated with the assistance of digital morphometry analysis using Infinity Analyze 6.1.0 (Luminera) and expressed as eosinophils per square millimeter
similar to a methodology described previously (23, 25). We further used the same software to measure epithelial cell layer thickness.

Analysis of epithelial cell proliferation. To determine the degree of epithelial cell proliferation, 5'-bromodeoxyuridine (BrdU) (Zymed Laboratories, San Francisco, CA) incorporation analysis was performed following the manufacturer’s protocol. In brief, saline or allergen-treated mice were injected intraperitoneally with 0.25 ml of BrdU (0.75 μg/ml) 3 h before death. The esophagus was fixed with 10% neutral buffered formalin (Sigma) for 24 h. After fixation, the tissue was embedded in paraffin, and 5-μm sections were processed using standard histological approaches. Tissues were digested with trypsin (0.125%) for 3 min at 37°C followed by incubation for 30 min at room temperature. The tissue was embedded in paraffin, and 5-μm sections were processed using standard histological approaches. Tissues were digested with trypsin (0.125%) for 3 min at 37°C followed by incubation for 30 min at room temperature. Sections were washed with PBS three times for 2 min and further incubated with monoclonal biotinylated anti-BrdU or anti-proliferating cell nuclear antigen (PCNA) antibodies for 60 min at room temperature. Negative controls included replacing the primary antibody with PBS, and positive controls were provided by the manufacturer. Anti-BrdU or anti-PCNA nuclear incorporated positive cells were detected with streptavidin-peroxidase and DAB substrate (Zymed Laboratories) followed by counterstaining with hematoxylin. Counting the percent BrdU+ or PCNA+ cells from the total epithelial cells of the basal layer quantified the epithelial cell proliferation.

Statistical analysis. Data are expressed as means ± SD. Statistical significance comparing different sets of mice was determined by unpaired InStat GraphPad t-tests.

Fig. 1. Resistin-like molecule (Relm)-α mRNA and protein expression following aerosol allergen or food allergen-induced experimental eosinophilic esophagitis (EoE). The experimental EoE was induced following aerosol allergen and food [ovalbumin (OVA) or peanut] allergen challenge in mice, and esophageal RNA and homogenate was obtained for Relm-α expression in the esophagus. The Relm-α mRNA expression and protein levels in the esophagus were examined by performing qPCR and ELISA analysis. Relm-α mRNA and protein in the esophagus from Aspergillus-challenged mice (A and D), OVA-challenged mice (B and E), and peanut-challenged mice (C and F) are shown. The data are expressed as means ± SD; n = 10 mice/group.

Fig. 2. Generation of doxycycline (DOX)-inducible CC-10-Relm-α bitransgenic mice and the analysis of Relm-α expression in the esophagus. The transgenic constructs present in the tetracycline reverse-transactivator transgenic mouse line CC10-rtTA (expressing rtTA cDNA under the regulation of the lung-specific, rat, 2.3-kb CC-10 promoter) and the tetO-Relm-α transgenic mice [containing germline Relm-α cDNA under the regulation of the tetracycline operator (tetO)] are shown (A). The Relm-α bitransgenic mice were fed DOX food or normal food (no DOX) for 6 wk, and esophageal RNA from homogenate was isolated and analyzed for Relm-α mRNA and protein expression via quantitative RT-PCR and ELISA, and data of Relm-α mRNA (B) and Relm-α protein (C) levels are shown. The data are expressed as means ± SD; n = 8 mice/group.
RESULTS

Relm-\( \alpha \) mRNA is induced in the esophagus following induction of allergen-induced experimental asthma. We previously reported that Relm-\( \alpha \) mRNA expression was significantly increased in two distinct models of experimental asthma [ovalbumin (OVA) and Aspergillus fumigatus] based on global quantitative microarray analysis of the lung (40). Peanut, OVA, and Aspergillus in the experimental murine asthma model also promotes EoE (9, 15, 22). Similarly, it has been shown previously that peanut-sensitized mice challenged with intranasal peanut also induces esophageal eosinophilia (33); therefore, we are now interested to know whether Relm-\( \alpha \) is also induced in the esophagus of Aspergillus, OVA, or peanut-induced experimental EoE. Accordingly, esophageal Relm-\( \alpha \) transcript and protein levels were analyzed in the esophagus. Our analysis demonstrated an \( \sim1.5\)- to 2.5-fold increase in relative Relm-\( \alpha \) mRNA expression and \( \sim2\)- to 6-fold increase in the Relm-\( \alpha \) protein levels in the esophagus of all allergen-challenged mice compared with their respective saline-challenged control mice (Fig. 1, A–F).

Relm-\( \alpha \) protein is induced in the esophagus of CC10-rtTA-Relm-\( \alpha \) bitransgenic mice following 6 wk of DOX food exposure. Furthermore, to examine the role of Relm-\( \alpha \) in promoting EoE pathogenesis, we used lung-specific, DOX-inducible Relm-\( \alpha \) bitransgenic mice (CC10-rtTA-Relm-\( \alpha \)) using the construct shown (Fig. 2A). Relm-\( \alpha \) mRNA and protein were analyzed in the esophagus of Relm-\( \alpha \) bitransgenic mice following 6 wk of normal (no DOX) and DOX food exposure. Our analysis shows that Relm-\( \alpha \) protein but not mRNA is induced significantly, not mRNA, in the esophagus of DOX food-exposed mice compared with no DOX-exposed mice (Fig. 2, B and C). The data indicate that CC10-rtTA-Relm-\( \alpha \) transgenic mice show a trend of increased levels of Relm-\( \alpha \) mRNA, but this was not statistically significant; however, a significant increase of Relm-\( \alpha \) protein is noted. It is possible that the Relm-\( \alpha \) protein in the esophagus may be deriving from the lung, since mice might swallow the protein produced in the lung of these transgenic mice.

Relm-\( \alpha \) overexpression induces esophageal-activated CD4\(^+\), CD4\(^-\) T cells, and eosinophils. Earlier it has been shown that Relm-\( \alpha \) is an allergen-induced cytokine and has a role in lung remodeling (27). We detected Relm-\( \alpha \) induction in the murine...
models of EoE; therefore, we next tested the hypothesis whether esophageal Relm-α overexpression in mice promotes number and activation of T cells and eosinophils in the esophagus. Accordingly, flow cytometry and immunostaining of tissue sections was performed to examine the induction of T cells and eosinophils in the esophagus of 6 wk DOX and no DOX-exposed Relm-α bitransgenic mice. Interestingly, our flow cytometry detected an induced number of activated CD4+ and CD4− T cells (Fig. 3, A–C) and esophageal eosinophils (Fig. 3, D and E) in DOX-treated compared with no DOX-exposed mice. Most of the eosinophils accumulated in the esophageal lamina propria of DOX-exposed mice (Fig. 3D). The absolute number of activated CD4+ and CD4− T cells was 65.5 ± 2.7 × 103 and 29.6 ± 3.2 × 103 in DOX-exposed Relm-α bitransgenic mice compared with 49.2 ± 2.1 × 104 and 20.8 ± 1.9 × 104 in no DOX-exposed Relm-α bitransgenic mice. Similarly, the increase of total eosinophil numbers in the esophagus of DOX-exposed Relm-α bitransgenic mice was 23.3 ± 7.1/mm² compared with 5.1 ± 1.4/mm² in no DOX-exposed Relm-α bitransgenic mice. Data are shown as means ± SD (n = 10–12).

Relm-α has no eosinophil chemotactic activity in vitro. The impressive accumulation of eosinophils in the esophagus of CC10-rTAg-Relm-α transgenic mice prompted us to examine the direct effect of recombinant Relm-α on the in vitro motility of eosinophils. Therefore, we tested the hypothesis that Relm-α might be a chemoattractant for eosinophils, and we performed an in vitro Transwell membrane chemoattraction analysis. Notably, Relm-α did not induce dose-dependent eosinophil movement through a Transwell membrane. No increase in the eosinophil movement was observed at any doses of Relm-α used in the experiment. Eotaxin-2 (100 ng/ml), a known eosinophil chemoattractant, was used as a positive control that showed an approximately sevenfold increase in eosinophil compared with Relm-α (100 ng/ml) or eotaxin-2 (0 ng/ml; data not shown).

Eosinophil active cytokines, IL-5 and IL-13, and chemokines, eotaxin-1 and eotaxin-2, are not induced in the esophagus of CC10-rTAg-Relm-α transgenic mice following DOX exposure. Next, we investigated whether eosinophil active cytokines and chemokines are induced in the esophagus of CC10-rTAg-Relm-α transgenic mice following 6 wk of DOX exposure. Accordingly, we performed real-time PCR analyses of IL-5, IL-13, eotaxin-1, and eotaxin-2 mRNA levels in the esophagus of Relm-α bitransgenic mice following 6 wk of no DOX and DOX-exposed mice were analyzed and are shown (A–D). The data are expressed as means ± SD; n = 10 mice/group.

![Graphs A-D](https://example.com/graphs.png)

Fig. 4. Eosinophil active cytokines and chemokines are not induced in the esophagus of 6 wk exposed Relm-α bitransgenic mice. The quantitative real-time PCR analyses of IL-5, IL-13, eotaxin-1, and eotaxin-2 mRNA levels in the esophagus of Relm-α bitransgenic mice following 6 wk of no DOX and DOX-exposed mice were analyzed and are shown (A–D). The data are expressed as means ± SD; n = 10 mice/group.

we also measured the thickness of the esophageal basal layer by morphometric analysis and found that following DOX exposure the thickness of the basal layer is also increased with the cell proliferation (Fig. 5, C and D). The BrdU positivity quantification indicated that DOX-exposed mice had an ~2.5-fold increase in BrdU-incorporated cells compared with mice exposed to no DOX food (Fig. 5, E and F). The BrdU+ cells in the basal layer of the esophagus of no DOX and DOX food-exposed mice was 10.1 ± 6.3 and 27.4 ± 8.3%, respectively (P < 0.01, mean ± SD; n = 12 mice/group). The no DOX and DOX food-exposed mice epithelial layer thickness was 2.06 ± 0.3 and 4.2 ± 0.8 μm, respectively (mean ± SD, P < 0.05; n = 12 mice/group). Furthermore, the epithelial cell proliferation in the allergen-challenged mouse model of EoE was also examined by immunostaining the esophageal tissue sections with anti-PCNA. The *Aspergillus*-challenged Relm-α gene-deficient mice showed a significant reduction in anti-PCNA+ esophageal epithelial cells compared with the *Aspergillus*-challenged wild-type mice, whereas a comparable number of anti-PCNA+ was observed in saline-challenged Relm-α gene-deficient and wild-type mice (Fig. 6A). The PCNA+ epithelial cells in the esophagus of *Aspergillus*-challenged wild-type and Relm-α gene-deficient mice were 31.2 ± 5.4 and 16.3 ± 7.2%, respectively (mean ± SD, n = 5–6; P < 0.05). Importantly, no significant changes in eosinophilic inflammation in the esophagus of *Aspergillus*-challenged Relm-α gene-deficient mice and wild-type mice were observed (Fig. 6B). These results establish that Relm-α promotes esophageal epithelial cell hyperplasia.

In vitro Relm-α promotes primary esophageal epithelial cell proliferation. Furthermore, we next would like to establish that indeed Relm-α promotes epithelial cell proliferation; therefore,
we examined in vitro esophageal epithelial cell proliferation in response to Relm-α exposure. The primary esophageal epithelial cells from mouse esophagus were isolated and cultured in complete RPMI 1640 medium in our laboratory as described earlier (39). We tested the characteristics of isolated cultured epithelial cells by staining with anti-cytokeratin Ab and analyzing with flow cytometry (Fig. 7A). Next, the in vitro experiments were performed in triplicate (2 × 10⁵ cells/well) in complete RPMI 1640 medium in the presence of 0, 10, 100, or 500 ng/ml Relm-α (Peprotech) and MTA reagent (200 μl) to measure the epithelial cell proliferation for 48 h at 37°C. The cells and cell supernatant were harvested at each time point. The cell numbers were counted by hemocytometer, and optical density of the supernatant was analyzed at 540 nm. A dose-dependent increase in cell proliferation was observed following Relm-α treatment. The optical density data at each concentration were shown in Fig. 7B and cell numbers in Fig. 7C.

**DISCUSSION**

Eosinophilic inflammation in the esophagus is a multifaceted phenomenon; however, a number of studies have shown that the mechanism operational in EoE pathogenesis is very similar in mice and humans (1, 22, 25, 35). To further understand the complex mechanisms involved in the pathogenesis of eosinophilic esophageal disorder, we selected the Relm-α gene for further testing because of previous data showing the association of this family of molecules’ prior association with inflammatory processes and our identification of Relm-α as an important cytokine involved in innate mucosal responses (5, 16, 19, 36). Despite the growing association of Relm family members with inflammatory conditions (11, 12, 18), there is a paucity of information concerning the function of this family of cytokines.

In this study, we found that esophageal inflammation induced by repeated intranasal aeroallergens or food (peanut) allergen-sensitized mouse models of experimental EoE en-
hanced Relm-α transcripts in the esophagus. These findings prompted us to examine the role of Relm in EoE pathogenesis. Accordingly, we first examined whether the CC10-Relm-α bitransgenic mice have induced Relm-α protein in the esophagus. It has been previously shown that the CC-10 promoter-driven bitransgenic mice have their protein induced in the esophagus (10, 41). We observed that Relm-α bitransgenic mice also express high levels of Relm-α protein in the esophagus of DOX food-exposed mice compared with normal food (no DOX)-exposed mice. Interestingly, CC-10 Relm-α bitransgenic mice showed a low expression of Relm-α mRNA in the esophagus but a significant increase of protein levels in the esophagus. A similar result of protein induction in the esophagus but a significant increase of protein levels in the esophagus and the lung that is swallowed by the mice; the current speculation is in accordance with the earlier reported studies (10, 41). Furthermore, we found that high expression of Relm-α promotes eosinophil accumulation in the esophagus. However, Relm-α bitransgenic mice do not have induced eosinophil active cytokines or chemokines following DOX exposure. The levels of eotaxin-1, eotaxin-2, IL-5, and IL-13 are comparable in DOX-exposed and no DOX-exposed mice. These data indicate that Relm-α overexpression may contribute to accumulation of eosinophils in the esophageal tissue. An earlier report indicated that Relm-α is a chemotactrant for eosinophils (29); however, our in vitro experimentation found no direct role of Relm-α in eosinophil chemotraction. This discrepancy may be due to the different source of the recombinant Relm-α protein that showed chemotraction to the eosinophils (29). Our in vivo data indicate that Relm-α overexpression recruits activated CD4+ and CD4− T cell subsets in the esophagus. These activated subsets of T cells may produce eosinophil active cytokines that are responsible for some eosinophil generation from the CD34+ eosinophil precursors present in the esophagus. Notably, the cytokine induction is local and not enough to be detected by our analysis. The most interesting finding we observed following the examination of esophageal histology of DOX-exposed Relm-α bitransgenic mice is a thickened esophageal epithelial layer in DOX-exposed Relm-α bitransgenic mice compared with the

Fig. 6. Epithelial cell hyperplasia in Aspergillus-challenged wild-type and Relm-α gene-deficient mice. The epithelial cell hyperplasia was measured in the esophagus of wild-type mice and Relm-α gene-deficient mice following saline and Aspergillus challenge by counting the percent anti-proliferating cell nuclear antigen (PCNA)-positive immunostained cells and shown in A. The anti-MBP+ eosinophils were also counted in the saline and Aspergillus-challenged wild-type (+/+ ) mice and Relm-α gene-deficient mice (−/−) and shown in B. Data is expressed as means ± SD; n = 12 mice.

Fig. 7. Mouse primary esophageal epithelial cell (mPEEC) proliferation following different concentrations of recombinant Relm-α treatment. The characteristics of isolated and cultured mouse primary epithelial cells (mPEEC) were verified by immunostaining with mouse anti-cytokeratin antibody and tested by performing FACS analysis (A). The mouse primary esophageal epithelial cell proliferation following 48 h of Relm-α (0, 1, 50, 100, 250, 500, and 1,000 ng/ml) exposure was examined, and the optical density data and cell counts of each concentration are shown (B and C). The three independent experiments were performed in triplicate. Data are expressed as means ± SD; n = 3 experiments.
esophageal epithelial layer of no DOX-exposed mice. This observation indicated that Relm-α might promote epithelial cell proliferation. To further substantiate our in vivo data, we performed in vitro kinetic analysis of primary esophageal epithelial cell proliferation in response to the different doses of Relm-α. To further establish Relm-α-induced in vivo epithelial cell proliferation, we performed BrdU incorporation analysis of epithelial cell proliferation in 6 wk no DOX- and DOX-exposed mice. The BrdU incorporation and morphometric analysis established that in vivo Relm-α overexpression promotes esophageal epithelial cell proliferation and thickening of the basal layer (basal layer hyperplasia). The thickened esophageal basal layer is noticed in both EoE and GERD. Furthermore, the data observed by inducing EoE in Relm-α gene-deficient mice and wild-type mice following Aspergillus challenge showed that both of these models promote esophageal eosinophilia, but Relm-α gene-deficient mice are protected from the induction of epithelial cell hyperplasia and basal layer thickening. The increase of esophageal eosinophilia in CC-10-Relm-α-overexpressed mice and lack of significant decrease in Relm-α gene-deficient mice indicate that Relm-α gene may not be critical in promoting esophageal eosinophilia but has an important role in basal layer hyperplasia. Taken together, these study data indicate Relm-α indeed promotes esophageal epithelial cell proliferation (hyperplasia) in the mouse model of EoE; however, Relm-α has a limited role in promoting esophageal eosinophilia.

Collectively, these findings augment our understanding of the function of Relm-α in the pathogenesis of allergen-induced EoE in several ways. First, Relm-α gene is induced in response to allergen exposure. Second, our results identify Relm-α as a proinflammatory cytokine with activity on T cell subsets and eosinophils. Third, our results identify Relm-α has no direct role in chemottracting eosinophils in vitro; however, Relm-α overexpression directly recruits activated CD4+ and CD4- T cell subsets in the esophagus and causes enhanced eosinophil accumulation in esophagus. Last, we identify the role of Relm-α in epithelial basal layer hyperplasia in the esophagus. In conclusion, we provide evidence that Relm-α overexpression has no major role in promoting esophageal eosinophilia but is responsible for epithelial layer thickening in experimental EoE.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: P.M. and R.N. performed experiments; P.M., R.N., P.D., A.Z., and J.S.S. analyzed data; P.D. and J.S.S. prepared figures; A.Z., T.K., and A.M. edited and revised manuscript; A.M. conception and design of research; A.M. approved final version of manuscript.

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