Resistin-like molecule alpha regulates IL-13-induced chemokine production but not allergen-induced airway responses

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Abstract

Resistin-like molecule alpha (Relm-α) is one of the most up-regulated gene products in allergen and parasite-associated Th2 responses. Localized to alternatively activated macrophages, Relm-α has been shown to have an anti-inflammatory effect in parasite-induced Th2 responses, but its role in experimental asthma remains unexplored. Herein, we analyzed the cellular source, IL-4 receptors required to stimulate Relm-α production, and the role of Relm-α following experimental asthma induction by IL-4, IL-13 or multiple experimental regimes, including OVA and *Aspergillus fumigatus* immunization. We demonstrate that Relm-α was secreted into the airway lumen, dependent upon both IL-13Rα1 and likely the type I IL-4 receptor, and differentially localized to epithelial cells and myeloid cells, depending upon the specific cytokine and/or aeroallergen trigger. Studies performed with Retnla gene-targeted mice demonstrated that Relm-α was largely redundant in terms of inducing Th2 cytokines, mucus and inflammatory cell infiltration into the lung. These results mirror the dispensable role that other alternatively-activated macrophage products (such as arginase 1) have in allergen induced experimental asthma and contrast with their role in the setting of parasitic infections. Taken together, our findings demonstrate distinct utilization of IL-4/IL-13 receptors for Relm-α induction in the lungs. Differential regulation of Relm-α expression is likely determined by the relative expression levels of IL-4, IL-13 and their corresponding receptors which are differentially expressed by divergent cells (i.e. epithelial cells and macrophages.) Finally, we identify a largely redundant functional role for Relm-α in acute experimental models of allergen-associated Th2-immune responses.


**Introduction**

Asthma is a chronic and complex inflammatory disease of the airways characterized by airflow obstruction, mucus production, airway hyperresponsiveness (AHR) and airway inflammation. It is the most common chronic illness of childhood affecting up to 20% of children and 7% of adults in Western countries with a combined prevalence of approximately 300 million people worldwide (1).

Asthmatic responses are associated with increased numbers of pulmonary inflammatory cells, including activated T-lymphocytes and eosinophils, which correlate with disease severity (2, 3). T-lymphocytes of the T helper 2 (Th2) phenotype are thought to induce asthma through the secretion of an array of cytokines; in particular, IL-4 and IL-13 (4, 5). These cytokines are produced at elevated levels in allergic tissue and are central regulators of many of the hallmark features of the disease such as IgE production, Th2 differentiation, eosinophilia, mucus hypersecretion, chemokine induction and airway hyperresponsiveness (6). Notably, IL-13 is considered to be more of an effector cytokine in pathogenesis of allergic airway disease compared with IL-4, since AHR and mucus production are predominantly IL-13-dependent (7, 8).

Inflammation triggered by IL-4/IL-13 is mediated by the IL-4 and IL-13 receptors that are expressed by multiple parenchymal cell types (including epithelial cells, smooth muscle and vascular endothelial cells), as well as infiltrative cells such as macrophages, dendritic cells and eosinophils (9). In bone marrow-derived cells, IL-4 exerts its activities by interacting with a
specific cell surface receptor comprised of IL-4Rα and the common γ (γc) chain (designated the type I IL-4R), which is shared by multiple cytokine receptors \(^2\). IL-4 can also utilize the type II IL-4R, comprised of IL-4Rα and the IL-13 receptor-α1 chain (IL-13Rα1) (9-11), a receptor complex that is also the cognate functional receptor for IL-13 (11). Of note, recent analyses of \(Il13ra1^{−/−}\) mice demonstrated an essential role for this receptor in mediating the effects of IL-13 and allergen in the lungs (12-14).

Resistin-like molecule alpha (Relm-α) belongs to a family of resistin-like molecules (Relms) including Relm-α, Relm-β and Relm-γ that are potent innate immune modulating molecules (15). Interestingly, Relm-α and Relm-β expression is tightly regulated by IL-13, IL-4 and STAT-6 (16-18). Indeed, Relm-family members are implicated in Th2-associated mucosal immune responses and fibrotic diseases (15). Relm-α was originally identified in inflammatory zones associated with an experimental allergic airway disease model and was therefore also called Found in Inflammatory Zones 1 (Flzz1) (19). Although Relm-α has not been identified in the human genome, the expression pattern of human resistin is more similar to Relm-α than to murine resistin (20) suggesting that information obtained about murine Relm-α may relate to human resistin, at least in part. Notably, Relm-α can regulate insulin resistance particular in the setting of intestinal inflammation (16, 21). The latter property may be a particularly important molecular link for connecting impaired metabolism (i.e. obesity) with inflammation.
Relm-α is a hallmark signature gene of murine alternatively activated macrophages (22-25). However, we (and others) have recently shown that intestinal epithelial cells and eosinophils can also express Relm-α (23, 26, 27). The exact function of Relm-α remains unclear, in part because its receptor has not been identified. We have shown a major pro-inflammatory role for Relm-α in dextran sodium sulfate (DSS)-induced colonic inflammation (21, 28), and that Relm-α has eosinophil chemoattractant activity (28). Notably, recent studies using Th2-associated parasite infection murine models have identified a surprising anti-inflammatory role for Relm-α as Retnla-/- mice display increased fibrosis, granuloma formation, IgE, and mucin gene expression (18, 23), consistent with the inhibition of Th2 cytokine production by Relm-α in some settings (23). Relm family members have key functions in pulmonary fibrosis (17, 29, 30). Yet, the function of Relm-α in the setting of experimental asthma, where it is one of the top induced genes (13, 31), has yet to be elucidated. In addition, the expression pattern (dependency on IL-4/IL-13 receptor components), and cellular source of Relm-α following induction of experimental asthma by distinct triggers is currently unknown. Herein, we aimed to define the regulation and role of Relm-α in allergic lung responses. We demonstrate that IL-13Rα1 predominantly regulates increased Relm-α expression following Aspergillus fumigatus (Asp) allergen challenge whereas following chicken egg ovalbumin (OVA)-challenge both IL-13Rα1 and probably the type I IL-4R contribute to the regulation of Relm-α expression. These findings were likely explained by the different sources of Relm-α expression and relatively higher IL-13:IL-4 ratio that was observed in the Asp model (14), so that IL-13 directly
induced Relm-α in lung epithelial cells and macrophages in vivo via the type II IL-4R. Surprisingly, Relm-α was largely dispensable for allergen-induced production of Th2 chemokines, cytokines, mucus production and eosinophilia. Taken together, our results demonstrate that Relm-α is not essential for acute Th2-associated allergen-driven lung responses, which contrasts its key role in helminth-induced pulmonary inflammation (18, 23).
Materials and methods

Mice
The generation of Retnla\textsuperscript{-/-}, Retnlb\textsuperscript{-/-} and Il13ra1\textsuperscript{-/-} mice has been previously described (13, 28). All experiments involving animals were approved by the Cincinnati Children’s Hospital Medical Center and Tel-Aviv University institutional animal experimentation ethics committees. BALB/c or C57BL/6 wild type mice were obtained from Charles River (Wilmington, MA) or Harlan (Rehovot, Israel) and housed under specific pathogen-free conditions.

Real time PCR
Lung RNA samples (0.5-1\textmu g) were subjected to reverse transcription analysis by using iScript reverse transcriptase (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer’s instructions. Mouse Retnla (Forward: cctccactgtaacgaagactc; Reverse: cacacccagtagcagtcatcc) and Hprt (Forward- cacactgaagagctattgtaatg; Reverse: cagactgaagagctattgtaatg) were quantified by means of real-time PCR with the iQ5 instrument or CFX96 and Bio-Rad SYBR Green supermix (Bio-Rad Laboratories, Hercules, CA). Results were then normalized to Hprt amplified from the same cDNA mix and expressed as fold-induction relative to Hprt (32).

Cytokine induced airway inflammation
Three doses (10 \textmu g/mouse) of IL-13 (Peprotech, Rocky Hill, NJ) were administered intratracheally every other day for 6 days. Mice were sacrificed 48 hours following the last cytokine administration.

Allergen Sensitization and Challenges
Experimental asthma was induced by challenging mice with Aspergillus
fumigatus (Asp) or by sensitizing the mice to chicken egg ovalbumin (OVA)
followed by two intranasal challenges as previously described (13, 31, 33). In
all experiments mice were sacrificed 24-48 hrs after the last intranasal
challenge.

**Bronchoalveolar lavage collection and analysis**

Bronchoalveolar lavage collection and cytospin preparations were stained and
determined as described (13, 33).

**ELISA**

Bronchoalveolar lavage fluid (BALF) cytokines and chemokines were
measured with Duo-Set kits (R&D Systems). Lower detection limits for
CCL11, CCL24, CCL2, CCL17, IL-4, IL-13 and IL-5 were 32.5, 15.6, 15.6, 3.9,
31.2, 6.25 and 31.2, respectively). The protein levels of Relm-α were
determined by ELISA using anti-Relm-α antibodies (Peprotech, NJ) according
to the manufacturers’ instructions as described (21, 28).

**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, CA), anti-Mac-3 (BD Pharmingen, San Jose,
CA), anti-MBP (kindly provided by James Lee, Mayo Clinic), anti-Relm-α
(Peprotech, Rocky Hill, NJ) (overnight, 4°C) followed by goat anti-rabbit alexa
488; 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 Corp., Jena, Germany) (21).

**Flow cytometry**

BALF cells were washed and incubated with anti-Gr-1-FITC and anti-F4/80-alexa fluor 647 (eBioscience). At least 10,000 events were acquired using FACSCalibur (BD Biosciences) and analyzed by FlowJo v.8.8.6 (Tree Star).

**Lung histopathology**

Hematoxylin and eosin or periodic acid–Schiff (PAS) staining were performed and quantified as previously described (13).

**Microarray data analysis**

Whole lung RNA was extracted and subjected to Affymetrix Gene Chip microarray analysis as previously reported (31).

**Statistical analysis**

Data were analyzed by ANOVA followed by Tukey post hoc test using GraphPad Prism 4 (San Diego, CA). Data are presented as mean ± SD and values of $p < 0.05$ were considered statistically significant.
Results

Regulation of Relm-α expression by IL-13Rα1

Previous reports have demonstrated that Relm-α is highly induced in settings of experimental asthma (34). Given the critical role of the IL-13:IL-13Rα1 pathway in experimental asthma, we aimed to identify allergen-induced genes, which were differentially regulated by IL-13Rα1 (13). Consistent with previous reports (13, 27, 31), Relm-α was highly induced following allergen (i.e. OVA) challenge (Figure 1A). Interestingly, although Relm-α was highly induced by direct administration of IL-13, the expression of Relm-α in OVA-induced experimental asthma was mostly IL-13Rα1-independent. In contrast, the induction of Relm-β following allergen challenge was entirely dependent on IL-13Rα1 (Figure 1B).

Given that IL-4 can also utilize IL-13Rα1 in order to elicit some of its effects (13, 35), we next examined whether IL-4-induced Relm-α and -β was IL-13Rα1-dependent. Indeed, IL-4 delivery substantially increased the expression of Relm-α and -β in the lungs (Figure 1A-B). Similar to our findings with OVA-induced experimental asthma, the expression of IL-4-induced Relm-α was predominantly independent of IL-13Rα1; whereas, Relm-β expression was partially IL-13Rα1-dependent (Figure 1A-B).

Relm-α expression and dependency on IL-13Rα1 was also assessed by real time PCR (qPCR) and ELISA. Following OVA-challenge, increased mRNA expression of Relm-α was predominantly independent of IL-13Rα1 (Figure
1C) and Relm-α protein was partially IL-13Rα1-dependent (Figure 1D). To define whether IL-13Rα1-independent regulation of Relm-α expression was allergen-specific, wild type and Il13ra1−/− mice were subjected to experimental asthma using a natural occurring Aeroallergen, Aspergillus fumigatus (Asp). While Asp-challenged wild type mice displayed a significant upregulation in Relm-α expression, Asp-challenged Il13ra1−/− mice showed no increase in Relm-α expression (Figure 1E). Notably, IL-13Rα1-dependent expression of Relm-α was observed at the protein level as well, as Asp-challenged Il13ra1−/− mice displayed no increase in expression of Relm-α protein (Figure 1F).

Collectively, these results indicate that Relm-α expression is largely IL-13Rα1-independent following OVA-challenge (and IL-4 delivery) but IL-13Rα1-dependent following Asp-challenge. These results likely reflect predominant utilization of type I and type II IL-4R by IL-4 and IL-13, respectively for Relm-α induction. Further, our results identify differential regulation of Relm-α compared with Relm-β, as the latter remains critically dependent on IL-13Rα1 (e.g. the type II IL-4 receptor) even following OVA challenge.

**Assessment of Relm-α cellular expression following IL-13 and allergen-challenge**

Recent data demonstrate expression of Relm-α in additional cells besides alternatively activated macrophages including epithelial cells and eosinophils (18, 21, 23, 28). As such, we aimed to define the cellular source for Relm-α expression following IL-13- or allergen-challenge. Under normal conditions,
Relm-α protein was expressed in epithelial cells but not alveolar macrophages or cells in the lung parenchyma (Figure 2A-B). Following IL-13 administration, Relm-α expression was highly upregulated in lung epithelial cells and was often detected in the airway lumen (Figure 2D, F). In addition, Relm-α was also expressed in alveolar macrophages (Figure 2E). Similarly, following allergen-challenge, Relm-α was upregulated in airway epithelial cells and macrophages but not infiltrating eosinophils although the latter displayed substantial mRNA levels of Relm-α (Figure 2G-L and data not shown). Importantly, staining of lung tissue with isotype control showed no staining (Figure 2C).

Analysis of Relm-α expression in allergen-challenged Il13ra1−/− mice revealed that allergen-induced Relm-α expression in epithelial cells was entirely dependent on IL-13Rα1 since airway epithelial cells in allergen-challenged Il13ra1−/− mice showed similar expression to saline-treated mice (Figure 3A-B and D-E, magnification X100). Nonetheless, following OVA-challenge, Relm-α expression in alveolar macrophages was IL-13Rα1-independent (Figure 3C, magnification X400), whereas its expression in macrophages following Asp-challenge was IL-13Rα1-dependent (Figure 3F, magnification X400).

**The roles of Relm-α in IL-13-induced lung inflammation**

Our findings defined a marked induction of Relm-α by IL-13- as well as two distinct allergens (OVA and Asp), raising the question of the role of Relm-α in Th2-associated lung responses. We first aimed to define the role of Relm-α in
IL-13-induced lung pathology (9, 36). In response to IL-13 challenge, Retnla−/− displayed elevated levels of CCL24, CCL2 and CCL11 (Figure 4B-C and data not shown) but decreased CCL17 and a trend towards decreased CCL22 expression (Figure 4A and 4D) compared with wild-type mice. Given the key role of chemokines in recruitment of inflammatory cells to the lung (37), we assessed the role of Relm-α in inflammatory cell recruitment. There was no difference in inflammatory cell recruitment in Retnla−/− in response to IL-13 (Figure 4E). Similarly, increased mucus production in response to IL-13 was similar between cytokine-challenged wild type and Retnla−/− mice (data not shown).

For comparison, we examined the role of Relm-β in IL-13-induced lung inflammation. Indeed, regulation of IL-13-induced CCL17 and CCL24 was similar between Retnla−/− and Retnlb−/− mice as both gene-targeted mice displayed decreased and augmented levels of CCL17 and CCL24, respectively in response to IL-13-challenge (Figure 5A-B). Interestingly, Relm-β and to lesser extent Relm-α enhanced IL-13-induced CCL22 as IL-13-induced CCL22 expression was significantly reduced in Retnlb−/− mice in comparison with Retnla−/− and WT mice (Figure 5C). In contrast, CCL2 production was significantly increased in IL-13-challenged Retnlb−/− mice compared to Retnla−/− mice (Figure 5D). Assessment of IL-13-induced cellular recruitment into the lungs revealed that IL-13-challenged Retnlb−/− mice displayed a significant decrease in neutrophil and eosinophil recruitment into the lungs (Figure 5E). These data were confirmed by flow cytometric analysis using the F4/80 and Gr-1 markers, inasmuch as F4/80/Gr1high (i.e.
neutrophils) and F4/80<sup>dim</sup>/Gr-1<sup>dim</sup> cells were significantly reduced in the BALF of IL-13-challenged Retnlb<sup>−/−</sup> but not IL-13-challenged Retnla<sup>−/−</sup> and wild type mice (Figure 5F-G).

**The role of Relm-α in OVA-induced experimental asthma**

OVA-challenged wild type mice displayed significantly increased CCL11, CCL24, CCL2 and CCL17 levels. Interestingly OVA-challenged Retnla<sup>−/−</sup> mice displayed significantly reduced CCL17 levels but comparable levels of CCL11, CCL22 and CCL24 to OVA-challenged wild type mice (Figure 6A-D). Consistent with this data, Retnla<sup>−/−</sup> mice displayed similar inflammatory cellular recruitment into the BALF as wild type mice (Figure 6E).

Assessment of the major Th2 cytokines in the BALF of OVA-challenged mice indicated that Retnla<sup>−/−</sup> mice displayed similar levels of IL-4, IL-13 and IL-5 as wild type mice (Figure 6F-G). Moreover, OVA-challenged wild type and Retnla<sup>−/−</sup> mice showed similar levels of IL-10 (Figure 5I).

Finally, assessment of OVA-induced epithelial cell pathology that is associated with asthma showed that OVA-challenged Retnla<sup>−/−</sup> mice displayed similar levels of goblet cell hyperplasia and mucus production as wild type mice (Figure 6J-K).

**The role of Relm-α in Asp-induced experimental asthma**

We hypothesized that the lack of activity for Relm-α in the “classical” OVA/Alum experimental asthma model may be due to the effects of artificial
adjuvant sensitization, which may override many features of natural mucosal sensitization and especially those driven by IL-13, which is more prominent in the Asp experimental asthma model. Thus, Retnla−/− mice were subjected to an additional experimental asthma model-using Asp. However, intranasal Asp delivery to Retnla−/− mice resulted in no significant alterations in Asp-induced CCL17 and CCL22 (Figure 7A-B) production. Similarly, the levels of CCL11 and CCL24 were found to be similar in Asp-challenged Retnla−/− mice (Figure 6C-D). Recruitment of eosinophils into the BALF was only modestly but statistically significantly reduced (35 ± 8.2%) in Retnla−/− mice (Figure 7E-F). Assessment of BALF and lung Th2 cytokine profile demonstrated no change in IL-4 and IL-13 levels (Figure 7G-H). In addition, Asp-challenged Retnla−/− mice revealed similar levels of goblet cell hyperplasia and mucus production compared to wild type mice (data not shown).
Discussion

The Relm family of proteins was originally identified in the lung and gastrointestinal tract and is strongly linked with the induction of Th2 immune responses and mucosal immunity including asthma, helminthic parasites and inflammatory bowel disease (15-17, 26, 38-40). Relm-α is a hallmark signature gene of murine alternatively activated macrophages (25). However, epithelial cells, eosinophils and adipose tissue may express Relm-α as well (16, 28, 34, 38). Surprisingly, and despite intensive research on Relm-α, the role of Relm-α in models of asthma is unknown.

In this study, we demonstrate several key and surprising results regarding the regulation and role(s) of Relm-α using acute models of experimental asthma. First, we demonstrate differential regulation of Relm-α by IL-13Rα1 and likely the type I IL-4R depending upon the experimental asthma model employed. In particular, we demonstrate that IL-13Rα1 critically regulates Relm-α expression following Asp-challenge. However, following OVA+Alum-induced experimental asthma, Relm-α expression is regulated both by IL-13Rα1 and most likely the type I IL-4R. Second, we demonstrate that baseline Relm-α expression is restricted to airway epithelial cells whereas following induction of experimental asthma airway epithelial cells and macrophages express Relm-α, consistent with the presence of alternatively activated macrophages only after induction of Th2-associated lung disease. Third, we demonstrate that Relm-α partially regulates IL-13-induced lung chemokine production. In particular, Relm-α deficient mice display elevated levels of CCL24, CCL2 and CCL11 and decreased CCL17 and CCL22. Of note, IL-13-challenged Retnlb-/-
mice displayed significantly more CCL2 and less CCL22 than IL-13-challenged Retnla−/− mice. Finally, using two models of experimental asthma, we demonstrate that Relm-α does not have a marked role in the overall Th2 response in the lung as assessed by production of lung Th2 cytokine, chemokine, cellular recruitment and mucus production.

Our results demonstrate that IL-13Rα1 differentially regulates Relm-α expression. Following OVA/Alum sensitization and consequent intranasal OVA challenge, Relm-α expression is predominantly IL-13Rα1-independent; whereas, following mucosal sensitization and challenge (using Asp extract) the expression of Relm-α was IL-13Rα1-dependent. While airway epithelial cells predominantly express the type II IL-4R, which mediates IL-4 and IL-13 signaling, infiltrating hematopoietic cells predominantly express the type I IL-4R (41). Indeed, we demonstrate that following allergen-challenge, Relm-α is expressed both by airway epithelial cells and macrophages. Therefore, the differential regulation and expression of Relm-α expression is likely driven by the marked differences between the OVA/Alum and Asp models and may result from differential IL-4 vs. IL-13 production (14), since the ratio between IL-13 to IL-4 is substantially higher following Asp inoculation than OVA inoculation (13, 14). Despite our finding that lung eosinophils express Relm-α mRNA and that gastrointestinal eosinophils express Relm-α protein, murine eosinophils did not express Relm-α protein following IL-13- or allergen-challenge. These results may indicate different roles for eosinophils and/or eosinophil-derived Relm-α in innate-immune gastrointestinal inflammatory settings compared with allergic airway inflammation.
Given the strong association between IL-13 and Relm-α induction and the ability of IL-13 to directly induce Relm-α, we hypothesized that Relm-α would regulate IL-13-induced lung responses. In particular, IL-13-treated Retnlα−/− mice displayed altered chemokine induction. CCL17 and CCL22 were decreased in Retnlα−/− mice indicating a role for Relm-α in the induction of these chemokines. CCL17 and CCL22 are mainly implicated in the recruitment of Th2 T cells (42). Although we could not detect any alterations in IL-13-induced T cell recruitment into the lung, it is possible that under different settings, Relm-α can modulate T cell responses by governing their chemotactic signals. Moreover, CCL24 [a hallmark eosinophil chemokine (43, 44)] and CCL2 [which recruits monocytes and dendritic cells (45)] were found increased in IL-13-challenged Retnlα−/− mice, indicating a suppressive role for Relm-α. Collectively, these data suggest that the effect of Relm-α was predominantly due to the effects of Relm-α on airway structural cells (such as epithelial cells) since these are responsive to IL-13 for induction of chemokine production (41). Furthermore, and given the structural similarities between Relm-α and -β (15, 40), we were interested in determining whether Relm-regulated IL-13-induced lung chemokine production was Relm-α specific. The regulatory effects of Relm-α and -β on IL-13-induced chemokine production were similar albeit Relm-β was more potent in increasing CCL24 and CCL2 and decreasing CCL17 and CCL22. Although the receptors for Relm-α and -β are still unknown, the different potency that Relm-α and Relm-β display in regards to IL-13-induced chemokine production may be in part due to their respective receptor expression and/or induction of intracellular signaling.
Recent studies indicate a key role for Relm-α in helminth induced Th2 responses (Table 1) (18). Indeed, N. brasiliensis-infected Retnla−/− mice display significantly increased Th2 cytokine production (including IL-4, IL-5 and IL-13) 7 days post infection. Furthermore, Schistosoma mansoni infected Retnla−/− mice displayed increased lung pathology (increased size of egg-induced granulomas, and elevated fibrosis) which was associated with elevated Th2 cytokines and IgE production (23). In fact macrophage-derived Relm-α has been shown to negatively regulate Th2 cytokine production from anti-CD3/anti-CD28–stimulated splenocytes. Collectively these data suggest that Relm-α would be a negative regulator of allergen-induced allergic airway inflammation as well. Nevertheless, in response to allergen challenge, using two distinct experimental asthma models, Retnla−/− mice displayed similar Th2 cytokine production compared with wild type mice. Furthermore, mucus production and chemokine induction was similar between allergen-exposed Retnla−/− and wild type mice.

Interestingly, following Asp-exposure, Retnla−/− displayed a minor (but statistically significant) decrease in lung eosinophilia. Similar to Relm-α, arginase I is another hallmark gene of alternatively activated macrophages which is induced following allergen-challenged and helminth-infection. While cationic amino acid transporter (CAT-2) and arginase I have been shown to possess key roles in response to helminth infection (46, 47), arginase I is not required for allergen-induced inflammation, airway hyperresponsiveness or collagen deposition (48). Furthermore, and similar to our data demonstrating a
role for Relm-α only in IL-13-induced responses but not allergen-challenge, RNAi interference targeting arginase 1 abrogated the development of IL-13-induced AHR (49), but not allergen-induced AHR (48).

Collectively, these data suggest that the classic and major products of alternatively activated macrophage (Relm-α and arginase I) have key roles in helminth-induced immune responses, but have limited roles in allergen-induced airway allergic inflammation (see Table 1). This effect could be due to the lack of chronicity of allergen/antigen exposure. While allergen exposure is rather limited, the exposure to helminth antigens driving the Th2 response may be more chronic. Since our experimental regimes were mainly conducted in models that mimic acute allergic airway inflammation, we cannot exclude the possibility that Relm-α may have a more important role following chronic exposure to allergens. In addition, the role of Relm-α may be dependent on its cellular source and synergy with other secreted molecules that may be present in the inflammatory milieu, which may differ in allergic and helminth infection. For example, we have recently established a pro-inflammatory and synergistic role for Relm-α in LPS-induced macrophage activation (28). In the presence of LPS, Relm-α induced macrophage IL-6 and TNF-α secretion while decreased IL-10 secretion (28). Thus, the role of Relm-α may be dependent on its synergism with LPS or other pattern recognition-dependent pathways, which may largely vary between allergic settings and parasitic infections. For example, recent studies highlight key roles for low dose of LPS and Toll-like receptor 4 in allergenicity. In contrast to allergens, which require narrow (but necessary) innate immune activation (50, 51), helminth infections
may activate numerous innate immune pathways including various pattern recognition receptors (e.g. Toll-like receptors, c type lectin receptors, protease activated receptors, nod-like receptors) (52). In fact, parasites can activate multiple innate immune components via chitin, proteases, lectins and secretory components (e.g. lacto-N-fucopentaose III, schistosome-derived lysophosphatidylserine, phosphorylcholine-rich glycoprotein); whereas, the repertoire of innate immune activation by allergens is more limited (50, 51, 53-59). Although not assessed yet, it would be interesting to determine whether any of the aforementioned components upregulate Relm-α expression and whether Relm-α regulates LPS-induced effects in the lungs. Additionally, given the proposed role of Relm-α in innate immune responses and in helminth induced Th2 responses (23, 26), it will be intriguing to assess its function in infection-associated asthma. For example, rats infected with localized pulmonary cryptococcal infection display increased IL-13 expression and consequent disease pathology (including AHR, mucus production) (60). Thus, it is possible that Relm-α may have a more significant role in disease settings, which involve increased IL-13 responsiveness.

In summary, we demonstrate that Relm-α is secreted into the airway lumen and differentially dependent upon IL-13Rα1 and the type I IL-4 receptors following OVA and Asp, respectively. The cellular source of Relm-α following these two allergens is likely explained by the differential IL-4R requirement and the relative induction of IL-4 and IL-13 in response to distinct allergens (14). Indeed, increased IL-13 levels will promote a predominant type II IL-4R-dependent response pathwa, as IL-13 induced Relm-α expression is mainly in
epithelial cells. Functionally, using Relm-α gene targeted mice, we show that Relm-α was largely redundant in terms of inducing Th2 cytokines, mucus and inflammatory cell infiltration into the lung. These results mirror the dispensable role that other alternatively-activated macrophage products (such as arginase 1) have in acute models of allergen induced experimental asthma and contrast their role in the setting of parasitic infections. These data suggest divergence between allergen-induced responses and helminth-induced Th2-type immunity, based on our collective data concerning Relm-α.
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Figure legends

**Figure 1. Regulation of Relm-α expression by IL-13Rα1.** Microarray analysis of whole lung (A) Relm-α and (B) Relm-β expression following allergen (OVA), IL-4 or IL-13 treatment of wild type (WT) and *Il13ra1*−/− mice. Real time PCR assessment of Relm-α expression following (C) OVA and (E) *Aspergillus fumigatus* (Asp) challenges in wild type (WT) and *Il13ra1*−/− mice. Assessment of Relm-α protein expression in the BALF following (D) OVA and (F) Asp challenges in wild type and *Il13ra1*−/− mice. Data are representative experiments of n=3, ns-non significant; *-p<0.05; **-p<0.01, ***-p<0.001

**Figure 2. Relm-α cellular expression following allergen challenge.** Assessment of Relm-α cellular expression in a model of IL-13- and allergen-induced airway inflammation. Forty-eight hours after the final saline, IL-13 (D-F), OVA (G-I) or *Aspergillus fumigatus* (Asp) (G-L) allergen challenge, the mice were sacrificed and Relm-α (Red) expression was assessed in Mac-1 positive cells (Green, B, E, H, K) or eosinophil major basic protein (green, I, L) using immunofluorescent analysis. Data are representative photomicrographs of n=3.

**Figure 3. Assessment of Relm-α cellular source in allergen-challenged *Il13ra1*−/− mice.** Frozen lung sections obtained from allergen-challenged *Il13ra1*−/− mice were stained for Relm-α (red) and Mac-1 (green) expression following OVA (A-C) or *Aspergillus fumigatus* (Asp) challenges (D-F) and analyzed by immunofluorescence. White arrowheads indicate Mac3+ cells in
the lung parenchyma, which are either positive (C) or negative (F) for Relm-α expression. Data are representative photomicrographs of n=3, numbers in parentheses indicate magnification.

**Figure 4. The effects of Relm-α on IL-13-induced lung responses.**
Assessment of Relm-α-mediated responses in a model of IL-13 induced airway inflammation. Forty-eight hours after the final IL-13 challenge, the mice were assessed for BALF chemokine expression (A-D) and BALF cellular infiltration (E). Data are representative of three experiments (six to eight mice per experimental group), ns- non significant; ** p< 0.01; ***-p < 0.001.

**Figure 5. The effects of Relm-β on IL-13-induced lung responses.**
Assessment of Relm-α- and Relm-β-mediated responses in a model of IL-13-induced airway inflammation. Forty-eight hours after the final IL-13 challenge, the mice were assessed for BALF chemokine expression (A–D), BALF cellular infiltration by differential cell counts (E) and cytometric analysis using anti-Gr-1 and F4/80 staining (F-G). Data are representative of three experiments (six to eight mice per experimental group), flow cytometric dot plots are representative of n=7, ns- non significant; *-p<0.05; ** p< 0.01.

**Figure 6. The effects of Relm-α on OVA-induced allergic airway inflammation.** Twenty-four hours after the final allergen challenge, the mice were examined for BALF chemokine (A–D) production as well as cellular infiltration (E), cytokine production (F-I) and mucus production (J and K). Data
are representative of one of three experiments (6–17 mice per experimental group). ns, not significant; ***-p< 0.001.

Figure 7. The effects of Relm-α on Aspergillus fumigatus-induced allergic airway inflammation. Forty-eight hours after the final allergen challenge, the mice were examined for BALF chemokine production (A–D) and BAL cells (E). In addition, paraffin-embedded allergen-challenged lungs were analyzed for eosinophilic infiltration using anti-eosinophil major basic protein immunohistochemistry (F). BAL samples were analyzed for IL-13 and IL-4 expression (G-H). Data are representative of one of three experiments (6–12 mice per experimental group). ns,-not significant; *- p<0.05; **-p< 0.01.
Table 1. A comprehensive summary comparing the effects regulated by Relm-α and arginase 1 in experimental asthma and helminth infections.

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<th>Property</th>
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<th>Arginase I</th>
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<tr>
<td></td>
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*-NA- not assessed; **- NR- non relevant; AHR-airway hyperreactivity response.
Results

Figure 1.
Figure 2.
Figure 3.

A. Saline (X100)
B. OVA (X100)
C. Asp (X400)

D. Saline (X100)
E. Asp (X100)
F. Asp (X400)
Figure 5.
Figure 6.
Figure 7.