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RNA-Binding Motif 3 (RBM3) regulates Type 2 Innate Lymphoid cell (ILC2) Cytokine Production

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RNA-Binding Motif 3 (RBM3) regulates Type 2 Innate Lymphoid cell (ILC2) Cytokine Production

A Thesis Submitted in Partial Satisfaction of the Requirements for the Degree

Master of Science

in

Biology

by

Jana Hicham Badrani

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2017
The Thesis of Jana Hicham Badrani is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2017
DEDICATION

This work is dedicated to my family: my parents, for their unwavering faith and drive, and my siblings, for their never ending curiosity and constant criticism. My parents have constantly stood by me and have always supported my dreams and desires. They have provided me with an appreciation of hard work and the drive to progress further than I ever could alone. They are, and always will be, my inspiration, my motivation, and my best friends. My younger siblings are constant reminders of life’s simple pleasures. They have shown me how far an active imagination can take you and have taught me all I know about patience.
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The content of this thesis may be submitted in the future for potential publication.

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ABSTRACT OF THE THESIS

RNA-Binding Motif 3 (RBM3) regulates Type 2 Innate Lymphoid cell (ILC2) Cytokine Production

by

Jana Hicham Badrani

Master of Science in Biology

University of California, San Diego, 2017

Associate Professor Taylor A. Doherty, Chair

Professor Li-Fan Lu, Co-Chair

Type 2 immune responses, such as asthma, occur after exposure to allergens and are characterized by the release of cytokines that include IL4, IL5, IL9, IL10, and IL13 (Spellberg et al. 2000). Until the last decade, these cytokines were thought to be produced by conventional CD4+ Th2 cells only and thus, were identified as Th2 cytokines. However, it was recently discovered that Type 2 Innate Lymphoid cells (ILC2s) also potently produce Th2 cytokines and play a role in Type 2 immune responses. Previous
research has also demonstrated that the protein, RNA Binding Motif 3 (RBM3), is one of ten highly expressed genes that contributes to airway hyperresponsiveness in an animal model of asthma. Our preliminary data has revealed that sorted ILC2s from mice challenged with the fungal allergen *Alternaria* have demonstrated higher expression of RBM3 when compared to PBS challenged controls. Thus, this study serves to determine the role of RBM3 in regulating ILC2 Th2 cytokine production and its contributions to type 2 inflammation. It was originally postulated that RBM3 serves to aide in cytokine production via Th2 cytokine mRNA stability; however, data acquired provides evidence of a different potential relationship between RBM3 and Th2 cytokine production. Utilizing a 7-day *Alternaria* model, wild-type and RBM3KO mice were challenged with *Alternaria* to induce asthmatic-like responses. RBM3KO mice expressed higher cell counts and percentages of eosinophils, IL5 expressing ILC2s, and IL13 expressing ILC2s than wild-type controls. *In vitro* data preliminarily supports that ILC2s lacking RBM3 are more active compared to WT ILC2s. These data suggest that RBM3 may be a negative regulator of ILC2 responses. This work may provide novel avenues for therapeutic approaches to asthma.
INTRODUCTION

Asthma is a disease estimated to affect about 5-10% of well-developed nations’ populations (Wenzel 2012), making it one of the most common diseases in the world. In recent years, the prevalence of asthma has become more common as communities become more urbanized. It is further estimated that by 2025, the urban population will increase from 45% to 59% and thus, it can be expected that asthma prevalence will also increase worldwide in that time (Masoli et al. 2004). Asthma has been linked to a wide array of phenotypes, most of which include allergic airway inflammation and increases in airway eosinophil counts. Asthma has largely been considered type 2 immune disease although other phenotypes exist, including neutrophilic asthma (Wenzel 2012).

Type 2 immune responses occur after exposure to allergens, such as pollens, mold, and dust mite, which lead to the release of cytokines including IL4, IL5, IL9, IL10, and IL13 by CD4+ Th2 cells (Spellberg et al. 2000). Asthma’s characteristic features include airway hyperreactivity and remodeling, airway inflammation, and increased mucus production and eosinophilia. IL5 and IL13 are critical cytokines in type 2 immune responses. IL5 increases eosinophil recruitment, activation, and survival. In the presence of IL5 and growth factors, hematopoietic progenitor cells differentiate into eosinophils. Eosinophils, as a characteristic of asthma, are closely associated with airway hyperreactivity and remodeling (Salter et al. 2017). Asthmatic patients demonstrate increased sputum eosinophil percentages (Hastie et al. 2013), which can serve as a way to measure asthma severity and mark airway inflammation. Further, IL13 increases mucus production and airway hyperreactivity and remodeling (van Rijt et al. 2016). Experiments
treated OVA challenged mice with an IL-13 receptor blocker showed decreases in airway inflammation, eosinophilia, mucus secretion, and airway hyperreactivity when compared to OVA challenged mice without the blocker, highlighting IL-13’s role in these processes. Importantly, IL-13 targeted therapy was recently approved for severe asthma after successful trials (Durham 2016). In mice, further decreases in challenged mice were found when both IL-13 and IL-25 receptors were blocked (Zhang et al. 2017). IL-4 is another Th2 cytokine and participates in IgE class switching and Th2 cell differentiation (Kabata 2015).

Prior to the last decade, the production of these Type 2 cytokines had been attributed to the role of Th2 cells. Since then, a multitude of mouse and human studies have demonstrated that type 2 innate lymphoid cells (ILC2s) that lack Th2 cell surface markers (including antigen receptors or TCR) are also capable of producing Th2 cytokines (Fort et al. 2001, Hurst et al. 2002). Very early experiments suggested the presence of a non-T cell that produced Th2 cytokines after IL25 was found to induce gene expression of IL4, IL5, and IL13 by non-T and non-B cells to generate the immune response (Fort et al. 2001). When testing IL25’s ability to induce increased Th2 cytokine expression in vitro, they determined that CD4+ T cells, both naïve and memory, did not contribute to Th2 cytokine production in the presence of IL25. However, slightly increased IL13 levels were detected in splenocytes from RAG2KO mice, which lack T and B cells, when exposed to even low levels of IL25. Fort et al. concluded that the Th2 cytokines produced after IL25 exposure was due to a non-B/non-T cell population. Through flow cytometry, they determined that this population was MHC classhigh, CD11cdull, F4/80low, and CD4-CD8α- (Fort et al. 2001). The newly identified
population was negative for the CD4 marker, which is a known marker for T helper cells; therefore, while the newly identified cell population did produce Th2 cytokines, it was not a part of the Th2 cell population.

Hurst et al. (2002) further confirmed the discovery of this novel cell population with their work with IL17 cytokine family members. They found that eosinophil responses due to IL25 exposure did not require T cells since RAG2KO mice could still generate an eosinophil response; however, the response still required IL5 and IL13. Therefore, the cells producing these Th2 cytokines could not be Th2 cells and had to be a previously unknown cell group. They determined that this cell population was CD45R/B220+, Thy-1+, NK1.1-, GR-1-, CD4-, CD3-, and c-kitnegative (Hurst et al. 2002). Hurst et al. further defined the identity of the surface markers for this new population and confirmed that they were neither CD4+, a component of the Th2 surface markers, nor CD3+, a component of the cytotoxic T cell surface markers. In 2010, three landmark articles determined the identity of ILC2s as lineage-negative lymphocytes that respond to IL-33 and IL-25 (Moro et al. 2010, Price et al. 2010, Neill et al. 2010). These cells have come to be known as Group 2 Innate Lymphoid cells, or ILC2s.

ILC2s are of lymphoid origin, arising from the common lymphoid progenitor (CLP) (Doherty 2015). CLPs are located within bone marrow and express the transcription factors Id2 and PLZF. These transcription factors are important for inhibiting B cell and T cell development and activating ILC development, respectively, and are representative of the ILC family (Suffiotti 2017). All members of the ILC family express the IL-7Rα receptor and have a TH cell counterpart (Zook et al. 2016). The ILC family is split into the cytotoxic NK cells and the non-cytotoxic ILCs, which is further
divided into ILC1, ILC2, and ILC3 groups. The members of the non-cytotoxic ILC family are differentiated by their expressed transcription factors and produced cytokines. ILC1s require expression of the T-Bet transcription factor, which aides in the production of IFN-γ. It functions similar to Th1 cells to ultimately activate macrophages and other phagocytes (Romagnani 2000). ILC3s require the transcription factor RORγt. The group consists of two subsets: one acts like Th22 and produces IL-22 and the other acts like Th17 and produces IL-17. ILC3 cells have been observed to play a role in protecting and maintaining the gut microbiome (Martinez-Gonzales 2015, Zook et al. 2016). ILC2s require the transcription factors GATA3, Bcl-11b, TCF1, ETS1, and RORα (Zook et al. 2016). ILC2s have the IL-33 receptor marker T1/ST2 and are activated by exposure to IL-33. They can also be activated by IL-25; however, they appear to respond more strongly to IL-33, which causes a more robust cytokine response (Barlow 2013).

Since the initial discovery of ILC2s, several mediators have been found to activate or inhibit their function, including cytokines and lipid mediators. The lipid mediators prostaglandin D2 and cysteinyl leukotrienes are increased in asthma and rapidly induce ILC2 activation as well as promote chemotaxis (Xue et al. 2014, Doherty et al. 2013). Further, CysLTs have been shown to synergistically activate ILC2s in concert with IL-33 and supports the idea that many factors can modulate ILC2 responses (Lund et al. 2017).

The discovery of ILC2 signaling events are also beginning to emerge. IL-33 binding to the IL-33 receptor involves the phosphorylation of NF-κB p65 and p38 MAPK and initiation of their respective signal transduction pathway. Inhibition of the p38 pathway has been demonstrated to suppress ILC2 production of IL5 and IL13 by
inhibiting phosphorylation of GATA3 and reducing its ability to bind to the IL5 and IL13 promoters (Furusawa et al. 2013). TSLP induces activation signals in ILC2s via STAT5 and enhances GATA3 expression (Mjosberg et al. 2012). CysLTs have recently been shown to induce NFAT signaling, leading to rapid ILC2 activation (Moltke et al. 2016). While ILC2s express a range of transcription factors, GATA3 is the most critical for their development and production of IL5 and IL13 (Mjosberg et al. 2012, Wolterink 2013).

Recent experiments have demonstrated that ILC2s may also play a role in initiating the adaptive Th2 response by contributing to Th2 cell differentiation via IL-13 induced dendritic cell migration to lymph nodes (Halim et al. 2014). ILC2s may also directly affect Th2 cell differentiation through ILC2 expression of type II major histocompatibility complexes that allow them to directly communicate with Th2 cells (Licona-Limon et al. 2013). It is also possible that the adaptive response, specifically the T-cell cytokines, may feedback and influence ILC2 activation and maintenance (Licona-Limon et al. 2013). As such, ILC2s may serve as a connecting and transitioning factor between the innate and adaptive responses.

ILC2s also play a role in airway hyper responsiveness (AHR), which is a critical feature of asthma largely dependent on IL-13 in animal models (Yang et al. 2004). Chang et al. (2011) utilized an influenza model to induce AHR and Rag2KO mice lacking B and T cells (but containing ILC2s) showed increased airway hyperreactivity after infection. Depletion of ILC2s abrogated the AHR and subsequent adoptive transfer of ILC2s could restore the hyperreactivity (Chang et al. 2011). Barlow et al. (2013) further confirmed that ILC2s promote airway hyper responsiveness. IL25 administered to IL13KO mice did not induce airway hyper responsiveness as expected and transfer of
wild-type ILC2s, as opposed to IL13KO ILC2s, restored AHR in the IL13KO mice (Barlow et al. 2013). Thus, AHR which is a significant cause of morbidity in asthma is driven by ILC2s in several animal models.

Importantly, Walker et al. (2005) discovered ten candidate genes of interest that were linked to the promotion of airway hyper responsiveness in mouse lung cells. Through a gene expression analysis of ovalbumin-induced allergic airway inflammatory disease, the authors identified highly expressed genes, of which RNA-binding motif 3, RBM3, was one (Walker et al. 2005). This suggests that RBM3 may be a gene, and protein, of interest when analyzing airway hyper responsiveness and ILC2s’ contribution to the phenotype.

The RBM3 gene encodes the RBM3 protein, a cold-shock protein that allows cells to adapt to harsh environments. RBM3 is a cytoplasmic protein consisting of a single RNA Recognition Motif (RRM) that binds to AU rich elements (AREs) of mRNAs and is a member of the glycine-rich RNA-binding protein family (Zhu et al. 2016). RBM3 has also been previously implicated in tumor growth (Sureban et al. 2008). This is supported by Wellman et al. who observed a direct relationship between RBM3 expression and embryonic kidney cell proliferation: increased RBM3 expression induced increased cell proliferation and decreased expression led to reduced proliferation (Wellman et al. 2010). RBM3 has recently been shown to have neuroprotective effects and its role in synaptic repair after Bastide et al. (2017) demonstrated that RBM3 binds to RTN3 mRNA and promoted translation and production of RTN3, a neuroprotective protein. RBM3’s ability to regulate cell expression of mRNA by binding to mRNA has
also been established (Bastide et al. 2017; Zhu et al. 2016). There have been no functional studies of RBM3 in asthma, ILC2s, or in any asthma model.

Taking into consideration ILC2s’ known role in airway hyperresponsiveness and Walker’s discovery of RBM3 as a gene also involved in airway hyperresponsiveness, we predicted a potential role of RBM3 in ILC2s. Our preliminary microarray analysis demonstrated higher RBM3 expression in sorted lung ILC2s from *Alternaria*-challenged mice than PBS-challenged controls. Based on this preliminary data and the known role of RBM3 in mRNA stability, we had originally postulated that RBM3 plays a role in positively regulating ILC2 Th2 cytokine production by stabilizing IL5 and IL13 mRNA which have predicted AREs that RBM3 could bind to. In order to test for the role of RBM3 in ILC2s’ Th2 cytokine production, we utilized an *Alternaria* challenge asthma model to assess ILC2 and inflammatory endpoints in RBM3KO mice and wild-type controls. Using Flow Cytometry, we determined levels of bronchoalveolar (BAL) and lung eosinophils, Th2 cells, ILC2s, ILC2 proliferation, and ILC2 IL5 and IL13 in order to identify how RBM3 affects ILC2 function and lung inflammation. To determine the direct effect of RBM3 on ILC2s, *in vitro* studies in which sorted WT and RBM3KO ILC2s were stimulated and their cytokine production was quantified. While much work is currently being done for ILC2s and their role in asthma, the role of RBM3 in ILC2s is unknown and novel. This current study has the potential to reveal new information regarding ILC2s, a relatively new cell type, and their role in asthma. Ultimately, this information may aide in uncovering new therapeutic targets for asthma and allergic diseases.
MATERIALS & METHODS

Mice: 6-12 week old female and male C57BL/6J wild-type (WT) mice were acquired from Jackson Laboratories (Bar Harbor, ME). WT mice were age and gender matched to RBM3KO mice acquired from Peter Vanderklish at TSRI and bred in house. All studies were approved by the University of California San Diego Institutional Animal Care and Use Committee.

Intranasal Challenges: All mice were challenged with Alternaria alternata extract (50µg; Greer). For all in vivo experiments, mice were challenged three times over a seven day period as depicted in Figure 2. For experiments in which mice cells were to be sorted and cultured, mice were challenged four times over a ten day period as shown in Figure 9 in order to expand the ILC2 population prior to sorting.

Lung and BAL Processing: After inserting a catheter into the trachea, BAL was collected through a total of 5 flushes with 2% BSA (Sigma, St. Louis, MO). The first flush used 0.5 mL 2% BSA, while all other flushes were in 0.6mL 2% BSA. After centrifugation, the supernatant of the first flush was set aside and frozen for future ELISA. The cells for all five flushes were combined and resuspended in 500µL FACS buffer. Lungs were collected and kept in RPMI; the lungs were dissociated into a single-cell suspension using the Miltenyi Lung Digest Kit and Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) as directed in the company’s protocol. The single cell suspensions were run through a filter, centrifuged, and resuspended in 1mL RPMI. Cells were counted via a flow cytometer (Novocyte).
**Flow Cytometry:** One million lung and BAL cells per tube were surfaced stained for Flow Cytometry; two million lung cells were used for intracellular stains. Cells were first Fc receptor blocked with an mAb to CD16/CD32 (24G.2) for 5 minutes. BAL and lung cells were stained for eosinophils (CD11c negative; SiglecF positive) and were stained with CD45.2 (PerCP), Siglec-F (PE), Gr-1 (APC), and CD11c (FITC). All these antibodies were obtained from eBioscience, San Diego, CA. Lung ILC2s (Lineage negative; Thy1.2 positive; lymphocytes) were stained using CD45.2 (PerCP) and Thy1.2 (APC) (Both obtained from eBioscience.) Lineage (FITC) staining was done by creating a lineage cocktail that included lineage cocktail (BioLegend), CD11c (eBioscience), NK1.1 (eBioscience), CD5 (BD Biosciences), FcεR1 (BioLegend), TCRβ (BD Biosciences), and TCRγδ (BioLegend). Lung TH2 cells (CD4 positive; T1/ST2 positive; lymphocytes) were stained using CD45.2 (PerCP), CD4 (FITC), and T1/ST2 (PE). (The first two antibodies were obtained from eBioscience; T1/ST2 was obtained from BD Biosciences.) For Ki-67 staining, cells were permeabilized using the FoxP3 kit (eBioscience). Cells were surface stained for ILC2s as described above and then intracellularly stained using Ki67 (eBioscience). Ten million lung cells were cultured with Cell Stimulation Cocktail (Life Technologies Corporation) for three hours. Cells were fixed and permeabilized using the BD kit (BD Biosciences) and stained for IL5 (BioLegend) and IL13 (Life Technologies) in PE. Flow Cytometry was performed using the Novocyt. Data was analyzed using FlowJo software (Tree Star, Ashland, OR).

**ILC2 Cell Sorting and Culture:** ILC2s from WT and RBM3KO mice were sorted using the BD Influx and BD FACSAnia II machines from UCSD’s Human Embryonic Stem Cell Core Facility. Sorted ILC2s were rested in 10ng/mL IL2 and IL7 (R&D Systems) in
T-cell media (TCM) in a 96-well plate (35,000 cells per well) for approximately 36 hours. The media before stimulation was collected for a future ELISA. ILC2s were then stimulated for 6 hours in three conditions: TCM and 10ng/mL IL2 and IL7; TCM, 10ng/mL IL2 and IL7, and 30ng/mL IL33 (AFFYMETRIX, INC.); and TCM, 10ng/mL IL2 and IL7, 30ng/mL IL33, and 10-6 M LTD4 (Cayman Chemical). Supernatant was collected for future ELISA cytokine analysis.

*ELISA:* BAL first flush supernatants and supernatants from *in vitro* stimulation were analyzed for IL5 and IL13 cytokines via ELISA (R&D Systems) and as per the manufacturer's instructions. Plates were read using a microplate reader model 680 (Bio-Rad Laboratories, Hercules, CA).

*Lung Histology:* For one experiment, only half the lung was used for stains. The left half was fixed using 4% paraformaldehyde. Hematoxylin and Eosin (H&E) and Periodic acid–Schiff (PAS) staining was performed at the Histology Core in UCSD’s Moore’s Cancer Center.

*Statistical Analysis:* Statistical analysis was performed with GraphPad Prism software (GraphPad Software, La Jolla, CA). P-values were obtained using the Mann-Whitney test and a P value of less than 0.05 was considered statistically significant. ELISA data was analyzed using the following website:http://www.elisaanalysis.com/app.
RESULTS

In order to identify genes unique to activated ILC2s, a microarray was performed on Alternaria-challenged ILC2s and compared to PBS-challenged ILC2s. Mice were challenged with either Alternaria Alternata or PBS and their lungs were harvested, processed, and sorted for a purified population of ILC2s (Figure 1A). Utilizing a microarray analysis, the gene expression of mouse ILC2s from the two conditions was analyzed and compared. RBM3 was found to be one of the highly expressed genes (Figure 1B). The increase in RBM3 expression was confirmed with qPCR (data not shown). Intracellular staining for RBM3 in ILC2s revealed that Alternaria challenged ILC2s had a higher expression of RBM3 than either PBS challenged ILC2s or naive (unchallenged) ILC2s (Figure 1C). This preliminary data, along with Walker et al.’s (2006) observation of RBM3’s role in airway hyperresponsiveness, introduced RBM3 as a gene of interest in ILC2 function and activation.

Utilizing a 7-day protocol (Figure 3), differences in RBM3KO mice and wild-type control mice were observed. Mice challenged with 50µg Alternaria on D0, D3, and D6. Lungs were harvested and BAL was collected on day 7. BAL was surface stained for eosinophils and lung single cell suspensions were surface stained for eosinophils, CD4+ TH2 cells, and ILC2s. ILC2s were then intracellularly stained for Ki67, a proliferation marker (Figure 3). Finally, lung cells were cultured for three hours with cell stimulation cocktail and surface stained for ILC2s. The next day, the cultured cells were intracellularly stained for IL5 and IL13 (Figure 3D).
Eosinophils, identified as CD11c negative, SiglecF positive hematopoietic cells (Figure 3A), were higher in challenged RBM3KO lungs and BAL (Figure 4A-D). The percentage of eosinophils in the BAL did not show a significant difference between groups; however, both groups had an average eosinophil percentage of about 70, demonstrating that both groups were well challenged and had a strong eosinophilic phenotype. H&E staining demonstrated a slight increase in infiltration in RBM3KO mice compared to WT mice (Figure 4E), which supports the eosinophil trends observed through Flow Cytometry.

There appeared to be a slight and insignificant decrease in the TH2 cells and ILC2s of RBM3KO mice (Figure 5A-C). There was no change in the number or percentage of Ki-67 expressing ILC2s between groups, signifying no difference in the proliferative abilities of wild-type and RBM3KO ILC2s (Figure 5D-E).

While there was no significant change in ILC2 numbers or percentages between groups, RBM3KO ILC2s did demonstrate a trending increase in IL5 and IL13 expression (Figure 6A-G), with the percentage of IL13 expression actually being a significant increase (p=0.03874).

In general, the 7-Day model used revealed a prominent increase in eosinophils and a trending increase in IL5 and IL13 expression in ILC2s for RBM3KO mice (Figure 7). In order to test the direct role of RBM3 in ILC2s, we then performed in vitro studies with purified WT and RBM3KO ILC2s, ILC2s were first expanded using a 10-Day *Alternaria* protocol (Figure 8), in which RBM3KO and wild-type mice were challenged with 50µg *Alternaria* on D0, D3, D6, and D9 and their lungs were harvested and
processed on D10. A sorted population of ILC2s was collected and cultured in 10ng/mL IL2 and IL7 in T-cell media (TCM) for 36 hours to rest the cells. The supernatant was collected before stimulation and saved as “pre-stimulation” data points. Cells were then stimulated in three separate conditions: 10ng/mL IL2 and IL7; 10ng/mL IL2 and IL7 and 30ng/mL IL33; 10ng/mL IL2 and IL7, 30ng/mL IL33, and 10-6M Leukotriene D4 (LTD4). Previous work has demonstrated that ILC2s can be rapidly activated with exposure to LTD4 (Doherty et al. 2013). By acting through a different signaling pathway (NFAT) than IL33, LTD4 can cause a synergistic ILC2 response when combined with IL33 (Lund 2017). After 6 hours, the supernatant (“post-stimulation”) was collected and ELISA was used to analyze the amount of IL5 and IL13 produced by ILC2s.

Without stimulation, RBM3KO ILC2s produced significantly more IL5 and IL13 than wild-type ILC2s (Figure 9A-B). RBM3KO ILC2s stimulated with IL33 and a combination of IL33 and LTD4 produced more IL5 than the wild-type counterpart for that condition. There was a general increase in the production of IL5 upon stimulation, with the greatest increase being for the combination of IL33 and LTD4 (Figure 9C). IL13 production followed similar trends as IL5 production; however, significance was only reached for the combination of IL33 and LTD4 in RBM3KO ILC2s when compared to unstimulated RBM3KO ILC2s (Figure 9D). This demonstrates that RBM3 directly negatively regulates ILC2 TH2 cytokine production.
DISCUSSION

We had originally postulated that RBM3 plays a role in regulating ILC2 TH2 cytokine production via mRNA stabilization. This hypothesis was based on RBM3’s known role as an mRNA stabilizer and preliminary data that suggested that RBM3 plays a part in ILC2s activation and function. RBM3KO mice, lacking RBM3, were expected to have decreased IL5 and IL13 mRNA stabilization. Based on this hypothesis, a decrease in IL5 and IL13 expressing ILC2s and, in turn, a decrease in lung eosinophil accumulation was expected.

In order to test our hypothesis and determine what, if any, role RBM3 plays in ILC2s, we utilized an innate Alternaria asthma model. The most prominent and consistent result of this model was an increase in BAL and lung eosinophils. This phenotype was also observed in several other models, both innate and adaptive (data not shown) and is the most convincing evidence of a role for RBM3 as a negative regulator in type 2 lung inflammation. The increase in eosinophil response suggested that there was also an increase in ILC2 IL5 and IL13 production, which was somewhat observed with the innate model. While only the percentage of IL13 expressing ILC2s was significantly increased, the corresponding IL13 expressing ILC2 numbers as well as the IL5 expressing ILC2 percentages and numbers demonstrated a consistent trend towards an increase.

While these observed results and trends were contrary to our initial hypothesis, the hypothesis was not initially discarded due to a caveat of the in vivo studies. These studies utilized RBM3KO mice, which had RBM3 knocked out of every cell, and was not cell specific to ILC2s. It was possible that ILC2s were acting as predicted; however, the
lack of RBM3 in a different cell type caused the observed trends and masked the ILC2s’ response. One distinct possibility was that a loss of RBM3 in eosinophils directly caused an increase in eosinophil recruitment, trafficking, or survival.

In order to more directly determine the effect of RBM3 in ILC2 function, ILC2s from wild-type and RBM3KO mice were sorted and stimulated in culture. RBM3KO ILC2s were found to have higher levels of IL5 and IL13 than wild-type controls. Upon stimulation with IL33, cytokine production for the RBM3KO ILC2s was also increased compared with WT ILC2s. Stimulation with a combination of IL33 and LTD4 caused an even greater increase with the highest levels of Th2 cytokines in the RBM3KO ILC2s.

This suggests that RBM3 may play a role in suppressing either LTD4’s effects through the NFAT pathway or the synergistic effects of leukotrienes and IL33, which have been observed to signal through different pathways (Lund 2017). Both before and after stimulation, there were higher levels of IL5 than IL13 and IL5 demonstrated more distinct differences between the RBM3KO group and wild-type controls. Even before stimulation, RBM3KO ILC2s produced more IL5 and IL13 than wild-type ILC2s, suggesting that RBM3 plays a role in establishing the basal levels of IL5 and IL13 produced by ILC2s. One important control issue is the lack of change in IL5 produced within the wild-type group between the cells in solely media and those stimulated with IL33. Previous experiments have shown that ILC2s, upon stimulation with IL33, should produce increase; however, this discrepancy may be due to the lot of IL33 used which we have found some variability.

The in vitro data partially support the in vivo data observed though the magnitude of ILC2 cytokine production differences (RBM3 vs WT) in vitro was greater than that
visualized *in vivo*. It is possible that another RBM3 in another cell type regulates ILC2s indirectly, which may account for the differences. Our early investigations have also found that RBM3KO mice have reduced IL-33 after *Alternaria* exposure (not shown) and perhaps this reduced IL-33 does not fully activate ILC2s as would be expected. Overall, RBM3 negatively influences ILC2 production of TH2 cytokines that could contribute to level of eosinophil accumulation via IL5 production.

The mechanism of RBM3 functioning in ILC2 and lung responses is unknown. RBM3 acts as more than mRNA stabilizing protein and other functions of RBM3 may serve to explain the observed results. RBM3 has been observed to have an inhibitory effect on the p38 pathway (Yang et al. 2017; Zhuang et al. 2017). In a study analyzing NO-induced apoptosis in human neuroblastoma cells, Yang et al. demonstrated that RBM3 has effects on ERK, p38, JNK, and AKT pathways. Specifically, with a NO stimuli, RBM3 overexpression caused a near complete inhibition of the p38 pathway (Yang et al. 2017). The p38 pathway is important for IL33 signaling as well as phosphorylation of GATA3 for production of IL5 and IL13. If the p38 pathway is suppressed, GATA3 remains unphosphorylated and there is a decreased production of IL5 and IL13. A loss of RBM3 would remove the suppression of the p38 pathway and phosphorylate GATA3 to increase production of IL5 and IL13 and ultimately increase ILC2 activation and lung eosinophilia. Further ILC2 signaling studies will need to be performed to determine which pathways RBM3 regulates during activation.

RBM3 has also demonstrated interactions with micro-RNAs that have been previously implicated in IL5 and IL13 production. One such micro-RNA is Let-7 (Pilotte 2011). Let-7a has been demonstrated to play a regulatory role in TH2 cells by regulating
the IL13 gene (Polikepahad 2010). In its interactions with RBM3, Let-7 may also play a regulatory role in ILC2s given their similar programming to Th2 cells for cytokine production. In both naïve and allergen challenged mice, the Let-7 family micro-RNAs made up the majority of total lung micro-RNA (Polikepahad 2010). While Let-7 has been observed to play a role in inflammation and asthma, the actual contribution to asthma appears complex. \textit{In vitro} experiments demonstrated that Let-7a regulates the IL13 gene. When Let-7a was suppressed in CD4+ Th2 cells, there was an increase in IL13 transcripts, which suggested an anti-inflammatory role for Let-7. However, \textit{in vivo} experiments demonstrated that CD4+ splenic Th2 cells of allergen challenged mice with reduced Let-7a expression had a decrease in IL13 transcripts, which supported a proinflammatory role for Let-7 (Polikepahad 2010). Other micro-RNAs, such as microRNA-155, play a role in ILC2 function and survival (Johansson et al. 2016) and a wide selection of micro-RNAs, short non-coding single stranded RNAs, are currently of interest because of their potential role in allergic inflammation and asthma (Dissanayake 2016). Thus, RBM3 likely has complex regulatory effects on ILC2 function that may involve micro-RNAs as well as stabilization of mRNA AU rich elements (ARE).

AREs are AUUA repeat regions in the 3’ UTR regions of mRNA, which can regulate mRNA translation and stability. RBM3 is not the only binding protein shown to bind to these AREs. Studies analyzing COX-2 mRNA found several proteins that bound to the same COX-2 ARE (Anant et al. 2009). Of the other ARE-binding proteins, Tristetraprolin (TTP) is one of interest because of its possible interaction with RBM3 and its role in destabilizing mRNA (Lai et al. 2006). TTP production is dependent on the p38 pathway (King et al. 2009) and is regulated by phosphorylation. When phosphorylated
(as it is when it is first produced via the p38 pathway), it is in its inactive form and does not negatively affect mRNA, but in its unphosphorylated form, it becomes active and is capable of binding to and destabilizing mRNA (Rahman et al. 2015). Studies analyzing the biphasic production of IL6 mRNA in TNFα stimulated airway smooth muscle cells determined a dynamic interaction between p38 MAPK, MKP-1 (a MAPK deactivator), and TTP that supported the complex temporal regulation of IL-6 mRNA (Prabhala et al. 2014). In summary, it was observed that p38 led to an increase in MKP-1 production and in phosphorylated TTP production, with a peak at 1 hour and 0.5 hours after stimulation, respectively. MKP-1, once accumulated to an appropriate level, deactivated the p38 pathway, and TTP, at that 1 hour mark, was primarily unphosphorylated and active. MKP-1 degradation decreases its inhibition of the p38 pathway, ultimately leading to the phosphorylation and inactivation of TTP. This temporal relationship between MKP-1, p38, and TTP regulates the initial increase in IL6 mRNA, subsequent decrease, and the final increase in mRNA expression at 24 hours after stimulation (Prabhala et al. 2014). Thus, the potential relationship between RBM3 and TTP as well as the relationship between RBM3 and the p38 pathway could likely display variable kinetics in regulating the production and stabilization of IL5 and IL13 cytokines.

Because of RBM3’s known and predicted relationships with multiple factors, including p38, TTP, and Let-7, as well as the possible relationships amongst those factors and the roles they play in mRNA regulation, RBM3’s effect on TH2 cytokine production in ILC2s may be more dynamic and complex than initially predicted. RBM3 appears to be at the center of a network of factors that can influence cytokine production and the removal of RBM3 within ILC2s may be influencing multiple factors in varying ways and
perhaps also at varying times to ultimately regulate Th2 cytokine production. As such, more studies are needed to expand upon the relationship between RBM3 and p38, TTP, and Let-7 and how these relationships could ultimately be regulating cytokine production.

In summary, our studies demonstrate that RBM3 negatively influences production of TH2 cytokines in ILC2s as well as lung eosinophilia in allergen challenged mice. Our data suggest that RBM3 plays an inhibitory or suppressive role in ILC2s. Ongoing work is being performed to determine precisely how RBM3 contributes to ILC2s and this novel research may eventually provide a new therapeutic target for future asthma treatments.

The content of this thesis may be submitted in the future for potential publication. Badrani, Jana; Doherty, Taylor; Vanderklish, Peter; Cavagnero, Kellen; Naji, Luay; Amadeo, Michael; Chirban, Ariana; Yee, Alicia; Tomka, Nancy.
Figure 1- RBM3 expression in sorted ILC2s after *Alternaria* challenges is high (A) Mice were challenged with *Alternaria* or PBS and lung cells were sorted to obtain a purified sample of ILC2s (B) Sorted mouse ILC2s were analyzed via a microarray analysis. Highly expressed genes are depicted in red. RBM3 (black arrow) exists within that red region. (C) Lung cells were intracellularly stained for RBM3 expression. Cells were surface stained for ILC2s. The isotype control is depicted in grey. *Alternaria* challenged cells, red, had a higher expression of RBM3 than PBS challenged, blue, and naive, black, cells.
Figure 2 - **WT and RBM3KO mice were challenged via a 7-day protocol.** Mice were challenged with 50µg *Alternaria* on D0, D3, and D6. Mice were sacked on D7 and lungs and BAL were collected. All surface stains (BAL and Lung eos and Lung TH2 and ILC2) and Lung ILC2 Ki67 were completed that same day. Lung cells were placed in culture for 3 hours with cell stimulation cocktail and then surface stained for ILC2s. Cells were fixed and then intracellularly stained for IL5 and IL13 the next day.
Figure 3 - Flow Cytometry Gating Schemas. (A) Eosinophils were gated as CD11c negative, Siglec F positive hematopoietic cells. Red arrow denotes the percentage values reported. (B) ILC2s were gated as lineage negative, Thy1.2 positive lymphocytes. Red arrow denotes the percentage values reported. (C) TH2 cells were gated as T1ST2 positive, CD4 positive lymphocytes. Red arrow denotes the percentage values reported. (D) IL5, IL13, and Ki67 were intracellular stains. As such, they were surface stained and gated for ILC2s as described previously. Red arrow denotes the percentage values reported. All are using the appropriate antibodies. IL5, IL13, and Ki67 were gated using an isotype control (not shown). Three different samples are depicted. Red arrows denote the percentage values reported.
Figure 3 - **Flow Cytometry Gating Schemas. (continue)** (A) Eosinophils were gated as CD11c negative, Siglec F positive hematopoietic cells. Red arrow denotes the percentage values reported. (B) ILC2s were gated as Lineage negative, Thy1.2 positive lymphocytes. Red arrow denotes the percentage values reported. (C) TH2 cells were gated as T1ST2 positive, CD4 positive lymphocytes. (D) IL5, IL13, and Ki67 were intracellular stains. As such, they were surface stained and gated for ILC2s as described previously (B) and were permeabilized and stained using the appropriate antibodies. IL5, IL13, and Ki67 were gated using an isotype control (not shown). Three different samples are depicted. Red arrows denote the percentage values reported.
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Figure 4 - **RBM3KO mice have higher eosinophil numbers in BAL and lungs and higher eos percentages in lungs.** (A) BAL eosinophils in challenged Wild-Type and RBM3KO mice. (B) Percent eosinophils of hematopoietic cells from BAL in challenged WT and RBM3KO mice. (C) Lung eosinophils in challenged WT and RBM3KO mice. (D) Percent eosinophils of hematopoietic cells from lungs in challenged WT and RBM3KO mice. Colors represent different separate experiments. Blue data points are from experiment for histology. (E) Fixed lung sections of challenged WT and RBM3KO mice stained with H&E at 20x magnification. * p < 0.05, *** p < 0.005, Mann Whitney Test.
Figure 5 - RBM3KO mice did not exhibit any significant changes in lung TH2 cell counts or lung ILC2 cell counts and percentages. There was no observed change in ILC2 proliferation. (A) Total numbers of lung TH2 cells. (B) Total numbers of Lung ILC2s. (C) Lung ILC2 percentages from WT and RBM3KO mice. (D) Total number of ILC2s expressing Ki67. (E) Percentages of Ki67 expressing ILC2s. Colors represent different separate experiments. * p < 0.05, *** p < 0.005, Mann Whitney Test.
Figure 6 - Challenged RBM3KO mice exhibited a trending increase in IL5 and IL13 expression in ILC2s. (A) Total numbers of lung ILC2s expressing IL5. (B) Percentages of IL5-expressing ILC2s. (C) Total numbers of Lung ILC2s expressing IL13. (D) Percentages of IL13-expressing ILC2s. (E) Representative sample FACS plots comparing Wild-Type and RBM3KO IL13 expression in ILC2s. (F) Representative sample FACS plots comparing Wild-Type and RBM3KO IL5 expression in ILC2s. (G) Sample FACS plot of isotype control used for gating for IL5 and IL13 expression. Colors represent different separate experiments. * p < 0.05, *** p < 0.005, Mann Whitney Test.
Figure 6 - **Challenged RBM3KO mice exhibited a trending increase in IL5 and IL13 expression in ILC2s. (continue)** (A) Total numbers of lung ILC2s expressing IL5. (B) Percentages of IL5-expressing ILC2s. (C) Total numbers of Lung ILC2s expressing IL13. (D) Percentages of IL13-expressing ILC2s. (E) Representative sample FACS plots comparing Wild-Type and RBM3KO IL13 expression in ILC2s. (F) Representative sample FACS plots comparing Wild-Type and RBM3KO IL5 expression in ILC2s. (G) Sample FACS plot of isotype control used for gating for IL5 and IL13 expression. Colors represent different separate experiments. * p < 0.05, *** p < 0.005, Mann Whitney Test.
Figure 7 - Summary of observed trends in cell numbers and percentages compared to expected trends based on the initial hypothesis. The initial hypothesis predicted a decrease in IL5 and IL13 production in RBM3KO ILC2s, which would lead to a decrease in eosinophilia. No predictions were made at the time for lung TH2, lung ILC2s, or Ki67 expression in ILC2s. Observed results were generally the opposite of the predicted results: IL5 and IL13 expression seemed to trend towards an increase and eos in lungs and BAL increased. One arrow signifies a non-significant increase or decrease and represent reproducible trends. Two arrows represent a significant change.

<table>
<thead>
<tr>
<th>RBM3KO vs WT</th>
<th>Expected Trends</th>
<th>Cell Numbers</th>
<th>Percentages</th>
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<tbody>
<tr>
<td>BAL eos</td>
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<tr>
<td>Lung eos</td>
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<td>Lung ILC2</td>
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<td>Lung ILC2 IL5</td>
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Figure 8 - WT and RBM3KO ILC2s were expanded, sorted, and stimulated per the above protocol. Mice were challenged with 50µg *Alternaria* on D0, D3, D6, and D9. Mice were sacked on D10 and the lungs were collected, processed, and stained for ILC2s. ILC2s were sorted and cultured. Cells were rested for approximately 36 hours and then stimulated as per the above conditions. Supernatants were collected before (“pre-stimulation”) and after (“post-stimulation”) stimulation. ELISA was run on collected supernatants.
Figure 9 - Sorted RBM3KO ILC2s produced significantly more IL5 and IL13 than Wild-Type ILC2s before stimulation. After stimulation, IL5 production was significantly higher for all conditions for RBM3KO ILC2s. ELISA data for (A) IL5 and (B) IL13 production before stimulation. ELISA data for (A) IL5 and (B) IL13 production after stimulation in three separate conditions. * p < 0.05, *** p < 0.005, Mann Whitney Test.
Figure 10 – **Summary of potential role RBM3 could play to bring about the observed results.** Blue lines signify activation or increased production. Red lines signify inhibition or degradation.
REFERENCES


3. Barlow, Jillian L.; Peel, Samantha; Fox, Jane; Panova, Veera; Hardman, Clare S.; Camelo, Ana et al. (2013): IL-33 is more potent than IL-25 in provoking IL-13-producing nuocytes (type 2 innate lymphoid cells) and airway contraction. In *The Journal of allergy and clinical immunology* 132 (4), pp. 933–941. DOI: 10.1016/j.jaci.2013.05.012.


15. Huang, Yuefeng; Guo, Liying; Qiu, Jin; Chen, Xi; Hu-Li, Jane; Siebenlist, Ulrich et al. (2015): IL-25-responsive, lineage-negative KLRG1(hi) cells are multipotential 'inflammatory' type 2 innate lymphoid cells. In *Nature immunology* 16 (2), pp. 161–169. DOI: 10.1038/ni.3078.


17. ILC2 Paper 2.


20. Klein Wolterink, Roel G. J.; Serafini, Nicolas; van Nimwegen, Menno; Vosshenrich, Christian A. J.; Bruijn, Marjolein J. W. de; Fonseca Pereira, Diogo et al. (2013): Essential, dose-dependent role for the transcription factor Gata3 in


30. Polikepahad, Sumanth; Knight, John M.; Naghavi, Arash O.; Opht, Toni; Creighton, Chad J.; Shaw, Chad et al. (2010): Proinflammatory role for let-7


36. Silver, Jonathan S.; Kearley, Jennifer; Copenhaver, Alan M.; Sanden, Caroline; Mori, Michiko; Yu, Li et al. (2016): Inflammatory triggers associated with exacerbations of COPD orchestrate plasticity of group 2 innate lymphoid cells in the lungs. In *Nature immunology* 17 (6), pp. 626–635. DOI: 10.1038/ni.3443.


43. Yang, Hai-Jie; Ju, Fei; Guo, Xin-Xin; Ma, Shuang-Ping; Wang, Lei; Cheng, Bin-Feng et al. (2017): RNA-binding protein RBM3 prevents NO-induced apoptosis in human neuroblastoma cells by modulating p38 signaling and miR-143. In *Scientific reports* 7, p. 41738. DOI: 10.1038/srep41738.

44. Yang, Gaoyun; Volk, Amy; Petley, Ted; Emmell, Eva; Giles-Komar, Jill; Shang, Xiaozhou et al. (2004): Anti-IL-13 monoclonal antibody inhibits airway hyperresponsiveness, inflammation and airway remodeling. In *Cytokine* 28 (6), pp. 224–232. DOI: 10.1016/j.cyto.2004.08.007.

45. Zhang, Fang-Qi; Han, Xin-Peng; Zhang, Fang; Ma, Xuan; Xiang, Dong; Yang, Xue-Min et al. (2017): Therapeutic efficacy of a co-blockade of IL-13 and IL-25 on airway inflammation and remodeling in a mouse model of asthma. In *International immunopharmacology* 46, pp. 133–140. DOI: 10.1016/j.intimp.2017.03.005.


47. Zhuang, Rui-Juan; Ma, Jian; Shi, Xiang; Ju, Fei; Ma, Shuang-Ping; Wang, Lei et al. (2017): Cold-Inducible Protein RBM3 Protects UV Irradiation-Induced Apoptosis in Neuroblastoma Cells by Affecting p38 and JNK Pathways and Bcl2 Family Proteins. In *Journal of molecular neuroscience : MN*. DOI: 10.1007/s12031-017-0964-3.