THE ROLE OF POSTTRANSCRIPTIONAL REGULATION IN THE PATHOGENESIS OF ALLERGIC ASTHMA

A Dissertation in
Biomedical Sciences
by
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Asthma is a highly prevalent disease characterized by chronic inflammation, airway remodeling, and mucus production. It involves a complex interplay of the airway epithelium, innate immune system, and adaptive immunity that is still not completely understood. Despite the increasing prevalence of asthma, we still have an incomplete understanding of this disease. Addressing this existing knowledge gap will potentially allow us to develop novel therapeutic strategies for the diagnosis and treatment of asthma.

Posttranscriptional regulation (PTR) has emerged as an essential control system for the regulation of gene expression. PTR mediates the fine tuning of protein expression by effecting changes in mRNA stability, availability, and translational efficiency. MicroRNAs (miRNAs) and RNA-binding proteins (RBPs) are crucial mediators of PTR. In this dissertation, we explored the role of these PTR mediators in the inflammatory pathogenesis of asthma. We demonstrated that miR-1248, a miRNA that is overexpressed in asthmatic individuals, positively regulates IL-5 cytokine expression. We also showed that circulating miRNA expression profiles can be used to diagnose and subtype asthma. Additionally, we established that tristetraprolin (TTP), an RBP, destabilizes the IL-13 mRNA and downregulates IL-13 cytokine production. Finally, we demonstrated that ablating TTP expression in the airway epithelium results in neutrophilic pulmonary inflammation. The studies described in this dissertation address key knowledge gaps in the pathogenesis of asthma. We shed light on the importance of PTR in asthma and demonstrate that PTR-based approaches can be beneficial in the clinical management of asthma.
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<tbody>
<tr>
<td>A23187</td>
<td>calcium ionophore</td>
</tr>
<tr>
<td>ACQ</td>
<td>Asthma Control Questionnaire</td>
</tr>
<tr>
<td>AHR</td>
<td>airway hyperresponsiveness</td>
</tr>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>AR</td>
<td>allergic rhinitis</td>
</tr>
<tr>
<td>ARE</td>
<td>AU-rich element</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>AUFI</td>
<td>AU-rich element RNA-binding protein 1</td>
</tr>
<tr>
<td>BALF</td>
<td>bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>BD</td>
<td>bronchodilator</td>
</tr>
<tr>
<td>CLIP-seq</td>
<td>cross-linking immunoprecipitation sequencing</td>
</tr>
<tr>
<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>FDR</td>
<td>false discovery rate</td>
</tr>
<tr>
<td>FEV1</td>
<td>forced expiratory volume in 1 second</td>
</tr>
<tr>
<td>FVC</td>
<td>forced vital capacity</td>
</tr>
<tr>
<td>GC</td>
<td>glucocorticoid</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>HDM</td>
<td>house dust mite</td>
</tr>
<tr>
<td>HuR</td>
<td>human antigen R</td>
</tr>
<tr>
<td>IC</td>
<td>isotype control</td>
</tr>
<tr>
<td>ICS</td>
<td>inhaled corticosteroid</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>KEGG</td>
<td>Kyoto Encyclopedia of Genes and Genomes</td>
</tr>
<tr>
<td>KSRP</td>
<td>KH domain RNA-binding protein</td>
</tr>
<tr>
<td>LABA</td>
<td>long-acting beta agonist</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>NPV</td>
<td>negative predictive value</td>
</tr>
<tr>
<td>PCA</td>
<td>principal component analysis</td>
</tr>
<tr>
<td>PFT</td>
<td>pulmonary function test</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PPV</td>
<td>positive predictive value</td>
</tr>
<tr>
<td>PTR</td>
<td>posttranscriptional regulation</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative real-time PCR</td>
</tr>
<tr>
<td>RBP</td>
<td>RNA-binding protein</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>RNP</td>
<td>ribonucleoprotein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RNP-IP</td>
<td>ribonucleoprotein immunoprecipitation</td>
</tr>
<tr>
<td>ROC</td>
<td>receiver operating characteristic</td>
</tr>
<tr>
<td>SCF</td>
<td>stem cell factor</td>
</tr>
<tr>
<td>TSLP</td>
<td>thymic stromal lymphoprotein</td>
</tr>
<tr>
<td>TTP</td>
<td>tristetraprolin</td>
</tr>
<tr>
<td>USER</td>
<td>untranslated sequence elements for regulation</td>
</tr>
</tbody>
</table>
This dissertation was built upon the work of previous and current members of the Ishmael Lab. Without their support and contribution, this would have been an impossible undertaking.

I owe my deepest gratitude to my supervisor and mentor, Dr. Faoud Ishmael. He has been an encouraging presence and a steady guiding hand throughout my years in the MD/PhD program. Dr. Ishmael has been an excellent role model who genuinely cared about my development as an aspiring physician scientist.

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Chapter 1

Introduction: Posttranscriptional regulation and the pathogenesis of asthma

Asthma is a highly prevalent disease that affects more than 300 million people worldwide\(^1\). In the United States alone, there are over 25 million people with asthma and this number is increasing every year\(^2\). Patients with asthma are often symptomatic and suffer significant impairment in their daily lives. In spite of current advances in asthma therapy, more than half of asthmatics fail to sufficiently control their symptoms. The annual cost of asthma in the US is at least $56 billion in hospitalizations and other medical costs, lost school and work days, and early deaths\(^3\).

Asthma is characterized by chronic inflammation, airway hyperresponsiveness (AHR), mucus production, and airway remodeling. These processes produce the well-recognized clinical symptoms of asthma such as shortness of breath, wheezing, coughing, and chest tightness. Exposure to various environmental factors such as allergens, pollution, and airway irritants can trigger these symptoms.

Despite the increasing prevalence of asthma and the annual costs associated with it, there are still several unmet clinical needs that exist within this disease. At present, the diagnosis of asthma is based on the patient’s clinical history and pulmonary function tests (PFTs). Although informative, the patient’s clinical history is not an objective test and while quantitative measurements can be derived from PFTs, different operational and procedural confounders can muddle their results. Thus, there is a need for objective biomarkers to diagnose and characterize this disease.

In addition to the lack of objective biomarkers, we currently have a limited treatment arsenal for asthma. Currently available therapeutic strategies for asthma include the use of beta-
agonists, anti-cholinergics, leukotriene blockers, corticosteroids, and anti-IgE antibodies. However, despite these options, a significant number of asthmatics still exhibit symptomatic disease. These therapeutic strategies can be thoroughly expanded by having a more complete understanding of the pathogenesis of asthma.

**Inflammatory Pathogenesis of Asthma**

The pathogenesis of asthma involves a complex interplay of environmental factors, airway epithelium, and the immune response\(^4\) (Figure 1-1). Allergens, pollution, and irritants present in the environment are recognized by pattern recognition receptors (PRRs)\(^5\) or proteinase-activated receptors\(^6\) present in the airway epithelium. These allergen-epithelium interactions can result in the disruption of the physical integrity of the airway epithelium as well as the release of various pro-inflammatory mediators.

In healthy individuals, the airway is a tightly regulated, impermeable physical barrier. The integrity of this barrier is maintained by tight junction proteins such as occludin, claudin, and zona occludens\(^7\). Although finger-like extensions from dendritic cells in the lamina propria can be exposed to airway allergens, most of the direct effects of allergens on the immune system are a result of disruption of the airway tight junctions\(^7,8\). Cysteine and serine protease allergens, e.g., from house dust mites (HDM), pollen grain, and fungi, can degrade tight junction proteins\(^9-11\). Indeed, bronchial biopsies obtained from patients with asthma often reveal a leaky and fragile airway epithelium with disrupted junction protein interactions\(^12\).

Furthermore, the airway epithelium plays a key role in the inflammatory pathogenesis of asthma by secreting pro-inflammatory mediators that activate both the adaptive and innate immune response. Disruptions in the physical integrity of the airway epithelium results in the secretion of interleukin (IL)-33, a cytokine that has been shown to play a crucial role in the propagation of Th2 inflammation\(^8,13\). Binding of allergens to PRRs expressed on the airway
epithelium can result in the production of thymic stromal lymphopoietin (TSLP) and granulocyte macrophage colony-stimulating factor (GM-CSF), both of which are potent dendritic cell activators. Dendritic cells are professional antigen presenting cells (APCs) that can induce Th2 differentiation of naïve T cells. The typical physiologic response to these antigens is usually immune tolerance; however, among individuals predisposed to atopic disease, these antigens induce immune sensitization. The mechanism behind this immunologic decision between tolerance versus sensitization is largely unknown. In turn, Th2-derived cytokines can also promote the production of several inflammatory mediators that act on innate immune cells such as eosinophils, neutrophils, basophils, mast cells, monocytes, and macrophages (Figure 1-1).

**Figure 1-1. The inflammatory pathogenesis of asthma.** Exposure of the airway epithelium to allergens and other environmental factors can compromise the physical integrity of the airway epithelium. This allows for antigen endocytosis by APCs present in the lamina propria. These APCs can activate Th2 cells and promote the production of IL-4, IL-5, IL-9, and IL-13. At the same time, the airway epithelium expresses PRRs on its apical side and these receptors can directly interact with allergens present in the airway. Allergen-airway epithelium interactions can result in the production of various inflammatory mediators such as CCL2, TSLP, IL-33, GM-CSF, SCF (stem cell factor), and TNF-α. Th2-derived cytokines such IL-9 and IL-13 can also stimulate the airway epithelium and further potentiate Th2 inflammation.
Activated Th2 cells are CD4+ cells that play a central role in the pathogenesis of asthma. These cells produce characteristic effector cytokines that include IL-4, IL-5, IL-9, IL-10, and IL-13. IL-4 promotes the Th2 differentiation of naïve helper T cells and thus acts as a positive feedback regulator of this process. IL-9 has been shown to act on mast cells, airway epithelial cells, and airway smooth muscle cells. IL-9, along with SCF produced by the airway epithelium, promotes the growth of mast cells. This cytokine also induces goblet cell metaplasia and upregulates mucus production by the airway epithelium. Finally, IL-9 can trigger the production of CCL11 by the airway smooth muscle cells. CCL11, which is also produced by the airway epithelium, is a potent eosinophil chemotactic protein.

Among the cytokines produced by Th2 cells, IL-10 is the only anti-inflammatory cytokine. IL-10 has been shown to inhibit differentiation of naïve T cells into Th1 cells; thus, although it has anti-inflammatory functions, IL-10 can indirectly promote Th2 cell differentiation. In spite of this, studies show that production of IL-10 is reduced in macrophages derived from asthmatic individuals. IL-10 can also antagonize cytokines that promote asthmatic inflammation such as CCL3, GM-CSF, and IFNγ. In this dissertation, we studied the regulation of both IL-5 and IL-13. A special emphasis on the role of these cytokines in the pathogenesis of asthma will be discussed in the succeeding sections of this chapter.

The main effectors of Th2 signaling are mast cells, basophils, eosinophils, and B cells. Mast cells and basophils are granulocytes which are both under the control of Th2 lymphokines. Both of these cells have IgE receptors present on their cell membranes. Binding of allergens to these IgE receptors promotes receptor crosslinking and cellular degranulation. During this event, mast cells and basophils secrete histamines, leukotrienes, prostaglandins and other cytokines. Histamine binding to its receptors present in the bronchial smooth muscles results in airway constriction. Histamine also promotes extravasation of immune cells from the blood by promoting vasodilation of pulmonary blood vessels. Similar to histamines, leukotrienes and prostaglandins are potent bronchoconstrictors. Furthermore, leukotrienes have been
demonstrated as neutrophil and eosinophil chemoattractants. Eosinophil infiltration is one of the most recognized features of asthma. Increased numbers of eosinophil cells have been shown in the airway, BALF (bronchioalveolar lavage fluid), and sputum of asthmatic individuals. Th2-derived IL-5 promotes the growth and maturation of eosinophils while chemokines such CCL11, CCL24, and CCL26 produced in the lungs facilitate eosinophil chemotaxis. Once in the airways, eosinophils can further upregulate asthmatic inflammation by producing other pro-inflammatory mediators. Lastly, the role of B cells in promoting asthma is well-established. IL-4 and IL-13 secreted by Th2 cells promote B cell class switching from IgG4 to IgE. These IgE antibodies become mast cell and basophil receptors that recognize allergens.

**IL-5 is a central mediator of eosinophilic inflammation in asthma**

The human IL-5 gene is located on the 5q31 locus and produces a 115-amino acid secreted glycoprotein. Active IL-5 cytokine is formed by homodimerization of two N-glycosylated IL-5 monomers. This cytokine is primarily produced by Th2 cells, mast cells, eosinophils, and NK cells. The IL-5 receptor (IL-5R) is formed by heterodimerization of the IL-5Rα subunit and the common βc chain. In addition to IL-5, both IL-3 and GM-CSF are also capable of binding the βc chain. The IL-5Rα subunit is exclusively expressed in eosinophils, basophils, and B cells.

Initially, IL-5 binds to IL-5Rα with low affinity but upon heterodimerization with the βc chain, a high affinity IL-5R is formed. Binding of IL-5 to IL-5R results in rapid induction of multiple signaling cascades such as the JAK-STAT, PI3K, MAPK, NF-κB, and Ras pathways. These signaling pathways promote eosinophil survival, differentiation, degranulation, and cytokine production. Furthermore, these pathways also result in eosinophil adhesion and chemotaxis.
Airway eosinophilia is one of the key characteristics of asthma and the role of IL-5 in this process is well-studied. For example, transgenic mice that overexpress IL-5 in the lung epithelium demonstrate marked airway eosinophilia\textsuperscript{39}. These mice also exhibit classic asthma features such as goblet cell hyperplasia, AHR, and airway fibrosis. Conversely, IL-5 knockout mice exhibit a significant decrease in airway eosinophilic infiltration\textsuperscript{40}. Because eosinophils produce TGF-β, IL-5 knockout mice also demonstrate downregulated airway remodeling secondary to the decreased airway eosinophilia.

In human asthma, the importance of IL-5 is well-supported by multiple previous studies. IL-5 mRNA expression has been shown to be elevated in T cells obtained from asthmatic individuals\textsuperscript{41, 42}. Moreover, IL-5 cytokine levels are increased in the BALF, sputum, and serum of asthmatic patients\textsuperscript{43}. Circulating IL-5 levels have been shown to correlate with asthmatic severity and thus allow for identification of patients with a predominantly IL-5-driven asthma\textsuperscript{44}.

Because of the key role of IL-5 in asthma, understanding the regulation of its production is of significant importance. The majority of the studies on IL-5 production focus on its transcriptional activation. The activating transcription factors C/EBP, NFAT, AP-1, and p300 have been demonstrated to act synergistically to promote IL-5 production\textsuperscript{45}. Furthermore, both the pro-inflammatory transcription factors GATA3 and NF-κB have also been demonstrated to trigger IL-5 synthesis\textsuperscript{46}. Finally, the glucocorticoid receptor has been shown to act as a transcription repressor of IL-5\textsuperscript{47}. Currently, approaches in anti-IL-5 therapy is focused on neutralizing the IL-5 cytokine or preventing the dimerization of the IL-5R. Identifying other regulatory control points and hijacking these molecular mechanisms may provide novel therapeutic strategies for asthma and other IL-5-driven diseases.
**IL-13 is a Th2 cytokine that plays a central role in the pathogenesis of asthma**

Human IL-13 cytokine is a 17-kD secreted protein primarily produced by Th2 cells. Other inflammatory cells that produce IL-13 are CD8+ T cells, NK cells, mast cells, basophils, and eosinophils. Interestingly, human airway smooth muscles have also been demonstrated to produce IL-13. Similar to IL-5, the IL-13 gene is situated on the 5q31 locus.

The cellular effects of IL-13 signaling is facilitated by the IL-13 receptor (IL-13R). The functional IL-13R is a transmembrane, JAK/STAT receptor composed of two subunits: IL-4Rα and IL-13Rα1. Unlike the IL-5R, this receptor is expressed by many different cell types such as B cells, macrophages, dendritic cells, eosinophils, endothelial cells, airway epithelial cells, and smooth muscle cells. As such, it is clear that IL-13 signaling can trigger gene expression changes in many different cell types.

Soluble IL-13 cytokine triggers the heterodimerization of IL-4Rα and IL-13Rα1. The JAK1 and TYK2 kinases associated with these receptors phosphorylate tyrosine residues on both IL-4Rα and IL-13Rα1, allowing for docking of STAT6. IL-13R-bound STAT6 is subsequently phosphorylated by JAK1. These phospho-STAT6 molecules are then able to homodimerize and translocate to the nucleus where they can promote the transcription of IL-13-regulated genes.

Although IL-4 and IL-13 both bind to the IL-4Rα subunit, these cytokines have independent roles in the pathogenesis of asthma. Landmark studies by Coyle et al have shown that IL-4 is primarily responsible for the initiation of allergic inflammation. Neutralization of IL-4 prior to allergic sensitization prevents allergic inflammation; however, neutralization of IL-4 after allergic sensitization does not inhibit the occurrence of allergic inflammation.

Compared to IL-4, IL-13 is mainly involved in AHR and mucus production. AHR, the exaggerated airway response to allergens, is one of the hallmarks of asthma. Allergen challenge of IL-13 knockout mice failed to exhibit AHR and mucus production despite high levels of IL-4 and IL-5 as well as substantial airway inflammation. In other words, while there is still a
significant amount of infiltrating inflammatory cells in the airways of the IL-13 knockout mice, these mice do not suffer from AHR and excessive mucus production. Adoptive transfer of T cells from IL-4 knockout mice still resulted in AHR and goblet cell hyperplasia. These studies reveal that even if IL-4 and IL-13 have significant functional overlap, IL-13 chiefly induces the main pathophysiological processes of AHR and mucus production in asthma.

Nevertheless, intranasal administration of IL-13 in mouse airways still recapitulates the asthmatic inflammatory phenotype in mouse models of asthma. IL-13 promotes the influx of eosinophils, monocytes, and lymphocytes in mouse bronchioles. This Th2 cytokine induces the expression of CCL2, CCL7, CCL8, and CCL12 – all of which are monocyte chemoattractant proteins. Furthermore, IL-13 also triggers the production of the eosinophil-specific chemoattractants such as CCL11 and CCL24. Finally, IL-13 promotes CCL17 synthesis in smooth muscle cells. CCL17 facilitates the recruitment of more Th2 cells and dendritic cells in the asthmatic airway.

Apart from upregulating the production of various chemoattractants, IL-13 has strong regulatory effects on many immune cells. IL-13 induces B cell growth and proliferation as well as B cell class switching from IgG4 to IgE. Furthermore, IL-13 promotes eosinophil survival and activation. This cytokine also facilitates IgE priming and activation of mast cells. Finally, IL-13 increases the production of other pro-inflammatory mediators such as adenosine and histamine which in turn upregulate IL-13 gene expression. This positive feedback loop is thought to contribute to the chronicity of asthma.

Another key feature of asthma in which IL-13 plays a central role is airway remodeling. Increased IL-13 expression in mouse airways induces a significant amount of subepithelial fibrosis. IL-13 has been demonstrated to promote fibroblast collagen synthesis, upregulate airway epithelial TGF-β secretion, and induce myofibroblast proliferation.

The robust inflammatory component of asthma has made glucocorticoids (GCs) the mainstay treatment for this disease. However, a small number of patients remain refractory to GC
treatment and these patients account for a large percentage of the overall cost of asthma. Previous studies by Kibe et al has demonstrated a potential role for IL-13 in GC-resistant asthma. They showed that although GCs attenuated pulmonary eosinophilia, they did not diminish IL-13-mediated AHR. Currently, a few anti-IL-13 agents are undergoing clinical trials and have shown promising results thus far.

Similar to IL-5, our current understanding of the molecular mechanisms that control IL-13 expression is largely focused on its transcriptional regulation. Moreover, because IL-5 and IL-13 are functionally related genes, it is unsurprising that they are regulated by the same transcription factors. GATA3, AP-1, and NFAT are among the transcription activators of IL-13 while the glucocorticoid receptor acts its transcriptional repressor. By contrast to IL-5, however, posttranscriptional regulation of IL-13 has been under increasing scrutiny. These studies will be discussed in the later portions of this chapter. Needless to say, these emerging mechanisms of IL-13 regulation can be a source of innovative therapeutic strategies for targeting IL-13 in asthma.

**Posttranscriptional Regulation and the Inflammatory Response**

The complex process of eukaryotic gene expression starts with transcription of DNA into RNA and ultimately results in the translation of a mature mRNA into a functional protein. Between these two molecular events is a series of highly regulated processes such as 5’ capping, splicing, 3’ polyadenylation, cytoplasmic translocation, translation initiation, mRNA destabilization, and mRNA decay. Together, these processes are referred to as posttranscriptional regulation (PTR).

In the presence of external stimuli, multiple transcription factors are usually activated. A single transcription factor is often capable of inducing the expression of a wide variety of genes that allow for a coordinated cellular response. During inflammatory stress, several transcription
factors such as NF-κB and AP-1 are activated. NF-κB alone is capable of targeting the expression of a couple hundred genes that collectively mount the immune response. In spite of the importance of transcription in establishing the immune response, posttranscriptional mechanisms are emerging as key regulatory factors in establishing, sustaining, and resolving inflammation.

Similar to transcriptional control, PTR tends to act on functionally related transcripts. This occurs because transcripts that encode functionally related proteins usually have common untranslated sequence elements for regulations (USERs). These USERs are recognized by regulatory factors such as RNA-binding proteins (RBPs) and microRNAs (miRNAs). Interactions of these regulatory factors with their target transcripts usually result in modulation of mRNA stability and translation efficiency. Seemingly minute changes in mRNA stability can often lead to significant differences in protein levels. These changes combined with additional control at the level of translation initiation can result in large changes in protein expression.

The importance of PTR in controlling the inflammatory response was convincingly demonstrated by the tristetraprolin (TTP) knockout mouse. TTP knockout mice exhibited a severe syndrome of growth retardation, cachexia, arthritis, and inflammation. Through subsequent studies, it was determined that TTP was an RBP that targets the TNF-α transcript for destabilization and degradation. This inflammatory phenotype was later recapitulated in mice with deletion of USERs in the TNF-α 3’ UTR.

In this dissertation, we explore the role of PTR in modulating the cytokines involved in asthmatic inflammation. Specifically, we looked at how miRNAs and the RBPs, TTP and human antigen R (HuR), affect the expression of cytokines that play a central role in the pathogenesis of asthma. The USERs that these RBPs bind to are AU-rich elements (AREs). An overwhelming majority of cytokines implicated in asthma contain these AREs. This supports the argument that ARE-binding proteins like TTP and HuR play a significant role in the pathogenesis of asthma.
HuR, an RBP that upregulates gene expression, is a posttranscriptional regulator of the immune response

One of the more extensively studied RBPs in chemokine PTR is HuR, the sole ubiquitous member of the Hu family of neuronal RBPs. HuR binds to AREs present in the 3’ UTR of numerous inflammatory transcripts. It is functionally defined as a positive regulator of RNA stability and/or translation, though mouse models for this factor suggest a more diverse functional spectrum with complex indirect effects. HuR is a well-recognized regulator of inflammatory genes, such as TNF-α, IL-3, IL-6, IL-8, GM-CSF, COX-2, VEGF, TGF-β, iNOS, CD154 (the CD40 ligand), and the β-adrenergic receptor. Among the cytokine genes clustered on chromosome 5q and therefore relevant for asthma pathogenesis and other Th2-driven, chronic inflammatory responses, IL-3, GM-CSF, IL-4, and IL-13 and the transcription factor GATA-3 are established targets of HuR.

HuR has been previously shown to play an extensive role in Th2 inflammation. Previous studies with human airway epithelial cells treated with IL-4 and TNF-α showed that these cells produce several eosinophilic chemokines such as CCL5, CCL11, and CCL13. Furthermore, upon stimulation with IL-4 and TNF-α, these cells exhibited increased HuR translocation from the nucleus to the cytoplasm. This led to an association of HuR with CCL11 mRNA, coupled with an increase in CCL11 mRNA stability and protein levels on transient overexpression of HuR.

Based on these findings and on the established role of HuR as a modulator of many inflammatory genes that are relevant for chronic allergic responses and epithelial activation, Fan and others utilized a ribonomics approach to test the role of HuR as a common regulatory factor of the chemokine-rich expression profile induced by TNF-α and IFN-γ. Treatment with these cytokines polarizes epithelial gene expression. This study identified a transcript pool containing a considerable cluster of chemokines and signaling molecules. In particular, a group of CCR2 ligands – the monocyte chemoattractants CCL2, CCL8, and CCL13 – and the neutrophilic...
chemokines CXCL1 and CXCL2 were among the most enriched HuR-associated mRNAs. After single gene validation of HuR association, sequence analysis indicated that these transcripts displayed in their 3’ UTRs diverse putative ARE-containing HuR binding sites\(^{107}\) (Figure 1-1A). Using biotinylated, full-length chemokine 3’ UTR and coding regions as probes for biotin pulldown experiments, transcript association with HuR was found to occur for the targets that selectively bind the ARE-containing 3’ UTR regions (Figure 1-1B).

Interestingly, only CCL2 and CCL8 displayed a stimulus-dependent increase in mRNA turnover and responded to transient HuR overexpression with concordant changes in mRNA levels in both primary human airway epithelial cells and the airway epithelial cell line BEAS-2B. Of notice, while on cytokine challenge, CCL2 mRNA was found to be mostly cytoplasmic, as it was HuR in activated cells, CXCL1 mRNA was detected predominantly in the nuclear RNA extract. This may indicate a more critical role for HuR in a stimulus-dependent increase of CCL2 expression, with the promotion of mRNA stability as well as nuclear export, whereas for CXCL1 – and possibly for the other HuR-associated chemokines with unchanged mRNA stability – additional signaling may need to be coupled to HuR association, affecting other factors participating in the ribonuclear protein (RNP) complexes in which HuR was detected.

HuR also exerts effects on translation\(^{84,85,108}\), and levels of targeted chemokines could reflect this additional layer of regulation by HuR\(^{109}\). Translation of CCL2 and other targets could be also influenced by HuR indirectly, by relieving miRNA-mediated translational repression, as in the case of HuR and CAT-1 mRNA\(^{110}\). Taken together, these data point to the complex composition of transcript-specific RNP complexes as a potential mechanism of PTR specificity for chemokine expression.
Figure 1-2. Association of Hu-antigen R (HuR) with chemokines mRNA through the 3’ untranslated region (UTR). (A) Putative HuR binding sites chemokine transcripts associated with HuR\textsuperscript{107}. (B) Western blot analysis showing HuR detection after biotin pull-down of BEAS-2B cell lysates with the biotinylated transcripts spanning either the coding regions (C) or the 3’ UTR (U) of the indicated chemokines and GAPDH (representative of n = 3). (C) Biotin pull-down assay using either the CCL2 mRNA 3’ UTR full length (nt 374–749) or segments (A–C) containing different putative HuR sites (indicated as 1 to 4 in the figure, sequences listed in the
box). Underlined is sequence 1, which is included in the biotinylated probe A that retained HuR binding. Modified from Fan et al.\textsuperscript{105}, reprinted with permission.

**TTP is an RBP that downregulates the expression of its target mRNAs and counteracts the effects of HuR**

TTP is a tandem CCCH zinc finger protein that was initially discovered as a rapidly induced gene in response to various stimuli\textsuperscript{80}. Some of the immune stimuli that have been shown to induce TTP include phorbol esters, LPS, and TNF-\(\alpha\)\textsuperscript{111,112}. TTP mRNA expression peaks at 30-45 minutes after induction and returns to baseline after about 2 hours\textsuperscript{113}. Thus, TTP was classified as an immediate early response gene.

The function of TTP was not identified until the development of the TTP knockout mouse\textsuperscript{81}. As previously mentioned, the TTP knockout mouse exhibited a severe inflammatory phenotype that resembled chronic TNF-\(\alpha\) expression. Treatment of the TTP knockout mouse with TNF-\(\alpha\) neutralizing antibodies resulted in the rescue of the inflammatory phenotype. Macrophages derived from this mouse exhibited upregulated TNF-\(\alpha\) secretion\textsuperscript{112}. These macrophages also demonstrated increased steady state levels of TNF-\(\alpha\) mRNA. As such, TTP was thought to act on either TNF-\(\alpha\) transcription or mRNA stability. Pulse-chase actinomycin D experiments later revealed that the TNF-\(\alpha\) transcript from TTP knockout macrophages had a significantly slower turnover rate while RNA-immunoprecipitation experiments later confirmed that TTP directly binds to AREs present in the 3’ UTR of the TNF-\(\alpha\) mRNA\textsuperscript{112}.

*In vitro* experiments with the tandem zinc finger domains of TTP revealed that TTP binds to the nonameric UUAUUUAUU sequence with highest affinity\textsuperscript{114}. Increasing or decreasing the number of U residues between the two A residues resulted in decreased binding affinity of TTP\textsuperscript{115}. Thus, the pentameric AUUUA sequence is often referred to as the core TTP binding site. The binding affinity of TTP is increased if the ARE in its target 3’ UTR is the heptameric WAUUAUAW or the nonameric WWUUUAUAWW where W can either be an A or a U\textsuperscript{116}.
Subsequent studies showed that TTP is an ARE-binding protein that downregulates its target transcript\textsuperscript{80}. Several studies have shown that TTP can interact with the CCR4/CAF1/NOT deadenylase complex, thereby facilitating mRNA deadenylation\textsuperscript{81}. Furthermore, TTP-mediated RNA deadenylation seems to be regulated by the p38-MAPK pathway\textsuperscript{117,118}. Phosphorylation of TTP at its Ser-52 and Ser-178 residues by MK-2, the downstream effector of p38-MAPK signaling, inhibits the recruitment of the deadenylase complex\textsuperscript{117}. Sun et al has shown that this phosphorylation can be reversed by PP2A\textsuperscript{119}. Moreover, TTP has also been implicated in the translation repression of its target transcripts. Previous studies have shown that TTP prevents the polysomal loading of its target mRNAs\textsuperscript{120-122}. More recently, TTP has been shown to recruit eIF4E2 to inhibit the translation of ARE-containing mRNAs\textsuperscript{123}.

Several genes and cytokines involved in asthma have been shown to be targeted by TTP. Apart from TNF-\(\alpha\), GM-CSF, CCL2, IL-6, and VEGF are among the pro-inflammatory cytokines regulated by TTP. Furthermore, TTP also regulates COX-2 which controls the production of prostaglandin D2 (PGD2). PGD2 is overexpressed in asthmatic airways and promotes contraction of the bronchioles. Finally, previous studies have determined that TTP plays a role in facilitating the anti-inflammatory effects of glucocorticoid. Smoak and Cidlowski have shown that TTP is induced by glucocorticoids in the A549 airway epithelial cell line and this results in decreased levels of TNF-\(\alpha\)\textsuperscript{124}. This glucocorticoid-mediated effect is blunted when TTP is silenced by shRNA\textsuperscript{124}. Later transcriptome-wide studies showed that 85% of glucocorticoid-mediated changes in gene expression of mouse embryonic fibroblasts is dependent on TTP\textsuperscript{125}.

TTP exerts its regulatory function on many HuR targets by binding to distinct but partially overlapping AREs that are conserved in their 3’ UTRs\textsuperscript{125-127}. Since TTP and HuR share similar binding sites, the interplay between these two RBPs has been the subject of multiple studies\textsuperscript{128-130}. It appears that under inflammatory conditions, the effects of HuR become dominant in the PTR process; while under conditions limiting the inflammatory cascade, TTP drives the RNP function toward acceleration of mRNA degradation. The study by Fan and others
identifying an UAUUUUAU sequence, which binds TTP\textsuperscript{115}, in the portion of the CCL2 3′ UTR associated with HuR (Figure 1-1C) supports the hypothesis that an interplay between these RBP\s may play a role in modulating chemokine levels. These events could occur in a condition of TNF-α and IFN-γ overexpression in the airway mucosa, for example, during a respiratory viral infection. The mechanisms that regulate these effects have not been fully characterized and are currently under research scrutiny.

MicroRNAs (miRNAs) are short noncoding RNAs with emerging roles in asthmatic inflammation

Another PTR factor that is heavily involved in controlling the initiation, maintenance, and resolution of inflammation are miRNAs. MiRNAs are short, non-coding RNAs which are synthesized from larger precursors and incorporated in the multiprotein RNA-induced silencing complex (RISC). The first miRNA, lin-4, was identified in \textit{C. elegans} in 1993\textsuperscript{131}. Since then, thousands of miRNAs have been identified in genomes of both plants and animals. A single miRNA can target hundreds of often functionally related mRNAs through imperfect pairing of the miRNA seed sequence with binding sites present in its target mRNA. Additionally, a single mRNA transcript can be targeted by multiple miRNAs.
Figure 1-3. Biogenesis of miRNAs. MiRNA genes are transcribed by RNA polymerase II or RNA polymerase III and a pri-miRNA transcript is formed. Stem-loop structures from this transcript are excised by the Drosha-DGRC8 microprocessor complex to form the pre-miRNA. Pre-miRNA is exported into the cytosol by Exportin 5 and is further processed by Dicer. The mature miRNA is then loaded onto RISC. This RNA-protein complex facilitates downregulation of gene expression through translation repression or mRNA degradation.

MiRNAs genes exist as introns from larger protein coding genes or as independent genes themselves\(^{132}\). During the synthesis of miRNAs, these miRNA genes are usually transcribed by RNA polymerase II\(^{133}\) but RNA polymerase III\(^{134}\) has also been implicated in this step (Figure 1-3). This results in the generation of a primary miRNA (pri-miRNA) transcript that contains stem loop structures. A single primary transcript can contain a single miRNA or multiple miRNAs. The stem loop structures present in the pri-miRNA are then processed by the microprocessor complex which is a heterodimer comprised of Drosha and Di George Syndrome Critical Region 8 (DGRC8)\(^{135}\). After microprocessor-mediated cleavage of the pri-miRNA, a 70-100 bp hairpin RNA is formed. This product is known as the pre-miRNA. Pre-miRNAs are shuttled from the nucleus into the cytoplasm via the RanGTP-dependent protein, Exportin 5\(^{136}\). Once in the cytoplasm, the pre-miRNA hairpins are further processed by the Dicer endoribonuclease to
produce a single-stranded, mature miRNA which is then loaded onto RISC via Argonaute (Ago) proteins\textsuperscript{137}.

The mature miRNA is 21-23 nucleotides long and contains a short seed sequence near its 5’ end. This seed sequence pairs with imperfect complementarity to the USERs present in the 3’ UTR of the target mRNA. This imperfect complementarity greatly expands the number of mRNAs a single miRNA can bind to. The exact mechanism by which miRNAs affect their target gene expression remains largely unknown. However, binding of miRNAs to its target transcript often results in downregulation of gene expression. Early studies have shown that this is likely due to repression of translation during the translation initiation step\textsuperscript{138}. More recently, the RISC-bound miRNA has been shown to promote deadenylation-dependent target mRNA decapping and degradation\textsuperscript{138}. MiRNA-mediated upregulation of gene expression has also been reported via miRNA association with the FXR protein\textsuperscript{139}.

There is mounting evidence that miRNAs play a significant role in the PTR of asthmatic inflammation. Multiple \textit{in vitro} and \textit{in vivo} studies have shown that miRNAs regulate various cytokines which play a central role in the pathogenesis of asthma. In the HDM mouse model of asthma, miR-16, miR-21, and miR-126 were significantly upregulated in the mouse airways\textsuperscript{140}. Later studies with the ovalbumin (OVA) model of asthma showed that inhibition of miR-126 results in decreased production of IL-5 and IL-13\textsuperscript{141}. Furthermore, Kumar et al has shown that the let-7 family of miRNAs targets IL-13 and that intranasal administration of let-7 mimic to mice with asthmatic airways results in decreased IL-13 expression, downregulated AHR and subepithelial fibrosis, as well as decreased mucus production\textsuperscript{142}. Finally, induction of asthma in the miR-155 knockout mouse results in diminished airway eosinophilia and decreased mucus production\textsuperscript{143}. The miR-155 knockout mouse also exhibited decreased Th2 cell numbers and consequent decreased Th2 cytokine production\textsuperscript{143}.

Adding to the evidence that miRNAs are important in the pathogenesis of asthma are multiple human studies that show differential miRNA expression in asthmatic patients. Solberg et
al has shown that miRNAs extracted from bronchial epithelial brushings showed differential expression of at least 200 miRNAs. They further showed that some of these miRNAs can be returned to normal levels with inhaled corticosteroid therapy. In a different study, Rijavec et al demonstrated that let-7a levels are significantly decreased in bronchial biopsies from patients with severe asthma. Lastly, studies in our lab have shown that miRNAs are differentially expressed in the exhaled breath condensates obtained from healthy individuals, patients with asthma, or patients with COPD.
Dissertation Overview

PTR has emerged as an important means of regulating gene expression. The key effectors of PTR are miRNAs and RBPs. TTP is an RBP that has been implicated in the PTR of the inflammatory response. Dysregulation of miRNA and RBP pathways may significantly contribute to the inflammatory pathogenesis of asthma. Importantly, posttranscriptional regulatory mechanisms are underexplored clinical targets for the management and treatment of this disease. Figure 1-4 highlights the chemokines and cytokines that are targeted by both TTP and HuR. All of these signaling molecules are additionally targeted by various miRNAs. Therefore, the goal of this dissertation is to characterize the posttranscriptional regulatory mechanisms involved in asthma. To accomplish this goal, the following aims were completed:

Identify miRNAs that play important roles in the pathogenesis of asthma.

A real-time quantitative PCR (RT-qPCR)-based approach was utilized to determine which miRNAs are differentially expressed in asthma. Previously described predictive algorithms were used to determine which genes are targeted by the differentially expressed miRNAs\(^{147-152}\). We also characterized how the differentially expressed miRNA, miR-1248, regulates IL-5. Finally, we used a machine learning algorithm to demonstrate that differentially expressed miRNA profiles can be used to diagnose and subtype asthmatic disease.

Evaluate the contribution of TTP to the pathogenesis of asthma.

Reports have indicated that the IL-13 mRNA is posttranscriptionally regulated by the let-7 family of miRNAs and stabilizing ARE-binding protein, HuR. However, the effects of TTP on
IL-13 PTR have never been studied. Using RNA-binding assays and functional studies, we showed that TTP destabilizes the IL-13 mRNA.

To further characterize the role of TTP in the pathogenesis of asthma, we generated an airway epithelium-specific TTP knockout mouse model of asthma. We demonstrated that ablation of TTP expression in the airway epithelium results in a glucocorticoid-resistant, neutrophilic inflammatory profile.

Figure 1-4. TTP, HuR, and miRNAs target key signaling molecules involved in the pathogenesis of asthma. MicroRNAs target all the chemokines and cytokines illustrated in the figure. Highlighted in red are experimentally determined targets of either TTP or HuR. Underlined cytokines are shown to be regulated by both RBPs.

Summary and conclusions

The studies described in this dissertation address key knowledge gaps in the pathogenesis of asthma. By addressing the aims outlined above, we shed light on the importance of PTR in asthma and demonstrated that PTR-based approaches can be beneficial in the clinical management of asthma.
Acknowledgements

Portions of this chapter have been published and is reprinted from Journal of Interferon and Cytokine Research, Panganiban RP, Vonakis BM, Ishmael FT, and Stellato C, Coordinated Post-Transcriptional Regulation of the Chemokine System: Messages from CCL2\textsuperscript{153}. 
Chapter 2

Differential microRNA expression asthma and the role of miR-1248 in regulation of IL-5

Abstract

Asthma is a chronic inflammatory disease that can be difficult to manage due to a lack of diagnostic biomarkers and an incomplete understanding of the molecular pathogenesis. MicroRNAs (miRNAs) are small, single-stranded, non-coding RNAs with increasing importance in regulation of immune function and as biomarkers. We profiled miRNAs in the serum of asthmatics and non-asthmatic controls to identify miRNAs that could serve as diagnostic markers and potential regulators of allergic inflammation. Differential expression of miR-1248, miR-26a, Let-7a, and Let-7d were observed in asthmatic patients compared to controls. Predictive algorithm analyses of these miRNAs revealed their specificity for different Th2 cytokines, including IL-5, which has not previously been shown to be post-transcriptionally regulated. Using multiple approaches, we showed that miR-1248 physically interacts with the IL-5 transcript in the 3’ untranslated region and serves as a positive regulator to increase IL-5 expression. Collectively, our results demonstrate a previously uncharacterized mode of regulation of IL-5 expression and potential use for miRNAs in the diagnosis and clinical management of asthma.
Introduction

Allergic asthma is a highly prevalent disease characterized by airway obstruction and bronchial hyper-responsiveness following various stimuli\(^{154}\). A complex interplay of the respiratory epithelium, innate immune system, and adaptive immunity produces these characteristics\(^4\). However, the molecular pathogenesis of asthma is not well defined, and regulatory mechanisms that govern the inflammatory processes are still unknown. In addition, diagnosis of asthma can be challenging, which is due in large part to a lack of objective and non-invasive biomarkers. Our overall goal is to identify biomarkers that may be involved in asthma pathogenesis and can be used in asthma diagnosis.

Central to the pathogenesis of allergic asthma is the Th2-mediated inflammatory response. Th2-predominant T-lymphocytes modulate allergic disease by secreting a host of proinflammatory cytokines. Canonical cytokines involved in the Th2 response are IL-4, IL-5, and IL-13. Physiologic effects of these interleukins result in the majority of the immunologic and histopathologic features of asthma. IL-4 and IL-13 promote class-switching in B-cells thereby upregulating their IgE production, while IL-5 is a strong signaling factor for eosinophil survival\(^4\). The latter cytokine is emerging as a key player in allergic disease as a target in difficult to control asthma\(^{155,156}\) and in eosinophilic diseases such as eosinophilic esophagitis\(^{157}\). The factors that regulate the "turning off" of mediators such as IL-5 are poorly characterized.

Regulation of cytokines post-transcriptionally, by the action of microRNAs (miRNAs) and RNA-binding proteins (RBPs) on stability or translation of transcripts, is emerging as a crucial means of regulating the inflammatory response. MiRNAs are short, single-stranded, non-coding RNA molecules that direct RBPs to multiple mRNA targets via partial complementarity. MiRNAs associate with members of the Argonaute family of proteins (such as Ago2) and form the central component of RNA-induced silencing complex (RISC)\(^{158}\). Binding of RISC to their target mRNA transcripts, usually in the 3’untranslated region (3’UTR), leads to mRNA instability.
or translational repression\textsuperscript{158}. In some cases, miRNA-Argonaute complex, possibly in association with a distinct set of regulatory proteins, can actually enhance gene expression\textsuperscript{139, 159}. Thus miRNAs may play diverse roles in the regulation of inflammatory mediators.

In addition, miRNAs have been shown to play a significant role in diverse disease processes\textsuperscript{160-162}. They have been found to be dysregulated in a number of diseases, either as a consequence of the disease process, or even as a pathogenic factor in disease progression\textsuperscript{160-162}. As over 2000 miRNAs have been identified, differential regulation patterns may serve as a molecular fingerprint to diagnose disease. Indeed, multiple studies have also demonstrated their utility as diagnostic or prognostic biomarkers\textsuperscript{163, 164}. Given their presence in bodily fluids such as serum and saliva, they carry tremendous diagnostic potential as a non-invasive biomarker\textsuperscript{165-167}.

In this study, we hypothesized that circulating miRNAs are differentially regulated in asthmatics compared to non-asthmatic controls. We found that several miRNAs are differentially expressed in serum of asthmatic subjects, and these are predicted to regulate Th2 mediators such as IL-5. We confirmed that IL-5 is regulated by miRNA, and identified miR-1248 as a positive regulator of its expression. The implications of these findings are described herein.
Methods

Patient selection

The study was approved by the institutional review board. All participants provided written informed consent. Patients were classified as asthmatic based on history and lung function, including forced expiratory volume in one second (FEV1) reversible by >12% and >200 ml post-bronchodilator, or airway hyper-responsiveness by methacholine (provocating concentration producing a 20% fall in FEV1 of less than 8 mg/ml). Patients were considered allergic if they had a history of aeroallergen sensitivity and at least one positive skin test to a standard panel of 40 relevant aeroallergens, and non-allergic if the skin test panel was negative.

Isolation and characterization of miRNAs

Blood was isolated by venipuncture in a red top tube, left at room temperature for 15-30 minutes, and then centrifuged at 3000 RPM in a clinical centrifuge to isolate serum. For isolation of total RNA, 500 μl of serum was mixed with 5 μl of DNase (Promega) for 30 min at 37°C, then 2 μl of 50 nM Cel-miR-39 (synthesized by Integrated DNA Technologies) was added as a "spike-in" normalization control\(^\text{168}\). Subsequently, 1.5 ml of TRIzol reagent (Life Technologies) was added, total RNA was extracted per the manufacturer’s protocol, and RNA concentration was measured by A280/260 using a NanoDrop Lite (Thermo Scientific).

For analysis by quantitative real time PCR (qPCR), 1 μg of RNA was reverse transcribed to cDNA using the miScript Reverse Transcription Kit (Qiagen) to add a universal adapter to the 3’ end of miRNAs. Quantitation of miRNAs by qPCR was performed on a MyIQ2 real time thermocycler (Bio-Rad) by using specific primers to miRNAs of interest (250 nM), miScript universal 3’ primer (250 nM), 1ul of cDNA (diluted 1:10), and the IQ SYBR Green Supermix
(Bio-Rad,) in a total volume of 10 μl. In addition to unknown samples, varying concentrations of synthetic Cel-miR-39 were run to generate a standard curve for absolute quantitation of samples in copy number/ml. A 2-step program was used, 95°C for 10 sec, 60°C for 30 sec, with 40 cycles. Sample cycle threshold (Ct) values were normalized to Cel-miR-39 expression to control for variability in RNA isolation and reverse transcription.

MicroRNA binding assays

The co-immunoprecipitation of mRNA with Ago2 was performed as previously described\textsuperscript{125, 169}. In short, Jurkat T-cells (10 x 106 cells) were lysed in polysomal lysis buffer (100 mM KCl, 5 mM MgCl\textsubscript{2}, 10 mM Hepes pH 7.0, 0.5% NP-40) for 8 min on ice, and then added to protein A sepharose beads (Sigma-Aldrich) pre-incubated with 10 μg of either an anti-Ago2 antibody (Millipore) or an IgG isotype control antibody (Santa Cruz Biotechnology). After 60 min, beads were washed thrice with NT2 buffer (50 mM Tris-HCl [pH 7.4], 150 mM Na\textsubscript{2}Cl, 1 mM MgCl\textsubscript{2}, and 0.05% Nonidet P-40). To confirm successful immunoprecipitation (IP), 10 μl of beads were removed, incubated with SDS-PAGE loading buffer, and analyzed by Western blot to confirm the presence of Ago2. The rest of the beads were subjected to DNase treatment, followed by phenol-chloroform extraction of RNA. Following reverse transcription with the High-Capacity Reverse Transcription Kit (Applied Biosystems), samples were analyzed by qPCR.

Isolation of miRNA bound specifically to IL-5 RNA was performed using a biotin pulldown procedure as previously described\textsuperscript{125, 169} with some modifications. Regions of DNA corresponding to the IL-5 3’UTR and CCL2 3’UTR (negative control) were PCR-amplified from total cDNA isolated from primary blood mononuclear cells (PBMCs) using the following primers: IL-5 3’UTR forward: 5’-CAA AGC TTC TAA TAC GAC TCA CTA TAG GGA GAC TAA ACT GGT TTG TTG CAG CC-3’; IL-5 3’UTR reverse: 5’-TCT CCA GAG AAA TGG GGA TG-3’; CCL2 3’UTR forward: 5’-CCA AGC TTC TAA TAC GAC TCA CTA TAG GGA...
GAA CAC TCA CTC CAC AAC CC-3’; CCL2 3’UTR reverse: 5’-TGT ACA AAA ATA TAT TTA TTT GGT GTA ATA GTT AC-3’. The products, which contain a T7 RNA polymerase initiation site, were reverse transcribed with the MAXIscript T7 kit (Ambion) in the presence of a 4:1 ratio of CTP to biotin-11-CTP (Roche Applied Sciences). The biotinylated RNA was incubated with fresh lysates of $10 \times 10^6$ Jurkat cells, and then purified using streptavidin Dynabeads (Life Technologies). The pulldown of Ago2 was confirmed by Western blot. For miRNA isolation, a phenol-chloroform extraction was performed on the beads, and miRNAs were reverse transcribed and analyzed by qPCR as described above.

**Reporter and PBMC assays**

To clone IL-5 3’UTR into the pmirGLO vector (Promega), the IL-5 3’UTR was amplified from total cDNA from PBMCs using the following primers: IL-5 3’UTR DraI forward: 5’-AAA TTT AAA AGA CTA AAC TGG TTT GTT GCA GC-3’; IL-5 3’UTR SalI reverse: 5’-AAA GTC GAC GAA CAG TTG TCT ATT TTT GTT TTA TTA GA-3’. The vector and PCR product were digested with DraI and SalI (both from New England Biolabs), gel purified, and ligated with Quick T4 DNA Ligase (New England Biolabs). Following transformation into NEB-5-α cells, samples were plated onto an ampicillin-containing agar plate, and colonies were selected and grown in 5 ml of Luria-Bertani broth. Presence of the correct product was confirmed by Sanger sequencing.

HEK293 cells were used for reporter assays. Cells were grown in DMEM containing 10% fetal bovine serum, 5% penicillin, and 5% streptomycin. The cells were seeded on 6-well tissue culture dishes and incubated at 37°C overnight. Co-transfections with the reporter plasmid and either the miRNA mimic or inhibitor were performed using the Attractene transfection reagent (Qiagen) as per manufacturer’s recommendations. For the reporter expression, HEK293 cells were transfected with either 50 ng of pmirGLO containing the IL-5 3’UTR (pmir-GLO-IL5-
3UTR) or 50 ng of pmirGLO empty vector (pmirGLO). For the miRNA precursor, 150 ng of miR-1248 precursor plasmid (GeneCopia) or its corresponding control vector was used. Alternatively, cells were transfected with 150 ng miR-1248 inhibitor plasmid (GeneCopia) or its corresponding control plasmid. The reporter assay was carried out using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions.

In order to determine whether miRNA-1248 had an effect on endogenous IL-5 levels, PBMCs were isolated from healthy donors by Ficoll isolation. The PBMCs (2 x 10^6 cells) were transfected with 20 nM miR-1248 mimic or negative miRNA control (both from Qiagen) by nucleofection using the Amaxa system (Lonza) per manufacturer recommendations. Cells were then stimulated with phytohaemagglutinin (1 μg/ml), cultured for 12 h in AIM-V media, and RNA was isolated by TRIzol.
Results

Patient characteristics

To determine whether miRNAs are differentially expressed in asthma, we isolated serum from 10 asthmatics and 10 control subjects (Table 2-1) for profiling. Patients were selected from our Allergy and Immunology clinic using the criteria defined in the methods section to classify into each group. The asthmatic group was composed of mostly allergic subjects (8/10) as defined by one or more positive skin tests to a panel of 40 aeroallergens. In contrast, the control group contained 3 allergic subjects. Lung function was higher in the healthy group, with a significantly higher percent predicted FEV1 (FEV1%, 91.3 vs. 80.5, p<0.05) and absolute FEV1 (3.41 L vs. 2.56 L, p<0.05). There were similar amounts of smokers in each group. Subjects in the asthma group were classified as either mild persistent or moderate persistent. In the asthma group, no patients were on an inhaled corticosteroid (ICS) only. Five patients were on a combined ICS and long acting beta agonist (LABA), and three of these were also using an anti-leukotriene (montelukast). Two of the latter patients were also using a long-acting anti-cholinergic inhaled agent (tiotropium bromide). The concentration of total RNA isolated from the serum of each group was not significantly different.

Table 2-1. Patient demographics.

<table>
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<tr>
<th>Characteristic</th>
<th>Normal n=10</th>
<th>Asthma n=10</th>
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</thead>
<tbody>
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<td>Male/Female</td>
<td>6/4</td>
<td>4/6</td>
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<tr>
<td>Age (y), mean (range)</td>
<td>38.4 (23-65)</td>
<td>49.2(22-64)</td>
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<td>FEV1% predicted, mean(SD)</td>
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<td>80.5 (24.1)</td>
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<td>FEV1 (L), mean (SD)</td>
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<td>2.56 (1.10)</td>
</tr>
<tr>
<td>Smoker (current/ex)</td>
<td>3/1</td>
<td>4/1</td>
</tr>
<tr>
<td>ICSa</td>
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</table>
MicroRNA profiling

A qPCR approach was utilized to measure expression of ten miRNAs (Let7a, Let7d, miR-21, miR-133a, miR-1248, miR-26a, miR-328, miR-126, miR-146a, miR-98) in the serum of each group (primers used are listed in Table 2-2). These miRNAs were selected based on either published studies that demonstrated a role in the inflammatory response or prediction algorithms that suggested that these miRNAs were involved in the regulation of cytokines. We observed differential expression patterns in four miRNAs: miR-1248, miR-26a, Let7a, and Let7d (Figure 2-1). Expression of miR-1248 was increased in asthmatics (mean ± SEM: 1.3x10^5 ± 3.5x10^4 copies/ml) relative to control (3.9x10^4 ± 9.3x10^3 copies/ml). However, all of the other miRNAs were down-regulated in the asthma group vs. controls as follows: Let7a, 2.4x10^4 ± 6.1x10^3 copies/ml vs. 1.7x10^5 ± 3.9x10^4 copies/ml; Let7d, 1.9x10^4 ± 5.4x10^3 copies/ml vs. 6.7x10^4 ± 1.9x10^4 copies/ml; miR-26a, 3.8x10^4 ± 6.2x10^3 copies/ml vs. 1.3x10^5 ± 3.2x10^4 copies/ml. For miR-328, a trend towards lower expression was observed in the asthma group, but this did not reach significance (p=0.1). MiRNA-21 is shown as a representative example of a miRNA without any difference between groups.
Figure 2-1. Differential expression of miRNAs in asthma. Box plots demonstrating miRNA expression (copy number/ml) in asthma and control subjects *p<0.05

Table 2-2. List of miRNA primers used for qPCR

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Primer Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Let-7a</td>
<td>TGAGGTAAGGGTGTAGTTAAGTTGTTAGTT</td>
</tr>
<tr>
<td>Let-7d</td>
<td>AGAGGTAAGGGTGCAGTAGTTAAGTTGTTAGTT</td>
</tr>
<tr>
<td>miR-26a</td>
<td>TTCAAGTAATCCAGGATAGGTAGGCT</td>
</tr>
<tr>
<td>miR-21</td>
<td>TAGCTTATCGACTGGTATAGTTAAGTTGTT</td>
</tr>
<tr>
<td>miR-1248</td>
<td>ACCTTCTTGTATAAGCAGCAGTGCTGCTAAA</td>
</tr>
<tr>
<td>miR-328</td>
<td>CTGGCCCTCTCTGCTCATTCGCTTCAGGA</td>
</tr>
<tr>
<td>miR-133a</td>
<td>TTTTGTCCTCTCTCAACCAGCTG</td>
</tr>
<tr>
<td>miR-126</td>
<td>TCGTACCGTGTAATAATTGCG</td>
</tr>
<tr>
<td>miR-98</td>
<td>TGAGGTAAGGGTTGTTAAGGTAGTT</td>
</tr>
<tr>
<td>miR-146a</td>
<td>TGAGAAGTCTGAATCCCATGGGTG</td>
</tr>
<tr>
<td>Cel-miR-39</td>
<td>TCACCGGGTGTAATCAAGCTTG</td>
</tr>
</tbody>
</table>
We next sought to determine whether miRNA expression level correlates with lung function. No association was observed for miR-1248 expression and FEV1% or Let7d and FEV1% in either the asthma or control groups (Figure 2-2A, 2-2B). However, there was a significant negative association observed between miR-26a expression and FEV1% in the asthma group (Pearson Correlation, R=-0.59, p=0.036) but not in the control group (R=-0.075, p=0.42) (Figure 2-2C). There was also a trend in let7a expression towards a negative correlation in the asthma group (R=-0.473, p=0.08) which was not apparent in the control group (R=-0.02, p=0.476) (Figure 2-2D).

![Graphs showing miRNA expression vs FEV1%](image)

**Figure 2-2. Negative correlation of serum miRNA expression with lung function.** miRNA expression in asthma (open squares) and control subjects are shown for miR-1248 (A), Let-7d (B), miR-26a (C), and Let-7a (D).

In order to determine whether there was functional significance of the miRNA expression patterns, we performed a prediction search for each miRNA to identify putative targets. We
performed a search on each miRNA using the following search algorithms (miRanda: http://www.microrna.org/microrna/home.do, MirTarget2: http://mirdb.org/miRDB/, PITA: http://genie.weizmann.ac.il/pubs/mi07/index.html, RNA hybrid: http://bibiserv.techfak.unibielefeld.de/rnahybrid/, and TargetScan: http://www.targetscan.org): Targets that were predicted by 3 or more algorithms were selected and subjected to a gene ontology analysis to identify immune targets. In addition, we also utilized the miRecords tool (http://mirecords.biolead.org/) and literature searches to identify experimentally validated targets, and the summary of these searches is summarized in Table 2-3.

Table 2-3. Prediction of miRNA targets

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Predicted Targets</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-1248</td>
<td>IL-5, IL-13, GATA3, FcεR1β, IL-1β, MMP-1, Mucin-1</td>
<td>0.031</td>
</tr>
<tr>
<td>miR-26a</td>
<td>TGF-β Receptor, CCR5, IL-4 Receptor, IL-6, Cox-2, TLR4, IFN-γ, ICOS</td>
<td>0.033</td>
</tr>
<tr>
<td>Let-7a, Let-7d</td>
<td>IL-13, TGF-β Receptor, TLR4</td>
<td>0.050, 0.040</td>
</tr>
</tbody>
</table>

Binding of IL-5 by miRNA

One of the targets predicted to be regulated by miR-1248 is IL-5, a cytokine that has not previously been demonstrated to be regulated by miRNAs. A prediction search indicated that numerous miRNAs can bind to the IL-5 3’UTR, including miR-1248 and miR-328. The predicted binding sites of each using RNA hybrid is depicted in Figure 2-3A. Given the important role of IL-5 in the allergic inflammatory response and as a stimulator of eosinophils, we sought to determine whether IL-5 is regulated by miRNA, and if miR-1248 plays a role in regulation of the cytokine.

In order to determine if the IL-5 transcript is capable of being bound by miRNAs, an IP was performed using anti-Ago2 to isolate the RISC complex associated with mRNA. This
technique allows the detection of endogenous protein-mRNA complexes formed in the cell by comparing enrichment of mRNA in samples immunoprecipitated (IP’d) with an antibody specific to a protein of interest vs. a non-specific isotype control (IC) as a measure of background. Using Jurkat T-cells, we successfully IP’d Ago2 using an Ago2-specific antibody (Figure 2-3B). An enrichment of IL-5 mRNA was observed by qPCR in the Ago2-IP over the IC-IP indicating that the transcript associates with the RISC (Figure 2-3C). As expected, there was no enrichment seen in GAPDH negative control, indicating no binding.

**Figure 2-3. Binding of Ago2 to IL-5 RNA.** A: Predicted sites of miRNA binding to IL-5 3’UTR are shown: binding site is shown in larger letters for miR-1248, and underlined for miR-328. B: Western blot demonstrating successful IP of Ago2 using a specific anti-Ago2 antibody but not with an isotype control (IC) antibody. C: IL-5 but not GAPDH mRNA is enriched with Ago2 IP, *p<0.05.

A complementary biotin-pulldown technique was utilized to confirm binding and to demonstrate miR-1248 association with IL-5 RNA. The 3’UTR of IL-5 was synthesized by in vitro transcription to incorporate biotinylated CTP. The biotinylated RNA was then mixed with a cell lysate of Jurkat cells, and streptavidin beads were used to isolate the RNA and binding
partners (Figure 2-4A). Western blot analysis confirmed that Ago2 interacted with the 3’UTR of IL-5 (Figure 2-4B). Mixture of the cell lysate with a biotinylated CCL2 3’UTR was used as a negative control, as we previously found that this did region not interact with Ago2 (unpublished data). To ensure that the presence of Ago2 was not due to non-specific retention of proteins from the lysate, the blot was re-probed with β-actin. As expected, The β-actin was present in the lysate, but only found in trace amounts in the pulldown samples.

RNA from pulldown sample was extracted, reverse transcribed, and analyzed by qPCR to assess whether miRNA was enriched in the IL-5 sample vs. the negative control. In the IL-5 pull-down, miR-1248 was readily amplified by qPCR, but undetectable in the negative control sample, indicating binding of the miRNA to IL-5 RNA (Figure 2-4C). The PCR products were then separated on an agarose gel to confirm that there was a single PCR band consistent with the expected size of the miRNA amplicon (~75 nt with the addition of a synthetic poly A tail and 3’ adaptor) (Figure 2-4D).

**MiR-1248 is a positive regulator of IL-5**

To determine whether miR-1248 is capable of regulating IL-5, we transfected a miRNA mimic into PBMCs from healthy donors (n=4) and measured the effect on IL-5 mRNA levels. We observed a decrease in Ct values (i.e. increase in gene expression) in each of the samples treated with miR-1248 (Figure 2-5A). Expressing the data as a fold change from a ddCt (difference in IL-5 Ct values normalized to GAPDH Ct values in samples treated with miR-1248 vs. negative control miRNA), this amounted to a 3.55 ± 0.76 fold increase in expression (p=0.03), indicating that the miRNA is a positive regulator of IL-5 (Figure 2-5B).
Figure 2-4. Association of miR-1248 with the IL-5 3’UTR. A: a biotin pulldown assay was used to isolate biotinylated IL-5 RNA (or negative control) bound to Ago2 and miRNA (SA, streptavidin; B, biotin). B: Western blot of the pulldown confirmed that Ago2 was bound to the IL-5 3’UTR. C: qPCR of the pulldown demonstrated enrichment of miR-1248, and separation by gel electrophoresis confirmed an appropriate size product.

In order to demonstrate that miR-1248 acts directly on the 3’UTR of IL-5, and to confirm the results in the PBMCs, the IL-5 3’UTR was cloned into a dual luciferase reporter (Figure 2-5C). In this system, the IL-5 3’UTR is placed downstream of the firefly luciferase, and the plasmid also contains an independent Renilla luciferase. This allows for normalization of firefly to Renilla luciferase signals to control for variability in transfection. Transfection of a miR-1248 mimic led to a 1.32 ± 0.14 fold increase in the pmirGLO-IL-5-3’UTR reporter expression compared to a 1.01 ± 0.09 fold change in the parent plasmid (p=0.03) (Figure 2-5D). Transfection of a miR-1248-antisense inhibitor yielded the opposite effect. Expression of luciferase in the pmirGLO-IL5-3’UTR resulted in a 0.8 ± 0.03 fold change with the inhibitor, compared to the
parent vector which showed a 1.0±0.02 fold change (p=0.01) (Figure 2-5E). Taken together, these data indicate that miR-1248 is increases IL-5 expression by acting through the 3’UTR.

**Figure 2-5.** miR-1248 increases expression of IL-5. A: transfection of miR-1248 into PBMCs demonstrated a decrease in normalized Ct as shown, corresponding fold increase shown in panel B, C: the IL-5 3’UTR was cloned into a dual luciferase reporter. D: transfection of miR-1248 increased expression of the IL-5 3’UTR luciferase reporter, while transfection of a miR-1248 antisense inhibitor (Panel E) decreased expression. *p<0.05.
Discussion

MiRNAs are emerging as crucial biomarkers in diseases and also as an important regulatory class of molecules that may be involved in the pathogenesis of many illnesses. Diagnosis of asthma is difficult due to a lack of non-invasive biomarkers, and the regulatory mechanisms that govern cytokine expression are not well defined. As such, we hypothesized that miRNAs are a potential biomarker in serum of asthmatics and that differentially expressed miRNAs regulate inflammatory mediators. In our study, we showed differential serum expression patterns of miR-1248, miR-26a, Let-7a, and Let-7d in asthmatic patients compared to non-asthmatic controls using qPCR analyses, demonstrating the potential of miRNA profiling in the diagnosis and management of asthma. In addition, we showed that miR-1248 regulates IL-5, a key allergic cytokine.

One of the challenges in defining biomarkers in asthma is the difficulty of finding molecules that are present in the blood that reflect the status of lung inflammation. In many cases, sampling of lung tissue by invasive methods such as bronchoscopy is required to assess the nature of lung inflammation176. The difference in miRNA expression we observed in asthmatics is an indication that serum markers have utility in this disease. To determine whether there was any correlation between expression of serum miRNA and lung function, we analyzed the relationship between miRNA expression and FEV1% in our subjects. Interestingly, we observed a negative Pearson correlation for miR-26a and lung function in asthma, but not control subjects. This trend was also observed for Let7a, though it did not reach significance. It was unexpected to find that miRNA expression in asthma decreased with increasing lung function. As expression of these two miRNAs is lower in asthma, we would have predicted the opposite finding, such that miRNA levels would decrease with lower lung function. Consequently, the nature of the relationship between serum miRNA expression and lung function remains unclear. However, we believe that this is an important observation, as it indicates that cellular events occurring in the lungs are
reflected systemically in the blood. It also raises the possibility that profiling of serum miRNA levels may be utilized to assess a patient’s disease based on its severity, phenotypic asthma differences, type of inflammation, or response to treatment.

The miRNAs that we found to be differentially expressed in asthma are predicted to regulate Th2 cytokines that play a crucial role in allergic inflammation. In particular, miR-1248 is predicted to regulate numerous cytokines, including IL-5. This cytokine has not previously been shown to be post-transcriptionally regulated. IL-5 is primarily responsible for eosinophilic survival, growth, differentiation, and recruitment. Not surprisingly, it plays a central role in asthma, eosinophilic esophagitis, and a variety of other allergic diseases, nasal polyposis, and hypereosinophilic syndromes. As such, IL-5 is an important target, and we aimed to determine whether IL-5 is indeed regulated by miRNA, and miR-1248 in particular.

Using ribonuclear protein immunoprecipitation experiments and biotin pulldown assays, we established that Ago-2 and miR-1248 physically interact with the IL-5 mRNA. These techniques may be a useful screen that can be applied to other systems to determine whether proteins are capable of being regulated by the RISC and miRNA. In addition, the modified biotin pulldown procedure that we developed is a simple tool to identify specific interacting miRNAs in any system. These approaches could be further combined with high throughput screening methods such as mass spectrometry and miRNA microarray/sequencing to globally identify proteins and RNAs bound to transcripts.

The functional effects of miR-1248 were probed to determine whether the miRNA were truly capable of regulating gene expression of IL-5. Transfection of a miRNA-1248 mimic into PBMCs isolated from healthy adults demonstrated that the miRNA actually increased expression of the IL-5 transcript. This was unexpected, as miRNAs typically downregulate gene expression. To validate these findings, demonstrate that there was a direct effect of miR-1248 on IL-5 expression, and show that this was mediated through the 3’UTR of the gene, a luciferase reporter construct was utilized. These assays confirmed the initial observation by showing that miR-1248
increased expression of the reporter, while an antisense inhibitor of miR-1248 decreased expression. Although a few studies have shown different miRNAs to function as positive regulators of their target’s expression, such reports are rare in the literature\textsuperscript{139,159,178}. Also, the mechanism of action of how miRNAs increase expression of IL-5 via its 3’UTR is not clear. There could be interactions with other RBPs, such as the mRNA-stabilizing protein HuR for instance\textsuperscript{110}, and this is an area that requires further exploration.

It is interesting to note that miR-1248 expression was increased in asthma, while all of the other miRNAs that we found to be differentially expressed were down-regulated. These findings raise the question of whether there might be pathogenic role of miR-1248 in asthma, such that its increased abundance in the disease could serve to elevate Th2 cytokine levels. In addition, it also suggests that antisense inhibitors could be used as potential therapeutic targets. As miRNAs that regulate IL-5 expression might be dysregulated in other diseases associated with eosinophilia, miR-1248 might also be a useful biomarker/target in these illnesses.

Our findings highlight the potential roles of miRNAs in the diagnosis, pathogenesis and treatment of asthma. We expect that expanding this methodology to profile miRNAs from a larger and more diverse asthma population, and analysis of a larger pool of miRNAs will be a useful technique in the clinical management of asthma. A fingerprint of miRNA expression differences could potentially be used to characterize phenotypic differences in asthma, monitor response to treatment, and personalize patient therapy.
Acknowledgements

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Chapter 3

Circulating microRNAs as biomarkers in allergic rhinitis and asthma

Abstract

**Background:** MicroRNAs (miRNAs) are emerging as important regulatory molecules which may be involved in the pathogenesis of various diseases. Circulating miRNAs may be non-invasive biomarkers to diagnose and characterize asthma and allergic rhinitis.

**Objective:** We sought to determine whether miRNAs are differentially expressed in the blood of asthmatic subjects compared to those expressed in the blood of non-asthmatic patients with allergic rhinitis and non-allergic, non-asthmatic patients. Furthermore, we sought to establish whether miRNAs could be used to characterize or subtype asthmatics.

**Methods:** Expression of plasma miRNAs were measured by real time quantitative PCR in 35 asthmatics, 25 non-asthmatic subjects with allergic rhinitis, and 19 non-allergic, non-asthmatic individuals. Differentially expressed miRNAs were identified using Kruskal-Wallis one-way analysis of variance with Bonferroni $P$ value adjustment to correct for multiple comparisons. A random forest classification algorithm combined with a leave-one-out cross-validation approach was implemented to assess the predictive capacities of the profiled miRNAs.

**Results:** We identified 30 miRNAs which were differentially expressed among healthy, allergic, and asthmatic individuals. These miRNAs fit into five different expression pattern groups. Among asthmatics, miRNA expression profiles identified two subtypes which differed by high or low peripheral eosinophil levels. Circulating miR-125b, miR-16, miR-299-5p, miR-126, miR-206, and miR-133b were most predictive of allergic and asthmatic status.
**Conclusions:** Subsets of circulating miRNAs are uniquely expressed in allergic rhinitis and asthma and have potential for use as non-invasive biomarkers to diagnose and characterize these diseases.
Introduction

Asthma is a heterogeneous disease comprised of numerous phenotypes which are difficult to characterize with current diagnostic tools. It involves a complex interplay of the airway epithelium, innate immune system, and adaptive immunity that is still not completely understood. There is a clear need for identification of non-invasive biomarkers to diagnose, characterize, and understand this disease. The aim of this study was to determine whether circulating microRNAs are differentially expressed in asthmatics compared to healthy controls and subjects with allergic rhinitis (AR), and whether their expression could be used as a tool to further characterize asthma.

MicroRNAs (miRNAs) are emerging as non-invasive biomarkers which play important roles in cytokine regulation and asthma pathogenesis. MiRNAs are short (20-25 nucleotides), single-stranded, non-coding RNAs that post-transcriptionally regulate gene expression via interactions with mRNAs. They associate with members of the Argonaute family of proteins and form the central component of RNA-induced silencing complex (RISC). Binding of RISC-miRNA to their target mRNA transcripts, usually in the 3’ untranslated region (3’ UTR), leads to downregulation of gene expression via destabilization of mRNA stability or translational repression. In some cases, the miRNA-Argonaute complex, possibly in association with a distinct set of regulatory proteins, can enhance gene expression. MiRNAs bind to their targets with partial complementarity, such that any miRNA is capable of binding hundreds or even thousands of targets. As miRNAs can regulate functionally related genes, it is possible that a few miRNAs or even a single miRNA could regulate entire pathways.

MiRNAs are produced by a wide variety of cells in different organs and are secreted into blood and other bodily fluids where they can exert biological effects. This also allows them to serve as non-invasive biomarkers. It is now evident that miRNAs play significant roles in diverse disease processes. Their expression is dysregulated in a number of diseases, and
they may play roles in disease pathogenesis. As approximately 150 miRNAs are detectable in the blood, differential expression patterns may serve as a molecular fingerprint to diagnose and characterize diseases. Indeed, multiple studies have already demonstrated their utility as diagnostic or prognostic biomarkers. In diseases such as cancer, circulating miRNAs can diagnose disease, characterize disease biology, predict response to different treatments, and serve as a target for novel therapeutics.

Circulating miRNAs are poorly studied in AR and asthma, but have great potential to diagnose and characterize these diseases. We have previously shown that miRNAs isolated from the lungs and blood have utility as biomarkers in asthma, and that differentially expressed miRNAs may be important regulators of Th2 cytokines. In this study, we sought to determine whether plasma miRNAs are differentially expressed in AR and asthma, to establish if they can be used as a tool to characterize asthma subtypes, and to identify candidate miRNAs which may play roles in disease pathogenesis.

We identified 30 miRNAs which are differentially expressed in the plasma of asthmatic patients, subjects with AR, and non-allergic, non-asthmatic individuals. These miRNAs can be classified into five groups that correlated with different patterns of expression in AR, asthma, or both. Bioinformatic analyses revealed that the differentially expressed miRNAs targeted genes involved in inflammatory pathways. Analysis of miRNA expression in asthmatics revealed two main clusters, which differed in peripheral eosinophil levels. Using random forest classification, we were able to implement a prediction model that is 91.1% accurate in predicting AR or asthmatic status. These findings indicate that plasma miRNAs could play roles in AR and asthma and may have potential as biomarkers. The implications of these findings are described herein.
Methods

Patient selection

The study was approved by the Penn State College of Medicine Institutional Review Board. All participants provided written informed consent. Patients were classified as asthmatic based on history and lung function, including forced expiratory volume in one second (FEV₁) reversible by >12% and >200 mL post-bronchodilator, or airway hyper-responsiveness by methacholine (provoking concentration producing a 20% fall in FEV₁ of less than 8 mg/mL). Patients were considered allergic if they had a clinical history of aeroallergen sensitivity and at least one positive skin test (3 mm larger than negative control) in a standard panel of 19 relevant aeroallergens, and non-allergic if the skin test panel was negative. Asthmatic patients were asked to answer the original 7-item Asthma Control Questionnaire (ACQ)\textsuperscript{184}.

Isolation and characterization of miRNAs.

Blood was collected by venipuncture in a purple top tube, and then centrifuged at 3000 RPM in a clinical centrifuge to isolate plasma. For isolation of total RNA, 2 μL of 50 nM synthetic cel-miR-39 was added as a “spike-in” normalization control to 500 μL of plasma\textsuperscript{168}. Afterwards, 1.5 mL of TRIzol (Life Technologies) reagent was added and total RNA was extracted according to the manufacturer’s protocol. RNA concentration was measured by A260/280 using a NanoDrop Lite Spectrophotometer (Thermo Scientific).

Expression of 420 miRNAs in plasma was screened using the Human miRNome v15 PCR array (System Biosciences). In short, 400 ng of total RNA was reverse transcribed using the QuantiMir RT Kit (Systems Biosciences), and expression of miRNAs was measured on the
CFX384 Real-Time System (Bio-Rad). Each reaction was run in triplicate and the average cycle threshold (Ct) value was used for analysis. For subsequent analysis by quantitative real-time PCR (qPCR), 250 ng of total RNA was reverse transcribed to cDNA using qScript microRNA cDNA Synthesis Kit (Quanta BioSciences). Quantification of miRNAs by qPCR was performed on the CFX384 Real-Time System by using specific primers to miRNAs of interest (250 nM) (Supplemental Table S1), 1 μL of diluted of cDNA (diluted 1:10), and the iTaq Universal SYBR Green Supermix (Bio-Rad) in a total volume of 10 μL. Primers to each miRNA was synthesized by Integrated DNA Technologies and placed in a unique position on 96-well plates at a stock concentration of 1 μM. A multichannel EDP-3 Plus electronic pipette (Bio-Rad) was used to transfer the primer to a 384-well plate, where they were mixed with cDNA, SYBR Green mix, and universal primer. Each sample was run in triplicate. A 2-step program was used as follows: 40 cycles of 95°C for 10 sec, 60°C for 30 sec. Sample Ct values were normalized to cel-miR-39 to control for variability in RNA isolation and reverse transcription, and then to total RNA expression as a means of normalizing expression data. To calculate copy numbers, real time PCR amplification of multiple dilutions of known concentrations of synthetically synthesized miRNAs (cel-miR-39, miR-155, Let-7a) was measured. An average of these 3 curves was used to generate a standard curve that could be used to calculate concentrations of unknowns as copy number/μL.

**Statistical analyses**

Normally distributed data was analyzed by one-way analysis of variance with Holm’s adjustment for pairwise comparisons or Student’s t-test where appropriate. Fisher’s exact tests were used for categorical binary variables.
Analysis of differentially expressed miRNAs

Hierarchical cluster analysis was performed in Cluster3.0 using the average-linkage method\textsuperscript{185}. Principal component analysis (PCA) and expression analysis were performed in R 3.2.0/Bioconductor\textsuperscript{186, 187}. Wilcoxon rank-sum test or Kruskal-Wallis one-way analysis of variance with Nemenyi post hoc test was used for non-normally distributed data\textsuperscript{188}. To control for multiple testing, we used the Benjamini and Hochberg false discovery rate (FDR) or Bonferroni procedure\textsuperscript{189}. When classifying miRNAs by their expression patterns, miRNAs are considered upregulated or downregulated when their median expression levels show at least 2-fold change between groups and demonstrate adjusted pairwise comparison $P$ values <0.05.

Bioinformatic analysis of pathways targeted by differentially expressed miRNAs

DIANA-miRPath v2.0 Web Service (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=mirpath) was utilized to identify the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of genes which are targeted by the each group of miRNAs\textsuperscript{190}. A network consisting of the top 20 pathways and the 30 differentially expressed miRNAs was constructed using Cytoscape 3.2.1\textsuperscript{191}. Cytoscape 3.2.1 was also utilized to create a network that comprised of inflammation-associated genes and the differentially expressed miRNAs that target these genes.

Evaluation of miRNAs as biomarkers for asthma and AR Random forest as implemented by the Python machine learning package, scikit-learn\textsuperscript{192}, was utilized to build a prediction model for asthma and allergic rhinitis based on the 39 profiled miRNAs and 5 available demographic characteristics (Supplemental Table S4). The accuracy and other performance measures of our predictive algorithm was determined with a leave-one-out cross-validation and the randomness of sample bootstrapping in random forest was controlled by taking the average of 10 runs.
Results

Selection of candidate plasma miRNAs for study

Our overall goal was to identify miRNA candidates in plasma that may be dysregulated in AR and asthma. First, we sought to determine which miRNAs could be readily and reproducibly detected in plasma using our real-time PCR array methodology. High throughput profiling of 420 miRNAs was performed on plasma isolated from five asthmatic and five non-asthmatic subjects. We found that 135 miRNAs were reproducibly detected in plasma (based on Ct values between 20 and 33 in all samples, melting curve analysis that showed a single product, and PCR amplification efficiency of 2.0 ± 0.1), consistent with other reported studies which have confirmed the expression of the majority of these miRNAs in blood \(^{193,194}\). We then measured the expression of these 135 miRNAs in the plasma of 12 allergic asthmatic and 12 non-allergic non-asthmatic subjects as a screen to identify candidate miRNAs that may be differentially expressed in AR and asthma. There were 30 miRNAs that were found to be differentially expressed with a greater than 2-fold difference between groups, a Wilcoxon rank-sum test significance cutoff of \(P\) value <0.05, and an FDR of 10% (Supplemental Figure S3-1). In addition to these miRNAs, we selected 9 additional miRNAs for further study that did not meet the criteria for significance. These included miRNAs that were not significantly different between the two groups that could be used as internal controls for normalization, as well as candidates that we previously identified as being differentially expressed in exhaled breath condensates in asthma\(^{146}\).

We assessed the quality of the qPCR data for these candidates by multiple methodologies. The presence of a single PCR product was confirmed by melting curve analysis and gel electrophoresis (Supplemental Figure S3-2), the latter of which showed a single product between 50 and 100 nucleotides in length (the expected size of the mature miRNA plus a poly(A) tail and 3’ adapter sequence as described in the Methods section). We also confirmed that our
miRNAs of interest had high amplification efficiency (90-105%) by analysis with LinRegPCR.

To identify miRNAs that might be associated with AR, asthma or both, the qPCR expression of these 39 miRNAs was subsequently profiled in a larger number of individuals (n=79) which included 35 asthmatics (29 of whom had AR), 25 non-asthmatic subjects with AR, and 19 non-allergic, non-asthmatic (healthy) subjects. Subject characteristics are presented in Table 1. Unsupervised hierarchical clustering of the miRNA expression data revealed the formation of three major groups that generally segregated based on the patients’ disease status (Figure 3-1A). Expression levels of 30 miRNAs were found to be statistically different among the three groups. These differentially expressed miRNAs could be classified into five expression pattern groups based on differences in expression between asthma and AR, AR and healthy, and asthma and healthy groups (Table 2, Figure 3-1B, and Supplemental Figure S3-3, miR-937 is shown as a representative non-differentially expressed miRNA).
Table 3-1. Subject characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Healthy (n=19) n(%) or mean ± SEM</th>
<th>Allergic (n=25) n(%) or mean ± SEM</th>
<th>Asthmatic (n=35) n(%) or mean ± SEM</th>
<th>P value</th>
</tr>
</thead>
<tbody>
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<td>Age, y</td>
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<td>43.9 ± 2.47</td>
<td>0.563&lt;sup&gt;1&lt;/sup&gt;</td>
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<td>Gender</td>
<td>Male/Female 8/11 (42/58)</td>
<td>11/14 (44/56)</td>
<td>14/21 (40/60)</td>
<td>0.957&lt;sup&gt;2&lt;/sup&gt;</td>
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<td>Ethnicity</td>
<td>Caucasian/Non-Caucasian 16/3 (85/15)</td>
<td>21/4 (84/16)</td>
<td>34/1 (97/3)</td>
<td>0.129&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
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<td>BMI</td>
<td>27.7 ± 1.64</td>
<td>28.3 ± 1.15</td>
<td>29.2 ± 1.09</td>
<td>0.701&lt;sup&gt;1&lt;/sup&gt;</td>
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<td>Smoker</td>
<td>5 (26)</td>
<td>2 (0.08)</td>
<td>5 (14)</td>
<td>0.268&lt;sup&gt;2&lt;/sup&gt;</td>
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<td>Allergic Rhinitis</td>
<td>0 (0)</td>
<td>25 (100)</td>
<td>29 (83)</td>
<td>&lt;0.001&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Spirometry</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FVC</td>
<td>3.94 ± 0.34</td>
<td>4.53 ± 0.32</td>
<td>3.70 ± 0.19</td>
<td>0.126&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>FVC%</td>
<td>93.3 ± 4.55</td>
<td>102.1 ± 3.83</td>
<td>89.9 ± 2.84</td>
<td>0.081&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>FEV1</td>
<td>3.05 ± 0.29</td>
<td>3.64 ± 0.29</td>
<td>2.68 ± 0.18</td>
<td>0.036&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>FEV1%</td>
<td>87.3 ± 4.94</td>
<td>98.5 ± 3.62</td>
<td>78.4 ± 3.85</td>
<td>0.008&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>FEV1/FVC</td>
<td>0.76 ± 0.02</td>
<td>0.79 ± 0.02</td>
<td>0.70 ± 0.02</td>
<td>0.041&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>Normally-distributed, continuous variables were analyzed by one-way ANOVA.

<sup>2</sup>Freeman-Halton extension of Fisher’s Exact Test was used to analyze categorical binary data.

In Group 1, the expression of miRNAs was statistically different between healthy subjects and those with AR, healthy subjects and asthmatics, and subjects with AR and those with AR.
asthma. In these cases, the magnitude of change in expression levels of miRNA was greater in asthma vs. healthy controls compared to AR subjects vs. healthy controls (Figure 3-1B, miR-125b is shown as a representative miRNA). The majority of miRNAs showed increased expression levels in allergic and asthmatic subjects vs. healthy controls (miR-125b, miR-126, miR-21, let-7b, let-7c, and let-7e) except for miR-1 which was decreased in both groups.

MiRNAs in Group 2 demonstrated expression differences that were unique to asthma. There was a significant difference in their expression in asthmatics vs. healthy subjects and asthmatics vs. AR subjects, but no difference in AR vs. healthy groups (Figure 3-1B, miR-16 panel). Among asthmatics, miR-16, miR-223, miR-148a, and miR-146a were upregulated while miR-299-5p, miR-570, and miR-150 are downregulated.

Group 3 microRNAs exhibited median expression levels that were similar between AR and asthmatic cohorts but were either downregulated or upregulated compared to healthy individuals (Figure 3-1B, miR-133b panel). Both miR-145 and miR-422 demonstrated increased plasma levels, while miR-133b, miR-133a, miR-26b, miR-1248, miR-330-5p, miR-29, miR-1291, and miR-144 showed decreased plasma levels.

MiRNAs in the Group 4 expression pattern showed similar median expression levels between healthy and asthmatic cohorts but are either downregulated or upregulated among patients with AR (Figure 3-1B, miR-206 panel). In the AR group, expression of both circulating miR-206 and miR-328 was upregulated, while miR-338-3p and miR-26a were both downregulated.

Finally, included in the Group 5 expression pattern are miR-106a and miR-155. Both miRNAs were significantly downregulated in AR and asthmatic patients compared to healthy individuals; however, their downregulation was more exaggerated among AR patients compared to asthmatic subjects (Figure 3-1B, miR-155 panel).
Figure 3-1. MicroRNA expression in healthy, allergic, and asthmatic individuals. A. Heatmap showing expression of 39 candidate miRNAs in 19 healthy, 25 allergic, and 35 asthmatic subjects after two-way unsupervised hierarchical clustering. B. Representative boxplots showing differential expression patterns of miRNAs among healthy, allergic, and asthmatic cohorts. MiR-937 is shown as a non-differentially expressed miRNA.
### Table 3-2. Differentially expressed miRNAs among healthy, allergic, and asthmatic cohorts

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Median miRNA Copy Number/µL</th>
<th>P value&lt;sup&gt;1&lt;/sup&gt;</th>
<th>H:AR&lt;sup&gt;2&lt;/sup&gt;</th>
<th>H:A&lt;sup&gt;2&lt;/sup&gt;</th>
<th>AR:A&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy</td>
<td>Allergic</td>
<td>Asthmatic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-125b</td>
<td>5.09E+03</td>
<td>1.73E+05</td>
<td>1.10E+06</td>
<td>&lt;0.001</td>
<td>0.003</td>
</tr>
<tr>
<td>miR-126</td>
<td>1.88E+04</td>
<td>3.44E+05</td>
<td>6.87E+06</td>
<td>&lt;0.001</td>
<td>0.011</td>
</tr>
<tr>
<td>miR-21</td>
<td>2.81E+05</td>
<td>1.99E+06</td>
<td>3.01E+07</td>
<td>&lt;0.001</td>
<td>0.008</td>
</tr>
<tr>
<td>Let-7b</td>
<td>2.31E+05</td>
<td>2.92E+06</td>
<td>2.17E+07</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Let-7c</td>
<td>2.90E+05</td>
<td>1.55E+06</td>
<td>6.31E+06</td>
<td>&lt;0.001</td>
<td>0.002</td>
</tr>
<tr>
<td>miR-1</td>
<td>1.17E+06</td>
<td>4.12E+04</td>
<td>7.48E+02</td>
<td>&lt;0.001</td>
<td>0.030</td>
</tr>
<tr>
<td>Let-7e</td>
<td>2.66E+05</td>
<td>7.58E+05</td>
<td>1.67E+06</td>
<td>&lt;0.001</td>
<td>0.013</td>
</tr>
<tr>
<td>miR-16</td>
<td>4.58E+05</td>
<td>4.41E+05</td>
<td>4.97E+07</td>
<td>&lt;0.001</td>
<td>0.993</td>
</tr>
<tr>
<td>miR-299-5p</td>
<td>2.71E+05</td>
<td>1.93E+06</td>
<td>3.17E+04</td>
<td>&lt;0.001</td>
<td>0.229</td>
</tr>
<tr>
<td>miR-223</td>
<td>2.59E+06</td>
<td>1.78E+07</td>
<td>1.24E+08</td>
<td>&lt;0.001</td>
<td>0.117</td>
</tr>
<tr>
<td>miR-570</td>
<td>6.35E+04</td>
<td>8.96E+04</td>
<td>1.06E+04</td>
<td>&lt;0.001</td>
<td>0.185</td>
</tr>
<tr>
<td>miR-148a</td>
<td>4.53E+05</td>
<td>2.91E+05</td>
<td>1.43E+06</td>
<td>&lt;0.001</td>
<td>0.894</td>
</tr>
<tr>
<td>miR-146a</td>
<td>4.24E+05</td>
<td>5.82E+05</td>
<td>4.73E+06</td>
<td>&lt;0.001</td>
<td>0.954</td>
</tr>
<tr>
<td>miR-150</td>
<td>1.13E+05</td>
<td>9.28E+05</td>
<td>4.66E+06</td>
<td>0.001</td>
<td>0.999</td>
</tr>
<tr>
<td>miR-133b</td>
<td>1.90E+06</td>
<td>7.06E+04</td>
<td>4.50E+04</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>miR-133a</td>
<td>2.32E+06</td>
<td>6.40E+04</td>
<td>1.58E+05</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>miR-26b</td>
<td>8.57E+06</td>
<td>4.94E+05</td>
<td>1.04E+06</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>miR-1248</td>
<td>1.23E+06</td>
<td>9.52E+04</td>
<td>3.45E+04</td>
<td>&lt;0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>miR-330-5p</td>
<td>6.24E+06</td>
<td>1.33E+05</td>
<td>4.98E+04</td>
<td>&lt;0.001</td>
<td>0.007</td>
</tr>
<tr>
<td>miR-145</td>
<td>6.64E+05</td>
<td>3.20E+06</td>
<td>4.21E+06</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>miR-29</td>
<td>2.30E+07</td>
<td>1.61E+06</td>
<td>5.71E+06</td>
<td>0.002</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>miR-422</td>
<td>2.04E+05</td>
<td>1.18E+06</td>
<td>5.61E+05</td>
<td>0.006</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>miR-1291</td>
<td>1.08E+06</td>
<td>2.81E+05</td>
<td>1.73E+05</td>
<td>0.011</td>
<td>0.039</td>
</tr>
<tr>
<td>miR-144</td>
<td>4.23E+05</td>
<td>1.01E+05</td>
<td>1.05E+04</td>
<td>0.018</td>
<td>0.036</td>
</tr>
<tr>
<td>miR-206</td>
<td>5.64E+04</td>
<td>3.06E+06</td>
<td>7.60E+04</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>miR-338-3p</td>
<td>7.16E+05</td>
<td>4.35E+04</td>
<td>2.71E+05</td>
<td>0.013</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>miR-328</td>
<td>1.58E+06</td>
<td>4.45E+06</td>
<td>1.66E+06</td>
<td>0.017</td>
<td>0.002</td>
</tr>
<tr>
<td>miR-26a</td>
<td>3.59E+06</td>
<td>1.64E+06</td>
<td>6.35E+06</td>
<td>0.034</td>
<td>0.036</td>
</tr>
<tr>
<td>miR-106a</td>
<td>2.79E+07</td>
<td>3.00E+06</td>
<td>8.08E+06</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>miR-155</td>
<td>1.89E+05</td>
<td>1.24E+04</td>
<td>4.33E+04</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

<sup>1</sup>Listed are Bonferroni-adjusted P values for Kruskal-Wallis one-way analysis of variance.

<sup>2</sup>Indicated are adjusted multiple pairwise comparison P values<sup>188</sup>. Shown in italics are P values that do not reach statistical significance (P >0.050). H: AR, H: A, AR: A refer to comparisons between healthy and allergic groups, healthy and asthmatic groups, and allergic and asthmatic groups respectively.
Identification of KEGG pathways regulated by different groups of miRNAs

Bioinformatic analyses using DIANA miRPath were used to analyze potential pathways and genes regulated by miRNAs in the five groups. A network map was generated to identify potential connections between miRNA groups and regulatory pathways, and to determine whether there were functional connections among the five groups of miRNAs (Supplemental Figure S3-4). The complete list of KEGG pathways regulated by each group of miRNAs and the specific gene targets within the pathways for each miRNA group are shown in the supplemental data (Supplemental Table S2). The top three KEGG pathways identified (based on number of genes regulated by miRNAs) were “PI3K-Akt signaling pathways”, “Pathways in cancers”, and “MAPK signaling pathways” such that miRNAs in each of the 5 groups were predicted to regulate multiple genes in each of these pathways (Supplemental Figures S4A, B). All five groups of miRNAs targeted multiple genes within these pathways (Supplemental Figures S3-4A, B). These genes included important inflammatory mediators, such as NF-κB, IL-8, STATs, AP-1, MAPK signaling molecules, and the TGF-β receptor (Figure 3-2). In some cases, multiple miRNAs within a group targeted a single gene (e.g., TGFBR1 as a target of Group 1 miRNAs). There were also cases where multiple miRNAs within each group targeted multiple genes within pathways, as exemplified by Group 2, whose miRNAs were predicted to regulate multiple components of the NF-κB pathway (Figure 3-2).
Identification of asthma subgroups based on miRNA expression

We hypothesized that miRNA expression could be used as a phenotypic tool to identify subsets of asthmatics. Using the 39 candidate miRNAs, we performed unsupervised cluster analyses of miRNA expression in the asthmatic group and identified two main clusters (cluster 1, n=16 and cluster 2, n=19) (Figure 3-3A). PCA was applied to reduce the dimensionality of the data set by constructing linear combinations of variables and focusing on the most relevant linear
combinations. PCA confirmed the formation of two major clusters (Supplemental Figure S3-5B). The first principal component (PC1) accounted for 25.1% of the variance in the data while the second principal component (PC2) accounted for 17.2% of the variance. There were 20 miRNAs differentially expressed between the two clusters (Supplemental Table S3-3 and Supplemental Figure S3-5A). Cluster 1 contained a higher level of blood eosinophils relative to the second cluster (mean ± SEM: 285.4 ± 44.4 vs 133.3 ± 23.4) (Figure 3-3B). All other characteristics, including demographics, lung function, AR status, aeroallergen sensitivity, ACQ score, and medication use, were similar in the two groups (Table 3).

**Figure 3-3. Cluster analysis of miRNA expression in asthmatics.** A. Heat map showing two main clusters of miRNA expression in asthmatics. B. Boxplot of peripheral eosinophil levels in Cluster 1 and Cluster 2.
Table 3-3. Cluster analysis of asthmatics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Cluster 1 (n=16) n(%) or mean ± SEM</th>
<th>Cluster 2 (n=19) n(%) or mean ± SEM</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>42.8 ± 3.13</td>
<td>44.8 ± 3.76</td>
<td>0.672</td>
</tr>
<tr>
<td>Gender, Male/Female, (%)</td>
<td>5 (31)/11 (69)</td>
<td>9 (47)/10 (53)</td>
<td>0.491</td>
</tr>
<tr>
<td>BMI</td>
<td>27.1 ± 1.41</td>
<td>30.9 ± 1.54</td>
<td>0.078</td>
</tr>
<tr>
<td>Current Smoker</td>
<td>3 (19)</td>
<td>2 (12)</td>
<td>0.642</td>
</tr>
<tr>
<td>FVC%</td>
<td>90.0 ± 3.91</td>
<td>89.7 ± 4.16</td>
<td>0.964</td>
</tr>
<tr>
<td>FEV1%</td>
<td>79.1 ± 5.20</td>
<td>77.8 ± 5.71</td>
<td>0.864</td>
</tr>
<tr>
<td>FEV1/FVC</td>
<td>0.71 ± 0.05</td>
<td>0.69 ± 0.04</td>
<td>0.752</td>
</tr>
<tr>
<td>FEV1ΔBD</td>
<td>13.9 ± 3.74</td>
<td>14.1 ± 3.04</td>
<td>0.967</td>
</tr>
<tr>
<td>ACQ Score</td>
<td>1.04 ± 0.14</td>
<td>0.95 ± 0.15</td>
<td>0.672</td>
</tr>
<tr>
<td>Allergic Rhinitis</td>
<td>13 (81)</td>
<td>16 (84)</td>
<td>1.000</td>
</tr>
<tr>
<td>Aeroallergen Sensitivity¹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trees</td>
<td>0.94 ± 0.35</td>
<td>0.78 ± 0.26</td>
<td>0.741</td>
</tr>
<tr>
<td>Grass</td>
<td>0.50 ± 0.16</td>
<td>0.21 ± 0.10</td>
<td>0.130</td>
</tr>
<tr>
<td>Weeds</td>
<td>0.63 ± 0.20</td>
<td>0.26 ± 0.10</td>
<td>0.124</td>
</tr>
<tr>
<td>Dust mites</td>
<td>1.00 ± 0.26</td>
<td>0.84 ± 0.23</td>
<td>0.653</td>
</tr>
<tr>
<td>Animals</td>
<td>0.88 ± 0.27</td>
<td>0.53 ± 0.23</td>
<td>0.339</td>
</tr>
<tr>
<td>Cockroach</td>
<td>0.13 ± 0.09</td>
<td>0.16 ± 0.09</td>
<td>0.787</td>
</tr>
<tr>
<td>Molds</td>
<td>0.44 ± 0.18</td>
<td>0.21 ± 0.12</td>
<td>0.310</td>
</tr>
<tr>
<td>Total</td>
<td>4.50 ± 0.90</td>
<td>3.00 ± 0.74</td>
<td>0.208</td>
</tr>
<tr>
<td>ICS use (mean daily dose, μg)</td>
<td>503 ± 103</td>
<td>715 ± 117</td>
<td>0.191</td>
</tr>
<tr>
<td>LABA, n (%)</td>
<td>7 (43.7)</td>
<td>8 (42.1)</td>
<td>1.000</td>
</tr>
<tr>
<td>Anti-leukotriene, n (%)</td>
<td>4 (25.0)</td>
<td>5 (26.3)</td>
<td>1.000</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>285.4 ± 44.4</td>
<td>133.3 ± 23.4</td>
<td>0.007</td>
</tr>
<tr>
<td>% Eosinophils</td>
<td>4.87 ± 0.81</td>
<td>2.02 ± 0.39</td>
<td>0.005</td>
</tr>
</tbody>
</table>

¹Mean number of positive skin tests in a panel of 19 aeroallergens

Evaluation of miRNAs as biomarkers for asthma and AR

We next sought to determine whether miRNA expression could be used diagnostically in AR and asthma with supervised machine learning classification. As a multiclass classification problem, 44 categories of patient information, including levels of 39 miRNAs and 5 demographic characteristics were utilized as features (Supplemental Table S4) for the 79 subjects to predict their disease status (healthy, AR, or asthma). Using a random forest model that constructed 100 decision trees, we were able to obtain the feature relevance of all features (Supplemental Table
Next, we performed manual feature selection to determine the most important miRNA in predicting disease status. The top six most relevant miRNAs (miR-125b, miR-16, miR-299-5p, miR-126, miR-206, and miR-133b) were determined to produce the model that was most accurate and resistant to overfitting (Supplemental Figure S3-6B). We also confirmed that the minimum number of decision trees for the random forest required to produce the most robust classification model was 100 (Supplemental Figure S3-6C).

With our most optimal model of random forest with the six most relevant miRNA features and 100 decision trees, we were able to correctly determine whether our subject was healthy, has AR, or asthmatic 92.4% of the time (73 out of 79 correct predictions) (Figure 3-4A). Taken as healthy vs diseased (has AR or is asthmatic), our model has high negative predictive value (NPV). Additionally, our model demonstrates high positive predictive value (PPV), with low false positive rates across all disease statuses (Figure 3-4B).

Figure 3-4. Results of the random forest model with 6 features and 100 decision trees. A. Plot describing the proportion of each predicted phenotype for subjects of each disease status. B. Multiclass ROC curve analysis with pairwise comparisons of one class vs. all other classes. The average AUC for the three ROC curves is 0.9736, indicating that the model performs well in discriminating between positive and negative instances.
Discussion

Our study is the first to demonstrate that circulating miRNAs are differentially expressed among individuals who are healthy, have asthma, or have AR. Specifically, we show that 30 miRNAs which we classified into five expression groups, are differentially expressed among these cohorts. Differential plasma miRNA expression in patients with asthma and AR is unlikely to be a mere epiphenomenon of these diseases. MiRNAs have been shown to directly or indirectly affect the expression of multiple genes involved in the inflammatory response\textsuperscript{196, 197}.

AR is an established risk factor for developing asthma in both children and adults\textsuperscript{198, 199}. Some studies have also shown that longer duration and increased severity of AR correlates with a higher prevalence of asthma\textsuperscript{200}. These clinical observations posit AR as an intermediate inflammatory phenotype between healthy and asthmatic states. Group 1 miRNAs exhibit changes in miRNA expression among patients with AR compared to healthy controls, and the magnitude of these changes is augmented by asthma. Expression of miRNAs in this group could represent a continuum of airway inflammation, with increased dysregulation as the asthmatic inflammatory changes are added to upper airway inflammation. Moreover, Group 1 miRNAs could also represent a profile of Th2 airway inflammation, as the high eosinophil cluster of asthmatics (Cluster 1) demonstrated similar expression patterns with increased expression of miR-126, miR-21, and let-7b and decreased levels of miR-1 compared to the low eosinophil cluster (Supplemental Table S3). It has been shown that miR-21 is upregulated in allergic airways and regulates eosinophil growth\textsuperscript{163, 171} and that the Let7 family regulates IL-13 expression\textsuperscript{142}. Furthermore, multiple miRNAs in this group were predicted to regulate different isoforms of the TGF-\(\beta\) receptor, raising the possibility that airway remodeling may be a target of these miRNAs. Thus, mRNA transcripts targeted by Group 1 miRNAs may give clues about the molecular mechanisms that underlie these clinical observations.
Group 2 contained miRNAs that were unique to asthma, as these were differentially expressed in asthma vs. AR and the healthy groups, but were similarly expressed in AR vs. healthy subjects. A majority of the Group 2 miRNAs have been implicated in asthma in various studies. MiR-16 has been found to be upregulated in airways of the house dust mite mouse model of asthma, although its mechanistic role remains unexplored. More recently, miR-570 has been shown to bind and regulate the expression of HuR, an RNA-binding protein that is involved in asthmatic inflammation. These miRNAs as a group were predicted to regulate prominent inflammatory genes and signal transduction regulators, such as MAP kinases, NF-κB components, and STATs, suggesting that they may regulate key components of inflammatory pathways. Because our study only compares asthma and AR, it is possible that Group 2 miRNAs are involved in other allergic diseases as well. MiR-223 is a Group 2 miRNA that has been shown to regulate the proliferation of eosinophil progenitors and its differential expression has been documented in eosinophilic esophagitis.

Group 4 miRNAs are particularly interesting because despite the fact that 83% (29 of 35) of our asthmatic cohort has AR, these miRNAs are only differentially expressed in non-asthmatic patients with AR. Empirical identification and further investigation of genes and signaling pathways that are exclusively targeted by either of these groups may untangle some of the biomolecular complexities of these diseases. These studies may also lead to more personalized approaches in AR and asthma therapies.

In our report, Group 3 miRNAs are those which are concordantly dysregulated by a similar magnitude in both diseases. As such, Group 3 miRNAs may be indicative of the common pathways involved in AR and asthma. AR and asthma are often thought of as a continuum of the same disorder and the Group 3 miRNA expression pattern is reflective of the shared molecular pathogenesis of AR and asthma. Indeed, the comorbidity between AR and asthma is well-recognized.
Similar to Group 1 miRNAs, Group 5 miRNAs show differential expression among the three cohorts in our study. However, Group 5 miRNAs demonstrate a greater magnitude of dysregulation among patients with AR compared to patients with asthma. Therefore, it is likely that targets of Group 5 miRNAs are more involved in AR-specific pathways.

One of the main limitations of our study is that we had a relatively small sample size given the heterogeneity of AR and asthma. Because of this, the expression patterns of some of the profiled miRNAs may be miscategorized. It is possible that with much larger cohorts, circulating miRNAs in our study could be classified under different expression patterns.

Nevertheless, even with our population size, we made a number of observations which may carry significant importance. Many of the differentially expressed miRNAs targeted pathways which are specific to inflammation. The role of the MAPK signaling pathway is well-known in the pathogenesis of AR and asthma\textsuperscript{207, 208}. The majority of the miRNA groups also target the PI3K-Akt pathway which is another particularly well-studied signaling cascade in Th2 inflammation\textsuperscript{209, 210}. Furthermore, there are emerging evidences in the literature that implicate neurotrophin\textsuperscript{211, 212} and insulin signaling in allergic lung diseases\textsuperscript{213}. Focal adhesion pathways which are targeted by Group 2 and Group 3 miRNAs in our study are likely involved in changes in airway basement membrane and airway smooth muscle in asthma\textsuperscript{214}.

Depending on the context of their biomolecular interactions, the differentially expressed miRNAs may play regulatory or pathologic roles. For example, miR-126 indirectly increases GATA3 expression in T cells, which could promote a Th2 response, and is consistent with our observation that this miRNA was elevated in the patients with AR and asthma\textsuperscript{141}. In addition, miR-21 has been shown to enhance eosinophilia by promoting eosinophil precursor growth and by inhibiting IL-12-mediated Th1 T-cell polarization\textsuperscript{171, 215}. MiR-21 is upregulated by at least 8-fold in the eosinophilic asthma cluster (Cluster 1).

On the other hand, since many miRNAs serve anti-inflamatory functions, it is also possible that some of the miRNAs upregulated in AR and asthma are secondarily induced by
chronic inflammation and do not promote disease pathogenesis. For instance, miR-146a is known to be stimulated by inflammatory stimuli and acts as a feedback mechanism to limit inflammatory responses by inhibiting NF-κB and cytokine signaling\textsuperscript{216}. Furthermore, the Let-7 family of miRNAs has also been shown to have important anti-inflammatory actions, primarily on IL-13 and Th2 responses\textsuperscript{142}. Thus, the increased plasma levels of these miRNAs in AR and asthmatic patients compared to healthy individuals could be an attempt to attenuate the inflammatory response. It is interesting to note that although these miRNAs were increased in the blood of asthmatics, we and others previously demonstrated that Let7 and miR-146a levels were decreased in the lungs of asthmatics compared to non-asthmatic controls (sources of miRNAs included bronchoalveolar lavage, exhaled breath condensates, and airway epithelial cells)\textsuperscript{144, 146, 217}.

MiRNAs are produced by most cells in the body and are highly secreted into exosomes, which enter circulation and traffic to distant sites where they can be taken up by other cells\textsuperscript{218, 219}. It is possible that abnormalities in the transfer of miRNAs between the blood and lungs, and/or impairment in the ability of lungs to express anti-inflammatory miRNAs contribute to asthma pathogenesis. The roles of these and other regulatory mechanisms which govern the synthesis and secretion of miRNAs will require further study as these areas have not been well-explored in asthma and AR.

In addition to shedding light on the roles of circulating miRNAs in the molecular pathogenesis of asthma and AR, our study has potential significance in the diagnosis and management of these diseases. The finding that the levels of many miRNAs clustered with asthmatics of high or low peripheral eosinophil counts indicates that expression profiling could be a useful tool to phenotype asthma. This may be important as patients with eosinophilic asthma may have different responses to glucocorticoids compared to non-eosinophilic asthmatics\textsuperscript{220}. Similar to asthma, subphenotyping of patients suffering from AR is of critical importance to the management and treatment of this disease\textsuperscript{205}. It is likely that circulating miRNAs could also be differentially expressed among patients with different subtypes of AR.
Our findings reveal that a random forest prediction model based on six circulating miRNAs is sufficient to determine an individual’s AR or asthmatic status. These miRNAs are miR-125b, miR-16, miR-299-5p, miR-126, miR-206, and miR-133b. Notably, our model could accurately differentiate healthy subjects from asthmatic and the few mislabeled cases were misclassified as AR. This goes hand in hand with the fact that many molecular processes implicated in the pathogenesis of AR also contribute to the pathogenesis of asthma. With high NPV and PPV, our model possesses excellent diagnostic potential for AR and asthma. One of the key shortcomings of our predictive model is that it cannot distinguish between asthmatics with AR and asthmatics without AR. Further, 91% (72/79) of our subjects were Caucasians. Our findings will need to be validated via studies with larger cohorts to overcome the phenotypic and demographic limitations of our study.

In conclusion, we show that circulating miRNAs have great potential for the diagnosis of AR and asthma as well characterization of asthma subtypes. Plasma miRNAs can be easily extracted from peripheral blood with minimal patient risk, and quantification of blood miRNA levels by qPCR is cheap, reproducible, and can be multiplexed for high throughput analyses. Circulating miRNAs are likely involved in different pathologic or regulatory components of AR and asthma. We have identified candidates which may be involved in regulation of Th2 inflammation and could serve as novel therapeutic targets.
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Supplemental Figure 3-1. Selection of candidate plasma miRNAs for study. A. Expression heat map of 135 miRNAs that were reproducibly detected in asthma. Two way unsupervised hierarchical clustering with the average linkage method was used. B. Volcano plot of the differentially expressed miRNAs in our screening.
Supplemental Figure 3-2 Validation of miRNA qPCR amplification. A. Representative raw data showing real-time PCR curves for miR-145, miR-148a, and let-7a run in triplicate from a random subject. B. Gel electrophoresis of the miRNA real-time PCR reaction shows one band at a size between 50-100 nucleotides, consistent with the expected size of the miRNA products.
**Supplemental Figure S3.** Boxplots of the 30 differentially expressed miRNAs. A. Group 1 miRNA expression pattern shows intermediate dysregulation in allergic patients and more exaggerated dysregulation in asthmatic individuals. B. Group 2 miRNA expression pattern shows no statistically significant difference between the healthy and allergic rhinitis cohorts. C. Group 3 miRNA expression pattern shows no difference in median miRNA expression levels between the allergic and asthmatic populations. D. Group 4 miRNA expression pattern shows no difference between healthy and asthmatic groups. E. Group 5 miRNA expression pattern shows intermediate dysregulation in asthmatic individuals and more exaggerated miRNA dysregulation in allergic subjects.
Supplemental Figure 3-4. MicroRNA-KEGG pathway network. A. 115 unique KEGG pathways are predicted to be regulated by the 30 differentially expressed miRNAs. The top 20 most regulated pathways and the miRNAs that target them are depicted in this network diagram. In general, there are extensive overlaps in the pathways targeted by each miRNA group. B. Bar graph of the top 10 KEGG pathways based on number of genes regulated by the differentially expressed miRNAs.
Supplemental Figure 3-5. MicroRNAs are differentially expressed in eosinophilic asthmatic patients compared to non-eosinophilic asthmatic subjects. A. The volcano plot illustrates which miRNAs are differentially expressed between Cluster 1 and Cluster 2. Log₂ fold change is calculated from the ratio of median miRNA copy/µL in Cluster 1 to Cluster 2. B. PCA mapping of the 35 asthmatic individuals using 39 candidate miRNAs confirms the formation of two major clusters. PC1 and PC2 account for 25.1% and 17.2% of the variance in the data, respectively.
Supplemental Figure 3-5. Establishment of the six miRNA prediction model. A. Example of a decision tree classification model. The tree is constructed with all 79 samples and 44 features as implemented by scikit-learn24. The split at each decision node is based on minimizing the Gini index61 which describes the probability of assigning the incorrect class labels to the set of samples at that particular node. The random forest classification we utilized takes the consensus of 10 to 100000 decision trees to predict a subject’s disease status. B. 44 different prediction models were generated by successively adding features in order of their predictive importance (Supplemental Tables S43 and S5). Our analyses reveal that a predictive model containing the top six most important miRNAs (highlighted in orange) performs with the highest accuracy (91.1%). Blue points represent outliers from 10 runs. C. The ideal number of trees for our prediction model was confirmed by performing random forest classification analyses with our top six miRNAs using 10, 100, 1000, 10000, and 100000 decision trees. A random forest algorithm with 100 trees (highlighted in orange) is sufficient to attain the highest predictive accuracy. Blue points represent outliers from 10 runs.
Chapter 4

Posttranscriptional Regulation of IL-13 by Tristetraprolin and Human Antigen R

Abstract

IL-13 is a pro-inflammatory cytokine that plays a central role in the pathogenesis of asthma, but the mechanisms that regulate its expression are not well elucidated. Mounting evidence indicates that the IL-13 transcript is subject to significant post-transcriptional regulation. We found that the destabilizing RNA-binding protein, tristetraprolin (TTP) was induced by inflammatory stimuli in CD4+ T -cells. Overexpression and siRNA-mediated silencing of TTP indicated that TTP inhibited IL-13 expression by destabilizing its mRNA. We demonstrated that TTP interacts with IL-13 mRNA, and mapped the binding site to the AU-Rich elements located between nucleotides 854 to 879 of the IL-13 3’ UTR. We also mapped the binding site of the RNA-binding protein human antigen R (HuR), which was previously shown to stabilize the IL-13 transcript. We discovered that HuR bound to the same region as TTP, but also a second region located between nucleotides 1122-1278. TTP binding to the IL-13 mRNA did not displace HuR, but the de-stabilizing effects of TTP were dominant nonetheless. These findings confirm the importance of post-transcriptional regulation in IL-13 regulation, and indicate that a balance between TTP and HuR may dictate IL-13 expression.
Introduction

IL-13 is a pro-inflammatory cytokine produced by T helper type 2 (Th2) cells that play a central role in the pathogenesis of asthma. The deleterious effects of IL-13 on the asthmatic airways have been well established. IL-13 can directly and indirectly facilitate the recruitment and activation of various inflammatory cells, including B cells, mast cells and eosinophils. Moreover, characteristic features of asthma such as bronchial hyperresponsiveness, increased mucus production and airway remodeling have been attributed to IL-13 signaling.

Although much is known about the role of IL-13 in asthma, there is still a lack of information about how IL-13 expression itself is regulated. Most of the studies regarding the regulation of IL-13 focus on its transcriptional regulation. Among the reported transcriptional activators of IL-13 expression are GATA3, AP-1, and NFAT. These transcription factors bind the IL-13 promoter in response to inflammatory stimuli. On the other hand, the glucocorticoid receptor has been demonstrated as a transcriptional repressor of IL-13. More recently, the post-transcriptional regulation of IL-13 expression has been subjected to increasing scrutiny.

Post-transcriptional regulation (PTR) has emerged as a crucial control mechanism for the establishment, maintenance and resolution of inflammation. Post-transcriptional regulation determines the fate of cellular mRNA transcripts by modulating their stability, availability and translational efficiency. As such, this process allows for rapid changes in gene expression in response to the changing cellular microenvironment. These changes in gene expression are mediated by the interaction of microRNAs (miRNAs) and RNA-binding proteins with their target mRNA transcripts, usually in the 3’ untranslated region (3’UTR).

There is little known about how IL-13 is regulated post-transcriptionally. Human antigen R (HuR), an AU-rich element (ARE)-binding protein, has been shown to upregulate the production of IL-13 cytokine by stabilizing the IL-13 mRNA. However, it is not clear whether
there are factors that promote mRNA degradation of IL-13 to inhibit its expression. The ability to “turn off” expression of inflammatory mediators may be crucial to limiting the extent or duration of an inflammatory response, and could have major implications to the pathogenesis and treatment of asthmatic inflammation. Notably, multiple previous studies have shown that HuR-mediated upregulation of cytokine expression is counteracted by tristetraprolin (TTP), an ARE-binding protein that downregulates the expression of its target transcripts by promoting mRNA degradation.

Tristetraprolin is an immediate early response gene that has been best characterized in monocytes and airway epithelial cells, where it acts as an anti-inflammatory protein. The binding sites of TTP are either an AUUUA pentamer, or a nonamer motif XXAUUUAXX, where the flanking nucleotides (X) can be A’s or U’s. These motifs are present at a high rate in the 3′UTR of many inflammatory mediators, suggesting that TTP could exert regulatory effects on cytokines and chemokines. Binding of TTP to AREs usually leads to mRNA deadenylation and decapping, which causes mRNA degradation and represses mRNA translational efficiency. The role of TTP in CD4+ T-cells has not been well studied, and it is not known whether IL-13 is a target.
Methods

Tissue culture and cell treatments

Human Jurkat T cells (ATCC) were cultured in Th2-skewing conditions by growing in RPMI (Cellgro) supplemented with 10% FBS and 50 ng/mL carrier free recombinant human IL-4 (R &; D Systems). Human HEK293T cells (ATCC) were grown in DMEM (Cellgro) supplemented with 10% FBS. Both cell lines were maintained in a 37°C incubator with 5% CO2. Cultures were routinely tested to ensure that they were mycoplasma-free.

Jurkat cells were transfected utilizing the Neon Transfection System (Thermo Fisher). For each transfection reaction, two million Jurkat cells were washed with 1x PBS without Ca2+ and Mg2+ and subsequently resuspended in 100 µL of Resuspension Buffer R (Thermo Fisher). For TTP overexpression experiments, 5 µg of pcDNA 3.1 empty vector or the previously described pcDNA-TTP overexpression plasmid was added to the resuspended cells. For the TTP knockdown experiments, 50 nM of AllStars Negative Control siRNA (Qiagen) or TTP-specific siRNA (Integrated DNA Technologies) was mixed with the resuspended cells. The sense and antisense siRNA sequences for TTP are: 5’- CAAGCUCAGUAUUCAUGAUGGUGG-3’ and 5’- CACCAUCAUGAAUACUGACUUGCAC-3, respectively. All electroporation reactions were performed by pulsing the cells three times at 1350 V for 10 milliseconds. Where indicated, Th-2 skewed Jurkat cells were stimulated with 50 ng/mL phorbol 12-myristate-13-acetate (PMA) (Acros Organics) and 250 ng/mL calcium ionophore (A23187) (Sigma). Actinomycin D was used at a concentration of 5 µg/mL.
**CD4+ T lymphocyte isolation and treatment**

Leukocyte reduction filters from healthy patients on no medications and no chemotherapy were retrieved from the Penn State Hershey Blood Donor Center (35 Hope Drive, Hershey, PA 17033). Filter packs were flushed with sterile, room-temperature PBS and the filtrate, containing diluted blood, layered on to a 15 mL Ficoll Paque (GE Healthcare) gradient. Centrifugation at 630 x g for 30 minutes differentially separated peripheral blood mononuclear cells (PBMCs). PBMCs were resuspended in AIM-V (Life Technologies). CD4+ T cells were then positively isolated from PBMCs using the Dynabeads CD4 Positive Isolation Kit (Invitrogen), following vendor protocol. CD4+ T cells were also resuspended in AIM-V media.

Where indicated, CD4+ T cells were stimulated with 50 ng/mL phorbol 12-myristate-13-acetate (PMA) (Acros Organics) and 250 ng/mL calcium ionophore (A23187) (Sigma). Where indicated, CD4+ T cells were activated with Dynabeads Human T-Activator CD3/CD28 beads (Life Technologies), added in a 1:1 beads-to-cell ratio.

**Dual luciferase reporter assay**

The luciferase reporter vector pmirGLO was purchased from Promega for expression of both firefly and Renilla luciferases from the same plasmid under the control of separate promoters. This reporter was used as both a reference control in transfection/luciferase assay experiments, described below, and as the backbone vector for the IL-13 3’UTR construct. First, genomic DNA from a healthy donor was used as a template for amplification of the target sequence by Phusion DNA polymerase (New England Biolabs) using the following primers (IDT DNA): 5’- CGAGGTGGCCCAGTTTGTAA-3’ and 5’ - GCCAAAATGAAAGACAAATACA-3’. The desired PCR product was gel-purified using the Zymo Gel DNA Recovery Kit (Zymo Research) and used as the template for a second round of PCR amplification with the following
primers to introduce the restriction endonuclease sites for XhoI and AccI (bases changed/added in lowercase, recognition sites underlined): 5'-aaaaactcgagAACTTCGAAAGCATCATT-3' and 5'-aaaaaaagaattacATCTGTCACCAACTTTAT-3'. Inserts were cloned via Quick T4 DNA Ligase (New England Biolabs), and plasmid DNA was isolated from multiple colonies using the Zyppy Plasmid Miniprep Kit (Zymo Research) for screening via restriction analysis. All positive clones were confirmed by sequencing at the Penn State University Nucleic Acid Facility. The final construct, pmirGLO-IL13-3UTR, consists of the pmirGLO vector with the complete human IL-13 3’UTR sequence (GenBank Accession #NM 002188.2) cloned downstream of the firefly luciferase coding region. Three minor sequence variants were noted in the cloned sequence (NCBI dbSNP ID rs1295685, rs848, and rs847), and reflect the major alleles found in the HapMap-CEU population (Caucasians of European ancestry). Deletion of the AREs present within positions 854-879 of the IL-13 3’UTR was accomplished using a multistep cloning strategy that replaced positions 854-879 with the SalI restriction site (5'-GTCGAC-3'). Sanger sequencing was performed to confirm this change. For the luciferase assays, HEK293T cells were grown to 70% confluence in 6-well plates and cotransfected with pmirGLO parent vector or pmirGLO-IL13-3UTR and pcDNA 3.1 empty vector (Thermo Fisher) or pcDNA-TTP overexpression vector. The Attractene Transfection Reagent (Qiagen) was used for the transfection of HEK293T cells.

**RNA isolation and quantitative real-time PCR (qRT-PCR) analysis**

Total RNA was isolated using TRIzol reagent (ThermoFisher Scientific) as per manufacturer’s instructions. Extracted RNA was quantified by UV absorbance at 260 nm and reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) as described by the manufacturer. qRT-PCR was performed using 1:10 diluted cDNA as template, 2 x iTaq Universal SYBR Green SuperMix (Bio-Rad), and gene-
specific primers listed in Supplemental Table 1. qRT-PCR reactions were run in either 96-well plates on the MyiQ2 Two-Color Real-Time Detection System (Bio-Rad) or 384-well plates on the CFX384 Touch Real-Time PCR Detection System (Bio-Rad). In both cases, the following program was used: 2 minute hot start at 95°C, 40 cycles of 15 seconds at 95°C, 60 seconds at 60°C, and 2 minutes at 10°C. Gene expression fold changes were calculated using the ∆∆Ct method with either GAPDH or Renilla luciferase as the normalization control where appropriate.

**In vitro biotin pulldown assay**

Biotinylated RNA oligos were synthesized from cDNA templates amplified with the primers listed in Supplemental Table 4-2. The templates were then in vitro transcribed with a 4:1 CTP to biotin-CTP ratio using the MAXIscript T7 kit (Thermo Fisher Scientific). This reaction was subjected to DNaseI (ThermoFisher Scientific) treatment. Subsequently, the in vitro synthesized, biotinylated RNA oligos were isolated using NucAway spin columns (Thermo Fisher Scientific). Biotin pulldown experiments were performed as previously described.

**RNA-immunoprecipitation (RNP-IP) assay**

For the RNP-IP, 10⁷ Jurkat T cells were stimulated for 24 hours and pelleted. Cell pellets were lysed in 300 μL 1× PLB (100 mM KCl, 5 mM MgCl₂, 10 mM HEPES, pH 7.0, 0.5% Nonidet P-40) for 10 minutes on ice in the presence of EDTA-free protease inhibitors (Roche Applied Science) and RNaseOUT (Thermo Fisher). Rabbit anti-TTP antibodies (ABE285, Millipore) or normal rabbit IgG (sc-2027, Santa Cruz Biotechnology) were immobilized on 75 μL of magnetic DynaBeads Protein A (Thermo Fisher) in PBST (PBS with 0.05% Tween 20) for 30 minutes at room temperature with gentle mixing. The resulting antibody-bead complexes were washed once with PBST and mixed with 150 μL of cell lysate per reaction. Each reaction was incubated for 15
minutes at room temperature with mixing. The beads were then washed three times with PBST. An aliquot of the beads from each RNP-IP reaction was collected from the third wash step for western blot analysis. Ribonuclear protein complexes were eluted from beads by proteinase K digestion and the eluate was then subjected to phenol-chloroform RNA isolation. The entire volume of isolated RNA was used for reverse transcription and subsequent qRT-PCR analysis. Fold enrichment was calculated by taking $2^{-\Delta C_t}$ where $\Delta C_t$ is the difference between the average Ct values between TTP immunoprecipitation and normal rabbit IgG immunoprecipitation for a given transcript.

**Measurement of secreted IL-13 cytokine by ELISA**

Secreted IL-13 cytokine in Jurkat cell culture media was quantified using the Human IL-13 Quantikine ELISA kit (R & D Systems) as per the manufacturer’s instructions. Samples were diluted 1:5 in the appropriate Calibrator Diluent and absorbance was measured on a GENios plate reader (Tecan). 4PL standard curves were generated using Assay Blaster! Data Analysis Software (Enzo Life Sciences).

**Immunoblotting**

Harvested cells were pelleted and lysed in 1x PLB (100 mm KCl, 5 mm MgCl2, 10 mm HEPES, pH 7.0, 0.5% Nonidet P-40) for 10 minutes on ice in EDTA-free protease inhibitors (Roche Applied Science). Lysates were cleared by centrifugation for 5 minutes at maximum speed (4 °C). The concentration of protein was determined by Bradford (Biorad Protein Assay) and used to normalize the amount of lysate loaded on gels. Samples were boiled for 5 min in SDS-PAGE sample buffer and run on a 10% polyacrylamide gel for 45 min at 45 mA. The following antibodies were used at a 1:1000 dilution: HuR (sc-5261; Santa Cruz Biotechnology),
TTP (T5327; Sigma), β-actin (A5441; Sigma), donkey anti-rabbit IgG (NA934; GE Healthcare), and goat anti-mouse IgG (31430; Thermo Fisher Scientific). Blots were developed using HyGlo chemiluminescent antibody detection reagent (E2500; Denville Scientific).

**Statistical analysis**

Data was analyzed using two tailed Student’s t-test when comparing two conditions, or ANOVA with Bonferroni post-hoc correction when comparing more than two conditions. Significance cutoff was set at p<0.05. All analyses were performed using Graphpad Prism.
Results

Inflammatory stimuli promotes T-cell expression of TTP and IL-13

Previous studies have shown that TTP expression is upregulated in response to various inflammatory stimuli. However, little is known about the role of TTP in TH2 inflammation. To determine if TTP plays a role in TH2 response, CD4+ cells were isolated from human subjects and then treated with combined anti-CD3 and anti-CD28 antibodies or isotype control to stimulate T-cell receptor signaling. Compared to control-treated cells, anti-CD3/CD28 treatment induced TTP expression in CD4+ cells (Figure 4-1a). We also found that the phorbol 12 myristate 13-acetate (PMA), a non-T-cell receptor-dependent stimulus and a known TTP inducer previously used as an inflammatory stimuli, also induces TTP expression in primary human CD4+ T-cells (Figure 4-1b).

To further characterize the role and mechanisms of TTP in TH2 inflammation, we utilized Jurkat T-cells cultured under TH2-skewing conditions. We chose Jurkat T-cells for subsequent studies, as this cell line can be efficiently transfected with plasmids or siRNA, which are crucial experiments to understand the mechanisms of TTP in PTR. Stimulation of TH2-skewed Jurkat cells with PMA increased expression of TTP mRNA and protein (Figure 4-1c and 4-1d). In addition, PMA also stimulated IL-13 expression in the Th2-skewed Jurkat cells, consistent with previous studies (Figure 4-1d). The IL-13 mRNA levels peaked at 6 h after the initial inflammatory stimuli while TTP protein levels remained elevated over the next 24 hours (Figure 1d), raising the questions about whether TTP limits the level of expression or duration of IL-13 expression.
Figure 4-1. Inflammatory stimuli induce T-cell expression of TTP and IL-13. A. One representative of three independently performed experiments is shown (a-c). Western blot analysis of TTP in a) primary human CD4+ T cells treated for 18 hr with anti-CD3 and anti-CD28 antibodies, b) primary human CD4+ T cells treated for 18 hr with PMA, and c) IL-4 skewed Jurkat T cells treated for 18 hr with PMA. d) Time course of IL-13 mRNA and TTP protein expression in IL-4 skewed Jurkat T cells over the course of 24 hours with representative western blot of TTP shown.

TTP overexpression results in decreased IL-13 cytokine production

As the accumulation of TTP protein coincided with the reduction of IL-13, we next asked whether TTP post-transcriptionally regulates IL-13 expression. The IL-13 3’UTR contains multiple AREs that are potential TTP binding sites (Supplemental Figure S4-1). As such, we investigated whether perturbations in TTP levels affected the expression of endogenous IL-13. Jurkat cells were electroporated with either pcDNA-control or pcDNA-TTP overexpression vector and allowed to recover overnight. The electroporated cells were then stimulated with PMA and calcium ionophore for 24 hours. Cell pellets and cell culture media were harvested for
western blot analysis and ELISA, respectively. Overexpression of TTP repressed IL-13 cytokine expression (Figure 4-2a). Moreover, silencing of TTP expression via electroporation with TTP-specific siRNA resulted in the upregulation of IL-13 cytokine expression (Figure 4-2b).

To further establish the effects of TTP on IL-13 expression, we performed a time course assay in which IL-13 mRNA levels were measured at different intervals in stimulated TTP-wild type or TTP-silenced Jurkat cells (Figure 4-2c). Our experiments revealed that silencing TTP led to a faster peak of IL-13 increase (4h in TTP-silenced vs. 6h in control-treated cells), but the duration of IL-13 expression was not significantly altered, as IL-13 levels drop by 12 h in both control and TTP-silenced cells. However, TTP-silenced Jurkat cells exhibit a 7.2 ± 1.0 fold increase in the peak expression of IL-13 mRNA, indicating that TTP limits the amplitude of TTP expression.

**Figure 4-2.** TTP overexpression and silencing after IL-13 expression. A) TTP was overexpressed in Jurkat T cells for 24 hr, followed by 18 hr stimulation with PMA and calcium ionophore. B) Jurkat T cells were electroporated with TTP-specific siRNA, followed by 18 hr stimulation with PMA and calcium ionophore. Representative western blot analysis (n=3) for overexpression and silencing is shown next to ELISA results of secreted IL-13 levels (n=6). ELISA data are represented as the mean ± SD. C) Time course of IL-13 mRNA expression in WT or TTP-silenced Jurkat cells stimulated with PMA and calcium ionophore. * p <0.0001.
TTP binds to the IL-13 3’UTR and destabilizes the IL-13 mRNA

We next sought to establish whether the effects of TTP were due to interaction with the IL-13 transcript, and if so, to map the binding site(s). Bioinformatic interrogation of the 827 nt long IL-13 mRNA 3’UTR reveals presence of two clusters of AU-rich elements (AREs) (Supplemental Fig. S1). Both of these clusters are potential TTP binding sites. A Co-IP of TTP-mRNA complexes was performed using TTP-specific antibodies or an IgG isotype antibody control mixed with cell lysates from stimulated Jurkat cells. The specificity of the TTP-IP was confirmed by Western blot (Figure 3). We used a qPCR-based approach to determine if the IL-13 mRNA is enriched in the TTP-IP versus the IgG-isotype control IP. Significant enrichment of the IL-13 transcript was observed in the TTP-IP, but not in the IgG isotype control-IP, thus confirming the interaction of IL-13 mRNA to TTP (Figure 4-3). As expected, the mRNA transcripts of established TTP targets, TNF-α and CCL2, were also enriched in the TTP-IP. In contrast, CCL5 and EMC7, genes that do not have AREs in their 3’UTRs, did not show significant enrichment.

Figure 4-3. TTP overexpression and silencing after IL-13 expression. Co-immunoprecipitation of Jurkat cell lysates using TTP-specific or isotype control antibodies, followed by RNA isolation and qPCR analysis shows enrichment of IL-13, TNF-α, and CCL2
mRNA. Representative Western blot inset (n=3) shows specific immunoprecipitation of TTP with the anti-TTP antibody, but not the isotype control antibody. Data are represented as the mean ± SD. * p < 0.05

Additionally, we used an in vitro-transcribed, biotinylated, IL-13 3’UTR RNA to isolate bound TTP from Jurkat cell lysates (biotin pulldown assay), confirming the TTP-IL-13 RNA interaction, and showing that the binding was mediated through the IL-13 3’UTR (Figure 4-4a)\textsuperscript{105}. The ARE-deficient GAPDH 3’UTR (negative control) failed to show TTP binding, while the CCL2 3’UTR (positive control) bound TTP\textsuperscript{125}.

The IL-13 mRNA contains two clusters of AREs, referred to as A1 and A2 (Figure 4-4a and supplemental Fig S4-2). A1 spans position 854 to 879 and contains two AREs both of which are present as the nonamer motif (UUAAUUAUU at position 854 and UUAAUUUAAA at position 871). A2 begins at position 1132 to 1158 and also contains two AREs: a nonamer motif (UUAAUUAUU at position 1132), and a heptamer motif (UAUUUAU at position 1152). To localize the site of TTP-IL-13 3’UTR interaction, we utilized biotin pulldown assays with two overlapping biotinylated fragments of in vitro-synthesized IL-13 3’UTR (Figure 4-4b). The first fragment, Fragment 1, spans positions 456 to 832 and does not contain any AREs. The second fragment, Fragment 2, spans positions 827 to 1282 and contains both A1 and A2. Both Fragment 1 and Fragment 2 were separately incubated with cell lysates from stimulated Jurkat cells and probed for TTP binding. We show that TTP is bound to the ARE-containing Fragment 2 but not the ARE-deficient Fragment 1. The CCL2 3’UTR was used as a positive control. Fragment 2 was then divided into three overlapping sub-fragments and used to probe TTP binding (Figure 4-4c). Fragment 2-1 spans positions 827 to 990 and contains the A1 AREs, while Fragment 2-3 spans position 1122 to 1282 and contains A2 AREs. Fragment 2-2 contains no AREs and corresponds to position 983-1134. TTP preferentially bound to Fragment 2-1 but not Fragment 2-2 or 2-3 (Figure 4-4c). To confirm the specificity of TTP binding to the A1 ARE, we created a deletion
mutant that removed this ARE (2-1Δ ARE) and performed the biotin pulldown assay. TTP failed to bind to the 2-1ΔARE RNA (Figure 4-4d).

Figure 4-4. TTP overexpression and silencing after IL-13 expression. A) Schematic of the IL-13 mRNA transcript with white arrows denoting AREs next to representative Western blot (n=3) showing the results of IL-13 3'UTR biotin pulldown assay. B) Binding of TTP was assessed by biotin pulldown assay using two fragments of the IL-13 3' UTR. C) Fragment 2 was further divided into three overlapping, sub-fragments and biotin pulldown assay was used to assess binding. D) A deletion construct removing the A1 ARE (nt 854-879, labeled 2-1ΔARE) was used to confirm TTP binding to this region.

TTP primarily exerts its effects by destabilizing its target mRNA\textsuperscript{126, 227, 228}. To test whether TTP destabilizes the IL-13 transcript, we measured changes in IL-13 mRNA half-life with TTP overexpression or silencing down using actinomycin D to arrest transcription and qPCR to quantify changes in IL-13 expression over time. A half-life of 3.96 h was observed in with TTP overexpression vs. 1.31 hours in the control (Figure 4-5a) TTP-silencing prevented the decay of IL-13, but it was not possible to determine a decay rate as there was an increase in IL-13 mRNA
overtime (Figure 4-5b). This is likely due to incomplete inhibition of transcription with actinomycin D at the concentration, which was chosen to maintain cell viability, but nevertheless indicates that TTP destabilizes the IL-13 mRNA.

**Figure 4-5. TTP overexpression destabilizes the IL-13 mRNA.** Decay of the IL-13 mRNA following actinomycin-D treatment in Jurkat cells with A) TTP overexpression or B) TTP silencing. C) Effect of increasing TTP levels on expression of an IL-13 3’ UTR luciferase reporter (n=5). D) Effect of TTP on an IL-13 3’ UTR luciferase reporter containing the 2-1 ∆ARE mutation (n=6).

**TTP downregulates IL-13 expression in a 3’UTR-dependent mechanism**

We next sought to confirm that the binding of TTP to the 3’UTR was functionally significant by using an IL-13 3’UTR reporter system. The entire IL-13 3’UTR was cloned into
the multiple cloning site of the firefly luciferase gene in the pmirGLO dual luciferase reporter vector, such that IL-13 3’UTR immediately downstream of the firefly luciferase coding region. Transcription of the firefly luciferase gene produces a fusion transcript that places the IL-13 3’UTR immediately downstream of the firefly luciferase coding region. HEK293T cells were co-transfected with either pmirGLO parent vector or pmirGLO-IL13-3UTR and pcDNA empty vector, or pcDNA-TTP overexpression vector at increasing concentrations (0-900 ng). TTP overexpression reduced firefly luciferase expression in a dose-dependent manner, indicating that TTP mediates a functional effect by acting on elements in the IL-13 3’UTR (Figure 4-5c).

We next sought to determine whether the 2-1ΔARE mutant was resistant to the effects of TTP, and cloned this mutation into the 3’UTR luciferase reporter. A statistically significant increase in luciferase expression was observed, indicating that TTP mediates effects on IL-13 expression by interacting with this region (Figure 4-5d). However, deletion of the A1 region did not completely restore luciferase activity to the level of the parent plasmid.

Interplay between TTP and HuR in the posttranscriptional regulation of IL-13

The RNA-binding protein HuR has been shown to up-regulate IL-13 expression by stabilizing the transcript. We sought to understand how TTP affects HuR function, and whether TTP exerts a dominant effect when both proteins are present. First, we mapped binding of HuR to the IL-13 3’UTR using the biotinylated RNA pulldown assay (Figure 6a). HuR bound to fragment 2 of the IL-13 3’UTR. Further mapping using the smaller fragments we used for probing TTP binding demonstrated that HuR had two distinct binding sites, one in fragment 2-1 and another in fragment 2-3 (Figure 6a). The binding of HuR was not altered when TTP was overexpressed, indicating that TTP did not displace HuR binding (Figure 6b). As TTP did not appear to displace HuR binding, we then explored whether TTP exerts a dominant effect on IL-13 expression over HuR. Increasing amounts of TTP (0, 150, 300, 600, 900 ng of plasmid) were
transfected into HEK293T cells co-transfected with pmiRGLO-IL-13’UTR and a constant amount of HuR (300 ng pcDNA-HuR plasmid). A concentration-dependent effect was observed as increasing levels of TTP had greater inhibitory effects on reporter expression (Figure 6c). However, while TTP was capable of inhibiting reporter expression at all concentrations, it was not able to completely mitigate the effects of HuR. Expression of the reporter was higher when HuR was present, indicating that HuR was still able exert some effects in the presence of TTP, though the magnitude of effect was small.

Figure 4-6. HuR also binds and regulates IL-13, but TTP function is dominant. a) Representative Western blot analysis (n=3) of RNA biotin pulldown assay to assess binding of HuR to fragments of the IL-13 3’ UTR. b) Representative Western blot analysis of RNA biotin (n=3) pulldown assay to map HuR binding to sub-fragments of fragment 2 and whether addition of TTP affected binding. c) Effect of addition of increasing concentrations of TTP on expression

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**Figure 4-6. HuR also binds and regulates IL-13, but TTP function is dominant.**

a) Representative Western blot analysis (n=3) of RNA biotin pulldown assay to assess binding of HuR to fragments of the IL-13 3’ UTR. b) Representative Western blot analysis of RNA biotin (n=3) pulldown assay to map HuR binding to sub-fragments of fragment 2 and whether addition of TTP affected binding. c) Effect of addition of increasing concentrations of TTP on expression.
of the IL-13 3' UTR luciferase reporter in the presence of HuR (n=6).*, p <0.05 for TTP + HuR effect vs TTP; †, p <0.05 for TTP + HuR effect vs no transfection control; ‡ p <0.05 for TTP effect vs. no transfection control.
**Discussion**

Post-transcriptional regulation has emerged as an important controller of immune and inflammatory processes. However, little is known about its role in asthma and its ability to regulate central inflammatory mediators that drive disease pathogenesis. As CD4+ T-cells play a central role in asthmatic inflammation, we sought to determine whether PTR controls gene expression in these cells. Our finding that TTP is induced by T-cell receptor stimulation in CD4+ T-cells is consistent with the known anti-inflammatory role of the protein, whereby inflammatory stimuli induce TTP expression as a form of feedback inhibition to limit the extent or duration of the inflammatory response. The kinetics of TTP expression, including a rapid induction within 60 minutes of cell stimulation and persistence in expression for 24 hours, is similar to that seen in other cell types such as monocytes and airway epithelial cells and suggests that TTP could have widespread effects conserved across many cell types.

In order to gain insight into the functional effects of TTP in CD4+ T-cells, we chose to study its effects on IL-13, a major effector cytokine produced primarily by these cells in allergic asthma. Our findings indicate that TTP acts primarily to limit the amplitude and kinetics of IL-13 expression, as TTP-silencing led to higher IL-13 mRNA levels that accumulated faster. These findings are in line with a recent study by Zeng et al., where TTP expression was negatively correlated with IL-13 expression in glioma tumorigenesis. The finding that altering TTP expression in glioma cells led to subsequent changes in IL-13 expressions indicates that the effect may be conserved in different cell types, and that post-transcriptional regulation of the cytokine has far reaching implications in different diseases.

Our work added a deeper mechanistic understanding of how TTP regulates IL-13 expression. First, although IL-13 mRNA has multiple AREs in the 3’UTR, TTP binding was limited to the ARE spanning nucleotides 854-879. Failure to bind to other AREs could be due to competition with other trans-factors, or absence of specific cis-elements, such as secondary
structure. As expected, mutation of the ARE in region 854-879 abolished TTP binding, but only had a marginal functional effect in a reporter system. We postulated that this site could be utilized by more than one RNA-binding protein, such that its disruption could alter not only TTP binding, but perhaps binding of an RNA-stabilizing protein as well, in principle cancelling out the effects. As HuR was previously shown to up-regulate IL-13 expression\textsuperscript{101}, we mapped the binding sites in the IL-13 3’UTR and investigated its interplay with TTP. We found that HuR did indeed bind to the same site as TTP, but also bound to a second ARE-containing site (nucleotides 1122-1278). Contrary to current models that suggest that TTP and HuR compete for a single binding site\textsuperscript{95,231}, we found that TTP does not displace HuR binding to the IL-13 3’UTR. Using IL-13 reporter constructs transfected with TTP +/- HuR, we found that the repressive effects of TTP predominate, but does not completely overcome the effects of HuR.

The combination of our findings and previous studies yield a dynamic model of TTP function in T-cells. T-cell stimulation leads to transcription of IL-13 and stabilization of the transcript by HuR. In parallel, TTP is induced, binds to nucleotides 854-879 of the IL-13 3’UTR, and destabilizes the transcript to limit expression of IL-13. Levels of IL-13 fall over the course of 24 h, and as TTP auto-regulates its own mRNA stability and is proteolytically degraded, levels return to baseline.

The balance between TTP and HuR may have important considerations in asthma pathogenesis and as potential therapeutic targets. Future studies will be aimed at determining whether this balance is altered in CD4+T-cells in allergic asthma, where IL-13 is over-expressed and central to chronic airway inflammation. Furthermore, the ability to shift this balance to promote TTP effects could represent a novel therapeutic strategy.
Acknowledgements

This chapter is a manuscript in preparation co-written by Panganiban RP, Lambert KA, Dziublenzki M, Hsu MH, Roff AR, and Ishmael FT.
**Supplemental Table 4-1.** Primers used for qRT-PCR analysis.

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<td>CCL2 Reverse</td>
<td>AGATCTCCTTGCCACAATG</td>
</tr>
<tr>
<td>CCL5 Forward</td>
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<td>EMC7 Reverse</td>
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**Supplemental Table 4-2.** Primers used to generate cDNA templates for biotin pulldown assay.

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<tr>
<td>Reverse</td>
<td>1122-1282</td>
<td>tcaccaactttattttctgtctc</td>
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<sup>a</sup>Letters in uppercase correspond to T7 transcription start site; letters in lower case indicate target-specific sequences.
Supplemental Figure 4-1. TTP potential binding sites in the IL-13 3’UTR. The IL-13 3’UTR is 827 nucleotides long and contains two pairs of AREs (underlined sequences).
Chapter 5

Ablation of airway epithelial tristetraprolin promotes neutrophilic inflammation in the ovalbumin model of asthma

Abstract

Background: Tristetraprolin (TTP) is an AU-rich element (ARE)-binding protein that posttranscriptionally downregulates the expression of many ARE-containing pro-inflammatory mediators involved in asthma. Although TTP has been studied in airway epithelial cells, its role in the in vivo mouse model of asthma has never been characterized.

Objective: We sought to establish the effects of airway epithelium (AE)-specific TTP ablation on the ovalbumin (ova) mouse model of asthma.

Methods: Conditional TTP knockout (TTP CKO) mice were generated by using the Cre-lox recombination system. Asthma was induced by ova challenge and sensitization. Bronchioalveolar lavage fluid (BALF), lung tissue, and serum were harvested to characterize the phenotype of the conditional TTP KO mouse.

Results: Differential cell count of BALFs from TTP CKO mice demonstrates significantly increased neutrophil levels compared to wild-type mice. TTP CKO also shows increased expression of GM-CSF and CXCL-1. This upregulated expression results in increased peribronchiolar neutrophil infiltration that is resistant to the effects of dexamethasone.
Conclusions: Our results indicate that TTP CKO mice produce a neutrophilic inflammatory asthma that is resistant to glucocorticoid treatment.
Introduction

Asthma is a highly prevalent disease characterized by chronic inflammation, airway remodeling, and mucus production. A complex interplay of the airway epithelium (AE), immune system, and external environment produces these characteristics. The AE is an immunologically active barrier that produces multiple pro-inflammatory mediators in response to pathogens, allergens, irritants, and other inflammatory stimuli. These pro-inflammatory mediators typically recruit and activate immune cells such as eosinophils, mast cells, neutrophils, lymphocytes, and macrophages. Airway epithelial cells also secrete growth factors that promote airway remodeling and subepithelial fibrosis. Finally, transdifferentiation of airway epithelial cells into goblet cells results in increased airway mucus production. As such, understanding the regulation of AE gene expression may provide novel insights into the pathogenesis of asthma.

Posttranscriptional regulation (PTR) has emerged as an important means of regulating gene expression. RNA-binding proteins (RBPs) are among the key effectors of PTR. Binding of RBPs to the 3’UTRs of their target mRNA transcripts usually results in the modulation of mRNA stability and translational efficiency. One of the more well-studied RBPs that has been implicated in the regulation of AE gene expression is the AU-rich element (ARE)-binding protein, tristetraprolin (TTP). TTP interacts with its target transcripts by binding to AUUUA motifs present in the mRNA 3’UTR. Binding of TTP to AREs promotes the recruitment of deadenylating and decapping protein complexes that subsequently results in decreased mRNA stability and downregulated mRNA translational efficiency. Multiple previous studies have demonstrated the importance of TTP in regulating AE gene expression. TTP downregulates the expression of IL-8 and GM-CSF in airway epithelial cells. Importantly, TTP has been shown to facilitate the anti-inflammatory effects of glucocorticoids on the AE. As such, TTP may play a role in the pathogenesis of glucocorticoid-resistant asthma.
Although multiple reports have shown that TTP posttranscriptionally regulates many AE-derived pro-inflammatory mediators, these findings have thus far been achieved with *in vitro* model systems. The role of TTP in airway inflammation has never been defined in an *in vivo* mouse model of asthma. In this chapter, we describe our preliminary data from experiments with the conditional knockout (KO) of TTP in the airway epithelial cells. We show that the AE-specific TTP KO mice exhibit significantly more neutrophilic inflammation compared to wild-type mice. We further demonstrate that this increase in neutrophil infiltration is not attenuated with glucocorticoid treatment. These findings suggest that TTP may have a crucial role in neutrophilic inflammation in asthma.
Methods

Animals

Wild-type C57BL/6 mice were purchased from The Jackson Laboratory while conditional TTP KO mice were obtained as described below. The mice were housed according to institutional guidelines. Our experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of The Pennsylvania State University College of Medicine.

The conditional TTP KO (TTP CKO) mice were generated by crossing the loxP-flanked TTP (TTP\textsuperscript{flox/flox})\textsuperscript{240} mice with mice expressing the Cre recombinase under the control of the Foxj1 promoter\textsuperscript{241}. The second exon of the TTP gene is flanked by loxP cassettes in the TTP\textsuperscript{flox/flox} mice while the Foxj1 gene is primarily expressed in ciliated epithelial cells\textsuperscript{242}. Consequently, the Foxj1 promoter-driven Cre recombinase excises the second exon of the TTP\textsuperscript{flox/flox} gene in the murine trachea, bronchi, and bronchioles. Knock-down was confirmed by immunohistochemical (IHC) staining.

Mice were routinely genotyped using the MyTaq Extract-PCR kit (Bioline). Genomic DNA was isolated from a 2 mm tail snip and subjected to end-point PCR analysis as per manufacturer’s instructions. The following primers were utilized for TTP genotyping: 5’-GAACCCCTCTCTCGATCGGGGATAC-3’ and 5’-GGATGGGAGTCCGAGTTTATGTTCGAAACATGC-3’\textsuperscript{240}. Foxj1-Cre-expressing mice were screened using the following primer pair: 5’-ATTTGGGCCAGCTAAACATGC-3’ and 5’-GCAAAAACAGGTGTATCGG-3’\textsuperscript{241}. 
Supplemental Figure 5-1. Anti-TTP IHC of murine airway epithelium. Top panel shows airway-epithelium-specific knockout mice. The bottom panel was obtained from wild-type mice.

**Induction of asthma in mice and dexamethasone treatment**

All mice were sensitized to ova by intraperitoneally injecting a 200 µL endotoxin-free 1× PBS (Cellgro) solution containing 200 µg of ova (Sigma-Aldrich, A5503) and 100 µL of Imject Alum Adjuvant (ThermoFisher Scientific). Ova sensitizations were performed during Day 0 and Day 7 of the experiment (Supplemental Figure 5-1). The mice were also given intraperitoneal injections of PBS (vehicle control) or 1.0 mg/kg dexamethasone (Sigma-Aldrich, D2915) during the mornings of Days 14, 15, 16, 17, and 18. Ova challenges were performed eight hours later on the same days by intranasal administration of 50 µL of 2 mg/mL ova solution in endotoxin-free 1× PBS. All mice were 6-8 weeks old during the experiments and were sacrificed using bell jar
isoflurane overdose on Day 19. Cardiac puncture was used both as a secondary method of euthanasia and to collect blood for serum isolation.

**Differential cell count of BALFs**

BALFs were obtained by flushing the lungs four times with 400 µL endotoxin-free, 1× PBS (Cellgro). The washes were combined in a single 1.7 mL tube. Subsequently, 200 µL of the combined washes was used to obtain Cytospin smears. A blinded, independent investigator performed differential white cell counts on Cytospin BALF smears stained with HEMA3 Solution (Fisher Scientific). A total of 200 cells per mouse was counted.

**Histology and immunohistochemistry**

After collection of the BALF, the left lung was excised, placed in a tissue cassette, and fixed with 10% neutral buffered formalin (Fisher Scientific). All tissue embedding, staining, and immunohistochemistry protocols were performed by the Molecular and Histopathology Core at The Pennsylvania State University College of Medicine. Immunohistochemical detection of TTP was accomplished using the anti-Tristetraprolin antibody (Abcam, ab33058). The NIMP-R14-specific, anti-neutrophil antibody (Abcam, ab2557) was used for detection of bronchial neutrophil infiltration. Neutrophil infiltration was scored by a blinded, independent investigator.

**ELISA assays**

Cytokines present in the BALF were quantified using the Quantikine ELISA Mouse Immunoassays for CCL2, TNF-α, GM-CSF, CXCL-1, and IL-6 (R&D Systems). Samples were
used as is and no dilution was performed. Absorbances were measured on a GENios plate reader (Tecan) and 4-PL standard curves were generated using Assay Blaster! Data Analysis Software (Enzo Life Sciences).

**Statistical analysis**

Data was analyzed using Student’s t-test or multiple measures ANOVA with post-hoc correction where appropriate.
Results

Knocking out TTP in the airway epithelium results in increased neutrophils in the BALF

To induce asthma in mice, we used a modified version of the ova model of asthma (Supplemental Figure 5-1A)

Wild-type or TTP CKO mice were sensitized to ova twice and challenged for five consecutive days with either endotoxin-free PBS (vehicle control) or 50 µL of 2mg/mL ova solution. Induction of asthma was confirmed by histological analysis (Supplemental Figure 5-1B). Compared to the lungs of PBS-challenged mice, hematoxylin and eosin (H&E) staining of lungs harvested from ova-challenged mice demonstrated increased peri-bronchiolar immune cell infiltration and narrowing of the small airway cross-sectional area. Periodic acid-Schiff (PAS)-stained histological sections of the mouse lungs from ova-challenged mice revealed significantly increased mucus production. Finally, whereas Cytospin BALF smears from the PBS-challenged mice mostly contained resident lung macrophages, BALF smears from ova-challenged mice indicated the increased presence of neutrophils and eosinophils.

Differential cell counts of BALFs obtained from asthmatic, TTP CKO mice showed increased presence of inflammatory cells compared to asthmatic, wild-type mice. (Figure 5-2A). In particular, there are significantly increased numbers of lymphocytes (1.00 x 10^5 vs 2.15 x 10^5 cells), neutrophils (5.40 x 10^4 vs 3.98 x 10^5 cells), and eosinophils (5.47 x 10^5 vs 7.60 x 10^5 cells) found the BALF of CKO mice. Previous studies have implicated TTP in the downregulation of several pro-inflammatory cytokines (Supplementary). Furthermore, in vitro studies have demonstrated that TTP plays a role in glucocorticoid-mediated gene regulation (124, 125). As such, we sought to determine whether the anti-inflammatory effects of glucocorticoids are perturbed in TTP CKO mice.

Both wild-type and TTP CKO mice were treated with PBS or dexamethasone (Supplementary Figure 5-1A). BALFs obtained from wild-type mice show that compared to
vehicle treatment, dexamethasone treatment results in the decreased presence of lymphocytes (1.00 x 10^5 vs 2.51 x 10^4 cells) and eosinophils (5.47 x 10^5 vs 2.05 x 10^5 cells). (Figure 5-2B). A similar phenomenon was observed in dexamethasone-treated TTP CKO mice (2.15 x 10^5 vs 5.79 x 10^4 lymphocytes and 7.60 x 10^5 vs 2.01 x 10^5 eosinophils) (Figure 5-2C). The number of neutrophils present in the BALF of either wild-type or TTP CKO mice did not significantly change in response to steroid treatment. However, TTP CKO mice showed a more neutrophilic inflammation compared to wild-type mice in the setting of ova challenge. (Figure 5-2B vs Figure 5-2C).

Figure 5-2. Differential cell count of Cytospin BALF smears from wild-type and conditional knockout mice. A. The increased presence of lymphocytes, neutrophils, and eosinophils are observed in asthmatic, conditional TTP knockout mice compared to asthmatic wild-type mice. B. Asthma-induced wild-type mice respond to anti-inflammatory effects of

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dexamethasone as evidenced by decreased lymphocytes and eosinophils in glucocorticoid-treated mice. C. The same effect is observed in asthma-induced conditional knockout mice. However, the BALF obtained from these mice show increased numbers of neutrophils both in control- and dexamethasone-treated mice.

**TTP CKO mice demonstrate increased production of neutrophil-specific cytokines.**

Because of the increased presence of neutrophils in TTP CKO mice, we investigated whether the expression neutrophil chemoattractants were increased in TTP CKO mice. Specifically, we investigated whether the expression of various well-established, TTP-regulated neutrophil chemoattractants such were changed in TTP CKO mice in comparison to wild-type mice.

Compared to BALF obtained from wild-type mice, BALF from TTP CKO mice contained increased levels of GM-CSF (301.70 ± 31.94 pg/ml vs 545.54 ± 55.12 pg/ml) and CXCL-1 (4594 ± 182.9 pg/ml vs 6028.44 ± 257.9 pg/ml) (Figure 5-3A). Additionally, an increase in IL-6 levels in TTP CKO mice was trending significance.

In light of these observations, we then asked whether the anti-inflammatory effects of glucocorticoids are perturbed by the lack of TTP expression. Among wild-type mice, pretreatment with glucocorticoids decrease the expression of CXCL-1 is by more than 3-fold (1698 ± 203.3 pg/ml vs 519.7 ± 64.24 pg/ml). This anti-inflammatory effect is eliminated in TTP CKO mice with glucocorticoid treatment resulting in an insignificant decrease in CXCL-1 expression (2929 ± 229.3 pg/ml vs 2332 ± 399.4 pg/ml).
Figure 5-3. TTP knockout mice have elevated BALF concentrations of GM-CSF and CXCL-1. A. Compared to wild type mice, TTP CKO mice show a 3.2- and 1.3-fold increase in GM-CSF and CXCL-1 concentrations respectively. B. TTP CKO mice have a decreased response to the anti-inflammatory effects of dexamethasone.

TTP CKO demonstrate decreased neutrophil response to dexamethasone

Although we show that there is no change in BALF neutrophil levels in both dexamethasone-treated wild-type and TTP CKO mice (Figure 5-2B and Figure 5-2C, we asked whether the absence of glucocorticoid-mediated CXCL-1 downregulation in TTP CKO mice have physiological consequences. In particular, we quantified whether there is a difference in peribronchiolar neutrophil infiltration in TTP CKO and wild-type mice. We observed that in wild-type mice, dexamethasone treatment results in decreased presence of neutrophils around bronchioles (2.4 ± 0.6 vs 1.2 ± 0.3 neutrophils/high power field, Figure 5-4). By comparison, TTP CKO mice do not exhibit such decrease in peribronchiolar neutrophil infiltration (2.5 ± 0.3 vs 2.9 ± 0.6 neutrophils/hpf).
Figure 5-4. Peribronchiolar neutrophilic infiltration is resistant to dexamethasone treatment in TTP CKO mice. A. Representative images of wild-type and TTP CKO mice bronchioles. B. Wild-type mice demonstrate decreased neutrophil infiltration in response to glucocorticoids whereas TTP CKO mice do not show the same response.
Discussion

Our results suggest that knocking down TTP in the airway epithelium significantly alters its inflammatory gene expression profile. In particular, our screening ELISA assay showed that the expression of neutrophil-specific chemoattractants such as GM-CSF and CXCL-1 are upregulated in TTP CKO mice. This is not surprising as both GM-CSF and CXCL1 have AREs present in their 3’UTRs to which TTP can bind to (Supplemental Figure 5-2). Additionally, both of these cytokines have been previously validated as TTP targets in in vitro experiment81.

The combined change in expression levels of GM-CSF and CXCL-1 results in the increased number of neutrophils present in the BALFs of TTP CKO mice. Further, this upregulation also led to increased peribronchiolar neutrophil inflammation and resistance to the anti-inflammatory effects of glucocorticoids.

The majority of asthma subtypes respond favorably to glucocorticoid treatment. Indeed, inhaled fluticasone and systematic prednisone are both mainstays of asthma treatment. Nonetheless, there are human asthma subtypes that are steroid-resistant. These asthma subtypes were characterized as having increased BALF neutrophils244. A study involving 205 patients demonstrated that sputum neutrophil numbers directly correlated with severity of airflow obstruction245. As such, our findings of increased BALF and peribronchiolar neutrophils in TTP CKO mice have physiological and symptomatic significance. Mutations in the TTP coding sequence that result in reduced TTP activity may account for these steroid-resistant asthma subtype. Moreover, mutations in 3’UTRs of TTP-regulated neutrophil-specific chemokines may also contribute to the of these severe asthma phenotypes. Augmenting the activity of TTP is therefore is therefore a novel and innovative asthma therapy.
Of the cytokines that we surveyed, GM-CSF and CXCL-1 were the only ones that exhibited differential expression in TTP CKO mice. This is despite the fact that most of the other cytokines such as TNF-α, CCL2, and IL-13 contain ARE in their 3’UTRs. These cytokines in particular are also well-validated TTP targets\(^81\). As such it is unclear why the expression of these cytokines remain unchanged with TTP downregulation. These findings suggest that the interactions between TTP and its target mRNAs may be influenced by tissue specificity.

The type of inflammatory stimulus may also influence TTP-mediated posttranscriptional regulation. Our experimental set-up takes advantage of the well-characterized alum/ovalbumin mouse model of asthma. Other models that use naturally occurring allergens such as house dust mite (HDM), cockroach, and *Alternaria alternata* may have inflammatory profiles that more closely resemble human allergic asthma. These separate models may also have a different response to the downregulation of TTP expression.

There are several other members of the CXC chemokine family that promote neutrophil chemotaxis such as, CXCL3, CXCL5, CXCL6, and CXCL7, that we were unable to test in our experiments\(^244\). Similarly, other members of the CC chemokine family such CCL6, CCL7, and CCL9 are potent neutrophil chemoattractants we were unable to investigate\(^244\). Determining the effects of TTP CKO on these cytokines will provide a more comprehensive inflammatory profile that can be used to establish novel asthma therapeutic targets.

At present, there are no other groups that have looked at airway epithelium-specific ablation of TTP in mice. Our system can be used to study the role of TTP in other inflammatory lung diseases such infection, ARDS, or pulmonary fibrosis as neutrophilic inflammation play a major role in the pathogenesis of these diseases.
Acknowledgements

This chapter is a manuscript in preparation co-written by Panganiban RP, Hsu MH, and Ishmael FT. Pathology studies including differential cell counts, H&E and PAS staining, and IHCs were performed by Timothy Cooper (Department of Comparative Medicine), Kang Li (Molecular and Histopathology Core), and Trey Bruggeman (Molecular and Histopathology Core).
**Supplemental Figure 5-1. Induction of the ovalbumin model of asthma in mice.** A. Experimental timeline of inducing asthma in knock-out and wild-type mice. B. HEMA-3 statined BALF, H&E and PAS stains of histological sections of lungs from ova-sensitized, PBS- or OVA-challenged mice. Ova-challenged mice demonstrate increased amount of inflammatory cells both in BALF and lung tissue. Ova-challenged mice also show increased mucous production compared to controls.
Supplemental Figure 5-2 3’UTRs of GM-CSF (top), IL-6 (middle), and CXCL-1(bottom). Bolded and underlined are AREs which ARE-BPs like TTP have been predicted or experimentally demonstrated to bind.
Chapter 6

Discussion: The role of posttranscriptional regulation in the pathogenesis, diagnosis, and management of asthma

Posttranscriptional regulation (PTR) has emerged as an essential control system for the regulation of gene expression. PTR mediates the fine tuning of protein expression by effecting changes in mRNA stability, availability, and translation efficiency. Multiple previous studies have shown that seemingly minute changes in mRNA stability can often result in significant changes in protein expression\textsuperscript{79}. Because posttranscriptional regulatory events can occur independent of transcriptional activation and repression, PTR allows for rapid cellular responses to changes in the cell’s internal and external microenvironments.

Fine control of the inflammatory response is critical for cellular homeostasis. It is thus unsurprising that PTR has been established as a crucial component of the inflammatory response. PTR has been implicated both in the establishment and resolution of inflammation\textsuperscript{221}. For example, P bodies, upon disassembly in response to inflammatory stress, can release sequestered, pre-made mRNAs and shuttle them to the cellular translation machinery which results in rapid upregulation of protein levels\textsuperscript{110, 246}. Conversely, decreasing the stability and translation efficiency of already synthesized RNA messages averts further unnecessary progression of the inflammatory response. Although pre-made pro-inflammatory proteins will still have to be degraded, PTR prevents extraneously transcribed mRNA from being translated into more protein. Whereas the role of PTR in controlling the inflammatory response is well-studied, much less is known about PTR in Th2-mediated inflammatory systems such as asthma. The work outlined in this dissertation explores the many roles of PTR in the pathogenesis, diagnosis, and management of asthma.
MicroRNAs regulate cytokines which are central to the pathogenesis of asthma

We and others have previously reported that miRNAs regulate the expression of several cytokines involved in asthmatic inflammation (Table 6-1). Specifically, in Chapter 2 of this dissertation, we demonstrated the miR-1248 binds to the 3’UTR of the IL-5 mRNA. We further showed that miR-1248 acts as a positive regulator of IL-5; overexpression or inhibition of miR-1248 results in the upregulation or downregulation of IL-5 3’UTR–controlled protein expression, respectively. For the most part, miRNAs downregulate the expression of their target transcripts and our report remains as one of the few studies that have shown that miRNAs can function as positive regulators of their target’s expression.139, 159, 179.

Table 6-1. Pro-inflammatory cytokines in asthma (Adapted from Barnes, 2008)247

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Function in Asthma</th>
<th>miRNA regulator</th>
<th>RNA-binding protein (RBP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4</td>
<td>Promotes IgE class-switching</td>
<td>None shown experimentally</td>
<td>HuR100</td>
</tr>
<tr>
<td>IL-5</td>
<td>Promotes eosinophil growth</td>
<td>miR-1248179</td>
<td>None shown experimentally</td>
</tr>
<tr>
<td>IL-13</td>
<td>Promotes IgE class-switching</td>
<td>Let-7142,173</td>
<td>TTP, HuR101</td>
</tr>
<tr>
<td>TNFα</td>
<td>Increases inflammation</td>
<td>miR-125b248</td>
<td>TTP82, HuR93</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Increases neutrophil infiltration</td>
<td>miR-133a249, miR-133b249</td>
<td>TTP126</td>
</tr>
<tr>
<td>VEGF</td>
<td>Promotes angiogenesis</td>
<td>miR-200b250, many others251</td>
<td>TTP252, HuR250</td>
</tr>
</tbody>
</table>

The molecular mechanism by which miR-1248 positively regulates IL-5 expression needs to be elucidated. To begin with, how miR-1248 affects the IL-5 mRNA needs to be determined. Stabilization of the IL-5 transcript, upregulation of IL-5 mRNA translation efficiency, and
alteration of the subcellular localization of the IL-5 transcript are all possible mechanisms by which miR-1248 can upregulate IL-5 gene expression.

There are many ways by which changes in RNA stability can be accomplished. Most commonly, mRNA decay is facilitated by the excision of the poly(A) tail by the CCR4-NOT1 deadenylase complex. Removal of the 5’ cap by the DCP1-DCP2 decapping enzyme can also result in mRNA degradation. Thus, it is possible that miR-1248 acts by inhibiting the binding of these protein complexes on the IL-5 3’UTR. Further, the IL-5 3’UTR contains multiple AREs and miR-1248 may also facilitate IL-5 transcript stabilization by promoting HuR binding while simultaneously inhibiting TTP interaction with these AREs. Because these molecular events are interconnected, it is likely that miR-1248 directly or indirectly affects all of these mechanisms.

Multiple assays can be used to establish if miR-1248 stabilizes IL-5 mRNA. Actinomycin D pulse-chase assays combined with miR-1248 overexpression or inhibition in PBMCs or isolated CD4+ T cells will allow for detection of changes in IL-5 mRNA half-life. Alternatively, the β-globin Tet-off transcriptional pulsing assay develop by Chen et al can be used to determine the effects of miR-1248 in the IL-5 3’UTR reporter system.

The effects of miR-1248 on IL-5 translation efficiency also needs to be ascertained. This can be accomplished via polysome profiling experiments. PBMCs or CD4+ T cells overexpressing miR-1248 can be fractionated by sucrose density gradient centrifugation. Because miR-1248 upregulates IL-5 cytokine production, increasing miR-1248 cellular levels should result in increased IL-5 mRNA copy number in the heavy polysome fractions of the sucrose density centrifugation experiment. Conversely, transfection of miR-1248 inhibitor should result in decreased IL-5 mRNA copy number in the heavy polysome fractions.

The majority of miRNAs downregulate protein expression by preventing the translation of their target transcript. Previous studies have demonstrated that miRNAs prevent the association
of eIF4E, a eukaryotic translation initiation factor, with the 5' RNA cap\textsuperscript{257}. MiRNAs have also been shown to prevent the association of the 40S and 60S subunits to form the 80S ribosome complex\textsuperscript{257, 258}. It is likely that miR-1248, bound to its own protein complex, competes with other miRNAs that downregulate IL-5 mRNA expression.

Lastly, it should be investigated whether miR-1248 affects IL-5 mRNA subcellular localization. Changes in miR-1248 levels may perturb the distribution of the IL-5 transcript in P bodies versus polysomes. P bodies are cytoplasmic foci composed of translationally silenced mRNAs complexed with RNA-interacting proteins. These proteins may facilitate translational repression or RNA destabilization. Messenger RNAs in P bodies have been demonstrated to dissociate from the P body complexes and reenter the translation process\textsuperscript{259}. Hence, it should be explored whether miR-1248 influences trafficking of the IL-5 mRNA. This can be accomplished using a variety of live cell imaging techniques such as RNA fluorescence \textit{in situ} hybridization (RNA-FISH) or by using any of the previously described RNA aptamers\textsuperscript{260}.

Our hypothesis is that miR-1248 exerts its effects directly on the IL-5 3'UTR. As previously mentioned, this can be achieved by stabilizing the IL-5 transcript, increasing the translation efficiency of the IL-5 mRNA, and altering the subcellular localization of the IL-5 message. Any single one of these processes or any combination of them contributing in varying degrees could be the molecular mechanism behind miR-1248-mediated PTR of IL-5. However, a key characteristic of cytokine PTR is that it tends to target functionally-related mRNAs and regulate entire pathways in a coordinated fashion\textsuperscript{153}. As such, it is also likely that miR-1248 also targets genes both upstream and downstream of the IL-5 signaling cascade. The changes in IL-5 mRNA levels may also be driven by indirect miR-1248 effects through any of its upstream regulators. From a biological systems standpoint, it is also possible that miR-1248 can target genes downstream of the IL-5 signaling cascade. Using high throughput mRNA expression
analyses techniques, the global transcriptomic effects of miR-1248 perturbations can be uncovered. These experiments will further shed light on the role of miR-1248 in allergic asthma.

In addition to determining how miR-1248 affects the IL-5 mRNA, protein mediators of miR-1248 function must be identified. Vasudevan and Steitz have shown that miR-369-3, in association with the FXR1 and Ago2 proteins, can enhance the translation efficiency of TNFα\textsuperscript{139}. Likewise, the same mechanism may also be involved in the miR-1248-mediated upregulation of IL-5 expression. RNA-immunoprecipitation coupled with qPCR, with or without UV crosslinking, can be utilized to test whether FXR1 interacts with miR-1248. Similarly, a biotinylated miR-1248 pulldown assay can be used to reveal a potential miR-1248-FXR1 interaction. If these approaches fail to demonstrate the involvement of FXR1 in miR-1248 function, the miR-1248-interacting protein complexes can still be determined from biotinylated miR-1248 pulldown experiments via mass spectrometry.

The in vitro experiments outlined above will significantly increase our understanding of miR-1248 molecular mechanisms. However, it is also imperative to study miR-1248 in an in vivo model of asthma. Kumar et al has shown that intranasal delivery of miRNAs can result in measurable physiological differences in the ovalbumin mouse model of asthma\textsuperscript{142}. A comparable approach can be used to study the effects of intranasal administration of miR-1248 mimic or inhibitor. Levels of IL-5 cytokine from BAL fluid or lung homogenate should be quantified. BAL fluid cytology should be performed to determine if there are differences in eosinophil levels which could be reflective of IL-5 cytokine levels. Finally, histopathologic analyses should be performed on the lung tissue from miR-1248 mimic- or inhibitor-treated asthmatic mice.

In general, examining the role of miRNAs in asthma will provide novel insights into the pathogenesis of this disease. For example, Ago2 CLIP-seq experiments in CD4\textsuperscript{+} T cells from severe asthmatics can uncover other differentially expressed and potentially dysregulated
miRNAs. This data set, combined with high throughput RNA expression studies, can reveal an overall picture of the role of miRNA-based PTR in severe asthma.

The studies performed in Chapter 2 of this dissertation and the experiments suggested above can be applied to investigate different subtypes of asthma such as glucocorticoid-resistant, exercise-induced asthma, and others. Further, because asthma involves an interplay between the immune system and the airway epithelium, smooth muscle, and airway epithelial cells, although outside the scope of this dissertation, the potential miRNA-mRNA interactions in immune, airway epithelial, bronchial smooth muscle, and exosomal miRNA crosstalk between these cells can also be interrogated.

Multiple different factors may lead to dysregulated miRNA expression in asthma.

miRNA decay have been mapped throughout the entire length of mature miRNAs. These cis-elements and Exo-5'UTR targets can reveal an overall picture of the role of miRNA-based PTR in severe asthma.

Recent reports show that miRNAs undergo accelerated decay. This leads to varying miRNA levels and hence changes in miRNA effects. Various cis-acting elements have correlated with rapid miRNA decay. A generally held view in miRNA biology is that miRNAs are stable molecules. However, in increased Exo-5'UTR targeting of miR-1248, complex. miRNA decay can also be achieved with deactivating mutations in miR-1248. The same molecular phenotype can also be achieved with deactivating mutations in miR-1248 transcription repressors. Further, in the miR-1248 symmetrical process, mutations in the miR-1248 promoter or in miR-1248 transcription activators can result in constitutive miR-1248 expression. If this is the case, then increased miR-1248 expression can be due to mutations anywhere along the miR-1248 gene. Multiple different factors may lead to dysregulated miRNA expression in asthma.
acting elements may act as binding sites by which various proteins can facilitate miRNA decay. Consequently, errors along the miR-1248 decay pathway could potentially result in decreased turnover of mature miR-1248.

In human cells, three exoribonucleases have been implicated in miRNA degradation. These enzymes are XRN1, RRP41, and PNPase. Failure of these enzymes to degrade mature miR-1248 can result in persistence of miR-1248 in the cytosolic space. There, it can exert its effects on IL-5 and other mRNA targets. Excess miR-1248 can also be packaged into exosomes and secreted into the bloodstream where it can be taken up by other cell types.

Alternatively, increased miR-1248 levels may be secondary to changes in the cellular milieu brought about by asthmatic inflammation. Multiple cytokines important in asthma have been shown to have a regulatory effect on the expression of various miRNAs. For example, IL-13 has been observed to induce expression of miR-21 while IL-4 has been demonstrated to upregulate miR-378-3p expression. Furthermore, TNFα has been shown to increase miR-155 and decrease miR-125b expression levels in mouse macrophages. In airway epithelial cells, TNFα upregulates the synthesis of miR-570-3p. Thus, it should be determined whether pro-inflammatory cytokines present in the extracellular environment can increase miR-1248 expression, thereby creating a positive feedback loop via miR-1248-dependent IL-5 gene upregulation.

**RBPs are crucial regulators of asthma-associated cytokines**

Apart from miRNA-facilitated PTR in asthma, in Chapter 4 of this dissertation we also reported that the destabilizing ARE-binding protein TTP is a crucial posttranscriptional regulator of IL-13, a canonical cytokine in Th2-driven inflammatory disease. In particular, we showed that TTP binds to the AREs present on the IL-13 3’UTR. We demonstrated that knocking out TTP in
Jurkat T cells results in the increased stability of the IL-13 mRNA which ultimately leads to increased production of secreted IL-13 cytokine.

The other effects of TTP on IL-13 cytokine production should be further explored. Similar to miRNAs, TTP can also alter the IL-13 mRNA translation efficiency and subcellular localization. There are multiple reported cases in the literature where TTP downregulates its targets’ translation efficiency\textsuperscript{123, 266, 267} and TTP is a known component of P bodies\textsuperscript{268}. Both of these molecular scenarios should be investigated in the context of IL-13 mRNA regulation.

Our data significantly contributes to the current understanding of IL-13 PTR. Two groups have independently shown that the Let-7 family of miRNAs regulate IL-13 expression\textsuperscript{142, 173}. Furthermore, Casolaro et al implicated HuR, a stabilizing ARE-binding protein, in IL-13 PTR\textsuperscript{101}. However, we have yet to explore interplay among these miRNA and RBP regulatory factors.

The antagonistic nature of TTP and HuR is well-established but there is still no consensus about the molecular mechanism behind their counteracting effects. Illustrated in Figure 6-1 are the multiple possible models by which TTP and HuR can interact with each other and the IL-13 mRNA. Model 1 presupposes that TTP and HuR compete with each other for binding sites on the IL-13 3’UTR. These ARE-binding proteins switch on and off the AREs on the IL-13 3’UTR. In this scenario, the interaction between TTP and HuR is either not present or only occurs transiently. On the other hand, models 2 and 3 suggest physical interactions between TTP and HuR. Model 2 hypothesizes that a ternary complex is formed by TTP and HuR as they compete for binding sites while model 3 illustrates that the complex formed by TTP and HuR is dependent on them binding to different sites on the IL-13 3’UTR.
Figure 6-1. Three potential models for the interaction of TTP and HuR with the IL-13 3’UTR. Model 1: TTP and HuR compete for binding sites on the 3’ UTR. Model 2: TTP and HuR form a ternary complex while competing for binding sites. Model 3: TTP and HuR bind to different sites on the IL-13 mRNA.

In a resting cell, the HuR protein is usually highly expressed and TTP is almost undetectable. In the presence of inflammatory stress, TTP, an immediate early gene, is rapidly transcribed and synthesized. Both HuR and TTP are regulated by the p38-MAPK pathway. HuR is a direct substrate of p38 and phosphorylation of HuR results in its increased cytoplasmic localization, thereby allowing it to function as a stabilizer of ARE-containing mRNAs. By contrast, TTP is phosphorylated by p38’s downstream effector, MK2. Phosphorylation of TTP results in its decreased affinity for AREs. During the resolution of inflammation, TTP is dephosphorylated by the PP2A phosphatase. It is likely that dephosphorylation of TTP increases its affinity for AREs and allows it to displace HuR, thereby resulting in the destabilization of ARE-containing mRNAs. As such, of the proposed models of TTP-HuR interaction, model 1 is likely to be the most plausible. However, it remains to be determined how TTP and HuR binding are affected by different types of ARE motifs. Furthermore, some mRNAs have more than one ARE and it should be established how the number of AREs affect the interplay between TTP and HuR.
By taking advantage of RNA aptamers and traditional protein affinity techniques, we can determine which of these proposed models correctly illustrates the molecular basis for TTP and HuR antagonism. Figure 6-2 briefly shows the experimental approach for identifying whether TTP-HuR interactions follow model 1, 2, or 3.

<table>
<thead>
<tr>
<th>MS2</th>
<th>MS2</th>
<th>MS2</th>
<th>MS2</th>
<th>MS2</th>
<th>MS2</th>
<th>IL-13 3’UTR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>pMS2-6x-IL13-UTR</td>
</tr>
</tbody>
</table>

Cotransfect with GST-fused MS2-binding protein expression plasmid in HEK293 cells.

1st purification: Affinity purification with glutathion-coated agarose beads

Confirm competitive TTP and HuR interaction by EMSA

Distinguish between model 2 and 3 by adding an RNase treatment step while RNA-protein complexes are immobilized on the magnetic beads.

Figure 6-2. Flow diagram of experimental approaches to characterize the interaction among TTP, HuR, and IL-13 mRNA. The IL-13 3’UTR will be cloned into the pMS2-6x plasmid to produce pMS2-6x-IL13-UTR. This newly synthesized construct will be cotransfected with a GST-fused, MS2-binding protein expression plasmid in HEK293 cells. Cotransfected cells will undergo two consecutive affinity purification steps. Glutathione-coated agarose beads will be used to isolate the synthetic IL-13 3’UTR. Magnetic beads coated with TTP antibodies or isotype control will then be used to pull down TTP-bound synthetic IL-13 3’UTR. Absence of HuR during this step is suggestive of competitive TTP-HuR interaction. Presence of HuR, however, will indicate the formation of a ternary TTP-HuR complex that may be RNA-dependent or RNA-independent.

The role of RBPs in the pathogenesis of asthma should be more closely scrutinized. Other RBPs apart from TTP and HuR may also play a role in the PTR of IL-13. Certainly, both TTP and HuR have been shown to target other cytokines involved in asthma (Table 6-1). Thus, there is
a need for high throughput proteomic and transcriptomic analyses to expand our understanding of
the role PTR plays in the pathogenesis of asthma. Mass spectrometry analyses can help identify
other members of the TTP and HuR protein complexes while next generation sequencing
expression studies can help establish a more complete gene library of TTP and HuR targets.

In addition to investigating the interplay among RBPs, the relationship between RBPs
and miRNAs should also be studied. Recently, Ma et al has shown that miR-4661 competes with
TTP for binding on the IL-10 3’UTR. Both miR-4661 and TTP destabilize the IL-10 transcript;
however, TTP has a stronger destabilizing effect and its displacement by miR-4661 results in the
overall stabilization of IL-10. Additionally, both HuR and miR-21 have been shown to bind the
PDCD4 3’UTR and regulate PDCD4 expression. Poria et al demonstrated that HuR binds and
sequesters miR-21, thereby preventing miR-21 from destabilizing the PDCD4 3’UTR.

We and others have shown that IL-5 and IL-13 are both regulated by miRNAs. Also, both TTP and HuR target IL-13 and our preliminary studies show that TTP and HuR also
bind to the AREs in IL-5 3’UTR. It should be determined whether miRNAs competitively inhibit
RBPs or if miRNAs are capable of directing RBPs to mRNA transcripts. None of the miRNAs we
studied overlap with any AREs on IL-5 and IL-13 3’UTR. Nevertheless, the mRNA 3’UTR is a
three-dimensional structure and it should be determined how miRNAs influence RBPs during the
PTR of various cytokines. Furthermore, it should be studied whether TTP and HuR can bind and
sequester various miRNAs and act in a similar mechanistic fashion to the findings of Poria et al.

The majority of the work described in this dissertation was done in in vitro systems. We
studied PTR primarily in PBMCs and Jurkat T cells. Nonetheless, Chapter 5 describes how we
attempted to elucidate the role of TTP in the ovalbumin mouse model of asthma. In contrast to the
majority of our experiments, we abrogated TTP expression in bronchial epithelial cells. Although
the results of our studies remain largely preliminary, there is an observable quantitative difference
in the inflammatory asthmatic characteristic of the conditional knockout mice. Conditional TTP
knockout mice seem to undergo a more neutrophilic asthmatic inflammation compared to TTP wildtype mice. In humans, neutrophilic asthma is usually associated with corticosteroid resistance and worse clinical outcomes\textsuperscript{273}. It is highly unlikely that human neutrophilic asthma is due to complete abrogation of TTP expression in bronchial epithelial cells. Nevertheless, it should be investigated how closely our animal model reflects the pathophysiology of human neutrophilic asthma. Interrogating the physiological characteristics of the conditional TTP knockout mouse model of asthma may provide clues for the pathogenesis of severe, treatment-resistant asthma.

**RBPs and miRNAs can mutually regulate each other**

As previously stated, the 5’ to 3’ exoribonuclease XRN1 has been implicated in miRNA degradation\textsuperscript{261}. Interestingly, this exoribonuclease has been shown to interact with TTP\textsuperscript{274}. Although this interaction has been determined to facilitate degradation of ARE-containing mRNA sequences, it should be explored whether TTP itself plays a role in miR-1248 decay or miRNA degradation in general. Additionally, the role of HuR and other RBPs in the regulation of miRNA expression should be established.

The regulation of miRNA expression by RBPs is not completely unprecedented. KSRP, another ARE-binding protein involved in PTR, has been shown to promote the maturation of a small subset of miRNAs\textsuperscript{275}. Additionally, knocking down GW182, an RBP that facilitates deadenylation of its target mRNAs, has been shown to destabilize various miRNAs\textsuperscript{276}.

On the other hand, miRNAs have been demonstrated to posttranscriptionally regulate the expression of RBPs. Both miR-27 and miR-29a bind the TTP 3’UTR and subsequently suppress TTP expression\textsuperscript{277, 278}. Further, miR-133b and miR-570-3p have been shown to negatively regulate HuR expression\textsuperscript{201, 279}.
Taking all the posttranscriptional mechanisms together, what emerges is a complex regulatory network (Figure 6-3). In this network, cytokines can trigger changes in miRNA expression levels and induce various posttranslational modifications in RBPs. RBPs can affect cytokine stability and control miRNA synthesis and degradation. Finally, miRNAs can regulate the expression of both cytokines and RBPs. Dysregulation of any part of this complex regulatory network may be a key contributing factor to the pathogenesis of asthma. It should be determined which specific cytokines, miRNAs, or RBPs act as master regulatory nodes and ideal potential therapeutic targets.

**Figure 6-3. Posttranscriptional regulatory network.** PTR is a complex mechanism wherein cytokines, RBPs, and miRNAs cross-regulate each other. Cytokine signaling can result in either activation or inhibition of RBPs as well as changes in miRNA expression levels. RBPs can stabilize or destabilize mRNA transcripts. RBPs can also affect the synthesis and degradation of miRNAs. Finally, miRNAs can bind the 3’UTRs of RBP and cytokine mRNAs.
PTR-based technologies have practical implications for the diagnosis and management of asthma

In this dissertation, we illustrate the importance of miRNAs and RBPs in the pathogenesis of asthma. We show that PTR plays a critical role in modulating the expression of key cytokines in asthma such as IL-5 and IL-13. Clearly, PTR plays a significant role in orchestrating the Th2 immune response. For these reasons, PTR is an excellent but under-utilized technology for the clinical management of asthma.

Our study, described in Chapter 3, is the first to show that plasma miRNAs are differentially expressed among subjects who are healthy, have asthma, or have allergic rhinitis. We further demonstrate that using only six differentially expressed miRNAs (miR-125b, miR-16, miR-299-5p, miR-126, miR-206, and miR-133b), we can design a predictive algorithm that can distinguish among healthy, allergic, and asthmatic patients with high specificity and sensitivity. A major drawback of the work presented here is that our predictive model is based only on 79 individuals, 90% of whom are of Caucasian ethnicity. Thus, the results presented need to be validated in larger cohorts with a more diverse demographic composition.

In that same study, we also found that among asthmatics, the levels of many miRNAs clustered with high or low peripheral eosinophil counts. This finding implies that we can possibly predict known asthma subtypes or even identify novel phenotypes based on their plasma miRNA profiles. This is especially important for making personalized treatment decisions. For instance, plasma miRNA biomarkers that associate with high eosinophil counts can be indicative of a more IL-5-driven disease. Patients in these cohorts may have better asthmatic control with targeted IL-5 therapies. Furthermore, plasma miRNAs can potentially be used to easily screen and identify steroid-resistant individuals. Such technology will permit cost-effective disease management of these patient populations.
Differential miRNA expression in patients with asthma is unlikely to be a mere epiphenomenon of the disease. Circulating miRNA levels are likely to reflect the ongoing molecular biology in airway epithelial cells and immune cells. Longitudinal cohort studies can be performed to determine if levels of plasma miRNAs correlate with symptom control, patient compliance, and disease exacerbation.

The clinical value of miRNAs in asthma is not limited to its promise as an ideal biomarker. Because of its extensive involvement in the pathogenesis of asthma, miRNAs themselves can be taken advantage of as novel therapeutic targets. Administration of miRNAs or miRNA inhibitors remains an unexploited approach for managing difficult-to-treat asthma. Currently, there are multiple miRNAs which are being studied as novel cancer therapeutic agents\textsuperscript{280}. Some of these miRNAs such as miR-34a and miR-122 have undergone Phase I clinical trials\textsuperscript{280, 281}. Intranasal or systemic delivery of miRNAs and miRNA inhibitors that can target key cytokines in asthma such as IL-4, IL-5, IL-13 and others should be explored (Table 6-1).

As previously mentioned, PTR tends to occur in a coordinated fashion whereby a single miRNA can target multiple different genes in interconnected pathways. This becomes a key advantage in which one miRNA or miRNA inhibitor can shut down the expression of multiple inflammatory genes at the same time. While this is a largely beneficial property of miRNA-based therapeutics, miRNA target promiscuity can lead to off-target effects which need to be meticulously identified and characterized. These principles are also applicable to RBPs.

Therapeutic strategies based on altering the equilibrium between TTP and HuR represent another avenue by which we can take advantage of the role of PTR in the pathogenesis of asthma. The interplay between TTP and HuR can be characterized as a balance between anti-inflammatory and pro-inflammatory posttranscriptional regulators. Tilting this balance in favor of TTP provides a unique method of arresting the inflammatory response. During inflammation, TTP is regulated by phosphorylation via the p38-MAPK pathway. p38’s downstream effector,
MAPK-activated protein kinase (MK-2) directly phosphorylates TTP\textsuperscript{121,266}. This phosphorylation event results in the downregulation of TTP-mediated mRNA decay and leads to increased stability of the TTP-targeted pro-inflammatory cytokines. Augmenting TTP activity using commercially available MK-2 small molecule inhibitors may lead to decreased pro-inflammatory cytokine production in cell culture and animal models. Additionally, the serine/threonine phosphatase PP2A has been shown to dephosphorylate TTP\textsuperscript{119}. PP2A inducers can therefore be used to upregulate TTP-mediated mRNA decay.

**Summary and conclusions**

Posttranscriptional mechanisms are critical regulatory control points in the inflammatory response. The studies presented in this dissertation demonstrate the emerging importance of miRNAs and RBPs in the posttranscriptional control of asthmatic inflammation.

We present a model in which during inflammatory stress, cytokines such as IL-5 and IL-13 are synthesized. During this stage, expression of TTP, an immediate early gene, is also upregulated. At the beginning of inflammation, HuR, which is constitutively expressed in cells, stabilizes the cytokine mRNAs. TTP, on the other hand, is phosphorylated by p38’s downstream effector, MK-2, and this posttranslational modification results in decreased TTP affinity for AREs. Upon resolution of inflammation, TTP is dephosphorylated and displaces HuR on the cytokine transcripts. TTP-bound RNA is destabilized and translationally silenced. We and others have shown that miRNAs regulate both IL-5 and IL-13. It is likely that stabilizing miRNAs like miR-1248 promote HuR binding to its target transcript while destabilizing miRNAs act in concert with TTP to deadenylate, decap, and translationally silence mRNA transcripts (Figure 6-4).

MiRNAs produced by immune and airway epithelial cells in response to inflammation are packaged into exosomes and secreted in the blood. Circulating miRNAs likely have the
capacity to exert posttranscriptional effects on both neighboring and distant cells. Thus, T cell-produced miRNAs are potentially capable of regulating the airway epithelial cell transcriptome. MiRNAs secreted by airway epithelial cells are also likely to affect T cell gene expression. This allows for coordinated establishment and resolution of the immune response. Defects in this process are likely to contribute to the pathogenesis of asthma.

Considering the extensive involvement of PTR in asthma, PTR-based technologies represent an excellent but unexploited approach in the clinical management of this disease. Circulating plasma miRNAs are convenient biomarkers that can be used to easily screen asthmatic patients and potentially classify their disease by its molecular pathogenesis. Altering the equilibrium of these posttranscriptional regulatory factors is a novel and potentially effective strategy to halt asthmatic inflammation.

**Figure 6-4. Schematic diagram of cytokine PTR in asthma.** RBPs and miRNAs regulate various cytokines which are central to the pathogenesis of asthma. During the establishment of the immune response, HuR binds the AREs in the cytokine mRNA and promotes its expression. Stabilizing miRNAs may also play a role during this initial phase. These events are controlled by the p38/MAPK pathway. Towards the resolution of inflammation, TTP, along with destabilizing miRNAs, displaces HuR and promotes mRNA destabilization as well as translational repression.
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