MICRORNA REGULATION ON THE EXPRESSION OF CD38 AND OTHER ASTHMA RELATED GENES IN HUMAN AIRWAY SMOOTH MUSCLE CELLS

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ABSTRACT

CD38 is a multifunctional enzyme that regulates intracellular calcium ($[Ca^{++}]_i$) homeostasis. It is expressed in airway smooth muscle (ASM) cells where it elevates $[Ca^{++}]_i$ through its enzymatic product cyclic ADPribose (cADPR) and increases ASM contractility. Increased expression of CD38 in the ASM cells derived from the asthmatic patients (AS-HASM) and attenuated airway hyperresponsiveness to contractile stimuli shown by $CD38^{-/-}$ mice implicate the importance of CD38 in asthma.

The proinflammatory cytokine tumor necrosis factor-alpha (TNF-α) is considered to be an important mediator for airway pathology in asthma. The reason for the differential expression of TNF-α-induced-CD38 in AS-HASM cells, does not involve transcriptional regulation of CD38 which is through signaling pathways mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K), and transcription factors nuclear factor kappa-B (NF-κB) and AP-I. Thus I hypothesized that post-transcriptional regulation of CD38 by microRNAs account for the differential expression of TNF-α induced -CD38 in AS-HASM cells.

Among the several potential microRNAs predicted for CD38 by microRNA target-predicting algorithms, I selected miR-140-3p and miR-708 for further studies, as these showed differential expression in the AS-HASM cells compared to those from healthy subjects.

Overexpression of these either microRNAs in ASM cells inhibited the TNF-α induced expression of CD38 at messenger RNA (mRNA) and protein levels. Luciferase-reporter assays with a mutated 3'UTR of the CD38 transcript confirmed the specific target sites for both microRNAs. Transcript stability assays revealed that

mRNA degradation is not the mechanism underlying regulation by microRNAs. Examination of the expression and activation levels of proteins in the upstream signaling pathways of CD38 revealed that miR-140-3p, by inactivating p38 MAPK and NF-κB, and miR-708, by inactivating c-Jun N-terminal kinase (JNK) MAPK and Akt by elevating the expression of their phosphatases MKP-1 and PTEN respectively, control the expression of CD38 indirectly.

Further, we found that miR-708 downregulates the expression of many chemokines and inhibits the serum induced proliferation of human ASM cells.

We conclude that both microRNAs have therapeutic potential in controlling asthma related symptoms through regulating the expression of CD38 and chemokines and controlling the proliferation of human ASM cells.

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CHAPTER i

Literature review and general introduction

1. Asthma

1.1 Definition, characteristic features and clinical signs

Asthma is a common chronic respiratory disorder affecting about 300 million people worldwide (282). It is characterized by chronic inflammation, reversible airflow limitation and remodeling of airway structures (238). According to GINA report, 2014, asthma is defined as a "*heterogeneous disease* that is *usually characterized by chronic airway inflammation with history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation*." These signs, including airflow typically change in magnitude over time. These features may resolve naturally or with treatments and remain absent for many months. Conversely, patients may experience episodic flare-ups of asthmatic symptoms that may pose a life-threatening risk. Under the microscope the stained tissue sections of lungs obtained from asthmatic patients show histo-pathological lesions such as disrupted epithelial cells, hyper plastic goblet cells, increased smooth muscle mass, increased fibrotic tissues, collagen deposition and metaplastic epithelial cells (23, 56, 140, 275, 292, 314).

1.2 Prevalence and economic importance

The prevalence of asthma is increasing every year and currently it is estimated that ~300 million people of all ages are affected world-wide (43). It is predicted that in 2025, an additional 100 million people will be affected by asthma (World Health Organization, 2007). In USA alone, the prevalence of asthma is increasing since 1980 and it is estimated that nearly 30 million people are

affected currently (U.S. Department of Health and Human Services Centers for Disease Control and Prevention). In 2010, 1 in 12 adults and 1 in 11 children were affected by asthma. According to a 2014 CDC report, the total cost of asthma (medical expenditures, untimely deaths, loss of productivity due to missed work days) in the US is $56 billion each year. In 2008 asthma caused 10.5 million missed days of school and 14.2 million missed days of work and in 2009 it accounted for 479,300 hospitalizations, 1.9 million emergency department visits and 8.9 million doctor visits. In the last decade there has been a ~15% increase in the number of people with asthma. Overall, health and economic burdens of asthma pose a serious concern in the US and the rest of the world.

1.3 Etiology

Both environmental factors and genetic elements influence the onset of asthma (75). Children are more prone to this disorder than adults (141) and it is the number one chronic condition responsible for school absenteeism of children (CDC). Male-children and female-adults are more susceptible to asthma (141). Other than gender and age, other genetic factors known to be involved in triggering asthma are obesity (40, 92, 115, 135, 214, 307), stress (27, 278, 341) , exercise (27) and pregnancy (61, 102, 104, 241, 339, 364).

Environmental factors

Environmental factors that induce asthmatic symptoms are allergens such as various pollens, animal hair and dander, cockroaches, chemicals, fumes and microbes. Asthma caused by allergens is the most common form in the US and it is estimated about 50% of asthmatic cases are due to allergic asthma (248).

Epigenetic modulations that influence the transcriptional activity of asthma-susceptible genes are histone modifications, DNA methylations and microRNA activities (295). Epigenetic modulations influence the expression of microRNAs such as let-7a, miR-9, miR-34a, miR-124, miR-137, miR-148 and miR-203 (296). On the other hand microRNAs can affect the expression of epigenetic modulators such as histone deacetylase, histone acetyl transferase and DNA methyl transferase by interfering with their gene expression (295, 296).

Genetic factors - Asthma susceptible genes

Asthma is not linked to only one gene but several genes that interact with each other and cause asthma pathogenesis (227). Thus far, more than 100 genes have been identified as asthma-related (315), though not all 100 genes may co-act to exhibit the asthma phenotype in an individual. While interplay of a group of genes may be involved in eliciting asthma symptoms in one individual, interaction of another group of genes may be responsible for inducing the asthma phenotype in another individual. Since asthma is associated with both genetic and environmental factors, carrying an asthma susceptible gene by itself may not result in inducing asthmatic symptoms. Exposure to and interaction with specific environmental triggers is also needed for an individual to demonstrate clinical signs. Candidate gene studies using animal models, positional cloning using linkage analyses, and genome wide associated studies (GWAS) have led to the identification of numerous asthma susceptibility genes. Candidate gene studies have identified many genes that are involved in the development of diverse biological effects such as T-helper cell-2 inflammation, regulatory T- cell function,

HLA locus/immunity and IgE response of B cells (184). Using linkage studies new genes have been recognized (227) . They are A disintegrin and metalloproteinase domain-containing protein 33 (ADAM33) (337) , DPP10 (8), PHF11(372), HLA-G(253), and GPRA (199) with a role in atopy, airway hyperresponsiveness (AHR) and elevated IgE levels. GWAS studies identified genes including IL1RL1(132), TSLP(35), IL33, HLA-DP (254), ORMDL3 (243), and Childhood Management Program cohort found genes including PDE4D, IRAK-3, PHF11, IL10, ITGB3 (287), IL13 (159) , and IL4R (159). These genes appear to be involved in irregular inflammatory/immunologic responses. Some of these genes have been already studied and have been shown to be involved in the pathogenic pathways of asthma (212). For example, the gene ADAM33 is associated with bronchial hyperresponsiveness (212). The ORLMD3 gene product is regulating ER-mediated Ca^{2+} signaling and cellular stress (55) as well as sphingolipid homeostasis (47) and is associated with the stress-related signaling molecule c-Jun N-terminal kinase (JNK) (336). Chemokines CCL11 and CCL5 are also identified as asthma susceptible genes (342). From these findings, it is clear that asthma is a complex disease involving many mechanisms and pathways.

2. Asthma phenotypes

Multiple etiological origins and differences in the pathogenesis of the disease cause heterogeneity in asthma phenotype and, there is no strong correlation between pathological features and clinical symptoms or treatment response. Many asthma phenotypes identified in humans are clustered according to

etiology, clinical severity, steroid treatment response, appearance of histo-pathological lesions and onset of asthma in life (42, 333) as shown in **Table 1**. Based on etiology, patients are grouped into allergic asthma, non-allergic asthma (viral infection, occupational asthma) and intrinsic asthma (exercise-induced, stress-induced asthma). Allergic asthma often starts in childhood and responds well to inhaled-gluco-corticosteroids (ICS). Induced sputum of these patients will have readily seen eosinophils. Most of the time, non-allergic patients do not respond well to ICS treatment and their induced sputum may have neutrophils or eosinophils or very few inflammatory cells. When clinical severity (frequency of asthma attacks, FEV1 and other asthma symptoms) is considered, patients are grouped into mild, moderate and severe. According to histo-pathological lesions, there are two sets of asthmatic patients, those who have eosinophilic asthma and those with neutrophilic asthma (346). Physicians sometimes categorize asthmatic patients , into steroid responders and steroid non-responders (229), and patients with childhood onset and adult onset asthma (346). Mostly, severe asthmatic patients need higher doses of ICS or systemic administration of steroids to control the symptoms of asthma or they do not respond to steroids at all. Patients with adult onset asthma, especially women, often show refractory asthma, a form of severe asthma that does not respond to treatments. Existence of a variety of phenotypes indicates that asthma therapy needs to be tailored to the phenotype and the pathogenic mechanism causing asthma.

Table 1: Asthma phenotypes

Etiology	Allergic asthma, non-allergic asthma, intrinsic asthma
Clinical severity	Mild, moderate, severe
Histo-pathological lesions	Eosinophilia, neutrophilia
Response to corticosteroid therapy	Steroid responders and steroid non-responders
Onset	Childhood onset and adult onset

3. Asthma diagnosis

Diagnosing asthma before starting any treatment is essential since many other disease conditions also present with apparently similar clinical signs. The steps involved in making a diagnosis of asthma are as follows:

a. History of clinical symptoms

Whether the clinical symptoms worsen at night, vary over time and in intensity, are increased by exposure to allergens/irritants, climate change, exercise or strong smoke (tobacco and cigarette smoke, indoor household solid fuels).

b. Physical examination

During the physical examination, expiratory wheezing on auscultation may be present. If the asthma is severe, it may not be possible to hear any wheeze due to severely limited airflow caused by increased obstruction of airways. While expiratory wheezing may be present in other respiratory diseases, inspiratory wheezing is not a feature of asthma.

c. Spirometry - Lung/Pulmonary function tests (PFT)

PFT provide information about the level of lung function impairment and serves as a tool to evaluate the level of improvement after treatment, i.e. treatment response. The values from PFT of asthmatic subjects are compared with values seen in normal healthy individuals of similar age, height, gender and race as well as with results of previous PFT. These tests help in the identification of diseases such as asthma, chronic obstructive pulmonary disease (COPD) and pulmonary fibrosis.

I. Peak Expiratory Flow (PEF).

This measures the amount of air one can exhale to the best of their capability. It is unreliable because measurements may differ from instrument to instrument by 20% resulting in false positive or false negative results.

ii. Forced Vital Capacity (FVC)

Here, one inhales as deeply as possible and then exhales as forcefully as possible. Measurement of the amount of exhaled air is called FVC. iii. Forced Expiratory Volume in 1 second (FEV_1)

This measures the maximum amount of air one can forcefully exhale in one second after full inspiration. An FEV_1 that is less or more than 12% of baseline (before the use of broncho dilator) and less or more than 200 ml from baseline (before the use of broncho dilator) after the administration of a bronchodilator is considered as improved lung function. Since reduced FEV_1 may be present in other disease conditions, a ratio of FEV_1/FVC yields a more reliable indication of

airflow limitation. A ratio that is lower than 0.75-0.8 is considered as being indicative of asthma. This is more trustworthy than peak expiratory flow.

iii. Provocation Test - Methacholine Challenge Test

This test is performed when clinical symptoms and spirometry results do not clearly establish a diagnosis of asthma. Methacholine is a spasmogen which causes asthmatic airways to contract involuntarily and constrict rapidly at concentrations much lower than in a healthy subject. This test enables detection of how responsive or irritable the airways are to methacholine. Spirometry is done before and after each dose of the methacholine challenge. A 20% reduction in FEV_1 relative to baseline is considered as a positive indication of asthma and the methacholine concentration that causes a positive reaction is referred to as PC_{20}. To distinguish asthmatic patients from healthy subjects, usually 8 to 16 mg/mL methacholine is used as an optimal cut off range. Inhaled histamine or exposure to cold air can also be used in this test instead of methacholine.

4. Asthma in animals

Dogs (330), cats (76) and horses(149) can develop asthma-like symptoms as in humans. However, cats and horses are more prone to develop these symptoms compared to dogs. The disorder is referred to as allergic bronchitis in dogs, feline asthma or allergic bronchitis in cats and recurrent airway obstruction or heaves in horses (149) (Merck Veterinary Manual). In these animals, the symptoms can be triggered by pollen, hay, straw, cigarette smoke, wood burning stoves, fire places, carpet or floor cleaners, deodorizers, air fresheners, dust, dry air, exercise, viruses, cold temperatures, rapid breathing due to exercise,

chemicals in the air, etc. Animals show symptoms such as difficulty in breathing, shortness of breath, wheezing, open-mouth breathing, cough (dry, hacking), pale mucous membranes (bluish gums and tongue), lethargy, exercise intolerance, lack of appetite and weight loss (Merck Veterinary Manual). Animals are treated with glucocorticoids, antihistamines and bronchodilators to relieve these symptoms (119) (Merck Veterinary Manual).

5. Animal asthma models

For asthma-related laboratory studies, mice are widely used as an animal model due to the following reasons: they are closely related to humans with respect to genome sequences, easier to handle, easier for genetic manipulation, comparatively less expensive, fast breeding due to a shorter gestation period and readily available reagents and tools for analysis. Further, mice are known to exhibit an asthma phenotype when exposed to pro-inflammatory cytokines (54, 188, 373).

Guinea pigs are also used in asthma studies. Advantage of using them is, they show immediate and late phase airway responsiveness, but the disadvantage is, their major anaphylactic antibody is IgG. While rabbits show immediate and late phase airway responses and advantageously produce IgE as their primary anaphylactic antibody but neonatal immunization is needed for late phase airway responses (302). Other animal models for asthma such as sheep, pigs and horses (7, 50, 156) also have been used. However, they are not very popular due to the lack of availability of appropriate tools and reagents for

analysis as well as cost ineffectiveness of conducting experiments using these models (302).

6. Functions of Airway Smooth Muscle (ASM) cells

ASM cells are important structural cells in the airways. They are known to maintain bronchial tone and mediate bronchoconstriction in response to foreign bodies and stimuli. ASM cells were recently recognized to have a role in inflammation. They secrete pro-inflammatory mediators such as cytokines, chemokines, growth factors, ECM proteins, etc., express receptors for these molecules and act as immuno-modulatory cells (358) that can function in an autocrine or paracrine manner (80). The secreted inflammatory mediators promote AHR during acute inflammation (53) and induce proliferation of ASM cells and altering the structure of the airway wall during chronic inflammation (185).

Structural changes in the airways are a significant component of the pathogenesis of asthma. The increased ASM mass in the airway wall is thought to be due to ASM cell proliferation, ASM cell hypertrophy, prolong survival of ASM cells, transformation and differentiation of fibroblasts into myofibroblasts and then to ASM cells. In addition, increased airway ASM mass could be caused by migration of vascular smooth muscle cells to airways, differentiation of stem cells in the peripheral blood or the airways into ASM cells, migration and transformation of epithelial cells into ASM cells via the process of epithelial mesenchymal transition. Since ASM cells participate in the development of all three cardinal features of asthma namely, AHR, inflammation and remodeling of

airway wall, they are considered to be a distinct and attractive therapeutic target for asthma.

7. Airway inflammation

7.1 Mediators and cells involved in pathogenesis

Asthma triggering agents induce and maintain chronic inflammation through a variety of mediators like histamine, immunoglobulin E (IgE), prostaglandins and leukotrienes (26). **Table 2** lists the different types of mediators and their functions in detail. This table is adapted from a review article published in 1998 by The American

Society for Pharmacology and Experimental Therapeutics (26). The cells involved in producing these mediators are epithelial cells, dendritic cells, T-cells, B-cells, mast cells, macrophages, eosinophils and ASM cells (28, 247). Epithelial cells produce Thymic Stromal Lymphopoietin, when they encounter allergens and induce dendritic cells to process the allergens and present them to T-lymphocytes (72). Once T-cells are activated by the antigen-priming, they produce interleukin IL-13 and IL-4 to induce B-cells and IL-5 to induce eosinophils (71, 143). Induced B-cells produce antigen specific immunoglobulin E (IgE) which binds to the surface of mast cells. Cross linking of IgEs by the allergen activates mast cells to de-granulate and various mediators such as histamines and prostaglandins and *de nova* synthesis and release of leukotrienes resulting in recruiting more inflammatory cells to the site (71). The above mechanism is depicted in **Figures 1 and 2**. IgE can also bind to macrophages and induce them to release inflammatory mediators (71).

Figure i: **Common mechanism for allergic asthma**

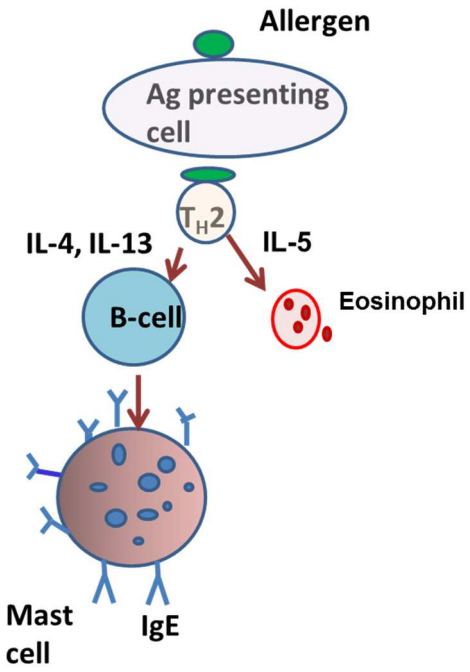
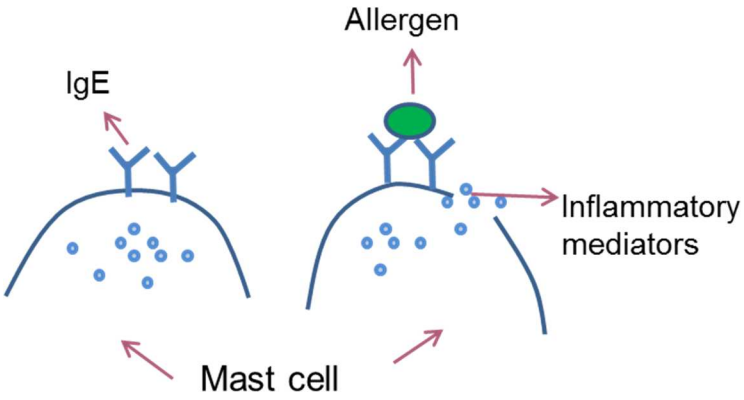


Figure ii: **Cross-linking of allergens with IgE**



7.2 TH2 biased inflammation and hygiene hypothesis

Changes in the environment along with epigenetic factors are thought to be the major causes for the recent enhancement in prevalence of asthma (144).

These factors influence the severity of the disease and responsiveness to therapy (247). The main hypotheses regarding increased prevalence of asthma are the hygiene hypothesis and the hypothesis based on TH1–TH2 imbalance (111, 332) as a result of increased urbanization. The “hygiene hypothesis” is a widely accepted hypothesis to explain the prevailing increased frequency of allergic asthma. It suggests that increased susceptibility to allergic diseases in urban children is due to less exposure to symbiotic microbes resulting in the suppression of natural development of the immune system relative to children living in a farm environment (130, 257). Diminished exposure to certain organisms such as Hepatitis A virus (235) Fungi (Eurotium and Penicillium) (1) and *Mycobacterium* (69, 303) in early life results in a less robust and defective immune system which in turn leads to many chronic inflammatory disorders including asthma. For thousands of years our ancestors have been exposed to helminths and harmless environmental saprophytes that live in decomposing matter. Continuous exposure of the gut to helminths and saprophytes enables regulatory dendritic cells to continuously sample the gut contents, allergens and self (290), thus keeping the immune system in check. A subsequent detection of a “known” pathogen of a specific pattern by Toll like receptors results in the recruitment of specific T-reg cells to act on the allergen and suppress the inflammation (333, 365). This process is called immune tolerance. Because of the recent Westernization associated with hygienic measures, these exposures have been minimized to a large extent in current conditions and changed the gut/airwaymicrobiome. The lack of early exposure to these beneficial organisms

leads to the defective development and regulation of the immune system, intolerance to allergens and endotoxins, and increased susceptibility to allergic inflammation (289).

The hypothesis about TH1 and TH2 imbalance states that people who lack induction of the TH1 pathway by viral or bacterial infections early in their life are prone to having an activated TH2 pathway later in their life (117, 176). Hepatitis A virus (HAV) infection, which occurs through orofecal route, has a receptor on human lymphocytes called ‘T cell immunoglobulin domain, mucin-like domain-1’ (TIM-1). This receptor is involved in regulating a subset of T lymphocytes. The binding of HAV to TIM-1 receptor on lymphocytes might suppress the TH2 response and enhance the T-reg response which is anti-inflammatory (235, 335).

Table ii. Inflammatory mediators and functions

Mediators	**names**	**Function**
Amine mediators		
	Histamine	Bronchoconstriction, airway secretion, plasma exudation, neural effects, chemotaxis
	Serotonin	Plasma exudation, neural effects
	Adenosine	Neural effects
Lipid-derived mediators		
Prostanoids	PGD_2 and $PGF_{2\alpha}$	Bronchoconstriction, airway secretion, neural effects
	PGE_2	Airway secretion, neural effects
Thromboxane (Tx)		Bronchoconstriction, plasma exudation, neural effects, AHR
Leukotrienes	LTB4	Chemotaxis
	LTC_4, LTD_4& LTE_4	Bronchoconstriction, airway secretion, plasma exudation, chemotaxis
Peptide mediators	Bradykinin	Plasma exudation, bronchoconstriction, airway secretion
	Tachykinins-Substance P	Neural effects, bronchoconstriction, airway secretion, plasma exudation
	Tachykinins-NKA	Bronchoconstriction, airway secretion, plasma exudation
	Endothelins	Bronchoconstriction, airway secretion, plasma exudation, neural effects
	Complement	Chemotaxis, bronchoconstriction, airway secretion, plasma exudation
Small molecules	Reactive oxygen species	Airway secretion, plasma exudation
	Nitric oxide	Airway secretion, neural effects,

		chemotaxis
Cytokines	Lymphokines - IL-2, IL-3, IL-4, IL-5, IL-IL-13, IL-15, IL-16, and IL-17	Eosinophil growth, maturation, activation and migration; T lymphocyte, growth, differentiation, proliferation and activation; activation of other cells like macrophages, B cells, neutrophils, epithelial cells, endothelial cells, fibroblasts.
	Pro-inflammatory cytokines-IL-1, TNF, IL-6, IL-11, GM-CSF, and SCF	Adhesion to vascular endothelium, growth factor for B cells, Th2 cells, neutrophil chemoattractant, T cell and epithelial cell activation, activation of fibroblasts, proliferation and maturation of hematopoietic cells, endothelial cell migration, growth factor for mast cells
	Inhibitory cytokines -IL-10, IL-1Rα, IFN-γ, IL-12, and 1L-18	Inhibits eosinophil survival, inhibits eosinophil influx after allergen, decrease the proliferation of TH1 and TH2 cells, decrease the activation of macrophages, increase the growth of B cells, natural killer cells and mast cells,
Growth factors	PDGF, TGF-β, FGF, EGF, and IGF	Fibroblast and ASM proliferation, release of collagen, fibroblast proliferation, chemoattractant for monocytes, fibroblasts, and mast cells,
Chemokines		Attract leukocytes into tissues
Proteases	Mast cell tryptase, mast cell chymase and matrix metalloproteinases	Tryptase increase the sensitiveness of airways to histamines, proliferation of ASM cells; chymase activates TGF-β and induces fibroblast proliferation

However, not all viral infections, i.e., measles, mumps, chickenpox and herpes viral infections, would provide protection against chronic inflammation by calming the TH2 responses (31, 232, 251).

7.3 Storage of inflammatory mediators

Many inflammatory mediators, cytokines and chemokines, are stored within intracellular compartments such as secretory granules, Weibel-Palade (WPB) body and vesicles in inflammatory cells and airway structural cells. These stored mediators can be differentially released depending on the pathways involved such as degranulation (piecemeal degranulation, anaphylactic degranulation), exocytosis, and secretion.

In endothelial cells, WPB stores eotaxin-3 and IL-8 whereas small granules other than WPB, store CXCL1 and CCL2 (81). Their exocytosis may be regulated by elevated levels of intracellular Ca^{++}, cAMP or phorbol esters (58). It has been found that these small granules are distinctly regulated by Protein Kinase A and Protein Kinase C compared to WPB in endothelial cells (58, 260).

Mast cells release inflammatory mediators and other substances through constitutive exocytosis and regulated exocytosis (99). Constitutive exocytosis happens throughout the lifetime of a cell without a stimulus. Regulated exocytosis occurs with a clear stimulus like pH, osmolarity changes, binding of ligands to receptors etc. Many secretory pathways are involved in differentially releasing the substances to various stimuli. Degranulation involves in readily releasing the preformed mediators that are stored in granules, to a stimulus. In

secretion, substances are moved from one containment to another through an active process. Exocytosis occurs when vesicles trafficking to the plasma membrane, fuse with it and release to the environment. In mast cells, two types of degranulation have been identified. One is piecemeal degranulation (PMD) and the other one is anaphylactic degranulation (AND) (98, 99). PMD involves selective release of substances from granules without fusion with plasma membrane or with other granules. In contrast, AND involves granule to granule and granule to plasma membrane fusion and a burst of release of granule contents mediated by soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) (245). Calcium flux is important for mast cell degranulation (107, 245). Several molecules are involved in mast cell degranulation such as Rac1, Rac2, Rab27b (305), Rab3d (285), Cdc42 (157), MARCKS (139), etc.

Many signaling pathways are known to participate in the expression and release of inflammatory mediators. **Table 3** shows various pathways involved in chemokine expression and release in different cell types.

Table iii. Pathways involved in chemokine expression and release

Inducer	**Pathway**	**Chemokine expression**	**Chemokine release**	**Reference**	**Cells**
IL-13	MAPK and STAT-6		CCL11	Peng et al, 2004	HASM
TSLP	MAPK and STAT-3	CCL11, CXCL8	CCL11, CXCL8	Shan *et al*, 2010	HASM
IL-1β	MAPK, NF-κB	CCL11, CCL2 and CCL7	CCL11, CCL2 and CCL7	Wuyts *et al*, 2003	HASM
TNF-α	JNK MAPK	CXCL1		Lo *et al*, 2014	Vascular

					endothelial cells
TNF-α	p38 MAPK and PI3K		CXCL1	Lo *et al*, 2014	Vascular endothelial cells
IL-17A	MAPK	CCL11	CCL11	Rahman *et al*, 2006	HASM
IL-17 + IL-1β	MAPK and PI3K	CXCL8	CXCL8	Dragon *et al*, 2007	HASM
IL-31	MAPK	CCL2	CCL2	Wai *et al*, 2007	Bronchial epithelial cells

7.4 Extracellular matrix

The ECM not only supports structural and immune cells (198) in the lung but actively participates in controlling ASM proliferation (152, 252, 313), ASM contractility (86, 153) and secretion of inflammatory mediators (60). Through changing the phenotypes of ASM cells, the ECM alters the functions of airway wall.

During asthma, ECM components such as collagens, proteoglycan, hyaluronan, tenascin-C, versican, elastin, fibronectin, and MMP-1 are aberrantly expressed (18, 274). Severe asthmatic patients with persistent asthma and permanent structural changes show increased deposition of fibronectin and collagen in the sub-epithelial matrix (153). Fibrillar forms of collagen present in the ECM bind to ASM cell integrins and influence the rigidity, distensibility and the structural stability of ASM cells (36). Consequential mechanical tension in the cell wall induces ASM cell differentiation. Furthermore, elastin, MMPs-1, 9 and -12 are highly expressed between the ASM bundles in asthmatic patients (19,

288). Altered concentration of the components of ECM in the lungs promotes ASM cell proliferation and migration.

8. Airway hyperresponsiveness (AHR)

Excessive ASM contraction occurs in asthmatics (223) due to increased actin–myosin coupling (261). Increased concentration of $[Ca^{++}]_i$ induces contraction of ASM cells through myosin light chain by two pathways. One is through calmodulin, which binds to Ca^{++} and activates the myosin light chain kinase (MYLK) by phosphorylating it. MYLK in turn activates myosin light chain (109, 370). The other pathway is through the small GTPase, RhoA. Increased $[Ca^{++}]_i$ induces loading of GTP onto RhoA, which in turn induces Rho associated coiled-coil–containing protein kinase (ROCK) to directly phosphorylate myosin light chain phosphatase and inactivate it (163). This causes increased phosphorylation of myosin light chain.

$[Ca^{++}]_i$ can be increased by acetylcholine released from parasympathetic nerves that innervate ASM (150). Acetylcholine binds to G-protein coupled M2 muscarinic receptors, which recruit Gq subunit intracellularly (150). This activates phospholipase C (PLC) which phosphorylates Phosphatidylinositol 4, 5-bisphosphate (PIP2) to produce Inositol trisphosphate (IP3) (105). IP3 is a potent second messenger for Ca^{++} released from the sarcoplasmic reticulum to cytoplasm.

Increased extracellular concentration of potassium ions causes opening of voltage-gated calcium channels in the plasma membrane and thus increases $[Ca^{++}]_I$ (105). During asthma, TH2 and TH1 cytokines at increased levels, bind to

G-protein coupled receptors (GPCR) and induce various signaling pathways to increase $[Ca^{++}]_i$(334).

Further, integrins are important transmembrane proteins that link the intracellular actin-myosin cytoskeleton to the ECM (136). Smooth muscle contraction due to the interaction between actin and myosin that is mediated by integrins and the ECM is translated into forceful narrowing of the airway wall. This is dependent on the strength of binding of ASM cells to the underlying ECM. Narrowing of the airway wall can occur with changes in the ECM even in the absence of changes in actin-myosin interactions (239, 369).

Increased airway wall thickness due to ASM cell proliferation can also cause increased airway contractility (13, 200, 353).

9. Airway remodeling

9.1 Pathological features

Airway remodeling in asthma has been recognized for over a hundred years. However many aspects of this pathological feature such as etiology, molecular/cellular mechanisms and relevance to physiology and clinical outcome are still not well understood. Among asthmatic patients, only 5-10% of them are severely affected (326). However, they are responsible for a large portion (>50%) of asthma-related costs (300). Most of the severely affected asthma patients have substantial remodeling of the airways. Currently available asthma therapies do not control airway remodeling, and this drives the need for continued research in this area to develop drugs that can resolve remodeled airways.

Characteristic features of airway remodeling include increased ASM mass, sub-epithelial fibrosis, goblet cell hyperplasia, sub-mucosal mucus gland hypertrophy, desquamation of epithelium, formation of new blood vessels and increase ECM deposition (63, 74, 259). Narrowing of the airway lumen and obstruction in airflow are a consequence of increased ASM mass through generation of greater force when airways contract. Meanwhile, persistent airway obstruction is also caused by hyperplasia of sub-epithelial fibroblasts (41, 242).

9.2 Role of ASM cells in remodeling

Changes in smooth muscle properties from contractile to proliferative due to several environmental stress are fundamental to the increase in ASM mass. Many studies have shown that asthmatic subjects exhibit increased ASM mass which is one of the important structural changes observed in airway remodeling (56). This could be due to smooth muscle hyperplasia, hypertrophy, decreased apoptosis, migration of smooth muscle cells from other places (vascular smooth muscle, stem cells) and differentiation of fibroblasts into smooth muscle cells. While TH2-derived chronic inflammation was thought to be the cause of airway remodeling, increasing evidence questions this belief. For instance, the augmented ASM mass observed in some children with severe asthma has not been associated with inflammation (38)

Human ASM cells derived from asthmatic or healthy subjects have been shown to proliferate in response to a wide array of mitogenic factors **(Table 4)**.

Table iv. Mitogenic factors for ASM cell proliferation

Mitogenic factor	Examples
Growth factors	Platelet derived growth factor (PDGF), epidermal growth factor (EGF)
Cytokines	TNF-α, TGF- β
Chemokines	CCL3, CCL5, CCL11
Inflammatory mediators	Histamine, endothelin, thromboxane A2, sphingosine 1-phosphate
Enzymes	Tryptase, thrombin, elastase, MMP
ECM components	Fibronectin, Collagen 1
Others	Reactive oxygen species, mechanical stretch

Mitogenic factors induce ASM proliferation by activating signaling pathways through the receptors such as receptor tyrosine kinase (RTK) or G-protein coupled receptors (GPCR). Two distinct downstream signaling pathways of these receptors, namely phosphoinositide 3-kinase (PI3K) and extracellular signal-regulated kinase (ERK), have been identified as major pathways that independently induce ASM cell proliferation (52, 195). Both pathways control the expression/activation of cyclin D to exert their proliferative function (263, 265). PI3K targets the downstream small GTPase Rac1 which activates the cyclin D1 promoter through the cAMP response element binding protein (CREB). This results in activation of the transcription factor (ATF)-2 binding site. Rac1 is a part

of the NADPH oxidase complex which produces H_2O_2 in response to mitogens. Cells treated with mitogens and antioxidants showed attenuated expression of cyclin D1 and H_2O_2, indicating that mitogens induce ASM cell proliferation through the PI3-kinase/Rac1/NADPH oxidase pathway (46, 264). It has been found that human ASM cells derived from asthmatic patients (AS-HASM) are more proliferative than cells derived from non-asthmatic healthy subjects (NA-HASM) (175). Furthermore, studies have shown that AS-HASM cell proliferation is regulated by the PI3K pathway and NA-HASM cell proliferation is controlled by the ERK MAPK pathway (52). This intrinsic defect in AS-HASM cells is thought to be due to decreased expression of CCAAT-enhancer-binding proteins (C/EBPα) (280) or changes in $[Ca^{++}]_i$ homeostasis (327). C/EBPα decreases the expression of cell-cycle inhibitor p21 which in turn increases ASM cell proliferation. Increased biogenesis of mitochondria leading to ASM cell proliferation was noted when there was an abnormal influx of extra cellular Ca^{++} along with activation of peroxisome proliferator-activated receptor γ-coactivator-1α (PGC-1α), nuclear respiratory factor-1 (NRF-1), and mitochondrial transcription factor A (mtTFA) (327).

TGF-β is increasingly expressed in the airways of severely affected asthmatic patients relative to healthy subjects (284). It induces fibroblasts to produce ECM and ASM to increase their cellular size(284). On the other hand, it was noted that TGF-β either increases or inhibits ASM cell proliferation (110).

10. Asthma therapy

10.1 Current treatment for asthma

While there is no current cure for asthma, it can be successfully controlled by avoiding exposure to asthma triggers, proper monitoring of asthma symptoms and preventive check-ups. Nasal nebulizers containing broncho-dilators such as short acting β_2-adrenergic receptor agonists or anti-cholinergics along with inhaled glucocorticoids are used to control exacerbations (58). Short acting β_2-adrenergic receptor agonists are given to relax the ASM, dilate airway walls, and increase air flow. This can be achieved within five minutes and lasts up to 3-6 hours. Use of β_2-adrenergic receptor agonists, alone in patients with severe and persistent asthma, leads to exacerbation of asthmatic symptoms with increased mortality (258). In addition, patients using this drug for extended periods of time may develop tolerance due to down regulation of β_2-adrenergic receptors. Simultaneous use of β_2-adrenergic receptor agonists and glucocorticoids has a beneficial effect as it increases the number of β_2-adrenergic receptors by inducing receptor gene expression. Glucocorticoids bind directly to the glucocorticoid response element (GRE) in the promoter region of β_2-adrenergic receptor gene and transcriptionally enhance gene expression (174). During asthma, the activity of histone deacetylase (HDAC) decreases while that of histone acetyl transferase (HAT) increases. Generally, this causes increased expression of pro-inflammatory genes. An added benefit of corticosteroid treatment is that it inhibits HAT by recruiting HDAC2 to the activated pro-

inflammatory gene complex and represses pro-inflammatory gene expression (25).

For long-term treatment, patients are prescribed inhaled long-acting β2-adrenergic receptor agonists (LABAs) with inhaled glucocorticoids and in severe asthmatic conditions, systemic glucocorticoids are used. Prolonged and frequent use of systemic glucocorticoids causes side effects such as suppression of hypothalamic-pituitary axis, reduced growth rate in children, obesity and reduced bone density (218). As alternative controller therapy, there are many other drugs available such as leukotriene modifiers, anti IgE therapy, theophylline or mast cell stabilizers (362). Leukotriene modifiers either antagonize leukotriene receptors or inhibit activation of the 5-lipoxygenase pathway thus decreasing the availability of bronchocontrictive peptidoleukotrienes (189, 213, 349). All of these alternate controller treatments are relatively less effective than glucocorticoids and not all patients respond to them. Non-medicinal treatments such as yoga, homeopathy, ayurveda, and acupuncture are also sometimes employed to control symptoms (57-59). The following table lists the various groups of available drugs, their names and function.

Table v: **Current treatment options for asthma**

Group	Sub- Group	Drug Examples	Action
Broncho-dilators	Short-acting β2-agonists, Long-acting β2-agonists, Anti-cholinergics	Salbutamol Formoterol, Salmeterol Tiotropium (Spiriva) and ipratropium bromide.	Relaxing smooth muscle cells
Glucocorticoids	Inhaled glucocorticoids	Prednisone	Transactivation of anti-inflammatory proteins by binding to GRE and Transrepression of pro-inflammatory proteins by blocking translocation of transcription factors to nucleus and recruiting HDAC2.
Leukotriene modifiers		Montelukast and Zafirlukast	Antagonism of cysteinyl-leukotriene type 1 receptors
Leukotriene modifiers		Pranlukast and Zileuton	Inhibition of the 5-lipoxygenase

			pathway of leukotriene metabolism
Mast cell stabilizers		Cromolyn	Inhibits degranulation
Monoclonal anti-IgE antibody		Omalizumab	Inhibits IgE binding to mast cells
PDE4 inhibitors		Rolipram/ Methylxanthines	Inhibits enzyme PDE and increase the concentration of cAMP
Monoclonal antibody to block receptor-IL4Rα		Dupilumab	Simultaneously blocking IL4 and IL13
Antibody against TSLP	In clinical trials	AMG 157	Blocks development of Th2-inflammation.
Serotonin antagonist	In clinical trials	5-HT (5-hydroxy tryptamine)	Reduces inflammation

Major limitations of current therapies for asthma are multiple etiologies and incomplete understanding of the underlying mechanisms of asthma development. In general, glucocorticoids are effective as an asthma symptom controller in about 90% of asthmatic patients. However, there is a certain

percentage of patients (5-10%) refractory to glucocorticoid therapy (256). Another disadvantage of steroid therapy is unwanted side effects as they are broad spectrum drugs. Severe asthmatic patients who use glucocorticoid inhalers for extended periods of time are known to develop systemic side effects such as decreased bone density, obesity, depression, retardation in growth and hormonal imbalances. Long time users of inhalers are also known to develop oral thrush (147). Using spacers may reduce this *Candida* infection since spacers reduce the deposition of drugs on oral surfaces.

10.2 Studies aimed at developing treatment options

Despite effective control of symptoms in a majority of asthmatic patients by corticosteroid therapy, in the small portion of severely affected patients that are refractory (256), even high oral doses of steroids or other maintenance therapies such as leukotriene receptor antagonists are ineffective. In recent years, many potential therapeutic targets have been evaluated and progressed to human clinical trials in order to develop new treatment strategies for severe asthmatics who are refractory to current therapies. New treatment approaches are aimed at reducing airway inflammatory cells (eosinophils and neutrophils) and ASM mass, optimizing airway caliber and blocking specific effector mechanisms (256).

Patients with refractory asthma have multiple phenotypes caused by various pathogenic origins that may be responsible for different mechanisms leading to the development and persistence of airway inflammation and

remodeling. Thus, targeting any single effector molecule or effector cell may control asthma symptoms in some patients, but may not be effective in others.

10.2.1 Enhancing bronchodilation

Studies have found that adding a long–acting muscarinic cholinergic receptor antagonist (LAMA), such as tiotropium or aclidinium bromide to the standard regimen of corticosteroids and long-actingβ_2-adrenergic receptor agonists (LABA), has improved lung function in severe asthmatic patients (125). Another study showed LAMA works better than LABA with corticosteroids in severe refractory asthma (256), although other studies have reported that tiotropium causes side effects such as difficulty in urination and retention (125).

10.2.2 Reducing ASM mass: Bronchial Thermoplasty

Drugs that are currently available for asthma are able to control airway inflammation and AHR. However, there are no medications available to reduce remodeling of structural tissues such as the increased ASM mass seen in severe asthma. Due to the presence of increased amount of smooth muscle mass and excessive contraction of airway smooth muscle cells in asthma, attempts have been made to reduce the muscle mass by a method called bronchial thermoplasty. It is a new, non-drug, minimally invasive procedure developed to treat patients with severe persistent asthma. Bronchial thermoplasty is performed by delivering a specific amount of radiofrequency thermal energy to the airway wall. A flexible bronchoscope is inserted through the mouth or nose and gently pushed to reachable airways (~3-10 mm) of the patient's lungs. A catheter system is extended through the tip of the bronchoscope to make contact

with the airway wall and expose it to thermal energy of ~ 65°C. Radiofrequency ablation has been used to treat cardiac arrhythmias and lung cancer as well. This technology was first tested in animal models before use in humans. In animal studies, it was found that bronchial thermoplasty destroys the actin-myosin interaction, disrupting the ASM contractile process (100).

This is performed in patients who are 18 years of age or older and have severe asthma that is not controlled by inhaled corticosteroids and long-acting β_2-adrenergic receptor agonists. The procedure is performed over three separate visits and has been found to be effective for up to five years (348).

This approach showed improvement in lung function (57, 170). But the disadvantage of this approach is that it can be done only in the larger airways and it could result in unwanted effects like collapse of lungs.

10.2.3. Decreasing airway inflammatory cells

In order to suppress eosinophilia in severe refractory asthma, specific antibodies and antisense RNAs have been employed. A monoclonal antibody against IL-5 (hMAb, mepolizumab) (345), antisense RNA against the common beta chain of cytokine receptors that regulates IL-5, IL-3 and GM-CSF(6) (needed for the eosinophil maturation and migration) and antisense RNA against the CCR3 receptor that regulates eosinophil recruitment mediated by the chemokine eotaxin have all been tested and shown to help in controlling asthmatic symptoms (123).

For suppression of neutrophilia, an antagonist of CXCR2, a receptor that mediates the effects of the chemokine IL-8 and an antagonist of chemoattractant

receptor-homologous molecule expressed on Th-2 cells (CRTh2) which gets activated by prostaglandin D2 have been evaluated. Blockade of these receptors was found to enhance lung function and reduce asthmatic symptoms (164).

10.2.4 Obstructing airway inflammatory effector mediators

Many studies have been carried out to prevent the binding of cytokines IL-4 and IL-13 to the alpha chain of their common receptor using antibodies. These cytokines are necessary for IgE production. Mixed results were obtained in different studies, with some reporting improvement in symptoms and others not. The existence of multiple mechanisms of asthma pathogenesis may explain the variation in the outcome of these studies (16).

Treatment with hMAb and antisense RNA against the mediator IL-5 effectively controlled asthma exacerbation (123, 345). Blocking of IgE binding to its receptor (Fc€R1) with MAb (omalizumab) also proved to be an effective therapy for refractory asthma (291, 299, 331). Recently antibodies against TSLP (124) and agonists at serotonin receptors (5HT2) (250) have also been shown to be beneficial in controlling asthma symptoms.

10.2.5 Immunotherapy

Immunotherapy is a new approach in which specific allergens are administered subcutaneously or sublingually for 5-6 years. At first the specific allergen is administered in small doses and then gradually increased to reach an optimal concentration. This enables the body to develop tolerance to the allergen, preventing a strong immune response when there is a subsequent encounter with the same allergen (22, 78, 113, 114). Immunotherapy is similar to

vaccination; the difference is that the former blunts the immune system and the latter provokes the immune system (33). This approach is directed towards the allergen and not the symptoms, and after 5-6 years of therapy patients have been shown to experience attenuated asthma-like symptoms. However, the disadvantage is that some patients develop severe anaphylactic responses during treatment (344).

11 Role of CD38

11.1 Background

CD38 is a 45 kDa transmembrane type II glycoprotein, ubiquitously expressed on the plasma membranes of cells (238). The CD38 gene with 70,776 bases is located on chromosome 4 in the plus strand (4p15). The mRNA of CD38 contains 1491 bases with 8 exons (NCBI). The CD38 protein has 300 amino acids (UniProtKB). It has a short cytoplasmic N-terminal region with 20 amino acids and an extensive extra-cellular C-terminal region with 256 amino acids. The extracellular domain has multiple enzymatic functions such as ADP-ribosyl cyclase activity that converts β-NAD into cADPR, cADPR hydrolase activity that converts cADPR into ADP ribose and NAD+ glycohydralase activity that converts NAD+ into ADP-ribose (205). The first exon of CD38 transcript is responsible for producing the N-terminal and transmembrane portions of the protein while exons 2-8 are encode the extra-cellular C-terminal region of the protein (249). CD38 expressed on the plasma membrane of cells has many functions such as cell adhesion, calcium signaling and signal transduction (238).

Receptor activity of CD38 in leukocytes (T and B lymphocytes) and dendritic cells promotes activities such as adhesion and proliferation. CD38 plays a role in antigen priming in T lymphocytes and in proliferation of B lymphocytes by initiating upstream signaling cascades (222). In dendritic cells, CD38 plays a role in cell trafficking from peripheral tissues to lymph nodes and in antigen presentation to T lymphocytes (267, 268). In bone tissue, CD38 induces osteoclasts to increase bone absorption (311). In pancreas, it stimulates beta islet cells to secrete insulin. Impairment or loss of CD38 in these cells leads to type II diabetes mellitus (361).

CD38 serves as a molecular marker in some disease states. It is used as a negative prognostic marker in chronic lymphocytic leukemia (CLL) (84). Presence of an increased number of circulating $CD38^{+}$ B lymphocytes indicates that they are immature and in a proliferative stage. Typically, only B lymphocytes that are found in germinal centers of secondary lymphoid organs possess CD38 (84). Once they come out of the germinal center and enter the marginal zone, they become CD38 negative. In CLL, because B cells proliferate rapidly, they escape normal maturation processes and enter the blood stream prematurely from lymph nodes. Therefore, the presence of CD38 on circulating B cells is an indicator of aberrant proliferation as seen in CLL. Similarly, increased expression of CD38 by regulatory T lymphocytes is also indicative of progressive CLL (219). In human immunodeficiency virus (HIV) infection, the amount of CD38 present on T lymphocytes correlates with disease progression (219).

11.2 Role of cyclic ADP-ribose (cADPR)

In ASM cells, the enzymatic activity of CD38 is important because it produces cADPR which is involved in ASM contraction and AHR. The extracellular multi-enzymatic region of CD38 generates the potent second messenger cADPR required for the release of $[Ca^{++}]_i$ from stores (204-208). There are many mechanisms proposed for the entry of extracellularly produced cADPR into the cytoplasm. Some of the suggested methods are via internalization by the plasma membrane (83), through connexin-43 channels (308), by a catalytically active channel created by 2 or 4 CD38 monomers (112) or by a nucleotide transporter (97).

cADPR, after entering the cytoplasm, binds to an accessary protein FKBP 12.6 and induces the ryanodine receptor to release Ca^{2++} into the cytoplasm from the sarco-endoplasmic reticulum, which serves as an intracellular calcium store (371). The released Ca^{++} in turn induces ryanodine receptors to release more calcium ions. This mechanism is referred to as “calcium induced calcium release (CICR)” (210)

11.3. Calcium homeostasis

Calcium concentrations within a cell are much lower (100-200 nM) than the extracellular compartment (1-2 mM) (96, 240). Normal calcium homeostasis in the intracellular compartment is strictly maintained. by the regulation of Ca^{++} “influx” from outside of the cell, “release” of Ca^{++} from intracellular stores and calcium re-uptake from cytoplasm into the sarco-endoplasmic reticulum through ‘sarco-endoplasmic reticulum calcium ATPase’ (SERCA). Influx of calcium ions

occurs through various ion exchangers, voltage gated channels, calcium-release channels, second messengers and pumps. Other channels involved in maintaining $[Ca^{++}]_i$ homeostasis are Store Operated Channels (SOC) and ORAI-STEMI channels through a mechanism called Store Operated Calcium Entry (SOCE) (304, 310).

11.4 Excitation-contraction coupling

Agonists of subunit–Gq tagged GPCR such as histamine, bradykinin, cysteinyl leukotrienes, thrombin, serotonin and acetylcholine released from parasympathetic nerve terminals are known to regulate the calcium dynamics of ASM cells and their contractility through receptors in the ASM cell membrane (193). When ASM cells are excited they elicit a biphasic intracellular calcium response (234, 276). After stimulation, $[Ca^{++}]_i$ concentration shows a sharp elevation followed by a rapid fall but is still above the basal level and in a steady state. Altered intracellular calcium dynamics triggers many downstream cell signaling pathways. Elevated Ca^{++} binds to the protein calmodulin which activates Myosin Light Chain Kinase (MLCK). Activated MLCK, in turn phosphorylates the myosin and promotes its binding to actin. Sliding of actin-myosin bridges causes the smooth muscle cells to contract (370). This explains the role CD38 plays in AHR (180).

12. MicroRNAs

12.1 Biogenesis and Mode of action

MicroRNAs are produced endogenously in the nucleus. They are non-coding, ~ 22 nucleotide long small RNAs involved in almost all cellular processes

including development, differentiation, metabolism, and organ functions as well as in many pathological processes such as inflammation, stress response, heart failures, renal failures, various forms of cancer and mental disorders (29). In mammals more than 50% of all protein coding genes are regulated by microRNAs. They exhibit a specific spatio-temporal expression pattern during environmental changes and developmental stages. An individual microRNA can inhibit the expression of hundreds of genes, fine tune them at different levels and maintain the cellular network. Aberrantly expressed microRNAs cause disturbances in the cellular network and lead to diseases. Up to now, about 1500 microRNAs have been discovered in the human genome. Thus, microRNAs are emerging gene regulators, playing roles in a variety of disorders. Their expression and function are controlled through transcriptional and posttranscriptional mechanisms (29). Aberrant expression of microRNAs could be due to a single nucleotide polymorphism or nucleotide deletions either on their mature sequence or at the cleavage sites where RNA-lll endonucleases Drosha and Dicer act to cleave pri-microRNAs and pre-microRNAs, respectively (29). Pathogenic processes could also be initiated due to a polymorphism at the 3'UTR of a target mRNA where microRNAs bind (297).

12.2 MicroRNAs in asthma

Accumulating evidence indicates that microRNAs play a crucial role in the development of asthma (21). Changes in the expression levels of microRNAs can be identified in airway structural tissues such as epithelial and smooth muscle cells and serum from asthmatic patients (338). Altered microRNA levels

play a part in the pathogenesis of asthma. Based on several microRNA-target-predicting algorithms, studies have proved that microRNAs control many signaling pathways involved in asthma pathogenesis such as expression of MMP, TGF-β signaling, inflammatory processes and NF-κB signaling (197, 338). Many studies in mouse models and with human cell cultures have revealed the role of microRNAs in asthma. A spontaneous development of asthma–like phenotype with inflammation and airway remodeling was observed in miR-155 knockout mice (262). Another study showed that increased expression of miR-126 is involved in TH2 inflammation and Toll-Like Receptor (TLR-4) activation in asthma (233). Chiba *et. al*. demonstrated that expression of miR-133a is reduced after IL-13 treatment of human bronchial smooth muscle cells (66). The above studies suggest that down-regulation of miR-155 or miR-133a and up-regulation of miR-126 may have a role in the pathogenesis of asthma.

MiR-146a is expressed in a variety of cell types such as alveolar epithelial cells, bronchial epithelial cells and bronchial smooth muscle cells and alters the expression of IL-1-β-induced chemokines such as IL-8 and RANTES (273). Thus MiR-146a may have a role in the pathogenesis of asthma as well.

Stimulation of human airway smooth muscle cells with IL-1β, IFN-γ and TNF-α down-regulates the expression of miR-25. Inhibitors of miR-25 decreased the expression of RANTES and eotaxin but increased the expression of TNF-α. With KLF-4 as a target protein, which in turn is a potent inhibitor of smooth muscle-specific gene expression (197) and playing a role in regulating many pro-inflammatory cytokines, miR-25 may play an important role in asthma

pathogenesis. MiR-708 plays a role in cell proliferation. In small cell lung carcinoma, highly expressed miR-708 down regulates the trans-membrane protein TMEM88 by directly binding to the 3'UTR of the transcript. Reduced level of TMEM88 protein, a negative regulator of the Wnt signaling pathway, induces cell proliferation in lung carcinoma (169). An anti-proliferative function of miR-708 was observed in renal cell carcinoma, where it targets survivin, a member of the inhibitor of apoptotic protein (IAP) family (294). Survivin plays a role in proliferation and inhibiting apoptosis. It is highly expressed in embryonic tissues, cancerous cells and vascular smooth muscle cells during vascular injuries (294). Based on these studies, I hypothesize that miR-708 may have a potential role in ASM cell proliferation.

12.3 MicroRNA therapy

MicroRNAs can regulate multiple genes of redundant pathogenic pathways of a disease, and fine tune them to varying degrees to gain a favorable effect. Thus, unlike other therapeutics that target only one gene or protein, microRNA therapy can target multiple genes resulting in a more effective and potent outcome (338).

Different approaches to microRNA therapy to reverse the disease condition include administration of synthetic mimics of specific microRNA or of viral vectors to replace the shortage of microRNAs that are down-regulated in the disease condition. Another option is delivery of antagomirs of microRNAs or microRNA sponges, which compete for the same target as the microRNA of interest into cells to block the microRNAs that are over-expressed due to a

pathological condition. For example, tumor development in a mouse model of non-small cell lung cancer was reduced when mimic of *let-7* was injected intra-tumorally. Likewise, administration of an antagomir of miR-21 to a mouse model of gliomas decreased the activity of MMPs (118). Yet another example is the complete regression of B lymphoid tumors by administration of miR-21 to mice in vivo (237).

Various chemical modifications such as, 2'-O-methyl, 2'-O-methoxyethyl, 2'-fluoro, RNA with a phosphorothioate modification, modified nucleotide or locked nucleic acid, have been employed to increase the cellular uptake and stability of synthetic oligonucleotides (mimic of miRNA or inhibitors of miRNA). These chemically modified therapeutic nucleotides were conjugated with cholesterol at their 3' end and wrapped in a lipid bilayer as lipid nanoparticles. These nanoparticles, when introduced into a tissue, fuse with the phospholipid bilayer of the cell membrane and gain entry to the cytoplasm. There they interact with endosomes at low pH and release therapeutic miRNAs intracellularly (44, 49).

In an experiment carried out to evaluate the efficacy of anti-miR-122 therapy in hepatic C viral (HCV) infection, antagomir of miR-122 (5 mg/kg) with Locked Nucleic acids (LNA) modifications was injected intravenously into chimpanzees once a week for 12 weeks. After 2 weeks from the end of treatment, the viral titer was dramatically lower (400 times lower in serum and 200 times lower in liver). In phase II trials in humans, administration of a chemically modified antagomir of miR-122 has shown successful results for HCV

infection. Recently, Regulus Therapeutics, Inc., announced that administration of chemically modified GalNac-conjugated anti-miR-122 at 2 mg/kg subcutaneously in patients with HCV as mono therapy reduced viral load by 4.1 log10 on day 29. They declared that the drug was safe, well-tolerated and has a promising pharmacokinetic profile.

Thus far, miR-34, a deregulated microRNA in many cancers, is the only microRNA tested in clinical trials as replacement therapy. A synthetic miRNA mimic of miR-34 named MRX34 is in phase I clinical trials (Mirna Therapeutics) for liver cancer in humans. Here MRX34 is incorporated into liposome as SMARTICLES (Marina Biotech) and delivered.

Other approaches to deliver microRNA *in vivo* are (i) cationic liposomes (PU-PEI, DOTMA) (68) that have low immunogenicity and high cellular uptake but retain cytotoxicity and are non-biodegradable, (ii) neutral liposomes (siPORT, SLNs) (77, 325, 352) with low immunogenicity but have non-specific uptake and toxicity that accumulate in the lung, (iii) viral vectors that are stable and have high transfection efficiency but are highly immunogenic and toxic, (194) that have low immunogenicity but have poor cellular uptake with rapid degradation and renal clearance.

While there are some draw backs and questions that remain unanswered about microRNA therapy such as their off-target effect, bio-stability, immunogenicity, toxicity and renal clearance, various reports have shown that the microRNA therapy can be well-tolerated in animals as well as in humans (39, 192, 352) since microRNAs are endogenous unlike siRNAs which are synthetic.

13. Previous findings from our laboratory

- Induction of CD38 expression by pro-inflammatory cytokines in ASM cells (87)
- Contribution of CD38 and cADPR to AHR (89).
- Allergen sensitized *Cd38* KO mice exhibit attenuated AHR relative to wild type mice (134).
- ASM cells obtained from *Cd38* KO mice showed reduced calcium responses compared to control mice in response to stimulation with spasmogens (89).
- In the presence of 8-Br-cADPR, an antagonist of cADPR, HASM cells showed reduced calcium responses to contractile agonists (87).
- Pro-inflammatory cytokine TNF-α-induced CD38 expression has transcriptional regulation through the MAP kinase and PI3 kinase pathway (180, 323).
- Transcriptional regulation of CD38 occurs through transcription factors NF-κB and AP-1 (323).
- Increased expression of CD38 in asthmatic HASM cells compared to NA-HASM cells (179).
- Class 1A PI3 kinases increase CD38 expression in human ASM cells following exposure to TNF-α (180).

14. Significance of the study

Asthma is a chronic respiratory disorder that is known for centuries. Despite considerable increase in knowledge regarding this disorder, several aspects about the pathogenic mechanism remain unclear and have hindered the development of completely effective treatment options or the prevention strategies. Currently, symptomatic treatment with corticosteroids is the first line of effective therapy for asthma, although a small number of patients are refractory to steroid therapy (256). Further, some asthmatic patients on therapy with other available drugs for asthma either do not respond well or show side effects. Thus, it is important to identify novel molecules that play a critical role in the pathogenesis of asthma and evaluate their function and regulation in order to define alternative targets for asthma therapy.

In this context, previous studies from this laboratory have identified CD38 on ASM cells as a potential asthma target. CD38, a transmembrane protein, is expressed in a variety of cells (191, 216, 224). ASM cells are known to play many important roles in the pathogenesis of asthma such as airway contractility, airway inflammation and remodeling (261). CD38 possesses multiple enzymatic activities. Its ADP-ribosyl-cyclase activity converts the nucleotide metabolite β-NAD into cADPR, which is a potent intracellular Ca^{++} releaser (180). In HASM cells, elevated Ca^{++} in the intracellular compartment causes contraction of smooth muscle cells resulting in AHR, one of the cardinal features of asthma (87-89, 133).

Previous studies from our laboratory indicate that signaling pathways associated with CD38 may provide novel therapeutic strategies in attenuating AHR and asthma. TNF-α and TH2 cytokines induce CD38 expression transcriptionally via transcription factors NF-κB and AP-1 and MAP Kinases (322, 323). CD38 protein and transcripts are differentially induced in ASM cells derived from asthmatic patients compared to non-asthmatic patients (178). Since expression of the above transcription factors and MAP Kinases are comparable between asthmatic and non-asthmatic ASM cells, the differentially induced CD38 expression could be due to post-transcriptional mechanisms which could potentially involve aberrantly regulated microRNAs in ASM cells during asthma.

In this investigation, I explored the potential role of microRNA in the regulation on CD38 expression in HASM cells and in a mouse model of allergic asthma. Further, the effect of microRNA on the proliferative function of ASM cells was investigated. The findings of this study revealed novel regulatory pathways of CD38 expression and identified other asthma-related genes that contribute to the overall pathogenesis of asthma.

CHAPTER ii

miR-140-3p regulation of TNF-alpha-induced CD38 expression in human airway smooth muscle cells

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Background

CD38, a membrane protein expressed in airway smooth muscle (ASM) cells, plays a role in cellular Ca^{2+} dynamics and ASM contractility. In human ASM (HASM) cells, TNF-α induces CD38 expression through activation of MAPKs, NF-κB, and AP-1, and its expression is differentially elevated in cells from asthmatic patients compared with cells from non-asthmatic subjects. The CD38 3′-untranslated region (UTR) has targets for miR-140-3p. We hypothesized that miR-140-3p regulates CD38 expression in HASM cells by altering CD38 mRNA stability. Basal and TNF-α-induced expression of miR-140-3p was determined in non-asthmatic human ASM (NA-HASM) and asthmatic human ASM (AS-HASM) cells. NA-HASM and AS-HASM cells were transfected with control, miR-140-3p mimic, or miR-140-3p antagomirs, and CD38 expression and CD38 mRNA stability were determined. Luciferase reporter assays were used to determine miR-140-3p binding to the CD38 3′-UTR. Activation of p38, ERK, and JNK MAPKs, NF-κB, and AP-1 was determined in miR-140-3p mimic-transfected NA-HASM. TNF-α attenuated miR-140-3p expression in NA-HASM and AS-HASM cells but at a greater magnitude in AS-HASM cells. CD38 mRNA expression was attenuated by miR-140-3p mimic at comparable magnitude in NA-HASM and AS-HASM cells. Mutated miR-140-3p target on the CD38 3′-UTR reversed the inhibition of luciferase activity by miR-140-3p mimic. CD38 mRNA stability was unaltered by miR-140-3p mimic in NA-HASM or AS-HASM cells following arrest of transcription. TNF-α-induced activation of p38 MAPK and NF-κB was attenuated by miR-140-3p mimic. The findings indicate that miR-140-3p

modulates CD38 expression in HASM cells through direct binding to the CD38 3′-UTR and indirect mechanisms involving activation of p38 MAPK and NF-κB. Furthermore, indirect mechanisms appear to play a major role in the regulation of CD38 expression.

Introduction

CD38 is a cell surface protein expressed in a variety of mammalian cells, including airway smooth muscle (ASM) cells (350). CD38 possesses multiple enzymatic activities, with ADP-ribosyl cyclase activity generating cyclic ADP-ribose (cADPR), a Ca^{2+}-mobilizing agent (158, 209). Past studies in our laboratory established that the CD38/cADPR pathway plays an important role in cellular Ca^{++} dynamics and ASM contractility (87, 89). In human ASM (HASM) cells, TNF-α induces CD38 expression through activation of the transcription factors NF-κB and AP-1 and MAPK kinases (322). Among the MAPKs, p38 and ERK MAPKs mediate TNF-α-induced CD38 expression through modulation of transcript stability (322). We recently reported that, in ASM cells from subjects with a history of asthma, TNF-α-induced CD38 expression was differentially elevated, although the mechanistic basis of this differential elevation was not clearly understood (178).

MicroRNAs (miRNAs) are noncoding small RNAs emerging as posttranscriptional regulators in various biological processes, including inflammation; they can regulate expression of their target genes by destabilizing the transcripts or by translational repression (29). Accumulating evidence has proven that miRNAs are associated with various pathological conditions in humans. Recent studies have shed light on the role of miRNAs in airway disorders such as asthma, chronic obstructive pulmonary disorder, and idiopathic pulmonary fibrosis. Recent studies report a role for miR-21 in determining the T helper (Th) type 1 (Th1)/Th2 immune response to antigen, thus playing a role in

the pathogenesis of allergic asthma (220, 221). Other studies have attempted to determine the role of specific miRNAs in airway inflammation and allergic airway hyperresponsiveness (AHR). Studies of the roles of let-7 and miR-155 in IL-13 signaling and the potential role of miR-133 in RhoA expression were among those that highlight the roles of miRNA in the pathogenesis of airway inflammatory disorders (66, 220, 221, 231). In the present study, bioinformatic tools were used to determine potential miRNA response elements in the 3′-untranslated region (UTR) of the human CD38 gene. Expression of miR-140-3p, which came as a top hit in one of the target prediction algorithms, and its functional role in CD38 expression were determined in asthmatic ASM (AS-HASM) and nonasthmatic ASM (NA-HASM) cells. We hypothesized that miR-140-3p downregulates CD38 expression in HASM cells through posttranscriptional mechanisms.

Materials and methods

Reagents.

Tris-base, glucose, HEPES, and other chemicals were purchased from Sigma Chemical (St. Louis, MO), unless otherwise noted. Human recombinant TNF-α (rhTNF-α) was purchased from R & D Systems (Minneapolis, MN); HBSS and DMEM from GIBCO-BRL (Grand Island, NY); TRIzol, SuperScript III reverse transcriptase, NCode miRNA first-strand synthesis kit, Platinum SYBR Green quantitative PCR (q-PCR) mix, and Lipofectamine RNAiMax from Invitrogen Life Technologies (Carlsbad, CA); miRVana RNA isolation kit from Ambion Life Technologies (Carlsbad, CA); NE-PER nuclear/cytoplasmic extraction kit from Pierce (Rockford, IL); antibodies for MAPKs and NF-κB and lamin A/C, actin, α-tubulin, MAPK kinase 3 (MKK3), the dual-specificity phosphatase MKP-1, and nuclear receptor-interacting protein (NRIP-1) from Cell Signaling Technology; TransAM ELISA kits (NF-κB and AP-1) from Active Motif (Carlsbad, CA); QuikChange Lightning Multi Site-Directed mutagenesis kit from Agilent Technologies (Santa Clara, CA); control (C.ele-miR-67), miR-140-3p mimic, and antagomir (hsa-miR-140-3p mature sequence 5′-UACCACAGGGUAGAACCACGG-3′) oligonucleotides from Dharmacon (Lafayette, CO); and chemiluminescent substrate for horseradish peroxidase (HRP) from Millipore (Billerica, MA).

HASM cell cultures and treatments.

Procedures for isolation and culture of HASM cells are described elsewhere (87, 178). Briefly, HASM cells were obtained from the laboratory of R. A. Panettieri,

Jr. The cells were from de-identified healthy donors (NA-HASM cells) and donors who died due to severe asthma (AS-HASM cells). All experiments were conducted in HASM cells in passage 4 or 5. In all the experiments, prior to exposure to TNF-α, the cells were growth-arrested for 48 h in arresting medium without serum, but in the presence of transferrin and insulin. In experiments to determine the expression of CD38 and miR-140-3p, the cells were exposed to vehicle (0.1% BSA in PBS) or 10 ng/ml rhTNF-α for 0–24 h. To determine CD38 mRNA stability, the cells were exposed to TNF-α for 12 h and then washed to remove TNF-α before addition of actinomycin D (5 μg/ml) to arrest transcription. Total RNA was collected at 0, 6, 12, and 24 h after transcriptional arrest. Data are reported for only 0- and 24-h time points for brevity. In experiments to determine the activation of NF-κB and MAPKs, cells were exposed to rhTNF-α (10 ng/ml) for 1 h and 15 min, respectively.

Extraction of total RNA and cDNA synthesis.

Total RNA was extracted from HASM cells following the manufacturer's instructions (miRVana, Ambion). Briefly, HASM cells ($5 \times 10^4 – 2 \times 10^5$) were collected in sterile PBS and centrifuged, and the pellet was homogenized in a buffer provided in the kit. Column-eluted total RNA was quantified in a bioanalyzer (Agilent NanoDrop). To synthesize cDNA from small RNA, 200 ng of total RNA were polyadenylated, and cDNA was synthesized using NCode first-strand synthesis kit according to the manufacturer's instructions. In parallel, 200 ng of total RNA were used to synthesize cDNA from larger RNA using the SuperScript III reverse transcription kit.

Extraction of whole cell/nuclear lysates.

Whole cell lysates were obtained by sonication of HASM cells (2–5 × 10^5) in lysis buffer (50 mM Tris, 100 mM NaCl, 50 mM NaF, 40 mM β-glycerol phosphate, 2 mM EDTA, 0.2 mM Na_3VO_4, 1% Triton X-100, and protease inhibitor cocktail, pH 7.4). Nuclear extracts were collected from HASM cells (5–6 × 10^5) using the NE-PER cytoplasmic and nuclear extraction kit, according to the manufacturer's protocols.

Transient transfection of HASM cells.

HASM cells were plated into appropriate formats and cell number (2 × 10^5 cells/well in a 6-well plate or 5 × 10^5 cells/plate in a 100-mm culture plate) 24 h prior to transfection. Transfection was performed using Lipofectamine RNAimax according to the manufacturer's instructions. Briefly, control (C.ele-miR-67), miR-140-3p mimic, or miR-140-3p antagomir oligonucleotides were transfected at 5–100 nM. Control oligonucleotide was transfected at 50 or 100 nM.

q-PCR.

To determine miR-140-3p expression, a forward primer specific to miR-140-3p and a universal primer targeting the poly (T) of the cDNA were used according to the manufacturer's instructions. Mammalian small nuclear RNA U6 was used for normalization of miR-140-3p expression. To determine CD38 expression, q-PCR was performed using Brilliant SYBR Green Master Mix, as described elsewhere (322). Cyclophilin was amplified as the housekeeping control.

Site-directed mutagenesis.

Site-directed mutagenesis was performed in luciferase-CD38 3′-UTR reporter plasmids. CD38–3′-UTR (481 bases long; UGC genome browser) has a predicted miR-140-3p target site at 8 bases after the stop codon in the CD38 mRNA. The first miR-140-3p target had a 7-base complementarity to the miR-140-3p. A second target site with a 6-base complementarity to miR-140-3p was found 21 bases from the first target site. The first target site (mutated 1) or both target sites (mutated 1+2) were mutated to determine the selectivity of miR-140-3p binding to these sites. The QuikChange Lightning Multi Site-Directed mutagenesis kit was used to mutate 4 bases (taGaGGa) at the first target site (CTGTGGT) or 3 bases (aGaGGa) at the second target site (TGTGGT) (**Fig. 2A, top**). Twenty bases flanking the target site with mutation were designed as primers for mutation. The primers for each mutagenesis were as follows: first target site mutation primer (TCTGAGATCTGAGCCAGTCGtaGaGGaTGTTTTAGCTCCTTGACTCC) and second target site mutation primer (TTTAGCTCCTTGACTCCTaGaGGaTTATGTCATCATACATGACTCAGC). Wild-type and mutant plasmids were expanded in Escherichia coli and sequenced to confirm mutations.

SDS-PAGE and Western blotting.

Total protein (10 μg) was resolved in a 4–20% Tris·HCl SDS gel and electrophoretically transferred onto a polyvinylidene difluoride membrane. The blot was blocked in 5% skim milk solution in PBS containing 0.05% Tween 20 for

≥4 h. The blot was probed with relevant primary antibodies and then incubated with HRP-conjugated secondary antibodies. After washes in PBS containing 0.05% Tween 20, the blots were treated with the chemiluminescent substrate for HRP and exposed to X-ray film for visualization of bands.

ELISA.

For determination of NF-κB or AP-1 activation, ELISA was performed according to the manufacturer's instructions (Activ Motif) and as previously described (178). Briefly, 3 μg of nuclear extracts from HASM cells were incubated in a multiwell plate coated with oligonucleotides carrying consensus NF-κB or AP-1 sequences. Competitor oligonucleotide (20 pmol, 20× excess) was added to some reactions to determine the specificity of the binding.

ADP-ribosyl cyclase assay.

The ADP-ribosyl cyclase activity of HASM cell lysates was quantified by measurement of the reverse cyclase activity of CD38. HASM whole cell lysates containing 5 μg of total protein were incubated for 1 h at 37°C with or without 10 mM nicotinamide in the presence of 0.45 mM cADPR. The reverse cyclase reaction was stopped by addition of 25 μl of 1 M HCl, vacuum-filtered through a 0.45-μm protein-binding membrane (Immobilon, Millipore), and neutralized with 15 μl of 2 M Tris-base. The filtrate was incubated with reagent mixture containing 2 μM rezasurin, 0.76% (vol/vol) ethanol, 4 μM flavin mononucleotide, 40 μg/ml alcohol dehydrogenase, and 0.04 U/ml diaphorase in NaH_2PO_4/Na_2HPO_4 buffer, pH 6.8, at room temperature. Fluorescence was quantified (excitation at 544 nm and emission at 590 nm) in a fluorometer (FLUO star Galaxy, BMG

Biotechnologies), and the rate of fluorescence emission was calculated. The quantity of NAD generated in the reaction was calculated from a standard curve generated from known NAD.

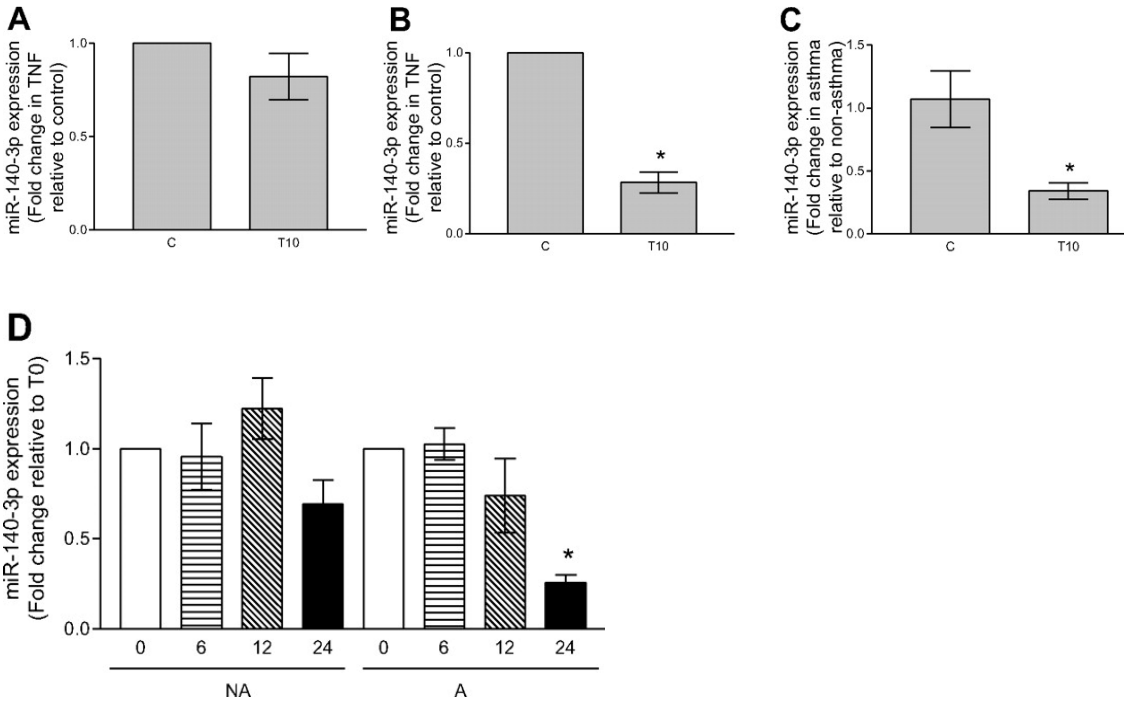
Data analysis.

Each experiment was performed three to six times (NA-HASM or AS-HASM cells obtained from 3–6 donors were used). Values are means ± SE. Data were statistically analyzed by Student's t-test or one-way ANOVA (depending on the number of experimental groups analyzed) using GraphPad Prism software. Differences were considered significant at $P \leq 0.05$.

Results

Expression of miR-140-3p in HASM cells.

HASM cells were exposed to vehicle (0.1% BSA in sterile PBS) or TNF-α (10 ng/ml) for 24 h, and expression of miR-140-3p and CD38 mRNA was determined. In TNF-α-treated NA-HASM cells, miR-140-3p expression was marginally reduced compared with vehicle-treated cells **(Fig. 1A)**. In TNF-α-treated AS-HASM cells, miR-140-3p expression was significantly reduced compared with vehicle-treated cells **(Fig. 1B).** The basal miR-140-3p expression levels were comparable in NA-HASM and AS-HASM cells **(Fig. 1C).** Exposure to TNF-α for 24 h resulted in downregulation of miR-140-3p expression in NA-HASM and AASM cells, but to a greater magnitude in AS-HASM cells **(Fig. 1C).** Exposure to IL-13, a Th2 cytokine with a critical role in asthma pathogenesis, did not alter miR-140-3p expression in either group of HASM cells (data not shown).



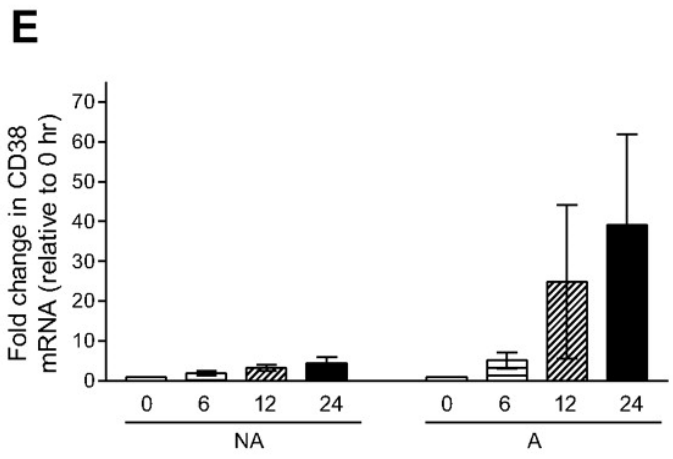


Fig. 1. Expression of miR-140-3p in vehicle-treated (C) and TNF-α-treated nonasthmatic airway smooth muscle (NA-HASM) and asthmatic airway smooth muscle (AS-HASM) cells. **A**: in the presence of TNF-α, miR-140-3p expression was marginally attenuated in NA-HASM cells compared with cells treated with vehicle (n = 5). **B**: in AS-HASM cells, TNF-α significantly attenuated miR-140-3p expression (n = 6). *P < 0.05. **C:** basal miR-140-3p expression levels were comparable between NA-HASM and AS-HASM cells. In the presence of TNF-α, miR-140-3p expression was significantly attenuated in AS-HASM cells compared with NA-HASM cells. *P < 0.05. (Data in A–C are from the same experiments.) **D**: when NA-HASM (NA) and AS-HASM (A) cells were exposed to TNF-α for 0–24 h, both showed attenuated miR-140-3p expression at 24 h, although reduction was statistically significant only in AS-HASM cells (n = 3). *P < 0.05. **E**: TNF-α produced a time-dependent increase in CD38 mRNA expression in NA-HASM and AS-HASM cells. Magnitude of CD38 induction in

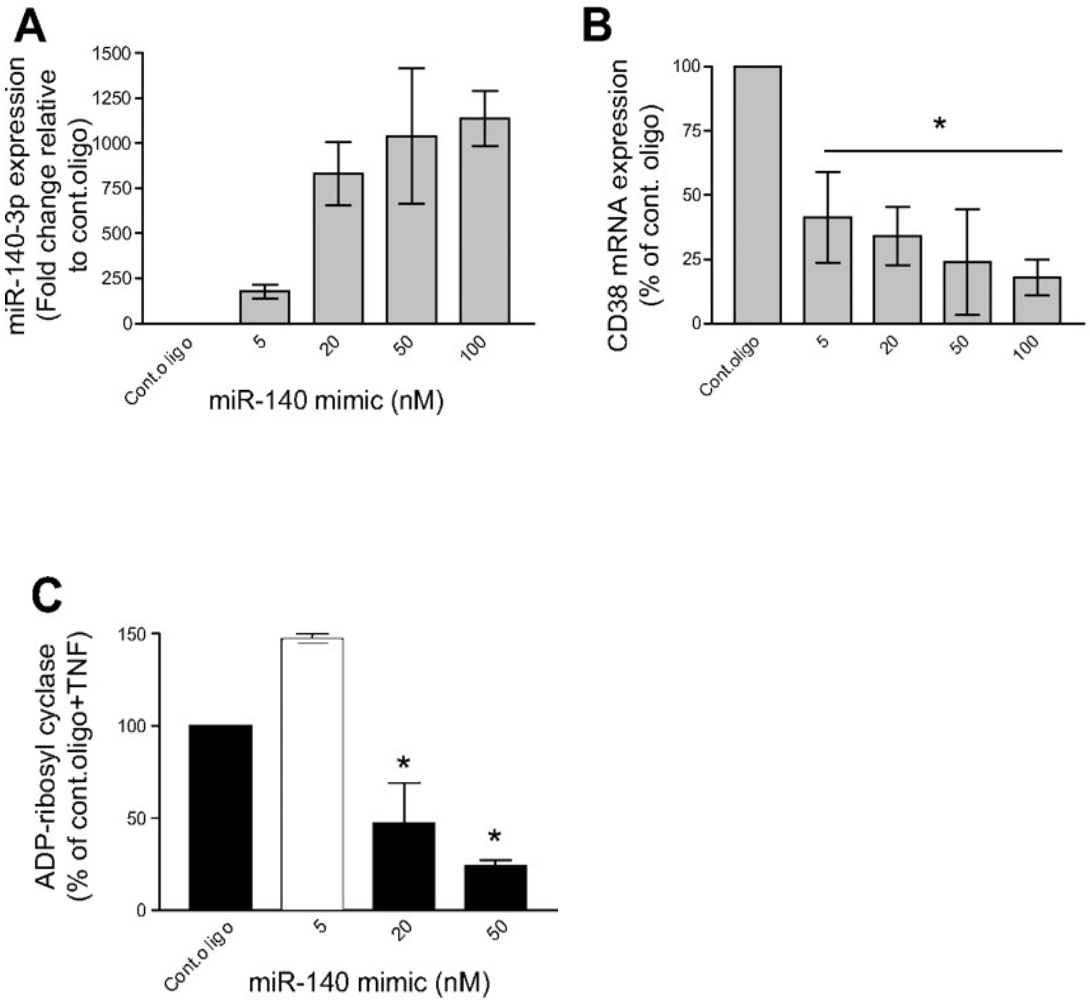
AS-HASM cells was higher in AS-HASM cells, although differential increase was not statistically significant because of larger standard error in AS-HASM cells.

In another set of experiments, miR-140-3p expression was determined at different times following exposure to TNF-α. Significant attenuation of miR-140-3p expression was noted at 24 h of TNF-α exposure in AS-HASM cells compared with NA-HASM cells **(Fig. 1D**). TNF-α induced CD38 mRNA expression in a time-dependent manner in NA-HASM and AS-HASM cells, with larger magnitudes of CD38 expression in AS-HASM cells, confirming our earlier findings (178) **(Fig. 1E).**

miR-140-3p mimic inhibits TNF-α-induced CD38 up-regulation in HASM cells.

To determine the functional role of miR-140-3p in CD38 expression in HASM cells, we transfected NA-HASM and AS-HASM cells with miR-140-3p mimic or control oligonucleotides and determined the effects on CD38 mRNA expression and ADP-ribosyl cyclase activity. Transfection of NA-HASM cells with a range of miR-140-3p mimic oligonucleotides resulted in a concentration-dependent increase in miR-140-3p expression **(Fig. 2A).** In cells transfected with miR-140-3p mimics, upregulation of TNF-α-induced CD38 mRNA was significantly attenuated in a concentration-dependent manner, although without a linear relationship to the mimic concentration **(Fig. 2B).** At higher concentrations of miR-140-3p mimic (20 and 50 nM), TNF-α-induced ADP-ribosyl cyclase activity was significantly reduced compared with the cells transfected with 50 nM control

oligonucleotide **(Fig. 2C).** At the optimal concentration (50 nM), miR-140-3p mimic transfection attenuated TNF-α-induced CD38 mRNA expression in NA-HASM and AS-HASM cells to a similar magnitude **(Fig. 2D).** Transfection of NA-HASM and AS-HASM cells with miR-140-3p mimic oligonucleotides also resulted in comparable inhibitory effects on TNF-α-induced ADP-ribosyl cyclase activity **(Fig. 2E).** Transfection of antagomirs for miR-140-3p did not alter basal or TNF-α-induced ADP-ribosyl cyclase activity in either group of HASM cells **(Fig. 2E).**



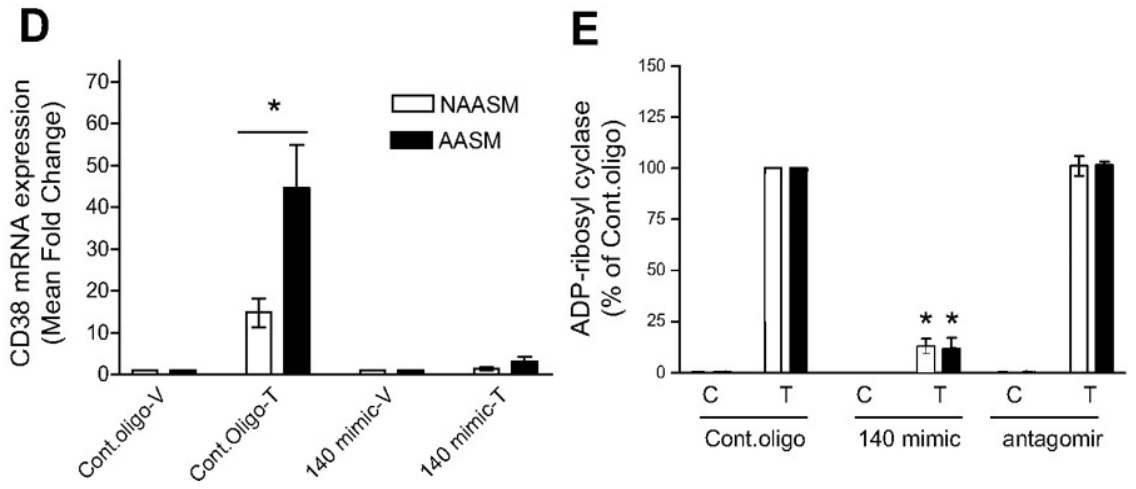


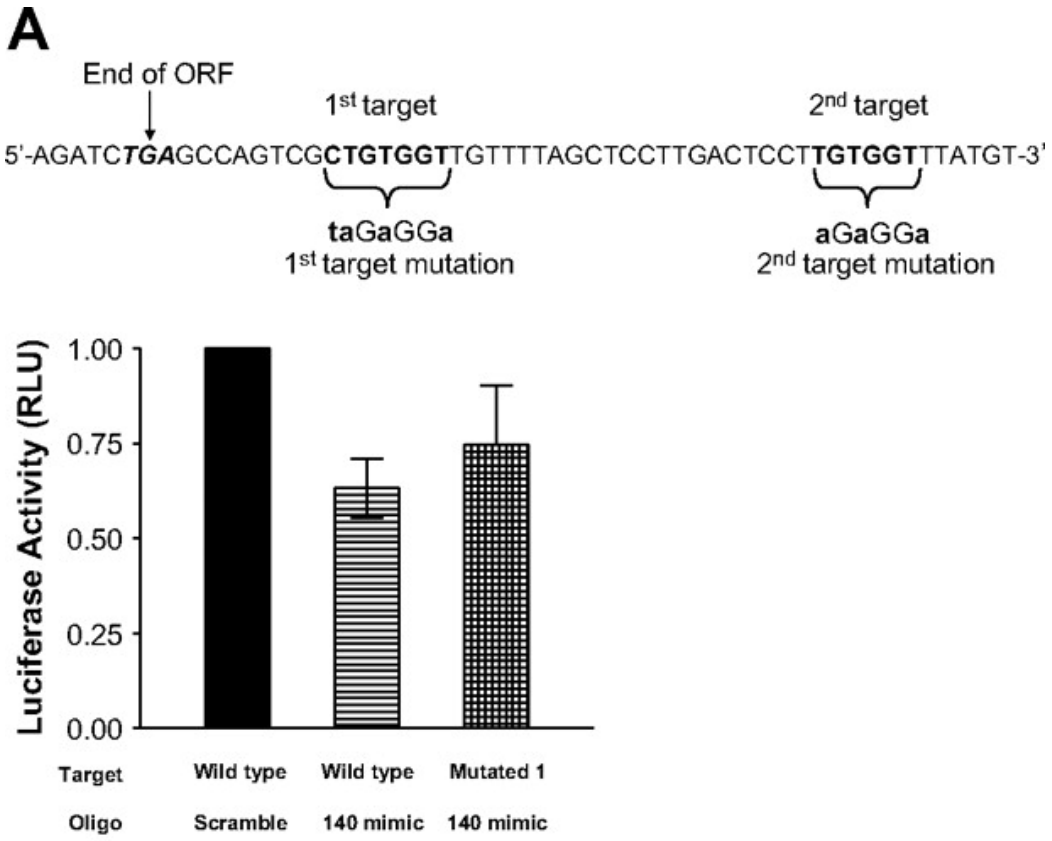
Fig. 2. Inhibition of TNF-α-induced CD38 expression in HASM cells by miR-140-3p mimic. Mimics of miR-140-3p (140 mimic) or antagomirs for the microRNA (miRNA) were transiently transfected into NA-HASM and AS-HASM cells, and CD38 mRNA expression and ADP-ribosyl cyclase activity were determined. **A**: transient transfection of NA-HASM cells with miR-140-3p mimic oligonucleotide resulted in a concentration-dependent increase in miR-140-3p expression (n = 3). **B**: transfection of miR-140-3p mimics in NA-HASM cells resulted in a concentration-dependent attenuation of TNF-α-induced CD38 mRNA expression, although the relationship between mimic concentration and CD38 expression was not linear (n = 3). *P < 0.05, all concentrations vs. control oligonucleotide (Cont oligo). **C**: in NA-HASM cells transiently transfected with miR-140-3p mimic oligonucleotides, ADP-riboysl cyclase activity was significantly attenuated at higher concentrations of the mimic (n = 3). *P < 0.05 vs. Cont oligo. **D**: when NA-HASM and AS-HASM cells were transiently transfected with 50 nM control oligonucleotide or miR-140-3p mimic oligonucleotide, CD38 mRNA expression was comparably attenuated in both groups of cells (n = 2). *P < 0.05.

E: TNF-α-induced ADP-ribosyl cyclase activity was attenuated to a comparable magnitude in NA-HASM and AS-HASM cells in the presence of miR-140-3p mimic oligonucleotides (n = 3). Basal [control (C)] and TNF-α-induced (T) ADP-ribosyl cyclase activities were unaltered in NA-HASM or AS-HASM cells transiently transfected with miR-140-3p antagomir oligonucleotides (n = 3). *P < 0.05 vs. Control oligonucleotide (T).

miR-140-3p targets the CD38 3′-UTR.

To determine whether miR-140-3p brings about its effects through direct binding to the 3′-UTR of CD38, dual luciferase reporter assays were performed in human embryonic kidney (HEK)-293 or NIH 3T3 cells. Cotransfection of cells with miR-140-3p mimic oligonucleotides and the wild-type Luc-CD38 3′-UTR resulted in a marginal 10–20% inhibition of luciferase activity in HEK-293 or NIH 3T3 cells. Site-directed mutation of the first miR-140-3p target on the CD38 3′-UTR partially reversed the inhibition by miR-140-3p mimic oligonucleotide in HEK-293 cells **(Fig. 3A)**. When the luciferase reporter assay studies were repeated in NIH 3T3 cells, site-directed mutation of the first miR-140-3p target completely reversed the luciferase inhibition by miR-140-3p mimic **(Fig. 3B)**. Mutation of both miR-140-3p targets on the CD38 3′-UTR resulted in elevated luciferase activity in the presence of miR-140-3p mimic oligonucleotides **(Fig. 3B).** To determine the effect of miR-140-3p mimic transfection on CD38 mRNA stability, NA-HASM and AS-HASM cells were transfected with 20 nM control oligonucleotide or miR-140-3p mimic. At 12 h following TNF-α exposure (0 h), mimic-transfected NA-HASM

and AS-HASM cells showed attenuated CD38 mRNA levels compared with control oligonucleotide-transfected cells **(Fig. 3C)**. There were no further reductions in CD38 mRNA levels in mimic-transfected cells at 6, 12, and 24 h in either group of HASM cells (**Fig. 3C**; 6- and 12-h data not shown).



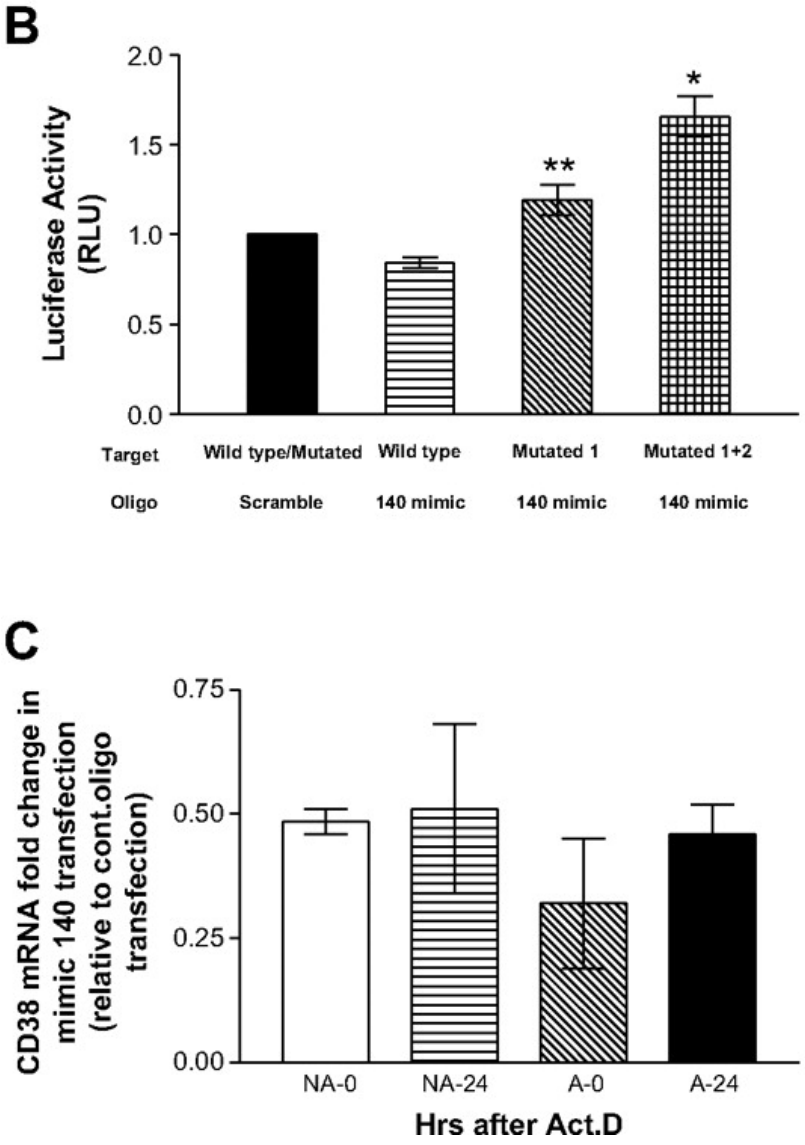
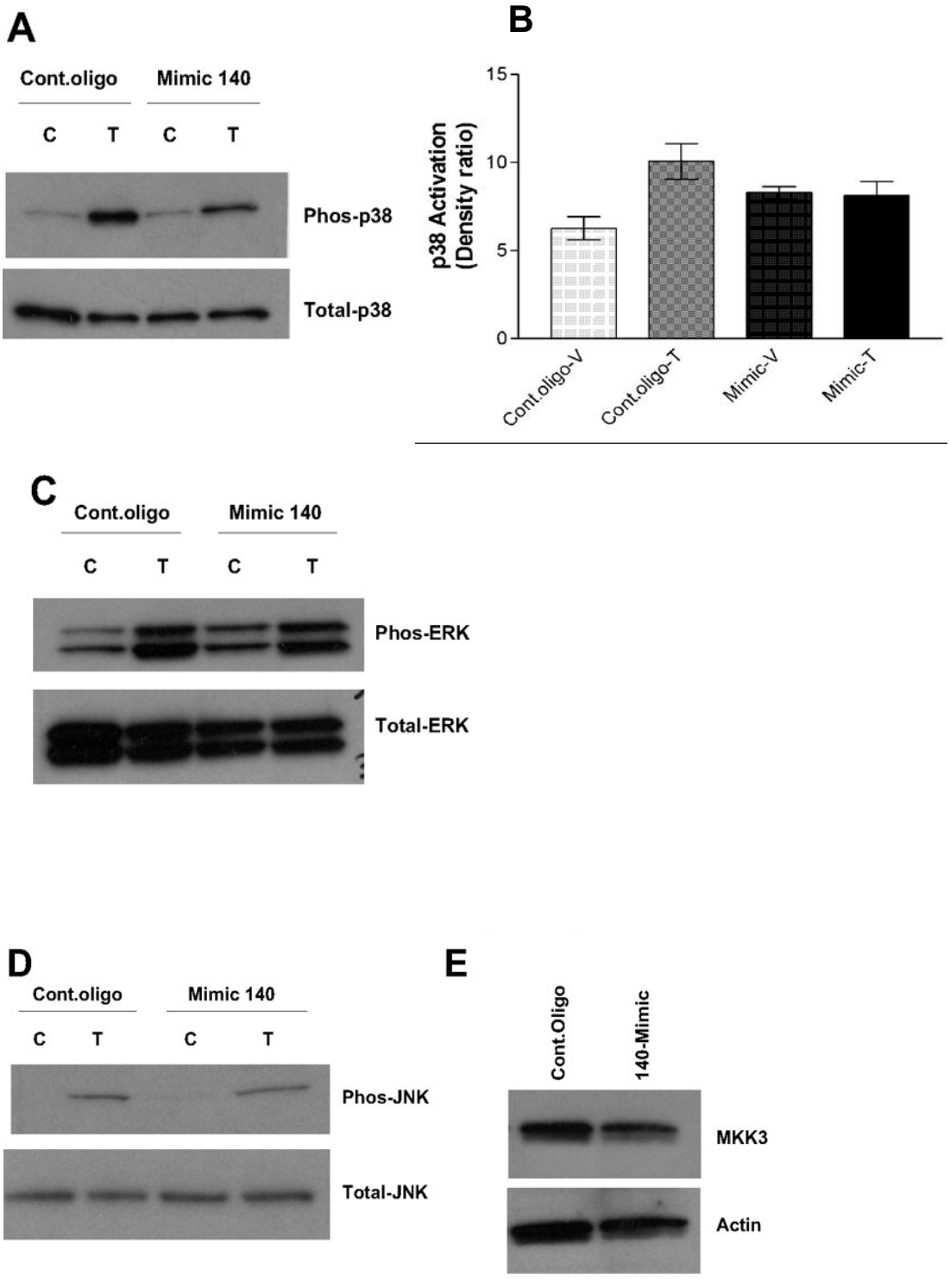


Fig. 3. miR-140-3p targets the CD38 3′-untranslated region (UTR). Luciferase reporter assays were performed in Luc-CD38-3′-UTR constructs containing wild-type or mutated (Mutated) target. **A**: 2 miR-140-3p targets on CD38 3′-UTR and mutations on each target (top). ORF, open reading frame. Bottom: in human embryonic kidney (HEK)-293 cells cotransfected with wild-type Luc-CD38-3′-UTR

and miR-140-3p mimic oligonucleotides, luciferase activity [relative light units (RLU)] was marginally reduced compared with cells cotransfected with scrambled-sequence oligonucleotides; in HEK-293 cells cotransfected with target-mutated Luc-CD38-3′-UTR and miR-140-3p mimic oligonucleotides, inhibition of luciferase activity was partially reversed (n = 3). **B**: in NIH 3T3 cells cotransfected with wild-type Luc-CD38-3′-UTR and miR-140-3p mimic oligonucleotides, luciferase activity was marginally reduced compared with cells cotransfected with scrambled-sequence oligonucleotides. When the first miR-140-3p target on Luc-CD38-3′-UTR was mutated (mutated 1), inhibition of luciferase activity was completely reversed. When both miR-140-3p targets on Luc-CD38-3′-UTR were mutated (mutated 1+2), luciferase activity was significantly elevated in the presence of miR-140-3p mimic oligonucleotides (n = 4). **C**: in NA-HASM and AS-HASM cells transfected with miR-140-3p mimic (mimic 140), CD38 mRNA levels were comparably attenuated at 0 h (NA-0 and A-0; following TNF-α removal and transcriptional arrest) compared with cells transfected with control oligonucleotides. There were no further reductions in CD38 mRNA levels at 6, 12, or 24 h (NA-24 and A-24) following transcriptional arrest in mimic-transfected cells of either group. *P < 0.05 vs. wild-type or mutated vector cotransfected with scramble oligonucleotide. **P < 0.05 vs. wild-type vector cotransfected with miR-140-3p mimic oligonucleotide. ActD, actinomycin D.

miR-140-3p mimic attenuates activation of p38 MAPK in HASM cells.

Results of the 3′-UTR luciferase reporter assay indicated that direct binding of miR-140-3p mimic on the 3′-UTR of CD38 only partially accounted for the inhibition of CD38 expression. We previously reported that the MAPKs mediate TNF-α-induced CD38 expression in HASM cells (322). Therefore, to determine whether changes in MAPK activation were involved in the miR-140-3p effect on CD38 expression, activation of p38, ERK, and JNK MAPKs was determined in HASM cells following transfection with miR-140-3p mimic oligonucleotides. Transfection with miR-140-3p mimic reduced the TNF-α-induced p38 phosphorylation, with no significant changes in the expression of total p38 **(Fig. 4, A and B)**. TNF-α-induced activation of ERK or JNK was not altered by transfection with miR-140-3p mimic **(Fig. 4, C and D).** Expression of MKK3, a Ser/Thr protein kinase upstream of p38 MAPK, was not altered in the presence of miR-140-3p mimic oligonucleotides **(Fig. 4E).** We also determined the expression of MKP-1, which is known to inactivate p38, ERK, and JNK MAPKs. Transfection of HASM cells with miR-140-3p antagomir or mimic oligonucleotides did not alter MKP-1 expression **(Fig. 4F).**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Cont.oligo
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Phos-p38
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15
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Mimic-V
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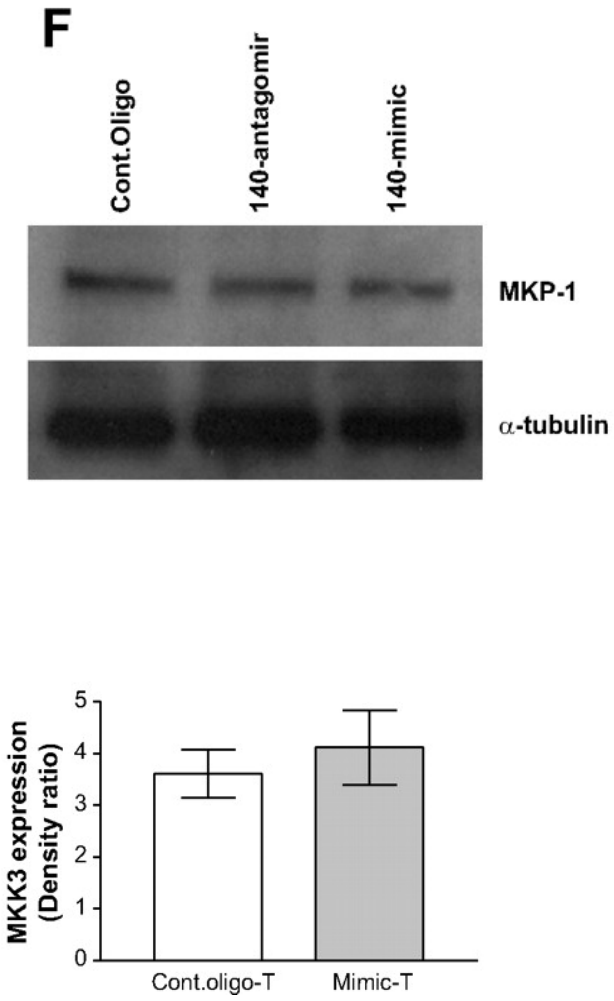
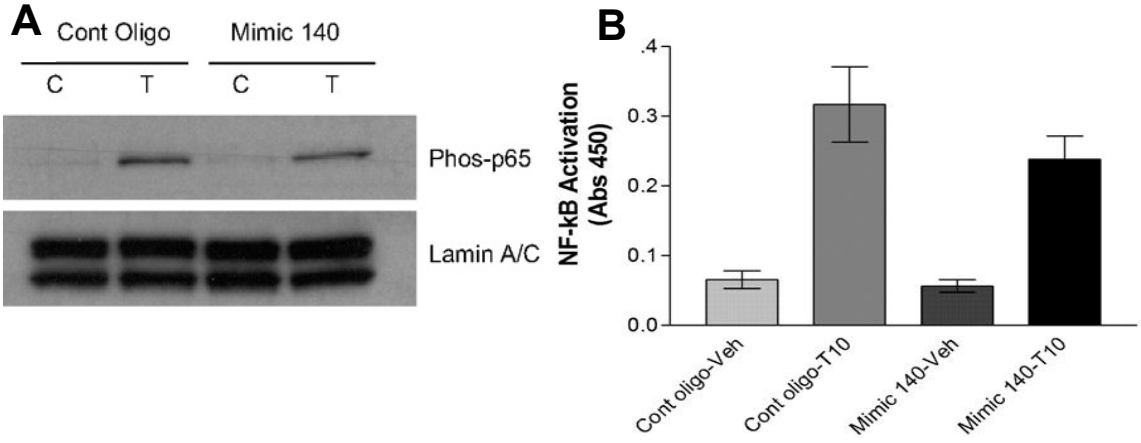


Fig. 4. miR-140-3p mimic attenuates activation of p38 MAPK in NA-HASM cells. TNF-α-induced activation of p38, ERK, and JNK MAPKs was determined in NA-HASM cells transiently transfected with 50 nM control oligonucleotides or miR-140-3p mimic oligonucleotide. **A:** representative Western blot showing marginally reduced TNF-α-induced p38 MAPK activation in miR-140-3p mimic transfection. Phos, phosphorylated. **B**: average relative densitometry measurement (n = 5) of Western blot showing marginally reduced p38 MAPK activation in miR-140-3p mimic-transfected NA-HASM cells. **C and D**: basal and

TNF-α-induced activation of ERK and JNK MAPKs was not altered in the presence of miR-140-3p mimics. Blots are representative of 5 independent experiments. **E:** expression of MAPK kinase 3 (MKK3), a kinase upstream of p38 MAPK, was not altered by transient transfection with miR-140-3p mimic oligonucleotides. Blot is representative of 3 independent experiments. **F:** representative blot showing unaltered expression of MKP-1, a dual-specificity phosphatase, in HASM cells transfected with miR-140-3p antagomir or miR-140-3p mimic oligonucleotides (n = 3).

miR-140-3p mimic attenuates activation of NF-κB in HASM cells.

We previously reported that the transcription factors NF-κB and AP-1 mediate TNF-α-induced CD38 expression in HASM cells (181, 322). Transient transfection of HASM cells with miR-140-3p mimic oligonucleotides marginally attenuated activation of the transcription factor NF-κB **(Fig. 5, A and B)**. Transfection with miR-140-3p did not have a significant effect on TNF-α-induced AP-1 activation **(Fig. 5C).** Expression of NRIP was not altered by miR-140-3p mimic transfection in HASM cells **(Fig. 5D).**



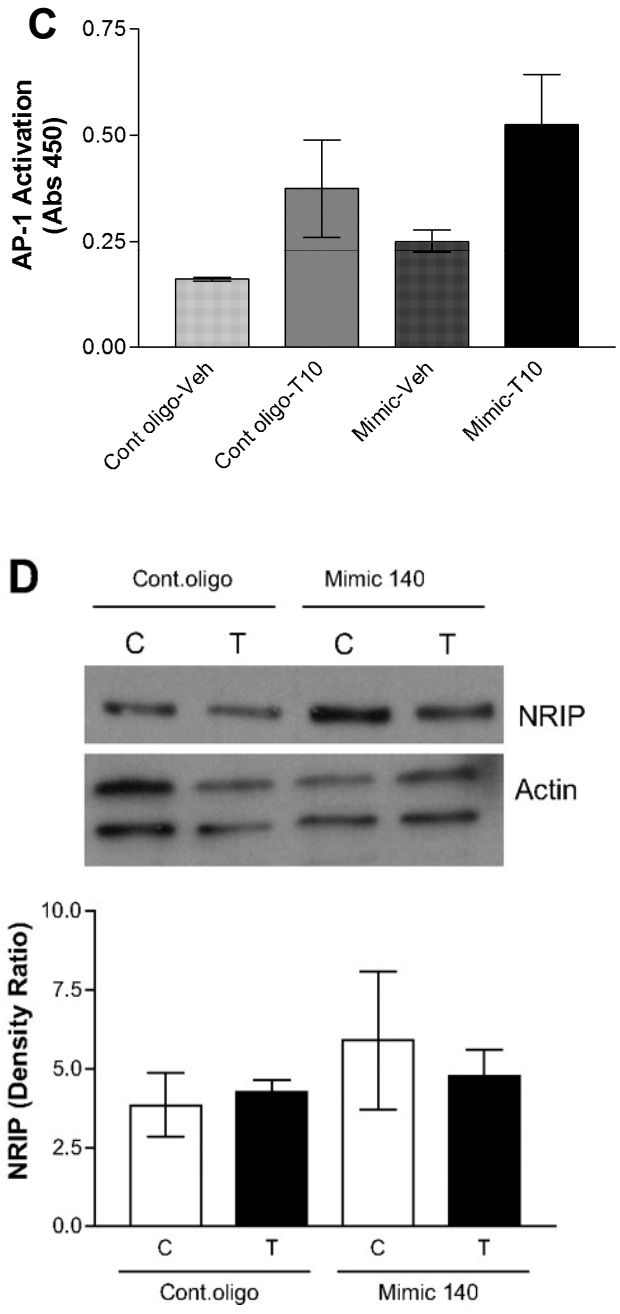
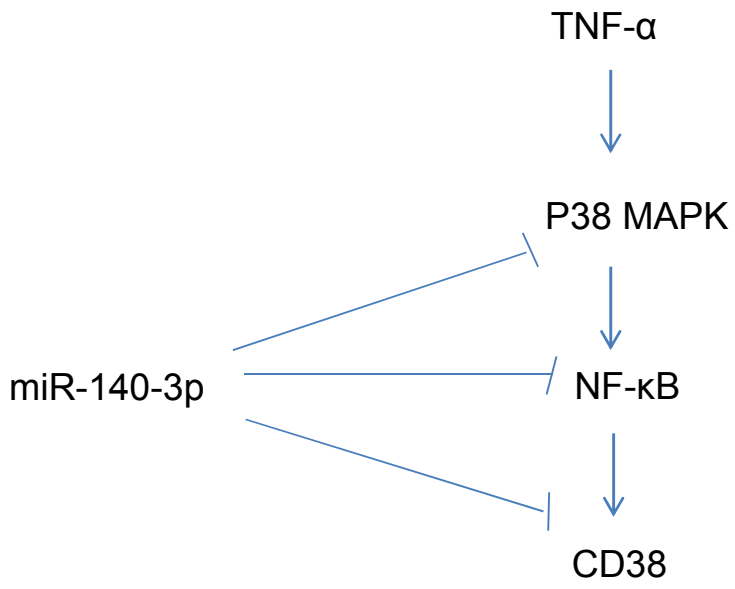


Fig. 5. miR-140-3p mimic attenuates activation of transcription factor NF-κB in NA-HASM cells. TNF-α-induced activation of NF-κB was determined in NA-HASM cells transiently transfected with 50 nM control oligonucleotide or miR-140-3p mimic oligonucleotides. **A:** TNF-α-induced nuclear translocation of NF-κB

(phosphorylated p65 subunit) was marginally attenuated in NA-HASM cells transfected with miR-140-3p mimic oligonucleotides. **B:** TNF-α-induced activation of NF-κB, measured as binding of p65 subunit to a consensus DNA motif [absorbance at 450 nm (Abs 450)], was marginally reduced in NAASM cells transfected with miR-140-3p mimic oligonucleotides (n = 5). **C:** TNF-α-induced activation of AP-1 (measured as binding of phosphorylated c-Jun to consensus DNA sequence) was unaltered in the presence of miR-140-3p mimic oligonucleotides (n = 4). **D:** expression of nuclear receptor-interacting protein (NRIP), which is associated with NF-κB activation in other cell systems, was not altered in HASM cells transfected with mimic 140 (n = 3). Blots (A and D) are representative of results from 3 independent experiments. C, vehicle; T, TNF-α.

Discussion

This is the first report of the regulatory role of miR-140-3p in CD38 expression in HASM cells. Our findings show that miR-140-3p expression is lower in HASM cells from donors with a history of severe asthma, in the presence of TNF-α. Our findings also indicate that the effects of miR-140-3p on CD38 expression are mediated through direct binding of miRNA to the CD38 transcript and indirect mechanisms involving activation of p38 MAPK and NF-κB. The net result of the modest inhibition of activation of p38 and NF-κB by miR-140-3p transfection is the robust attenuation of CD38 expression in HASM cells.



Previous studies conducted in our laboratory established that TNF-α-induced CD38 expression in HASM cells is mediated at the transcriptional and posttranscriptional levels (181, 322). The MAPKs ERK1/2 and p38 play a role in posttranscriptional regulation, whereas p38 and JNK MAPKs have a role in transcriptional regulation of the CD38 gene (322). One of the objectives of the current study was to determine the role of miR-140-3p in the regulation of CD38 expression. Studies by other investigators revealed that specific miRNAs, such as miR-21 and miR-133a, contribute to the pathogenesis of airway inflammatory disorders (66, 221). Chiba et al. (66) showed that IL-13, a Th2 cytokine with a prominent role in allergic asthma, downregulates the expression of miR-133a. It is suggested that the downregulation of miR-133a leads to elevated RhoA, a procontractile protein in the ASM cells. CD38 contributes to the development of AHR in mouse models of asthma (133, 134). The CD38-null mouse developed significantly lower levels of airway responsiveness than the wild-type mouse in response to the contractile agonist methacholine (89). The CD38-null mouse also developed reduced AHR compared with the wild-type mouse following brief exposure to TNF-α or IL-13 (133, 134). Therefore, investigating the miRNAs that target CD38 gene expression in HASM cells may lead to an understanding of the signaling pathways involved in the pathogenesis of AHR and asthma.

Multiple web-based target prediction algorithms [Target Scan (www.targetscan.org), miRWalk (www.ma.uni-heidelberg.de), and miRbase (www.mirbase.org)] were used to determine potential miRNA targets in the CD38 3′-UTR. From the list of predicted miRNAs, miR-140-3p was chosen for further

investigation, because it has been reported as one of the highly expressed miRNAs in HASM cells (354). Furthermore, in HASM cells exposed to a mixture of inflammatory cytokines including TNF-α, there is significant downregulation of several miRNAs, including miR-140 and some miRNAs involved in the regulation of the smooth muscle phenotype (197). In earlier publications by other investigators, miR-140 has been reported as a cartilage-specific miRNA in mouse and zebrafish (329, 351). However, these reports were largely referring to miR-140-5p, one of the two mature miRNAs originating from the precursor miR-140. In the present study, we focused on miR-140-3p and its role in regulation of CD38 expression.

An altered miRNA expression profile has been reported in T cells obtained from patients with severe asthma, indicating that miRNAs are among the mechanisms involved in the cellular phenotypic changes observed in asthma (328). Attenuation of miR-140-3p expression in AS-HASM cells by TNF-α suggests that this miRNA may have a role in the asthmatic phenotype in ASM cells. The mechanisms involved in miR-140-3p downregulation by TNF-α in HASM cells are yet to be determined. Recent studies in human chondrocytes showed that the proximal upstream region of pri-miR-140 has functional response elements for chondrogenic transcription factors Sox5/Sox6/Sox9, indicating transcriptional regulation of miR-140-3p and miR-140-5p expressions (363). Different transcriptional regulators and epigenetic mechanisms such as DNA methylation may be involved in the altered miR-140-3p expression in AS-HASM cells. The finding that miR-140-3p expression is attenuated to a larger

magnitude in AS-HASM cells in the presence of TNF-α suggests an anti-inflammatory role for this miRNA. The potential anti-inflammatory role for miR-140-3p is supported by previously reported findings of downregulated miR-140 expression in whole lung lysate from rats exposed to cigarette smoke extract (167). We also found that IL-13, a Th2 cytokine with a major role in asthma, did not alter the expression levels of miR-140-3p in NA-HASM or AS-HASM cells (data not shown). These observations suggest that miR-140-3p may have a functional role selective to TNF-α signaling. TNF-α is a cytokine with a major role in the pathogenesis of asthma (4, 321). TNF-α expression is elevated in the airways of asthmatic patients, and some recent therapeutics for asthma target TNF-α and its receptors in lungs (34, 45, 160, 367). Therefore, defining the role of miRNAs involved in the regulation of TNF-α-induced genes in ASM may have a therapeutic potential. Although TNF-α attenuates miR-140-3p expression to a greater magnitude in AS-HASM cells, it does not appear to solely contribute to the differential induction of CD38 expression by TNF-α that we reported in a recent study (178).

Although the inhibitory effect of miR-140-3p mimic on luciferase activity was modest, mutation of both miR-140-3p target sites reversed the inhibition, indicating binding of the miRNA to the 3′-UTR of CD38. Since the 3′-UTR of the CD38 transcript possesses targets for other miRNAs as well, a significant effect on stability or translatability of the CD38 transcript may require multiple miRNAs. However, the significant inhibition of CD38 mRNA and protein expression by miR-140-3p mimic oligonucleotides suggests that additional mechanisms are

involved in miR-140-3p regulation of CD38 expression in HASM cells. In this context, we found that miR-140-3p mimic attenuated p38 MAPK activation with no apparent effects on ERK or JNK MAPKs, demonstrating the selectivity of miR-140-3p for specific targets on cell signaling pathways in HASM cells. The marginal inhibitory effect of miR-140-3p mimic on p38 MAPK activation does not appear to be related to the upstream kinase MKK3 or the MAPK dual-specificity phosphatase. However, it remains to be determined whether other MAPK phosphatases with higher substrate specificity to p38 MAPK mediate the miR-140-3p-mediated attenuation of p38 activation.

The significant attenuation of TNF-α-induced CD38 mRNA expression by miR-140-3p mimic suggests that the regulation by miRNA may be through transcription of the CD38 gene in HASM cells. The transcription factors NF-κB and AP-1 mediate TNF-α-induced CD38 expression in HASM cells (181). The role of NF-κB signaling in airway inflammatory disorders has been demonstrated through in vitro studies and animal models (90, 318). For this reason and because of the availability of numerous synthetic inhibitors of NF-κB signaling, this pathway remains an attractive therapeutic target in airway inflammatory disorders such as asthma and chronic obstructive pulmonary disorder. Although the DNA binding activity of NF-κB was only marginally reduced by miR-140-3p mimic transfection, the reduction was a consistent finding in five independent experiments. Furthermore, a recent study reported attenuation of NF-κB activation by miR-140-3p in hepatocytes, primarily by targeting NRIP-1, a coactivator of NF-κB (316). This is an unlikely mechanism in HASM cells, since

NRIP-1 expression was unaltered in cells transfected with miR-140-3p. The results of CD38 mRNA stability also showed that, following transcription arrest, CD38 mRNA levels were maintained for up to 24 h. The expression levels of miR-140-3p remain high for up to 72 h following transfection. If the effects of miR-140-3p on CD38 transcript levels were due to binding to 3′-UTR target sites, we would have seen a significant decline in transcript levels following transcription arrest. A lack of correlation between miR-140-3p and CD38 expression following TNF-α treatment strengthens the argument that miR-140-3p effects are largely indirect and time-dependent. Furthermore, overexpression of miR-140-3p causes a significant decline in CD38 transcript levels before arrest of transcription, with no further decline in transcript levels for up to 24 h, favoring a mechanism that involves indirect transcriptional regulation of CD38 expression in HASM cells.

In summary, miR-140-3p regulates TNF-α-induced CD38 expression in HASM cells through direct interaction with the 3′-UTR of CD38 mRNA and indirect mechanisms involving activation of p38 MAPK and the transcription factor NF-κB. However, we cannot rule out other indirect mechanisms, such as competing endogenous RNAs (320), in the regulation of CD38 expression in HASM cells. Understanding the signaling pathways involved in miR-140-3p regulation of CD38 expression in HASM cells may reveal novel therapeutic targets for AHR and asthma.

Grants

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CHAPTER iii

MicroRNA-708 regulates CD38 expression through signaling pathways JNK MAP kinase and PTEN/AKT in human airway smooth muscle cells

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Background

The cell-surface protein CD38 mediates airway smooth muscle (ASM) contractility by generating cyclic ADP-ribose, a calcium-mobilizing molecule. In human ASM cells, TNF-α augments *CD38* expression transcriptionally by NF-κB and AP-1 activation through inducing MAPK and PI3K signaling. *CD38*$^{-/-}$ mice develop attenuated airway hyperresponsiveness following allergen or cytokine challenge. The post-transcriptional regulation of *CD38* expression in ASM is relatively less understood. In ASM, microRNAs (miRNAs) regulate inflammation, contractility, and hyperproliferation. The 3' Untranslated Region (3'UTR) of *CD38* has multiple miRNA binding sites, including a site for miR-708. MiR-708 is known to regulate PI3K/AKT signaling and hyperproliferation of other cell types. We investigated miR-708 expression, its regulation of CD38 expression and the underlying mechanisms involved in such regulation in human ASM cells.

Growth-arrested human ASM cells from asthmatic and non-asthmatic donors were used. MiRNA and mRNA expression were measured by quantitative real-time PCR. CD38 enzymatic activity was measured by a reverse cyclase assay. Total and phosphorylated MAPKs and PI3K/AKT as well as enzymes that regulate their activation were determined by Western blot analysis of cell lysates following miRNA transfection and TNF-α stimulation. Dual luciferase reporter assays were performed to determine whether miR-708 binds directly to *CD38* 3'UTR to alter gene expression.

Using target prediction algorithms, we identified several miRNAs with potential *CD38* 3'UTR target sites and determined miR-708 as a potential

candidate for regulation of *CD38* expression based on its expression and regulation by TNF-α. TNF-α caused a decrease in miR-708 expression in cells from non-asthmatics while it increased miR-708 expression in cells from asthmatics. Dual luciferase reporter assays in NIH-3 T3 cells revealed regulation of expression by direct binding of miR-708 to *CD38* 3'UTR. In ASM cells, miR-708 decreased *CD38* expression by decreasing phosphorylation of JNK MAPK and AKT. These effects were associated with increased expression of MKP-1, a MAP kinase phosphatase and PTEN, a phosphatase that terminates PI3 kinase signaling.

In human ASM cells, miR-708 regulates the TNF-α-induced-*CD38* expression directly by binding to 3'UTR and indirectly by regulating JNK MAPK and PI3K/AKT signaling and has the potential to control airway inflammation, ASM contractility and proliferation.

Introduction

The cell-surface protein CD38 mediates calcium signaling in airway smooth muscle (ASM) and innate immune responses (268). The ADP-ribosyl cyclase activity associated with CD38 converts β-nicotinamide adenine dinucleotide (β-NAD) to cyclic adenosine diphosphoribose (cADPR) and adenosine diphosphoribose (ADPR) (205). In previous investigations in ASM, we showed that cADPR mediates calcium release from the sarcoplasmic reticulum and promotes contractility (87, 88). Furthermore, *CD38*$^{-/-}$ mice exhibit attenuated methacholine responsiveness and airway hyperresponsiveness (AHR) following allergen sensitization and challenge as well as after intranasal IL-13 challenge (89, 120, 134). ASM cells obtained from *CD38*$^{-/-}$ mice exhibit attenuated intracellular calcium responses to contractile agonists relative to cells obtained from wild-type mice (89). These observations indicate a prominent role of *CD38* in AHR, a cardinal feature of asthma in humans.

The transcriptional regulation of *CD38* in ASM involves the transcription factors NF-κB and AP-1 and signal transduction mechanisms involving activation of MAP kinases and PI3 kinase (179, 322). *CD38* is ubiquitously expressed in many cell types in addition to ASM cells and its expression is augmented by inflammatory and Th2 cytokines including TNF-α and IL-13 (87),(24) (2, 67, 93, 116, 186, 196). While the transcriptional regulation of *CD38* expression has been thoroughly investigated in mammalian cells, there is a paucity of information regarding post-transcriptional regulation of its expression. In this regard, we recently reported evidence for such regulation involving the microRNA (miRNA) miR-140-3p (177).

In human ASM cells, human recombinant TNF-α-(*rh*-TNF-α)-induced *CD38* expression is attenuated by miR-140-3p through both direct binding to the 3' Untranslated Region (3'UTR) of *CD38* as well as indirect mechanisms involving activation of p38 MAP kinase and the transcription factor NF-κB. *CD38* 3'UTR is ~481b long and has multiple miRNA binding sites, including a site for miR-708. Prior studies have revealed a prominent regulatory role of miR-708 in the expression of phosphatase and tensin homolog (*PTEN*), which in turn regulates PI3 kinase signaling through activation of AKT(340). In other cell types, PI3 kinase/AKT signaling regulates expression of target genes by activating the proinflammatory transcription factor NF-κB (215). An effect of miR-708 on *PTEN* expression is expected to profoundly affect cytokine-induced *CD38* expression in ASM cells by modulating PI3 kinase signaling. In ASM cells obtained from asthmatics, *rh*-TNF-α induces significantly greater *CD38* expression compared to its expression in cells from non-asthmatics(178). In this study, we investigated the expression of miR-708, its potential additive role with miR-140-3p in the regulation of *CD38* expression and the underlying mechanisms involved in such regulation in human ASM cells. We also examined miR-708 expression and its effects on *CD38* expression in ASM cells obtained from asthmatics to determine whether the augmented cytokine-mediated *CD38* expression stems from altered regulation through miR-708.

Materials and methods

Reagents

DMEM was from GIBCO-BRL (Grand Island, NY); *rh*-TNF-α was from R&D Systems (Minneapolis, MN); TRIzol, SuperScript III reverse transcriptase, NCode miRNA first-strand synthesis kit, Platinum SYBR Green quantitative PCR (q-PCR) mix, Opti-MEM® reduced serum medium and Lipofectamine® RNAiMax transfection reagent were from Invitrogen Life Technologies (Carlsbad, CA); Brilliant lll Ultra-Fast SYBR Green q-PCR Master Mix from Agilent Technologies, Inc (Santa Clara CA); Fugene HD transfection reagent was from Roche Diagnostics (Indianapolis, IN); QuikChange Lightning Multi Site-Directed mutagenesis kit was from Agilent Technologies, Inc (Santa Clara, CA); control oligo (scrambled sequence mimic), miR-708 mimic (mature miR-708 sequence: 5'-AAGGAGCUUACAAUCUAGCUGGG-3'), and antagomir oligonucleotides were from Dharmacon (Lafayette, CO); Dual Luciferase Reporter Assay System was from Promega (San Luis Obispo, CA); NIH-3 T3 cells were from ATCC (#CRL-1658, Manassas, VA); chemiluminescent substrate for horseradish peroxidase (HRP) was from Millipore (Billerica, MA); rabbit primary antibodies against major MAPK family, PTEN, AKT2, AKT and β-actin as well as anti-rabbit secondary antibody were from Cell Signaling Technology (Danvers, MA); mouse primary antibodies for MKP-1, α-actin and goat-anti mouse antibodies were from Santa Cruz Biotechnology (Dallas, TX). Tris-base, glucose, HEPES and other chemicals were from Sigma Chemical Co. (St. Louis, MO) unless otherwise mentioned.

Culture and transfection of HASM cells

Isolation, culture and maintenance of HASM cells was carried out as described in our previous publications (87, 178, 323) Briefly, HASM cells obtained from unidentified healthy donors or from fatal asthmatics were maintained in culture and used until the smooth muscle phenotype is sustained (2–5 passages). DMEM supplemented with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 g/ml amphotericin B was used to grow the HASM cells up to 80% confluency. Rh-TNF-α (10 ng/ml) was used to induce the expression of CD38 in HASM cells while control cells were treated with 0.1% BSA in PBS. Since human cells used in this study were obtained from unidentified donors, it is considered Exempt under National Institutes of Health guidelines. Drs. Panettieri and Kannan have Institutional Review Board approval for the use of these cells in the study.

Transient transfection of primary HASM cells with miR-708 mimic, antagomir or scrambled sequence mimic at 10–100 nM was carried out in the presence of Lipofectamine® RNAiMax transfection reagent. Reduced serum Opti-MEM® medium was used as a base to prepare the transfection complex containing Lipofectamine and the oligonucleotides. After 20 min of incubation at room temperature the transfection complex was gently dropped on to the cell suspension (1.5-2.0 × 10^5 cells/well in 24 well plates or 2.5-3.0 × 10^5 cells/well in 6 well plates) which was seeded few minutes (<3 min) prior to the addition of transfection complex. Transfected cells were incubated at 37°C for 24 h, growth

arrested for 24 h, exposed to *rh*-TNF-α for another 24 h before isolation of total RNA or total protein.

Total RNA isolation

PureLink and *mir*Vana RNA isolation kits (Ambion Life Technologies, Carlsbad, CA) were used to isolate total large RNA and small RNA, respectively, according to the manufacturer's instruction.

cDNA synthesis and quantitative q-PCR

NCode™ miRNA First-Strand-cDNA synthesis kit was used to synthesize cDNA followed by q-PCR for miRNAs using the Platinum q-PCR Kit according to the manufacturer's instruction. Briefly, expression of miR-708 at constitutive levels and after induction with *rh*-TNF-α was measured by q-PCR after poly-adenylation. Whole miR-708 sequence was employed as a specific forward primer and universal q-PCR primer was used as a reverse primer. Mammalian small nuclear RNA U6, a spliceosomal RNA, served as a control housekeeping gene. To measure changes in the expression of *CD38 (178, 322)* and *JNK* at transcript levels (following miR-708 mimic or scrambled sequence mimic transfection), q-PCR was performed using Brilliant SYBR Green Master Mix. Primers for JNK were selected using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and performed in the Stratagene Mx3000p q-PCR system, under the following conditions: 1 cycle of 95°C for 5-min segment, 40 cycles of 95°C for 30 s, 59°C for 30 s, 72°C for 45 s, and a final 1 cycle of 95°C for 1 min, 59°C for 30s and 95°C for 30s (JNK forward primer 5'-CACCACCAAAGATCCCTGACA-3'; JNK reverse primer 5'-

CTGTGCTAAAGGAGAGGGCT-3'). Expression level of *cyclophilin* was used to normalize the expressions of *CD38* and *JNK*.

mRNA stability

HASM cells were transfected with miR-708 mimic or scrambled sequence mimic. After 24 h, transfection medium was replaced with growth-arrest medium for 24 h. Cells were then exposed to *rh*-TNF-α for 12 h in fresh growth arrest medium which was replaced with fresh growth arrest medium containing actinomycin D (5 μg/ml) to inhibit transcription. At different time points (0, 6, 12 and 24 h), cells were collected for isolation of total RNA to evaluate the rate of decay of CD38 transcript by q-PCR.

Western blot

Twenty four hours after transfection of ASM cells with miRNA, cell growth was arrested for 24 h (with serum-free media containing transferrin and insulin) followed by treatment with *rh*-TNF-α for an additional 24 h. Cells were collected in the lysis buffer (50 mM Tris, 100 mM NaCl, 50 mM NaF, 40 mM β-glycerol phosphate, 2 mM EDTA, 0.2 mM Na_3VO_4, 1% Triton X-100, and protease inhibitor cocktail, pH 7.4), lysed by sonication and total protein concentration in the lysates estimated by Bradford assay (Bio-Rad Laboratories, Inc, Hercules, CA). Equal amounts of total protein (5–15 μg) were separated by SDS-PAGE (4-20% gradient gels), transferred onto a PVDF membrane and blocked overnight in 5% skim milk in PBS containing 0.05% Tween 20 (PBST) at 4°C. Incubation of membranes with primary antibodies was carried out in 1% skim milk in PBST at 4°C overnight. After three 5-minute washes with PBST, membranes were

incubated with HRP-conjugated secondary antibodies in 1% milk for 1 h at room temperature. Primary rabbit antibodies against all major MAPKs, β-actin, PTEN, Pan AKT and AKT2 were used at a dilution of 1:1000 except antibodies to detect p38 and phospho-p38 which were used at a dilution of 1:500. Primary mouse antibodies (smooth muscle-specific α-actin and MKP-1) were used at a dilution of 1:700. Anti-rabbit secondary antibodies were used at a dilution of 1:5000, while goat anti-mouse secondary antibodies were used at a dilution of 1:4000. To determine the activation of MAPKs and AKT following miR-708 transfection, cells were collected at 20 min or 2 h respectively after treatment with *rh*-TNF-α and lysed. After electrophoresis, same membranes were probed for phosphorylated and total protein expression, stripping membranes (Restore Plus Western Blot Stripping Buffer [Thermo Fisher Scientific Co., Pittsburgh], 30 min) between blots. Intensity of protein bands was measured using ImageJ image analysis software and the level of expression of phosphorylated protein relative to total protein was considered as a measure of activation. In the case of MKP-1, PTEN and total MAP kinase protein expression, cells were collected 24 h after stimulation with *rh*-TNF-α and changes in expression level relative to loading control (smooth muscle specific α-actin or β- actin) were measured.

Reverse ADP-ribosyl cyclase assay

The enzymatic function of CD38 was quantified by reverse cyclase assay as described in our earlier publication (178). Briefly, equal amount of (5 -15 μg) total proteins in human ASM (HASM) cell lysates were incubated at 37°C for 1 h with cADPR (0.45 mM) in the presence or absence of nicotinamide (10 mM). The

enzymatic reaction was terminated by adding 25 µl of 1 M HCl, and contents were vacuum filtered (0.45 µm, Immobilon, Millipore), neutralized (15 µl of 2 M Tris-base), and incubated at room temperature with a mixture of the following reagents: rezasurin (2 µM), ethanol, flavin mononucleotide (4 µM), alcohol dehydrogenase (40 µg/ml) and diaphorase (0.04 U/ml) in phosphate buffer (Na_2HPO_4/NaH_2PO_4). FLUO star Galaxy fluorometer was used to quantify the rate of fluorescence emitted at 590 nm which is proportionate to the amount of NAD generated by the CD38 present in 5–15 µg of total protein.

Mutation of miR-708 target site and dual luciferase reporter assay

Location of miR-708 target site at the 3'UTR of CD38 transcript is 21 bases away from the stop codon. Mutation of four bases (ctCgCCg) at the target site (AGCTCCT) specific for miR-708 was achieved using the QuikChange Lightning Multi Site-Directed mutagenesis kit. The primer sequence designed for the mutation consists of target sequence and 14 bases flanking the target sequence (5'-GCTGTGGTTGTTTT***CTCGCCG***TGACTCCTTGTGGT-3'). XL10-GOLD Ultra competent *E. coli* was used to expand the wild-type and mutant plasmids. NIH-3 T3 cells seeded in 24 well plates (1.5-2.0 × 10^5 cells/well) were co-transfected with miR-708 mimic or scrambled sequence mimic (50 nM), wild-type (or mutated) firefly Luc-CD38-3'UTR-reporter plasmid (200 ng/well) and Renilla luciferase plasmid (control) (30 ng/well), in culture medium (Opti-MEM® reduced serum medium; 100 µl), facilitated by Fugene HD transfection reagent (1 µl/well). After 24 h, cells were collected in lysis buffer and luciferase activity in triplicate samples for each condition was measured with a luminometer according to the

manufacturer's recommendation (Synergy 2 microplate reader, BioTek, Winooski, VT). Renilla luciferase activity was used to normalize the firefly luciferase activity.

Statistical analysis

HASM cells obtained from 3–8 different donors were used. Values represent means ± SEM. Data were analyzed for statistical significance by Student's *t*-test or one-way ANOVA (depending on the number of experimental groups analyzed) using GraphPad Prism 6 Software. Differences were considered significant at $p < 0.05$.

Results

Differential expression of miR-708 in HASM cells

In order to investigate the potential regulation of *CD38* expression by multiple miRNAs, we first analyzed the *CD38* 3'UTR for miRNA binding sites using target prediction algorithms. MiRNAs miR-1272, miR-548, miR-208a, miR-1298, miR-708 and miR140-3p were predicted to bind to the *CD383*'UTR with high context score. Expression levels of these miRNAs in HASM cells were evaluated by q-PCR. As co-expression of miRNA(s) along with its target gene transcript is required for regulation, using a cycle threshold (ct) cut-off, miRNAs with ct values above 34 in HASM cells were filtered out **(Figure 1A).** Other miRNAs (miR-708 and miR140-3p) which showed ct values ranging from 20–30 **(Figure 1A)** were selected for further studies.

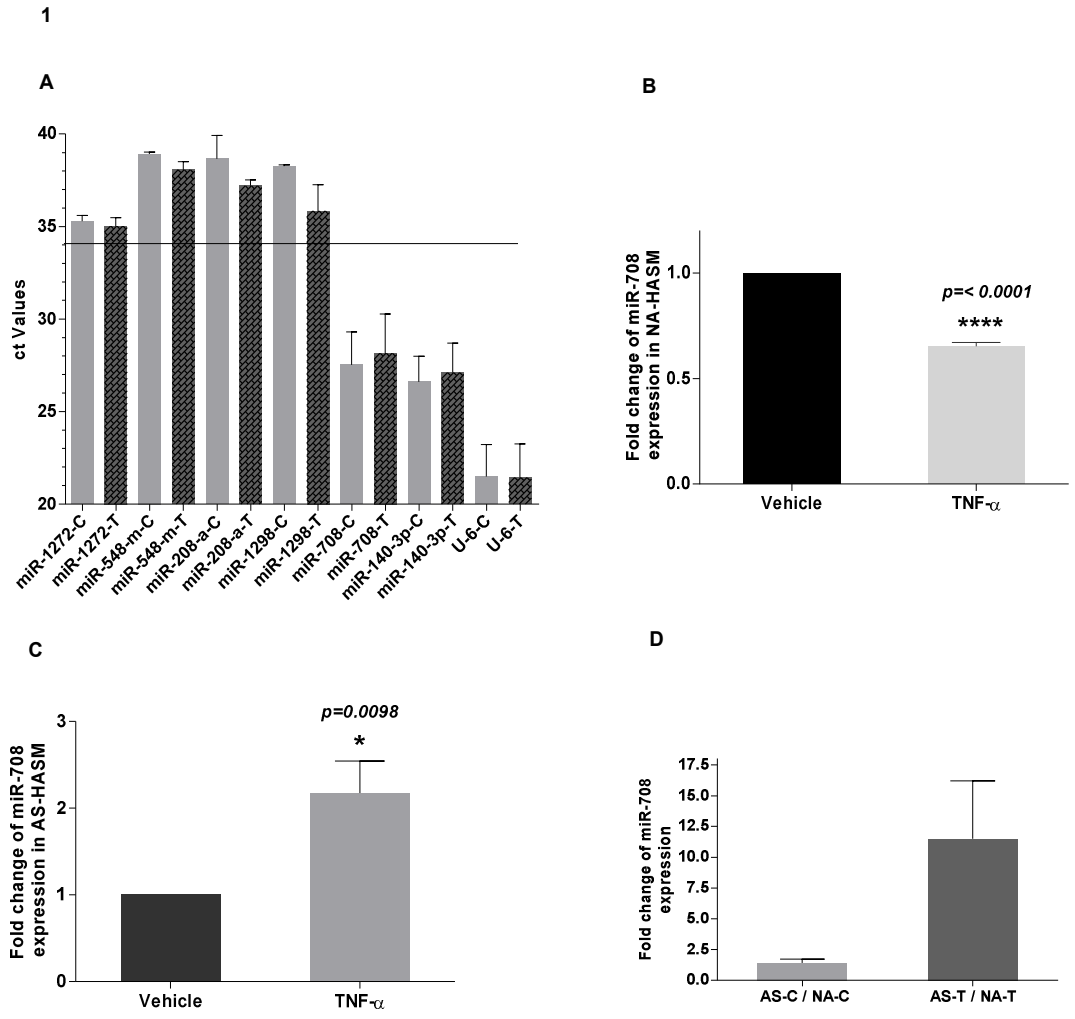


Figure 1. Expression of miR-708 in *rh*-TNF-α-treated ASM cells. A*:*Analysis of miRNAs that are predicted to target *CD38* by q-PCR. Expression levels of these miRNAs in HASM cells with and without TNF-α treatment were normalized with small nuclear RNA U6. miRNAs with ct values above and below 34 in HASM cells are indicated by the horizontal line (n = 3–4 donors). **B** and **C***:* miR-708 expression in HASM cells from non-asthmatics (NA-HASM) and asthmatics (AS-HASM), respectively. *Rh*-TNF-α produced a significant reduction in miR-708 expression in growth-arrested NA-HASM cells compared with cells treated with vehicle (n = 8 donors), while it produced a significant increase in its expression in

AS-HASM cells (n = 6 donors). **D***:* miR-708 expression in AS-HASM cells is higher than in NA-HASM cells both under vehicle and TNF-α-treated conditions (3–5 donors/group). Values are shown as mean ± SEM. C-vehicle-treated cells (0.1% BSA); T- *rh*-TNF-α (10 ng/ml) treated cells.

To evaluate the expression of miR-708 in HASM cells obtained from non-asthmatic and asthmatic donors in the presence of *rh*-TNF-α, q-PCR was performed. In non-asthmatic HASM (NA-HASM) cells, *rh*-TNF-α produced a significant (*p < 0.0001*) reduction in the expression of miR-708 **(Figure 1B)** compared to expression in unstimulated (vehicle-treated) cells. In contrast, *rh*-TNF-α exposure significantly increased the expression of miR-708 *(p = 0.0098)* in asthmatic-HASM (AS-HASM) cells **(Figure 1C).** Further, expression of miR-708 in AS-HASM cells was found to be higher in vehicle treated (~2-fold) and *rh*-TNF-α treated (>10-fold) cells when compared to NA-HASM cells **(Figure 1D).**

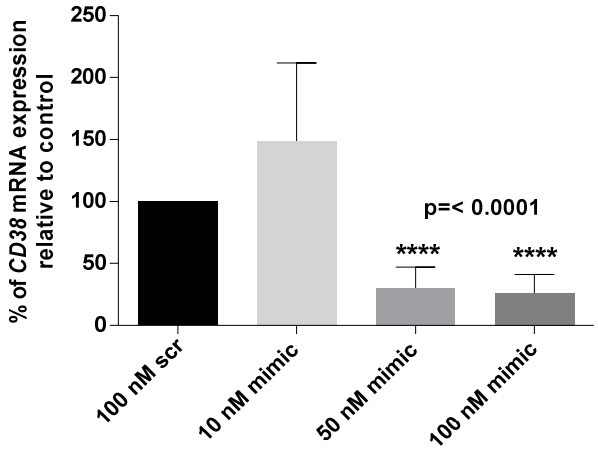
miR-708 inhibits *CD38* expression and its enzymatic activity in HASM cells

To examine whether miR-708 alters the expression level of *CD38*, NA-HASM cells were transiently transfected with different concentrations (10nM, 50nM and 100nM) of miR-708 mimic. Over-expression of miR-708 mimic at 50 nM and 100 nM concentrations significantly decreased CD38 transcript level **(Figure 2A).** This finding was further confirmed in AS-HASM where miR-708 mimic at 50 nM significantly inhibited CD38 transcript level relative to the scrambled sequence mimic **(Figure 2B).** Further, transfection of AS-HASM cells and NA-HASM cells with miR-708 at a concentration of 50 nM decreased the enzymatic activity of

CD38 measured by ADP-ribosyl cyclase assay **(Figure 2C)**. To establish the specificity of the inhibitory effect of endogenous miR-708 on *CD38* expression, NA-HASM cells were transfected with miR-708 mimic, scrambled sequence mimic or the antagomir of miR-708 mimic. *Rh*-TNF-α-induced *CD38* expression following miR-708-antagomir transfection was similar to the expression in cells transfected with scrambled sequence mimic **(Figure 2D).**

2

A



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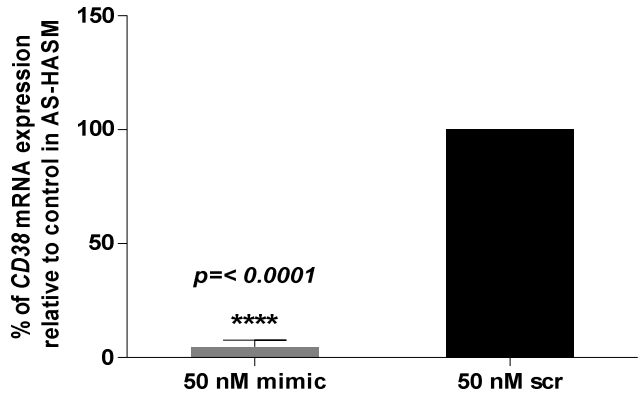


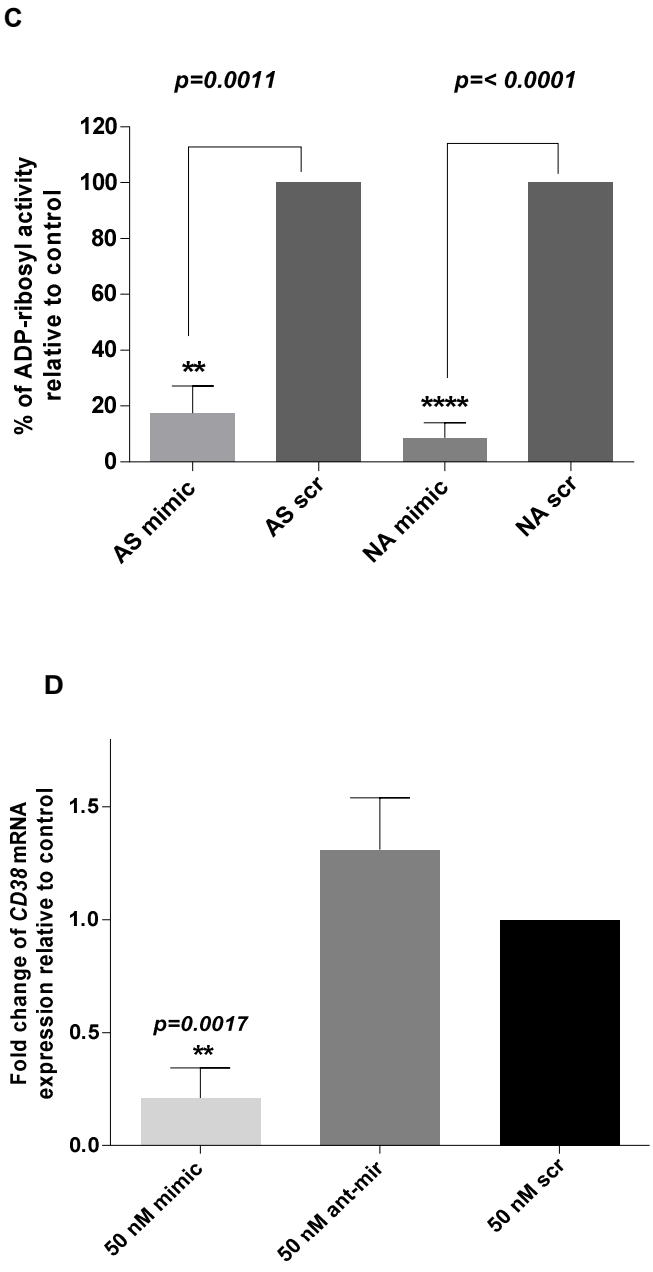
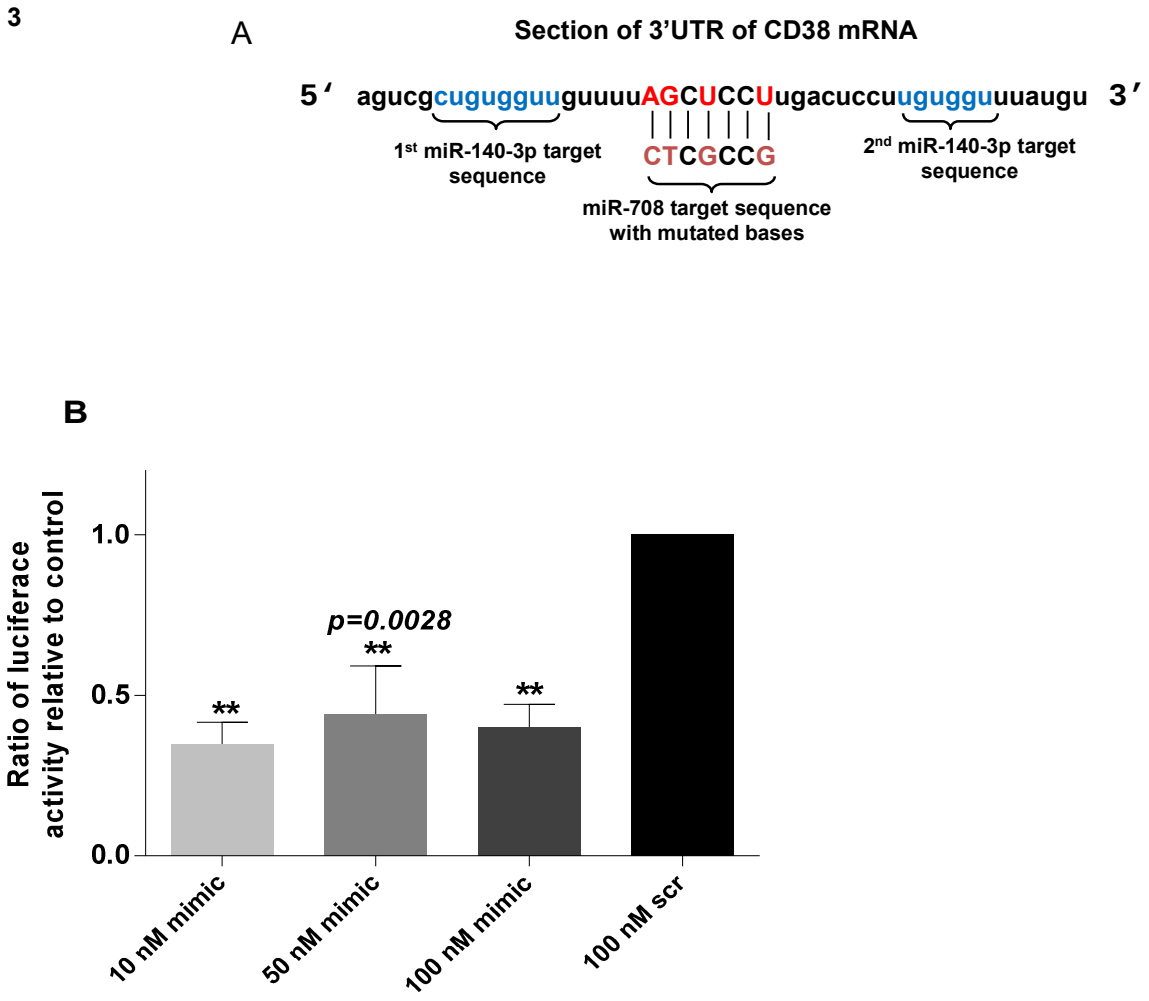


Figure 2. miR-708 inhibits *CD38* expression in HASM cells. A: NA-HASM cells were transfected with different concentrations of miR-708 mimic (mimic) or scrambled sequence mimic (scr), followed by exposure to 10 ng/ml *rh*-TNF-α. CD38 transcript levels were measured by q-PCR. Results are shown as % CD38

mRNA relative to scrambled sequence mimic (scr) transfection. Significant inhibition of *CD38* expression was observed following transfection of miR-708 mimic at concentrations ≥ 50 nM (n = 3–5 donors).**B**: CD38 transcript levels following transfection with miR-708 mimic (50 nM) or scrambled sequence mimic in AS-HASM cells. Note significant attenuation of *CD38* expression by miR-708 mimic transfection (n = 3 donors); **C**: ADP-ribosyl cyclase activity in AS-HASM (AS) and NA-HASM (NA) cells following transfection with miR-708 mimic or scrambled sequence mimic and exposure to *rh*-TNF-α (MT and ST, respectively, (n = 3 donors). Note significant attenuation of enzyme activity in cells from AS-HASM and NA-HASM after transfection with miR-708 mimic. **D**: CD38 transcript levels in NA-HASM cells following transfection with miR-708 mimic (50 nM), scrambled sequence mimic or antagomir for the miRNA (ant-mir) (n = 3 donors). Note the lack of inhibition of *CD38* expression in cells transfected with ant-miR. Data represent mean ± SEM.

Our previous studies have identified a role for miR-140-3p in the regulation of cytokine-induced CD38 gene expression and enzyme activity independent of miR-708(177). Since the target sites of these miRNAs are closely situated in the 3'UTR of CD38 transcript (see **Figure 3A**), we examined whether transfection of HASM cells with both miR-140-3p and miR-708 would amplify the inhibitory effect on enzymatic activity of CD38. Co-transfection of HASM cell with miR-140-3p and miR-708 at 50 nM and 100 nM (equimolar concentrations) significantly inhibited CD38 enzymatic activity relative to cells transfected with scrambled

sequence mimic. However there was no evidence of an additive effect **(Figure 4).**



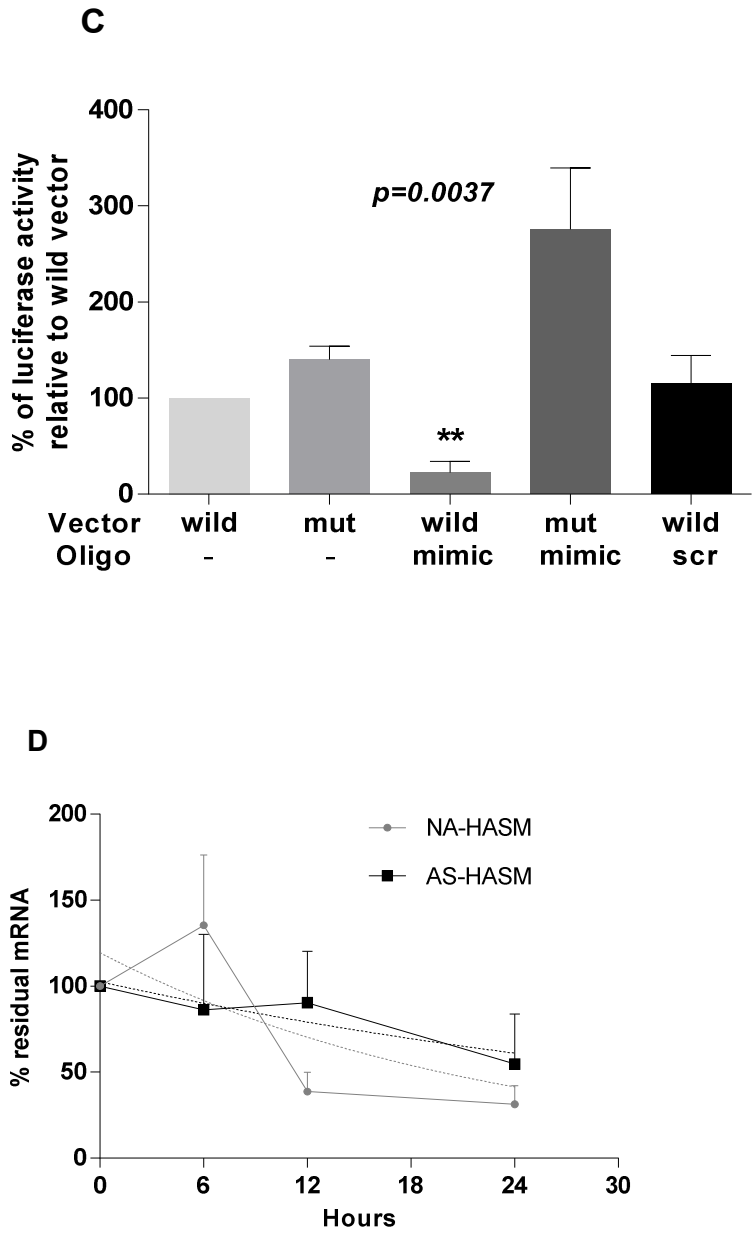


Figure 3. Interaction of miR-708 with the 3'UTR of CD38. **A**: Predicted binding sites for miR-708 and miR-140-3p in 3'UTR of CD38. Shown are the mutated bases in the miR-708 target site of the 3'UTR of*CD38*. **B**: Relative luciferase activity in NIH-3 T3 cells co-transfected with reporter plasmid and different concentrations of miR-708 mimic (mimic) or scrambled sequence mimic

(scr) at the highest concentration (n = 3, experiments in triplicate). Note significant reduction in luciferase activity following transfection with miR-708 mimic at all concentrations. **C**: Relative luciferase activity in cells co-transfected with reporter plasmid containing either wild-type (wild) or mutant (mut) *CD38* 3'UTR followed by transfection with 50 nM of miR-708 mimic (mimic) or scrambled sequence mimic (scr) (n = 3, experiments in triplicate). Note no inhibition of luciferase activity in cells co-transfected with mutant *CD38* 3'UTR. **D**: CD38 mRNA stability measured in growth-arrested HASM cells. Cells were transfected with 50 nM miR-708 and exposed to *rh*-TNF-α in the presence of actinomycin D. At time points indicated, CD38 transcript levels were quantified by q-PCR (n = 2–5 donors). Graph shows plots of non-linear regression of mRNA decay over time for NA-HASM and AS-HASM cells, with the dotted lines indicating the best fit. Values are means ± SEM for results shown.

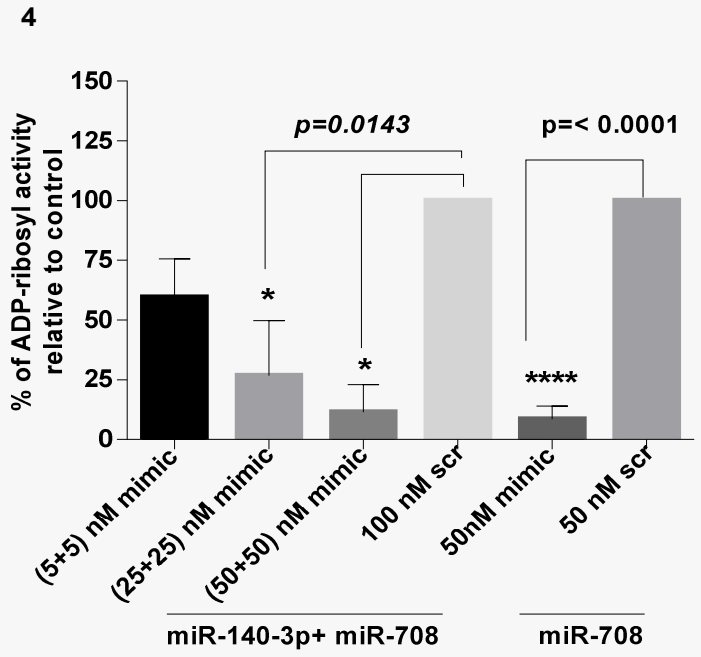


Figure 4. Effect of miR-708 in combination with miR-140-3p on ADP-ribosyl cyclase activity in NA-HASM cells. Cells (n = 3 donors) were co-transfected with equimolar concentrations of miR-708 and miR-140-3p, miR-708 mimic alone or the corresponding control scrambled sequence mimic at the indicated concentrations followed by exposure to 10 ng/ml *rh*-TNF-α (T) and measurement of ADP-ribosyl cyclase activity. Data represents mean ± SEM. Note that no additive effect was observed in cells transfected with equimolar concentrations of miR-708 and miR-140-3p relative to miR-708 alone.

miR-708 directly binds to 3'UTR of *CD38*

MiRNAs can inhibit gene expression directly by binding to the target gene at the 3'UTR or indirectly by inhibiting multiple components in the signaling pathway (30, 244). We first examined whether miR-708 regulates the expression of CD38 by directly binding to its 3'UTR by performing dual luciferase reporter assays in a heterologous cell system (NIH-3 T3 cells). Four bases in the target sequence on the CD38 3'UTR were mutated to establish specificity of miR-708 target binding **(Figure 3A).** A significant reduction in relative luciferase activity was noted when miR-708 mimic and reporter plasmid were co-transfected compared to co-transfection with a scrambled sequence mimic **(Figure 3B).** Mutation of four bases in the target sequence on the *CD38* 3'UTR reversed the inhibitory effect of miR-708 on luciferase activity confirming the specificity of its target binding at the 3'UTR of *CD38* **(Figure 3C).** To examine whether this binding leads to mRNA degradation, CD38 mRNA stability was measured in HASM cells transfected with

miR-708 mimic or scrambled sequence mimic and exposed to *rh*-TNF-α followed by actinomycin D for transcriptional arrest. For analysis of mRNA stability, the amount of CD38 mRNA remaining at each time point up to 24 h relative to 0 h was determined by q-PCR using the $2^{-\Delta\Delta Ct}$ calculation method and plotting against time. Nonlinear regression analysis of biological replicate samples was fitted using one-phase decay kinetics (GraphPad Prism 6). In NA- and AS-ASM cells transfected with miR-708 mimic or the scrambled sequence mimic, there was comparable decay in CD38 mRNA content, with half-life ranging from 16–32 h **(Figure 3D).**

Transcriptional regulation of *CD38* expression by miR-708

In prior studies, we reported that cytokine-induced changes in *CD38* expression in HASM cells involve activation of MAP kinases and PI3 kinases. Among the MAP kinases, p38 and JNK MAP kinases were found to regulate expression transcriptionally while ERK MAP kinase was involved in regulation post-transcriptionally through transcript stability (322). In this study, we examined the effect of miR-708 transfection following TNF-α exposure (20 min) on levels of total MAP kinase protein as well as phosphorylated (activated) protein in order to determine whether decreased MAP kinase activation is an underlying mechanism in the regulation of *CD38* expression. Cells were transfected with miR-708 mimic or its scrambled sequence mimic, exposed to *rh*-TNF-α for 24 h and expression levels of the MAP kinases determined by Western blot analysis of cell lysates. Transfection of HASM cells with miR-708 mimic had little effect on levels of phosphorylated or total ERK and p38 **(Figure 5)**. A significant reduction,

however, was noted in the level of phosphorylated JNK MAP kinase in growth-arrested, TNF-α exposed (20 min) cells following miR-708 transfection **(Figure 6A).** Further, although total JNK MAP kinase expression remained unaltered in these cells **(Figure 6B),** there was a significant reduction in JNK mRNA expression **(Figure 6C).** We next examined whether reduced JNK phosphorylation might be a consequence of increased expression of a phosphatase. Expression of a MAP kinase phosphatase, MKP-1, was measured by Western blot in miR-708-transfected cells following *rh*-TNF-α exposure. MKP-1 expression was significantly higher in cells transfected with miR-708 mimic compared to cells transfected with the scrambled sequence mimic **(Figure 6D).** These results suggest that decreased JNK MAP kinase phosphorylation caused by increased expression of MKP-1 may be involved in miR-708 regulation of *CD38* expression in HASM cells.

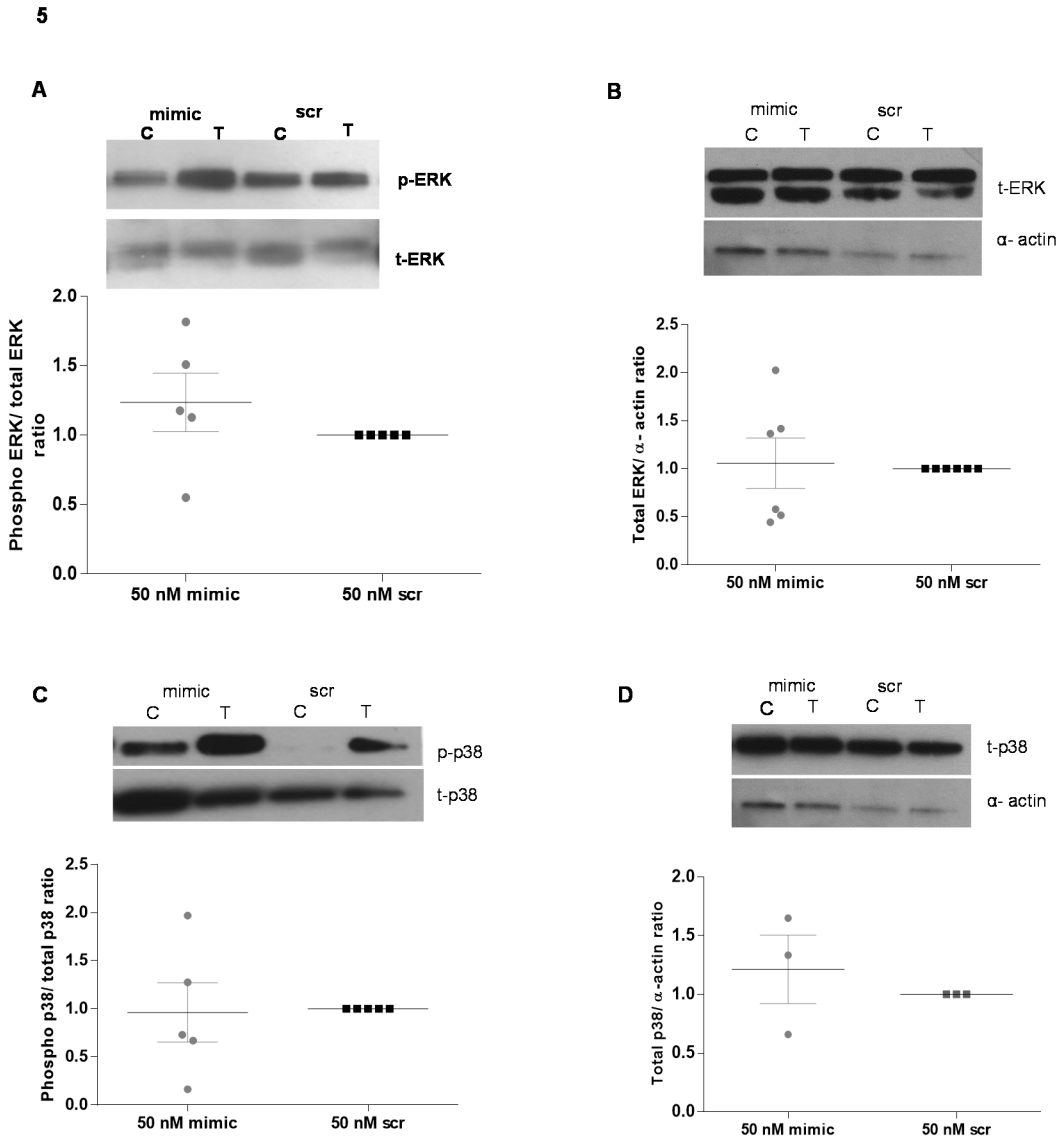


Figure 5. Effect of miR-708 transfection on ERK and p38 MAP kinase activation and expression in NA-HASM cells. Western blot analysis of cell lysates following transfection with 50 nM miR-708 mimic (mimic) or scrambled mimic (scr) and treatment with *rh*-TNF-α for 20 min (for total and phosphorylated levels) or 24 h (for total expression levels) were performed with antibodies against phosphorylated and total ERK **(A and B)** as well as p38 **(C and D)**, respectively. Note no significant change in expression of total or phosphorylated

ERK (n = 5–6 donors) and p38 (n = 3–5 donors) following miR-708 mimic transfection. Each Western blot is a representative blot from a single donor. Values are means ± SEM for results shown. C-vehicle-treated cells (0.1%BSA); T- *rh*-TNF-α (10 ng/ml) treated cells.

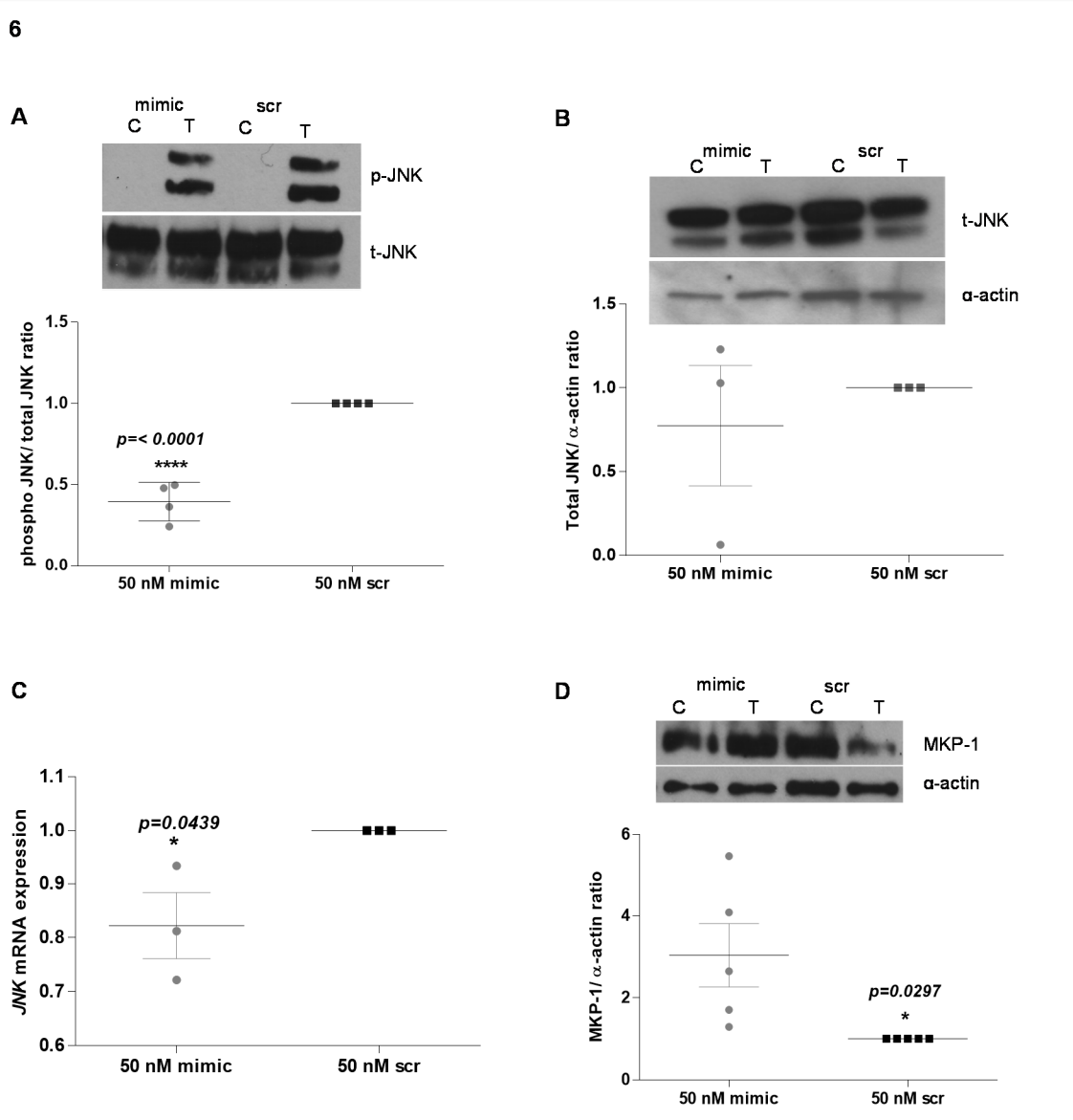


Figure 6. Effect of miR-708 transfection on JNK MAP kinase signaling in NA-HASM cells. A *and* **B**: Western blot analysis of cell lysates following transfection with 50 nM miR-708 mimic (mimic) or scrambled sequence mimic

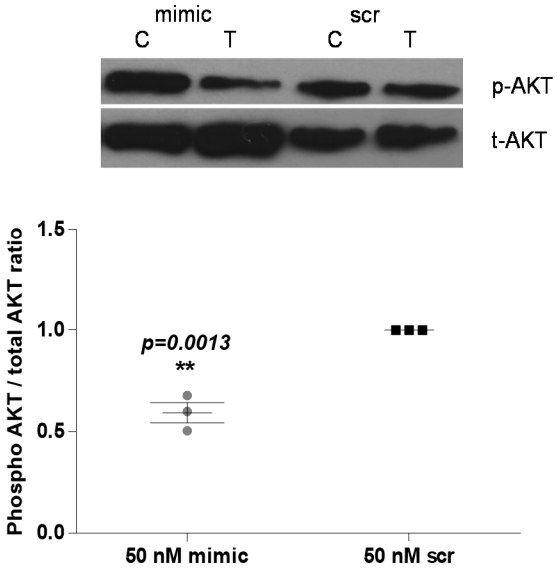
(scr) was performed as described in Figure 5 to detect JNK MAP kinase after *rh*-TNF-α treatment for 20 min (total and phosphorylated levels) (n = 4 donors) as well as total JNK expression after *rh*-TNF-α treatment for 24 h (n = 3 donors). Note significant down-regulation of JNK MAP kinase phosphorylation but no change in total JNK protein expression in mimic-transfected cells compared to cells transfected with scr. **C**: Total RNA extracted from cells transfected with mimic or scr and treated with *rh*-TNF-α (24 h) was analyzed for JNK mRNA expression by q-PCR (n = 3 donors). Note significant down-regulation of JNK expression by mimic. **D**: MKP-1 expression in cells transfected with mimic or scr and treated with *rh*-TNF-α for 24 h was examined by Western blot analysis (n = 5 donors). Note significant up-regulation of MKP-1 expression in mimic-transfected cells compared to scr. Each Western blot is a representative blot from a single donor. Values are means + SEM for results shown. C-vehicle-treated cells (0.1% BSA); T-TNF-α (10 ng/ml) treated cells.

In a prior study, we reported that regulation of *CD38* expression in HASM cells involves activation of PI3 kinases(179). Furthermore, PI3 kinase signaling is regulated by other miRNAs, including miR-708 (293). Therefore, we measured levels of phosphorylated AKT, a molecule in the PI3 kinase signaling cascade, as well as levels of PTEN, a phosphatase that terminates PI3 kinase signaling. In cells transfected with miR-708 mimic, the level of phosphorylated AKT was significantly lower relative to cells transfected with the scrambled sequence mimic **(Figure 7A).** Further, a significant reduction was also noted in the

expression of AKT2 **(Figure 7B),** an isoform of AKT that has a 3'UTR binding site for miR-708 (293). In consistence with an overall attenuation of PI3 kinase signaling in miR-708-mimic-transfected cells, there was a significant increase in PTEN expression compared to expression in cells transfected with the scrambled sequence mimic **(Figure 7C)**. A similar increase in PTEN expression was observed in AS-HASM cells following miR-708 mimic transfection compared to cells transfected with the scrambled sequence mimic (Figure 7D).

7

A



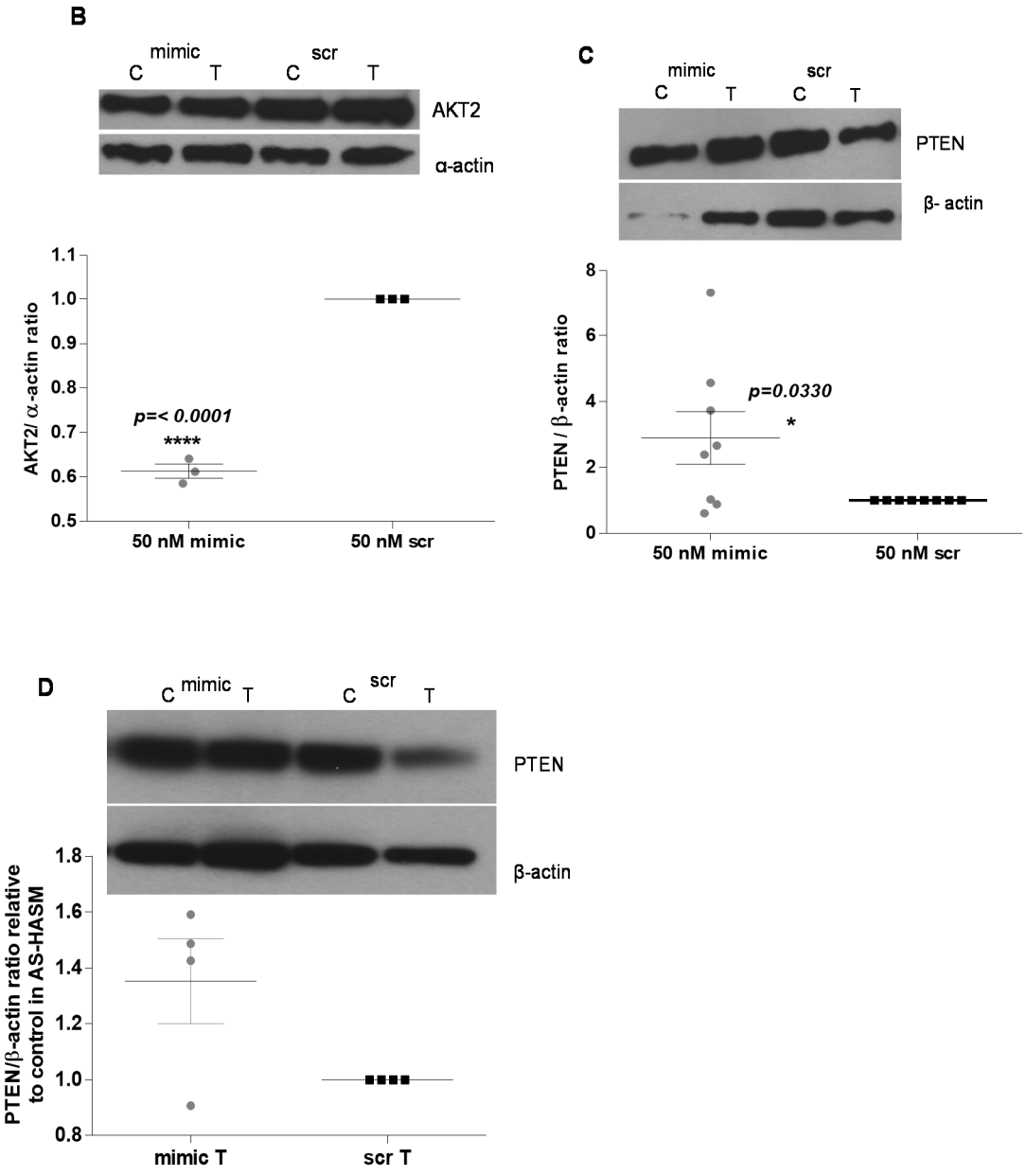


Figure 7. Effect of miR-708 transfection on PTEN/AKT signaling. A,**B** and **C**: NA-HASM cells were transfected with 50 nM miR-708 mimic (mimic) or scrambled sequence mimic (scr) and treated with *rh*-TNF-α for 2 h to measure total and phosphorylated AKT (n = 3 donors) or 24 h to measure AKT2 (n = 3 donors) and PTEN expression (n = 8 donors) by Western blot analysis of cell lysates. Note significant down-regulation of AKT phosphorylation and AKT2

expression and up-regulation of PTEN expression. **D**: PTEN expression in AS-HASM cells was higher (not significant) in samples from 3 of 4 donors. Each Western blot is a representative blot from a single donor. Values are means ± SEM for results shown. C-vehicle-treated cells (0.1% BSA); T-TNF-α (10 ng/ml) treated cells.

Discussion

The cell-surface protein CD38 is known to contribute to calcium regulation and contractility of ASM (87). In prior investigations, we and others have shown that in ASM cells calcium release through ryanodine receptor channels in the sarcoplasmic reticulum during agonist stimulation involves cyclic ADP-ribose (cADPR) (87, 95, 187). This involves the enzymatic activity of CD38, i.e., ADP-ribosyl cyclase, which converts β-NAD to cADPR (205, 350). Furthermore, exposure of ASM cells to inflammatory cytokines results in significantly augmented *CD38* expression and cADPR-mediated intracellular calcium release (87). These observations strongly implicate the CD38/cADPR pathway of calcium signaling in ASM hyperresponsiveness, a hallmark of asthma. *CD38* deficient mice are also hyporesponsive to inhaled methacholine following sensitization and challenge with allergen (120) as well as following challenge with inhaled inflammatory cytokines (133, 134). These mice develop a robust airway inflammatory response following allergen or cytokine challenge, although ASM obtained from these mice exhibit significantly attenuated contractile responses to relevant airway spasmogens (133). Furthermore, ASM cells obtained from *CD38* deficient mice show attenuated intracellular calcium responses to spasmogens (89). Together, these results indicate a significant role for *CD38* in calcium signaling and contractility of ASM and AHR. Therefore, delineating the mechanisms that regulate its expression can lead to better understanding of changes in ASM function in diseases such as asthma.

MiRNAs are known to play critical roles in the regulation of gene expression and in disease pathogenesis (30). Recent studies have identified several miRNAs whose expression in ASM cells is down-regulated by inflammatory cytokines (66, 177, 197). There is also evidence for miRNA regulation of ASM contractility (66) and relaxation (347) as well as ASM phenotype (162, 197). Increased ASM mass due to growth factor-driven ASM cell hypertrophy and hyperplasia is also a feature of diseases such as asthma (3, 62, 161, 230) and COPD (366). Recent reports have identified the specific role of miR-221 and miR-10a in the regulation of ASM proliferation (162, 272). It is interesting to note that miR-10a targets two key signaling molecules involved in cell proliferation, i.e., PI3 kinase and ERK MAP kinase. MiR-10a suppresses the expression of the catalytic subunit of PI3 kinase, leading to decreased AKT phosphorylation.

In this study, we identified miR-708 as a regulator of *CD38* in HASM cells. We further characterized miR-708 in HASM on the basis that its expression in HASM cells is regulated by the inflammatory cytokine *rh*-TNF-α, which is elevated during allergic asthma (59, 146), as well as its differential expression in cells from asthmatics versus non-asthmatics, and therefore its potential to regulate the expression of genes involved in signaling mechanisms regulating inflammation. We report that miR-708 down-regulates *CD38* expression through mechanisms that involve direct binding to the 3'UTR as well as indirectly by regulating JNK MAP kinase and PI3 kinase signaling in HASM cells.

Among the numerous potential *CD38* 3'UTR binding targets that we have identified, miR-708 and miR-140-3p (177) appear to play major roles in the regulation of *CD38* expression in HASM cells. We also observed that transfection of cells with both miR-140-3p and miR-708 has no additive or synergistic effects on CD38 enzymatic activity, suggesting that either miRNA is capable of independently regulating *CD38* expression in HASM cells.

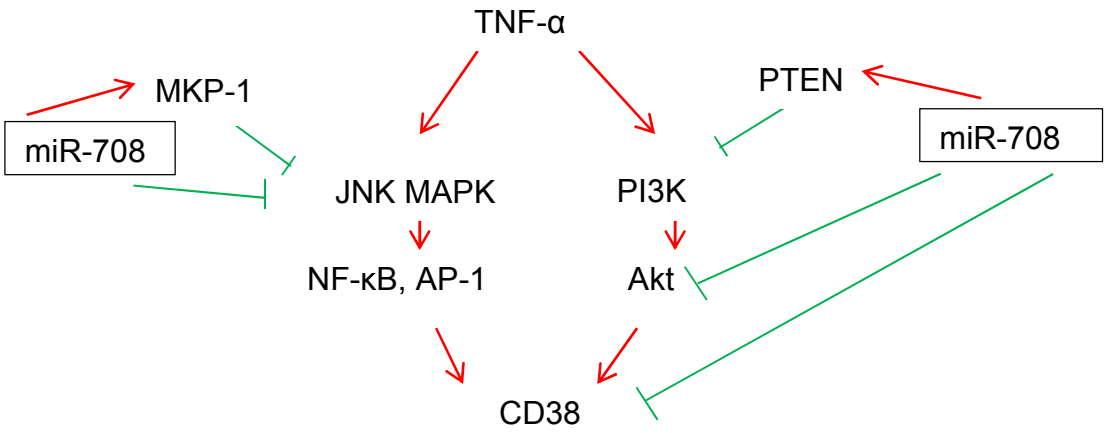
In the current study, we have shown that over-expression of miR-708 through transfection causes increased PTEN expression and an associated decrease in AKT phosphorylation. *PTEN* expression in certain cancers is regulated by miRNAs. A recent study reported that miR-221 and miR-222 inhibit *PTEN* expression by binding to the 3'UTR, while knockdown of these miRNAs causes induction of *PTEN* expression (70). *PTEN* expression in human hepatocellular cancer cells is also under the regulation of miR-21 (79). Knockdown of miR-21 causes increased *PTEN* expression in these cells. Regulation of *PTEN* expression in these cancer cell lines by miRNAs has a direct effect on tumor cell proliferation, migration and invasion. These studies suggest that miRNA regulation of PI3 kinase signaling in ASM cells by altering the expression of *PTEN* would have a profound impact on cell proliferation. In this context, a previous study demonstrated that in ASM cells obtained from asthmatics, the PI3 kinase/AKT pathway has a major contribution to cell proliferation (52). However, in ASM cells obtained from non-asthmatics, cell proliferation appears to be regulated by ERK MAP kinase signaling (52). In the current study, we found that miR-708 also regulates the phosphorylation of JNK

MAP kinase in NA-HASM cells. This effect appears to be a consequence of increased expression of MKP-1, a MAP kinase phosphatase. Why this enhanced MKP-1 expression is not accompanied by decreased phosphorylation of p38 and ERK MAP kinases is not clear. However, it is very likely that the expression of some of the upstream kinases involved in the p38 and ERK MAP kinase pathways may be increased, compensating for the increased MKP-1 expression. It is worth noting that loss of *PTEN* in certain cancer cells results in increased JNK activation independent of AKT and implicating JNK as a component of the PTEN/PI3 kinase signaling cascade (343). Thus, it is likely that the decreased JNK MAP kinase phosphorylation observed in this study may stem from increased PTEN expression. Decreased JNK activation in ASM cells has been shown to have an effect on *CD38* expression through transcription factors NF-κB and AP-1 (322). Our study also shows that ASM cells obtained from asthmatics have increased constitutive as well as *rh*-TNF-α-induced expression of miR-708 compared to expression in cells from non-asthmatics. The functional effect of increased miR-708 expression in these cells remains to be determined. In a prior study, we reported a differential induction of *CD38* expression in response to *rh*-TNF-α treatment in ASM cells from asthmatics (178) although an enhanced half-life of CD38 transcript appears not to be an underlying mechanism for this differential induction.

In summary, this study provides evidence for miR-708 regulation of *CD38* expression in human ASM cells. This regulation stems from direct 3'UTR binding to the transcript as well as through regulation of signaling

involving PI3 kinase and JNK MAP kinase pathways. The inhibition of PI3 kinase signaling by miR-708 involves induction of *PTEN* expression which is likely to have a significant impact on ASM cell proliferation and AHR, the latter through inhibition of expression of *CD38* and potentially other pro-inflammatory genes. Our findings suggest the possibility that miR-708 can be used as a potential therapeutic strategy to inhibit ASM cell proliferation and contractility.

Summary figure:



Acknowledgement

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CHAPTER iv

microRNA miR-708 mediated chemokine responses in human airway smooth muscle cells

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Background

Human airway smooth muscle cells are a source of cytokines and chemokines. The expression of chemokine genes is regulated by transcription factors NF-κB and AP-1 and involves activation of the MAP kinases and PI3 kinases. In a recent study, we reported that miR-708 transfection of HASM cells results in significant augmentation of expression of DUSP-1, a MAP kinase phosphatase and PTEN, a phosphatase that dephosphorylates Akt to inhibit the PI3 kinase signaling. Both these signaling pathways are critical for the expression of several pro-inflammatory genes and for regulating cell proliferation. In this study, we investigated the regulation of inflammatory gene expression, chemokine release and cell proliferation in HASM cells following transfection with miR-708. Gene expression results revealed significant downregulation of expression of several chemokine genes as well as some genes associated with the asthmatic phenotype and functional and pathway analysis showed changes in the expression of many of the IL-17 proinflammatory pathway as well as genes involved in cell cycle regulation. Validation of the microarray results using q-PCR revealed significant downregulation of expression of CCL11, CXCL10, CCL2 and CXCL8 as well as asthma related genes such as CD44, ADAM33 and RARRESS2. Of the chemokines that were assayed, only CCL11 and CXCL12 release was inhibited by miR-708 transfection. Serum-induced HASM cell proliferation was inhibited by miR-708 transfection. These results indicate a profound anti-inflammatory effect of miR-708 in HASM cells with potential to

inhibit recruitment of inflammatory cells into the airways and inhibition of cell proliferation.

Introduction

Several recent reports have provided evidence that airway smooth muscle (ASM) has strong proinflammatory and immunomodulatory functions (80, 85, 148, 324). These properties of ASM are mediated through its synthetic function as well as through expression of a variety of cell-surface molecules, integrins (129, 136, 319), and Toll-like receptors (101, 226). During acute airway inflammation, mediators and cytokines released from structural and inflammatory cells alter ASM contractile function (51, 73, 271, 359). However, during persistent airway inflammation, cytokines and chemokines produced by inflammatory cells and ASM can cause ASM proliferation, leading to structural changes in the airways, often referred to as airway remodeling (142, 185). During chronic airway inflammation, the immunomodulatory role of ASM may be more significant in establishing structural changes within the airways than its contractile function. In this context, recent reports show that ASM is capable of releasing cytokines such as IL-5 (173, 203), IL-6 (11, 236), IL-33 (277), TSLP (368), GM-CSF (298) and VEGF (190); chemokines such as RANTES (173), Fractalkine (103), CCL11 (127), CXCL10 (5, 48, 121), CXCL8(171); adhesion molecules such as ICAM-1(15, 266), VCAM-1(202, 266), CD44 (201) and LFA-1(131); and growth factors such as IGF-1 (255, 281) and stem cell factor (155). Cytokines released by immune cells recruited into the lungs during allergic inflammation may also stimulate ASM cells to alter the expression of proinflammatory genes in an autocrine or paracrine manner. There is also evidence for hypersecretion of chemokines both constitutively and in response to cytokines in ASM cells

obtained from asthmatics than in cells from non-asthmatics (142, 306). There is also increased chemotaxis of mast cells toward ASM cells from asthmatics both *in vivo* and *in vitro* (9, 48, 261, 312). Other studies have examined the transcriptional regulation of expression of chemokine genes in human ASM cells (12, 171). While such transcriptional regulation of expression of chemokines is better understood, the post-transcriptional regulation is an emerging area of investigation. In this context, recent studies provide evidence for specific microRNAs in the regulation of ASM proliferation (162, 272), ASM phenotype (197) and airway inflammation (231, 328).

MicroRNAs (miRNAs) are small non-coding ~22nt RNAs that regulate gene expression by binding to the 3'-Untranslated Region (3'UTR) of target mRNAs to cause mRNA degradation and/or translational repression (29). Since binding of miRNAs to target sequences is dependent on its 'seed' sequence, a single miRNA can potentially regulate a large number of genes. However, specific miRNAs are known to regulate cellular functions such as differentiation, proliferation, and apoptosis and dysregulation of miRNA expression is known in controlling inflammation (29, 137, 138). In a recent report we identified miR-708 in the post-transcriptional regulation of expression of a cell-surface protein CD38 through two major signaling pathways (91). Transfection of human ASM cells with miR-708 causes the induction of phosphatase and tensin homolog (*PTEN*), which regulates PI3K/AKT signaling by decreasing Akt phosphorylation and is involved in NF-κB-regulated genes. MiR-708 also induces DUSP-1, a dual specificity phosphatase leading to JNK MAPK dephosphorylation (91). The net

effect of *PTEN* and *DUSP-1* induction in ASM cells should lead to modulation of two key signaling pathways involved in inflammation and cell proliferation. There is evidence that the expression of several chemokine genes in human ASM cells, the release of chemokines and cell proliferation are also regulated by these same signaling pathways (52, 94, 217, 279, 317). Therefore, in the present study, we evaluated differentially expressed genes in human ASM cells following miR-708 transfection and stimulation with the inflammatory cytokine TNF-α, with particular emphasis on the expression of cytokine/chemokine genes, other pro-inflammatory genes, and those involved in the asthmatic phenotype compared to scrambled sequence of mimic transfected cells. From the microarray results of differentially expressed genes following miR-708 transfection, we confirmed the expression levels of cytokine/chemokine genes, other pro-inflammatory genes, and those involved in the asthmatic phenotype using q-PCR. Since many of these chemokines are involved in the recruitment of inflammatory cells such as eosinophils, basophils, mast cells and T lymphocytes into the airways during allergic airway disease, we also measured their release from cells stimulated with the inflammatory cytokine TNF-α and following transfection with miR-708.

Materials & methods

Reagents

Reagents used in the current study: DMEM from GIBCO-BRL (Grand Island, NY); rh-TNF-α from R&D Systems (Minneapolis, MN); TRIzol, SuperScript III reverse transcriptase, Opti-MEM® reduced serum medium and Lipofectamine® RNAiMax transfection reagent from Invitrogen Life Technologies (Carlsbad, CA); Brilliant lll Ultra-Fast SYBR Green q-PCR Master Mix from Agilent Technologies Inc (Santa Clara CA); control oligo (scrambled sequence mimic) and miR-708 mimic (mature miR-708 sequence: 5'-AAGGAGCUUACAAUCUAGCUGGG-3') from Dharmacon (Lafayette, CO); CyQUANT® NF Cell Proliferation Assay Kit from Thermo Fisher Scientific brand Life technologies (Eugene, Oregon 97402); Tris-base, glucose, HEPES and other chemicals from Sigma Chemical Co. (St. Louis, MO).

Microarray sample preparation

ASM cells, derived from three de-identified healthy donors, used between 2-5th passages, were seeded at 1.5 X 10^5 cells/well and transfected with mimic or scrambled sequence mimic of miR-708 at 50 nM concentration (91). We used the same concentration which was previously determined to be optimal to inhibit the expression of CD38 (91). Cells that were growth arrested (24 h) after transfection, were induced with pro-inflammatory cytokines TNF-α at 10 ng/ml (24 h). Total RNA was harvested using PureLink RNA isolation kit according to the manufacturer's instructions. Purity of the RNA was determined with a Nanometer 2000C for the ratios 260/280 and 260/230. For each condition (mimic

or scrambled oligo treatment) 1000 ng of total RNA was subjected to microarray analysis. Genome-wide changes in gene expression in transfected cells were generated using Illumina human (HT-12) arrays and analyzed using BeadStudio version 3.1.1.

Data Analysis

For statistical analysis and clustering, we used the Partek Genomics Suite software package (Partek Inc., St. Louis, MO, USA). We performed a paired *t*-test with donor ID and mimic/control as the nominal variables. Before comparison analysis and clustering, we filtered extremely low and non-variant genes out of the datasets. Significance cutoff filters were set at $P < 0.05$ and an expression change of at least 2-fold. For functional and pathway analyses we used Ingenuity Pathway Analysis (IPA) software (Qiagen, Redwood City, CA, USA). IPA employs a right-tailed Fisher exact test to calculate a *P* value corresponding to the probability that a biologic function not relevant to the input dataset is falsely identified as relevant. A Benjamini–Hochberg false discovery rate of 0.05 was used to correct such *P* values.

Validation of genes by q-PCR

As described in the “Microarray sample preparation”, total RNA was isolated and cDNA was prepared using reverse transcription kit from Invitrogen Life Technologies (Carlsbad, CA). cDNAs were subjected to q-PCR analysis using Brilliant SYBR Green Master Mix and Stratagene Mx3000p q-PCR system (Foster City, CA). Primer sequences and conditions for the genes tested are

provided in **Table 1**. The β-actin gene was used as a housekeeping gene to normalize the expressions of other genes.

Table 1: Primer sequences for chemokine genes

Gene	primer sequences
CXCL10	F: 5' - 3' GAACTGTACGCTGTACCTGCA
	R: 5' - 3' TTGATGGCCTTCGATTCTGGA
CXCL8	F: 5' - 3' ACTGAGAGTGATTGAGAGTGGAC
	R: 5' - 3' AACCCTCTGCACCCAGTTTTC
CCL5	F: 5' - 3' CAGTCGTCTTTGTCACCCGAA
	R: 5' - 3' TCCCAAGCTAGGACAAGAGCA
CCL2	F: 5' - 3' AGGTGACTGGGGCATTGAT
	R: 5' - 3' GCCTCCAGCATGAAAGTCTC
CXCL12	F: 5' - 3' TGCCAGAGCCAACGTCAAG
	R: 5' - 3' CAGCCGGGCTACAATCTGAA
CCL11	F: 5' - 3' CCCCAGAAAGCTGTGATCTTCA
	R: 5' - 3' GGAGTTGGAGATTTTTGGTCCAGAT
RARRES2	F: 5' - 3' GAGGGACTGGAAGAAACCCG
	R: 5' - 3' CATGGCTGGGGATAGAACGG
ADAM33	F: 5' - 3' GACCTAGAATGGTGTGCCAGA
	R: 5' - 3' AGCCTGGC TTGTCACAGAAG
CD44	F: 5' - 3' AGCATCGGATTTGAGACCTG
	R: 5' - 3' GTCCACATTCTGCAGGTTCC
β-Actin	F: 5' - 3' ACACTGTGCCCATCTACGAGG
	R: 5' - 3' AGGGGCCGGACTCGTCATACT

Chemokine Release assay

Human ASM cells were transfected with mimic or scrambled sequence mimic of miR-708 or were untransfected (control) as described in an earlier publication (91). Cells were then growth arrested and treated with 10ng/ml TNF-α. Cell culture supernatants were collected at different time points ranging from 6-48 h. Collected supernatants were aliquoted and immediately stored at -80 ^{o} F until assayed. Chemokines in the culture supernatants were quantified using ELISA kits from R&D system according to the manufacturer's instructions (Minneapolis, MN 55413).

Proliferation assay

Human ASM cells derived from healthy donors were transfected with mimic or scrambled sequence mimic of miR-708 and seeded at a concentration of ~7000 cells/well in 96 well plates. RNAimax was used to facilitate the transfection according to the manufacturer's instruction (Invitrogen Life Technologies). Twenty four hours after plating, the cells were growth arrested with serum deprived media that was supplemented with insulin and transferrin. After 24 h, cells were treated with 10% fetal bovine serum for 48 h and the number of cells enumerated using the Cyquant assay kit according to the manufacturer's instructions (20) (Thermo Fisher Scientific brand Life technologies).

Results

Microarray results

Gene expression results are shown as a heatmap (**Figure 1**). Visual inspection easily identifies differential patterns of expression between samples treated with a miR-708 mimic (purple bar) versus a scrambled control (orange bar). Principal Component Analysis (PCA) confirmed the mimic as the primary differential component (**Figure 2**). Our analysis found that 821 genes were differentially expressed (348 upregulated and 473 downregulated) in HASM cells transfected with a miR-708 mimic versus a scrambled control sequence (paired t-test, $P < 0.05$). **Table 2** summarizes the differentially expressed chemokines/cytokines, transcription factors, extracellular matrix components, calcium signaling molecules, growth factors and other genes related to airway hyperresponsiveness. The complete list of genes is available as **Supplemental Table 1**.

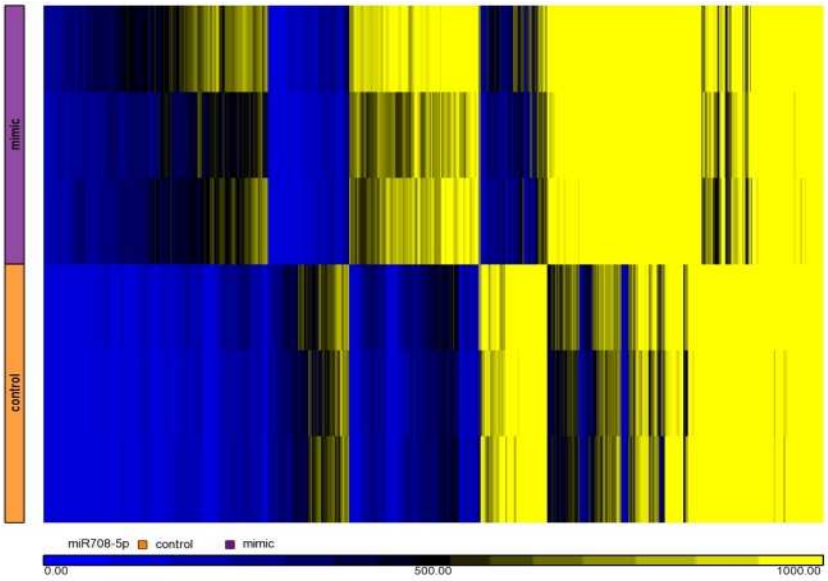

Figure 1: **Heatmap of mRNA microarray expression data**. Purple bar indicates samples treated with the miR-708 mimic; orange bar indicates samples treated with a scrambled control. Sample rows are arranged in the same donor-order (i.e. donor 1 samples are rows 1 and 4).

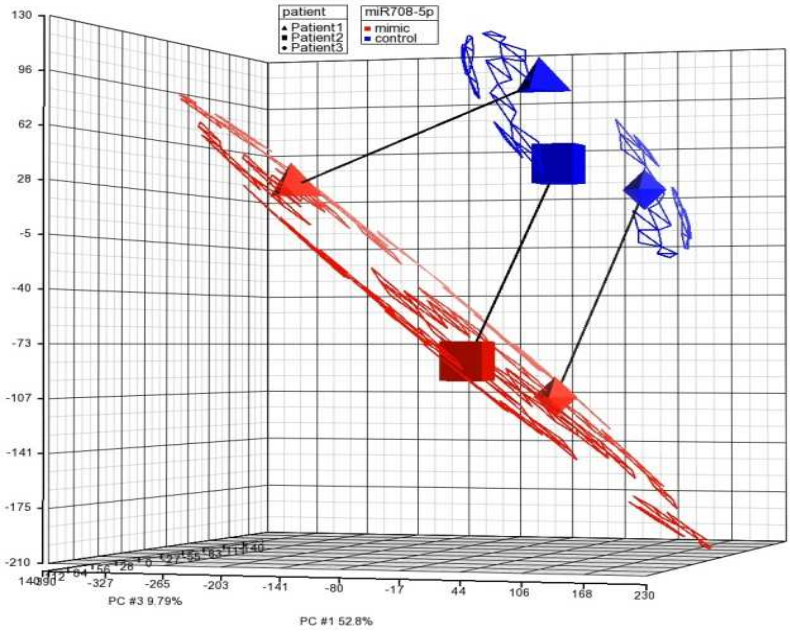

Figure 2. PCA Analysis showing a clear primary separation of samples based on miR-708 mimic versus scrambled control. Secondary separation was by donor ID.

Table 2. Summary of differentially expressed genes

	Gene ID	fold change	Function
Inflammatory mediators			
Chemokines	*CXCL10*	-2.695	Chemoattracts mast cell
	CCL8	-5.38	Chemoattracts monocytes
	CCL11	-8.968	Chemoattracts Eosinophils
	CXCL12	-5.254	Chemoattractant T-lymphocytes & monocytes
	CCL5	-3.52	Chemoattracts Eosinophil
	CCL2	-3.21	Chemoattracts monocytes, fibrocytes and basophils
	CXCL8	-2.03	Chemoattracts neutrophils, basophils and T-cells
	CXCL16	-2.688	Scavenger receptor on macrophages
	CXCL5	-2.389	Activates neutrophils
	CXCL9	-2.375	Chemoattracts activated T-cells
	CXCL11	-2.151	Chemoattracts interleukin-activated T-cells
	CXCL6	-2.039	Chemoattracts neutrophil, granulocytes
Cytokines			
	IL18BP	-4.028	Inhibits the early TH1 cytokine response
	IL6	-1.832	Stimulates the differentiation of B-cells and acts as a myokine.

	TNFSF13B	-4.216	Stimulates B- and T-cell function
Genes associated with ECM			
	VCAM1	-20.59	Enhances leukocyte-endothelial cell adhesion and T cell inflammatory functions
	COL3A1	-9.808	Activates RhoA pathway
	COL6A1	-3.185	A major structural component of microfibrils
	CD44	-4.25	Increases airway hyperresponsiveness (122); leads to inflammation (182, 183) by interacting with T-cell (201) and mast cell (128); increases ASM cell proliferation (3)
	THBS1	-4.797	Increases IL-8 production (165)
	MXRA5	-4.581	Associates with matrix-remodeling protein
	ADAMTS-1	6.158	Increases FEV1 (269)
	TAPBP	-4.38	Increases antigen processing and assembly of MHC class I (17)
Transcription factors			
	NFκB1	-1.876	Regulates immune response
	RELA	-1.728	Regulates immune response
Calcium signaling			
	CD38	-2.286	Increases cell adhesion, signal

			transduction, AHR and calcium signaling.
	BDKRB1	-1.994	Increases chronic and acute inflammatory responses
	FKBP10	-2.198	Regulates [Ca2+]i dynamics
Growth factors and related genes			
	IGFBP5	-4.009	Prolongs the half-life of the IGFs
	PDGFRL	-4.258	Increases proliferation
	EGFL6	-3.886	Regulates of cell cycle & induces proliferation
Airway hyperresponsiveness			
	ACTG2	-17.181	Increases muscle contraction
	TAGLN	-4.691	Increases calcium interactions and contractility
	MYLK	-2.12	Increases Smooth muscle contraction
	PDE5A	-2.50	Inactivates of cGMP (108)
Genes associated with proliferation	*F2F7*	6.8	Blocks proliferation (82)
	IL24	11.6	Anti-proliferative (360)
	COL1A1	-7.474	Increases ASM cell proliferation (151)
	DUSP6	10.915	Decreases ASM cell proliferation
	UBE2C	18.96	Increases cell proliferation
	CDC20	16.459	Increases cell proliferation

	ID1	16.089	Increases cell proliferation
	ANGPTL4	12.633	Increases ASM cell proliferation (309)
	CDK1	5.387	Increases cell proliferation

Functional and Pathway Analysis

The most significant biologic functions for this differential gene set included decreased inflammatory response, cytokine expression and signaling. In particular, many components of the IL-17 proinflammatory pathway were downregulated. Multiple pathways and biologic functions related to cell cycle progression were predicted to be upregulated.

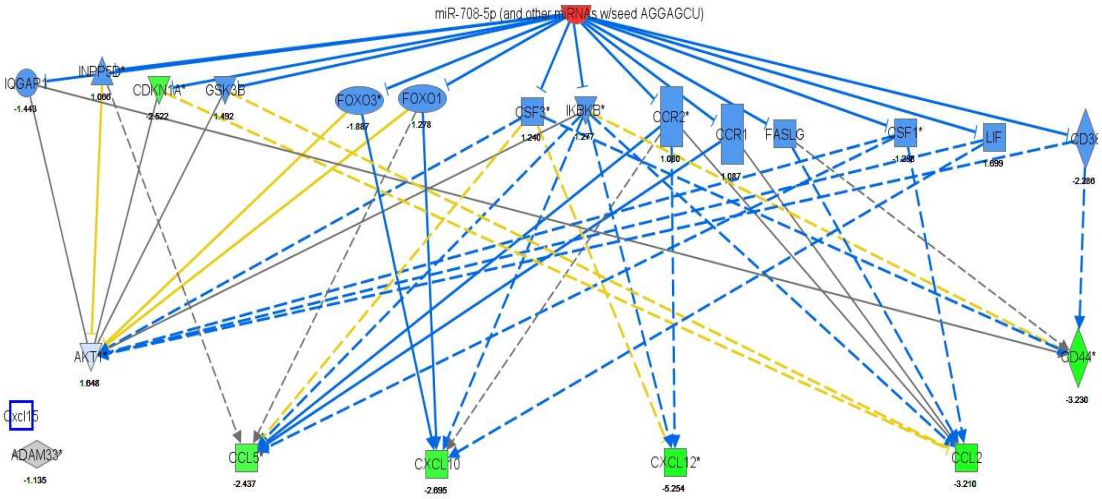

Figure 3. Network diagram showing potential regulatory pathways connecting miR-708 and down-regulated molecules of interest in human ASM cells. Note significant down-regulation of several chemokine genes, CD44, and CD38. Genes are colored either by observed expression changes in the paired t-test (Green, fold decrease given below gene name) or by predicted activation status (Blue = predicted inhibition) based on the assumption of increased miR-708 (Red). Potential relationships are indicated by solid (direct interaction) or dotted (indirect interaction) lines. Interaction lines are colored based on whether the predicted relationship leads to inhibition (Blue), leads to

activation (Yellow; inconsistent with observed results), or effect was not able to be predicted (Gray).

miR-708 inhibits chemokine mRNA expression and other asthma related genes

Results of gene expression analysis revealed significant downregulation of expression of several chemokine genes as well as some genes associated with the asthmatic phenotype. Therefore, we used q-PCR analysis to validate the microarray results of expression of these genes. Human ASM cells were transfected with miR-708 mimic oligonucleotides or the scrambled control and then treated with TNF-α. Twenty four hours following the addition of TNF-α, total RNA was collected from the cells and subjected to q-PCR analysis. There was significant inhibition in the expression of chemokine genes CCL11 (*p=<0.0001*), CXCL10 (*p=0.0308*), CCL2 (*p=0.0422*) and CXCL8 (*p=0.0156*) **(Figure 4)** as well as other 'asthma related' genes such as CD44 (*p=0.0328),* ADAM33 (*p=0.0016*) and RARRESS2 (*p=0.0006)* **(Figure 5)**. On the other hand, the mRNA expression levels for chemokine genes CCL5 (*p=0.0549*) and CXCL12 following transfection with the miR-708 mimic were not significantly different from expression in scrambled miR-708 oligonucleotide-transfected cells **(Figure 5)**.

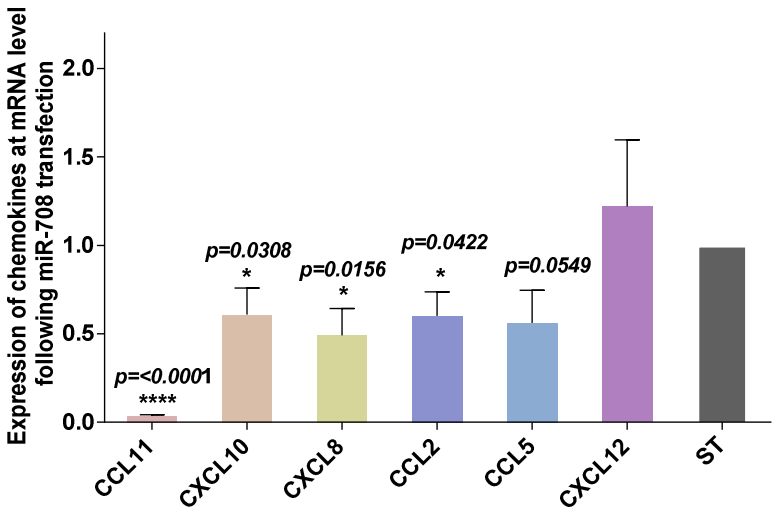

Figure 4. Downregulation of chemokine mRNA expression following miR-708 transfection. Human ASM cells derived from 3-5 donors were transfected with mimic or scrambled (Scr) sequence mimic of miR-708 followed by exposure to TNF-α (10ng/ml) to measure chemokine mRNA expression. Note the significant inhibition in the expression of CCL11, CXCL10, CXCL8 and CCL2 following miR-708 mimic transfection compared to expression in cells transfected with scrambled sequence. Data represent mean±SEM.

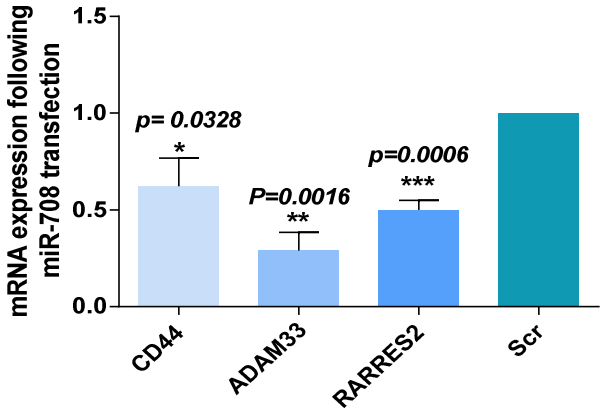

Figure 5. Down regulation of other 'asthma related' genes by miR-708. HASM cells obtained from 3-5 donors were transfected with mimic or scrambled sequence mimic (control) of miR-708 followed by treatment with TNF-α (10ng/ml/). Note significant inhibition of expression of CD44, ADAM33 and RARRES2 transcripts in miR-708 mimic-transfected cells compared to expression in scrambled sequence transfected cells. Data represent mean±SEM.

miR-708 transfection and release of chemokines.

To determine whether changes in the mRNA expression of chemokines were reflected in their protein expression, we measured their release in HASM cell culture supernatant following miR-708 mimic or scrambled sequence mimic transfection and TNF-α induction. As a control, we collected the culture supernatant from untransfected but TNF-α treated HASM cells. Of the chemokines that were assayed, only CCL11 release exhibited significant down regulation of release in mimic miR-708-transfected cells compared to release from cells transfected with the scrambled miR-708 oligonucleotides or from control cells at all-time points examined **(Figure 6)**. CXCL12 release on the other hand exhibited significant inhibition following addition of TNF-α at 24 h but not at earlier time points **(Figure 6)**.

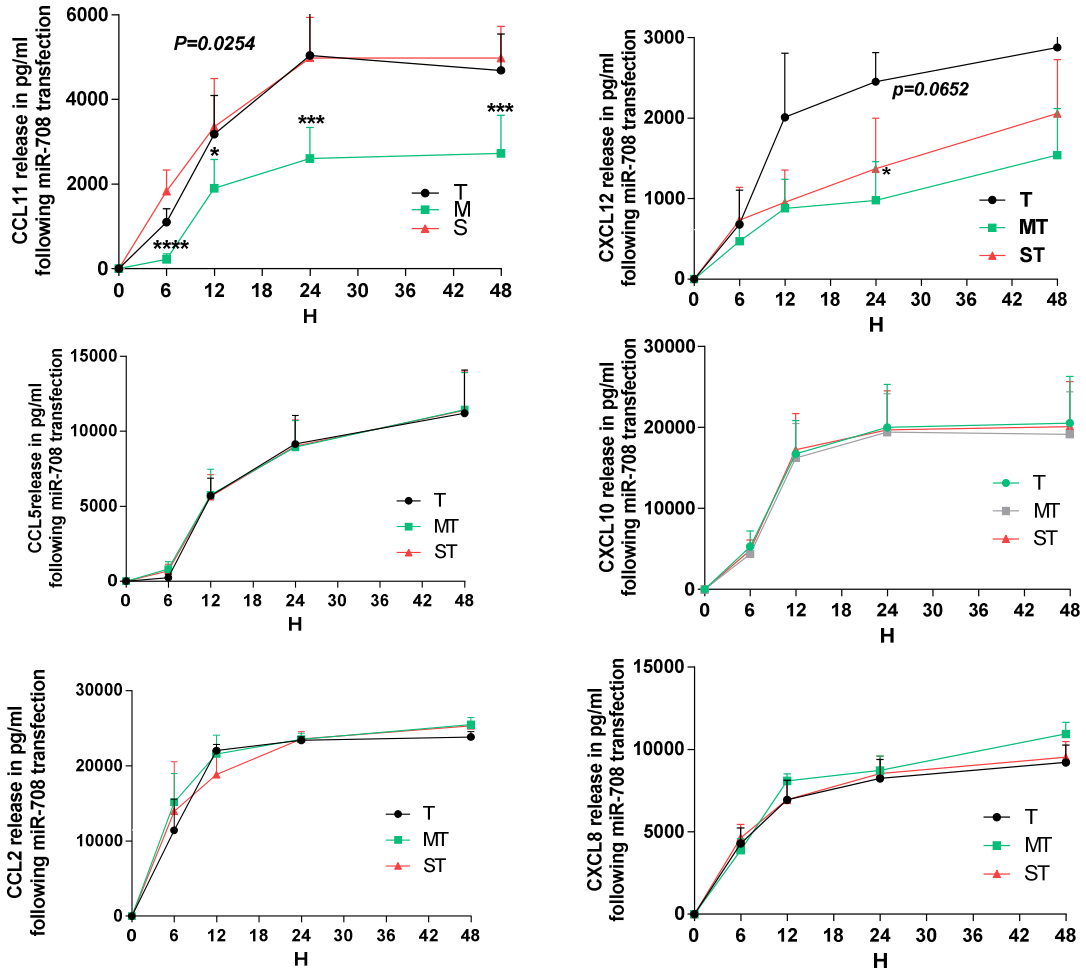

Figure 6. C**hemokine release from HASM cells following miR-708 transfection**. HASM cells from 3-6 donors were transfected with mimic or scrambled sequence mimic of miR-708 and treated with TNF-α (10ng/ml) following growth arrest of cells. Twenty hours later cell culture supernatants were collected for the measurement of chemokines. Note the release of CCL11 was significantly inhibited at every time point following miR-708 transfection when compared to scrambled sequence mimic transfection. The release of CXCL12 was significantly inhibited only at 24 h, relative to control. Data represent mean±SEM.

miR-708 inhibits proliferation of HASM cells

Functional and pathway analysis of differentially expressed genes following miR-708 transfection revealed changes in the expression of several genes involved in cell cycle progression/regulation. Therefore, we examined the effect of miR-708mimic transfection on serum-induced HASM cell proliferation. HASM cells were transfected with mimic or scrambled sequence of mimic miR-708 oligonucleotides and exposed to 10% serum for 48 h. Cell proliferation measured using the Cyquant assay showed significant inhibition of proliferation in cells transfected with the mimic miR-708 as compared to cells transfected with the scrambled miR-708 oligonucleotides (**Figure 7**).

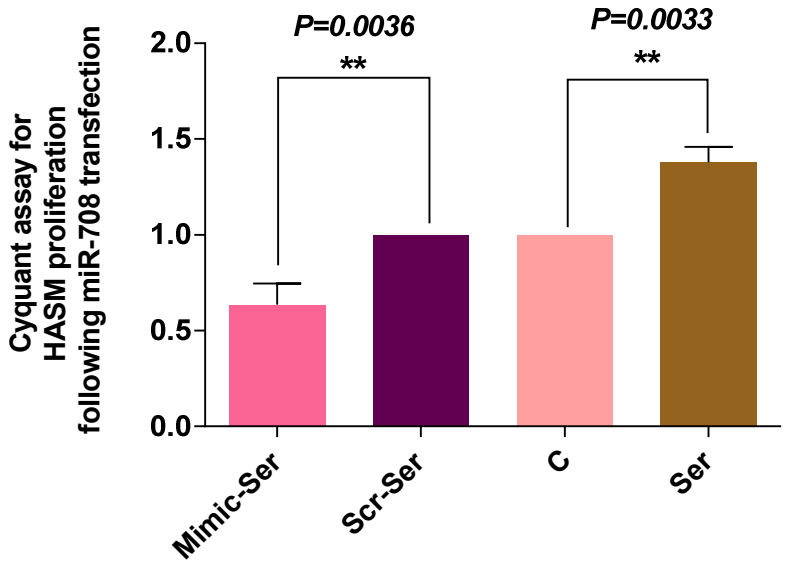

Figure 7. miR-708 decreased the proliferation of HASM cells. HASM cells derived from 4-6 donors were transfected with mimic or scrambled sequence mimic of miR-708 followed by treatment with 10% bovine fetal serum for 48 h. Proliferation of cells was measured using Cyquant proliferation assay kit. Note significant inhibition of proliferation of cells transfected with mimic of miR-708

relative to cells that were transfected with scrambled sequence mimic of miR-708. Data represent mean±SEM.

Discussion

Using a transcriptomics-based approach, we investigated differentially expressed genes in human ASM cells treated with TNF-α following miR-708 transfection compared to expression in cells transfected with the scrambled mimic oligonucleotides. This analysis revealed significant changes in the expression of several genes, including those for chemokines / cytokines, extracellular matrix proteins, transcription factors, calcium signaling molecules, growth factors, and genes associated with airway hyperresponsiveness. Several genes involved in cell cycle regulation were up-regulated, although E2F7, DUSP6 and IL-24, genes that block cell proliferation, were significantly up-regulated. The net functional effect of these changes in gene expression was reflected in inhibition of serum-induced cell proliferation. There was down-regulation of expression of JNK MAP kinase which is involved in serum-induced ASM cell proliferation (64). The changes in the expression of chemokine genes revealed in this approach were confirmed by q-PCR. In addition, miR-708 also produced down-regulation of expression of several 'asthma-related' genes such as CD44 (182, 201), ADAM33 (154, 337) and RARRES2 (37, 145, 228). Prior reports have shown that CD44 is involved in mast cell-ASM cell adherence through Type I collagen and this adherence is greater during airway inflammation as well as in ASM cells derived from asthmatics (128).

In the present study, we examined the post-transcriptional regulation of expression of several inflammatory genes in human ASM cells by miR-708. In a recent study in human ASM cells stimulated with the inflammatory cytokine TNF-

α, we showed that transfection with miR-708 caused a significant augmentation of PTEN and DUSP-1 expression, with concomitant decreased activation of Akt and JNK MAP kinase respectively (91). The PI3 kinase/Akt and MAP kinase signaling mechanisms are clearly involved in airway inflammation by activating transcription factors such as NF-κB and AP-1 (106, 211, 283, 357). Recent reports have shown that this signaling is involved in the hyperproliferative phenotype of ASM cells from asthmatics (52). The promoter regions of several chemokine genes contain binding sites for NF-κB and AP-1 as well as for other transcription factors (10, 126, 286). Furthermore, TNF-α is capable of inducing the expression and release of these chemokines from human ASM cells as well (9, 12, 14, 32, 127). Although the mechanisms by which miR-708 decreased the expression of the chemokine genes that we examined are not addressed in this study, miRNA target prediction algorithms showed 3'UTR targets for miR-708 for CXCL12 and CCL5 but not for the other chemokines examined. It is very likely that the inhibition of expression of chemokine genes following miR-708 transfection resulted from indirect mechanisms of decreased activation of transcription factors and MAP kinases involved in their regulation.

We examined the expression and release of several chemokines from human ASM cells stimulated with TNF-α in this study. There was significant inhibition of expression of chemokines involved in the recruitment of eosinophils, mast cells, T lymphocytes and fibrocytes following miR-708 transfection of human ASM cells. However, chemokine release studies revealed that there was inhibition of release of CCL11 and CXCL12. It is interesting to note that our

microarray results showed a high level of down-regulation of CXCL12 expression, while the q-PCR results did not show any change in CXCL12 transcript levels following miRNA transfection. It is very likely that miR-708 may regulate transcription and release of some chemokines while it may have a dominant effect on release for others. It is also known that production of specific chemokines in ASM cells may involve unique signaling pathways and stimuli, as has been shown for CXCL10 release (9). In this study, the authors demonstrated that in human ASM cells exposed to TNF-α or IL-1β, CXCL10 production required JNK MAP kinase activation, while its release was induced by p38 MAP kinase activation. The results of the microarray analysis of differentially expressed genes and q-PCR results confirmed selective down-regulation of JNK MAP kinase expression by miR-708, with decreased JNK MAP kinase phosphorylation. Recent investigations have shown that stimulation of ASM cells with a mixture of cytokines causes significantly higher amounts of chemokine release than following exposure to individual cytokines (172, 317). It should be emphasized that in our study chemokine release was measured from cells following miRNA transfection and growth-arrest, before stimulation with TNF-α. It will be interesting to examine release of chemokines in response to a mixture of cytokines following miR-708 transfection.

There was also attenuation of serum-induced proliferative response of ASM cells following miR-708 transfection. Although the molecular mechanisms underlying this attenuation are not addressed in this study, it is likely that it stemmed from inhibition of JNK MAPK and PI3 kinase/Akt activation by miR-708

that we reported in our earlier study (91) and confirmed in this study for JNK MAPK.

In conclusion, this study demonstrates a profound anti-inflammatory effect of miR-708 in human ASM cells stimulated with the inflammatory cytokine TNF-α, with significant down-regulation of expression and release of several important chemokines involved in the recruitment of inflammatory cells into the airways during allergic airway inflammation. Serum-induced ASM cell proliferation is also attenuated by miR-708 transfection, which should have an impact on airway wall remodeling in asthma.

Acknowledgement

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CHAPTER v

Conclusions and future directions

General Conclusions and Future Directions

My goal was to determine whether microRNAs play a role in regulating the expression of CD38 and altering the phenotype of human airway smooth muscle (HASM) cells. Using bio informatics/ algorithms such as TargetScan, MIRDB, DIANA and PITA, the following microRNAs predicted to have target sites at the 3'-UTR of CD38 mRNA were selected based on high context score: miR-140-3p, miR-20a, miR-20b, miR-708, miR-1272, miR-208a, miR-548 and miR-1298. The pattern of expression of these microRNAs in HASM cells in the presence of the pro-inflammatory cytokine TNF-α (or vehicle as control) was determined by q-PCR. MicroRNAs miR-140-3p and miR-708 were differentially expressed in HASM cells in the presence of TNF-α, showing a CT value of 35 or lower in the q-PCR analysis and were selected for further studies. My work is primarily based on the regulation of the expression of CD38 and other asthma related genes by miR-708 in HASM cells.

MicroRNAs are powerful gene modulators (29). Aberrantly expressed microRNAs cause disturbances in cellular processes, which lead to diseases. Several microRNAs have been implicated in the pathophysiology of asthma, such as miR-133a in bronchial hyperresponsiveness (65) as well as miR-10a (162), miR-221 (272) and miR-25 (197) in airway smooth muscle hyperplasia. Since miR-708 was predicted to have a target site at the 3'UTR of CD38, a known player in AHR, we sought to determine whether miR-708 might play a regulatory role in the expression of CD38. Accordingly, I first transiently transfected the mimic of miR-708 or a control microRNA (scrambled sequence

mimic) at different concentrations into HASM cells and measured the expression of CD38 at mRNA and protein levels, using q-PCR and reverse cyclase assays, respectively. I found that miR-708 inhibits the expression of CD38 at 50 nM and 100nM but not at 10nM. This concentration (10nM) may not be the optimum (threshold) concentration for miR-708 to initiate its inhibitory effect on the expression of CD38.

Conclusion 1:

miR-708 regulates the expression of CD38 at mRNA and protein level

MicroRNAs exert their inhibitory effect mostly by directly binding to the 3'UTR of their target mRNA (29, 30). Therefore, to determine whether miR-708 directly binds to the predicted target site at the 3'UTR of CD38 and regulates gene expression, NIH-3T3 cells were transiently transfected with luciferase-reporter-plasmids with CD38-3'UTR constructs and mimic of miR-708 or scrambled sequence mimic as a control. Here I used a heterologous cell system (NIH-3T3) to perform this assay because HASM cells are primary cells and are technically challenging to transfect with larger plasmids. I found that the relative luciferase activity in cell lysates obtained from cells transfected with the mimic of miR-708 was lower compared to lysates of cells transfected with scrambled sequence mimic or the luciferase-reporter–plasmid alone. The specificity of target binding by miR-708 was confirmed by mutating four bases in the target sequence which reversed the inhibitory effect of miR-708 on luciferase gene expression.

Conclusion 2:

miR-708 has specific functional and putative target sites at the 3'UTR of CD38.

MicroRNAs regulate the expression of genes post-transcriptionally by binding to their target site at the 3'UTR of mRNA and degrading it or repressing translation, depending on the degree of sequence complementarity (29, 30). We performed mRNA stability assays to determine whether miR-708 inhibits the expression of CD38 by degrading the transcript. After transfection with mimic of miR-708 and growth arrest, HASM cells were treated with TNF-α for 12 h to induce CD38 expression. Actinomycin D was used to arrest further transcription and total RNA was collected at different time points to analyze the expression level of CD38 relative to 0 time point using non-linear regression analysis. I found that binding of miR-708 to its target site did not result in degradation of CD38 mRNA, therefore miR-708 should have caused translational repression.

Conclusion 3:

miR-708 does not regulate the expression of CD38 through degradation of CD38 mRNA.

MicroRNAs can indirectly regulate the expression of a target gene by regulating multiple constituents in signaling pathways (168). Since the expression of CD38 at mRNA level was significantly inhibited by miR-708, I sought to determine whether miR-708 regulates expression of CD38 at the level of transcription. Because MAPK and PI3K signaling pathways transcriptionally

regulate the expression of CD38 in the presence of TNF-α, I examined the activation and expression level of genes in these pathways. I found that following miR-708 transfection, activation of JNK MAPK and Akt in TNF-α-treated HASM cells was inhibited and the expression of their phosphatases, MKP-1 and PTEN, respectively was elevated.

Conclusion 4:

miR-708 transcriptionally regulates the expression of CD38 through inactivating JNK MAPK and Akt and increasing the expression of their inhibitors MKP-1 and PTEN.

Since miR-140-3p is also differentially expressed in HASM cells treated with TNF-α, I examined whether this microRNA plays a role in regulating the expression of CD38. Over-expression of miR-140-3p in cells transfected with the mimic of this microRNA inhibited the expression of CD38 in a concentration-dependent manner at mRNA and protein level. Luciferase reporter assays as described earlier with miR-708 confirmed direct and specific (by site-directed mutagenesis) interaction of miR-140-3p with the 3'-UTR target binding site of CD38. Further, I observed that as in the case of miR-708, miR-140-3p transfection did not alter CD38 mRNA stability. Since transcript degradation was not the cause for decreased levels of CD38 mRNA, I sought to determine whether miR-140-3p exerts its regulatory effect on CD38 expression at a transcriptional level.

Western blot analysis to detect the level of expression and activation status of signaling molecules in the MAPK and PI3K pathway as well as of the

transcription factors NF-κB and AP-1 revealed that over expression of miR-140-3p decreases the activation of p38 MAPK and NF-κB (figure 1).

Conclusion 5:

MiR-140-3p regulates the expression of CD38 post-transcriptionally by binding to its putative target site and transcriptionally by inactivating p38 MAPK and transcription factor NF-κB.

MicroRNAs simultaneously regulate several genes that accomplish a cellular process or a tissue function. In order to determine whether miR-708 transfection alters expression of other asthma related genes, I performed a microarray analysis of total RNA isolated from HASM cells that were transfected with mimic of miR-708 or scrambled sequence mimic and treated with TNF-α. Interestingly, I found that several inflammation-associated genes such as chemokines, cytokines, genes associated with extracellular matrix components and genes related to TNF-α and NF-κB were down-regulated. Decreased expression of some of these inflammatory genes such as CCL11, CXCL10, CXCL8, CCL2, RARRES2 (retinoic acid receptor responder 2) and extra cellular matrix associated genes such as ADAM33 (a disintergrin and metalloprotease 33) and CD44 was validated by q-PCR. Chemokines secreted by HASM cells play an important role during inflammation by attracting/recruiting inflammatory cells to the airways. Chemokines CCL11, CXCL10, CXCL8, CCL2 and RARRES2 have been shown by other investigators to recruit eosinophils, mast cells, neutrophils, monocytes, dendritic cells and macrophages, respectively. We measured the release of CCL11, CCL5, CXCL10, CXCL8 and CXCL12 in the

culture supernatant of HASM cells transfected with mimic of miR-708 or scrambled sequence mimic at different time points ranging from 6-48h. We found that the release of only CCL11 was inhibited by miR-708 transfection with a coordinated decrease at the mRNA level. Although the expression of other chemokines was inhibited at the mRNA level, there was no corresponding decrease at the protein level. This suggests that these chemokines might be stored in vesicles or in pre-formed granules as seen in other cell types such as eosinophils, endothelial cells, mast cells, etc., and when TNF-α is added to the cell culture they are readily released. Expression and release of these chemokines from HASM cells is known to be controlled by various signaling pathways depending on the type of trigger. IL-17A-induced expression of CCL11 involves the MAPK pathway (279), while TSLP-induced expression of CC/CXC chemokines involves MAPK and STAT3 pathways (301). Likewise, IL-β-induced release of CCL11, CCL2 and CCL7 involves activation of the MAPK pathway as well as the transcription factor NF-κB (357) and IL-13-induced release of CCL11 involves MAPK and STAT-6 signaling pathways (270). Therefore it is possible that signaling pathways that are not regulated by miR-708 may be involved in the release of the chemokines CCL5, CXCL10, CXCL8 and CXCL12 and when the cells are exposed to TNF-α, irrespective of whether miR-708 is present or not, HASM cells readily released these chemokines. On the other hand, it is also possible that mRNAs of these chemokines that were transcribed prior to miR-708 transfection/over-expression undergo protein translation and are released. In this case, differences in the release of these chemokines following miR-708

transfection may be detected in the culture supernatant at later time points. However, taking into consideration that this is a transient transfection system and the half-life of microRNAs within cells after transfection in vitro is about 3-5 days, we collected supernatants only up to 48h. Studies from other laboratories suggest that using a mixture of cytokines (cytomix) containing IFN-γ, IL1-β, and TNF-α instead of TNF-α alone may enable detection of differences in the release of these other chemokines compared to control (cells transfected with scrambled sequence mimic) (9, 91). Establishing stable transfection using viral vectors is technically challenging since the procedure entails several passages of cells for the selection of successfully transected cells which may lead to loss of smooth muscle phenotype in HASM primary cells.

CD44, a cell surface glycoprotein, is a key receptor for hyaluronan and a major component of the extra cellular matrix which plays a role in airway remodeling and AHR. Further, CD44 has been shown to promote the homing of activated T-lymphocytes to asthmatic airways (201). Studies have shown that allergen-challenged CD44-deficient mice exhibit markedly attenuated AHR compared to wild type (WT) mice and that administration of antibodies against CD44 to allergen-challenged WT mice results in reduced AHR (122). Thus, inhibition of the expression of CD44 by miR-708 is likely to have a beneficial effect on asthma-related symptoms. Likewise, down-regulation of ADAM33 and RARRES2 gene expression by miR-708 is also likely to be beneficial in asthma since both have been implicated in asthma. ADAM33 is an asthma susceptibility gene strongly associated with bronchial hyperresponsiveness with a role in

airway remodeling by promoting fibroblast hyperplasia (212, 225, 246, 337) and RARRES2, is a chemoattractant for dendritic cells and macrophages (166, 355, 356).

Microarray analysis of miR-708-transfected cells also revealed inhibition of expression of various other genes that could impact inflammation such as (i) those associated with extracellular matrix, i.e., collagen types 1,3 and 6, VCAM, THBS1, MXRA5; (ii) TNF-α related genes, i.e., TNFRSF11B and TNFSF13B; (iii) genes related to smooth muscle contraction, i.e., TAGLN, MYLK, ACTG2; (iv) transcription factors, i.e., NF-κB1 and RELA; (v) growth factor related genes such as IGFBP5, PDGFRL, EGFL6. In addition, up regulation of important genes such as PPIL5 that suppress the activation of NF-κB, DUSP6 that inactivates MAPKs, BMP2 that up-regulates PTEN and IL-24 that has anti-inflammatory and anti-proliferative properties was also noted.

Conclusion 6:

miR-708 inhibits the expression of several inflammatory genes including chemokines CCL11, CXCL10, CXCL8, CCL2 and RARRES2, extracellular matrix components CD44 and ADAM33 as well as the release of CCL11 by HASM cells.

Proliferation of HASM cells causes thickening of the airway wall and obstruction of airflow. Several studies have shown that thickening of the airway wall due to increased smooth muscle mass increases airway resistance. Further, studies have also shown that the MAPK and PI3K signaling pathways regulate the proliferation of HASM cells. MicroRNAs such as miR-10a and miR-221 have

been shown to play a role in the proliferation of HASM cells. miR-708, a focus of the current study, has been implicated in many cancer-related studies and is shown to have anti-proliferative as well as pro-proliferative properties, depending on the type of cancer. Given the role of MAPK and PI3K signaling pathways in regulating the proliferation of HASM cells, participation of miR-708 in down-regulating these signaling pathways as shown in the current study may have an inhibitory effect on proliferation of HASM cells (figure 1). In the microarray data, we found that expression of an almost equal number of pro-proliferative and anti-proliferative genes were altered in HASM cells transfected with mimic of miR-708 and treated with TNF-α. To further, explore this, we performed proliferation assays using the CYQUANT cell proliferation assay kit with HASM cells following transfection with mimic of miR-708 or scrambled sequence mimic in the presence of TNF-α or 10% fetal bovine serum. miR-708 was found to inhibit the proliferation of NA-HASM cells by about 37% in the presence of fetal bovine serum.

Conclusion 7:

miR-708 has an anti-proliferative function in HASM cells.

Figure 1. Proposed model for the microRNA regulation of CD38 and other asthma related genes, based on the findings of the present investigation.

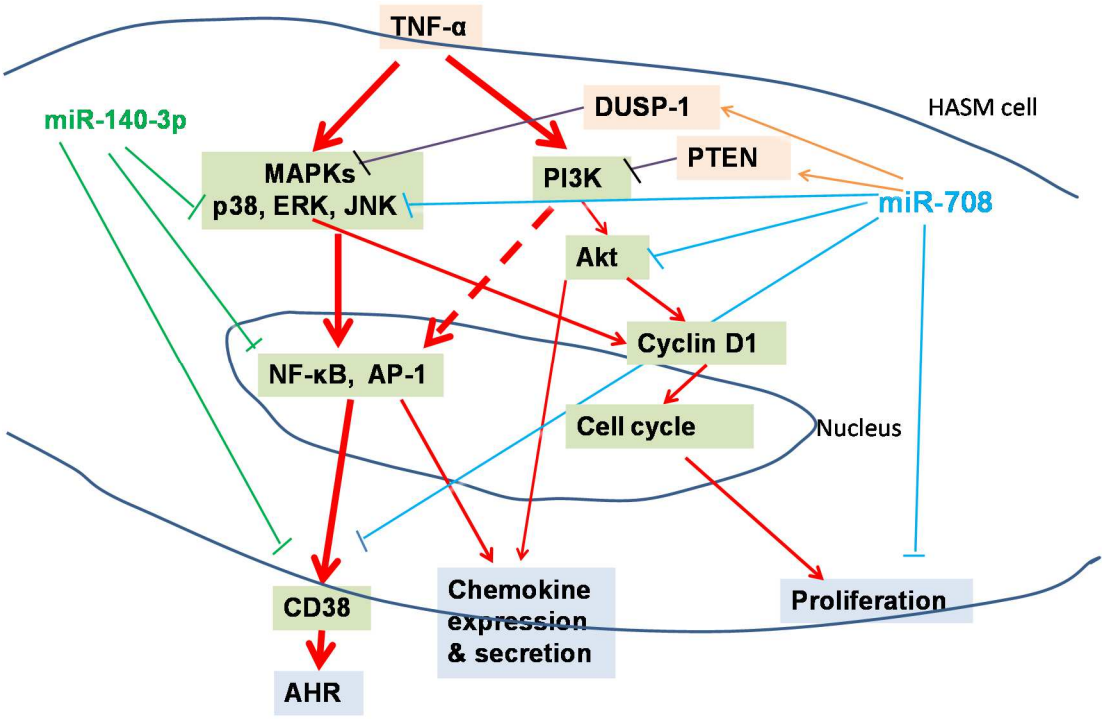

Figure 1: Model depicting regulation of chemokine gene expression and proliferation of human airway smooth muscle cells:

Exposoure of human airway smooth muscle cells to the inflammatory cytokine TNF-α causes activation of the MAP kinases and PI3 kinase signaling pathways. These results in augmented expression of the cell-surface protein CD38 through transcription factors NF-κB and AP-1 to cause increased calcium signaling, contractility and airway hyperresponsiveness (AHR). TNF-α also causes increased ASM cell proliferation through MAP kinase and PI3 kinase/Akt signaling pathways. The expression and secretion of several chemokines from

ASM cells is significantly increased on exposure to TNF-α. MiR-708, by increasing the expression of a dual specificity phosphatase (DUSP-1) decreases JNK MAP kinase phosphorylation, resulting in decreased expression of CD38 and several chemokine genes. By inducing the expression of PTEN, a phosphatase that inactivates Akt, miR-708 can inhibit cell proliferation as well as CD38 expression. MiR-140-3p, on the other hand, decreases activation of p38 MAP kinase and NF-κB resulting in decreased CD38 expression. MiR-140-3p effects on inhibition of chemokine gene expression and secretion may also be exerted through decreased activation of p38 MAP kinase and NF-κB.

Future Direction 1:

Test efficacy of miR-708 in down-regulating allergen-induced airway inflammation and AHR in mouse models: Cholesterol-incorporated, nucleotide-locked miR-708 will be administered intranasally or intravenously to asthmatic mice and pulmonary function assays (plethysmography) as well as airway inflammation based on total and differential cell counts in BAL fluid will be determined. To compare miR-708 versus an inhibitor of CD38 in controlling AHR and inflammation, the CD38 inhibitor (modified NAD) will be delivered intranasally to allergen-challenged WT mice and airway inflammation will be assessed. I anticipate that allergen-challenged mice administered with miR-708 will exhibit lower AHR than mice that received CD38 inhibitors though species differences and species specific functions may interfere with the results.

Future Direction 2:

Previous studies from our laboratory have shown that asthmatic $CD38^{-/-}$ chimeric mice that were transplanted with WT bone marrow demonstrate partial rescue in airway resistance to contractile agonists. To determine whether CD38 expressed by inflammatory cells have a role in controlling airway contractility, an inhibitor of CD38 will be administered to these mice and pulmonary function will be measured. Here I anticipate that chimeric mice that received the inhibitor will exhibit less airway contractility compared to mice that received the vehicle alone.

Future Direction 3:

Establish the ability of miR-708 to inhibit cellular recruitment by HASM cells: Since miR-708 and miR-140-3p inhibit release of CCL11 and CXCL12 by HASM cells, respectively, migration assays will be performed with culture supernatants of HASM cells transfected with mimic of miR-708, mimic of miR140-3p or scrambled sequence mimic and activated with TNF-α to examine recruitment of human eosinophils and Jurkat cells (a human T cell line), respectively, in Transwell chamber plates. We anticipate that there will be less cellular migration with culture supernatants from cells transfected with mimic of miR-708 or miR140-3p relative to culture supernatants from cells transfected with scrambled sequence mimic.

Perspectives of the current study

- Previous studies from our laboratory showed that airways of allergen challenged CD38 $^{-/-}$ mice were hyporesponsive to contractile agonist methacholine compared to that of wild type mice. Airway smooth muscle (ASM) cells derived from CD38 $^{-/-}$ mice also showed reduced calcium responses to contractile agonists compared to that of wild type mice. These studies revealed the significant role of CD38 in calcium signaling and airway hyperresponsiveness (AHR). Further, it has been found that signaling pathways MAPK and PI3K regulate TNF-α induced- CD38 expression and ASM cells of asthmatic patients have increased expression of CD38 when compared to ASM cells from healthy subjects. In the current studies, microRNAs miR-708 and miR-140-3p are shown to control the expression of CD38 directly by binding to the 3'UTR and indirectly through MAPK and PI3K signaling pathways. Collectively, these data suggest that the microRNAs miR-708 and miR-140-3p may have a potential role in attenuating AHR by controlling the expression of CD38.
- The signaling pathways MAPK and PI3K also regulate the expression and release of many proinflammatory cytokines and chemokines. In asthma, proinflammatory mediators trigger these signaling pathways to produce tremendous amounts of chemokines and cytokines in the airways that augment and sustain the inflammation. Administration of

pharmacological inhibitors of MAP kinases or PI3kinases in allergen induced mouse asthma models reduces the airway inflammation. In the current study I found that microRNA miR-708 controls the signaling pathways MAPK and PI3K by inactivating JNK MAPK and AKT and increases the expression of their phosphatases MKP-1 and PTEN respectively. Thus introduction of mimics of miR-708 in the airways to target ASM cells would cause a high impact in controlling airway inflammation. Validation of the reduced mRNA expression of many chemokines and the reduced release of CCL11 following miR-708 transfection in human ASM cells compared to control, further support this hypothesis. Likewise, microRNA miR-140-3p inactivates p38 MAPK and transcription factor NF-κB, and inhibits the mRNA expression of many chemokines and the release of CXCL12. Thus miR-140-3p may also have a therapeutic potential in controlling airway inflammation. Further it has been shown by other laboratories that CD38 is essential for dendritic cell trafficking from peripheral tissues to regional lymph nodes, T-cell antigen priming and chemotactic migration of neutrophils. Thus regulating the expression of CD38 in these cells by microRNAs miR-708 and miR-140-3p, we might be able to control the airway inflammation.

- Another aspect of CD38 in contributing to the pathogenesis of airway inflammation would be through production of a purine nucleoside, adenosine. It is known to be produced from ATP and hypothesize to be

produced through the enzymatic activity of CD38 from the substrate NAD, which also acts as a source for the production cADPR, a potent Ca^{++} releaser from intracellular Ca^{++}storages. Adenosine is highly expressed in the airways of asthmatic patients and has been shown to elevate the expression of IL6 through STA3 activation. IL6 is involved in the proliferation of fibroblasts and ASM. Further adenosine binds to its receptor A1R on the membrane of ASM cells and induce the production of IP3 (inositol triphosphate) another secondary messenger for Ca^{++}release from intracellular Ca^{++}storages. Therefore, by regulating the expression of CD38 by microRNAs miR-708 and miR-140-3p, we may potentially control the airway hyperresponsiveness by blocking adenosine-IP3 pathway, airway inflammation by blocking adenosine production itself and airway remodeling by hindering the adenosine - IL6 pathway.

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