Interaction Between $CD4^+$ T cells and Airway Smooth Muscle cells: Implications in Asthma

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I. Acknowledgments

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II. Contributions

Abstract:

The abstract was translated into French by Julia Chronopoulos and Erwan Pernet.

Chapter 6:

Soroor Farahnak, along with Michelle Kim and Ana Wang, aided in the collection of mRNA samples and the running of qPCR experiments. Soroor Farahnak and Ruchi Sharan both contributed to proliferation in co-culture as well as afatinib treated conditions. Ruchi Sharan was responsible for running and analyzing calcium experiments.

Chapter 7:

Rui Sun transduced ASM cells with a lentivirus to expressed PDHA-1-tRFP in order to study mitochondrial transfer.

Chapter 8:

Gjis Ijpma contributed ideas towards the creation of a novel Nanotube assay. Megan Hammell, from Dr. Anne Marie Lauzon's lab, performed optical tweezer experiments.

Nurse Cathy Fugere drew blood for all experiments requiring $CD4^+$ T cells. Both Soroor Farahnak and Rui Sun aided in the isolation of $CD4^+$ T cells throughout the study.

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III. Abstract

Asthma is a respiratory disease that is characterized by exaggerated narrowing of the airways in response to a contractile agonist or airway irritants. This characteristic is called airway hyperresponsiveness (AHR) and is accompanied by airway remodeling which consists of thickening of the airway wall caused by an increase in airway smooth muscle (ASM) mass and fibrosis. Remodeling of the ASM may be the key to AHR as increased muscle mass would produce more force to overcome intrinsic impedances to airway narrowing. Some evidence suggests that individual ASM cells are also more contractile. Infiltration of the airways by CD4+ T cells has been associated with disease severity. CD4+ T cells localize to airway smooth muscle bundles in the airways where they may mediate increases in ASM mass and AHR. ASM cells have also demonstrated the potential to enhance CD4+ T cell responses but the mechanisms behind the bi-directional cellular modulation is unclear.

The purpose of the current study was to determine the changes in ASM and T cell phenotypes resulting from contact dependent interaction of the two cells types. To achieve this objective, we used *in vitro* co-culture methods to elucidate some of the pertinent mechanisms. Analysis of BrdU incorporation showed CD4+ T cells increased ASM cell proliferation. By measuring fluorescence of the calcium binding dye, Fura-2, we determined that ASM cells in co-culture with CD4+ T cells had a weaker peak calcium response to histamine. A reduction in the expression of contractile factors was also observed in ASM cells. These effects were contact dependent as they did not occur in Transwell cultures. Inhibition of ICAM-1 using an antagonistic antibody inhibited CD4+ T cell induced reduction in contractile factors suggesting ICAM-1 is responsible for physical contact mediated changes in ASM phenotype.

CD4+ T cells were hyper-reactive to CD3/28 re-stimulation after co-culture with ASM cells as measured by cytokine expression, exhibiting a memory like response. Whether or not CD4+ T cells have transitioned into a memory T cell requires further experimentation. Previous studies demonstrated ASM cells pass mitochondria to CD4+ T cells through membrane nanotubes, thus

the role of mitochondrial mechanisms was explored in mediating CD4+ T cells hyper-reactivity. Re-stimulation induced greater mitochondrial reactive oxygen species synthesis in co-cultured CD4+ T cells as measured by mitoSOX. Seahorse analysis showed oxygen consumption was maintained after re-stimulation while glycolytic processes uncharacteristically declined. Preliminary analysis suggests ASM cells increase caspase-1 activation in CD4+ T cells upon re-stimulation, which could be mediated by inflammasome activation by ROS. IL-1β release due to this process can enhance CD4+ T cells responses to CD3/28 stimulation, however, this needs to be confirmed.

To more efficiently visualize membrane nanotube, a polydimethylsiloxane (PDMS) based microfluidic device was designed and commercially manufactured. Two cell chambers were separated by microchannels that were 1µm wide and ranged from 10-200µm long. Cultures using tumor associated fibroblasts and a breast cancer cell line demonstrated that fibroblasts could form tube like structures towards breast cancer cells through the microchannels. CD4+ T cells, however, failed to do so. Using optical tweezers to physically control CD4+ T cell movement, membrane nanotube formation was visualized without prior contact with the ASM cells. Preliminary testing demonstrated membrane nanotube formation in this system is reproducible.

These results demonstrate CD4+ T cells induce a pro-proliferative and anti-contractile phenotype in ASM cells, while ASM cells induce hyper-reactive responses by CD4+ T cells to CD3/28 re-stimulation. Also, the use of optical tweezers may be a viable method of studying nanotubes formed from non-adherent cells.

Sommaire

L'asthme est une maladie respiratoire caractérisée par un rétrécissement des voies respiratoires (VR) en réponse à un agoniste contractile ou à l'inhalation d'agents irritants. Ce phénomène est appelé hyperréactivité des voies respiratoires (HVR) et s'accompagne d'un remodelage des voies aériennes, consistant en un épaississement de la paroi des VR, conséquence d'une augmentation de la masse du muscle lisse (ML) et à la fibrose. Le remodelage du ML pourrait jouer un rôle important dans l'HVR car une augmentation de la masse musculaire produirait plus de force pour surmonter les impédances intrinsèques au rétrécissement des VR. De plus, il a été suggéré que les cellules du ML sont également plus contractiles. L'infiltration des VR par les cellules T CD4+, localisées dans les faisceaux du ML, a été associée à la sévérité de la maladie en contribuant potentiellement à l'augmentation de la masse du ML et l'HVR. Les réponses des cellules T CD4+ peuvent également être augmentées par les cellules du ML, mais les mécanismes impliqués dans cette modulation bidirectionnelle ne sont pas clairs.

Le but de cette étude est de déterminer les changements du ML des voies respiratoires et des phénotypes des cellules T CD4+ après contact direct entre les deux types cellulaires. Pour cela, nous avons utilisé des techniques de coculture *in vitro* afin d'identifier les mécanismes sous-jacents. Nos résultats indiquent que les cellules T CD4+ augmentent la prolifération des cellules du ML et que les cellules du ML cultivées en contact direct avec les cellules T CD4+ présentaient une réponse calcique maximale diminuée en réponse à une stimulation par l'histamine et une réduction de l'expression des facteurs contractiles. Ce dernier phénomène peut être inversé en utilisant un anticorps neutralisant contre ICAM-1, suggérant qu'ICAM-1 est responsable des changements du phénotype du ML induite par contact direct avec les cellules T CD4+.

Après coculture avec les cellules du ML, les cellules T CD4+ restimulées sont hyper-réactives, présentant une réponse de type mémoire, qui devra être confirmé. Comme les cellules du ML transmettent des mitochondries au cellules T CD4+ par des nanotubes membranaires, nous avons analysé le rôle des mécanismes mitochondriaux dans l'hyper-réactivité des cellules T CD4+.

Après re-stimulation, les cellules T CD4+ cultivées avec les cellules du ML présentent une augmentation de la production d'espèces réactives de l'oxygène (ERO) mitochondriales. L'analyse du métabolisme cellulaire a montré une réduction des processus glycolytiques alors que la consommation d'oxygène est maintenue après re-stimulation. Une analyse préliminaire suggère que les cellules du ML augmentent l'activation de la caspase-1 dans les cellules T CD4+, ce qui pourrait être favorisé par l'activation de l'inflammasome par les ERO. La sécrétion d'IL-1β due à ce processus pourrait améliorer les réponses des cellules T CD4+, mais cela doit être confirmé.

Pour visualiser plus efficacement les nanotubes membranaires, un dispositif microfluidique à base de PDMS a été conçu en utilisant deux chambres de cellules séparées par des microcanaux d'une largeur de 1 µm et d'une longueur de 10 à 200 µm. Des études précédentes ont démontré que les fibroblastes, mais pas les cellules T CD4+, peuvent former des structures ressemblant à des tubes en direction de cellules cancéreuses par les microcanaux. En utilisant des pinces optiques pour contrôler physiquement le mouvement des cellules T CD4+, la formation de nanotubes membranaires a été visualisée sans contact préalable avec les cellules de ML de façon reproductible.

Ces résultats démontrent que les cellules T CD4+ induisent un phénotype pro-prolifératif et anti-contractile dans les cellules de ML, tandis que ces dernières induisent des réponses hyper-réactives des cellules T CD4+. De plus, l'utilisation de pinces optiques peut être une méthode viable pour étudier des nanotubes formés à partir des cellules non adhérentes.

1. Introduction to Asthma

Asthma is a chronic respiratory disease that inflict people of all ages. It has been estimated to affect 235 million people worldwide and is the most common chronic disease amongst children [1]. The prevalence of asthma has been decreasing globally; some countries, such as the United States, are seeing yearly increases in persons suffering from asthma [2]. The disease presents with signs of respiratory distress such as wheezing, coughing and shortness of breath that can be debilitating. Acute exacerbations can also occur when asthmatics inhale irritants, such as cigarette smoke, causing excessive narrowing of airways and damage to the airways. Acute attacks can even be fatal but rarely are. Asthmatics are also more susceptible to respiratory infection and exhibit high levels of inflammation in the airways which have both been demonstrated to worsen the respiratory condition. Viral respiratory tract infections are the predominant cause of acute exacerbations [3]. Although many steps have been taken to treat and prevent asthma, our understanding of the disease is still incomplete.

1.1 Airway Hyperresponsiveness

Asthmatic patients are defined by the intermittent development of narrowed airways causing airflow limitation, the degree of which is measured by a forced expiratory maneuver. The predisposition to narrow the airways or airway hyperresponsiveness (AHR) is quantifiable by methacholine challenge. Methacholine, a muscarinic receptor agonist, induces narrowing of the airways allowing the prediction of the response to an inhaled irritant or to other triggers. Methacholine delivered by aerosol in a standardized manner and the airway narrowing evoked is quantified from the forced expiratory volume in the first second of the expiration (FEV_1). Asthmatics exhibit an exaggerated response with narrowing of the airways that causes significantly obstructing airflow compared to a small or absent response to inhaled methacholine in healthy subjects. The phenomenon of AHR is a characteristic that defines asthma. The severity of AHR correlates directly with the severity of asthma [4-6], suggesting that treating the causes of AHR is the key to treating asthmatics.

The causes of AHR are currently unknown but clinical evidence points towards two possible culprits. The first is inflammation. Asthmatic airways are infiltrated with inflammatory cells including eosinophils, mast cells and T cells that can augment airway contraction in multiple ways [7, 8]. Mast cells store the contractile agonist histamine which is released through degranulation when mast cells are activated through IgE cross-linking by antigen [9]. Cysteinyl-leukotrienes or prostaglandins (PG), such as PGD_2, are synthesized do novo and signal through specific receptors that trigger airway smooth muscle (ASM) contraction. Leukocytes can also modulate airway contraction indirectly through interactions with structural cells, inducing a phenomenon called airway remodeling. Like AHR, levels of infiltrating leukocytes and inflammatory mediators, such as interleukin (IL)-13, correlate with the severity of asthma and thus, the severity of AHR [8, 10-13]. Furthermore, reducing this inflammation using inhaled or, in severe cases, oral corticosteroids can suppress or even abolish AHR in some asthmatic patients [14].

Asthmatic airways are frequently characterized by airway remodeling where structural changes lead to a thickening of the airway wall [15, 16]. One key component of this remodeling is a thickening of ASM bundles [17-19], which is the second effective target to treat AHR. The ASM was predicted to be responsible for the enhanced narrowing of the airways by a computational model of bronchoconstriction [20]. This can occur through two possible mechanisms. First, an increase in ASM mass will result in an increase in contractile units causing larger contractions [21]. In so far as an increase in ASM may encroach upon the airway lumen the airways may be in a chronically narrowed state. An increase in extracellular matrix (ECM) deposition around the ASM will exacerbate this problem as well [22]. The second mechanism by which ASM can cause AHR is by a modulation in its phenotype such that each individual ASM cell is more contractile. This could be achieved through an enhanced expression of contractile proteins. The variety of endotypes seen within the asthmatic population suggests that either mechanism or a combination of both is possible. Relaxing the ASM by targeting beta-adrenergic receptors with selective beta2-agonists is a standard approach to treating acute asthma attacks by dilating the

airways. Long term administration of beta-agonists has also been demonstrated to reduce AHR suggesting increased ASM tone is also a contributor to AHR [23, 24].

1.2 Airway remodeling

Airway remodeling involves more than just the ASM. Alterations in other components of the subepithelial space as well as the epithelium occur that can exacerbate asthmatic pathology. Changes to the epithelium include increased shedding, goblet cell hyperplasia and loss of ciliated cells [25]. Epithelial shedding destroys the barrier between the outside environment and the lung, causing an increased susceptibility to allergen and microbes, particularly viruses, thus increasing airway inflammation. An increase in goblet cells, the mucus producing cells of the airways, may also narrow the airways themselves [26]. A loss of ciliation will exacerbate the problems caused by mucus because of the lack of clearance by the ciliated cells. Subepithelial fibrosis is another hallmark of airway remodeling [27]. This is caused by increased collagen deposition at the basement membrane of the epithelium by fibroblasts as well as a reduced expression ratio of metalloproteinases to tissue inhibitor of metalloproteinase [28]. An increase in myofibroblasts that are contractile like the ASM and pro-fibrotic like fibroblasts, can also contribute to remodeling [29]. These two components can lead to stiffening of the airways and organ malfunction respectively [30].

Similar to AHR, the severity of airway remodeling correlates directly with the severity of asthma. Increases in ASM mass, epithelial shedding, mucus production and fibrosis are all signs of a worsening asthmatic condition [25, 27]. AHR may be the physiological manifestation of airway remodeling, thus it has been proposed that reversing the structural changes in the airways may be the key to treating asthma. The causes behind airway remodeling are unknown, but to give further evidence to the above theory, airway remodeling can be treated using the same methods used to target AHR. It is theorized that ASM can be “trained” like skeletal muscle to induce hyperplasia and hypertrophy and thus preventing contraction may lead a “de-training” phenomenon. Airway contraction has also been shown to induce the release of

growth factors, such as HB-EGF, that can act on various airway cells to promote remodeling [31]. In culture, treating ASM cells with a beta-agonist can promote proliferation [32].

Administration of corticosteroids to reduce inflammation can also revert the airways to a healthy state [14, 33]. Chronic inflammation is currently the leading theory behind airway remodeling. In equine asthma, corticosteroids have been shown to reverse, in part, the increase in airway smooth muscle [34] that is characteristic of horses with so-called “heaves”, a severe form of equine asthma. Inflammatory mediators such as cytokines and prostaglandins have been implicated in all aspects of remodeling from the epithelium to the ASM. IL-13, for example, is a known modulator of ASM phenotype promoting the expression of contractile proteins [35]. The structural cells such as the ASM and epithelium, are known to be pro-inflammatory themselves but infiltrating leukocytes seem to be the major mediators of inflammation. Inhibiting immune cells such as eosinophils and $CD4^+$ T cells have shown to have restorative effects in *in vivo* studies [36, 37]. It is possible that inflammatory cells can directly contribute to AHR, but evidence points to the interactions with and modulation of structural cells as the most important role of inflammation in asthma. It is most likely a combination of both the autologous changes to the ASM through contraction and the external influence of inflammation that lead to both airway remodeling and AHR.

2. Airway Smooth Muscle

2.1 Airway Smooth Muscle Phenotypes

As mentioned above, hyperproliferation and hypercontractility of the ASM are two properties that are hypothesized to be the key to AHR. Therefore, it is pertinent to understand how these changes in the ASM occur. ASM cells have been described to adopt two distinct phenotypes that align with these possible changes. The first is a contractile phenotype, thought to be a fully mature ASM cell within a tissue. As the name suggests, the contractile phenotype is marked by high expression levels of contractile proteins and an enhanced shortening response to contractile agonists, such as acetylcholine [38]. ASM cells of the contractile phenotype are also

characterized by a reduced capacity to proliferate, which is in direct contrast to their second phenotype, the synthetic/proliferative (proliferative) phenotype. ASM cells of this phenotype are capable of proliferating and have limited expression of contractile proteins [39]. The proliferative phenotype is also characterized by increased expression and release of various signaling proteins such as cytokines, growth factors and prostaglandins [39].

The distinction between a contractile and proliferative phenotype were originally made on vascular smooth muscle cells by Chamley-Campbell [40]. Similar studies performed subsequently comparing ASM cells in culture to freshly isolated ASM found similar results [38]. ASM cells demonstrated a declining expression of contractile proteins the longer they were kept *in vitro* while their proliferative rate increased [39]. When ASM cells reached confluence, however, cells were growth arrested and an increased expression of contractile factors was observed [39]. This suggests that limiting ASM cell growth can induce maturation. This may be a phenomenon only seen in *in vitro* conditions, however there is some evidence suggesting that the contractile and proliferative phenotypes are naturally occurring competing expression patterns within the ASM [41, 42]. Myocardin (MYOCD) is a transcription factor described to be the master regulator of ASM contractility [41]. Transcription of α-smooth muscle actin (ACTA), smooth muscle heavy chain 11 (MHC11), calponin (CNN) and myosin light chain kinase (MYLK), the components of the ASM contractile apparatus, are all regulated by MYOCD [41]. MYOCD requires binding to another transcriptions factor, serum response factor (SRF), in order to bind to its promoter [42]. SRF is part of the MADS-box gene family, which is an evolutionarily conserved family of proteins that is known to regulate cell differentiation. In ASM cells, SRF will also competitively bind to ETS domain-containing protein Elk-1 (ELK-1) that has been demonstrated to not only prevent MYOCD interactions with SRF but will actively suppress the transcription of MYOCD target genes as well [42].

The regulation of the two ASM cell phenotypes may be the key to remodeling the ASM as a shift toward the proliferative phenotype can lead to an increase in ASM mass, and an

enhancement of the contractile phenotype can produce stronger contractions. A balance of both phenotypes may be at play, where a cycle of proliferation and maturation into an enhanced contractile phenotype occur. Multiple studies have been done to tease out the possible regulators of these pathways and the nature of these transcription factors present multiple possibilities. ELK-1, SRF and MYOCD are known to be activated or suppressed, through variable phosphorylation by the mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) pathways [43, 44]. Both pathways are commonly converged on when cells are exposed to various stimuli, from growth factors to cytokines and from cell-to-cell interactions to cell-extracellular matrix (ECM) interactions [45, 46]. Combined with *in vivo* measurements of these factors within the lung, as well as *in vitro* studies on the effects of these factors on ASM phenotype, the major players in regulating the phenotypic switch between contractility and proliferation have been identified and are possible mediators of the asthmatic condition.

2.2 Mechanical Modulation of Phenotype

One modifier of ASM cell phenotype is the mechanical load, such as the strain ASM senses during airway contraction. Early studies made use of compressible membranes coated in collagen to mimic *in vivo* contraction. Canine ASM cells isolated from the trachea, were grown on these membranes and subjected to short cycles of pressure by a vacuum applied to the membrane so as to deform it and thus applying mechanical strain to the cells [47]. The system used was found to apply the greatest strain at the periphery of the membrane creating a gradient toward the center [47]. ASM cells stimulated with high strain were found to express more MHC11 and MYLK compared to control ASM cells which were unstimulated [47]. No differences were found with a low strain stimulation compared to the control, demonstrating that the level of strain can dictate ASM cell phenotype [47]. A high strain stimulation, in contrast to the control, also demonstrated an increase in desmin expression, a cytoskeletal protein associated with fully differentiated cells and the contractile phenotype of ASM cells [47]. However, no differences in vimentin were found, another cytoskeletal protein associated with the epithelial-mesenchymal transition and the proliferative phenotype of ASM cells [47].

This shift towards the contractile phenotype was demonstrated to have functional consequences as well. In the same *in vitro* compression system, cell lysates from strained cells demonstrated a greater ability to phosphorylate LC20, the regulatory chain on the myosin light chain (MLC) and target of MYLK [48]. ATPase activity was also assessed with and without the presence of calcium (Ca^{2+}) in order to differentiate MYLK dependent ATPase activity from other ATPase dependent cellular processes. MYLK requires binding of Ca^{2+} activated calmodulin in order to carry out its kinase function. Only MYLK dependent ATPase activity was elevated in strained ASM cells lysates compared to the control [48]. These studies have shown that ASM cells respond to mechanical strain by shifting towards a contractile phenotype which results in greater activity of contractile activators.

Recent studies have elucidated a mechanism behind the ASM cells response to mechanical strain. Strips of ASM were dissected from canine trachea and were placed vertically in a force transducer. ASM strips were then subjected to a 1g or 0.5g weight to induce a strain on the strips [49]. A 1g load induced greater activation of an integrin bound protein, focal adhesion kinase (FAK) compared to a 0.5g load [49]. This resulted in a reduced activation Protein kinase B (AKT), a kinase that has been shown to inhibit SRF binding to its promoter [49]. Greater inhibition of AKT at the 1g load resulted in increased MHC11 expression compared to ASM strips stimulated with a 0.5g load [49]. To test their synthetic activity, ASM was treated with IL-13 to induce eotaxin release. ASM strips under the 1g load demonstrated a reduced response release of eotaxin in response to IL-13 [49]. This suggests a greater load is not only inducing a contractile phenotype, but it is also inhibiting the proliferative phenotype. Treating tissues with elastase inhibited effects of mechanical strain on ASM strips. Elastase cleave ECM fibers, preventing the transfer of force between cells through integrins [50]. This is significant in the context of asthma, as asthmatic sputum was found to contain high levels of neutrophil elastase [51]. Elastase can also induce AHR in animal models, suggesting a possible mechanism for the induction of remodeling [52]. Other cell types can also interact with ASM through various integrins, such as $CD4^+$ T cells, providing other potential routes of ASM phenotype modulation [53].

2.3 Growth Factor Influence on Phenotype

Growth factors have the potential to have a major influence on ASM cell phenotype. These molecules are known to act on proliferative and differentiating pathways of various cell types, and they play a similar role in ASM cells. Three growth factors/family of growth factors have been identified as potent modulators of ASM phenotype and they are also found in higher levels in the bronchoalveolar lavage fluid from asthmatic patients compared to healthy subjects. These growth factors are transforming growth factor-β (TGF-β), platelet derived growth factor (PDGF) and the epidermal growth factor receptor (EGFR) ligands: epidermal growth factor (EGF), heparin binding epidermal growth factor (HB-EGF), and amphiregulin (AREG).

Many sources of the abovementioned growth factors have been identified, from structural cells to infiltrating leukocytes, and their expression has both chronic and acute kinetics related to asthmatic pathology. Epithelial cells, for example. have been found to have elevated expression in asthmatics as assessed through immunohistochemical staining of tissue sections [54, 55]. A spike of growth factor release has also been demonstrated after an acute asthma attack which has been linked to airway contraction [56]. Both mechanical and chemical mechanisms have been proposed for growth factor release within the airways. Inducing contraction of airways through methacholine, a contractile agonist thought to only act on ASM cells, can induce the release of TGF-β [31]. Application of transcellular pressure on epithelial cells can induce the release of HB-EGF [57]. A similar effect can be achieved using histamine, an airway contractile agonist, without the presence of any contractile load [58]. Functionally these growth factors from stimulated epithelial cells can modulate ASM phenotype in both pro-proliferative or pro-contractile directions.

2.3.1 TGF-β

TGF-β is a growth factor that acts on a serine/threonine kinase receptor, causing a signaling cascade involving the Smads and mitogen-activated protein kinase (MAPK) pathways that regulate cellular proliferation, differentiation, migration and apoptosis [59]. In the airways, many sources of TGF-β have been identified, but in the asthmatic condition, the major producers are infiltrating eosinophils [60]. These inflammatory leukocytes contribute to Th2 responses in the airways and are a characteristic cellular marker of asthmatics. Multiple other sources of TGF-β can be found within the mucosal and submucosal regions of the airway including epithelial cells, lymphocytes and ASM cells [61-63]. TGF-β, paradoxically, has been demonstrated to act as both a proliferative and contractile stimulus on ASM cells but this may be explained by divergent signaling cascades activated in response to TGF-β.

It has been demonstrated in multiple studies that treating ASM cells with TGF-β in culture induces proliferation [64-66]. This response seems to be mediated by the phosphorylation/activation of MAPK and subsequent phosphorylation/activation of extracellular signal-regulated kinase-1/2 (ERK1/2) upon activation of the TGF-β receptor [64, 65]. It is possible that this pathway converges on the activation of ELK-1 as it is a known target of ERK-1/2 [67]. Treating ASM cells with an NFκB inhibitors or a corticosteroid such as dexamethasone can also inhibit TGF-β stimulated proliferation implicating a possible role of inflammation [68]. There is some evidence that this augmentation in proliferation is independent of the Smad pathway as knocking out these proteins had no effect on ASM proliferation [68]. However, a separate study demonstrated that TGF-β can activate Smad2/3 resulting in an increase expression and activation of histone deacetylase-4 (HDAC4) causing the upregulation of cyclin D1, a pro-proliferative regulator [66]. Activation of this pathway did result in ASM cell proliferation thus it is possible that multiple factors regulate the effect of TGF-β on ASM cells and further research is required to elucidate the differences in these studies.

Cellular metabolism is a regulator of the TGF-β response. The proliferation induced by TGF-β induces a Warburg-like effect, shifting ASM cell metabolism sharply towards glycolysis [69, 70]. This is accompanied by an increase in glucose consumption, an upregulation of glycolytic enzymes and a down regulation of cellular respiratory activators [69, 70]. TGF-β stimulated proliferation can be inhibited by replacing glucose in the medium with 2-deoxy-D-glucose, a glucose analog that cannot be metabolized by glycolytic enzymes [69, 70]. This may be a simple energy imbalance, but some signaling consequences have been found as well. Activation of AMP-activated protein kinase (AMPK), an enzyme active during times of low [ATP] and high [AMP], by metformin can inhibit the activation of HDAC4 and subsequent induction of proliferation by TGF-β [66]. TGF-β has also been shown to have anti-proliferative effects on ASM treated with EGF and thrombin [71]. Interestingly, this effect was mediated through the inhibition of MAPK activation, therefore the effects of TGF-β seem to be context dependent.

Not surprisingly TGF-β modulates ASM contractile activation. By mounting murine tracheal rings on a force transducer, an increase in force was found in response to bradykinin with TGF-β treatment [72]. An increase in peak calcium concentration in response to a contractile agonist demonstrated an augmentation in contractile activation had occurred. Recently, another mechanism has been proposed involving the activation of the Smad3 pathway [73]. Using human precision cut lung slices (PCLS) investigators determined that TGF-β enhanced MLC phosphorylation through inhibition of myosin phosphatase (MLP) causing enhanced narrowing of airways [73]. An increase in cell stiffness was also found after TGF-β treatment [73]. This is dependent on the activation of Smad3 and subsequent activation of ROCK, a negative regulator of MLP [73].

TGF-β not only enhances contractile activation, it can induce a phenotype change leading an enhanced response attributable to effects downstream of contractile activation. Treating ASM cells in culture with TGF-β induces hypertrophy and increases protein synthesis, including ACTA, MHC11 and MYLK [74, 75]. ASM cells were not just bigger, but there was an increase in length,

taking on a more muscle-like morphology [74-76]. ACTA filaments were thicker and more organized in TGF-β treated cells suggesting that cytoskeletal rearrangement into contractile filaments had occurred [75, 76]. Functionally, TGF-β treated cells were stiffer than untreated cells and had an enhanced shortening response to a contractile agonist [75, 76]. Similar to its proliferative effects, multiple pathways have been found to be involved in the enhancement of contraction by TGF-β. Activation of PI3K is required for the inhibition of Eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP) a general translational repressor [75]. Other mechanisms have been proposed through the study of vascular smooth muscle since TGF-β has a similar pro-contractile effect in this tissue. TGF-β induces the activation of Ras homolog family member A (Rho-A) which seems to promote MAPK activation as well as Smad3 translocation into the nucleus [77, 78]. These processes lead to an increase in SRF expression and binding to serum response elements respectively, resulting in increased contractile gene expression [79-81].

The above evidence demonstrates that TGF-β is a regulator of ASM phenotype and is a potential target for therapy. However, the variable effects of TGF-β also suggest that modulating other aspects of TGF-β signaling, such as nutrition, may be an alternative or a required supplement to molecular therapies.

2.3.2 PDGF

PDGF is another well studied modulator of ASM cells for its pro-proliferative properties. PDGF is known as a mitogen for a variety of cells, such as the epithelial cells and fibroblasts. It comes in four isoforms that homodimerize to bind to its receptors, PDGF receptor (PDGFR) α and β. PDGFR is a receptor tyrosine kinase (RTK) that dimerizes upon ligand binding and initiates the PI3K pathway [82]. Early reports found only elevated levels of PDGF in eosinophils from asthmatics [83, 84]. However, more recent studies have identified epithelial cells, along with eosinophils, as a source of increased PDGF in chronic rhinosinusitis (CRS) patients with asthma

compared to CRS patients without asthma [85]. Further studies are needed to elucidate the sources and elevation of PDGF expression in asthma but its effects on ASM are significant.

PDGF has been demonstrated as a possible mediator of ASM remodeling and AHR in asthma models. Intranasal administration of an adenovirus with PDGF encoded in the adenovirus genome to overexpress PDGF in the airways of mice was sufficient to induce ASM proliferation and an increase in ASM area [86]. An analysis of gene expression showed a downregulation of ASM contractile genes, however no changes in ASM cell size occurred with PDGF overexpression [86]. An increase in airway responsiveness was determined using methacholine challenges indicating that PDGF overexpression was sufficient to cause AHR [86]. PDGF was also found in the BAL of wild type ovalbumin (OVA) sensitized mice after chronic exposure to OVA to induce an asthma phenotype [86]. Chronic exposure to OVA also resulted in the same ASM outcomes as overexpressing PDGF in these mice [86]. In another asthma mouse model using exposure to diesel exhaust particulate matter, neutralizing PDGF through an antagonizing antibody inhibited increases in airway resistance and airway thickening [87]. Epithelial cells and macrophages were observed sources of PDGF in these mice [87].

PDGF has been observed as a pro-proliferative growth factor for ASM cells [86, 88-93]. ASM strips treated with PDGF produce less force in response to methacholine [93]. Similar to *in vivo* results, reduced contraction by the ASM paralleled reduced expression of contractile genes [93]. ASM from asthmatic rats have a greater proliferative response to PDGF compared to healthy rat ASM [92]. However, no differences in PDGFR phosphorylation was observed suggesting downstream signaling mechanisms are responsible for PDGF induce proliferation augmentation [92]. Two major pathways have been determined to regulate PDGF-induced proliferation: the PI3K pathway and the Janus Kinase (JAK)-signal transducer and activator of transcription proteins (STAT) pathway. PI3K is a common target of RTK such as the PDGFR, and transduces its signal through activation of kinases, ERK1/2 and AKT, and through calcium-dependent signaling. PDGF causes the activation of both ERK1/2 and AKT in ASM cells, as well

as increasing intracellular calcium concentrations [88, 92, 94-96]. Inhibition of any of these of proteins and knocking out Orai1 or STIM1, regulators of the store-operated calcium entry response, prevents PDGF induced proliferation [88, 94-96]. PDGF also activates JAK2-STAT3 leading to expression and suppression of STAT3 target genes cyclin D1 (pro-proliferative) and p27 (anti-proliferative), respectively [90, 97].

Other cellular processes can also regulate the effectiveness of PDGF pro-proliferative processes. One of the targets of AKT is mammalian target of rapamycin (mTOR), a nutrient sensor that regulates PDGF induced proliferation [95]. AMPK activation through metformin can inhibit PDGF induced proliferation through inhibition of mTOR, suggesting nutrient levels or rates of cellular metabolism can affect PDGF induced proliferation of ASM cells [95, 98]. Metformin had no measurable effects on ASM stiffness [98]. Casticin, an anti-inflammatory found in certain plants, can also inhibit PDGF induced proliferation through inhibition of ERK1/2 and NFκB [94]. It was recently found that the pro-inflammatory regulator NFkB can also be a major regulator of PDGF signaling in ASM as it appears to be essential for PDGF induced proliferation and downregulation of contractile genes [94, 99]. Dimethyl sulfoxide a modulator of the antioxidant response, can also reduce PDGF induced proliferation through enhanced heme oxygenase-1 (HO-1) expression [89]. Increase in this antioxidant mediator is significant because activation of STATs can be regulated by reactive oxygen species (ROS) [100]. Similar to TGF-β, addressing separate cellular processes, such as inflammation, may resolve or suppress PDGF related pathologies.

2.3.3 EGFR Ligands

The EGFR ligands are a major family of growth factors commonly known as modulators of ASM phenotype. Like the PDGFR, the EGFR is a RTK with 4 isoforms that dimerize upon ligand binding, initiating a signaling cascade. Three ligands of the EGFR have been associated with asthma and airway remodeling: EGF, HB-EGF and AREG. In asthma, EGFR signaling has been mainly associated with epithelial cell repair and differentiation into mucus-producing goblet

cells [101-104]. Epithelial cells are also known as the major sources of EGFR ligands when stimulated with various stressors, such as epithelial shedding, physical contraction and cytokine signaling [55, 58, 103, 105]. ASM cells also have a similar upregulation of EGFR ligand expression in response to these stressors [58, 106, 107]. EGFR ligands can also be stored within the extracellular space bound to extracellular matrix (ECM) proteins that are released upon shearing of ECM fibers through the action of MMPs [108]. Activation of the EGFR is a pro-proliferative process and has been demonstrated as a driver of ASM remodeling both *in vivo* and *in vitro*.

The potential for EGFR ligands to drive ASM remodeling has been elucidated in both human and animal model studies. Histological analysis of bronchoscopic biopsies revealed that ASM in biopsies from severe asthmatics expressed more HB-EGF than in healthy airway samples [107]. Rat models of asthma revealed increased expression of HB-EGF in the epithelium upon repeated OVA challenges, along with increased ASM mass and AHR [109]. The ASM remodeling was inhibited by pretreating these rats with an EGFR inhibitor, AG1478 [109, 110]. ASM thickening was enhanced with intranasal treatment with HB-EGF along with OVA challenges in a mouse model of asthma [111]. A similar study in the rat found that increased MHC11 expression in ASM of the large airways by OVA challenge can also be inhibited by AG1478 [110]. Conversely, this study did not find increased HB-EGF expression in the epithelium, but elevated levels of HB-EGF and AREG were detected in BAL samples [110]. The effects on ASM remodeling by inhibiting EGFR was mirrored in a mouse study using house dust mite challenges and EGFR inhibition through erlotinib or by the expression of a dominant negative EGFR expressing mouse [112]. Elevated levels of EGF and AREG were also found in sputum samples from asthmatics after an acute asthma attack, demonstrating that challenge of the airways in humans also induces increased EGFR expression [104]. Various other animal model studies have demonstrated a release of EGFR ligands in the airways upon exposure to various insults, such as microbes, allergens and environmental pollutants, however the authors did not make any correlations to changes in the ASM [105, 113, 114].

Activation of the EGFR is known to induce proliferation of ASM cells *in vitro*. HB-EGF, EGF and AREG were all found to induce proliferation of ASM cells through similar pathways [58, 104, 109, 110, 115-117]. EGFR, being an RTK, will activate the PI3K pathway leading to subsequent activation of ERK1/2, AKT and MAPK. These three kinases are the major downstream regulators of this pathway and are commonly used as markers of PI3K activation. Treating ASM cells with EGFR ligands was found to induce phosphorylation, and therefore activation, of all these kinases [104, 113, 117]. To further strengthen this evidence, inhibition of the proto-oncogene tyrosine-protein kinase Src (Src), a necessary activator of PI3K, inhibited EGF-induced proliferation [118]. Activation of the PI3K pathway was also found in whole lung lysates of OVA sensitized mice [113]. The possible mechanisms behind PI3K modulation of the ASM phenotype have been described above.

Similar to TGF-β and PDGF induced proliferation, secondary factors, such as inflammation, seem to play a role in EGFR mediated proliferation by ASM cells. $CD4^+$ T cells are possible mediators of EGFR activation as $CD4^+$ T cell-induced ASM proliferation can be inhibited by afatinib, another EGFR inhibitor [119]. Furthermore, $CD4^+$ T cells have been shown to mediate remodeling in murine asthma models and possibly human asthmatics as well [120, 121]. Treating ASM cells with fluticasone or dexamethasone, two anti-inflammatories, enhanced EGF binding to the EGFR [122]. It is unclear of the functional consequences of enhanced binding since dexamethasone is also known to inhibit EGF-induced proliferation of ASM cells [116]. A β2-agonist was demonstrated to reduce the binding of EGF to the EGFR suggesting that the contractile state or stiffness of the muscle may modulate proliferative responses [122]. EGF induced proliferation was also inhibited by β2-agonists in a cyclic-adenosine-monophosphate (AMP) dependent manner [123]. Again, effects of growth factors on ASM phenotype are dependent on several other factors and treating asthmatics with anti-inflammatories and bronchodilators, as in current practice, may have growth factor-dependent benefits outside of their intended actions.

2.4 Role of Inflammation and Cytokine Signaling in Phenotype Modulation

Inflammation plays a key role in asthma pathology. Various pro-inflammatory mediators, such as cytokines, have been found at elevated levels in asthmatic airway samples and have been implicated in driving the disease in animal models. Various sources of inflammation can be found within the airways from infiltrating leukocytes to the structural cells within the airway. This pro-inflammatory environment can modulate the ASM phenotype in multiple ways. As described above, it can modulate growth factor signaling but it can also initiate growth factor release from both ASM and the epithelium. Studies have also shown that cytokines and pro-inflammatory lipid mediators may modulate ASM phenotype directly. Three families of pro-inflammatory mediators have been implicated as the major regulators of ASM: Th2/17 cytokines, IL-1β and TNFα, and lipid mediators.

2.4.1 *T-Helper Cytokines*

T-helper cytokines get their name for being distinct markers of T-helper subset activity but they are known to be expressed by various other leukocytes and structural cells, including the ASM. The Th2 phenotype is one of the better recognized endotypes of asthma as it is known to mediate the allergic response, including immunoglobulin class switching. Th2 cells are thought to be the major source of Th2 cytokines in asthma but mast cells, type II innate-like lymphoid cells (ILC2), epithelial cells and ASM cells are also contributing sources. IL-4, IL-13 and IL-8 have been studied as regulators of ASM phenotype and have been shown to drive asthma in multiple asthma models. Th17 cells, characterized by IL-17 expression, have also been implicated in asthma and ASM remodeling in murine asthma models. Evidence shows that T-helper cytokines, and thus $CD4^+$ T cells, are possible drivers of asthma pathology.

2.4.1.1 IL-4 and IL-13

IL-4 and IL-13 are the prototypical Th2 cytokines and are commonly linked in their activity as they share a receptor subunit, IL-4 receptor (IL-4R) α. IL-4 uses the common gamma chain as its

second subunit while IL-13 uses the IL-13 receptor α1. Signaling by these two cytokines converges on the activation of STAT6, a transcription factor, but IL-4 is capable of initiating the PI3K pathway through the common gamma chain subunit. There is some evidence for IL-13 also activating PI3K in colon cancer cells but it was dependent on signaling through scaffold proteins and FAK [124]. This may have implications in ASM as FAK activation has also been implicated in ASM phenotype modulation by mechanical stimulation [125]. IL-13 also binds the IL-13 receptor α2, however, this subunit is described as a decoy receptor, sequestering IL-13. The Th2 responses are known to play a role in the clearance of bacteria and are critical for the formation of mature immunoglobulin producing B cells. This axis of the T helper response is also associated with allergic responses, like those seen in asthma pathology.

The effects of IL-4 and IL-13 on asthma pathology have not been completely elucidated, however, it is clear they are able to promote AHR. Analysis of bronchial biopsies showed an increased number of IL4Rα expressing cells in asthmatics, however, there was no difference in IL4Rα$^+$ cells within ASM bundles [126]. The effect of IL4Rα varies as IL-4 is dependent on IL4Rα signaling, while IL-13 can induce AHR independently. By knocking out Stat6 in an OVA sensitized murine model of asthma, the development of AHR can be prevented [127]. Inhibiting IL-4 and IL-13 using antagonists produces a similar effect [128]. The two cytokines have had implications for AHR independently of one another. Treating mice with IL-4 intratracheally could induce AHR and this phenomenon was independent of knocking out IL-13 [129]. Inhibiting IL4Rα however, prevented IL-4 induced AHR [129]. By transferring IL-13-null CD4$^+$ T cells into mice and stimulating them with OVA, researchers demonstrated that IL-13, specifically from CD4$^+$ T cells, is required for allergen induced AHR [130]. Interestingly, this effect was not inhibited by knocking out IL4Rα or STAT6, demonstrating that IL-13 can induce AHR in an IL-4 and IL-4Rα independent manner [130]. The effects on ASM remodeling were not assessed in these studies.

The implications of IL4Rα signaling in ASM were studied in a set of experiments using selective expression or knockout of IL4Rα in ASM cells. First, a cre-lox system was created where a flox-

IL4Rα was cleaved by a cre-recombinase controlled by the MYH11 promoter resulting in mice lacking IL4Rα in the ASM only [131]. These mice developed AHR after sensitization and challenge with OVA, suggesting IL4Rα was not necessary for AHR [131]. No differences in any other asthma marker, such as airway inflammation or goblet cell hyperplasia, were found [131]. A separate study demonstrated the sufficiency of IL4Rα signaling in ASM to induce AHR. A mouse expressing a transgene for IL4Rα controlled by the smooth muscle specific promoter, SMP8, was bred with an IL4Rα knockout mouse creating a model where IL4Rα was only active in ASM ($IL4R\alpha^{ASM}$) [132]. Intratracheal treatment with IL-13 induced AHR in these mice while an $IL4R\alpha^{-/-}$ mouse similarly treated did not show the same effect [132]. The level of AHR, however, was not as great as $IL4R\alpha^{+/-}$ mice treated with IL-13 [132]. IL-4 had a similar effect, except $IL4R\alpha^{ASM}$ mice showed the highest level of airway responsiveness [132]. Challenging mice with the allergen, house dust mite, reproduced the effects of IL-4 treatment, showing IL-4 signaling, at the very least, is involved in allergen mediated AHR [132]. IL-13 and IL-4 were still able to induce AHR in a selective knock out of IL4Rα in this study, however IL-13 did so to a lesser degree [132]. These experiments show a sufficiency but necessity of IL4Rα signaling in ASM to induce AHR.

In vitro studies on IL-4 and IL-13 have enabled the proposing of possible mechanisms by which these cytokines can modulate the ASM phenotype and cause the above-mentioned pathologies. Both cytokines are known to inhibit growth factor induced proliferation of ASM cells through an inhibition of cyclin D1 upregulation [133, 134]. They can contribute to synthetic activities of the ASM. When given to ASM cells in combination with either TNFα or IL-1β, IL-4 and IL-13 can enhance eotaxin release [135, 136]. Inhibiting IL4Rα and the ERK/MAPK pathway prevents the response demonstrating that IL4Rα signaling activates the ERK/MAPK pathway [135, 136]. The context dependence of this pathway is again exemplified, as this pathway has been shown to be pro-proliferative in response to growth factors. A separate study showed IL-13 itself can induce eotaxin and, interestingly, IL-13 pre-treatment can inhibit this response [137]. Desensitization to IL-13 occurs through an upregulation of IL-13Rα2 [137]. This receptor is known as a decoy receptor but its expression was required for IL-13 to inhibit PDGF induced

proliferation [133]. Recent studies have discovered that IL-13Rα2 has signaling properties through interactions with the EGFR and activator protein-1, however, these findings were in the context of glioblastoma tumor cells [138, 139]. Further research is required to elucidate the significance of IL-13Rα2 expression by ASM.

IL-4 and IL-13 can also modulate contractile properties of ASM but they may have opposing effects. IL-13 is known as a pro-contractile factor while IL-4 can be both pro- and anti-contractile. Force production by murine ASM tissue in response to acetylcholine was significantly enhanced by IL-13 pre-treatment [35]. ASM tissue from mice intranasally treated with IL-13 was also able to produce greater force than without IL-13 [35]. The increased expression of RhoA induced by IL-13 was postulated as a possible mediator of this response [35]. Using gel contraction assays, further proof was obtained of IL-13's pro-contractile properties and IL-4's opposing contractile properties. IL-13 pre-treatment enhanced gel contraction in response to adenosine-triphosphate (ATP) while IL-4 diminished contraction [140]. However at high concentrations IL-13 slightly abrogated the enhanced contractile response [140]. IL-4 at low concentrations, however, augmented contractile responses [140]. It was determined that PI3K dependent expression of matrix metalloproteinase (MMP)-1 was required for both the IL-13 and IL-4 mediated contractile modulation [140]. It is not clear how MMP-1 can both enhance and diminish contractility, but it appears to be concentration dependent. The authors suggested that MMP-1 can induce a reorganization of the ECM to allow for more efficient contraction but an overabundance can detach ECM connections from the ASM cell, reducing force transfer [140]. This theory was supported by data showing IL-4 inducing higher expression of MMP-1 compared to IL-13, as well as analysis of greater ECM density by electron microscopy with IL-13 treatment compared to IL-4 [140]. It was also shown IL-4 can be more pro-contractile than IL-13 when MMP-1 was knocked down in ASM cells [140].

Different signaling outcomes may account for the differences between IL-13 and IL-4. IL-13 enhanced peak calcium response to histamine by ASM cells while IL-4 reduced peak calcium

responses to carbachol [133, 141]. IL-4 reduced calcium response was dependent on activation of PI3K [141]. No force measurements were made on IL-4 treated cells but the stiffness of IL-13 treated cells was measured through atomic force microscopy after histamine stimulation. IL-13 treated cells were significantly stiffer than cells without treatment but no differences in contractile protein expression was found [133]. This suggests that IL-13 can modulate ASM contractile responses without enhancing expression of the contractile apparatus, but it may still be able to. A recent study comparing IL-13 responses by ASM from healthy and cystic fibrosis patients showed IL-13 can upregulate MYLK expression [142]. Activation kinetics of STAT6 also differ slightly between IL-13 and IL-4 and ASM cells. Phosphorylation of STAT6 peaks at 15min and 1h with IL-4 and IL-13 treatment respectively [143]. Activation kinetics of the downstream kinase, ERK, however, were not different [143]. IL-13 diminished the relaxation response to isoproterenol in ASM which was determined to be ERK dependent [143]. IL-4 did change responses to isoproterenol, even though a similar degree of activation of ERK to IL-13 was achieved [143]. The effects of IL-4 are still unclear but IL-13 has proven to be a pro-contractile factor for ASM.

2.4.1.2 IL-8

IL-8, also known as chemokine (C-X-C motif) ligand (CXCL)8, is a chemoattractant that is not commonly associated with a specific T-helper response, but it has implications in Th2 immunity as eosinophils have been identified as a major source in asthmatics [144]. Other sources of IL-8 in the airways include macrophages and epithelial cells [145, 146]. IL-8 is best known for its ability to attract neutrophils and is correlated with airway neutrophilia in asthmatics [147, 148]. The highest levels of IL-8 in the serum are found in patients during an asthma attack [149]. This is significant as neutrophils are one of the earliest infiltrating leukocytes during an airway challenge. IL-8 has properties in addition to chemoattraction. Its receptors include C-X-C motif chemokine receptor (CXCR) 1 and 2. These G-protein coupled receptors (GPCR) induce a cascade of G-protein signaling that leads to the activation of PI3K and ERK, a pathway common to many modulators of ASM phenotype.

The effects of IL-8 on ASM or AHR have not been greatly studied *in vivo* outside of neutrophilic effects but much *in vitro* research has demonstrated IL-8 as a modulator of the ASM phenotype. One *in vivo* study implicated CXCR1/2 in mediating neutrophilia and AHR in IL-33 and IL-17 induced AHR, however, this study did not examine the effects of IL-8 directly [150]. *In vitro*, ASM cells have been demonstrated to be able to produce IL-8 with various stimuli, such as antimicrobial peptides, cigarette smoke extracts, cytokines and growth factors [151-154]. Interestingly, Th2 cytokines, IL-4,13 and 10, repressed IL-1β induced IL-8 expression while the Th17 cytokine IL-17, enhanced it [152, 155]. IL-8 expression by ASM might have implications for airway neutrophilia as its expression is regulated by neutrophil elastase, but IL-8 stimulation seems to have both pro-contractile and pro-proliferative consequences for ASM cells [156]. It was determined that IL-8 itself can act as a contractile agonist. IL-8 induces phospholipase C dependent release of intracellular calcium stores, resulting in the activation of actin-myosin cross-bridging and cellular contraction [157, 158]. IL-8 is also a mitogen for ASM [158]. Airway epithelial cells have been shown to express high level of IL-8 when exposed to Areca nuts, an allergen associated with increased asthma risk [159-161]. Conditioned medium from these cells was able to induce ASM cell proliferation in an IL-8 dependent manner [159]. These results show possible mechanisms by which IL-8 can contribute to remodeling and AHR outside of its chemotactic functions.

2.4.1.3 IL-17

IL-17 is a marker of the Th17 phenotype of $CD4^+$ T cells and is associated with the clearance of fungal infections. Although there is some evidence that severe asthma may be associated with fungal colonization, IL-17 has implications beyond fungal involvement in driving the disease. High levels of IL-17 can be found in the BAL or sputum of asthmatics [162-165]. Biopsies reveal an increase in $IL\text{-}17^+$ cells as well. IL-17 also correlates with functional outcomes of asthmatics [163]. Levels of IL-17 in asthmatic sputum samples correlated positively with the level of AHR [165]. IL-17 mRNA in the sputum also correlates with the level of airway neutrophilia [164]. It may even regulate steroid insensitivity in some asthma patients [162, 166]. Although Th17 $CD4^+$

T cells are known to be the main producers of IL-17 in the airways, other sources have been identified from epithelial cells to other leukocytes such as ILC3s, B cells and γδ T cells [163, 167-169].

Many *in vivo* studies have demonstrated that IL-17 production is vital for the induction of AHR in various models. Ozone-induced AHR in mice can be prevented by knocking out the IL-17 receptor (IL-17R) [170]. A similar effect can be achieved using an intranasal sensitization to an allergen, such as OVA or house dust mite, and again knocking out IL-17R or treating mice with an antagonistic antibody for IL-17 [171-173]. Cytokine induced AHR can be exacerbated with the addition of IL-17. Intranasal administration of IL-13 induced AHR but it also increased the number of IL-17 producing CD4 and γδ T cells [174]. By co-administering IL-13 and IL-17, AHR was exacerbated but only at low levels of IL-17 as high levels had an opposing effect [174]. A combination of IL-33 and IL-17 can also induce greater AHR than either cytokine alone [150]. Many of these studies have found that IL-17 treatment enhances neutrophilia and CXCR2 signaling which is necessary for IL-17's effects on AHR [150, 170-172]. It is commonly known that IL-17 does induce IL-8 release in various cells but it may also play a role in remodeling. Airway remodeling, including ASM and goblet cell hyperplasia, induced by OVA sensitization in mice can be amplified by adoptively transferring Th17 cells [175]. The level of remodeling and AHR even correlated with Th17 cells in the lung [175]. This remodeling was determined to be EGFR dependent with HB-EGF being the likely activating ligand, demonstrating Th17 cells are also capable of inducing HB-EGF expression [175]. Th17 cells were able to induce HB-EGF expression by epithelial cells when placed in co-culture, which was then shown to induce ASM proliferation [175].

In vitro studies have implicated IL-17 as a regulator of many ASM cell functions including the expression of IL-8, potentially contributing to neutrophilia [176-178]. The IL-17 receptor uses TNF receptor associated factor (TRAF) 6 and NF-κB activator 1 (Act1) adaptor proteins to induce a signaling cascades down both the NF-κB and MAPK pathways. IL-17-induced IL-8 expression

was shown to be dependent on both the NF-κB and MAPK pathways [179]. As discussed previously, ERK1/2 are 2 of the major kinases in the MAPK pathway and regulate ASM phenotype. IL-17 was shown to induce proliferation of ASM cells which was inhibited by knocking down or antagonizing ERK1/2 [180]. Inhibition of NF-κB however, did not prevent IL-17 induced proliferation, but it may play a role in modulation of ASM contractility [180]. Treating tracheal rings with IL-17 increased contractile force production in response to methacholine [171, 181, 182]. NF-κB-mediated upregulation of RhoA and ROCK was responsible for the increased contraction [171, 181]. Recently, a second mechanism independent of NF-κB was found. IL-17 was found to activate Ras-related protein Rab-35 (Rab35) by recruitment to Act1 [182]. Through adaptor proteins, Rab35 recruits and activates Protein Kinase C (PKC) alpha which in turn recruits and binds to Fascin [182]. Fascin is a protein that bundles actin filaments, preventing them from forming contractile units with myosin. The recruitment of Fascin to PKCα freed actin filaments to interact with myosin filaments, increasing contractile force production by ASM [182]. It is clear that IL-17 has various potential mechanisms by which it may mediate ASM remodeling in asthmatics.

2.4.2 Il-1β and TNFα

IL-1β and TNFα are pro-inflammatory cytokines that are key regulators of inflammatory processes in clearing infections and inflammatory diseases. Both cytokines are mainly expressed by cells in the innate arm of the immune response such as macrophages and mast cells [183, 184]. These leukocytes use IL-1β and TNFα to induce pro-inflammatory cytokine expression locally and systemically. IL-1β and TNFα are released early during infections and are associated with initiating immune responses at the site of infection but they are also released systemically mediating symptoms such as fever [183, 184]. They can also regulate other cellular processes such as differentiation and apoptosis [183, 184]. IL-1β and its regulator, an inflammatory protein complex called the inflammasome, also regulates a specialized apoptotic process associated with high levels of inflammation named pyroptosis [183].

Elevated levels of IL-1β and TNFα have been found in various compartments of the lung and body including BAL, sputum and serum [185-194]. Expression analysis of cells from the BAL and sputum, as well as histological analysis of bronchial biopsies, revealed various cells that express IL-1β and TNFα in asthmatics. The major producers of IL-1β and TNFα are macrophages and mast cells respectively, but neutrophils, eosinophils and epithelial cells are also capable of producing these cytokines [186, 188-195]. Both cytokines have been associated with levels of airway neutrophilia, particularly in severe asthmatics [186, 196-198]. Levels of NACHT, LRR and PYD domain-containing protein 3 (NLRP3), an essential component of the inflammasome, and IL-1β in the sputum and BAL correlated with neutrophilic asthmatic patients, but not eosinophilic [186, 198]. Experiments where healthy and mild asthmatic subjects were treated with inhaled TNFα both increased AHR and neutrophils in sputum samples [199, 200].

Studies done on animal models have revealed functional IL-1β and TNFα roles *in vivo* that correlate with the above human findings. Intratracheal treatment with either cytokine is capable of inducing AHR in mice that was accompanied by a significant increase in neutrophils within the BAL [201-204]. Both cytokines have been found to play essential roles in various other animal models of asthma. Allergen sensitization increased both TNFα and IL-1β levels in the BAL of mice [201, 204-206]. Inhibiting these cytokines prevents the development of AHR and in the case of TNFα, mast cells were the key source [201, 204-208]. Preventing mast cell development through knock out of KIT proto-oncogene RTK was sufficient to reduce AHR to levels similar to TNFα inhibition [204]. One study did find that knocking out TNFα exacerbated OVA induced AHR while overexpression of TNFα prevented AHR [209]. This effect was dependent on γδ T cells as inhibiting their activity abolished the protective effects of TNFα, suggesting context-dependent roles of TNFα [209]. Knocking out the inflammasome components, NLRP3 or Apoptosis-associated speck like protein containing a caspase recruitment domain (ASC), or the IL-1 receptor also mimicked IL-1β inhibition in allergen models of asthma [205, 208]. Chemically induced AHR using pollutants such as ozone can also be prevented by antagonizing IL-1β or TNFα [210-213].

IL-1β and TNFα can also have significant effects on ASM function, giving these cytokines multiple possible roles in mediating AHR other than just regulating inflammatory responses. Studies have also shown that they have synergistic functions in augmenting ASM contractility. Both cytokines have been demonstrated to increase shortening and force production by ASM while inhibiting proliferation [203, 214-217]. One study did find that IL-1β can have mitogenic effects through the MAPK pathway but the exact same signaling cascade has been implicated in IL-1β mediated enhanced contractility [216, 218]. Both cytokines were found to enhance the contractile response to 5-hydroxytryptamine (5-HT; serotonin) which induces weak contractile response in baseline conditions [203, 216]. Oddly, IL-1β reduces contractile force production in response to histamine [219]. The synergistic effects of IL-1β and TNFα have been mainly studied in the context of relaxant responses. Various studies have shown that ASM maintain their stiffness in response to isoproterenol when treated with either IL-1β or TNFα but not to the degree of a combined treatment [220-223]. This effect was mediated by an increase in cyclo-oxygenase 2 (COX2) expression leading to an increased synthesis and autocrine signaling of PGE_2 [221, 222]. Increased force production and shortening was also measured but its connection to COX2 and PGE_2 is unclear as PGE_2 is a known relaxant of ASM cells [221, 223, 224]. Transcriptomic analysis revealed that with combined treatment ASM upregulates many genes in response to IL-1β and TNFα [221]. These include various cytokines and adhesion molecules that have functional consequences on interacting cells [221]. Supernatant from IL-1β and TNFα-stimulated ASM can enhance eosinophil viability through GM-CSF [225, 226]. Upregulation of adhesion molecules such as Intercellular Adhesion Molecule (ICAM)-1 increases interactions with $CD4^+$ T cells [227, 228].

The presented evidence demonstrates IL-1β and TNFα as complex and diverse mediators of asthma pathology. They can have direct effects on ASM to induce AHR, but they can also mediate inflammatory processes in the lung, that can then act on ASM to induce AHR.

2.4.3 Lipid Mediators: Leukotrienes and Prostaglandins

Leukotrienes (LT) and prostaglandins (PG) are lipid-based molecules and are part of the eicosanoid family that mediate pro-inflammatory responses. Both eicosanoids are synthesized through various enzymatic reactions on arachidonic acid. PGs require COX-1 or 2 in conjunction with PGE synthase to form PGE_2 or PGD synthase to form PGD_2 while LTs depend on 5-lipoxygenase (5LO) to form LTA_4 which is either hydrolyzed by LTA_4 hydrolase (LTA_4H) to form LTB_4 or is bound to a glutathione molecule by LTC_4 synthase (LTC_4S) to form the cysteinyl (Cys) LTs: LTC_4, LTD_4, and LTE_4. Synthesis of LTs is limited to certain cell types due to the limited expression of their synthetic enzymes. The complete synthetic pathway is typically found in leukocytes but LTA_4's ability to cross cellular membranes allows for a process called transcellular biosynthesis. Mice lacking 5LO transplanted with bone marrow cells from LTA_4H and LTC_4S lacking mice expressed normal levels of LTs in response to Zymosan [229].

Both LTs and PGs have been found at elevated levels in asthmatics, but they may play opposing roles as LTs have been associated with exacerbating the disease, PGs seem to have protective capabilities. Phospholipase A_2 (PLA_2), a secreted enzyme responsible for the synthesis of arachidonic acid is found at elevated levels in BAL of asthmatics suggesting an increase potential for the production of PGs and LTs [230, 231]. Staining of bronchoscopy samples found epithelial cells and macrophages to be the major producers of $PLA_{2\,[230]}$. LTB_4, CysLTs, PGE_2 and PGD_2 have also been measured at elevated levels in asthmatic BAL and sputum with or without a respiratory challenge such as an allergen or an exercise test [232-237]. CysLTs can even be found in exhaled condensates from asthmatics [232, 234, 238]. The sources of these eicosanoids require consensus but various cells from mast cells and macrophages to epithelial cells and ASM cells have been proposed [239, 240]. The levels of LTs and PGs tended to correlate with opposing inflammatory and lung function readouts. Sputum levels of PGE_2 were highest in patients without eosinophilia, while CysLT levels trended with the eosinophil count [233]. PGE_2 and CysLTs correlated with FEV_1 in a similar manner, thus suggesting protective and harmful roles of PGs and LTs respectively [233]. CysLT levels are also correlated with asthma severity and the magnitude of the fall in FEV_1 with exercise induced bronchoconstriction [232, 235]. Treatment of patients with inhaled LTs or PGE_2 gave definitive evidence for their opposing

roles. LTD_4 inhalation did not significantly exacerbate AHR but it did enhance eosinophilia while PGE_2 inhibited early and late phase responses to allergen [241, 242]. Inhalation of CysLTs was able to exacerbate AHR in asthmatics subjects only [243].

2.4.3.1 Leukotrienes in Experimental Models

Modulation of LTB_4 and CysLT expression and signaling in animal models of asthma have further demonstrated their harmful role in asthma. In dogs, inhaled treatment of LTB_4 was sufficient to induce AHR and neutrophilia but LTD_4 failed to do the same in mice [244, 245]. LTD_4 is able to exacerbate AHR, neutrophilia and eosinophilia when given in combination with OVA [245]. OVA sensitization was shown to increase levels of both LTD_4 and CysLTs in the BAL and lung tissue, respectively [246, 247]. An irritant model of asthma using inhaled chlorine demonstrated that chlorine elevates CysLT levels in the BAL and enhances CysLT receptor (CysLTR) 1 expression in the lung [248]. AHR brought on by both OVA and chlorine can be prevented using various methods of LT inhibition [247, 249-252]. OVA sensitization fails to induce AHR, eosinophilia and neutrophilia in LTB_4 receptor (BLT) 1 or LTA_4H knockout mice [247, 249-252]. This effect was dependent on BLT1 expression on $CD4^+$ T cells and dendritic cells (DC), required to induce proper Th2 responses and thus LTB_4 may not have a direct action on ASM [247, 249].

CysLTs may play a more direct role in ASM modulation. Inhibition of CysLT signaling using CysLTR1 inhibitors, montelukast or 36ranlukast, confers a similar inhibition of OVA induced pathologies as knocking out BLT1 and LTA_4H [245, 246, 253, 254]. These effects are mimicked in studies using chlorine inhalation [248]. CysLTR1 inhibition was also shown to prevent airway remodeling in OVA sensitized mice [246, 253, 254]. Both a reduction in ASM area and goblet cell numbers were observed [246, 253, 254]. No changes in ASM cell area was found with montelukast treatment but reduced DNA synthesis was discovered as a marker of reduced proliferation with pranlukast [246, 253]. This suggests that CysLTs are involved in ASM hyperplasia but not hypertrophy. Overexpression of CysLTR1 on ASM induced spontaneous AHR in mice, however, it had no effect on allergen induced AHR [255]. The findings in irritant asthma

caused by chlorine involving innate immune mechanisms differ from findings from allergen driven models involving adaptive immunity. Knocking out CysLTR1 exacerbates rather than protects against chlorine induced airway inflammation and AHR [256]. CysLTs appear to be required for a functional antioxidant response by epithelial cells, demonstrated to be a protective mechanism against chlorine [256].

In vitro studies demonstrate both proliferative and contractile functions of LTB_4 and CysLTs. Both LTB_4 and LTD_4 have shown mitogenic properties in ASM cells through activation of the PI3K, ERK and MAPK pathways, however, LTD_4 acts indirectly on these pathways [257]. LTD_4-induced proliferation was dependent on the activation of the EGFR and subsequent activation of ERK [258-260]. Through the same pathway, LTD_4 can potentiate EGF and TGF-β induced ASM cell proliferation [258-260]. Other studies have shown that LTD_4 cannot induce ASM cell proliferation alone, thus its proliferative effects may be dependent on the presence of EGFR ligands or LTD_4 may have the ability to induce EGFR ligand expression in certain contexts [259, 260]. Indirect induction of proliferation has yet to be studied using LTB_4. LTB_4 and LTD_4 were found to induce increases in intracellular calcium concentrations and subsequent ASM contraction by stimulating ASM tissue and tracheal rings [255, 260-263]. Overexpressing the CysLTR1 in ASM cells can even augment force generation in response to LTD_4 [255]. LTD_4 also induces actin cytoskeletal rearrangement through Rho GTPases, forming organized filaments associated with increased contractility [264]. The mechanism underlying LTB_4 induced contraction has yet to be elucidated.

2.4.3.2 Prostaglandins in Experimental Models

In vivo studies on PGs have been mostly conducted through the genetic and chemical inhibition of PG synthetic enzymes, however protective effects of PGE_2 have also been demonstrated through the administration of exogenous PGE_2 and a PGE_2 receptor (EP2)-agonist, butaprost. Allergen induced AHR and eosinophilia using house dust mites can be inhibited by intranasal administration of either PGE_2 or butaprost [265]. This effect of PGE_2 was suggested to be

dependent on mast cells [265]. Inhibition of COX1/2 using indomethacin has been shown to exacerbate OVA induced AHR in mice that was accompanied by increased eosinophilia and lymphocytosis [266, 267]. IL-13 and IL-5 were the only T-helper cytokines found in elevated levels in the lung tissue [266, 267]. Knocking out EP_2 in mice mirrored results seen with indomethacin except levels of IL-4 and IL-17 were also elevated in the BAL [268]. PGE_2 can also cause bronchodilation which is prevented in EP_2 knockout mice [269]. COX1 and 2 appear to differ in their effects. One study found that a selective COX1 inhibitor, FR122047, reduced AHR in response to OVA sensitization [270]. However, inhibition of neither COX1 nor 2 worsened eosinophilia [270]. The COX2 selective inhibitor, lumiracoxib, actually reduced eosinophilia and levels of total inflammation [270]. A follow up study showed either COX1 or 2 inhibition exacerbated OVA induced AHR whereas only COX2 inhibition reduced total cell numbers but not eosinophil numbers in the BAL [271]. Inhibition of both COX1 and 2 are required to evoke elevated levels of IL-13 in the BAL [270, 271]. Neither COX1 or 2 inhibition had any effect on aspects of remodeling, including increases in ASM area and goblet cell hyperplasia [271].

In vitro, ASM cells have been shown to express COX2 and release PGE_2 in response to various stimuli. Along with the above-mentioned cytokines IL-1β and TNFα, bradykinin, protease-activated receptor (PAR) stimulation, trypsin, and the growth factors EGF and PDGF also induced COX2 expression and PGE_2 release [239, 272-275]. PGE_2 seems to have inhibitory functions in ASM cells as it has shown to both reduce their proliferation and contractility, although the data on contraction have been contradictory. Both EGF and FBS induced proliferation of ASM cells are inhibited by PGE_2 through activation of EP2 and not EP3 [276-278]. Asthmatic ASM cells expressed higher levels of both receptors and were more sensitive to the anti-proliferative effects of PGE_2 [277]. These effects were mediated by the activation of PKA and were correlated with the inhibition of PI3K activation by EGF [276]. PGE_2 is also a known relaxing and contractile agonist of ASM [275-283]. Asthmatics may be more resistant to the relaxant effects as rabbit tracheas incubated with human asthmatic serum maintained greater tension despite treatment with PGE_2 compared to serum stimulation from a healthy subject [282]. Contractile effects were dependent on the activation of Rho-Kinase [280]. PGE_2

can also augment contractile responses to histamine and carbachol [283]. These opposing effects can be explained by variety of PGE_2 receptors expressed by ASM. EP_1 was demonstrated as pro-contractile receptor while EP_2 acted to relax the muscle [279]. The modulation of contraction by PGE_2 can thus be modulated through the use of EP_1 and EP_2 specific inhibitors [279].

3. Immune Cell Involvement in Asthma

It is evident that inflammation is a key factor in mediating and regulating AHR and airway remodeling. Although structural cells within the airways contribute to the inflammatory milieu, leukocytes are the major mediators of the inflammatory mechanisms in asthma. Analysis of BAL, sputum and histological samples from asthmatics shows their lungs are infiltrated by all subsets of leukocytes but not all asthmatics present the same profile of inflammatory cells. The recent concept of endotypes has been proposed to better characterize asthmatics with the hope to clarify the different mechanisms of asthma onset and exacerbation, as well as personalize therapies [284, 285]. A consensus set of endotypes has yet to be determined but the proposed distinguishing factors have commonly been levels of eosinophilia and neutrophilia, and whether the T-helper response is or is not Th2 dominant [284, 285]. Eosinophilia is commonly associated with Th2-asthma while neutrophilia is associated with non-Th2-asthma however both processes can occur in the same patient [284, 285]. These leukocytes have been demonstrated to have significant consequential roles in mediating both AHR and airway remodeling, including direct interactions with ASM cells.

Both direct co-cultures between neutrophils or eosinophils with ASM or stimulating ASM with neutrophil and eosinophil specific products have been shown to modulate ASM proliferation and contractility. Eosinophils make direct contact with ASM via very late antigen (VLA)-4 and Integrin alpha M (ITGAM) which induces TGF-β production and release by ASM leading to proliferation through autocrine signaling [36, 286, 287]. Neutrophil elastase induces ASM proliferation by a similar TGF-β mediated mechanism [288, 289]. However neutrophils directly

co-cultured with ASM cause apoptosis via neutrophil elastase [290]. Direct contact between these two cells was not necessary, demonstrating opposing roles of neutrophil elastase on ASM [290]. Exosome derived from neutrophils were taken up by ASM cells after treating them with LPS [291]. This led to proliferation of ASM, showing a novel mechanism of signaling between ASM and neutrophils [291]. Eosinophils also enhanced expression of αSMA and collagen gel contraction by ASM and eosinophils isolated from asthmatic subjects had even greater effects [292]. Inflammatory mediators such as cytokines and eicosanoids are clearly not the only pathological consequence of inflammatory cell infiltration into the lung.

3.1 $CD4^+$ T cells

$CD4^+$ T cells, also known as T-helper cells, are part of the adaptive immune system which functions as immune modulators, directing the immune responses of various cells to aid in efficient clearance of infections or repairing damaged tissue. They are antigen specific cells that require activation through binding of their T cell receptor (TCR) to a specific protein epitope. A subset of $CD4^+$ T cells known as follicular helper cells are responsible for inducing immunoglobulin class switching and antibody expression by B cells that are specific to the same protein epitope. The other major T-helper subsets include Th2, Th1, Th17 and Treg, each being induced by different types of infections. Bacterial, viral and fungal infections clearance require Th2, Th1 and Th17 responses respectively, while Treg cells are responsible dampening the immune response post clearance [293]. The adaptive arm of the immune response is also responsible for immune memory. One of the last stages of the immune response is the transition of $CD4^+$ T cells from effector cells to memory cells, a dormant cell that localizes to the site of infection and lymph nodes [293]. Upon recognition of their specific antigen, these memory cells induce a stronger immune response than the response to the original insults, allowing for more efficient clearing of the infection.

As described above, asthmatics can be grouped by whether a Th2 dominant response is present in the lungs. One measure of Th2 responses is based on the cytokine expression profile of $CD4^+$

T cells present in the lungs. $CD4^+$ T cells can be found at elevated levels in BAL, sputum bronchial biopsies and peripheral blood of asthmatics and which were also found to be express higher levels of activation markers [7, 294-301]. The majority of studies has found $CD4^+$ T cells express predominantly express Th2 cytokines, particularly IL-4 and IL-5 [295, 297, 298]. IFN-γ expressing $CD4^+$ T cells, associated with a Th1 phenotype, have also been found in asthmatic samples but it seems that the Th1 phenotype may play a bigger role in infant asthma [302]. Functional correlations with $CD4^+$ T cells have also been identified in asthmatics. Levels of $CD4^+$ T cells seem to correlate with levels of eosinophilia [7, 296, 299]. Levels of $CD4^+$ T cell, particularly Th17 cells, are correlated with FEV_1 levels, and thus asthma severity [297, 300]. Depletion of $CD4^+$ T cells using an anti-CD4 antibody, keliximab, increased peak expiratory flow rates indicating an improvement of lung function [37]. Histological analysis of bronchial biopsies revealed $CD4^+$ T cells localized to sub-epithelial regions of the airways, even being found either within or in between ASM bundles [300, 301]. Their elevated levels and possible interactions with ASM make $CD4^+$ T cells a potential player in mediating or modulating AHR and/or ASM remodeling in asthma.

The consequences of $CD4^+$ T cell infiltration on AHR have been elucidated through depletion and adoptive transfer experiments in murine models of asthma. Allergen specific $CD4^+$ T cells can be found at elevated levels in the BAL, sub-epithelial compartments or cervical lymph nodes of mice or rats sensitized with an allergen to induce AHR [303-308]. Adoptive transfer of these $CD4^+$ T cells was able to induce AHR in naïve rats after a single allergen challenge [303, 307, 308]. Transfer of $CD8^+$ T cells failed to replicate these findings [303]. Intravenous staining of Thy1 allowed researchers to identify tissue resident T cells as they would be protected from the Thy1 antibody [306]. After a 3-week house dust mite sensitization protocol a significant increase in tissue resident $CD4^+$ T cells was found that localized around the airways [306]. Levels of tissue resident $CD4^+$ T cells were maintained for 4-6 weeks of rest after sensitization [306]. These T cells demonstrated an enhanced ability to produce Th1, Th2 and Th17 cytokines when isolated and challenged with house dust mite in culture [306]. Challenging rested mice showed AHR was also maintained but could be abolished when depleted of $CD4^+$ T cells using a Thy1

antibody [306]. These results suggest a robust tissue resident memory response by $CD4^+$ T cells is present in this asthma model and is required for late phase AHR. To strengthen this theory, knocking out CD4 in mice does not prevent OVA induced AHR after an initial sensitization period, however, AHR is not maintained after 6 weeks of rest after the last challenge [309]. Depletion of $CD4^+$ T cells was also associated with a loss of eosinophilia which seems to be due to the high production of RANTES and IL-5 by $CD4^+$ T cells [303, 304, 309, 310].

Effects of $CD4^+$ T cells on airway remodeling have also been studied in murine models. Adoptive transfer of $CD4^+$ T cells from OVA sensitized rat induced increases in ASM mass after a three OVA challenges in naive rats [311]. $CD4^+$ T cells from a naïve rat did not replicate these findings [311]. Increased ASM mass was accompanied by increased proliferation and decreased apoptosis of both ASM cells and epithelial cells [311]. Increased thickening of the airway wall induced by OVA sensitization in mice was shown to be inhibited by CD4 depleting antibody [305]. The presented evidence suggests that $CD4^+$ T cells may be mediating both AHR chronically and remodeling, although they may be connected, in asthmatics. This aligns with their physiologic function of mediating immune memory, thus, regulating $CD4^+$ T cell responses may be key to preventing asthma progression.

3.1.1 Interactions with ASM cells

In vitro studies have revealed many modes of interactions between $CD4^+$ T cells and ASM cells. Possible effects of paracrine factors such as cytokines were addressed above, but $CD4^+$ T cells also physically interact with ASM cells through various adhesion mechanisms. ASM cells are known to constitutively express CD44, an adhesion molecule capable of binding CD44 on another cell through hyaluronic acid [53]. Stimulated with either IL-1β or TNFα induces the expression of ICAM-1 and Vascular Adhesion Molecule (VCAM)-1, adhesion molecules that binds Lymphocyte Function-Associated Antigen (LFA)-1 and Very Late Antigen (VLA)-4 respectively, which are commonly associated with extravasation of leukocytes [53]. Furthermore, co-culturing ASM cells with $CD4^+$ T cells activated with CD3/28 also induced ICAM-

1 expression [312]. $CD4^+$ T cells adherence to ASM stimulated with TNFα increased, which could be inhibited to varying degrees by antagonizing the above-mentioned adhesion molecules [53, 312]. LFA-1 antagonists had the strongest effects alone, but combinations of CD44/ICAM-1/VCAM-1/LFA-1/VLA-4 antagonists had additive effects on adhesion [53]. ASM cells also express Fcε-receptors, allowing ASM cells to be stimulated with IgE which was found to induce the expression of CD40, CD86 and CD11a [313, 314]. These are co-stimulatory molecules that can mediate adhesion but are better known for mediating secondary signaling during T cell activation. Inhibition of these molecules reduced $CD4^+$ T cells adhesion to ASM cells as well [313].

Functionally, adhesion between $CD4^+$ T cells and ASM cells induces changes in phenotype that favor AHR and airway remodeling activities. Co-culture with $CD4^+$ T cells induces proliferation of ASM cells which can be inhibited by antagonizing CD44 [53, 315]. Activated $CD4^+$ T cells express EGFR ligands that can also mediate proliferation of ASM cells [315]. Cleavage and release of the EGFR ligands were suggested to be dependent on MMP9 sequestration on CD44 [315]. Rabbit ASM tissue produced more force in response to acetylcholine after co-culture with activated $CD4^+$ T cells [313]. Relaxant responses to isoproterenol were also inhibited by $CD4^+$ T cells [313]. Inhibition of both CD40 and CD86 or inhibition of CD11a abolished contractile changes caused by $CD4^+$ T cells [313]. These results were not replicated in studies of rat ASM tissue, but an enhancement of shortening velocity was measured with $CD4^+$ T cells in co-culture [316]. Co-culturing in a Transwell® system, where $CD4^+$ T cells were physical separated from the ASM using a porous membrane, did not increase shortening velocity of the ASM [316]. $CD4^+$ T cells also increased MLCK expression by ASM, resulting in increased LC20 phosphorylation following stimulation with methacholine [316]. Further *in vitro* studies are required for more detailed mechanistic explanations for these effects which *in vivo* evidence suggests would potentially provide effective therapies against asthma progression.

ASM cells also play modulator roles on $CD4^+$ T cells. Activated $CD4^+$ T cells induce major histocompatibility complex (MHC) II expression on ASM cells via IFN-γ suggesting ASM cells may present antigen and activate $CD4^+$ T cells [312, 313]. This is a surprising finding as structural cells are not generally regarded as having antigen presenting capabilities. IFN-γ stimulated ASM cells were not able to induce proliferation of resting $CD4^+$ T cells, suggesting they were not capable of activating $CD4^+$ T cells [312]. A follow up study did find an increase in T cell activation markers on $CD4^+$ T cells co-cultured with IgE stimulated ASM cells [313]. Treating ASM cell with staphylococcal enterotoxin A (SEA) in combination with IFN-γ enhanced activation marker expression on $CD4^+$ T cells after co-culture [317]. Imaging experiments surprisingly showed an ASM cell binding to $CD4^+$ T cells via a CD3/MHC II complex [317]. The combination of SEA and IFN-γ also enhanced $CD4^+$ T cell adhesion to ASM cells [317]. A Th2-like response was initiated as an increase in IL-13 expression was found in $CD4^+$ T cells that in turn increased force production by ASM tissue [317].

The last mode of physical interaction discovered between $CD4^+$ T cells and ASM cells is through membrane nanotubes (MNT) [318, 319]. MNTs are a method of cellular communication through tubular cellular projections that was originally thought to be exclusive to single celled organisms. However, recent studies have identified that various mammalian cells have the capacity to form MNTs. In particular, immune cells, including $CD4^+$ T cells, dendritic cells and macrophages, have been the most commonly discovered MNT producers but MNTs from structural cells such as cancer cells and tumor associated fibroblasts have also been described. MNTs are capable of transferring various molecules to neighboring cells, such as DNA and RNA, proteins, organelles and membrane components. MNTs have yet to be found in *in vivo* studies, except for one instance where MNTs from DC were discovered inside a mouse cornea. However, mesenchymal stem cells have been shown to produce MNTs toward macrophages and pass mitochondria to them. It was then found that macrophages received mesenchymal derived mitochondria in the alveoli, however the method by which this transfer occurred was not verified. $CD4^+$ T cells have been shown to transfer HIV particles through MNTs demonstrating a possible mode of HIV progression [320]. Between ASM cells, $CD4^+$ T cells form

tubulin dense MNTs that range in length from 2.9-60.5µm and 1.3-2µm in width [318]. Fibroblastic growth factor-2b (FGF2b) from ASM was partially responsible for inducing MNT formation by $CD4^+$ T cells [318].

The functional consequences of communication via MNTs are unclear but the package being delivered may give some clues. $CD4^+$ T cells receive anti-apoptotic proteins, B-cell lymphoma-2 and myeloid leukemia cell differentiation protein-1, enhancing $CD4^+$ T cell viability [319]. Inhibition of CD44 reduced the enhancement of viability by ASM cells, suggesting that $CD4^+$ T cells may still need direct contact with ASM cells to form MNTs [319]. Intracellular calcium release in ASM during a contractile response to histamine was also transferred through MNTs, increasing $CD4^+$ T cell calcium concentrations [319]. The consequences of increased calcium in the $CD4^+$ cells are not clear, but calcium regulates many pro-inflammatory processes in $CD4^+$ T cells, including increased NFκB translocation. Normal activation processes in $CD4^+$ T cells induces increases in calcium concentrations as well. Mitochondria are donated to $CD4^+$ T cells by ASM cells through MNTs [318]. Similar to increased calcium concentrations, the consequences of mitochondrial transfer need further research, but various potential pro-inflammatory mechanisms exist. ASM cells isolated from asthmatic lungs expressed more FGF2b and also induced more MNT formation by $CD4^+$ T cells suggesting MNTs may play a role in asthma [318].

It is clear that ASM-$CD4^+$ T cell interactions have biologically interesting and consequential implications in asthma but multiple pieces of research are incomplete in this field. Modulation of ASM phenotype has major implications in mediating AHR and remodeling, but the mechanism by which $CD4^+$ T cells accomplish this eludes us. Modulating $CD4^+$ T cell activity, particularly through the transfer of a vital metabolic and signaling organelle like the mitochondrion, can also have major implications in mediating airway inflammation and progression of asthma. However, the $CD4^+$ T cell side of this interaction is greatly understudied.

Progression in this field has great potential to greatly progress our understanding of asthma which will in turn lead to better therapies.

4. Engineering Solutions to Studying Cellular Projections

The study of membrane nanotubes has been limited by current available techniques. Some efforts have been made to isolate a small number of cells in multiple chambers using a microfluidic system for high-throughput analysis, but most studies are constrained to simple co-culture methods [321]. This requires researchers to image and scan large culture areas in the hope of finding MNTs and if used to study formation mechanics or kinetics, they would need to catch an MNT during its induction. When working with cells with high rates of MNT formation, this may not be a significant hindrance, but less than half of $CD4^+$ T cells can be observed producing MNTs with ASM cells at a time [318]. $CD4^+$ T cell to ASM cell MNTs are also short lived. Observations of $CD4^+$ T cells forming MNTs, connecting to ASM cells and releasing from ASM cells showed that MNT kinetics are in a time scale of seconds (unpublished data). An assay where the location of MNT formation can be guided would greatly increase the efficiency of MNT research.

The use of microfluidics has been commonly implemented to study other cellular projections, particularly neuronal projections. These devices separate two chambers by microfluidic channels, generally ranging from 10-1000µm wide and 200µm-2mm long [322]. They are generally constructed by pouring polydimethylsiloxane (PDMS) over a micro-patterned mold [322]. These molds can be made using a variety of techniques such as photolithography. Photolithography uses a photoactive material that crosslinks upon exposure to UV light which can be done at micron to nanoscale dimensions [323]. However, the commonly generated dimensions are too large to restrain $CD4^+$ T cells as they can pass through a 10µm gap and their MNTs only achieve a length of 60µm. Although $CD4^+$ T cells are approximately 10µm wide, they have been shown to squeeze through a 3µm pore, requiring a microfluidic channel <2µm to prevent their migration [324]. Micro-patterning cell adherent areas can also be implemented to

allow for precise ASM cell placement, thus creating predictable zones of MNT formation. Again, a photoactivated material can be used to create micropatterns using UV light, but cell adherence needs to be accounted for [325]. For example, collagen molecules have been conjugated to photoactive molecules, which process allows the crosslinking of collagen using UV light instead of heat [326]. Regions with crosslinked collagen would allow for ASM adherence while collagen free regions will be devoid of cells. Micro manipulation of $CD4^+$ T cells using optical tweezers is another means of studying MNTs. By isolating single cells and pulling them towards ASM cells, MNT formation can be predictably and efficiently visualized. Various potential methods exist to increase the efficiency of MNT research, but manufacturing devices with micron details will have its limitations as it requires extreme precision.

5. Hypothesis

Multiple pieces of evidence have shown that $CD4^+$ T cell interactions with ASM cells can play a major role in mediating and/or exacerbating the asthma condition. However, studies on the mechanisms by which $CD4^+$ T cells and ASM cells modulate each other are very limited. The purpose of this thesis was to elucidate some of these mechanisms and further demonstrate the importance and consequential nature of $CD4^+$ T cells and ASM cell interaction. I purpose these interactions will have implications on exacerbating asthma. This will be mediated by $CD4^+$ T cell alterations to ASM phenotype as to promote AHR and/or airway remodeling, properties which will include enhanced proliferative and contractile capacities. Concurrently, ASM cells will induce a pro-inflammatory phenotype in $CD4^+$ T cells particularly through a mitochondrial transfer-dependent mechanism. In order to study mitochondrial transfer, I will describe the experimental work performed to create a novel assay using engineering techniques to better study the cellular interactions.

6. Modulation of ASM Cell Phenotype by $CD4^+$ T cells through ICAM-1

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[1]Performed qPCR experiments to measure expression of ASM contractile factors and measured proliferation of ASM cells with afatinib treatment

[2]Performed qPCR experiments to measure expression of adhesion molecules

[3]Performed all calcium experiments

6.1 Abstract

AHR, the excessive narrowing of the airways and defining characteristic of asthma, is thought to be caused by increased ASM mass and/or differentiation of ASM cells into a hypercontractile phenotype. Understanding the mechanisms inducing ASM remodeling is the key to understanding and treating asthma. $CD4^+$ T cells have been found at elevated levels in the airways of asthmatics and localize around the ASM in the subepithelial compartment. *In vitro* experiments have demonstrated $CD4^+$ T cells interact with ASM through various integrins leading to ASM proliferation and hyper-contractility. This study used co-culture methods to elucidate the mechanisms behind this process. $CD4^+$ T cells enhanced proliferation and reduced calcium responses to histamine of ASM cells. These effects were not present in Transwell cultures. A reduction in expression of ASM contractile factors was also induced by $CD4^+$ T cells. Inhibition of ICAM-1 reversed the suppression of contractile factors, which may be mediated by FAK and AKT. These results demonstrated $CD4^+$ T cells induce a pro-proliferative phenotype in ASM cells through ICAM-1.

6.2 Introduction

Asthmatics are characterized by a phenomenon called AHR, which is an excessive narrowing of the airways in response to a contractile agonist. AHR is potentially mediated by airway remodeling, particularly of the ASM. ASM bundles in asthmatic airways are enlarged which is thought to allow enhanced force production causing excessive narrowing of the airways [1]. The phenotype of ASM cells themselves is also thought to be modulated to produce more contractile force. The mechanisms underlying airway remodeling are unclear, but many factors have been proposed. $CD4^+$ T cells are among the most promising cellular elements that may drive remodeling. Various studies have found $CD4^+$ T cells at elevated levels in the subepithelial compartment of asthmatic airways [2-4]. Studies employing murine models driven by allergen sensitization and challenge have also shown that $CD4^+$ T cells are sufficient to induce airway remodeling and AHR [5]. $CD4^+$cells were also demonstrated to be necessary for AHR maintenance after the initial sensitization and challenge [6]. Very few experiments have been

done to model the effects of $CD4^+$ cells in culture, however, $CD4^+$ T cells have been shown to increase proliferation of ASM cells [7-9]. They can also increase force production by ASM tissue [10, 11]. Although $CD4^+$ T cells are major producers of cytokines, their proliferative and contractile effects have been linked to growth factor expression and integrin binding [8, 9]. We aim to elucidate the modality of interaction and subsequent signaling mechanisms required in $CD4^+$ T cell modulation ASM phenotype.

ASM cells and $CD4^+$ T cells bind using various adhesion molecules including, ICAM-1, VLA-4 and CD44 [9]. Previous experiments have shown that inhibition of CD44 can reduce $CD4^+$ T cell induced ASM proliferation but inhibition of ICAM-1 resulted in the greatest binding inhibition [9]. ICAM-1 is also responsible for ASM tissue enhancement of force production [11]. The signaling mechanisms by which these integrins mediate these changes in ASM cells have not been further explored, but CD44 was determined to mediate HB-EGF release, which is the actual molecule inducing rat ASM proliferation [8]. Signaling downstream of integrins has also been demonstrated to have phenotype modulator roles in the context of mechanical stimuli. When ASM strips are put under a mechanical load, such as by attaching a weight, mechanical forces are transduced from cell to cell through integrins [12]. It was determined that heavier loads induced greater FAK activation that was localized to surface integrins. AKT was subsequently inhibited which resulted in SRF translocation into the nucleus and increased expression of contractile factors [12]. Binding of other cells, such as $CD4^+$ T cells, to ICAM-1 or VLA-4 may modulate a similar FAK dependent pathway.

The current study has demonstrated that $CD4^+$ T cells enhance ASM proliferation in a contact-dependent process as Transwell conditions do not enhance proliferation. Inhibition of CD44 or the EGFR was not sufficient to inhibit these pro-proliferative effects. Analysis of calcium responses to histamine showed that ASM cells were less responsive to contractile stimulation, both in proportion of responders and peak calcium release after co-culture with $CD4^+$ T cells. A reduction in expression of ASM contractile factors was also observed after co-culture. These

effects were again contact dependent. $CD4^+$ T cells activated AKT and inhibited FAK activation, mimicking ASM strips under a light load. Inhibition of ICAM-1 prevented suppression of the contractile factors. Preliminary results demonstrated $CD4^+$ T cells induce a proliferative phenotype in ASM cells via interactions with ICAM-1

6.3 Methods

ASM cell culture

ASM cells were isolated from transplant grade control lungs, procured by the International Institute for the Advancement of Medicine (IIAM, USA). ASM tissues were dissected out and incubated in 0.4mg/ml collagenase IV diluted in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) overnight to dissociate cells. Large pieces of tissue were removed, and the remaining cell suspension was centrifuged at 1500 rpm for 5 min. The pellet of ASM cells was re-suspended in growth medium (DMEM supplemented with 10% fetal bovine serum (FBS, Gibco) and 100 U/ml penicillin, 100 µg/ml streptomycin and 2500 ng/ml amphotericin B (PSA, Gibco)) and kept in a humidified atmosphere of 95% air -5% CO2 at 37°C. Growth medium was changed every 2-3 days until cells were approximately 80-90% confluent before being passaged for experimentation. All cells used for experimentation were between passage 2-5.

PBMC Activation and $CD4^+$ T cell Isolation

Following informed consent, human peripheral blood was collected from healthy volunteers in heparin-coated tubes and was diluted with PBS in at 1:2 ratio (blood: PBS). Diluted blood was deposited on Ficoll-Paque PLUS (GE Healthcare) and centrifuged at 1400 rpm for 35 minutes. The peripheral blood mononuclear cell (PBMC) layer was isolated from the centrifuged blood. PBMCs were cultured in RPMI 1640 (Gibco) supplemented with 10 % FBS and 1% PSA, 20 ng/ml of PMA and 250 nM ionomycin (Thermo Fisher Scientific) for 48 hours. CD4 T cells were isolated from PBMCs by CD4 negative selections using manufacturers recommended protocol (MACS)

Co-culture

25,000 ASM cells were plated in 6 well plates in complete media and allowed to adhere overnight. They were starved using 0.5% FBS medium the next day for 24h before co-culture. 500,000 $CD4^+$ T cells were co-cultured with ASM cells in a 1 to 1 DMEM:RPMI mixture containing 0.5% FBS. Cells were co-cultured for 48 hours before analysis.

Proliferation assay

Cell proliferation was evaluated by the incorporation of bromodeoxyuridine (BrdU) (BD Biosciences). BrdU (3mg/ml in PBS) was added 18h before harvest for a final concentration of 10µM. Cells were collected using trypsin, then fixed and stained with FITC conjugated anti-BrdU antibodies following manufacturer's recommended (BD Bioscience). BrdU incorporation was assessed by flow cytometry using FACSCanto II (BD Biosciences).

IL-10, ICAM-1 and CD44 antagonism

For ICAM-1 and CD44 antagonism, ASM cells were incubated with 1µg/ml anti-ICAM-1 antibodies (Thermo Fisher Scientific) or 0.5-2µg/ml anti-CD44 antibodies (Thermo Fisher Scientific) for 1 hour. When co-cultured, cells were treated with 1µg/ml anti-ICAM-1, 1 µg/ml anti-IL-10 antibodies (Thermo Fisher Scientific) or 0.5-2µg/ml anti-CD44 antibodies were used.

Afatinib Treatment

To Inhibit the EGFR, ASM cells were pre-treated with 1nM afatinib (Tocris), a chemical EGFR inhibitor, for 2 hours before co-culture. Co-cultures were also carried out in medium with 1nM afatinib.

Calcium assay

Co-cultures were done in a 6 well plate with a glass coverslip on the bottom. After co-culture, cells were washed with HBSS and incubated for 30 minutes at 37°C with 1mM Fura-2 AM (Thermo Fisher Scientific). Cells were again washed and incubated in HBSS for 15min to allow for complete esterification. Fresh HBSS was added and cells were fluorescently imaged using a deltaRAM (Horiba) which allows for rapid imaging at the 340nm and 380nm excitation

wavelengths. Calcium responses were evaluated by performing a ratiometric analysis of 340/380 values collected from individual ASM cells. Individual cells were hand traced.

RT-qPCR

$CD4^+$ T cells and ASM cells were separated by collecting supernatant containing T cells and multiple washes of the ASM cells using PBS. RNA was extracted using a RNeasy mini-kit (Qiagen) and 500ng were retrotranscribed by AffinityScript qPCR cDNA synthesis kit (ABM). Quantitative real-time PCR was performed using CFX96 (Bio-Rad) with BrightGreen Mastermix (ABM), respectively to check mRNA expression levels. Relative mRNA expression was calculated using the "ΔΔCt" method. Individual data were normalized against the housekeeping gene ribosomal S9. Results were expressed as a fold change in expression compared to a calibrator sample.

Western Blot

$CD4^+$ T cells and ASM cells were separated by collecting supernatant containing T cells and multiple washes of the ASM cells using PBS. Cells were washed with ice-cold PBS and lysed using NP40 Cell lysis buffer (Life Technologies) supplemented with a protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). Cell lysates were centrifuged at 13 000 rpm for 10 minutes, and the supernatant was evaluated for protein concentration by Bradford assay (Biorad).

Five µg of protein were re-suspended in loading buffer (Bio-Rad Laboratories) containing Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) and incubated at 95°C for 6 minutes to denature proteins. Proteins were separated on a precast Tris/glycine gel (Bio-Rad Laboratories) and then transferred onto a PVDF membrane (Bio-Rad Laboratories) for immunoblotting. The membrane was blocked for 1 hour at room temperature in 5% BSA in Tris-buffered saline with 0,1% Tween (TBS-T). Membranes were incubated with primary antibodies against: vimentin (Cell Signaling Technologies), α-smooth muscle actin (αSMA, Abcam), smooth muscle myosin heavy chain (MHC, Abcam) , myosin light chain kinase (MLCK, Abcam), phospho/total protein kinase B (AKT, Cell Signaling), phospho/total Iκβ and phospho/total FAK (Cell Signaling Technologies), diluted in TBST, overnight at 4°C. After washing, membranes were incubated with an HRP linked secondary antibody (Cell Signaling Technologies) for 1 hour at room temperature and then

visualized using the ChemiDoc^{TM} MP Imaging System (Bio-Rad Laboratories) after ECL Plus Western blotting detection (Bio-Rad Laboratories). Densitometric analysis was performed with Image Lab (Bio-Rad Laboratories). Protein expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Flow cytometry

When analyzing cytokine expression, cells were treated with Golgi-Stop (BD bioscience) for 4 hours before harvest. Cells were collected by trypsinization and stained with viability dye efluor780 for 10min. Cells were then fixed and permeabilized for 30min and stained with CD4, CD45, TNF-α, IL-5 and IL-10 antibodies (Biolegend) for 1h. Cells were washed and analyzed by flow cytometry using FACSCanto II (BD Biosciences).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software. Data are presented as the mean ± SEM, unless otherwise specified. A one-way ANOVA followed by Tukey's posttest was implemented. The p values < 0.05(*), 0.01(**) and 0.001(***) were considered significant.

6.4 Results

CD4$^+$ T cells Enhance ASM cell Proliferation and Calcium Responses

To study proliferative effects of CD4$^+$ T cells on ASM cells, CD4$^+$ T cells isolated from PMA/Ionomycin activated PBMCs were co-cultured with ASM cells for 48 hours and incubated with BrdU for the last 18h. Analysis by flow cytometry showed more ASM cells incorporated with BrdU when co-cultured with CD4$^+$ T cells, indicating more cells have proliferated (Fig.1A). Co-culturing ASM cells with CD4$^+$ T cells in a Transwell system had no significant effects on proliferation (Fig.1A). Previous findings demonstrated adherence through CD44 and activation of the EGFR mediates CD4$^+$ T cells induced proliferation of ASM cells in rats [8]. To explore this mechanism in humans, co-cultures were treated with afatinib and anti-CD44 antibodies to inhibit the EGFR and CD44 respectively. Neither afatinib nor anti-CD44 antibodies had any

effect on $CD4^+$ T cell-induced proliferation of ASM cells (Fig.1B and 1C), differing from previous results on rat ASM cells.

Contractile activation of ASM cells was inferred by measuring intracellular calcium release in response to histamine using Fura-2, a calcium binding dye. Fewer ASM cells responded to histamine when co-cultured with $CD4^+$ T cells (Fig.2A). Peak calcium release was also reduced when co-cultured with T cells. These findings were not replicable in Transwell cultures, suggesting a need for direct contact (Fig.2B). These results suggest an ASM cell proliferative phenotype is induced by $CD4^+$ T cells.

$CD4^+$ T cells suppress ASM cell contractile phenotype

To assess phenotypic changes in ASM cells, ASM cell proliferative and contractile phenotype markers were assessed by qPCR and western blot. mRNA expression of contractile factors, CNN and MYOCD, was significantly reduced by co-cultures with $CD4^+$ T cells (Fig3A and 3B). MLCK mRNA levels trended in the same way but MLCK protein was not significant reduced (Fig3D and 3F). These results were not replicable in Transwell cultures. No changes in αSMA were observed (Fig3C and 3E). No significant differences in proliferative markers were found (Fig4A-D). This data suggests that only a suppression of contractile factors was induced by $CD4^+$ T cells.

$CD4^+$ T cells induce cytokine expression by ASM cells

Cytokine and growth factor expression by either ASM cells or $CD4^+$ T cells after co-culture was measured by qPCR and flow cytometry. qPCR analysis showed $CD4^+$ T cells increased the expression of IL-5 and TNF-α transcripts in ASM cells (Fig.5D and 5E), however, flow cytometry analysis showed no difference in protein expression (Fig.5G and 5H). IL-10 expression, however, was found to be significantly increased by flow cytometry in ASM cells (Fig.5F and 5I). ASM cells seem to reduce a broad range of cytokine expression by $CD4^+$ T cells. TNF-α and IL-5 mRNA were significantly reduced in $CD4^+$ T cells after co-culture (Fig.6D and 6E) while IL-10 trended downward (Fig.6F). No significant differences in cytokine expression by $CD4^+$ T cells were found

by flow cytometry (Fig.6G-I). All reported results were contact dependent and not replicable in Transwell cultures. No differences in HB-EGF and TGF-β expression were found in either cell type (Fig.5A, 5B, 6A and 6B). This data suggests autocrine signaling by ASM cells are the only significant source of cytokines.

IL-10 is not responsible for contractile phenotype suppression

IL-10 has been demonstrated in the past to act as both a pro-proliferative and pro-contractile factor for ASM cells. We explored whether autocrine signaling of IL-10 by ASM cells is responsible for $CD4^+$ T cells induced contractile phenotype suppression by inhibiting IL-10 using an antagonistic antibody. Inhibition of IL-10 did inhibit $CD4^+$ T cell-induced MLCK expression by ASM cells, but it also significantly reduced basal levels of MLCK expression (Fig.7A and 7B). IL-10 inhibition may reduce MLCK expression to levels that cannot be further down regulated by $CD4^+$ T cells. Along with the fact that IL-10 inhibition caused a reduction of MLCK expression, IL-10 does not seem to be responsible for the suppression of the contractile phenotype (Fig.7A).

$CD4^+$ T cells induce expression of ICAM-1 and VCAM-1 by ASM cells

Next, we examined the expression of various adhesion molecules by ASM cells to determine which are likely candidates that $CD4^+$ T cells use to bind to ASM cells as well as mediate phenotypic modulation of ASM cells by $CD4^+$ T cells. qPCR analysis showed low levels of ICAM-1 and VCAM-1 expression at basal levels but a significant increase in ICAM-1 was seen after co-culture (Fig.8A-C). CD44 expression was only increased in the Transwell condition (Fig.8A).

ICAM-1 is involved in contractile phenotype suppression

ICAM-1 has been previously demonstrated to be one of the stronger binding adhesion molecules in the interaction between $CD4^+$ T cells and ASM cells although their phenotype modulatory functions have yet to be described [9]. Preliminary experiments antagonizing ICAM-

1 prevented down regulation of MLCK protein in ASM cells by $CD4^+$ T cells and enhanced basal expression of MLCK (Fig9A). No trends were observed in MHC11 expression with co-culture in these preliminary experiments, however inhibiting ICAM-1 seemed to upregulate MHC11 (Fig.9B). ICAM-1 seems to be a candidate for mediating contact dependent modulation of ASM cell contractile phenotype by $CD4^+$ T cells.

AKT and FAK activation by $CD4^+$ T cells

Previous studies have shown that mechanical stimulation modulates ASM cell phenotype and the changes are transduced through integrin-dependent signaling. Activation of AKT and FAK was measured in ASM cells to test whether $CD4^+$ T cells activate similar pathways. A significantly increased phosphorylation of AKT was induced by $CD4^+$ T cells (Fig.10B). Preliminary results suggest a reduction in FAK phosphorylation by $CD4^+$ T cells was inhibited by ICAM-1 inhibition (Fig.11E and 11F). Preliminary results with AKT showed AKT phosphorylation was not enhanced by $CD4^+$ T cells when treated with an isotype antibody (Fig.11B). ICAM-1 antagonists increased AKT expression and phosphorylation (Fig.11A and 11B). It is currently unclear what signaling mechanisms are responsible for increasing ASM proliferation and suppressing their contractile phenotype.

6.5 Conclusion

This study has demonstrated that $CD4^+$ T cells promote a pro-proliferative and anti-contractile phenotype in ASM cells. Increased proliferation and reduced peak calcium responses were found in ASM after co-culture (Fig.1A and 2). A reduction in expression of ASM contractile factors was accompanied by increased expression of TNF-α and IL-5 transcripts and IL-10 protein as measured by qPCR and flow cytometry (Fig.3 and 5). A generalized down-regulation of cytokines seems to occur in $CD4^+$ T cells after co-culture with ASM (Fig.6). Co-culturing in a Transwell system did not result in any of these phenotypic changes, suggesting that direct contact between the two cell types is key. Inhibition of IL-10 caused a reduction of MLCK,

demonstrating opposing effects of IL-10 and $CD4^+$ T cells on ASM cells (Fig.7). A significant increase in ICAM-1 expression by ASM cells was also induced by $CD4^+$ T cells (Fig.8). To assess the role of ICAM-1, ICAM-1 was inhibited using an antagonistic antibody. Preliminary results showed ICAM-1 inhibition prevented MLCK down-regulation (Fig.9A). In further probing mechanisms we observed an increase in AKT activation in ASM cells co-cultured with $CD4^+$ T cells (Fig.10). Both the ICAM-1 and isotype antibodies seem to greatly influence the activity and expression of AKT making it difficult to assess specific effects on ASM-$CD4^+$ T cell co-cultures (Fig.11A-C). FAK activation was shown to be reduced by $CD4^+$ T cells, possibly through ICAM-1 but these were again preliminary results and need further exploration (Fig.11D-F).

The results of this study have confirmed other reports of $CD4^+$ T cell induced proliferation of ASM cells that is mediated by physical contact and engagement of adhesion molecules. ICAM-1 is a possible candidate molecule mediating the proliferative effects of $CD4^+$ T cells. FAK dependent pathways have been described as one of the intracellular signaling pathways down stream of ICAM-1 [13]. Recently, it was discovered that the level of mechanical strain on ASM tissue influences ASM phenotype through FAK. Heavier loads induced a more contractile phenotype, while lighter loads enhanced synthetic properties and inhibited contractility [12]. It was determined that ASM cells transduce the mechanical stimulus to neighboring cells through integrin connections [14]. Increased strain on the integrin connection induced FAK activation which was localized to the intracellular tail of the beta-subunit of integrins [12]. FAK activity leads to inhibition of AKT, reducing pro-synthetic effects of IL-13 on ASM cells [12]. Although ICAM-1 does not contain a beta-subunit, studies have demonstrated that ICAM-1 can modulate FAK through Rho [13]. Thus, we hypothesized that ICAM-1 mediated phenotypic changes in ASM through a similar pathway that ASM cell to cell interactions use. Preliminary results showed that $CD4^+$ T cells actually reduced FAK activation that was reversed by ICAM-1 inhibition (Fig.11D-F). The effects of ICAM-1 on AKT were confounded by effects of antibody treatment (Fig.11A-C). Nevertheless, it is also possible that ICAM-1 activation on ASM cells can have graded effects on FAK, similar to mechanical strain.

Previous research done on ASM tissue has revealed that ASM and $CD4^+$ T cell interactions through ICAM-1 induce opposite effects to those reported on cells in the current study. $CD4^+$ T cells enhanced contractile force production in response to methacholine and reduced relaxation responses to isoproterenol [11]. Inhibition of ICAM-1 prevented this augmentation of contractility [11]. This may be due to various factors that differentiate tissue and cell cultures. One possible confounding factor is the ratio of $CD4^+$ T cells to ASM cells. Although precise cell counts of ASM tissue was not determined and precise number of $CD4^+$ T cells used were not reported, the use of ASM tissue would significantly increase the number of ASM cells present compared to that of this study. Tissues are also 3-dimensional structures, thus $CD4^+$ T cells are only interacting with the outer layer of ASM cells while all ASM cells in culture were exposed to T cells. Another significant factor is the starting phenotype of the ASM cells before co-culture. Previous studies have demonstrated that culturing ASM cells reduces their contractile capacity and induces a pro-proliferative change [15]. ASM cells in different states may respond to $CD4^+$ T cells differently, possibly promoting their current phenotype.

$CD4^+$ T cells clearly play a role in modulating ASM cell contractile and proliferative phenotype and its mechanisms and outcomes seem to be context dependent. ICAM-1 is a candidate adhesion molecule that mediates their interaction and phenotypic modulation. With the importance that $CD4^+$ T cells seem to play in asthma, further research should be done on their interaction with ASM cells.

6.6 Figures

Figure 1: Proliferation of ASM cells after co-culture with $CD4^+$ T cells

Proliferation was measured by analyzing BrdU incorporation by flow cytometry. (A) $CD4^+$ T cells increased the proliferation of ASM cells but not in a Transwell system. (B,C) Inhibition of the

EGFR and CD44 using afatinib and an antagonistic antibody respectively did not affect $CD4^+$ T cell induced proliferation.

Figure 2: Calcium responses to histamine

Calcium responses were recorded by fluorescently imaging cells stained with Fura-2. Data is presented as a ratiometric analysis of 340/380nm converted to [Ca] using the Grynkiewicz equation. (A) A reduced peak response was observed in ASM cell co-cultured with $CD4^+$ T cells. (B) Fewer $CD4^+$ T cells responded as well.

Figure 3: Suppression of ASM cell contractile phenotype

Expression ASM cell contractile factors CNN, MYOD, αSMA and MLCK was measured by qPCR. Expression of αSMA and MLCK was assessed by Western blot. (A-D) qPCR results showed significant reduction in CNN and MYOD with co-culture with $CD4^+$ T cells. (E-F) Western blot analysis showed a down regulation in MLCK protein but not αSMA with co-culture. All effects on contractile factor expression were contact dependent.

Figure 4: Expression of ASM cell proliferative factors

Expression of ASM cell contractile factors ELK-1, KLF-4 and Vimentin was measured by qPCR. Expression of vimentin was also assessed by Western blot. (A-D) No differences in expression of proliferative factors were found at any level.

Figure 5: Growth factor and cytokine expression by ASM

Expression of various growth factors and cytokines was assessed by qPCR and flow cytometry. (A-B) No significant differences in HB-EGF or TGFβ expression by ASM cells was induced by $CD4^+$ T cells. (C) No statistical test could be run on IFN-γ due to the fact only one control sample

expressed detectable levels of IFN-γ transcript. (D-E) ASM upregulated IL-5 and TNF-α mRNA expression after co-culture. (F) IL-10 showed trending increases, but sample size needs to be increased. Transwell conditions failed to replicate co-culture results. (G-H) IL-5 and TNFα protein expression as measured by flow cytometry did not increase with co-culture. (I) IL-10 protein was significantly upregulated in ASM cells by $CD4^+$ T cells.

Figure 6: Growth factor and cytokine expression by $CD4^+$ T cells

Panel of growth factors and cytokines measured in ASM cells was also measured in $CD4^+$ T cells. (A-B) No differences in HB-EGF and TGFβ expression were determined by qPCR. (D-E) Significant reductions in IL-5 and TNF-α transcripts were found. (C-F) No significant differences in IFN-γ and IL-10 transcripts were measured, however, IL-10 measurements are lacking in samples. (G-I) No differences in IL-5, TNF-α or IL-10 expression was found by flow cytometry.

Figure 7: Effects of IL-10 antagonists on MLCK expression

Effects of IL-10 on the interaction between ASM and $CD4^+$ T cells was assessed by inhibiting IL-10 using 1μg/ml of an antagonistic antibody. (A) Expression of MLCK was significantly reduced by IL-10 inhibition without co-culture. (B) Relative reduction of MLCK expression by $CD4^+$ T cells was inhibited by IL-10 inhibition.

Figure 8: Expression of Adhesion Molecules Mediating $CD4^+$ T cell Interactions

Expression of CD44, ICAM-1 and VCAM-1 by ASM cells was assessed by qPCR. Transwell but not co-culture conditions induced an upregulation of CD44. No differences in ICAM-1 or VCAM-1 expression were detected.

Figure 9: Effects of ICAM-1 antagonists on ASM contractile proteins

1µg/ml of ICAM-1 antagonistic antibodies was added to co-cultures to assess the role of ICAM-1 in the modulation of MLCK and MHC11 expression. Levels of MLCK and MHC11 in ASM cells was measured by Western blot. (A-B) Preliminary results demonstrate a slight increase in both MLCK and MHC11 expression with ICAM-1 inhibition in control conditions.

Figure 10: Activation of AKT by $CD4^+$ T cells

Activation of AKT was inferred by its phosphorylation and measured by Western blot. (A) No difference in total AKT expression was induced by $CD4^+$ T cells. (B) Co-culture but not Transwell conditions induced a significant increase in pAKT.

Figure 11: Modulation of AKT and FAK signaling pathways by ICAM-1

Effects of ICAM-1 on AKT and FAK activation were assessed by measuring phosphorylation levels by Western blot. (A) Preliminary results showed ICAM-1 inhibition increased total AKT expression. (B) Isotype antibodies seem to be affecting activation of AKT as it seemed to have inhibited $CD4^+$ T cell induced phosphorylation. ICAM-1 inhibition, however, did not inhibit AKT activation. (C) Total FAK expression was down-regulated by co-culture which may have been inhibited by ICAM-1 inhibition. (D) Activation of FAK seemed to also be reduced with co-culture, which is again, unclear whether ICAM-1 is involved.

Figure 1

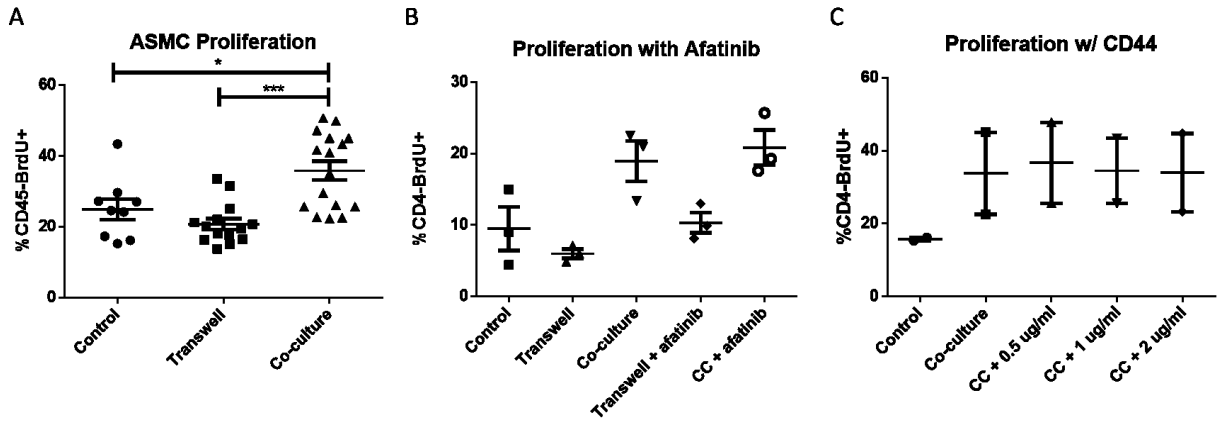
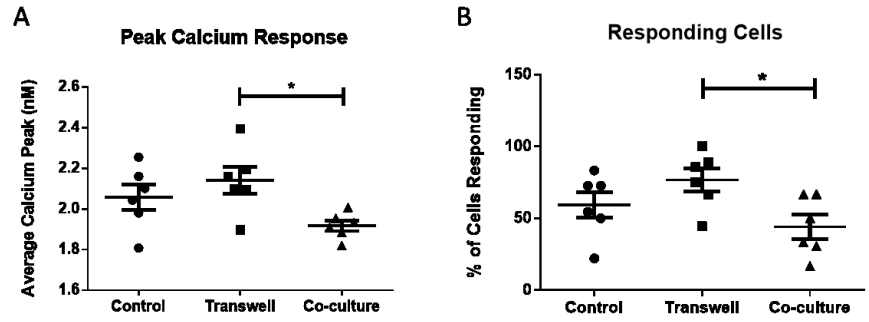


Figure 2



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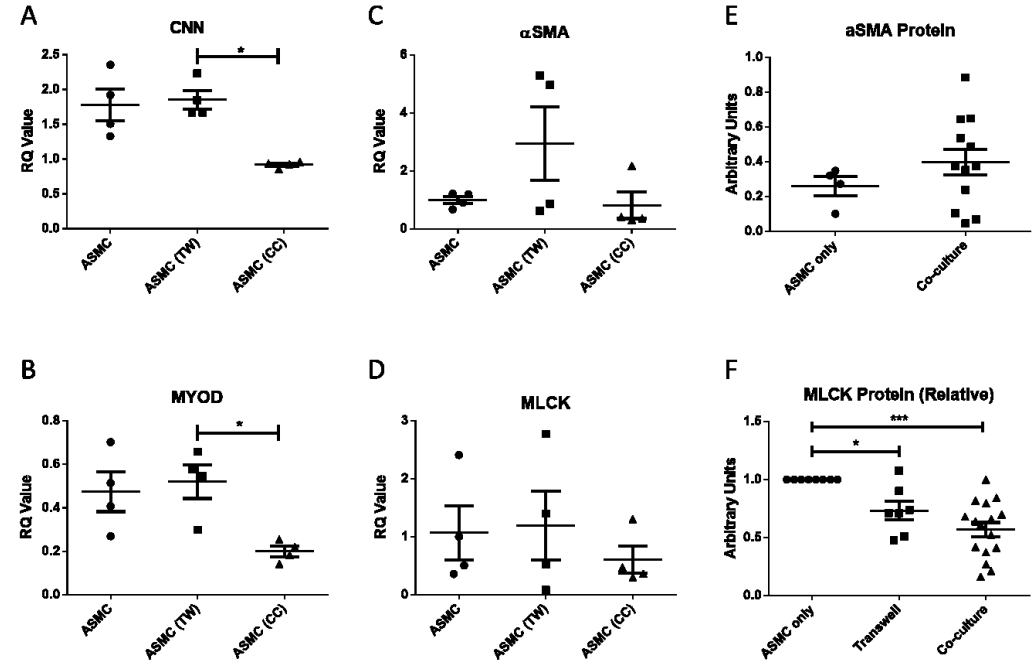

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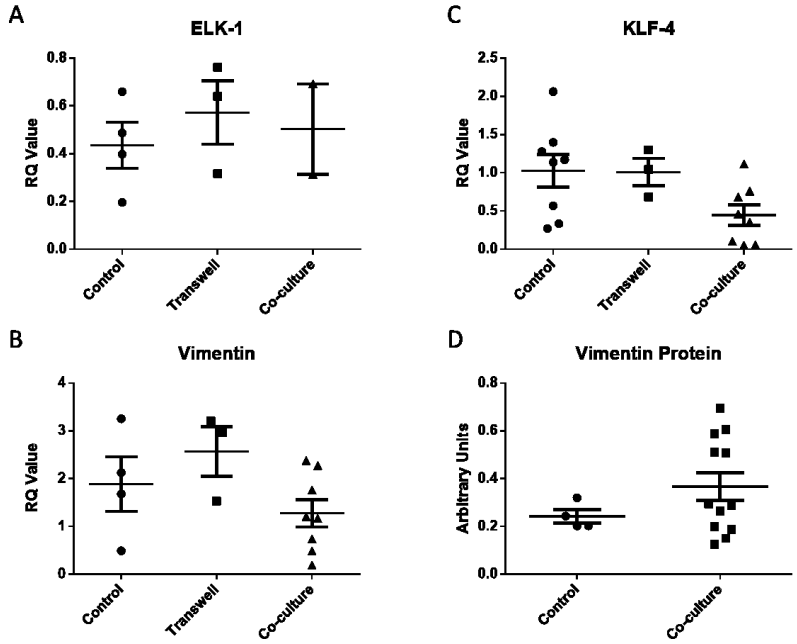
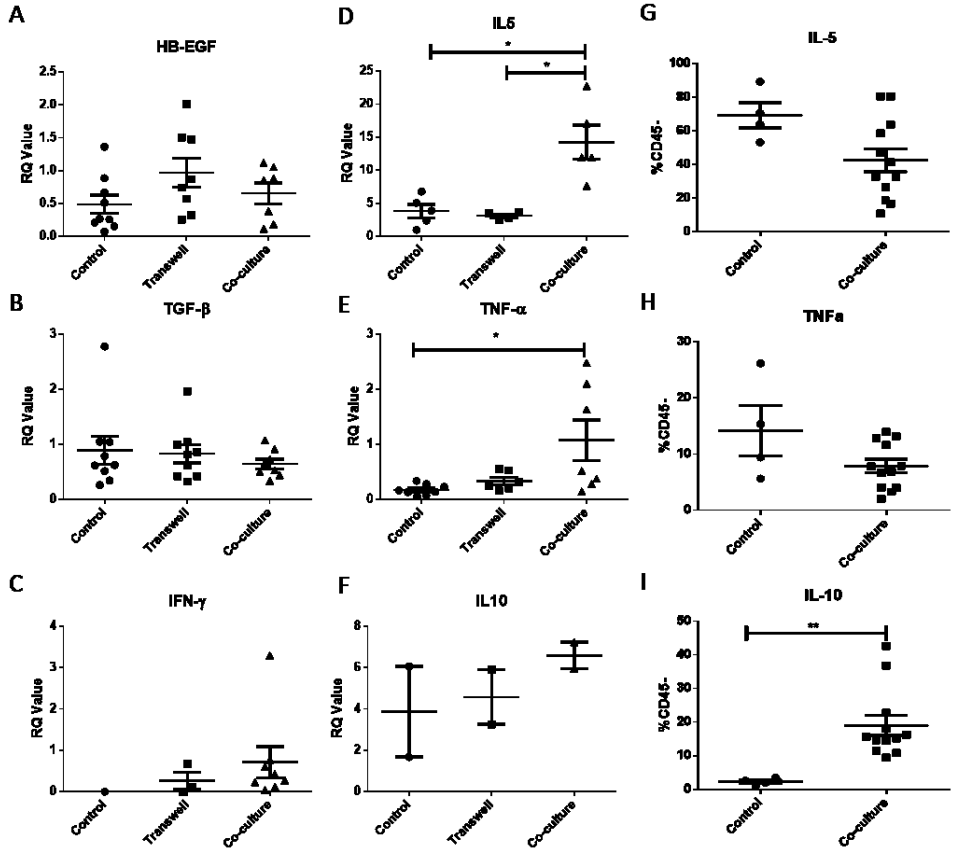


Figure 5



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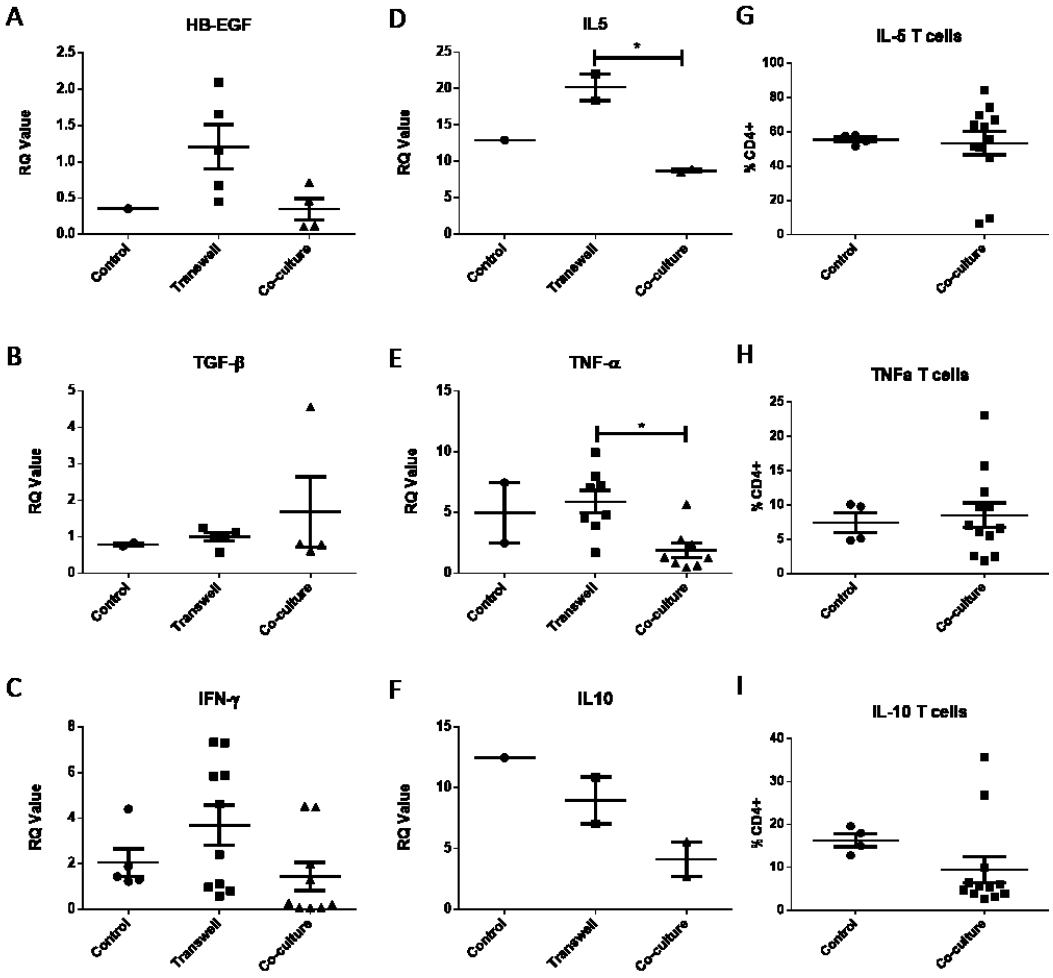


Figure 7

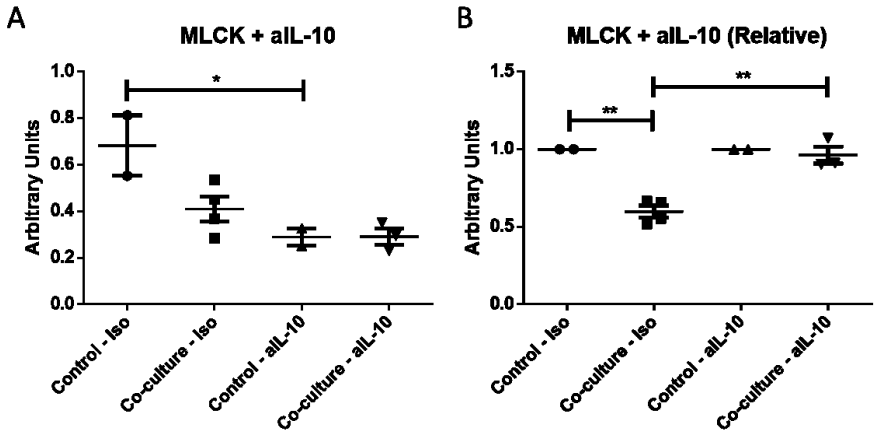


Figure 8

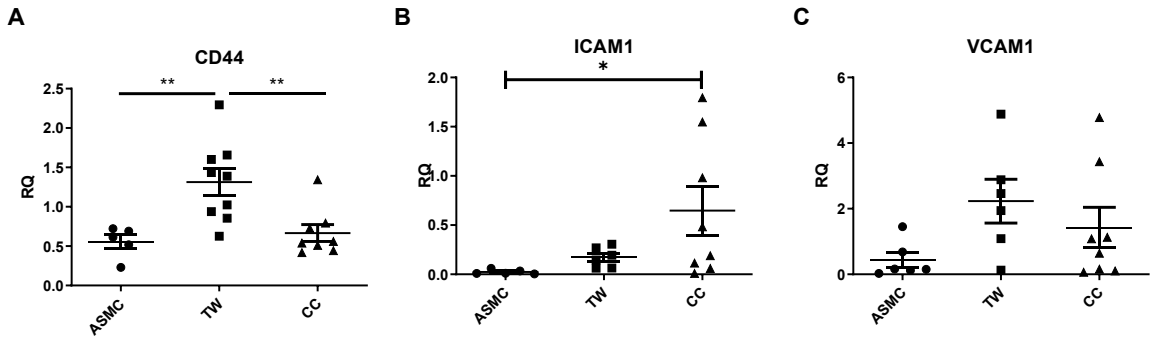


Figure 9

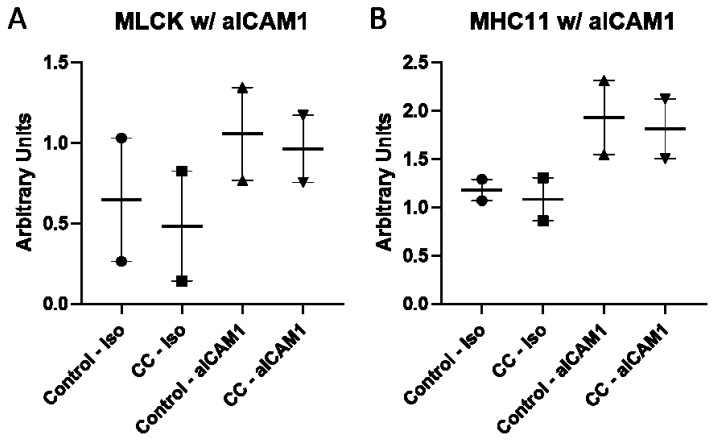


Figure 10

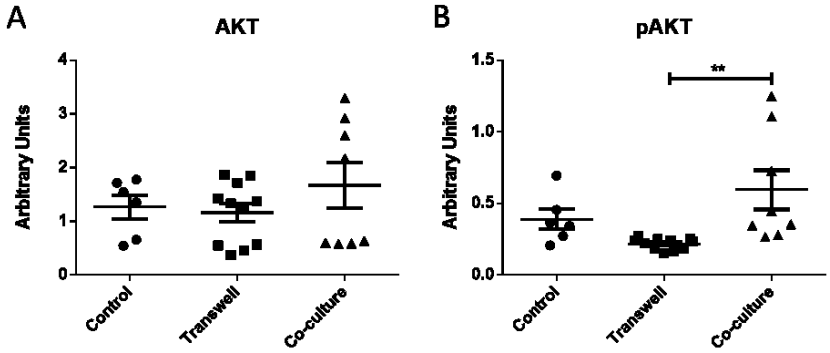
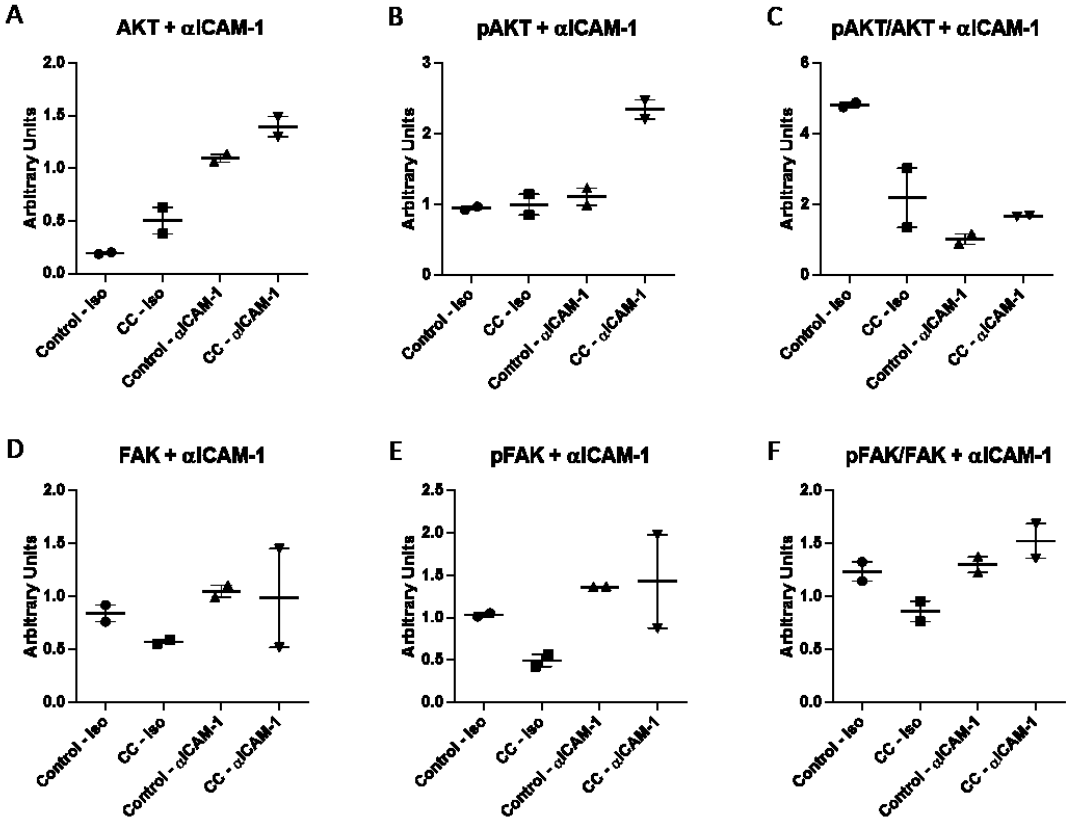


Figure 11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Control - αICAM-1
CC - αICAM-1
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Control - Iso
CC - Iso
Control - αICAM-1
CC - αICAM-1

6.7 References

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After studying the effects of $CD4^+$ T cells on modulating their proliferative and contractile phenotypes of ASM cells, we then looked at changes in $CD4^+$ T cells. The interaction between the two cells may also be inducing inflammatory changes in $CD4^+$ T cells and thus contributing to the inflammatory milieu of asthmatic airways. Enhancement in inflammatory activity in $CD4^+$ T cells may also enhance the modulation of ASM cells. These two processes are mechanisms by which $CD4^+$ T cells can exacerbate asthma with the influence of ASM cells.

7. Induction of Hyper-reactive $CD4^+$ T cells by ASM Cells: Implications of Mitochondrial Transfer

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[1]Transduced ASM cells with a lentivirus to express PDHA-1-tRFP

7.1 Abstract

$CD4^+$ T cells are found in abundance in the airways of asthmatic patients and they are drivers of the inflammatory milieu and progression of the disease. Some of their effects on the ASM have been documented, but the mechanisms by which ASM cells can influence $CD4^+$ T cells are understudied. The current study employed co-culture techniques to study the effects of ASM cells on $CD4^+$ T cells, to enhance their ability to exacerbate asthma. $CD4^+$ T cells were hyper-reactive to CD3/28 re-stimulation after co-culture with ASM cells. No differences in memory T cell markers were found, however, the lymph node homing chemoreceptor, CCR7, was increased in memory cells with re-stimulation in co-cultured $CD4^+$ T cells. Co-culture also enhanced metabolic activity of $CD4^+$ T cells, with significant enhancement of cellular respiration upon re-stimulation. Increased production of mitochondrial ROS and activation of caspase-1 were also measured in co-cultured $CD4^+$ T cells with re-stimulation. ASM cells induce a hyper-reactive response to re-stimulation in $CD4^+$ T cells, possibly through mitochondrial ROS dependent activation of the inflammasome.

7.2 Introduction

Asthma is widely recognized as an inflammatory disease. Inflammatory factors such as cytokines or immune cells have been linked to AHR and airway remodeling in asthmatics. The severity of these processes defines the severity of the disease and have been correlated to the levels of inflammatory factors in the airways [1]. $CD4^+$ T cells has been particularly implicated as their Th2 subset mediates the allergic response, including the maturation of B cells to produce immunoglobulins. $CD4^+$ T cells are also major producers of inflammatory cytokines such as IL-4, IL-13 and IL-17, all factors that have strong modulatory effects on the tissues of the airways that promote AHR and airway remodeling [2, 3]. In particular, the ASM is a key tissue that increases in mass and/or becomes hypercontractile [4]. Although a few studies have discovered various mechanisms by which $CD4^+$ T cells interact with and induce these changes in the ASM, not much is known about the effects of ASM on $CD4^+$ T cells. This study aims to explore the effects

of ASM and $CD4^+$ T cell interactions has on $CD4^+$ T cells, focusing on pro-inflammatory processes that may contribute to asthma onset or progression.

$CD4^+$ T cells play a crucial role in the immune response, which is to mediate immune memory. $CD4^+$ T cells are activated in by antigen presenting cells (APCs) expressing an MHC II with an antigen that matches the T cell receptor of that T cell. These $CD4^+$ T cells will contract after clearance of the infection, leaving a small population of memory $CD4^+$ T cells that remain dormant within tissues or the lymph nodes until re-introduced to the same initiating antigen. These memory cells are also more reactive to the antigen compared to their original response. As a reflection of this memory property, $CD4^+$ T cells have been shown to mediate AHR during secondary challenges to allergen in murine asthma models [5]. A tissue-specific subset of memory $CD4^+$ T cells has also been discovered within the lungs allowing for immediate responses to lung insults [6]. $CD4^+$ T cells localize to the subepithelial compartments of the airways and have been found near and within ASM bundles [7]. This observation triggered research into the modulatory roles of $CD4^+$ T cells on ASM, but mechanisms by which $CD4^+$ T cells are affected also have emerged.

Various modalities of interactions have been discovered between $CD4^+$ T cells and ASM cells. Paracrine factors such as cytokines and growth factors are commonly described modulators of either cell type but $CD4^+$ T cells and ASM cells make many physical bonds. Adhesion molecules such as ICAM-1, VCAM-1 and CD44 on ASM cells have been shown to mediate $CD4^+$ T cells binding through LFA-1, VLA-4 and CD44/hyaluronic acid [8]. These molecules are commonly associated with T cell migration, but they can have consequences for T cell processes such as activation. ASM cells have also been identified as APCs capable of presenting superantigen to $CD4^+$ T cells causing their activation [9]. Recently, a tubular membrane protrusion called membrane nanotubes (MNTs), produced by $CD4^+$ T cells were discovered to connect with ASM cells allowing for transfer of various cellular products [10, 11]. Anti-apoptotic proteins B cell lymphoma-2 and induced myeloid leukemia cell differentiation protein are transferred from

ASM cells to $CD4^+$ T cells through MNTs, enhancing $CD4^+$ T cell survival [10]. Mitochondria are also passed from ASM cell to $CD4^+$ T cell, but the consequences of this process need further elucidation [11].

In the current study, ASM cells were co-cultured with activated $CD4^+$ T cells to study immunomodulatory effects that occur in the T cells. $CD4^+$ T cells co-cultured with ASM cells were hyper-reactive to CD3/28 re-stimulation. There were no changes in memory T cell markers in $CD4^+$ T cells with co-culture, however, expression of the lymph node homing chemoreceptor, CCR7, was increased in memory cells upon re-stimulation, unlike control cells. Co-cultured $CD4^+$ T cells were glycolytically and mitochondrial more active. Upon re-stimulation, enhancement of their cellular respiration was greater in co-cultured $CD4^+$ T cells but glycolytic enhancements were similar. Interestingly, control $CD4^+$ T cells maintained their glycolytic activity better than co-cultured cells with re-stimulation. Re-stimulation of co-cultured cells also induced greater mitochondrial ROS production. An increased activation of caspase-1 accompanied the mitochondrial ROS, suggesting a possible inflammasome-dependent pathway. However, IL-1β was not detected in co-culture and re-stimulation cultures after 24 hours. These results point to an enhancement of $CD4^+$ T cell immune responses by ASM cells, possibly mediating asthma exacerbations.

7.3 Methods

ASM cell culture

See '*ASM cell culture*' in section 6.3 above.

$CD4^+$ T cell Isolation and Activation

For isolation of PBMCs from peripheral blood, see '*PBMC activation and $CD4^+$ T cell Isolation*' in section 6.3 above. $CD4^+$ T cells were isolated from PBMCs using CD4 positive selection (MACs) using the manufacturer's recommended protocol. 5 million $CD4^+$ T cells were activated in a 6

well plate using a 1:1 ratio of CD3/28 Dynabeads (Thermo Fisher Scientific) to cells for 72 hours. $CD4^+$ T cells were separated from beads using magnetic separation and cultured in complete RPMI (Gibco).

ASM-T cell co-culture

50,000 ASM cells were plated in a 6-well plate, 24 hours before co-culture. 1 million activated $CD4^+$ T cells were co-cultured with ASMC in a 1:1 mixture of complete DMEM and RPMI for 24 hours.

Proliferation Assay

Cell proliferation was evaluated by the incorporation of bromodeoxyuridine (BrdU) (BD Biosciences). BrdU (3mg/ml in PBS) was added 18h before harvest for a final concentration of 10µM. Cells were collected using trypsin, then fixed and stained with FITC conjugated anti-BrdU antibodies following manufacturer's recommended (BD Bioscience). BrdU incorporation was assessed by flow cytometry using FACSCanto II (BD Biosciences).

RT-qPCR

$CD4^+$ T cells and ASMC were separated by collecting supernatant containing T cells and multiple washes of the ASMC using PBS. RNA was extracted using a RNeasy mini-kit (Qiagen, Venlo, Netherlands) and 500ng were retrotranscribed by AffinityScript qPCR cDNA synthesis kit (ABM). Quantitative real-time PCR was performed using CFX96 (Bio-Rad, Hercules, CA) with BrightGreen Mastermix (ABM), respectively to check mRNA expression levels. Relative mRNA expression was calculated using the "ΔΔCt" method. Individual data were normalized against the housekeeping gene ribosomal S9. Results were expressed as a fold change in expression compared to a reference sample.

Flow cytometry

When analyzing cytokine expression, cells were treated with Golgi-Stop (BD Bioscience) for 4 hours before harvest. Cells were collected by trypsinization and stained with viability dye efluor780 for 10min. For analysis of mitochondrial ROS, cells were incubated with 10µM

solution of mitoSOX (Thermo Fisher Scientific) for 15 min before fixation. Cells were then fixed and permeabilized for 30min and stained with CD4, CD45, CD45RO, CD45RA, CCR7, IL-4, IL-5, IFN-γ, IL-10, TGF-β, T-Bet, GATA3, FOXP3 and RORγT antibodies (Biolegend) for 1h. Cells were washed and analyzed by flow cytometry using FACSCanto II (BD Biosciences).

Re-stimulation

$CD4^+$ T cells with and without co-culture were collected and resuspended in a complete RPMI. 1 million $CD4^+$ T cells were then re-stimulated using a 1:1 ratio of CD3/28 Dynabeads to cells for 24h.

Seahorse Assay

The Seahorse™ Assay (Agilent) was used to measure the extracellular acidification rate (ECAR), to assess glycolysis, and oxygen consumption (OCR), a measurement of mitochondrial respiration. 300,000 $CD4^+$ T cells with or without co-culture were collected and seeded in a Seahorse 96 well plate. After an initial baseline measurement, wells were injected with $1.2x10^6$ CD3/28 Dynabeads. Metabolic measurements were taken every 5 min for 2 hours. Data were collected using the Seahorse Analyzer XF96 (Agilent).

Western Blot

$CD4^+$ T cells and ASMC were separated by collecting supernatant containing T cells and multiple washes of the ASMC using PBS. Cells were washed with ice-cold PBS and lysed using NP40 Cell lysis buffer (Life Technologies) supplemented with a protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). Cell lysates were centrifuged at 13 000 rpm for 10 minutes, and the supernatant was evaluated for protein concentration by Bradford assay (Biorad Laboratories).

Five µg of protein were re-suspended in loading buffer (Bio-Rad Laboratories) containing Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) and incubated at 95°C for 6 minutes to denature proteins. Proteins were separated on a precast Tris/glycine gel (Bio-Rad Laboratories) and then transferred onto a PVDF membrane (Bio-Rad Laboratories) for immunoblotting. The membrane was blocked for 1 hour at room temperature in 5% BSA in Tris-buffered saline with 0,1% Tween

(TBS-T). Membranes were incubated with primary antibodies against phospho/total Iκβ and caspase-1 (Cell Signaling Technologies), diluted in TBST, overnight at 4°C. After washing, membranes were incubated with an HRP linked secondary antibody (Cell Signaling Technologies) for 1 hour at room temperature and then visualized using the ChemiDoc™ MP Imaging System (Bio-Rad Laboratories) after ECL Plus Western blotting detection (Bio-Rad Laboratories). Densitometric analysis was performed with Image Lab (Bio-Rad Laboratories). Protein expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

IL-1β ELISA

Supernatants from co-cultures and re-stimulation experiments were collected and spun down to remove T cells. The supernatant was analyzed for IL-1β content using an ELISA (R&D).

PDHA-1-tRFP expressing ASM cells

A pre-packaged lentiviruses containing a Pyruvate dehydrogenase E1 component subunit alpha (PDHA-1) gene conjugated to turboRFP (tRFP) controlled by a CMV promoter was purchased from Origene. PDHA-1 is a mitochondrial membrane bound protein. To transduce ASM cells, 40,000 cells were plated in a 6 well plate in complete media. Cells were transduced the following day in complete DMEM without PSA and supplemented with 8µg/ml polybrene (Thermo Fisher Scientific). An MOI of 20 particles/cell was used. Viral particles were washed away after 20 hours of transduction and ASM cells were allowed to grow to 80-90% confluency in complete media. Successfully transduced cells were selected using through fluorescence-activated cell sorting (FACS).

Mitochondrial Transfer

To study mitochondrial transfer, the same co-culture protocol was employed, except mitochondrion-tagged ASM cells were used. A Transwell (Fischer Scientific) culture was implemented to control for non-MNT mediated mitochondrial transfer. Frequency of $CD4^{+}tRFP^{+}$ cells were measured by flow cytometry.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 software. Data are presented as the mean ± SEM, unless otherwise specified. Either a Student t-test or a one-way ANOVA followed by Tukey's posthoc test was used to compare either the means of two or more groups, respectively. The p values < 0.05(*), 0.01(**), 0.001(***) and 0.0001(****) were considered significant.

7.4 Results

Viability of $CD4^+$ T cells after co-culture with ASM cells

To study the effects of ASM cells on $CD4^+$ T cells, activated $CD4^+$ T cells were co-cultured with ASM cells for 24 hours before collecting for analysis. Cultures were treated with BrdU 18 hours before harvest to infer proliferation from BrdU incorporation. Apoptosis was assessed by Annexin V staining. $CD4^+$ T cells reduced their incorporation of BrdU when co-cultured with ASM cells and had fewer cells positive for Annexin V (Fig.1A and 1B). These results indicate that $CD4^+$ T cells are more viable after co-culture with ASM cells, but they proliferate less.

Characterization of $CD4^+$ T cells Immune Activity after Co-culture with ASM cells

We next assessed cytokine expression by $CD4^+$ T cells by qPCR and flow cytometry to see if their immune activity reflects their proliferation. No significant differences in transcript levels of IL-5, IFN-γ, IL-10 and TNFα was measured in $CD4^+$ T cells after co-culture with ASM cells (Fig.2A-D). By flow cytometry, $CD4^+$ T cells expressed more IL-5 when co-cultured with ASM cells (Fig.3B). An increase in IL-5 suggests that a Th2 like phenotype was induced, thus we separated T helper subsets by their defining transcription factors. No differences in T-BET, GATA3, FOXP3 or RORγT were found (Fig.4).

A lack of proliferation and an increase in cytokine expression suggests that ASM cells are inducing post-expansion effector $CD4^+$ T cells. To confirm this observation, we measured the

expression of activation markers CD69, CD44 and CD25 as well as Iκβ phosphorylation, and thus inactivation, to infer NF-κB activation. There were no differences in the proportion of cells expressing any of the activation markers between control $CD4^+$ T cells and co-cultured cells (Data not shown). The MFI of the early activation marker CD69 was significantly reduced after co-culture while no changes in the late activation marker CD25, were found (Fig.5A and 5C). These results suggest either $CD4^+$ T cells have progressed through their activation process, leading to the eventual loss of CD69 expression or immune activity has been inhibited. The MFI of the antigen-experience marker CD44 was increased suggesting that $CD4^+$ T cell immune activity may be enhanced but CD44 may also be upregulated to enhance adhesion to ASM cells (Fig.5B). ASM cells also increased phosphorylation of Iκβ, a key component in Nf-κB signaling, in $CD4^+$ T cells as measured by Western blot (Fig.5D). Analysis of activation markers, cytokine expression and NF-κB signaling by $CD4^+$ T cells suggest they may be more inflammatory.

Re-stimulation of CD4+ T cells

To test whether ASM cells modulate $CD4^+$ T cell responses to re-stimulation, $CD4^+$ T cells were separated from ASM cells or collected under control conditions and incubated with CD3/28 Dynabeads. Cytokine expression with or without CD3/28 re-stimulation was assessed by flow cytometry. Co-cultured $CD4^+$ T cells significantly expressed more IL-4, IFN-γ and TGF-β with re-stimulation than control $CD4^+$ T cells that were not co-cultured (Fig.6A, 6B and 6D). A significant increase in IL-10 expression was observed with re-stimulation in co-cultured $CD4^+$ T cells that was not replicated by control $CD4^+$ T cells (Fig.6C). ASM cells induced hyper-reactive $CD4^+$ T cells to CD3/28 re-stimulation, a property of memory T cells.

Modulation of Effector and Memory Markers in $CD4^+$ T cells by ASM cells

Expression of memory T cell markers was measured by flow cytometry after co-culture and re-stimulation. CD45RA and CD45RO were used as naïve and memory T cell markers respectively while CCR7 were used as markers of lymphoid homing central memory T cells. 24 hours of co-

culture with ASM cells had no effect on CD45RA or CD45RO expression of $CD4^+$ T cells demonstrating that no enhancement or loss of memory T cells occurred (Fig.7A). No differences between central or effector memory cells, as distinguished by $CCR7^{+/-}$, were found either (Fig.7B and 7C). When re-stimulated, control $CD4^+$ T cells exhibited a predictable drop in CCR7 in $CD45RA^+$ cells (Fig.8C). However, co-cultured $CD4^+$ T cells retained CCR7 in $CD45RA^+$ sub-populations (Fig.8C). In $CD45RO^+CD4^+$ T cells, CCR7 expression was significantly increased with re-stimulation in co-cultured cells compared to control cells (Fig.8D). This suggests that $CD4^+$ T cells that have interacted with ASM cells are destined for the draining lymph nodes.

Mitochondrial transfer from ASM cells to $CD4^+$ T cells

To measure mitochondrial transfer from ASM cells to $CD4^+$ T cells, a line of ASM cells with fluorescently tagged mitochondria was established. Prepackaged lentiviral particles were designed by and purchased from Origene. PDHA-1, a mitochondrial membrane protein, conjugated to tRFP controlled by the CMV promoter was used as the mitochondrial tag. After transduction with MOI of 20 particles/cell, approximately 20% of the ASM cells expressed tRFP and were sorted by FACS. Transduced ASM cells were co-cultured with activated $CD4^+$ T cells to measure mitochondrial transfer by flow cytometry. After 24 hours, a range of 4% to 60% of $CD4^+$ T cells stained positive for tRFP, indicating they had received mitochondria (Fig.9A). This confirms previous reports of mitochondrial transfer from ASM cells to $CD4^+$ t cells (Fig.9B).

Metabolic activity of $CD4^+$ T cells with re-stimulation

Since we observed that $CD4^+$ T cells received mitochondria from ASM cells we aimed next to assess whether co-cultured $CD4^+$ T cells had more activated cellular respiration. Using the Seahorse, the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured as proxies for glycolytic rates and cellular respiration, respectively. After baseline measurements, control and co-cultured $CD4^+$ T cells were re-stimulated and metabolic measurements were taken for 2 hours (Fig10A-D). Co-cultured $CD4^+$ T cells had a higher

baseline ECAR and OCR (Fig10E and 10H). Upon re-stimulation, both groups had an equivalent fold increase in their ECAR but co-cultured $CD4^+$ T cells had greater enhancement of OCR (Fig10F and 10I). The increase in OCR was maintained for 2 hours, while ECAR unexpectedly dropped in co-cultured $CD4^+$ T cells while control cells were more stable (Fig.10J and 10G). Co-cultured $CD4^+$ T cells have greater mitochondrial activity that may be relied upon for ATP production over glycolysis.

Activation of Caspase-1 with re-stimulation

IL-1β has been shown to enhance $CD4^+$ T cell responses to TCR stimulation, so we postulated that the inflammasome activates caspase-1 in a mitochondria dependent process, leading to the cleavage and release of IL-1β [12]. To test this hypothesis, levels of mitochondrial ROS were measured by flow cytometry using MitoSOX, a mitochondrial ROS specific dye, and the expression and activation of caspase-1 were measured by qPCR and Western blot. $CD4^+$ T cells did not produce more mitochondrial ROS after co-culture, however, they had a greater enhancement of mitochondrial ROS production upon re-stimulation (Fig.11A-C). Co-cultured $CD4^+$ T cells also expressed more caspase-1 mRNA (Fig.12A); however, this was not mirrored at the protein level (Fig.12B). Activation of caspase-1, as measured through levels of cleaved P20 caspase-1, did not differ with co-culture, however, a significant loss of pro-caspase-1 was observed (Fig.12D and 12C). Preliminary results on re-stimulation experiments suggest that CD3/28 treatment induces a greater caspase-1 activation in co-cultured $CD4^+$ T cells (Fig.12G). We then examined whether IL-1β can be detected in culture supernatants by ELISA. Supernatants collected from co-culture and re-stimulation experiments did not contain measurable levels IL-1β at a 24-hour time point (Data not shown). Further analysis of IL-1β at early time points is needed to implicate inflammasome activation in $CD4^+$ T cells hyper-reactivity.

7.5 Conclusions

This study has demonstrated ASM cells ability to enhance $CD4^+$ T cell responses to CD3/28 re-stimulation. $CD4^+$ T cell Proliferation was reduced but viability was enhanced by co-culture with ASM cells (Fig.1). Expression of IL-5 was also increased by ASM cells (Fig.3B). A down regulation and upregulation of activation markers CD69 and CD44 was difficult to interpret, but an increase in Iκβ phosphorylation suggests $CD4^+$ T cells are more inflammatory after co-culture (Fig.5). Re-stimulating $CD4^+$ T cells with anti-CD3/28 Dynabeads showed co-cultured $CD4^+$ T cells were hyper-reactive, similar to memory T cell responses (Fig.6). Neither the expression of the memory marker CD45RO, nor the lymphoid homing receptor CCR7 changed with co-culture, however, CCR7 expression was increased after re-stimulation (Fig.7 and 8D). It was also confirmed that $CD4^+$ T cells received mitochondria from ASM cells (Fig.9). Analysis of their metabolism by Seahorse revealed co-cultured $CD4^+$ T cells were both glycolytically and oxidatively more active (Fig.10E and H). Re-stimulation induced a greater enhancement of the OCR in co-cultured $CD4^+$ T cells compared to control cells (Fig.10I). The difference in OCR was maintained (Fig.10J). ECAR also increased with re-stimulation, however, co-cultured and control $CD4^+$ T cell ECARs rose to similar levels (Fig.10F). Co-cultured $CD4^+$ T cells had an uncharacteristic drop of their ECAR over time (Fig.10G). Activated T cells are known to have a strong glycolytic response [13]. Synthesis of mitochondrial ROS was also enhanced by re-stimulation and co-cultured $CD4^+$ T cells were capable of a greater enhancement (Fig.11B and C). Increased expression of caspase-1 mRNA was found thus an inflammasome dependent release of IL-1β was explored (Fig.12A). Preliminary results show an increased activation of caspase-1 upon re-stimulation however IL-1β was not detected in either control or co-cultured supernatants with or without re-stimulation for 24 hours (Fig.12G). These results show $CD4^+$ T cells are hyper-reactive after co-culture, possibly through a caspase-1 dependent mechanism.

$CD4^+$ T cells were hyper-reactive but their pro-inflammatory state right after co-culture was unclear. qPCR results showed no differences in IL-5 expression, but IL-5 protein expression was increased as measured by flow cytometry (Fig.2A and 3B). However, with the lack of significance in other cytokines, a conclusion that the $CD4^+$ cells are more pro-inflammatory is tentative but is supported by the increase in Iκβ phosphorylation (Fig.5D). The upregulation of

IL-5 may indicate a Th2 like phenotype is being induced although expression of GATA3 did not differ (Fig3B and 4). Further analysis into $CD4^+$ T cells inflammatory state after co-culture with ASM cells is required and more detailed exploration of the time course of changes in transcription factors and protein expression is warranted.

The hyper-reactivity of $CD4^+$ T cells is very reminiscent of memory T cell responses. Memory like processes seem to be pertinent for $CD4^+$ T cells in asthma as they mediate AHR in response to secondary challenges [5]. Since $CD4^+$ T cells also localize around ASM bundles in the airways, it would make sense for ASM cells to play a role in the process of memory induction within the airways [14]. However, no increases in memory markers were found, both after co-culture and with re-stimulation. It is unlikely for the memory transition to occur within the 24 hours of co-culture we studied therefore we may have analyzed $CD4^+$ T cells in the process of transitioning. An increased expression of CCR7 after re-stimulation may indicate that co-cultured $CD4^+$ T cells are more memory like (Fig.8D). CCR7 is a common marker for central memory T cells that reside in the lymph nodes, awaiting re-stimulation by their specific antigen. This notion conflicts with $CD4^+$ T cells localizing to the ASM but this may be a dynamic process whereby $CD4^+$ T cells localize to but do not reside permanently within the ASM. $CD4^+$ T cells also receive mitochondria from ASM cells, and this study showed co-cultured $CD4^+$ T cells seem to rely on their cellular respiration more than glycolysis (Fig.9 and 10). These are metabolic features of memory T cells as well [13]. Longer co-cultures may reveal that a fully differentiated memory phenotype is achieved.

Another possible mechanism mediating hyper-reactivity of $CD4^+$ T cells is through IL-1β. IL-1β is a cytokine that has been demonstrated to augment $CD4^+$ T cell responses to antigen presentation, similar to CD3/28 stimulation [15]. IL-1β is thought of as an innate cytokine as its production is dependent on the inflammasome. The inflammasome is an inflammatory protein complex containing caspase-1 that can be more commonly found in innate immune cells but lymphocyte-derived inflammasomes have been found [16]. Their function in lymphocytes is

unknown, but in innate cells they regulate a special apoptotic process known as pyroptosis and are responsible for the cleavage of pro-IL-1β and pro-IL-18 into their active forms [17]. The expression of inflammasome components is regulated by NF-κB and its activation can be induced by mitochondrial ROS, both being upregulated in co-cultured $CD4^+$ T cells and with re-stimulation respectively (Fig.5D, 11B and C) [18]. The increase in mitochondrial ROS still needs explanation, but it may be related to increased mitochondrial mass via transfer from ASM cells. The increase in caspase-1 activation provides more evidence that co-cultured $CD4^+$ T cells are producing inflammasome components and upon re-stimulation, an increase in mitochondrial ROS activates the inflammasome leading to the cleavage and release of IL-1β (Fig.12G). IL-1β can then finally autologously act on $CD4^+$ T cells to enhance their response to CD3/28 re-stimulation.

ASM cells are clearly not the only cell that is modulated in its interactions with $CD4^+$ T cells. The hyper-reactive response induced by the ASM cells, suggest the they may contribute to $CD4^+$ T cell mediated asthma exacerbations. One asthmatic process that $CD4^+$ T cells mediate is ASM phenotype changes, thus the bidirectional modulations of $CD4^+$ T cells and ASM cells may be a key process in asthma. More research in this area may reveal to have significant benefits on both understanding and treating asthma.

7.6 Figures

Figure 1: Proliferation and viability of $CD4^+$ T cells

Incorporation of BrdU was analyzed by flow cytometry as a proxy for proliferation. Annexin V and PI staining was used as markers of proliferation and necrosis, respectively. (A) $CD4^+$ T cells were less proliferative after co-culture. (B) $CD4^+$ T cells were also more viable after co-culture as fewer cells were apoptotic.

Figure 2: Cytokine mRNA expression by $CD4^+$ T cells

Cytokine expression by $CD4^+$ T cells was measured by qPCR. (A) IL-5 was assessed as a Th2 cytokine. (B,D) IFN-γ and TNF-α were assessed as Th1 cytokines. (C) IL-10 was assessed as a Treg cytokine. No differences in any of these cytokines were induced by ASM cells.

Figure 3: Cytokine Protein Expression by $CD4^+$ T cells

Protein expression of cytokines by $CD4^+$ T cells was measured by flow cytometry. (A-B) IL-4 and IL-5 were assessed as Th2 cytokines. (C) IFN-γ was assessed as a Th1 cytokine. (D-E) IL-10 and TGFβ were assessed as Treg cytokines. MFI values were reported. (B) Only IL-5 was significantly upregulated with co-culture.

Figure 4: ASM cells do not Induce Th2 $CD4^+$ T cells

Expression of T-Bet, GATA3, FOXP3 and RORγT was used as markers of Th1, Th2, Treg and Th17 subsets. Expression of these transcription factors was assessed by flow cytometry. No consistent changes in T helper subsets were found after co-culture.

Figure 5: Activation of $CD4^+$ T cells

Expression of activation T cell activation markers and activation of NFκB, as measured by phosphorylation of Iκβ, was analyzed to assess $CD4^+$ T cell activation state. CD69, CD44 and CD25 were measured by flow cytometry and phosphorylation of Iκβ was measured by western blot. (A) Expression of CD69 was significantly reduced after co-culture. (B) ASM cells also increased expression of CD44 by $CD4^+$ T cells. (C) No difference in CD25 was found. (D) Increased phosphorylation of Iκβ, and thus increased activation of NFκB, was induced by ASM cells.

Figure 6: Re-stimulation of $CD4^+$ T cells using CD3/28 Dynabeads

$CD4^+$ T cells were separated from ASM cells by washing and re-stimulated with CD3/28 Dynabeads for 24 hours. Cytokine expression was measured by flow cytometry. (A-D) Re-stimulation increased IL-4, IFNγ, IL-10 and TGFβ expression in co-cultured $CD4^+$ T cells. (A-B, D) Enhanced expression of IL-4, IFN-γ and TGFβ by re-stimulation was greater in co-cultured $CD4^+$ T cells.

Figure 7: Expression of memory markers post co-culture

Expression of CD45RO and CCR7 was measured by flow cytometry as memory and lymphoid homing markers, respectively. CCR7 distinguishes $CD45RO^+$ memory cells as either central or effector memory cells. (A-C) No differences in any memory subset were found.

Figure 8: Expression of memory markers after re-stimulation

Expression of CD45RO, CD45RA and CCR7 was measured by flow cytometry as memory, naïve and lymphoid homing makers. CCR7 distinguishes central memory cells in $CD45RO^+$ cells while $CD45RA^+$ cells lose CCR7 upon activation [19].(A-C) No significant differences in naïve or $CD45RO^+$ populations. (D) CCR7 was significantly higher in re-stimulated cells in the co-culture conditions compared to control conditions.

Figure 9: Mitochondrial transfer from ASM cells to $CD4^+$ T cells

ASM cells transduced with a lentivirus to express a tRFP tagged mitochondrial protein, PDHA-1, was used to measure mitochondrial transfer by ASM cells to $CD4^+$ T cells. After co-culture, cells were collected and analyzed by flow cytometry for tRFP positivity. (A) A range of 3-40% of $CD4^+$ T cells received tRFP tagged mitochondria. (B) Representative flow plots demonstrate mitochondrial transfer did not occur in Transwell cultures.

Figure 10: Cellular metabolism of $CD4^+$ T cells

Measurements of ECAR and OCR were made to assess the metabolic activity using the Seahorse. CD3/28 Dynabeads were injected after baseline measurements. (A,C) Raw time course of ECAR and OCR is shown. (B,D) ECAR and OCR time course normalized to the baseline. (E,H) Baseline measurements showed co-cultured $CD4^+$ T cells were more glycolytically and mitochondrially more active. (F) Control and co-cultured $CD4^+$ T cells had equivalent relative spike in ECAR with re-stimulation. (I) Co-cultured $CD4^+$ T cells had a greater relative spike in OCR after re-stimulation. (G) ECAR in co-cultured $CD4^+$ T cells declined faster than control cells. (J) Enhanced OCR by co-cultured $CD4^+$ T cells was maintained for the duration of the assay.

Figure 11: Synthesis of Mitochondrial Reactive Oxygen Species

Synthesis of mitochondria ROS was measured using a mitochondrial ROS specific dye, MitoSOX, and analyzing by flow cytometry. (A) Co-cultured $CD4^+$ T cells did not produce more mitochondrial ROS. (B-C) Co-cultured $CD4^+$ T cells were induced to produce more mitochondrial ROS with re-stimulation than control cells.

Figure 12: Activation of Caspase-1

Expression of total Caspase-1 was assessed by qPCR and Western blot. Caspase-1 activation was measured as the quantity of its activated isoform, P20 and the reduction of its pro-isoform. Levels of each isoform was measured by western blot. (A) ASM cells induced caspase-1 transcription in $CD4^+$ T cells. (B) No difference in total caspase-1 protein was detected. (C-D) Significant reduction in pro-caspase-1 was detected, however, no difference in P20 was found. (E-F) Preliminary results may indicate that an increase in P20 isoform and a reduction in the pro-isoform of capsase-1 is present in co-cultured $CD4^+$ T cells with re-stimulation.

Figure 1

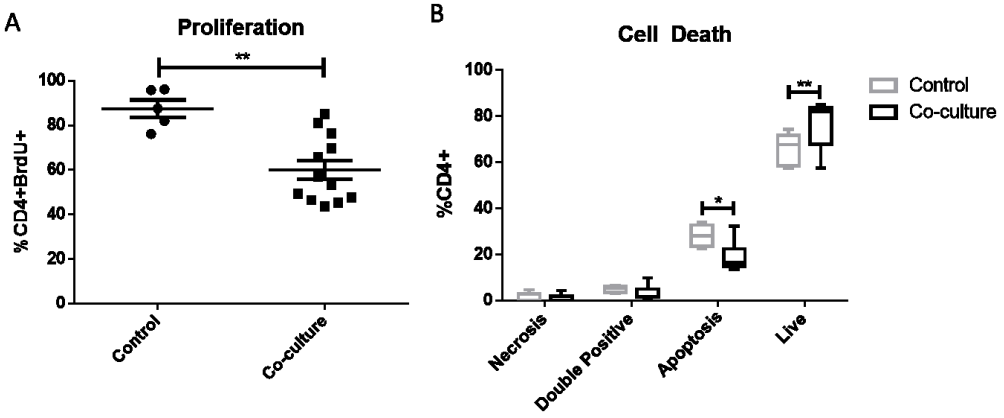


Figure 2

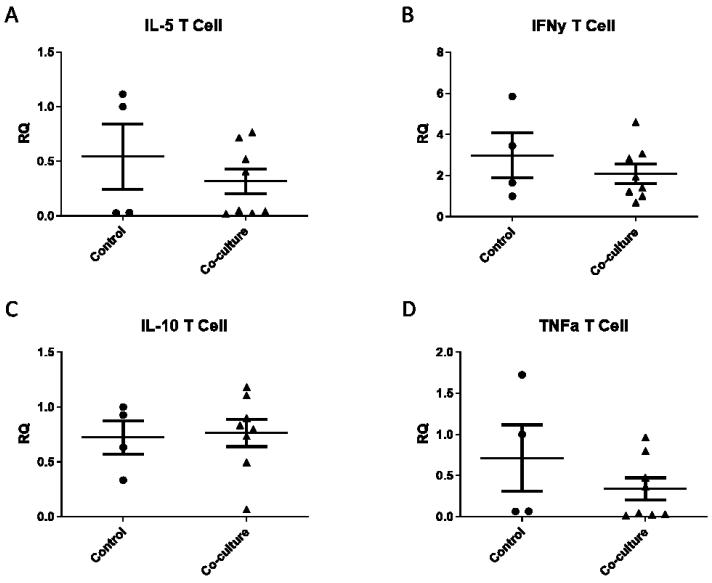


Figure 3

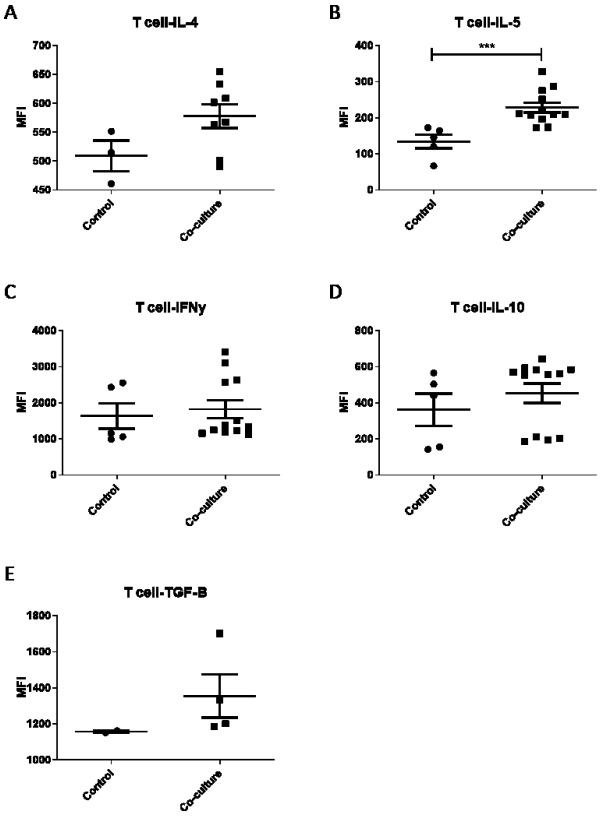


Figure 4

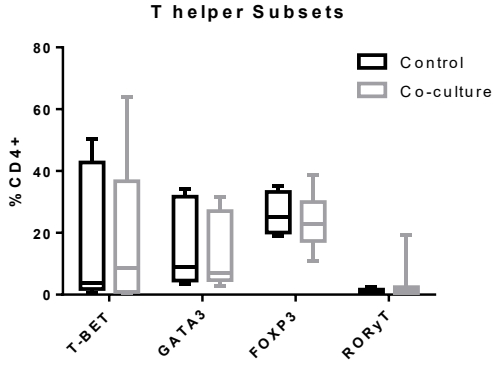
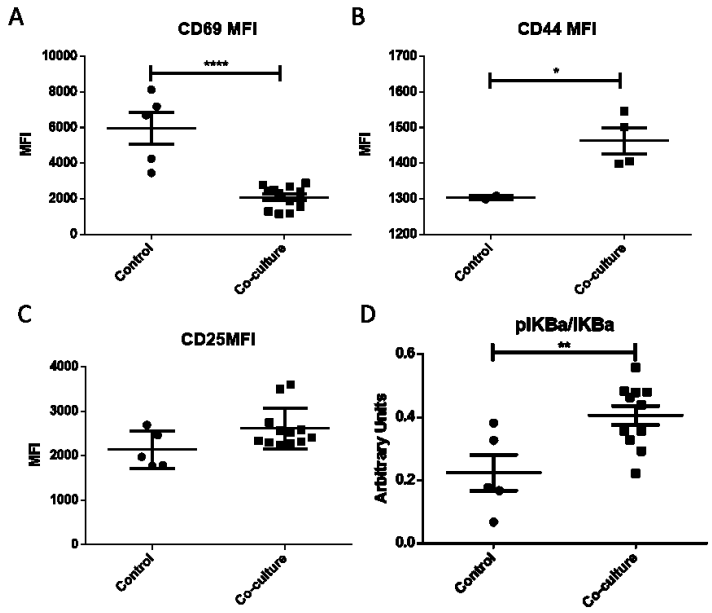


Figure 5



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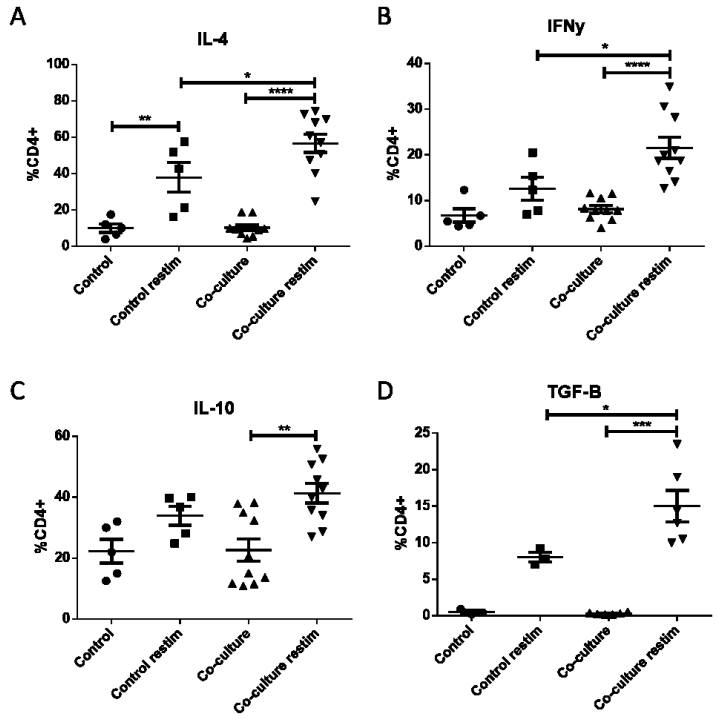

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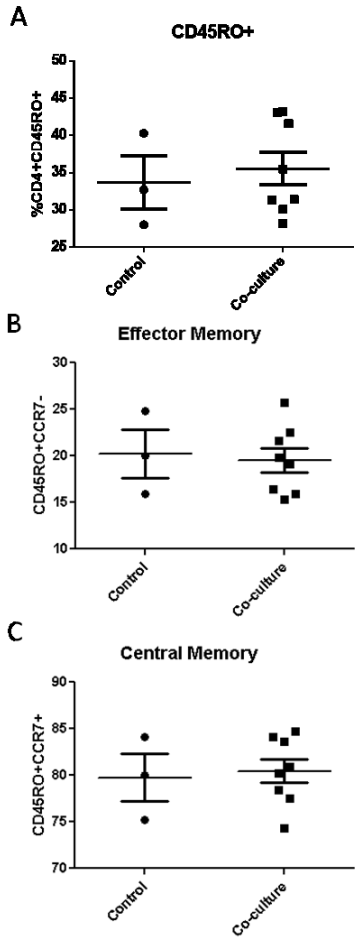

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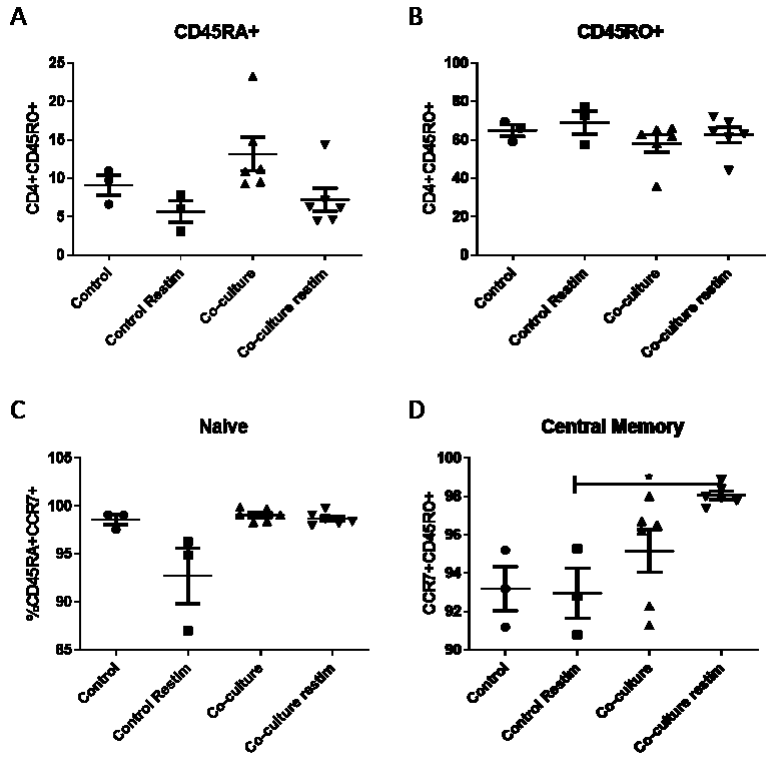

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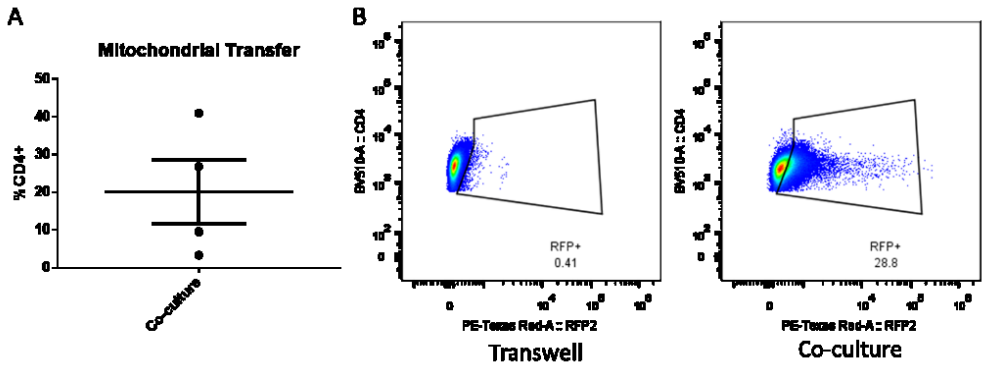
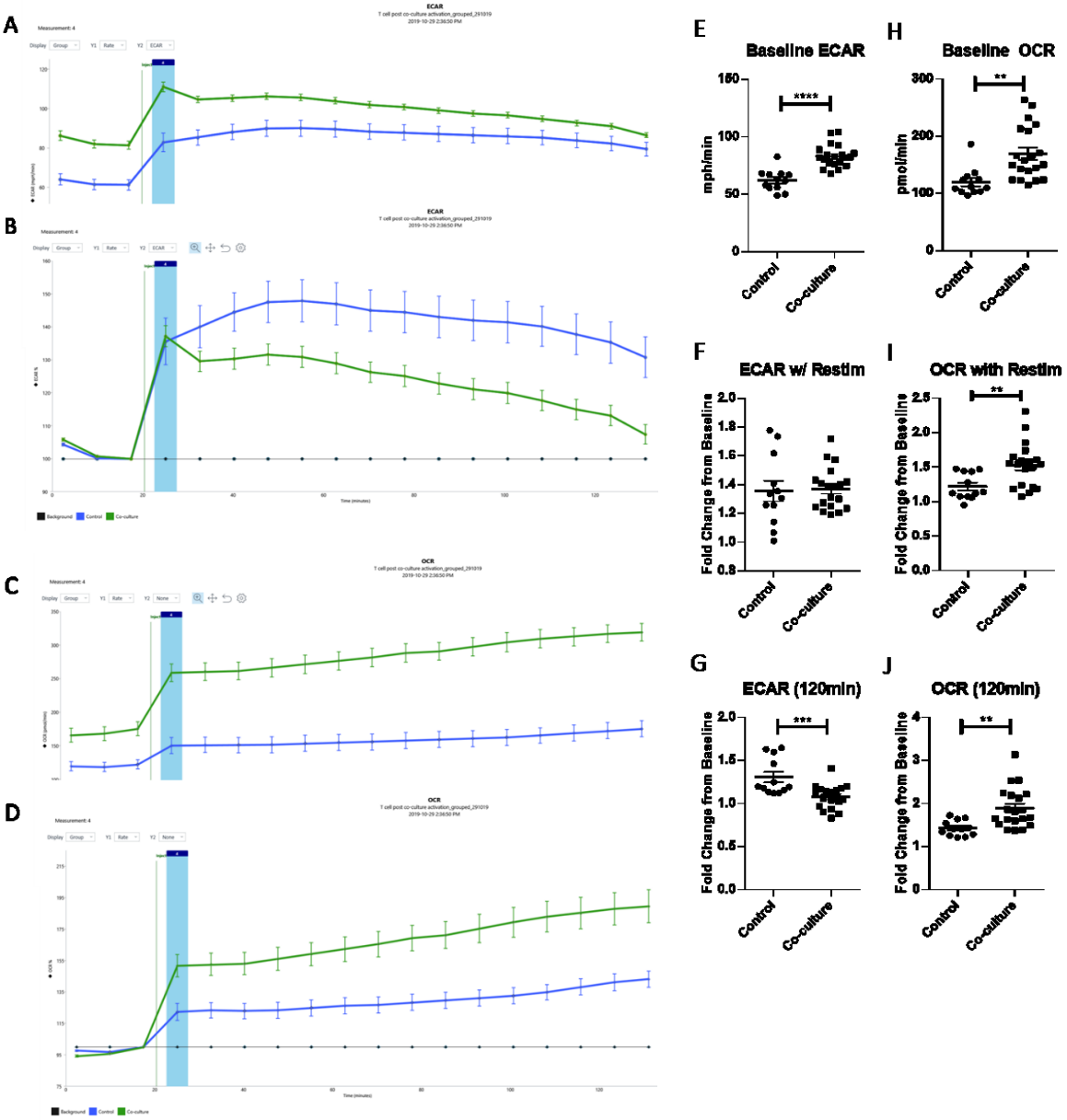


Figure 10



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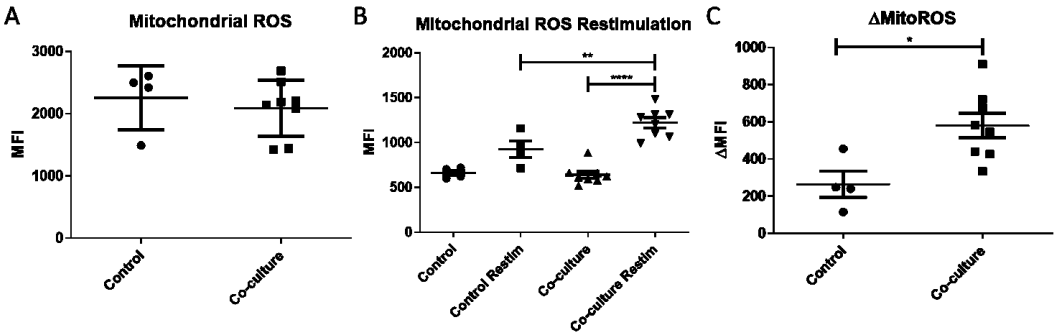
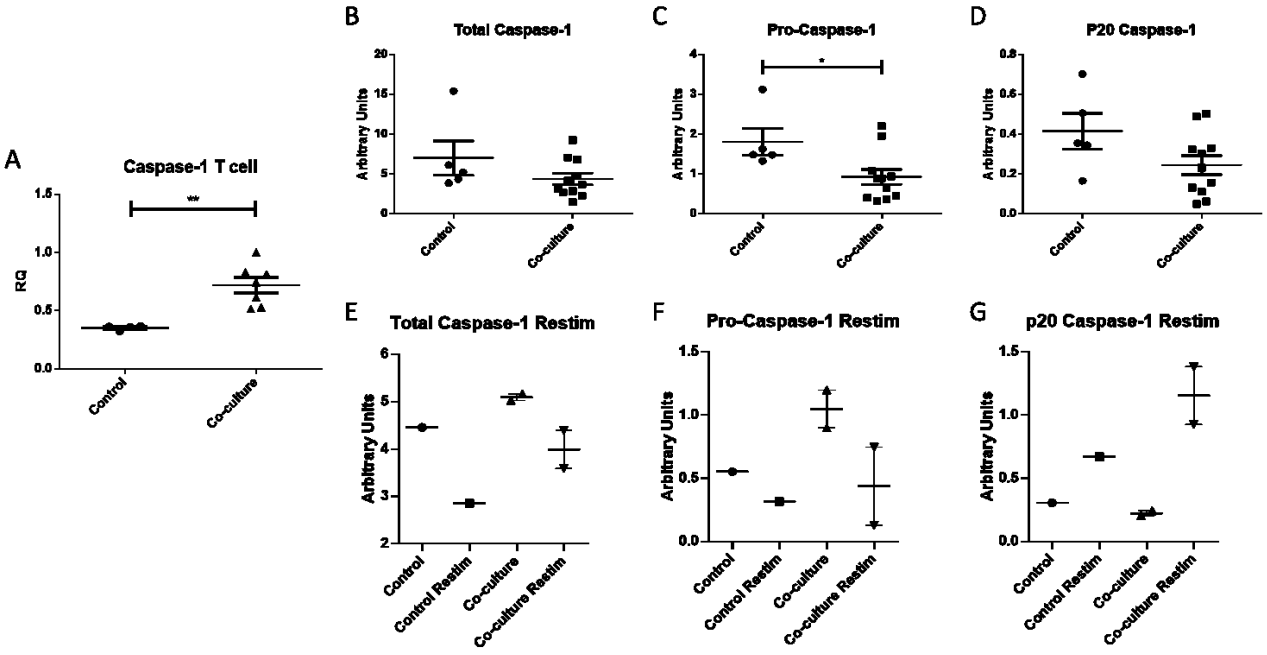


Figure 12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After observing mitochondrial transfer from ASM cells to $CD4^+$ T cells, we aimed to study the formation of MNTs, the structures responsible for the transfer. Understanding the conditions required for MNT formation may give more clues to their function. The infrequency and short kinetics of $CD4^+$ T cell MNT formation make them difficult and time consuming to study. We sought to devise an assay where MNT formation can be predictably located, imaged and analyzed, in order to enhance the efficiency of studying MNTs.

8. Novel Assay to Study Membrane Nanotubes Using Optical Tweezers

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[3]Performed optical tweezer experiments

8.1 Abstract

MNT's are tubular cellular protrusions that can be used to transfer various molecules and cellular structures from one cell to another. These delivered packages can enhance tumor cell resistance to cytotoxic agents, enhance macrophage phagocytic capabilities and even spread HIV in CD4+ T cells. The study of MNTs is limited to simple co-culture techniques limiting the ability to probe mechanism, making MNT research inefficient and time consuming. This study aims to create a novel assay to studying nanotubes, particularly from non-adherent cells. CD4+ T cell derived MNTs towards ASM cells was used as the model system. A microfluidic device with 1µm high channels of various lengths was designed and manufactured with PDMS. Tumor associate fibroblasts were able to make MNTs through the micro channels in this device, while CD4+ T cell were not. By holding CD4+ T cells close to but separated from ASM cells, MNT formation by the CD4+ T cell was visualized. Initial contact between the two cell types was not necessary to induce MNT formation. This study demonstrated that the use of optical tweezers may be a viable tool to study MNT formation.

8.2 Introduction

MNTs are cellular structures that were previously thought to be exclusive to single celled organisms, but human cell-derived MNTs have been recently discovered. Various cell types have been shown to produce MNTs in culture such as tumor cells, macrophages and $CD4^+$ T cells [1-3]. MNTs are tube-like cellular protrusions that connect two or more neighboring cells allowing them to transfer molecules such as nucleic acids and proteins, as well as larger structures such as organelles and membrane components [1, 2, 4, 5]. This method of cellular communication has various significant consequences. Transfer of mitochondria to cardiomyocyte has been demonstrated to be a survival mechanism against cellular damage from hypoxia [6]. Viruses such as HIV can be transferred between two $CD4^+$ T cells introducing a novel method of viral infection [7]. Macrophages receiving mitochondria from mesenchymal stem cells increased their phagocytic capabilities and was crucial for antimicrobial effects in a

model of acute respiratory distress syndrome [8]. ASM cells pass anti-apoptotic proteins to $CD4^+$ T cell, enhancing their survival [2].

Studying nanotubes has been limited to simple co-culture methods where researchers must scan cultures in the hope of finding MNTs. In the case of MNTs connecting $CD4^+$ T cells and ASM cells, less than 20% of the total $CD4^+$ T cells can be observed to make MNTs after 24h of co-culture [5]. The transient nature of these structures makes it difficult and time consuming to catch MNTs during their formation (unpublished data). These properties of $CD4^+$ T cell MNTs make it difficult and time consuming to study MNT formation. Not many reported steps have been taken to increase the efficiency of MNT research, but techniques can be taken from studying neuronal axon outgrowth. Microfluidic devices have been used in the past to connect two cell chambers by micro channels. This system forces axons to grow through the micro channels to form a synapse with cells on the other side [9]. This system may work for larger cells such as tumor cells, however, for smaller cells such as leukocytes, dimensions of the micro channels may be too large. For example, $CD4^+$ T cell bodies are approximately 10µm, but they are known to migrate through a membrane with 3µm pores [10]. Manufacturing devices at these scales are difficult but can be done now with current 3D printing techniques.

Previous experiments on our laboratory have used optical tweezers to discover MNTs. Optical tweezers are a tool to make micro-manipulations of micro-structures such as microbeads or cells [11]. A laser is used to create a 'trap' in a liquid medium which can then be used to translate objects within the trap zone. In this case, a $CD4^+$ T cell bound to an ASM cell was trapped and pulled away from the ASM cell. When released, the $CD4^+$ T cell sprang back towards the ASM cell, leading to the discovery of a tubular structure connecting these two cell types (unpublished data). These were later confirmed to be MNTs by electron microscopy and by observing transfer of proteins and mitochondria from ASM cell to $CD4^+$ T cells [5]. Isolating and manipulating $CD4^+$ T cells using the optical tweezers may be a viable method of studying MNTs from non-adherent cells, but it has yet to be explored.

This study aimed to optimize a technique to more efficiently study MNTs in culture using $CD4^+$ T cells and ASM cells as the model system. A microfluidic device was designed to contain micro channels 1µm high and 3µm wide with lengths ranging from 10µm to 200µm. These devices were commercially manufactured and adhered to a polyester membrane with 0.4µm pores to aid in cell migration. Fluorescently tagged fetal lung fibroblasts, IMR-90, and breast cancer adenocarcinoma cell line, MDA-231, was used for preliminary testing. Initial tests demonstrated that IMR-90 cells can make MNTs through the microchannels toward MDA-231 cells but $CD4^+$ T cells failed to with ASM cells. Using optical tweezers, $CD4^+$ T cells were trapped and brought close to an ASM cells, but no physical contact was made. Under these conditions, $CD4^+$ T cells were visualized to produce MNTs, demonstrating that $CD4^+$ T cells do not require direct contact to initiate MNT formation. The use of optical tweezers may be a viable method for studying MNT formation.

8.3 Methods

Cell culture

For ASM cell and $CD4^+$ T cell culture, see '*ASM cell Culture*' and '*$CD4^+$ T cell Isolation and Activation*' in sections 6.3 and 7.3 above. Jurkat cells were obtained from ATCC and cultured in complete RPMI. Jurkat cells were activated with a 1:1 ratio of CD3/28 Dynabeads to cells for 72 hours before co-culture. Activated Jurkat cells were cultured on Transwells™ with 1µm and 3µm pores to determine the physical limits of T cell migration. Whether T cells can be found on the basolateral side of the Transwell was observed. MDA-231 and IMR-90, fluorescently tagged breast cancer adenocarcinoma cells and fetal lung fibroblasts were cultured in complete DMEM. MDA-231 cells' nucleus were tagged using a histone GFP tag and the IMR-90 cells' cytoplasm was tagged with mCherry.

Microfluidic Design and Manufacturing

A microfluidic device with 3µm wide and 1µm high channels with lengths of 10, 20, 50, 100 and 200 µm were designed using Inventor Pro (Autodesk). PDMS based devices were manufactured

by µFluidix. A 2mm inlet ports were punched out and PDMS surfaces were activated using plasma chamber to enhance bonding to culture surfaces. Devices were then bound to either glass slide or a polyester membrane with 0.4µm pores (Sterlitech). Devices bound to porous membranes were cultured on Transwells. Jurkat cells and ASM cells were stained with calcein green and red respectively before co-culture. 5,000 ASM cells and 15,000 activated Jurkat cells in 10µL RPMI:DMEM each, were loaded into the microfluidic devices. MNT formation was visualized using fluorescent microscopy. For MDA-231- mCherry and IMR-90 cell experiments, approximately 5,000 cells of each type in 10ul were loaded into separate cell chambers and allowed to adhere overnight. Cells well were visualized by fluorescence microscopy (Olympus).

Optical Tweezers

Our single beam laser trap assay was built around the Laser Tweezers Workstation (Molecular Machines & Industries) with an inverted microscope (TI, Nikon). A diode pumped Nd:YAG solid-state laser (TEM00, 8 W, 1070 nm) was used to create the trap. A temporary PDMS well was adhered to a sterile glass cover slip. 10,000 ASM cells were plated on the coverslip in complete DMEM the day before the assay. The cover slip was prepped for forming a glass chamber by aspirating the medium and removing the PDMS well. The glass coverslip was adhered onto a glass slide using double sided tape, creating a chamber that holds liquids through capillary action. Approximately 20,000 – 30,000 activated $CD4^+$ T cells, stained with calcein-red/orange, in 40-70µL of 50:50 complete media. To perform the assay, $CD4^+$ T cells visualized in bright field by a charge coupled device (CCD) camera (MXF285c, Molecular Machines & Industries) were captured in the laser trap. $CD4^+$ T cells were brought close to an ASM cell and visualized by fluorescence (KP-E500, Hitachi) and bright field imaging while the $CD4^+$ T cells was trapped or freed.

8.4 Results

Design of Microfluidic Device for Studying MNTs

To more efficiently study MNT fromation, strategies for studying other cellular protrusions, such as neuronal axons were implemented. A microfluidic device with two cell chambers separated by micro channels would allow ASM cell and $CD4^+$ T cell bodies to be distant while allowing for paracrine communication and nanotube formation (Figure 1A). Dimensions of the micro channels were tuned to allow for MNT formation while preventing small cells such as a T cells from entering. A Transwells with 1µm pores was determined to prevent T cell migration while 3µm pores did not (Data not shown). Micro channels were thus designed to be 1µm high and 3µm wide channels with variable lengths: 10µm, 20µm, 50µm, 100µm and 200µm (Fig.1A). The device was manufactured by µFluidix, by curing PDMS onto a silicon mold that was 3D printed using proprietary methods. Inlet ports were punched out of the PDMS device using a 3mm hole punch. PDMS devices were determined to form a liquid tight seal against a glass slide without any treatment (Fig.1B).

Optimization of Cell Loading into the Microfluidic Device

For initial testing, fluorescently tagged breast cancer cells, MDA-231, and fetal lung fibroblasts, IMR-90, were used. IMR-90 cells were visualized to form MNTs toward MDA-231 cells previously by other lab members. 5,000 cells were loaded in separate cell chambers in 10µL of complete DMEM. Loading cells by pipette or by syringe pump did not allow for enough cell migration towards the center micro channels and cells aggregated in the inlet ports (Fig.2A). We hypothesized that the small enclosed area of the cell chambers did not allow for adequate nutrient supply and/or oxygen diffusion. To address these problems, the device was adhered to a polyester membrane with 0.4µm pores and again, no treatment was required. The device-membrane system was cultured in a Transwell to allow for fresh medium diffusion through the bottom of the chamber (Fig.1C). This increased cell migration toward the center micro channels (Fig.2B). We now had a working system to test whether cells can form MNTs through the micro channels.

Formation of MNT by Tumor Associated Fibroblasts but not $CD4^+$ T cells through Micro Channels

Initial testing of the microfluidic device with IMR-90 and MDA-231 cells showed IMR-90 were able to make cellular protrusions through the micro channels toward MDA-231 cells (Fig.3A). These tubular structures were not confirmed to be MNTs; however, a proof of concept was established that cells can form structures through these micro channels. When the devices were tested using ASM cells and activated Jurkat cells, cells were stained with calcein-green and calcein-red/orange for easy visualization. Jurkat cells were not seen making MNTs through the micro channels toward ASM cells (Fig.3B). This assay may be a viable method for studying nanotubes by other cell types, but it was not adequate for T cells.

Controlling $CD4^+$ T cell Movement and Visualizing MNT Formation using Optical Tweezers

Optical tweezers, a method of trapping and manipulating small structures, such as micro-beads or cells, using a laser, was implemented as a possible tool for studying MNT formation. We hypothesized that by holding $CD4^+$ T cells at variable distances away from an ASM cell, we can reproduce the conditions of the microfluidic device without the microchannels. Activated $CD4^+$ T cells were flowed into a glass chamber containing ASM cells. Using the optical tweezers, a $CD4^+$ T cell was trapped and moved close to an ASM cell and was visualized with the $CD4^+$ T cell both trapped by the laser and freed. Under both trapped and freed conditions, $CD4^+$ T cells formed MNTs that can connect them to ASM cells (Fig.4). Initial contact between the two cell types was not required (Fig.4). Optical tweezers have been demonstrated to be a feasible tool for studying MNT formation by non-adherent cells, like T cells.

8.5 Conclusions

To devise an optimal technique for studying MNTs, a microfluidic device was designed to have microchannels that are limited to a height of 1µm was designed (Fig.1A). These microchannels ranged from 10µm to 200µm in length. These microfluidic devices were commercially manufactured out of PDMS and required assisted nutrient and/or oxygen delivery (Fig.2A). To achieve this support, the devices were adhered to a microporous membrane and cultured on a

Transwell allowing for nutrient and oxygenated medium diffusion through the bottom of the culture system (Fig.1B and C). This is system allowed for sufficient cell migration to the microchannels to study MNT formation (Fig.2B). Initial testing using IMR-90 and MDA-231 cells demonstrated that IMR-90 cells can form what appeared to be MNTs through the microchannels toward MDA-231 cells (Fig.3A). However, $CD4^+$ T cells did not produce MNTs toward ASM cells in these devices (Fig.3B). Using optical tweezers, $CD4^+$ T cells were trapped and placed near ASM cells without allowing them to touch (Fig.4). Using this technique, it was shown that $CD4^+$ T cells could form MNTs towards ASM cells without the need of initial contact (Fig.4). This study has demonstrated the feasibility of using optical tweezers to study MNTs formed by non-adherent cells.

Simple culture and scan methods to study MNTs are inefficient for the study of these transient structures, particularly for $CD4^+$ T cells as less than half of $CD4^+$ T cells can be found making MNTs toward ASM cells at one time [5]. Placing T cells close to but not in contact with ASM cells using optical tweezers allowed us to predictably locate MNTs as we could control the exact position of trapped $CD4^+$ T cells. Being able to reliably visualize the MNTs as they form will also greatly ease studies on mechanisms of their formation. Drug treatments can be easily done in the glass chamber by adding the desired drugs to the T cell suspension before flowing into chamber. The negative aspect of working with optical tweezers is that it is a low throughput technique. Multiple cells can be trapped and manipulated at one time, but it is limited by the number of lasers within the setup. Therefore, this assay may be more suitable for mechanistic studies on MNT formation, and not for functional studies of the effects of MNT.

It is unclear why $CD4^+$ T cells were not able to form MNTs through the microchannels, as IMR-90 cells were (Fig.3). It is difficult to compare $CD4^+$ T cells and IMR-90 cells as one is a non-adherent leukocyte and the other is a cancer associated adherent fibroblast. The nature of MNT induction and formation may differ widely between these two cell types. One possibility is that the 10µm minimum distance given between $CD4^+$ T cells and ASM cells may have been too far

for proper MNT signaling to occur. This hypothesis can be tested using the optical tweezer technique developed by holding $CD4^+$ T cells various distances from an ASM cell.

Both microfluidic devices and optical tweezers were demonstrated to be viable methods for studying MNTs, however each has their limitations. The use of optical tweezers will be exclusively limited to non-adherent cell types. The use of these techniques will hopefully further advance the understanding of this mechanism of mammalian cell communication.

8.6 Figures

Figure 1: Design of Microfluidic Devices

(A) A microfluidic device with two cell chambers separated by microchannels was designed and sent to µFluidix for manufacturing. Channels were designed to be 3µm wide and 1µm high with lengths of 10, 20, 50, 100 and 200µm. (B-C) A schematic of the two culture methods implemented.

Figure 2: Cell migration with a Glass Slide or Transwell

Cell loading and migration was viewed using fluorescence microscopy. Red cells are IMR-90 cells that were fluorescently tagged with mCherry. Green cells are MDA-231 cells with a nuclear GFP tag. (A) Adhering microfluidic devices to glass resulted in only a few cells reaching the microchannels. (B) Using a microporous membrane and culturing on a Transwell, more cells reached the microchannels after loading.

Figure 3: Formation of IMR-90 derived MNTs through Microchannels

The formation of MNTs through the microchannels was imaged by live fluorescence microscopy. (A) IMR-90 cells produced tube like structures toward MDA-231 cells through the

microchannels. (B) $CD4^+$ T cells and ASM cells were stained with calcein-green and calcein-red/orange respectively for easier visualization. $CD4^+$ T cells failed to form MNTs toward ASM cells in the microfluidic device.

Figure 4: CD4+ T cells trapped by Optical Tweezers form MNTs

CD4+ T cells and ASM cells were co-cultured in a custom glass chamber designed for use with Optical Tweezers and visualized using phase contrast microscopy. CD4+ T cells were trapped by the optical tweezers and brought close to an ASM cell without making physical contact. A time course of 6 second intervals is shown. CD4+ T cells were kept in the trap until approximately the 18s time point. Black arrow indicates the first MNT formed. The Red arrow indicates the second MNT formed. No initial contact was required, and MNTs only lasted 30-36 seconds.

Figure 1

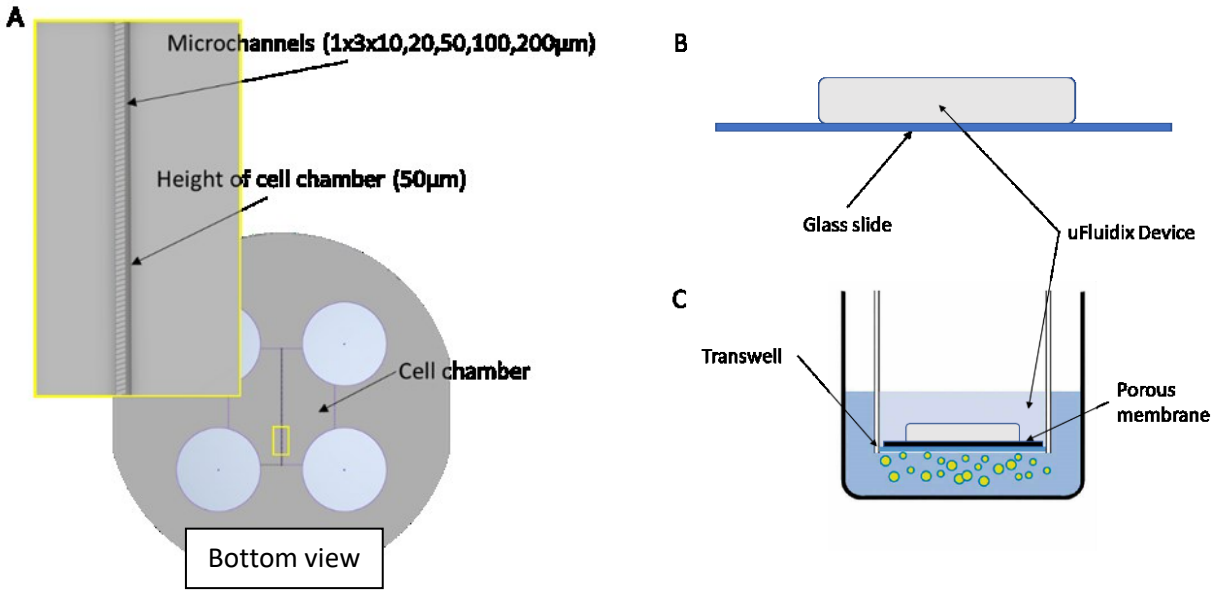
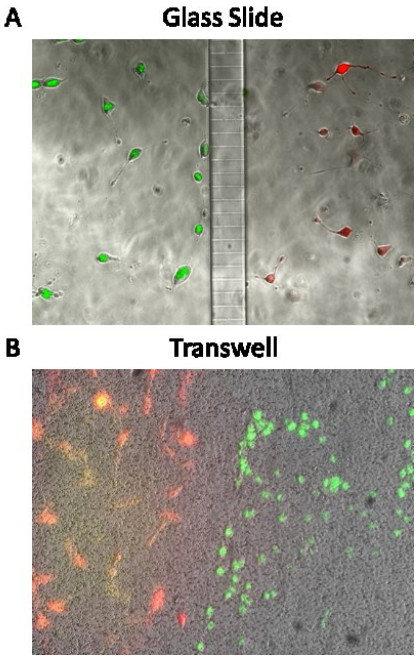


Figure 2



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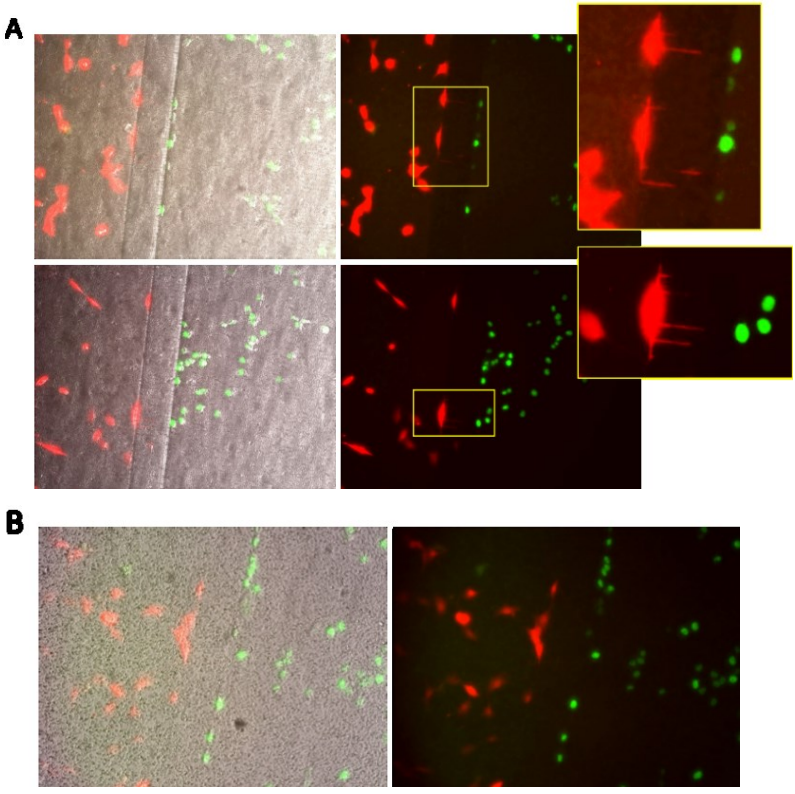
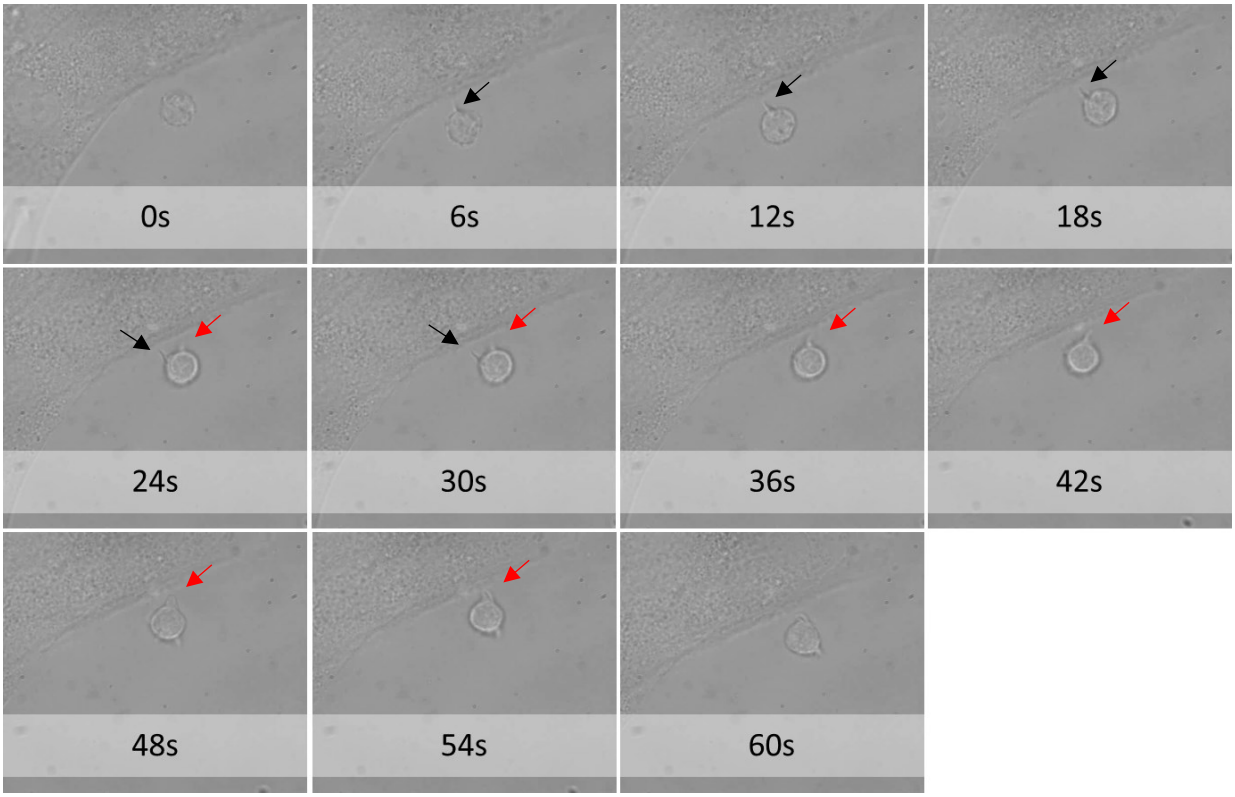


Figure 4



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9. Discussion

Throughout this thesis, evidence for various mechanisms by which ASM cells and $CD4^+$ T cells interact was presented. These mechanisms resulted in phenotypes that can have consequential impacts on asthma. ASM cells co-cultured with $CD4^+$ T cells were more proliferative. Following the dual phenotype theory of ASM cells, $CD4^+$ T cells also reduced ASM cell contractility. This was determined by the findings of a reduction in peak calcium responses to histamine and a down regulation of ASM contractile factors. Physical contact between the cells was key to mediating these changes and ICAM-1 is possibly mediating these changes. With $CD4^+$ T cells localizing around or within ASM bundles in asthmatics, this pro-proliferative interaction may occur in vivo leading to ASM thickening. Thickening of the ASM is a key component of airway remodeling and thought to be responsible for AHR. Allergen stimulated $CD4^+$ T cells have already been shown to be necessary to cause ASM mass increases and ICAM-1 is required for the induction of AHR with allergen sensitizations in mice. The interaction between $CD4^+$ T cells and ASM cells via ICAM-1 may be a link to these findings.

Changes in $CD4^+$ T cells can have significant consequences on the progression of the disease. ASM cells induced hyper-reactive responses by $CD4^+$ T cells to CD3/28 re-stimulation as measured by increased cytokine expression. This stimulation is meant to mimic the activation of $CD4^+$ T cells through TCR-MHC II interactions. This suggests that the interaction between $CD4^+$ T cells and ASM cells can have significant impact on asthmatics with repeated exposure to allergens such as ragweed. As $CD4^+$ T cells are significant contributors of inflammatory mediators in the airways, thus an enhancement of $CD4^+$ T cell responses can exacerbate inflammation in an already inflammatory disease. This mechanism has already been demonstrated in murine models of asthma. Initial challenge of sensitized mice with OVA was able to induce AHR regardless of the presence of $CD4^+$ T cells, however, secondary challenge induced AHR was dependent on $CD4^+$ T cells [309]. Even though these mice were rested after the initial sensitization and challenge to restore normal lung functions, a single secondary challenge was able to induce AHR [309]. Hyper-reactive $CD4^+$ T cells formed from the initial challenge may be mediating the maintenance of AHR in response to allergen.

The mechanism by which this is occurring is unclear, but this study has outlined two possibilities. The first mechanism proposed is the induction of memory $CD4^+$ T cells by ASM cells. A hyper-reactive $CD4^+$ T cell to re-stimulation is one of the key functions of memory T cells to confer rapid protection to repeat infections. No upregulation of memory $CD4^+$ T cell markers was found but CCR7 expression was increased after re-stimulation. This lymphoid homing chemoreceptor may indicate that $CD4^+$ T cells are destined for the lymph nodes after co-culture with $CD4^+$ T cells where central memory $CD4^+$ T cells reside. Although they would not be within the airways ready for an allergen challenge, the store of memory $CD4^+$ T cells in the lymph nodes would not require T cell selection and expansion mechanisms leading to immediate migration to the lungs. Further experimentation is required to confirm these findings. For example, only 24 hours of co-culture may be insufficient to induce a complete transition into the memory phenotype by $CD4^+$ T cells. The second proposed mechanism was through an activation of the inflammasome that is enhanced by mitochondrial transfer from ASM cells. $CD4^+$ T cells form MNTs toward ASM cells, through which mitochondria is transferred to $CD4^+$ T cells. Upon re-stimulation an increase in synthesis of mitochondrial ROS was detected, which is a known inflammasome activator. IL-1β, a product of inflammasome activation, has been demonstrated to enhance $CD4^+$ T cells response to TCR stimulation, similar to effects seen in this experiment [335]. IL-1β, however, was not detected in culture supernatants, but it is possible the correct time point was not analyzed.

These results demonstrate a cyclic mechanism by which $CD4^+$ T cell and ASM cell interaction can exacerbate asthma pathology. Repeated insults to the lungs by some allergen will eventually lead to allergen induced asthma. Through this process $CD4^+$ T cells will infiltrate the lungs, as suggested by increased $CD4^+$ T cell in asthmatic lungs [300, 303]. $CD4^+$ T cells can now induce ASM proliferation leading to increased ASM mass. $CD4^+$ T cells were become hyper-reactive due to their interaction with ASM cells. Upon reintroduction and re-stimulation to the allergen, $CD4^+$ T cells will be hyper-active, leading to more interactions with and proliferation of

ASM cells. Cytokines produced by these hyper-active $CD4^+$ T cells, such as IL-4, can also exacerbate ASM cell proliferation and/or contractility. This cycle can continue upon re-exposure to the allergen leading progressively more severe asthma conditions.

$CD4^+$ T cells and ASM cells interacting through MNTs may play important roles in the process outline above but the lack of efficient assays to study MNTs may have retarded research in this area. Using optical tweezers, MNT formation by non-adherent cells, such as $CD4^+$ T cells, can be studied in a controlled manner. Optimization of the assay is still ongoing; however, this assay may significantly progress the study MNTs and the formation in particular.

This thesis sought to demonstrate the importance of $CD4^+$ T cell and ASM interactions and the mechanisms by which these interactions happen. Processes that can play significant roles in airway remodeling and progression of asthma have been outlined but it is still very preliminary findings in the context of this field. The bi-directional modulation of $CD4^+$ T cells and ASM cells has been severely understudied as, based on a literature search in PubMed only 8 articles have been published on this topic. Hopefully, this study will convince asthma researchers of the importance and need for continued work in this field. It not only has the potential to progress our understanding of asthma but may also lead to novel therapies.

10. References

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