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ROLE OF ALTERNATIVE MACROPHAGE ACTIVATION IN MEDIATING FIBROSIS IN *PSEUDOMONAS AERUGINOSA* PNEUMONIA

DISSERTATION

A dissertation submitted in partial fulfillment of the
Requirements for the degree of Doctor of Philosophy in the
College of Pharmacy at the University of Kentucky

By
Susan Elizabeth Birket

Lexington, Kentucky

Co-Directors: Dr. David Feola Professor of Pharmacy Practice and Science
and Dr. Patrick McNamara Professor of Pharmaceutical Sciences

Lexington, Kentucky

2012

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ABSTRACT OF DISSERTATION

ROLE OF ALTERNATIVE MACROPHAGE ACTIVATION IN MEDIATING FIBROSIS IN *PSEUDOMONAS AERUGINOSA* PNEUMONIA

Patients with cystic fibrosis who are infected with the pathogen *Pseudomonas aeruginosa* have shown favorable responses to the drug azithromycin (AZM). This drug works in an anti-inflammatory capacity, improving clinical outcomes and improving quality of life in this population. The drug has also been shown to affect macrophage polarization by shifting these cells away from an inflammatory phenotype toward an alternatively activated anti-inflammatory phenotype. The full impact of this phenotypic change is not well understood in the context of the response to *P. aeruginosa* infection, or the overall immune response in cystic fibrosis.

To understand how the AZM-polarized macrophage affects other types of cells, we utilized a co-culture *in vitro* system, with macrophages and fibroblasts incubating together. In this system, we determined that AZM causes upregulation of the pro-fibrotic mediator transforming growth factor-β as well as the extracellular matrix (ECM) protein fibronectin. The mediator of ECM turnover, matrix metalloproteinase (MMP)-9 was upregulated in this system as well. In an *in vivo* model of *P. aeruginosa* infection, MMP-9 and fibronectin were increased in the bronchoalveolar lavage 7 days post-infection in mice that were treated with AZM. This was accompanied by a decrease in damage to the lung tissue, determine by histological examination. To determine if these changes would continue in human subjects with cystic fibrosis, a clinical study was done in this population. Subjects with AZM treatment had decreased TGF-β levels, but no differences in MMP-9 or fibronectin. Interestingly, correlations between certain fibrotic mediators and inflammatory cytokines, specifically interleukin -1β, were different in subjects with AZM treatment compared to subjects without AZM therapy. Together, these data indicate that AZM alters the fibrotic response from the macrophages, as well as the interaction of the inflammatory response and fibrosis development.

Keywords: Azithromycin, alternative macrophage, transforming growth factor-β, matrix metalloproteinase-9, *Pseudomonas aeruginosa*

Susan Elizabeth Birket

July 23, 2012

ROLE OF ALTERNATIVE MACROPHAGE ACTIVATION IN MEDIATING FIBROSIS IN *PSEUDOMONAS AERUGINOSA* PNEUMONIA

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LIST OF ABBREVIATIONS

CF, cystic fibrosis; **CFTR**, cystic fibrosis transmembrane regulator; **ASL**, airway surface liquid; **MRSA**, methicillin-resistant *Staphylococcus aureus*; **NF-κB**, nuclear factor kappa B; **TLR-4**, toll-like receptor 4; **MMP**, matrix metalloproteinase; **NE**, neutrophil elastase; **IL**, interleukin; **IFN-γ**, interferon gamma; **TNF**, tumor necrosis factor; **CAM**, classically activated macrophage; **AAM**, alternatively activated macrophage; **LPS**, lipopolysaccharide; **iNOS**, iducible nitric oxide synthase; **NO**, nitric oxide; **TGF-β**, transforming growth factor beta; **ECM**, extracellular matrix; **CCL-18**, chemokine (CC motif) ligand 18; **IPF**, idiopathic pulmonary fibrosis; **LAP**, latent associated protein; **COPD**, chronic obstructive pulmonary disease; **BALF**, bronchoalveolar lavage fluid; **TIMP**, tissue inhibitor of metalloproteases; **VAP**, ventilator associated protein; **AZM**, azithromycin; **FEV1**, forced expiratory volume in 1 second; **FVC**, forced vital capacity; **ARF**, acute respiratory failure; **MR**, mannose receptor; **BEC**, S-(2-boronoethyl)-L-cysteine; **CFU**, colony forming units.

Chapter 1: Introduction

A. Pulmonary Infection in Cystic Fibrosis

Overview

Cystic fibrosis (CF) is a genetic disorder affecting 30,000 people in the United States. The mutation in the Cystic Fibrosis Transmembrane Regulator gene (CFTR) causes a defect in the chloride transporter that it encodes. Over 1,000 mutations in the CFTR gene have been identified. This defect results in reduced movement of chloride ions, subsequently altering sodium transport and water movement, changing the airway surface liquid (ASL) depth.[1] The mutation affects multiple organs, but the impact is most prominent in the lungs, most likely due to the changes in ASL and the associated consequences. The most common mutation, ΔF508, results in a missense mutation which causes a defect in processing, rendering the protein unable to reach the cell membrane. [2] Other missense mutations, such as G551D, result in a protein at the cell membrane that is improperly regulated, and therefore non-functional. The variation in mutations that lead to disease result in a variety of disease phenotypes, ranging from mild to severe disease.[3] Those mutations which cause mild pulmonary disease may leave other organs unaffected. The more severe mutations cause other clinical manifestations, including pancreatic insufficiency.[4] Cystic fibrosis pathology is characterized by increased mucus secretions in the pulmonary spaces, which provide an excellent environment for frequent bacterial infection.[5] Patients with CF are colonized with a wide variety of bacteria. Infections early in the progression of the disease are commonly caused by pathogens that also commonly cause disease in non-CF children and adults, such as methicillin-sensitive *Staphylcoccus aureus* and *Haemophilus influenzae*.[6] However, by their teen years and into adulthood, most patients become infected with more serious pathogens that produce damaging virulence factors and

multiple resistance mechanisms, such as methicillin-resistant *S. aureus* (MRSA)*, P. aeruginosa,* and *Burkholderia cepacia* complex.[5] These organisms are not commonly observed to cause infection in the normal, immunocompetent population. They produce exacerbations that are extremely damaging to lung function and quality of life in this patient population. Especially in the case of *P. aeruginosa* (and more recently MRSA) patients with CF have extreme difficulty clearing these pathogens, resulting in chronic colonization.[7, 8] In fact, according to the most recent epidemiological data from the Cystic Fibrosis Registry, a national database that tracks infection incidence, up to 80% of patients in their late teens to early twenties typically become chronically infected with *Pseudomonas aeruginosa*.[9] As approximately 85% of CF mortality is due to pulmonary complications, this is an important focus for research efforts.

The mutation in the CFTR gene is the direct cause of the increased mucus secretions in the lung. As stated above, the damage to the pulmonary system can cause significant morbidity and mortality to the CF population. Damage to the lungs during the course of disease occurs in a cyclical fashion (Figure 1.1); mucus buildup provides an ideal environment for bacterial infection, which results in a hyper-inflammatory response from the CF immune system. This response, in clearing the bacterial infection, causes damage to the patient's pulmonary tissue as a result. Neutrophils, the major infiltrating cell type, release a multitude of peptidases and elastases, causing damage to the alveolar interstitium.[10] As the inflammation begins to resolve, if damage is severe enough, the repair processes orchestrate remodeling that results in scarring, causing an even greater difficulty in clearance of mucus, which leads to more bacterial infections.[11] By the time the patient is nearing adulthood, they are subject to chronic infections, a buildup of fibrosis and scarring in their lungs, and a quantity of mucus that is difficult to dissipate. This cumulative damage ultimately results in pulmonary function decline, which is measured with pulmonary function tests. Forced

expiratory volume in one second (FEV1), tracks the compliance of the lung, and is used as a clinical measurement of lung function, and corresponding disease severity. As FEV1 declines, patients are less able to move air through their lungs, resulting in severe morbidity and ultimately death of the patient. [12]

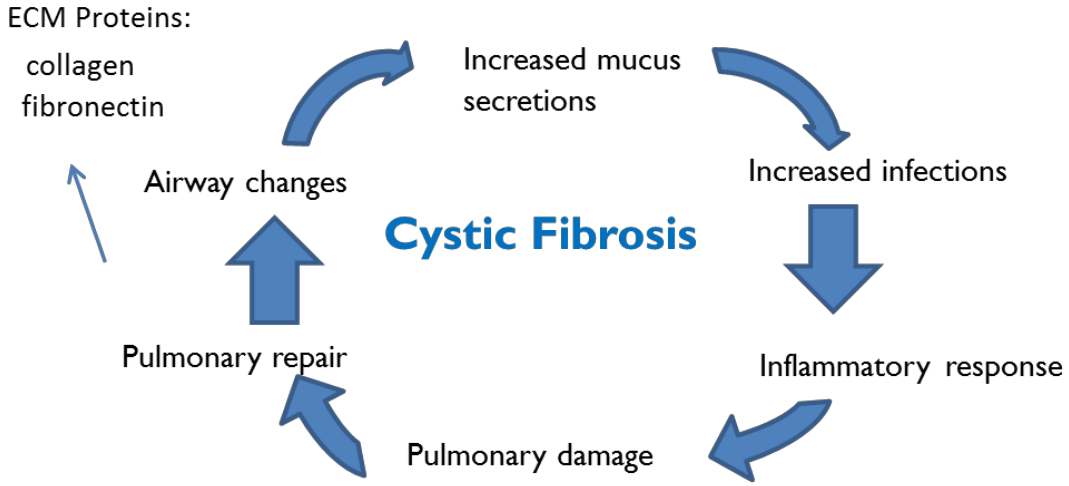

Figure 1.1. Cystic fibrosis pulmonary disease progresses in a cyclical fashion.

Inflammatory Response

In addition to the increased susceptibility to bacterial infection, patients exhibit vigorous inflammation early in their disease; the inflammatory response causes widespread tissue damage in addition to killing the bacteria. It has been shown that the CFTR is directly linked to this hyperinflammation; lack of the CFTR has been shown to cause upregulation of nuclear factor (NF)-κB and toll-like receptor (TLR)-4 mistrafficking, both of which lead to an increase in inflammatory cytokine expression.[11, 13, 14] The bacterial cell wall component lipopolysaccharide (LPS) binds to the TLR-4 receptor, triggering downstream signaling that ultimately activates NF-κB. In macrophages that have the CFTR mutation, TLR-4 degradation is inhibited, resulting in prolonged activation of downstream molecules, including NF-κB.[14] In addition, deregulated

signaling from interleukin (IL)-1β and tumor necrosis factor (TNF) receptors also prolong NF-κB activation.[13] This mistrafficking is demonstrated in macrophages and epithelial cells; the result is that they produce cytokines and chemokines which recruit more inflammatory cells to the airways.[15-17] The cellular infiltrates are predominately neutrophils, which release proteases, such as matrix metalloprotease(MMP)-9 and neutrophil elastase (NE).[18] While these proteases are vital for bacterial clearance, in the CF airway the burden of protease overcomes the presence of naturally produced anti-proteases, shifting the effects toward airway damage and ultimately bronchiectasis.[19] In fact, neutrophils and their associated cytokines are increased in BAL from patients with CF when infection cannot be detected.[20] Because of the continued NF-kB signaling, constant influx of neutrophils and release of proteases, the inflammation characteristic of the disease is more of a prolonged acute response rather than chronic inflammation.[21]

While neutrophils are the primary mediating cell type, T cells are also involved in the exaggerated inflammatory response in patients with CF. Infection leads to a subset of CD4+ T cell response known as Th1, which involves inflammatory cytokines such as IL-1β, IL-6, IFN-γ, and TNF.[22] This response is characteristic of these patients early in the course of their disease (Figure 1.2). The highly inflammatory response, while vigorous, is necessary for clearance of the bacteria. Furthermore, a Th1 response has been associated with better outcomes in *P. aeruginosa* infection, as opposed to a Th2-predominant response.[23] However, as disease progresses, patients' immune responses tend to switch to a subset of T cell response known as Th2-mediated polarization.[24, 25] Despite being associated with repair instead of inflammation, a Th2 response is associated with worse outcomes for *P. aeruginosa* infection[23]. In contrast to a Th1 response, Th2 responses are induced by the cytokines IL-4 and IL-13 (Figure

1.2). T cell response is of primary interest here because the cytokines produced by T cells can influence cells of the innate immune response, including macrophages.

Macrophages can respond to cytokines produced by T cells, and can affect the adaptive immune system in return. Because they are an important part of the innate immune response, these cells respond initially to pathogens, but are also present throughout the infection. In response to the cytokines produced by Th1 and Th2 type T cells, macrophages are polarized to two distinct phenotypes; classically activated macrophages (CAM) or alternatively activated macrophages (AAM), respectively. [26]

IFN-γ, produced by the Th1 response, polarizes macrophages to the CAM phenotype, especially when combined with a pathogen trigger such as lipopolysaccharide (LPS), as shown in Figure 1.3.[27] The primary effector protein of the CAM is inducible nitric oxide synthase (iNOS), which metabolizes L-arginine to produce nitric oxide (NO). Along with oxygen radicals, NO is one of the major bactericidal components of the lysosomes in the CAM, and mainly responsible for its efficiency at bacterial killing.[28] Furthermore, the signaling cascades induced by IFN-γ and LPS stimulate the NF-κB pathway, upregulating the production of inflammatory cytokines. Therefore, this macrophage phenotype is associated with inflammation, bacterial clearance, and damage.[29]

The macrophages that are alternatively activated can be stimulated by a variety of triggers; IL-4 and IL-13, TGF-β, IL-10, and glucocorticoids, each one resulting in a different phenotype and different gene expression patterns.[30] Some of these AAM subsets are summarized in Table 1.1. As noted in the table, some of the triggers and products can overlap between the subsets, making characterization of the specific macrophage phenotype complex. Here, the subset induced by IL-4 and IL-13 will be the primary focus. Macrophages stimulated by IL-4/13 downregulate iNOS and increase production of the effector protein arginase in its place, as shown in Figure 1.3.[26] The

enzyme metabolizes the same substrate, L-arginine, to produce L-ornithine.[31] Without products such as NO, the macrophages are less efficient at killing bacteria, and become involved in clearing away cellular debris.[32, 33] Further, the products from arginase are further converted to prolines and polyamines, which are precursors to collagen production.[29] In this way, AAM are associated with an increase in collagen deposition and therefore participate in mechanisms of tissue repair, as discussed below. While T cells are terminally differentiated into Th1 or Th2 subtypes, macrophages retain plasticity and can be polarized into another phenotype. Because alveolar macrophages can influence the immune environment and response to bacterial infection in the pulmonary spaces, AAM are a target of investigation in multiple infection models, including those caused by fungal pathogens and respiratory syncytial virus.[34] This dissertation will examine, in part, the role of the AAM response to infection with *Pseudomonas aeruginosa.*

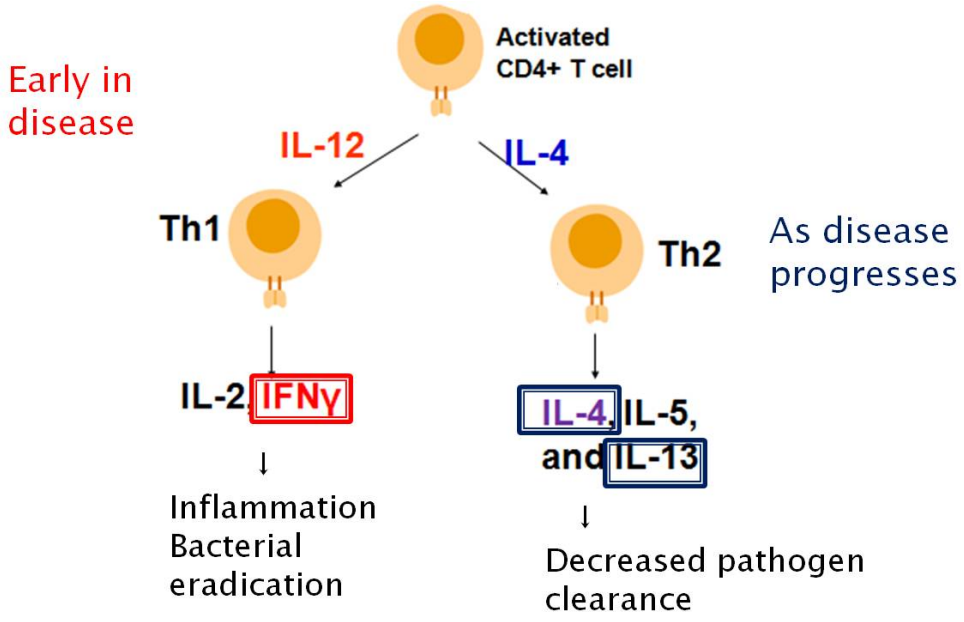

Figure 1.2. Response of T cell subsets in CF disease progression.

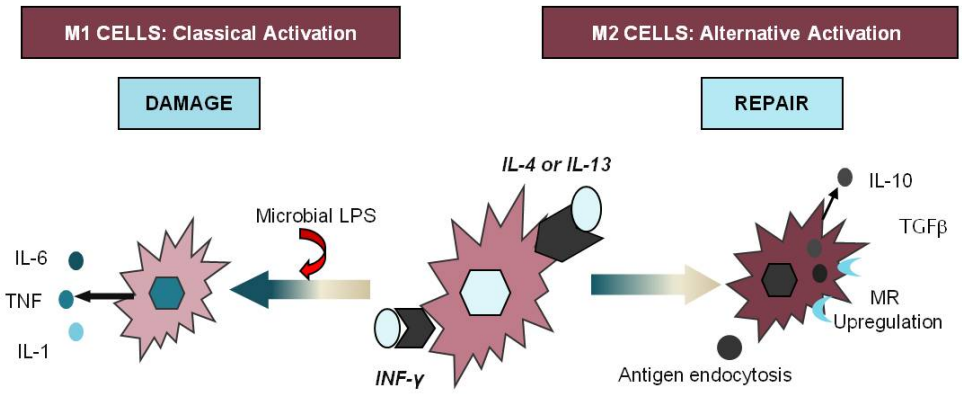

Figure 1.3. Polarization subtypes of macrophages.

Cell Type	Stimulus	Function	Surface Markers	Functional markers	Cytokines produced
M1 (Classically Activated Macrophage)	IFNγ, LPS	Phagocytosis, destruction of intracellular pathogens	TLR4 CD80 CCR7	iNOS ROS	IL-1 IL-6 IL-12 IL-23 TNFα
M2a (Alternatively Activated Macrophage)	IL4, IL13	Tissue repair, encapsulation of parasites	MR CD23	Arginase I Polyamines IL1ra	IL-10
M2b (Type II Macrophage)	Immune Complexes, TLR signaling	Th2 activation, Immuno-regulation	Scavenger Receptor (SR) 1, SR2 CD86		IL-10 IL-1 IL-6 TNFα
M2c (Deactivated Macrophage)	IL-10	Matrix deposition, Tissue remodeling	MR CD14	Polyamines	IL-10 TGFβ

Table 1.1. Function and markers of different macrophage phenotypes. Listed are the macrophage phenotype, the stimulation necessary to polarize an inactive macrophage to that phenotype, the function of the cell, surface markers, functional markers, and the cytokines produced (adapted from [27, 35, 36].

B. Alternatively Activated Macrophages and Fibrosis

Alternatively activated macrophages, the phenotype of macrophage induced by IL-4 and IL-13, produced primarily by Th-2 polarized T cells, have very distinct effector proteins produced. Typically they are identified by increased arginase activity and upregulated mannose receptor expression. The AAM subtype is also marked by higher

rates of antigen endocytosis, the expression molecule found in inflammatory zone 1 (FIZZ-1) and the molecule Ym1.[27, 37, 38] Other markers include increased expression of IL-10, CD23, and chemokine (C-C motif) ligand (CCL)-18.[32] Paired with these changes are downregulation of inflammatory mediators and enzymes, such as iNOS, CD80, and the chemokine receptor CCR7, that are CAM markers.

Without iNOS, which produces NO and other oxygen radicals that are the molecules that allow classically activated macrophages to be efficient bacterial killers, AAMs are more effective at clearing debris than killing pathogens.[39] Along with the decrease in inflammatory and bactericidal effects are an association of AAMs with increased mediators of extracellular matrix (ECM) production through the upregulation of arginase, a fibrotic precursor.[40] Arginase catalyzes the conversion of L-arginine to urea and L-ornithine; L-ornithine is a precursor to prolines and polyamines, which are then used to make collagens and other fibrogenic proteins that make up the ECM.[27] [41]

In fact, alveolar macrophages isolated from patients with several types of pulmonary fibrotic diseases display characteristics of alternative activation. These cells also cause normal lung fibroblasts to increase collagen production when the two cells types are incubated *ex vivo*. [40] This effect on fibroblasts appears to be through the macrophage production of chemokine CCL-18, which is increased in AAM and can recruit naïve T cells.[32] Gene expression of CCL-18 was increased after Th2 cytokine exposure, suggesting this chemokine may be part of a positive feedback loop of fibrosis perpetuation by fibroblasts. In this study by Prasse et al, the effect on fibrosis increase occurred with incubation of Th2 cytokines and presence of an infectious agent was not necessary.[40]

Th2 cytokines are also associated with fibrosis as demonstrated in a murine model of pulmonary fibrosis using a strain of transgenic mouse which over expresses IL-

13. In this study, fibrosis was increased directly through increased activation of transforming growth factor (TGF) –β, a chemokine with both anti-inflammatory and pro-fibrotic properties. [42] Here, the development of fibrosis was dependent solely on the overexpression of IL-13 in the absence of infection, and was blocked through the administration of a TGF-β antagonist. Here, TGF-β was localized to macrophages by histology, showing that macrophages do upregulate this fibrotic mediator after exposure to IL-13, a primary Th2 cytokine.

Likewise, arginase and TGF-β have been associated with fibrogenesis in multiple models of lung injury and repair. This suggests that the alternatively polarized macrophages are major mediators of pulmonary fibrogenesis. Genetically engineered hTGF-β-1 transgenic mice, which overexpress human bioactive TGF-β, were treated with doxycycline to induce a pulmonary fibrosis phenotype, displayed increased arginase produced by mononuclear cells, identified by the common macrophage marker F4/80. [43] These mononuclear cells producing arginase were isolated from bronchoalveolar lavage fluid, showing that they were alveolar macrophages participating in the pulmonary fibrosis response. Likewise, in a model of murine allergic asthma, subepithelial fibrosis was shown to be reduced by the small molecule mepacrine.[44] Administration resulted in reduced arginase and TGF-β expression in the allergic asthma model, likely secondary to the reduced levels of IL-4 and IL-13 also seen with mepacrine treatment. Also, pirfenidone, a drug under investigation for IPF, has been shown to inhibit both arginase and TGF-β, though the mechanism is unclear. [45]

Combined, this evidence shows that the prolonged presence of AAM could be detrimental to the pulmonary environment and participate in the upregulation of fibrotic mediators. However, it is important to note that macrophage polarization is not terminal, and once polarized to one phenotype, the cell can be polarized to another phenotype when the cytokines present are altered. When Th1 cytokines are added to AAM cells,

those cells lose their alternative markers and take on classical characteristics.[46] It is also important to remember that, while the AAM can be pro-fibrotic, their anti-inflammatory properties may make them more protective than harmful long-term.[27]

C. Transforming Growth Factor-β

As mentioned above, TGF-β is one of the mediators responsible for AAM-induced fibrogenesis. It is a cytokine with multiple influences, responsible for cell proliferation, differentiation, adhesion, migration and apoptosis of various cells types.[47] It is produced from most cell types, including those of the immune system such a neutrophils, macrophages, and dendritic cells.[48] TGF-β is a critical molecule in pulmonary homeostasis: it blunts inflammatory response to injury and infection, while also coordinating the wound healing process by upregulating matrix genes.[49] TGF-β is produced as a latent precursor bound to latent associated protein (LAP), where it is cross-linked to the ECM of most organs to be activated when necessary[50, 51]; when activated, TGF-β attracts fibroblasts and induces them to produce a number of fibrotic components, including collagen and fibronectin.[52, 53] It can be activated by cleavage of LAP by a variety of proteases, such as thrombospondin, plasmin, and integrin β6, as examples.[49, 54, 55] Once activated, the protein is free to move about the pulmonary spaces and bind to the TGFβRII receptor.[56] When bound to its receptor, TGF-β signals through a primarily Smad-dependent manner, as outlined in Figure1.1, to upregulate matrix-associated gene production.[47]

TGF-β is necessary for pulmonary development – in early stages of growth it is responsible for the formation of the architecture of the airways and alveolar sacs. [57] It is also a necessary component of the normal immune response. It is activated late in the response to infection in order to downregulate the production of inflammatory

cytokines.[51] Activation of the inactive form is the primary means of regulation of TGF-β, and this is triggered by the upregulation of its activators, which are typically released during hyperinflammatory states.[58] However, this is a molecule that must be maintained in correct balance; too little TGF-β causes hyperinflammation, while too much TGF-β results in lung fibrosis and scarring.[59, 60] When there is too much TGF-β present post-development, the mechanisms that are crucial in forming the architecture of the lung are re-initiated, leading to deposition of connective tissue where it is harmful. In this way, TGF-β is upregulated in multiple models of lung repair; it is released by multiple types of AAM (Table 1.1), and is significantly increased in models of bleomycin-induced fibrosis [52, 61]. Other chronic inflammatory and fibrotic pulmonary diseases, such as chronic obstructive pulmonary disorder (COPD) and idiopathic pulmonary fibrosis (IPF), have reported increases in TGF-β mRNA and protein production as well.[62]

In IPF, TGF-β increases fibrosis accumulation. This appears to be promoting activation and recruitment of fibroblasts that leads to the fibrotic characteristics of the disease.[53] In lung biopsy samples from patients with advanced IPF, immunohistochemistry analysis shows that TGF-β is localized to areas of dense fibrosis and connective tissue deposition.[63] This occurrs in both bronchiolar epithelial cells and in type II pneumocytes. These cell types showed hyperplasticity, which is characteristic of the disease. Another study also evaluated biopsy specimens from patients with IPF via immunohistochemistry.[64] Investigators found TGF-β not only in epithelial cells, but also in myofibroblasts, indicating that both types of cells are being induced to produce matrix proteins. In addition, the cells that stained TGF-β positive persisted over a period of 14 days, while cells that stained positive for TNF-α diminished over that time. Interestingly, the results showed an interaction of the interstitium and the alveolar epithelium during IPF, based on TGF-β localization.

Broekelmann, et al, went a step further in evaluating the localization of TGF-β.[65] This study showed that macrophages that stained positive for TGF-β production by *in situ* hybridization and immunohistochemistry were localized to the site of activated fibroblasts that were expressing high amounts of matrix proteins, including fibronectin, procollagen, and smooth muscle actin. This demonstrated a direct correlation that cells of the immune system, and not just cytokines, were involved in development of IPF and the resulting matrix accumulation.

TGF-β appears to have similar effects in COPD. While the initial causes of IPF are still unknown, COPD is primarily a disease caused by environmental exposure to inhaled chemicals; most often those associated with cigarette smoke.[66] Most of the damage caused by the chemicals is concentrated in areas of the small airways. In the case of emphysema, an obstructive disease under the umbrella of COPD, the damage is more generally located to the alveoli.[67] In all cases of damage, however, TGF-β upregulation acts as a function of wound healing, leading to fibrosis, as a consequence.[68] This has been demonstrated in several studies. TGF-β was examined in subjects with or without COPD who underwent lobectomy for cancer; subjects were either current or ex-smokers.[62] In the lung tissue specimens from subjects with COPD, TGF-β mRNA was positively correlated with bronchial and bronchiolar epithelial cells, while this correlation was not as strong in subjects without COPD. The authors suggest that this increase correlated to the recruitment of mast cells and macrophages to sites of damage, as TGF-β is known to be chemotactic. Takizawa, et al, correlated TGF-β mRNA not only with COPD but also with smoking. [69] This study compared subjects with COPD to healthy subjects who were smokers and healthy subjects who never smoked. TGF-β mRNA was upregulated in smoking subjects with and without COPD, and correlated positively with subjects' duration of smoking (in terms of pack-years). This suggests that TGF-β is directly increased as

damage from exposure occurs, and that it could be a major mechanism of fibrosis buildup during disease.

TGF-β has also been investigated as a mitigating factor in CF pulmonary disease. There is some evidence that TGF-β may have an interaction with the CFTR. A study performed at University of California at San Francisco showed that TGF-β reduces β_2-adrenergic dependent chloride transport, resulting from CFTR reduced activity. This appears to be acting in a cAMP-dependent manner.[70]

Importantly, TGF-β expression has been shown to be increased in patients with CF compared to patients with a non-CF pulmonary exacerbation.[71] In this study that enrolled patients with CF investigators found a correlation between high TGF-β protein levels in BALF and reduced lung function. Investigators also demonstrated that TGF-β levels were not different in patients colonized with *P. aeruginosa* compared to CF patients who had not cultured this pathogen. An additional study from this group found that levels of TGF-β in the plasma correlated to BALF TGF-β levels, and that plasma TGF-β was actually increased in patients positive for *P. aeruginosa* colonization.[72] Treatment for the infection decreased plasma levels of TGF-β, and post-treatment measurements of TGF-β were inversely correlated with lung function. The implications for TGF-β in CF become more interesting when polymorphisms are examined: a phenotypic gene study in the Czech Republic showed that patients with CF who had TGF-β concentrations at both high and low extremes in their lungs had the worst outcomes.[73] Although the polymorphisms were not associated with lung function, they were associated with other organ disease.

The studies summarized here demonstrate that TGF-β is of critical importance in various diseases of fibrosis, including CF. It is a protein produced by with AAMs, which have also been associated with diseases of fibrosis, as noted above. There is also evidence that in the absence of TGF-β, presence of AAM is reduced.[74] This study will

continue to investigate TGF-β in the development of fibrosis, in the context of *P. aeruginosa* infection.

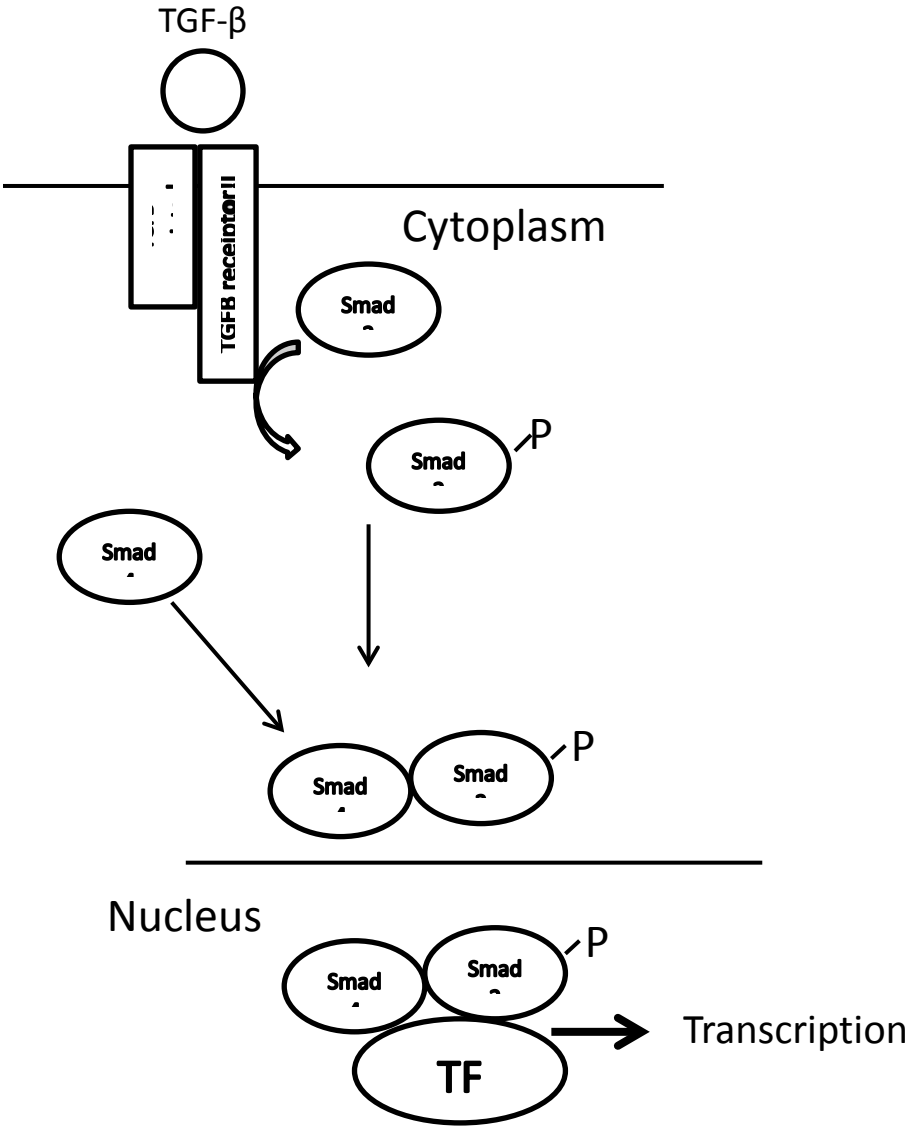

Figure 1.4. Activation of the TGF-β Smad-dependent signaling pathway. TGF-β isoforms binds to TGF-βRI and II coupled, which then phosphorylate Smad2. Phosphorylated Smad2 binds Smad4, and the complex travels to the nucleus to bind to a transcription factor and begin transcription of TGF-β dependent genes.

D. Matrix Metalloproteinase-9

In many of the aforementioned pulmonary diseases, TGF-β is linked with MMP function.[42] TGF-β can suppress the production of these proteases that are produced

by monocytes and macrophages, which could lead to a reduction in ECM turnover.[75] This, in turn, could lead to clearance of ECM proteins before they are accumulated to a degree that would lead to scar tissue and pulmonary function decline.'

One member of this family is MMP-9. It is a protease that is also known as gelatinase B, as the first known function was to bind and cleave gelatin. Other substrates have since been identified, including collagen IV, α1-antiproteinase, latent TGF-β1, latent VEGF, fibrin, and fibronectin.[76, 77]. MMP-9 has similar activity to another member of the family, MMP-2, also known as gelatinase A. Like TGF-β, these proteases are produced in an inactive form, and are then activated among the tissues. Activation of the pro-forms, or zymogens, is generally based on availability of substrates, however, rather than other catalytic enzymes.[78] Once activated, the enzymes are able to execute their main function of cleaving other proteins.

In the lung, MMP-9 is primarily involved in remodeling. Many types of cells can produce this enzyme, including bronchial epithelial cells, alveolar type II cells, fibroblasts, endothelial cells, and immune cells; neutrophils, macrophages, lymphocytes, NK cells, and dendritic cells have all been shown to produce MMP-9.[79] There is typically little to no MMP-9 in healthy tissue, but it is released quite readily upon injury. [76] One function is that it appears to facilitate cell migration. The degradation of collagen IV, a basement membrane protein, allows architecture to be changed in order for immune cells to migrate to the site of inflammation.[80] It also allows for remodeling of structure after or during inflammation for epithelial cells. [81]

One type of immune cell that produces MMP-9 in large amounts is neutrophils. These cells produce MMP-9-filled granules during bone-marrow maturation and release the granules during migration toward sites of inflammation.[79] Therefore, MMP-9 is often associated with inflammatory diseases. For example, in ventilator-induced lung injury, in which there is an influx of neutrophils, MMP-9 has been shown to be increased

in response to ventilation.[82] Furthermore, this study showed that MMP-9 activity was associated with neutrophil influx, as well as degree of acute lung injury. The investigators also demonstrated that when MMP-9 was inhibited, this effect was prevented, suggesting that MMP-9 may be promoting neutrophil influx in this model.

In a model of emphysema, the result of chronic inflammation, MMP-9 was again associated with inflammation. Mice which had inactivation of the surfactant protein D gene developed emphysema and had increased activity of MMP-9 compared with mice that had function surfactant protein D.[83] MMP-9 has also been associated with bronchiectasis, which is a result of chronic inflammation in the airways. In this inflammatory disease, bronchial walls are destroyed, often as the result of damage by neutrophilic-associated lytic enzymes. Among these were MMP-9 and MMP-8; these two proteins were detected by immunostaining in bronchial biopsy specimens of subjects with bronchiectasis, and were highly correlated with number of neutrophils present.[84] This study provides evidence that excess MMPs may cause damage to the airways.

However because MMP-9 has the ability to assist in remodeling pathways, it is also an important molecule in diseases of fibrosis. In a model of bleomycin-induced lung fibrosis, MMP-9, as well as MMP-8, concentration was increased in the BAL of mice treated with bleomycin.[85] In these same mice, hydroxyproline, a precursor of collagen, was increased at day 14 post-administration. However, when the neutrophils were depleted, MMP-9 concentrations were reduced, compared with that found in the bleomycin treated mice. Conversely, hydroxproline was increased in the neutrophil-depleted group, suggesting that the upregulation of MMP-9 in fibrotic processes assists in turnover of the accumulation of fibrotic proteins. Also supportive of that notion, a group of investigators showed that pro-MMP-9 secretion from macrophages is increased by fragmented fibronectin.[77] When monocytes isolated from human peripheral blood were incubated with TNFα and incubated with either native fibronectin or fibronectin

degraded into specific fragments, the degraded fibronectin elicited more of a response from the macrophages to produce pro-MMP-9.[77] Because MMP-9 can degrade fibronectin directly[79], this data is likely indicative of a feedback mechanism, wherein MMP-9 can be stimulated to continue to degrade ECM. This suggests a role for MMP-9 in controlling the buildup of fibrosis. It also suggests that moderate increases in MMP-9 concentrations may have positive outcomes, by degrading fibronectin before it accumulates to cause scarring.

Lastly, MMP-9 has also been studied in patients with ventilator associated pneumonia (VAP) who are infected with *Pseudomonas aeruginosa*. In these patients, serial BALF samples were collected and analyzed for MMP-9, MMP-8, and tissue inhibitor of metalloproteinases (TIMP)-1 concentrations. TIMP-1 is the natural inhibitor of the MMPs, which circulates in order to counteract the activity of the proteases. Both MMP-9 and -8 concentrations were significantly higher in the BALF of patients with VAP compared with patients ventilated only for airway protection.[86] Patients were further analyzed for microbiology profiles, to see if their cultures possessed type III secretion system (TTSS) characteristics. The TTSS is the mechanism in *P. aeruginosa* that secretes toxins that cause damage to the host. When analyzed this way, the authors found that MMP-9 was increased in patients whose bacterial growth showed TTSS positive characteristics, compared to patients whose bacterial growth was TTSS negative. Lastly, patients with higher MMP-9/TIMP-1 ratios had a higher risk of death compared to those with lower MMP-9/TIMP-1 ratios. Because there is more protease compared to its inhibitor, this suggests that more MMP-9 activity, most likely in response to more virulent bacteria, is detrimental to the outcome VAP patients.[86] The authors suggest that proteases, such as MMP-9 and -8, are released more readily as the toxicity level of the bacterial infections increases, causing heightened risk of airway damage and death.

MMP-9 also plays an important role in the pathophysiology of CF, a result that is not surprising given the data underlining its importance in diseases of neutrophilic inflammation, fibrosis, and *P. aeruginosa* pneumonia. Increases in MMP-9 production is associated with occurrence of pulmonary exacerbation, airway breakdown leading to remodeling, and in response to infection with *Burkholderia cenocepacia* in patients with CF, all of which can decrease survival.[87-89] Sagel, et al, assessed induced sputum samples from healthy controls and subjects with CF for levels of MMP-9 and TIMP-1.[90] This study found that concentrations of both molecules were significantly higher in the CF subject samples, as was the ratio of MMP-9/TIMP-1. MMP-9 was also negatively correlated with FEV_1, and therefore positively correlated with disease severity, in these subjects. Lastly, both MMP-9 and TIMP-1 were positively correlated to neutrophil counts, and levels of IL-8 in the sputum samples.

Another study examined lower airway secretions of subjects with CF, comparing the results to subjects with non-CF acute respiratory failure(ARF) as the controls. Gaggar, et al, showed that subjects with CF had increased active MMP-9 concentrations, while subjects with ARF had increased active MMP-8 concentrations in samples taken from endotracheal suctioning. [19] The study went on to show that the increase in CF MMP-9 activity is constitutive, while subjects with ARF had mostly inactive, pro-MMP-9 forms of the enzyme in their samples. However, subjects with CF had lower levels of TIMP-1 in their secretions than did the ARF subjects, and higher MMP-9/TIMP-1 ratios. This indicates dysregulation, and potential hyperactivity of the protease in the CF airways.

Lastly, Ratjen et al examined MMP-9 levels and their potential modulation by a commonly used CF treatment.[91] In this study, the expected increases in MMP-9, MMP-8, and TIMP-1 in BALF of subjects with CF compared with healthy controls were demonstrated. However, when the concentrations of these three molecules were

measured in BALF 18 months later, treatment with dornase alfa had reduced levels of all three markers after compared to subjects not receiving the drug. Dornase alfa cleaves the DNA that accumulates in the mucus of patients with CF[92]; it has been shown to increase FEV_1. This study suggests that preventing tissue destruction by MMPs may be one of the ways in which this is being accomplished.

Taken together, previous study of MMP-9 shows its importance in the process of fibrotic disease. Overabundance of MMP-9 in relationship to its inhibitor predisposes toward damage and bronchiectasis, yet the protease is still necessary to turnover of ECM proteins. However, whether this molecule is causing fibrosis by initiating the damage that requires subsequent repair, or whether it is upregulated after fibrosis is initiated in order to clear away the buildup of repair molecules is unclear.

E. Anti-inflammatory Therapy for CF

Patients with CF are treated using a number of different therapeutic approaches. Mucolytics, such as dornase alfa (Pulmozyme) and acetylecysteine (Mucomyst) help to decrease viscosity and clear away mucus. Oral, intravenous, and aerosolized antimicrobial agents are used during exacerbations caused by infecting organisms and routinely for bacterial clearance and suppression.[92] However, many additional therapies for CF now focus on influencing the hyperinflammatory state of the immune system. Inhaled and oral corticosteroids, used in a multitude of other disease states for inflammation, are not actually recommended for CF.[92] While these drugs can be very effective for chronic inflammatory disease states such as asthma, studies have shown that decline in FEV_1 is not improved in CF patients, nor are there decreases in exacerbations in patients taking corticosteroids.[93, 94] In addition, patients with chronic steroid use experienced glucose abnormalities, growth retardation, and cataracts.[95]

Oral nonsteroidal anti-inflammatory drugs (NSAIDs) have also been studied in patients with CF, mostly ibuprofen. This drug must be dosed significantly higher than typically recommended, and must be given on a daily basis. Studies have demonstrated slowed decreases in FEV_1 compared to placebo controls, an effect that is dramatic for subjects less than 13 years of age.[96] Yet no changes in hospitalizations rates, an indicator of exacerbation, have been demonstrated. However, studies from this group also suggest that sub-therapeutic levels of ibuprofen may actually exacerbate inflammation, and therefore recommends pharmacokinetic monitoring for each patient. This recommendation makes chronic treatment of ibuprofen difficult for many patients. Another concern for long-term therapy with ibuprofen is the potential for renal damage and gastrointestinal toxicity; the study done was not powered to detect these effects. The limitations of these interventions leave a need for the development of effective anti-inflammatory therapies to treat the hyper-immune response observed in CF.

The latest class of drugs to be examined for its anti-inflammatory effects in CF is the macrolides. These antimicrobials target the 50S ribosomal subunit in bacteria, thus disrupting protein translation. Azithromycin (AZM) is the macrolide that is most often used, due to its reduced side effects, ease of administration, and broad spectrum.[97] AZM has activity against mainly gram negative bacteria, but also atypical organisms, like *Legionella pneumonia*, and some aerobic and facultative gram positive organisms, such as *S. pneumonia*; however it has no bactericidal activity against *P. aeruginosa* or *S. aureus.* [98, 99] While AZM is a common therapeutic agent used to treat bronchitis, sinusitis, and other upper and lower respiratory infections, because of its spectrum of activity it is ineffective in treating many of the types of infections that CF patients are prone to. Instead, AZM is employed as a long-term therapy for its effects on the immune system. AZM is extensively partitioned into the cells, leaving serum concentrations low, and has a long half-life, which allows for daily or every other day dosing.[100] In this

way, AZM is able to affect cell signaling of macrophages. These drugs, especially AZM, are shown to have direct anti-inflammatory effects, including alterations in cytokine production profiles and changes in NF-κB nuclear translocation.[101] AZM has minimal adverse effects and has not been associated with the issues found when using corticosteroids and NSAIDs for chronic treatment.

To that end, continuous treatment with AZM has now been studied in multiple clinical trials. Subjects treated with AZM have increased FEV1, decreased pulmonary exacerbations, and reported better quality of life compared to placebo controls.[50, 102] The first study by Saiman, et al, recruited subjects with CF who were colonized with *P. aeruginosa*.[50] Patients receiving AZM for 168 days reported higher increases in FEV_1, less incidence of exacerbation, and increased weight as compared to baseline at the end of the study compared to patients receiving placebo. While antibacterial resistance is a concern for this treatment, Saiman, et al, showed in this study that treatment with AZM did not change resistance profiles in multiple bacteria compared to placebo control subjects. The two groups had similar resistance rates of current infections and growth of new infections at the end of the trial, including MRSA, suggesting that AZM does not pose a significant threat toward inducing antibacterial resistance in this population.[50]

While AZM was initially studied in patients who were already infected with *P. aeruginosa*, this group repeated their study in patients negative for *P. aeruginosa* colonization.[103] Once again, they showed that AZM treatment over 168 days reduced the risk of exacerbation compared to placebo. Here, AZM did not improve pulmonary function in this subset, but this study, like the previous, showed no changes in microbiology or in treatment-emergent microbiology. This data was important to show that AZM is safe to use long-term in this cohort from a microbial resistance standpoint.[103]

Equi, et al, performed a 15-month crossover trial with AZM treatment in pediatric patients with CF in 2002.[104] Subjects received AZM or placebo for six months, followed by a two-month washout period, and then received the opposite therapy. Results showed that subjects required fewer days of oral antibiotic therapy when on AZM, suggesting AZM could reduce the incidence of exacerbations. Though this study did not demonstrate a statistically significant effect on FEV_1, it did show that FEV_1 improved on AZM but returned to baseline at the end of a washout period. This provides some justification for the chronic dosing of the drug. This study also showed no differences in sputum bacterial densities, which is further support that AZM does not affect microbial resistance. Importantly, there were no subjective reports of side effects, and liver tests showed no evidence of toxicity. [104]

Finally, Wolter, et al, found that patients who received AZM for three months had decreased number of IV antibiotic courses, as well as decreased total numbers of days on IV antibiotic therapy. This complements the study done by Equi, et al, to corroborate the evidence that AZM can reduce the number of exacerbations when given over the long term. Subjects in this trial also reported significantly improved quality of life, assessed by the Chronic Respiratory Disease Questionnaire.[105] Lastly, these subjects had reduced levels of CRP, a general marker of inflammation.[104]

While AZM is not bactericidal against *P. aeruginosa*, there is some evidence that it can affect the bacterium in other ways. Quorum sensing gene production, a regulatory mechanism that allows the bacteria to coordinate growth, metabolism, and protein production based on density or growth, is disrupted by AZM.[106] When sub-MIC levels of the drug are added to bacterial cultures, organisms undergo reduction in quorum sensing gene production and reduced motility. Also through disruption of quorum sensing circuits, AZM blocks alginate and other biofilm component production and therefore prevents biofilm to aid the survival of bacteria grown *in vitro* and *in vivo*.[107,

108] This was demonstrated both with susceptibility assays in cell culture and in pulmonary infection with *P. aeruginosa* in CFTR$^{-/-}$ mice. Interestingly, AZM has been recently shown to affect some small RNAs upstream of the quorum sensing pathway, adding complexity to the impact of the drug on the ability of the bacteria to communicate.[109] Together, these data suggest that, while the drug may not be acting directly to kill the bacteria in the CF population, long-term treatment may have some overall benefit in disrupting the bacterium's ability to maintain high-density function.

Azithromycin and Alternatively Activated Macrophages

Because AZM is utilized in patients with CF for its anti-inflammatory properties, it is important to identify the effects of the drug on specific cells. Published work has shown that AZM can affect the NF-κB inflammatory cascade in monocytes and bronchial epithelial cells.[110, 111] However, these authors did not address how the monocytes are affected once they become activated into macrophages. To extend these studies, our lab demonstrated that AZM treatment alters surface marker expression and cytokine production of macrophages; increasing MR, and decreasing CCR7. AZM treatment increased the effector protein of AAMs, arginase, as well as switching the cytokine profile from IL-12 to IL-10 producing, *in vitro* (Figure 1.5).[112] Together, these data suggest that AZM is polarizing macrophages toward an alternatively activated phenotype. This is confirmed further, by evidence that AZM increases arginase *in vitro* and *in vivo* (Figure 1.6), and decreases iNOS, associated with CAM activity.[112] Preliminary data from our lab also shows that AZM decreases IKK-β, a molecule in the signaling cascade of the toll-like receptors. When phosphorylated, IKK-β releases NF-κB to translocate to the nucleus and begin transcription of inflammatory proteins. Importantly, in a study conducted by Fong, et al, IKK-β has also been shown to inhibit Stat-1, and may be decreasing the CAM effector proteins in this way.[113]

Our data, combined with this evidence of IKK-β/Stat-1 interactions, suggest that this may be the mechanism by which AZM can alter the polarization of macrophages. Furthermore, AZM could be resulting in full AAM function, upregulating ECM mediators and components. If that is the case, and the AZM- polarized macrophages are functioning to increase fibrosis, the implications could be important. As stated above, there are quite a few studies showing the association between AAM and fibrosis development in either mouse models or human disease. If patients with CF have increased Th2-driven immune responses as their disease progresses, they have the natural propensity to have increased numbers of AAM in their pulmonary spaces without AZM treatment, an effect that our group also previously characterized and published.[114] Adding another stimulus for AAM may not be beneficial in the long term. Alternatively, the increased presence of AAM may inhibit the hyperinflammatory response characteristic of the CF pulmonary response. The full effects of the drug on the macrophage are unknown; therefore it is important to examine the full functionality of the AZM-polarized macrophage for long-term safety implications.

To that end, in this project, we studied the pro-fibrotic potential of the AZM-polarized macrophage. This macrophage phenotype was examined for its effects on other cell types and the immune response to *P. aeruginosa* in general. Because of the AAM-like properties of the AZM-polarized macrophages, and the fibrotic properties of the AAM, we proposed the following hypothesis for this project: **AZM-polarized macrophages contribute to fibrosis by increasing the production of TGF-β and decreasing the production of MMP-9 in the context of *P. aeruginosa* infection.**

As stated above, understanding the full mechanism of AZM is important. The effects on the macrophage population could be wide ranging and could affect the way the macrophages interact with other immune cell types. The following dissertation examines this interaction in detail.

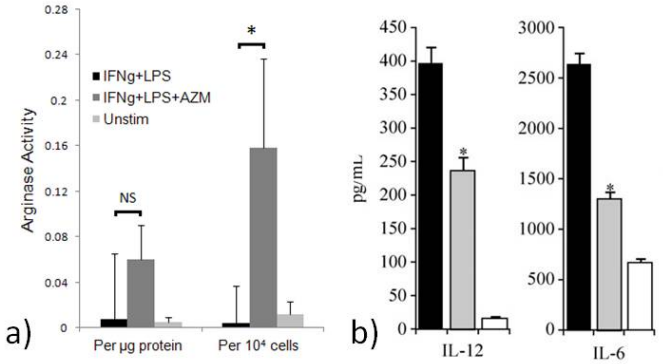

Figure 1.5. AZM polarizes macrophages to an alternative phenotype *in vitro.* Macrophages were treated with INF-γ, with or without AZM. After overnight incubation, LPS was added for stimulation. In a), arginase activity was measured and normalized per µg protein and per 10^4 cells. In b), IL-12 and IL-6 was measured from the supernatants by CBA. Data is represented as mean ± SD. Data were analyzed via one-way ANOVA. Significance of < 0.05 is indicated with *.
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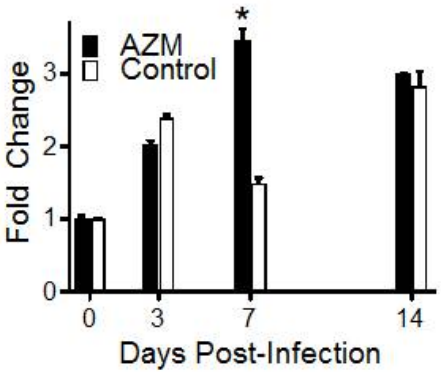

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Figure 1.6. AZM polarizes macrophages to an alternative phenotype *in vivo.* Macrophages were treated with INF-γ, with or without AZM. After overnight incubation, LPS was added for stimulation. Arginase activity was measured and normalized per µg protein. Data is represented as mean ± SD. Data were analyzed via two-way ANOVA. Significance of < 0.05 is indicated with *.

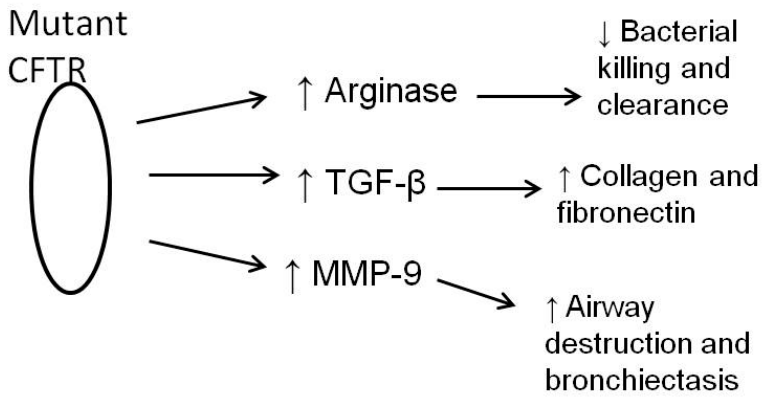

Figure 1.7. How molecules of interest are altered in CF pathology. Arginase has been shown to be increased in CF, reducing the amount of NO available to kill bacteria.[115] TGF-β is increased in patients with CF compared to healthy controls, causing increases of ECM products.[71] MMP-9 is increased in patients with CF compared to healthy controls, resulting in increased airway damage.[90]

F. Project Overview

In order to determine whether AZM-polarized macrophages are affecting fibrosis through TGF-β and MMP-9 production, we employed three approaches. First, we used an *in vitro* co-culture system to examine the direct effects of AZM-polarized macrophages on fibroblasts. Using this system, we were able to examine the resulting fibrotic mediator production in response to stimulation with *P. aeruginosa.* This model was used to examine cell-cell interactions and protein-protein interactions. We also utilized inhibitors of molecules important in fibrosis mediation, in order to determine the mechanisms by which AZM is affecting the macrophage's impact on fibrosis. Secondly, we use a murine model to examine the role of AZM-polarized macrophages in the immune response to *P. aeruginosa* pneumonia. We studied the immune response overall, as well as the interactions of the molecules identified as important from the cell culture results, including MMP-9 and fibronectin. In this model, we were able to establish a lasting infection, isolating *P. aeruginosa* from the mice as late as 14 days after inoculation. We also utilized genetically-engineered mice to examine AZM activity

and the importance of the AAM in both the response to *P. aeruginosa* infection and the subsequent fibrosis development. Finally, this project incorporated a clinical study involving subjects with CF. Sputum samples were collected from these patients, and concentrations of fibrotic proteins were measured and correlated to inflammatory markers and markers of alternative macrophage activation. These factors were examined in patient groups stratified by both AZM therapy and *P. aeruginosa* infection. Results from the human study give up insights not only to how AZM is affecting macrophages in patients, but also which appear to be most important in terms of severity of disease.

The overarching hypothesis for this project is that AZM-polarized macrophages contribute to fibrosis by increasing the production of TGF- and decreasing production of MMP-9 in the context of *P. aeruginosa* infection. Each approach to this study produced complementary results that allow for a better understanding of the role of AZM-polarized macrophages in the development fibrosis. The co-culture model showed increased production of fibrotic mediators from cells treated with AZM, including TGF-β, MMP-9, and fibronectin. When the mice infected with *P. aeruginosa* were examined, we found that those treated with AZM had increased concentrations of MMP-9 and fibronectin in the BAL, as well as increased accumulation of collagen. The human study underlined the importance of the interaction between infection and AZM treatment, with increased arginase and TGF-β production in the sputum of those subjects both receiving AZM and positive for bacterial colonization. This study also highlighted important relationships between these proteins and lung function.

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Chapter 2: Azithromycin-polarized macrophages increase fibrosis mediators in co-culture with fibroblasts

A. Introduction

Alveolar macrophages are a component of the innate immune response, and responsible for being first line of airway defense for pulmonary infections. The macrophages that are involved in bacterial killing and clearance are classically activated; these cells upregulate molecules such as inducible nitric oxide synthase (iNOS), which produces nitric oxide. Nitric oxide, along with other products of CAMs, participates in bacterial killing in the lysosomes.[26] These cells are traditionally activated by IFN-γ and a microbial trigger, such as LPS. Conversly, AAMs function to coordinate debris clearance, repair, and tissue remodeling.[116, 117] They produce arginase as a primary effector protein, in direct contrast to iNOS, which metabolizes the same substrate, L-arginine, to urea and L-ornithine, which can then be converted to proline and polyamines. These molecules are then incorporated into collagen and fibronectin, key components in extracellular matrix.[27] Further, arginase production has been associated with TGF-β activation, which also increases fibrotic mediators.[41] This has been shown in multiple models of pulmonary repair.[41, 45]

MMP-9 is a protease, released from multiple cells, which is able to assist in turnover of the ECM by degrading proteins such as fibronectin and collagen.[75] In this way, it is able to counteract the pro-fibrotic effects of TGF-β. However, TGF-β is able to decrease expression of MMP-9 from monocytes, as a mechanism of reducing ECM turnover.[118] Therefore, if AZM affects TGF-β, it is important to know if the drug will affect MMP-9 as well. It is this set of effector proteins that this chapter's work is focused on.

Previous work in our lab has shown that AZM is able to polarize macrophages toward an alternatively activated-like phenotype. When macrophages were treated with AZM in addition to LPS and IFN-γ, there was an alteration in the phenotype compared to macrophages treated only with LPS and IFN-γ.[112] Macrophages which were treated with AZM demonstrated significantly reduced production of the inflammatory cytokines IL-6 and IL-12, as well as increased production of the anti-inflammatory cytokine IL-10. These AZM-treated macrophages had a shift in surface receptor expression, with increases in mannose receptor and CD23, and decreased expression of CCR7, a protein profile that is indicative of alternative phenotype.[27] The cells treated with AZM also produced decreased amounts of the CAM effector protein iNOS compared to the cells treated with IFN-γ and LPS alone; the AZM-treated cells upregulated arginase, the effector protein of the alternative macrophage phenotype. AZM was able to polarize these macrophages toward the alternative phenotype even in the presence of the classical cytokine IFN-γ[112]

This alteration of macrophage function may be the means by which this drug is able to positively affect patient's clinical outcomes.[114, 119] CAMs produce molecules that are damaging the CF lung in response to the repeated and/or chronic infection in these patients, and AAMs have been shown to directly inhibit these inflammatory functions.[26, 32] However, as mentioned above, CF patients are already primed to have an increase in AAMs, as they have increased concentrations of cytokines such as IL-4 and IL-13.[22] This may be a compensatory protective mechanism as the disease severity progresses, in an attempt by the body to minimize the danger. AZM-polarized macrophages have not been evaluated for the effects that they may exert upon other cell types involved in the remodeling process. Therefore, it was important to investigate not only the anti-inflammatory response of the macrophage to the bacterium *P. aeruginosa*, but also the effect of these AZM-polarized AAMs on other cell types. Fibroblasts are

important in the lung during remodeling process, and are a major cell type involved in the production of fibrotic mediators. Therefore, we chose to examine these two types of cells *in vitro* in order to determine whether AZM-polarized macrophages are able to induce production of fibrotic mediators from fibroblasts.

Lastly, we sought to determine a mechanism by which AZM affects the shift in polarization in the macrophages. Previous work in our lab has identified alterations in the NF-κB signaling cascade as a possible mechanism for the decreased inflammatory cytokine production already mentioned.[112] One of the proteins altered in this cascade, IKK-β, was identified as a potential target for AZM during the polarization of the macrophage. To this end, we investigated whether the increase in fibrosis was also occurring through this molecule.

For these experiments, macrophages and fibroblasts were co-cultured together, according to the design in Figure 1 below. The effect of AZM in an environment that would otherwise stimulate toward a CAM phenotype was studied. As discussed previously, TGF-β and MMP-9 are both molecules that have been associated with diseases in which fibrosis is a pathologic component.[40, 77] TGF-β signaling upregulates fibrotic proteins, such as fibronectin, from fibroblasts,[65], while MMP-9 can degrade these same molecules and result in ECM turnover.[85] These molecules have also been associated with the AAM phenotype in a model of fibrotic disease.[42] Therefore, we postulated that TGF-β and MMP-9 may be important molecules in the mechanism of AZM's effect on the macrophage. We hypothesized that **AZM-polarized macrophages induce ECM buildup during *P. aeruginosa* infection by increasing production of TGF-β and MMP-9.**

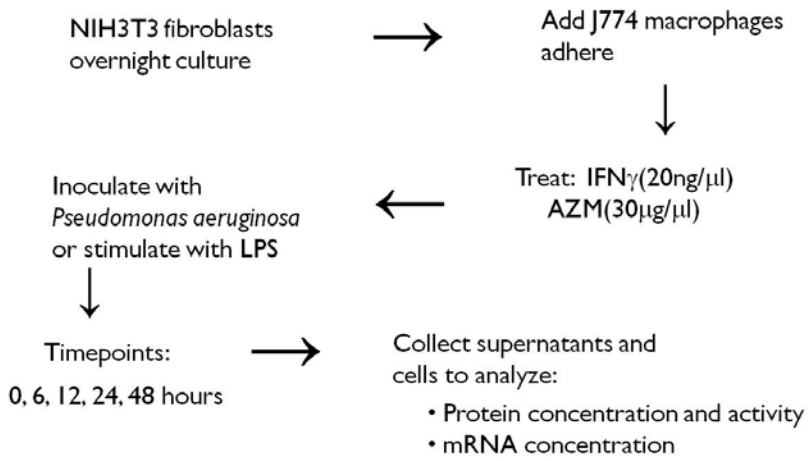

Figure 2.1. Schematic of experimental methods used for the co-culture experiments.

B. Materials and Methods

Fibroblast/Macrophage Co-Culture System

The mouse cell lines NIH/3T3 and J774A.1 (ATCC, Manassas, VA, USA) were used for the co-culture system. The NIH/3T3 line (ATCC #CRL-1568) is a fibroblast cell line originally obtained from embryonic cultures of NIH Swiss mice.[120] The J774 (J774A.1, ATCC #TIB-67) cell line is an immortalized macrophage cell line derived from BALB/cN adult mice.[121] Cells were grown in RPMI 1640 media (Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal calf serum and $2x10^{-5}$M 2-mercaptoethanol at 37°C and 5% CO_2. At time 0 of the experiment, NIH/3T3 cells were added to culture treated 6 well plates at a density of 2.5 X 10^5 cells/ml, and allowed to incubate overnight. The media was then removed and replaced with 5 mL of medium along with 2.5 X 10^5 J774 cells/5ml, and again allowed to incubate overnight. The next morning, cells were stimulated by adding interferon-γ (R&D systems, Minneapolis, MN, USA) (IFNγ) (100ng/mL) and 2.5 X 10^5 *P. aeruginosa*, of the strain PA39018, a non-biofilm producing strain obtained from ATCC (Manassas, VA, USA). Certain wells were additionally

treated with 30μM azithromycin (Sigma Aldrich, St. Louis, MO, USA) (AZM). The optimal concentration of AZM had already been determined, based on dose response studies, as 30μM is enough to elicit the maximum response of the drug – with regard to arginase and mannose receptor expression. Cells were incubated and harvested after 0, 6, 12, 24, or 48 hours. At these time points, cells were dislodged via scraping and centrifuged, and 1mL of supernatant was saved for collagen and TGF-β analyses. Cells were washed, enumerated with trypan blue staining to assess viability, and aliquots were taken for the arginase activity assay and qRT-PCR. Supernatants and cells were frozen at -80°C for later analysis. Cells were additionally suspended in 150μl of RIPA buffer with Roche Mini-tablet protease inhibitor cocktail for lysis upon thawing, and aliquots of cells were frozen in RNAlater (Applied Biosystems, Foster City, CA, USA).

Inhibitor Experiments

In a subset of experiments, neutralizing antibodies and/or small molecule inhibitors were used to mechanistically investigate the role of effector proteins in the fibrosis process and the impact AZM has on these processes. Materials used included: S-(2-boronoethyl)-L-cysteine (BEC), used at 500μM, was utilized to inhibit arginase activity. TGFβ-1,2,3 neutralizing antibody (R&D systems, Minneapolis, MN, USA) was used at 0.25μg/mL, as recommended by the company. MMP-9 inhibitor SB 3CT (Enzo Life Sciences, Farmingdale, NY, USA), was used at 600nM, which had been determined by other groups to be the concentration to specifically block MMP-9 and no other MMPs. [122] Lastly, the IKKβ inhibitor IKK16 (Tocris Biosciences, Ellisville, MO, USA) was used at 100nM, which was determined through previous experiments to optimally inhibit arginase production by the macrophages.

Arginase Activity

Arginase activity was quantified by measuring the conversion of L-arginine to urea [123]. 10mM $MnCl_2$ in 50mM Tris-HCl was added to the lysed sample and the mixture was then incubated at 55°C to activate the arginase enzyme. L-arginine was then added and samples were incubated overnight at 37°C. Each reaction was terminated by the addition an acid solution. The colorometric indicator 9% alpha-isonitrosopropiophenone (ISPF) in 100% ethanol was then added to each tube, heated to 95°C, and the OD of each sample was read using a 490nm filter. Readings were compared to a standard curve of known urea concentration. One unit of arginase activity converts 1μmole of L-arginine to urea per minute. Values were normalized to total protein concentration as assayed by the bicinchoninic acid protein assay (BCA) (Pierce Biotechnology, Rockford, IL, USA).

TGF-β Activity

Active TGF-β in cell co-culture supernatants were analyzed by ELISA using the $TGF\beta_1$ E_{max} ImmunoAssay System (Promega, Madison, WI, USA). Samples were diluted in PBS per manufacturer's instructions. Plates were coated with a monoclonal antibody specific for bioactive TGFβ, and samples were added to the wells along with a standard curve prepared using supplied $TGF\beta_1$ standard. Polyclonal anti-$TGF\beta_1$ antibodies were applied, followed by washing and incubation with horseradish peroxidase conjugate. Development solution was applied to the samples, and the OD readings were obtained at 450nm and compared to the TGFβ standard curve to find the concentration of the active form of TGFβ in each test sample, then normalized to cell count.

qRT-PCR

RNA was isolated using RNeasy mini kits (Qiagen, Valencia, CA, USA) and quantified using a Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA). Reverse transcription was performed on equal amounts of RNA utilizing Taqman Reverse Transcriptase Reagents (Applied Biosystems, Foster City, CA, USA) according to manufacturer's protocols. Quantitative real-time PCR was initiated utilizing Taqman gene expression arrays for murine *Tgfb*, MMP-9, and GAPDH using an ABI Prism 7000 (Applied Biosystems, Foster City, CA, USA).

ELISA assay

Concentrations of effector proteins of interest in cell culture supernatants were quantified by indirect ELISA. Samples were diluted in coating buffer and incubated at 4°C overnight for adherence to the ELISA plate. Wells were blocked, then incubated with antibodies specific to MMP-9 (Abcam, Cambridge, MA, USA) or fibronectin (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA), followed by an anti-rabbit HRP conjugated secondary antibody (Millipore, CA, USA). Wells were analyzed by OD reading at 450nm, using OptEIA detection reagents (BD, CA, USA). Readings were compared to a standard curve using MMP-9 or fibronectin recombinant protein.

Western Blot

Cell supernatants or cell lysates were run with β-mercaptoethanol, in denaturing conditions, on 10% SDS-PAGE gels, and then transferred to PVDF membrane at 100V for 1 hour. Membranes were blocked in 5% dry milk or bovine serum albumin (BSA), in the case of phospho-antibodies in TBS for at least four hours. Primary antibodies were diluted 1:1000 in 1% dry milk or BSA in TBS, and incubated overnight. Blots were

washed and incubated for two hours at room temperature in secondary-HRP conjugate at a 1:4000 dilution. Signal was detected using Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL, USA). Primary antibodies used were specific for Smad2 and pSmad2 (Cell Signaling Technology, Boston, MA, USA) and Collagen I and Collagen III (Abcam, Cambridge, MA, USA). Secondary antibodies used were goat anti-rabbit HRP conjugated (Millipore, CA, USA), and goat anti-mouse HRP conjugated (BD Pharmingen, San Diego, CA, USA).

Statistical Analysis

Results are reported as mean ± SD and compared using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Data were compared via one- or two-way ANOVA where appropriate, followed by Bonferroni's post-test for individual comparisons. Differences were deemed statistically significant at a p value <0.05.

C. Results

Arginase Activity

The first parameter analyzed in the co-culture system was arginase. Previously we have shown that azithromycin can polarize macrophages exposed to IFN-γ to display characteristics of alternative activation when stimulated with LPS isolated from *E. coli*.[112] We observed a significant increase in arginase concentrations in cell lysates after 24 ($p<0.01$) and 48 ($p<0.01$) hours of bacterial stimulation in cells treated with azithromycin, suggesting an increase in alternatively activated macrophage polarization (Figure 2.2a). Arginase concentrations predictably decreased when the organism was introduced, as this shifts macrophages toward a pro-inflammatory state. Exposure to azithromycin caused stabilization in this decrease in arginase production, so that by 24

hours the concentration of arginase rebounded to a significantly higher level compared to the control wells. Furthermore, when assessed with Trypan blue staining, there was no statistically significant impact upon cell number or viability between azithromycin-treated and the control cells (Figure 2.2b). Therefore, this suggests that azithromycin is able to polarize macrophages to an alternative-like phenotype in the presence of fibroblasts when stimulated with *P. aeruginosa*. This data confirms that the drug exerts similar effects on macrophage polarization using this co-culture design that we reported previously for J774 macrophages cultured alone. While the differences in arginase production are small, data discussed later in this chapter indicate the they are likely biologically relevant.

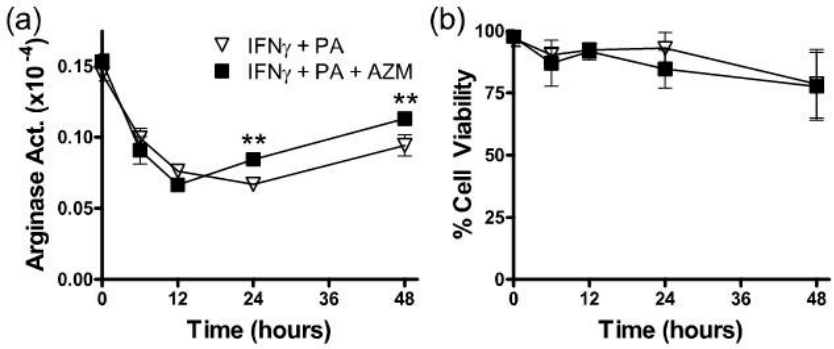

Figure 2.2. Azithromycin increases arginase production in macrophages when co-cultured with fibroblasts. NIH/3T3 and J774 cells were cultured together overnight with IFNγ with or without 30μM azithromycin (AZM), and stimulated the following day with *P. aeruginosa* (PA). (a) Cells were harvested at 0, 6, 12, 24, and 48 hours, and arginase activity, a measure of arginase concentration, was assessed in cell lysates. (b) Cell viability was determined using Trypan blue staining and manual counting. AZM does not significantly affect cell viability, and does not appear to be cytotoxic at 30μM. Samples were run in triplicate, and data is shown as mean ± SD. Data is representative of 3 replicated experiments, and was analyzed using one-way ANOVA with Bonferroni's post-test. ** indicates a p value of < 0.01. [124]

TGF-β

Because arginase and TGF-β have been correlated in models of pulmonary fibrosis, [41, 125] we measured active TGFβ concentrations in the co-culture supernatants, expecting an arginase-induced increase in production of TGFβ from the alternatively polarized macrophages. Supernatants from co-culture conditions were measured for TGF-β activity using the TGF-β_1 E_{max} ImmunoAssay System (Promega, Madison, WI, USA). Samples were run neat to determine endogenous active TGF-β and acid-treated, to determine the amount of total TGF-β present. Total concentrations were increased at 48 hours ($p < 0.01$) in cells treated with AZM (Figure 2.3a). Active TGFβ concentrations in the supernatants were increased in the azithromycin-treated group, Figure 2.3b, after 24 and 48 hours of stimulation as compared to baseline (with p values <0.05 and < 0.01 respectively). Cells treated with IFN-γ and LPS had concentrations of active TGFβ that fell below baseline by 48 hours after stimulation.

When the ratio of active/total TGF-β is plotted over time, AZM treated cells had higher ratios compared to controls at 12, 24, and 48 hour timepoints (Figure 2.3c). The difference over time comparing each treatment had an overall p value of 0.01. This shows that the TGF-β that is present is activated more readily in the cells that had the additional treatment of AZM.

In order to determine if AZM was only affecting activation, as opposed to production, of TGFβ, we additionally assessed *Tfgβ* gene expression via qPCR over time after *P. aeruginosa* stimulation (Figure 2.3d). Relative to time 0, *Tfgβ* mRNA expression at 6 hours increased to a higher degree in the azithromycin-treated cells than that observed in the absence of azithromycin. Following this, there was a down-regulation of *Tfgβ* expression in both groups. The azithromycin-treated cells maintained higher levels of mRNA throughout the experiment, although differences did not reach statistical significance. This result, in conjunction with higher activated TGFβ

concentrations (Figure 2.2b), indicates that azithromycin is likely affecting production of the protein, but does not rule out the possibility that in vitro activation is also influenced.

Because arginase and TGF-β have both been increased in other studies of AAM [45], we wanted to determine whether the effect on TGF-β activation was a result of the increased arginase production induced by AZM treatment. To do this we utilized the small molecule inhibitor BEC, which has the ability to inhibit arginase activity.[126] When BEC was added to the co-culture at a concentration of 500μM, total TGF-β concentrations were not changed by arginase inhibition (Figure 2.3e). However, activated TGF-β concentrations induced by AZM were reduced in cells treated with BEC (Figure 2.3f). As expected, the cells treated with IFN-γ, LPS, and AZM had increased concentrations of active TGF-β at the 24 hour timepoint, $p < 0.05$, as in the previous experiments. The result of this experiment confirms previous studies associating arginase and TGF-β in models of pulmonary injury and repair, although other studies have not employed the use of arginase inhibition. Altogether these data show that AZM alters TGF-β mRNA transcription as well as protein translation and activation, and that this is dependent upon the ability of AZM to increase arginase.

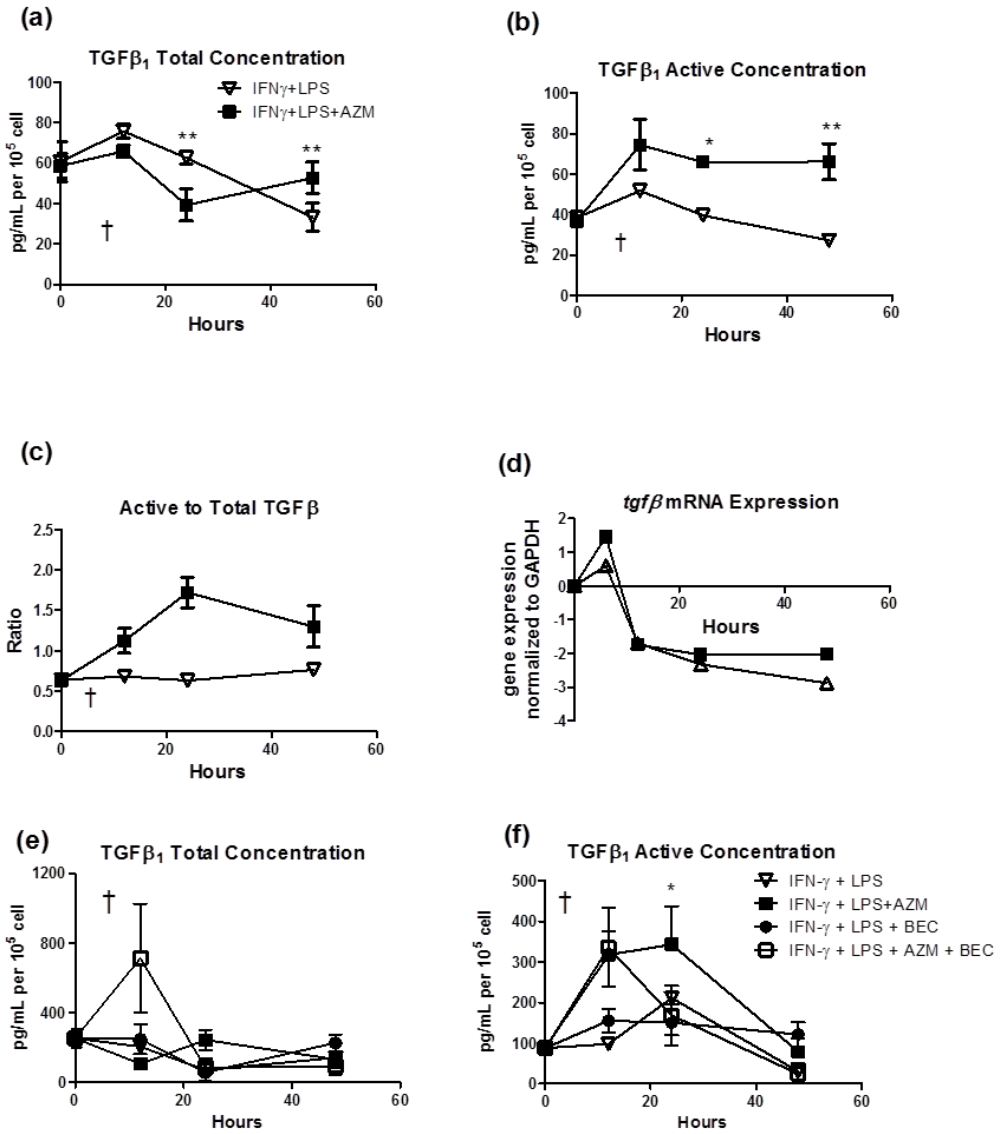

Figure 2.3. Azithromycin increases active TGF-β and *Tgfβ* mRNA in the co-culture system. Macrophages were co-cultured with fibroblasts and treated with IFNγ overnight then stimulated with LPS in the presence or absence of azithromycin (AZM). Cells and supernatants were collected at hours 6, 12, 24, and 48 after the addition of LPS. (a) Total TGFβ concentrations in the culture supernatant were measured by the TGF-β_1 E_{max} ImmunoAssay System. (b) Activated TGF-β concentrations in the culture supernatant as measured by TGFβ_1 E_{max} ImmunoAssay System are graphed over time for the azithromycin treatment and non-treatment groups. (c) Ratios of active to total TGF-β were determined, normalized as compared to time zero, and graphed over time. (d) qPCR analysis of *Tgfβ* mRNA was normalized to GAPDH expression in each sample, and then normalized to expression of *Tgfβ* at time zero. (e) Total TGF-β concentrations and (f) active TGF-β concentrations were measured in the same conditions as the upper graphs, but with the additions of the arginase inhibitor BEC. Data was analyzed using two-way ANOVA with Bonferroni's post-test. Significance is indicated for p values < 0.05 (*), < 0.01 (**), and < 0.001 (***), and p < 0.05 overall between groups (†).[124]

MMP-9

MMP-9 is a protease that functions as an effector important in the turnover of ECM through its ability to cleave fibronectin and collagen. It is also capable of directly activating TGF-β.[76, 77, 127] This is accomplished by cleaving away TGF-β from its latent protein while still bound to the matrix, at which point TGF-β is released for downstream initiation of fibrogenesis.[55] Further, MMP-9 is capable of directly degrading fibronectin, collagen III, and collagen IV.[76] Other macrolides, notably erythromycin, have been shown to cause reduced levels of MMP-9 protein production, making it important to study the effect of AZM on this molecule as well.[128] Therefore, we examined MMP-9 using our macrophage/fibroblast co-culture experiment. Macrophages and fibroblasts were co-cultured in the presence or absence of *P. aeruginosa*, azithromycin, and cytokine treatment (IFN-γ or IL-4/IL-13), and supernatants were collected 4 hours after exposure to the bacteria. Figure 2.4a shows that MMP-9 secretion was significantly increased at 4 hours by the addition of azithromycin ($p < 0.001$), even in the absence of cytokine treatment. Cells exposed to IFN-γ, *P. aeruginosa*, and azithromycin produced concentrations of MMP-9 that were significantly higher than cells treated with azithromycin alone ($p < 0.01$), the CAM control condition of IFN-γ plus *P. aeruginosa* ($p < 0.01$), and the AAM control condition of IL-4/13 plus *P. aeruginosa* ($p < 0.01$). To further examine the modulation of MMP-9 expression in the presence of azithromycin, the co-culture was then treated with conditions to induce a CAM phenotype (IFNγ plus *P. aeruginosa*), both with and without azithromycin, and analyzed over time. While cells began with similar levels of MMP-9 in the supernatants, by 12 hours the concentration had dropped to levels that would be maintained through 72 hours (Figure 2.5a). The steady-state level of MMP-9 was significantly higher in the cells treated with azithromycin. A similar trend was found when mRNA expression of MMP-9 was determined under these conditions at earlier timepoints. Azithromycin

treatment prevented the decrease in MMP-9 mRNA expression over time in the first 6 hours of cells treated with IFNγ and *P. aeruginosa* (Figure 2.5b). Taken together, these results suggest that while azithromycin eliminated the transient decrease in MMP-9 production induced by the bacterium, the production to degradation ratio of MMP-9 was thereafter maintained between the 2 conditions. We then examined the impact of azithromycin exposure upon MMP-9 secretion from each individual cell line. The drug dramatically increased the concentration of MMP-9 in the supernatant of the J774 macrophages, with a statistically significant difference at the 24 hour timepoint (Figure 2.5c). While production of MMP-9 from the fibroblast cell line was high (Figure 2.5d), results were inconsistent across experimental timepoints as to the impact of azithromycin directly on this cell type. These results suggest that the ability of azithromycin to act requires interaction between these 2 cell types.

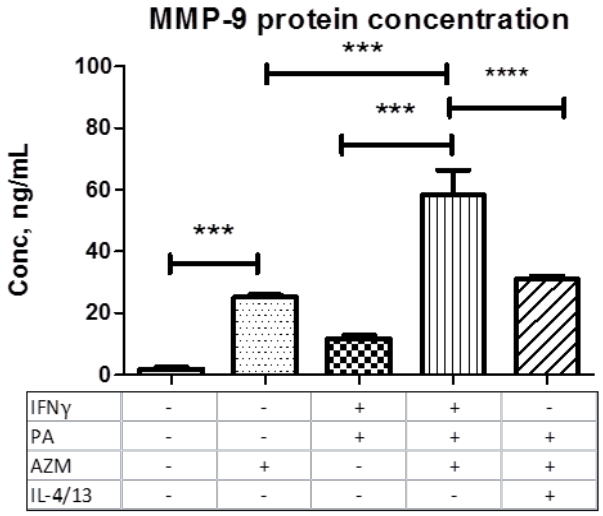

Figure 2.4. Azithromycin increases MMP-9 protein concentration. (a) Macrophages were co-cultured with fibroblasts and treated with INFγ, IL-4/IL-13, or media alone overnight. At time 0, azithromycin and/or *P. aeruginosa* (PA) were added, and the cells were incubated for 4 hours. Supernatants were collected to measure MMP-9 protein concentrations by indirect ELISA. Data was analyzed with one-way ANOVA with Bonferroni's post-test. Significance is indicated for p value < 0.001 (***).

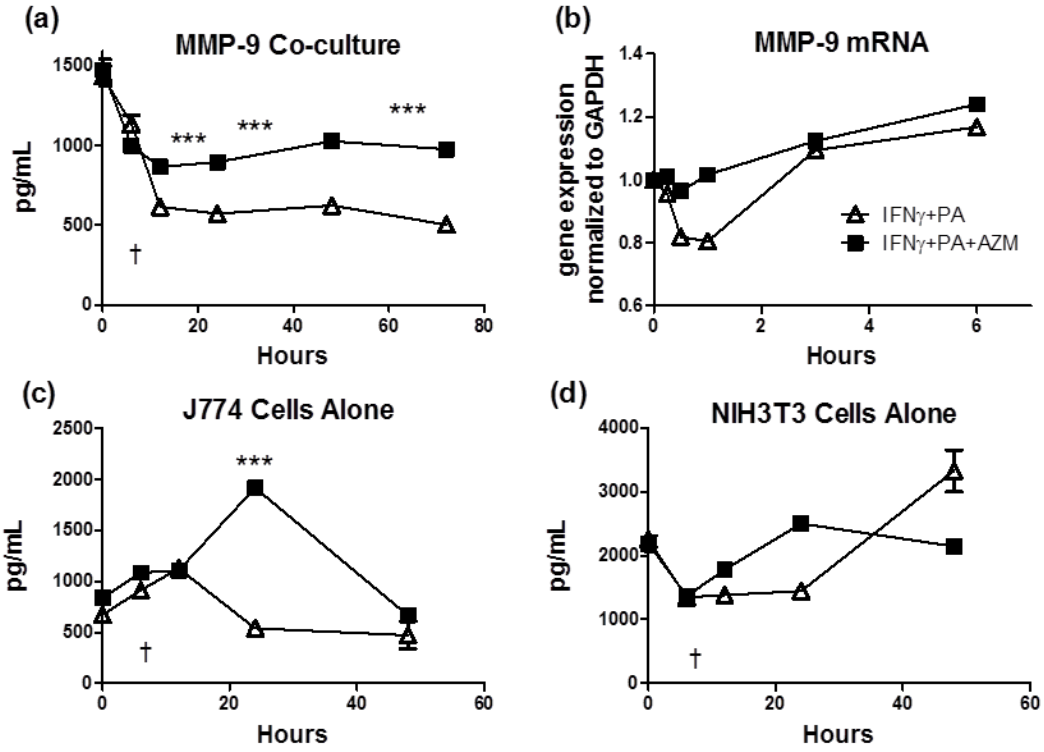

Figure 2.5. Azithromycin increases MMP-9 protein and mRNA expression. (a) Co-culture cells were treated with IFNγ overnight and stimulated with PA in the presence or absence of azithromycin (AZM). Cells and supernatants collected at hours 0, 6, 12, 24, and 48. Supernatants were analyzed for MMP-9 protein concentration via indirect ELISA. (b) Co-cultured cells were treated as in (a), but harvested at earlier timepoints to measure mRNA for MMP-9. Cells were collected at timepoints 0, 15 min, 30 min, 1 hour, 3 hours, and 6 hours after the addition of PA, to determine the impact of azithromycin treatment. Single cell controls were treated similarly to measure MMP-9 concentration in (c) J774 macrophages alone and (d) NIH3T3 fibroblasts alone. All data was analyzed with two-way ANOVA with Bonferroni's post-test. Significance is indicated for p values < 0.05 (*), < 0.01 (**), and < 0.001 (***), and p < 0.05 overall between groups (†).

Fibronectin

Fibronectin is a protein incorporated into the ECM during the process of fibrogenesis, remodeling, and scar formation. TGF-β has been shown in other models to increase the production of fibronectin [129]. Therefore, fibronectin concentrations were examined as an endpoint for ECM increase in the co-culture model. While fibronectin

was measured in the supernatant samples, it was found to be already incorporated into the ECM in the tissue culture plate, which was then spun down with the cell after tissue harvest. Fibronectin was therefore measured with the cellular lysates preparations. At specified timepoints, cells were harvested from tissue culture wells, pelleted via centrifugation, and then lysed. Fibronectin was then quantified by indirect ELISA and normalized to total protein concentration for each condition. Upon examination in the co-cultured cells over time, significantly higher fibronectin concentrations were observed at 12 and 24 hours after stimulation with LPS in cells exposed to IFN-γ plus azithromycin as compared to IFN-γ alone ($p<0.001$ and $p<0.05$, respectively, Figure 2.6a). Most of the fibronectin in the cultures was found aggregated within the cell pellet, as opposed to suspended in the supernatant, suggesting that fibronectin had already been incorporated into the matrix component in the extracellular portion of the wells. The drug had no effect on fibronectin concentration when either J774 or NIH/3T3 cells were separately cultured and stimulated with LPS (Figures 2.6b and c). This data suggests that azithromycin is affecting the control that the macrophages exert upon the fibroblasts in the model. Furthermore, the addition of BEC reduced the increase in fibronectin production induced by AZM (Figure 2.6d). This demonstrates that the increase in arginase caused by addition of AZM is stimulating fibronectin upregulation, likely through TGF-β activation.

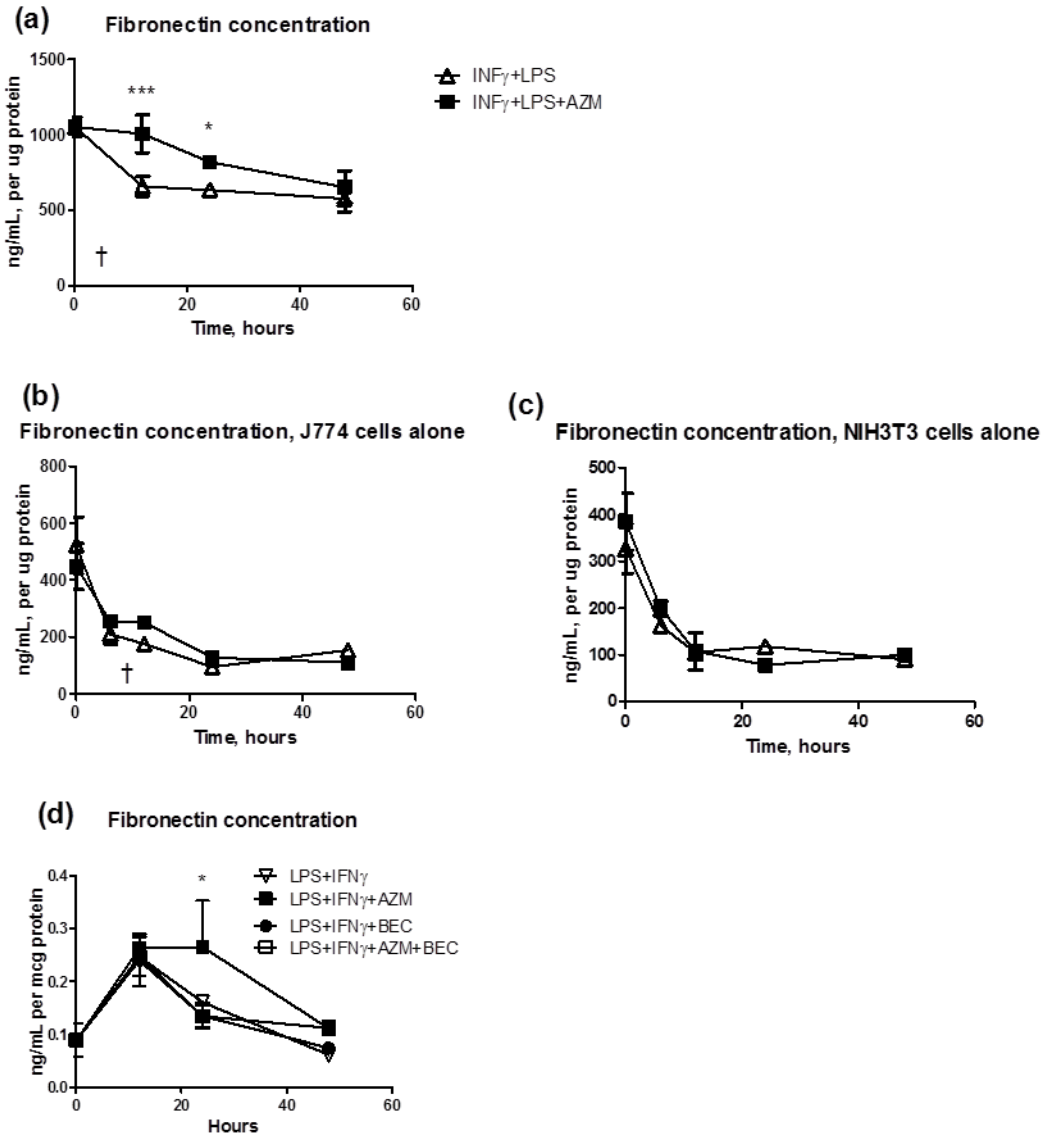

Figure 2.6. Azithromycin increases fibronectin concentration. Macrophages and fibroblasts were co-cultured and treated with IFNγ and LPS, with or without 30μM azithromycin (AZM) from time zero. Cells and supernatants were collected at hours 0, 6, 12, 24, and 48 hours after stimulation. (a) Fibronectin concentration in the cell pellet, as a measure of protein incorporated into the matrix, was determined by indirect ELISA and depicted over time. Macrophages (b) or fibroblasts (c) cultured alone and treated as in (a) with fibronectin concentration shown over time post-stimulation with LPS. Fibronectin concentration is also decreased with the addition of the arginase inhibitor BEC (d). Results are normalized to μg protein in each sample. All data was analyzed with two-way ANOVA with Bonferroni's post-test. Significance is indicated for p values < 0.05 (*), < 0.01 (**), and < 0.001 (***), and p < 0.05 overall between groups (†).[124]

TGF-β Neutralization

Because TGF-β has the ability to increase the production of ECM components, we hypothesized that the effect of AZM on fibronectin was caused by the drug's ability to increase active TGF-β concentrations. Therefore, we conducted a TGFβ neutralization experiment to determine if loss of activity would affect fibronectin production from the co-cultured cells. The anti-TGFβ antibody utilized for this purpose selectively binds to the activated form of the protein only. The neutralizing antibody was added to select wells of co-cultured cells at the same time as LPS. Fibronectin and MMP-9 concentrations were again assessed in the co-culture cell pellets and supernatants, respectively, over time. While fibronectin concentrations were increased by azithromycin at 24 and 48 hours compared to the control condition ($p < 0.001$ and $p < 0.05$, respectively, Figure 2.7a), the addition of anti-TGFβ antibody resulted in fibronectin concentrations that were nearly identical between the treatment groups, ablating the effect of the drug (Figure 2.7b). MMP-9 concentrations again decreased over time after stimulation with LPS, with azithromycin causing MMP-9 concentrations to stabilize at a higher level than that of the control condition (Figure 2.7c). The addition of the TGFβ-blocking antibody did not change the impact of azithromycin treatment on MMP-9 concentration, as the MMP-9 levels in the supernatants mirrored what was observed in the wells with functional TGFβ over time (Figure 2.7d). To ensure that the neutralizing antibody was indeed effective in blocking TGFβ function, we performed a Western Blot to determine the impact of neutralization on Smad2 activation. Smad2 is the main cell signaling molecule that is phosphorylated when TGFβ binds to its receptor. Indeed we observed that Smad2 phosphorylation was inhibited by the addition of the neutralizing antibody (Figure 2.7e). Taken together, these results suggest that the ability of azithromycin to impact MMP-9 concentration is independent of its effect on TGFβ activation, while the drug's ability to increase fibronectin concentration does rely on increases in TGFβ activation.

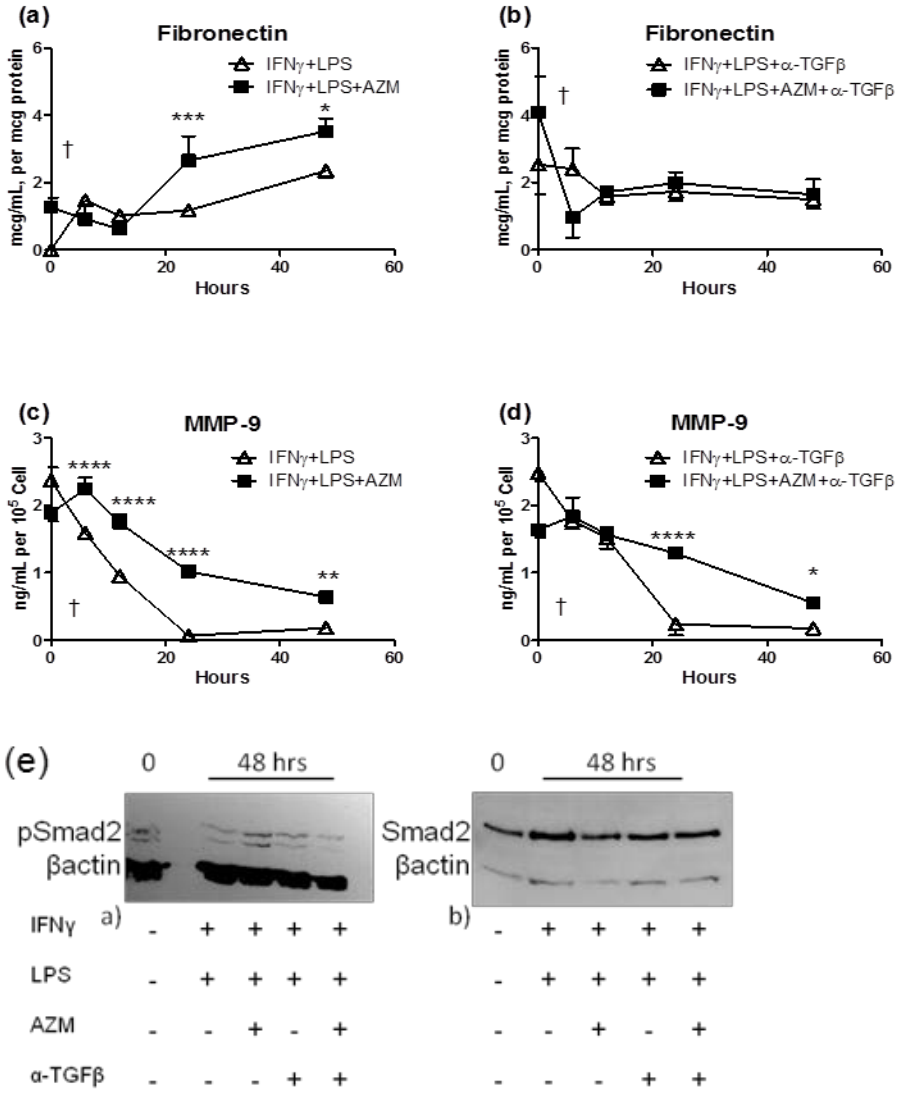

Figure 2.7. TGF-β neutralizing antibody reverses the ability of azithromycin to affect fibronectin but not MMP-9. Macrophages and fibroblasts were co-cultured, treated with INFγ, and stimulated with *P. aeruginosa* (PA), with or without azithromycin (AZM). Cells and supernatants were harvested over time to measure fibronectin with (a) and without (b) the addition of TGFβ neutralizing antibody by indirect ELISA. MMP-9 concentrations were measured in culture over time with (c) and without (d) the addition of TGFβ neutralizing antibody by indirect ELISA. (e) TGFβ-neutralizing antibody's effect upon phosphorylation of Smad2. Smad and pSmad expression at 0 and 48 hours of culture (with β-actin as a loading control) in cells co-cultured with LPS, IFNγ, and azithromycin. Significance is indicated for p values < 0.05 (*), < 0.01 (**), and < 0.001 (***), and p < 0.05 overall between groups (†).

Collagen

In addition to fibronectin, TGF-β can coordinate the production of the ECM protein collagen.[129] For that reason, we also examined the amounts of collagen I and III by Western blot to determine whether AZM-induced changed in TGF-β activity would influence collagen production in the co-culture. We hypothesized that AZM would have the same effect on the different forms of collagen, increasing their production as it did fibronectin. However, western blot analysis determined that collagen I was actually decreased by the addition of AZM when compared to cells that only received IFN-γ and LPS. Additionally, the TGF-β neutralizing antibody did not affect the signal detected (Figure 2.8a). Likewise, when AZM was added to the co-culture, collagen III concentration was not altered compared to cells that were treated with IFN-γ and LPS alone. However, when TGF-β neutralizing antibody was added to INF-γ, LPS, and AZM, the amount of collagen III was increased in cell lysates (Figure 2.8b), suggesting that TGF-β signaling may be causing a decrease in collagen protein production in the co-culture system.

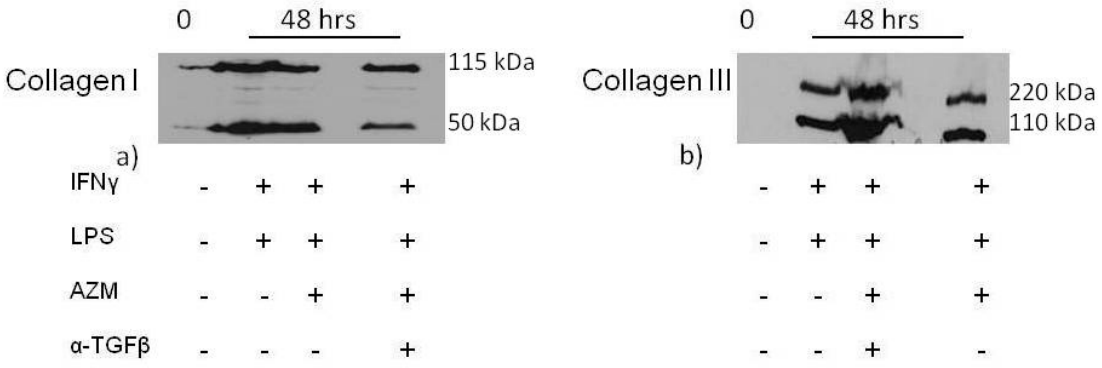

Figure 2.8. The effect of AZM and TGFβ-neutralizing antibody upon collagen I and collagen III expression. Western blots of collagen I (a) and collagen III (b) expression at 0 and 48 hours of culture in cell lysates co-cultured with LPS, IFN-γ, and azithromycin and TGF-β neutralizing antibody, as indicated below. Results are representative of multiple experiments.

MMP-9 Inhibition

Because the increase in MMP-9 concentrations was not dependent on TGF-β as demonstrated in the TGF-β neutralizing experiments, we hypothesized that AZM may actually be increasing MMP-9 first, which is then activating TGF-β directly. MMP-9 has been shown to have this ability. [127] In order to determine if this is the case, an MMP-9 inhibitor, SB3CT (Enzo Life Sciences, Farmingdale, NY, USA), was added to the co-culture at 600nM, which is a concentration specific for inhibiting MMP-9. [122] In these experiments, when the levels of active TGF-β were measured by the TGF-β_1 E_{max} ImmunoAssay System, there was no decrease in TGF-β activation with MMP-9 inhibition, as would be expected if our hypothesis was correct. In fact, TGF-β activation was even higher in those cells treated with the MMP-9 inhibitor added, at the 48 hour timepoint Figure 2.9b Further, fibronectin concentrations were decreased with the addition of the MMP-9 inhibitor, at 24 and 28 hours, ($p < 0.0001$ and < 0.001, respectively, Figure 2.9c). This is the opposite result expected, considering that activated TGF-β is increased in the same conditions, and data above has shown that fibronectin is directly dependent on TGF-β. The data shows that MMP-9 is not the major mechanism by which TGF-β is being activated in this co-culture system, but also suggests that MMP-9 may actually be involved in some signaling mechanisms whereby AZM is increasing production of fibronectin in concert with activated TGF-β.

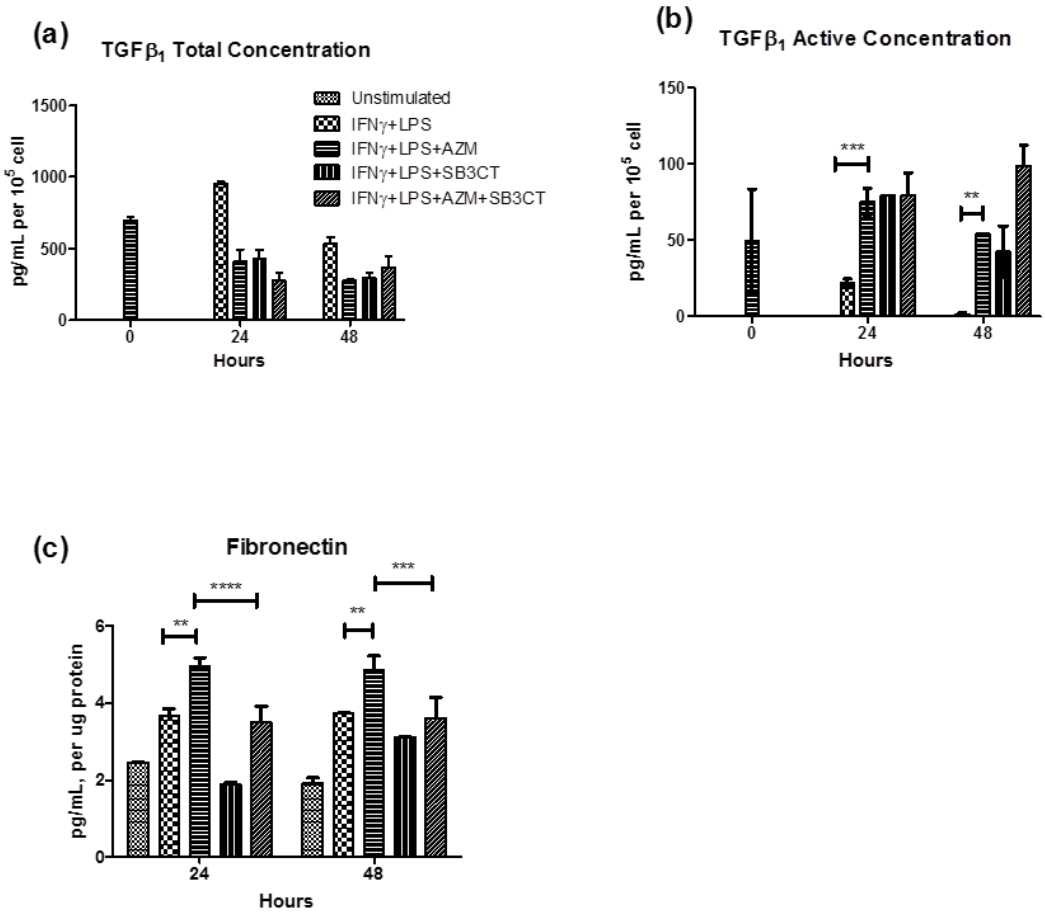

Figure 2.9. MMP-9 inhibitor reverses ability of azithromycin to increase fibronectin but does not inhibit activation of TGF-β. Macrophages and fibroblasts were co-cultured, treated with INFγ, and stimulated with LPS, in the presence or absence of azithromycin (AZM) 30μM. Cells and supernatants were harvested over time to measure fibronectin with (a) addition of the MMP-9 inhibitor SB3CT by indirect ELISA. Likewise, TGF-β activity was measured over time with (c) addition of the MMP-9 inhibitor SB3CT using a TGF-β_1 E_{max} ImmunoAssay System. Significance is indicated for p values < 0.01 (**),< 0.001 (***), and < 0.0001 (****).

IKKβ Inhibition

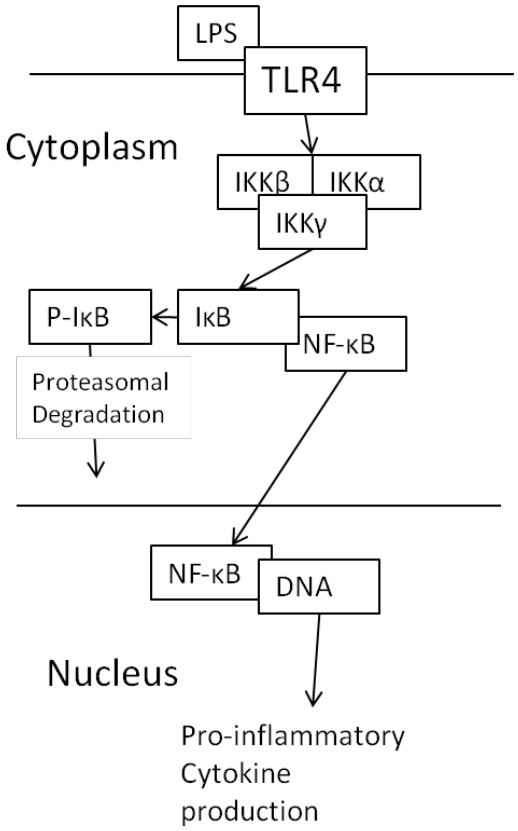

Figure 2.10. Activation of the NF-κB signaling cascade. LPS binds to TLR4, eventually leading to activation of the IKK complex. Upon activation of the IKK complex, it phosphorylates IκB, leading to the destruction of IκB. IκB is bound in its resting state to NF-κB, preventing it from translocating to the nucleus of the cell. When NFκB translocates to the nucleus, it binds to DNA leading to the transcription of genes associated with inflammation. [124]

While the experiments utilizing the TGF-β neutralizing antibody and the MMP-9 inhibitor identified fibrotic mechanisms affected by AZM, we also wanted to investigate the mechanism by which the drug affects macrophage polarization. It has been shown that AZM has anti-inflammatory affects through decreased NF-κB signaling and subsequent downregulation in inflammatory cytokine production.[101, 111] Yet these studies do not investigate the entire NF-κB signaling cascade. Previous work in our lab has shown that AZM alters multiple proteins in this signaling cascade. When TLR-4 is stimulated by LPS, IKKβ is phosphorylated, causing phosphorylation of IκB, which

ultimately allows NF-κB to translocate to the nucleus to initiate transcription of inflammatory cytokines (Figure 2.10). Treatment with AZM decreases NF-κB activation over time, while increasing IKKβ protein production. These changes are most dramatic at the 10min timepoint post LPS-stimulus, as detected by protein concentration on Western blot. Importantly, IKK-β has been demonstrated to exhibit cross-talk with the Stat-1 pathway, decreasing Stat-1 phosphorylation.[113] This could have important implications as to the reason why AZM alters the polarization of macrophages. Therefore, we hypothesized that this is the mechanism by which AZM is affecting macrophage polarization.

To confirm this hypothesis, we added the IKKβ inhibitor, IKK16, to macrophages and fibroblasts. IKK16 is a small molecule inhibitor with specificity to the beta form of IKK. Inhibition of IKKβ will result in inhibition of NF-κB downstream (Figure 2.10), by reduced degradation of IκB. Previously, we had identified a target concentration for IKK16. We had found that when the cells were treated with either IFN-γ and AZM, or IL-4 and IL-13, and stimulated with LPS, the addition of IKK16 blunted the increase in arginase production induced by AZM when added to cells treated with INF-γ and LPS. IKK16 was unable to block arginase upregulation in cells treated with IL-4/13, the traditional way to stimulate the alternative macrophage.[27] Therefore, IKK-β inhibition appears to be blunting the effects on arginase only through AZM, which strongly supports our hypothesis that IKK-β is involved in the mechanism by which AZM shift macrophage polarization.

To expand our investigation of this mechanism, we hypothesized that the inhibition of macrophage polarization by IKK16 would also inhibit downstream fibrosis development in the co-culture system. Therefore, the inhibitor was incubated with first fibroblasts alone, and then the cells in co-culture, with cells treated with IFN-γ, AZM, and

stimulated with LPS. Fibroblasts alone (Figure 2.11a) did not show an increase in arginase activity in response to AZM until after 48 hours of exposure. This was significantly different than the response from macrophages, which began to increase arginase production while exposed to AZM in response to LPS stimulation within 6 hours. However, the increase of arginase production in fibroblasts was blunted by the addition of IKK16. Co-culture cells (Figure 2.11b) had increases in arginase activity after 24 and 48 hours of incubation when treated with IFN-γ, LPS, and AZM compared to cells treated with IFN-γ and LPS alone; IKK16 added to cells treated with AZM achieved a statistically significant reduction in arginase activity at both 24 and 48 hours (all p values < 0.0001). This suggests that increasing IKKβ activation likely plays a role in the mechanism by which AZM is affecting these cell types.

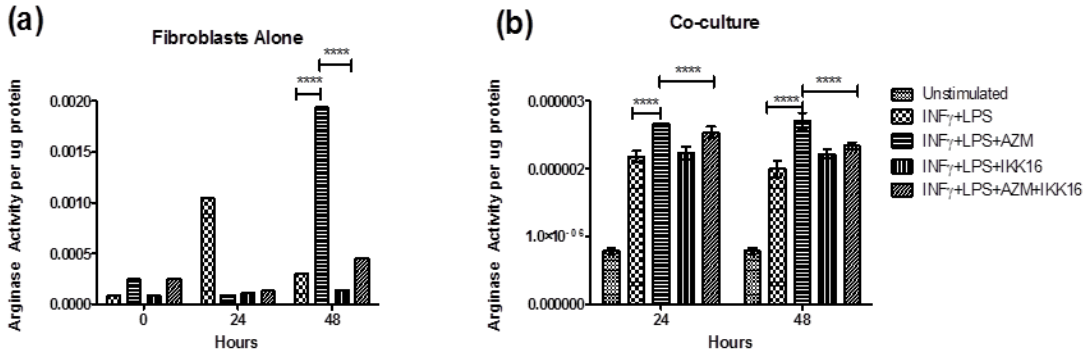

Figure 2.11. Arginase activity in the presence of an IKKβ inhibitor. NIH3T3 fibroblasts were cultured alone (a), and J774 macrophages were co-cultured with NIH3T3 fibroblasts (b), and both were stimulated overnight with IFN, AZM, and the IKKβ inhibitor IKK-16. Cells were then stimulated with LPS up to 48 hours. Cells were then lysed, and lysates were utilized for the arginase assay. Statistical analysis was performed with a one way ANOVA with Bonferroni's post-test. Arginase samples were run in triplicate, and data represents mean ± SD.

To determine if downstream markers of fibrosis were reduced by the inhibition of IKKβ, TGF-β activation, and MMP-9 and fibronectin concentrations were measured in the co-culture treated with IKK16. Both TGF-β total concentrations and activation were increased by addition of AZM to IFN-γ and LPS, but this effect of AZM was blocked at 24

hours (Figure 2.12a,b), by the addition of the inhibitor of IKK-β, compared to cells that were not exposed to inhibitor (Figure 2.12a,b), although this trend did not hold true at 48 hours. The concentration of MMP-9 was increased by AZM at 24 hours ($p < 0.0001$), an effect that was again blocked by IKK16. Although the level of MMP-9 was not increased by AZM at 48 hours compared to the control conditions, the concentrations of MMP-9 in the sample treated with AZM+IKK16 were lower than the concentration in the condition with only AZM ($p < 0.0001$). Fibronectin production was not increased by AZM until 48 hours of exposure, at which point the addition of IKK16 blunted the effect of AZM ($p < 0.05$). These data taken together suggest a mechanism by which AZM is acting through, but identifies a pathway by which this may be occurring, and reverses the effect by the addition of an inhibitor.

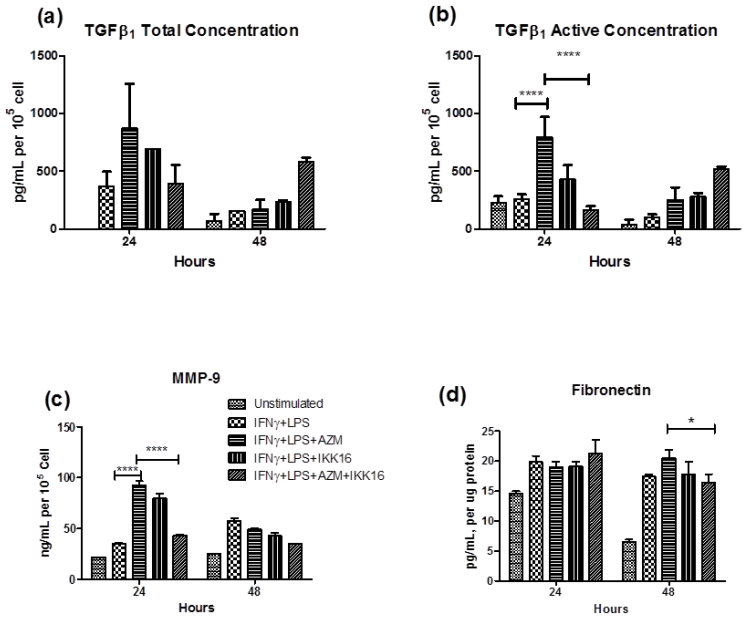

Figure 2.12. IKK16 inhibits TGF-β activation and MMP-9 and fibronectin increases induced by AZM. Macrophages and fibroblasts were co-cultured, treated with INFγ, and stimulated with LPS, in the presence or absence of azithromycin (AZM) 30μM. Cells and supernatants were harvested over time to measure TGF-β total (a) and active concentrations (b) by TGF-β_1 E_{max} ImmunoAssay System , and MMP-9 (c) and fibronectin (d) by indirect ELISA, with an without the addition of the IKKβ inhibitor IKK16. Significance is indicated for p values < 0.05 (*) and < 0.0001 (****).

D. Conclusions

Several important conclusions can be drawn from the data presented in this chapter. It had been demonstrated that AZM was able to polarize macrophages over to an alternative-like phenotype *in vitro*, as marked by an increase of arginase and mannose receptor.[112] Here we show that AZM-polarized cells also have the ability to function in the same manner as AAM by participating in stimulating the production of fibrotic proteins. Our hypothesis of how this occurs is depicted in Figure 2.13. When cultured with fibroblasts *in vitro,* AZM-polarized macrophages increase production and activation of TGF-β, in addition to increasing the production of the AAM effector protein arginase. These macrophages also appear to cause fibroblasts to increase production of fibronectin, an effect which appears to be dependent on the drug's impact upon TGF-β. This data is consistent with published reports of AAM in pulmonary repair and fibrosis, where these cells are correlated with increases in arginase, TGF-β, and fibronectin, with the novelty of the AZM-induction of these effects.[40-43] We have also demonstrated here a direct dependence of arginase production on TGF-β activation, which is a novel finding. However, the AZM-polarized cells are also increasing MMP-9 production, a result that was not expected (Figure 2.13). As shown by the TGF-β neutralization experiments, this is not dependent on TGF-β activation, whereas increased fibronectin production is. In fact, fibronectin shows a dependence upon TGF-β activity. This is demonstrated both when TGF-β is reduced indirectly, through the arginase inhibitor BEC, and directly, by a TGF-β neutralizing antibody. Because MMP-9 appears to be independent of these mechanisms, we hypothesized that MMP-9 may be participating in the activation of TGF-β. When MMP-9 itself is inhibited, the expected decrease in activated TGF-β did not occur, suggesting an alternate mechanism through which TGF-β is being activated in this co-culture system. However, fibronectin is also decreased when the MMP-9 inhibitor is added. This indicates that MMP-9 does have

some role in the increase in fibronectin caused by AZM treatment. Whether this effect is direct or indirect is unknown.

As discussed in Chapter 1, TGF-β signaling causes upregulation of fibrotic proteins from cells such as fibroblasts.[40, 130] Conversely, MMP-9 is able to degrade the same products to cause ECM turnover.[77, 85] Here, we show that AZM is causing both activation of TGF-β and increased production of MMP-9. Therefore, it is possible that AZM has caused a form of its own negative-feedback; in that MMP-9 is available to degrade excess fibrotic proteins produced by TGF-β signaling. While fibronectin concentrations are increased in the system, collagen concentrations are not, lending support to this theory. Furthermore, if increases in both TGF-β and MMP-9 lead to a homeostasis in the lung, rather than a shift to either fibrosis accumulation or excess damage, this may explain why patients with CF do well on AZM.[50, 102, 104]

Another surprising result from these experiments is that AZM does not appear to cause an increase in production of collagen I or III from the co-culture. Collagen I and III are two forms of collagen that are upregulated and secreted by epithelial cells, macrophages, and fibroblasts in response to pulmonary injury, and are often associated with fibrin and fibronectin in the phases of ECM remodeling.[56] Therefore, these collagens were expected to be affected in the same manner as fibronectin, which was found to be untrue. Indeed, collagen III is even increased, as measured by western blot, when the TGF-β neutralizing antibody is added to the cells treated with AZM. While the results regarding collagen were unexpected, this molecule will be of further study in Chapter 3.

The last conclusions to be made from these data concern the potential mechanism of AZM. We previously demonstrated that AZM increased IKK-β expression early after drug exposure (10min), while decreasing NF-κB expression at that same time. These results are interesting, because typically an increase of IKK-β is accompanied by

an increase in NF-κB activation, while AZM is causing changes in the activation of these molecules in opposing directions. It has been shown, by Fong, et al, that IKKβ can inhibit activation of the classical macrophage, by crosstalk through Stat1 pathway. [113] The Stat1 pathway provides signaling from IFN-γ to induce the markers of CAM, such as iNOS. Therefore, we hypothesized that AZM may be increasing IKKβ, which is inhibiting the activation of the CAM in order to allow the alternative-like phenotype to take its place. We are currently evaluating the effect of AZM upon Stat-1 phosphorylation in our *in vitro* experiments.

We have previously demonstrated that inhibiting IKK-β with IKK16 caused a reversal of the effect AZM on arginase activity in the macrophages. However, these data show that IKK16 not only reverses AZM's effect on arginase activity in macrophages, but also in fibroblasts alone and co-cultured with macrophages. Further, IKK16 reverses AZM's effect on TGF-β activation, as well as increases in MMP-9 and fibronectin concentrations. This indicates that IKK-β may play a role in AZM-induced macrophage polarization and the resultant increase in fibrotic mediators (Figure 2.13).

The data generated in these experiments become important for further analyses. If patients with CF have an increase in this type of macrophage when they are treated with AZM, they might also have increases in the pro-fibrotic protein production which could increase pathologic progression. Alternatively, the suppression of the CAM phenotype may prevent some of the hyperinflammation inherent in the disease, which may be beneficial. However, it is unknown how these pro-fibrotic changes may develop in the presence of multiple other cell types. Therefore, the next chapter will study AZM treatment in the setting of *P. aeruginosa* infection *in vivo.*

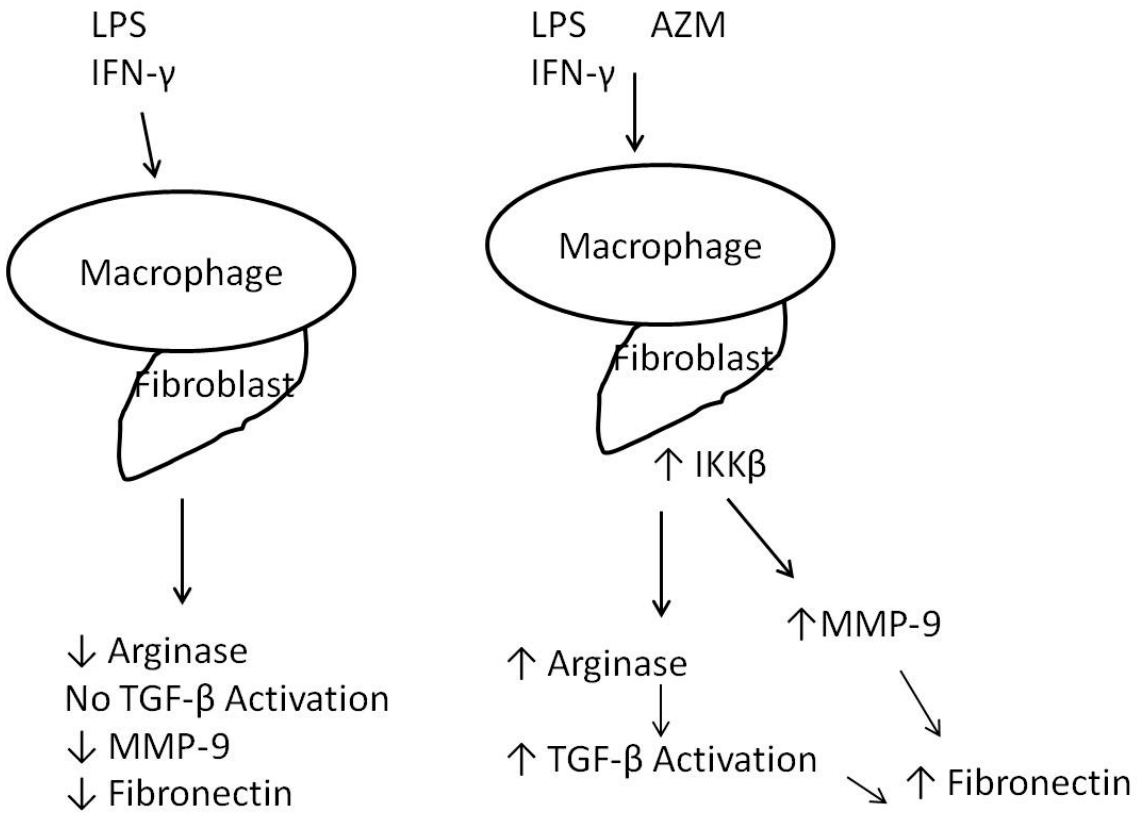

Figure 2.13. Hypothetical schematic of the production of the fibrotic mediators from macrophages co-cultured with fibroblasts with and without treatment with AZM.

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Chapter 3: Azithromycin alters fibrotic response to *P. aeruginosa* infection

A. Introduction

While the experiments outlined in Chapter 2 established that AZM-polarized macrophages can affect fibroblasts and cause increases in production of fibrotic mediators, it is also important to understand how AZM-polarized macrophages influence other components of the immune system in response to infection with *P. aeruginosa.* In order to examine the effects on macrophage phenotype, fibrotic mediators, and pulmonary damage, a murine infection model was utilized.

Previous experiments with the murine model demonstrated that AZM has similar effects on the macrophages *in vivo* as it did *in vitro.* When C57Bl/6 mice were pre-treated with methylcellulose vehicle or AZM and infected with *P. aeruginosa*, the AZM group had higher survival rates, compared to control treated mice, although body weight and bacterial burden were not significantly different.[114] In these experiments, macrophages were also found to show higher surface expression of mannose receptor and lower surface expression of CD80 in AZM treated mice 7 days after infection, indicating a shift toward the alternative phenotype. This was confirmed with increased arginase activity at the same timepoint in AZM treated mice. Lastly, histological examination determined that treatment with AZM shifted the infiltrating cellular response to infection from neutrophilic (in the control mice) to monocytic. This is significant, because, as mentioned previously, CF is a neutrophilic-mediated inflammatory disease. Furthermore, the switch to a monocytic response was concurrent with peak bacterial burdens.[114]

Previous work in our lab established infection models with this bacterium in multiple strains of mice. In experiments to determine the proper infectious dose of bacteria, mice were infected with CFUs ranging from 10^5 to 10^{10}. C57Bl/6 mice bias

toward a Th-1 cytokine response to infection and are generally more susceptible to the generation of fibrosis. [131] These mice responded to infection with *P. aeruginosa* with a 50% lethal dose (LD_{50}) of about 10^6 CFUs. In contrast, BALB/c mice have a bias toward producing Th-2 cytokines in response to infection and are typically more resistant to fibrosis development. [131] Likewise, they were also more resistant to the *P. aeruginosa* infections. All of the BALB/c mice infected with CFUs up to 10^9 survived, yet all mice infected with CFUs of 10^{10} succumbed, showing a more resistant phenotype, as reflected by the high LD_{50}. The third strain of mouse used was chosen as part of our investigations into AZM mechanism; the IL-4rα$^{-/-}$ mouse strain. This genetic knockout mouse lacks part of the IL-4 receptor in immune cells, and is unable to respond to either IL-4 or IL-13, as both cytokine receptors require the IL-4rα domain to function. This strain was bred onto a BALB/c background, however the IL-4rα$^{-\backslash-}$ mice responded to infection with *P. aeruginosa* much more similarly to the C57Bl/6 – with an LD_{50} in the range of 10^6. These mice also lose the resistance to fibrosis that the BALB/c strain demonstrates, suggesting that IL-4 and IL-13 signaling, perhaps through the AAM, plays a role in preventing fibrosis development. This suggests that activity of alternative macrophage activation is protective upon challenge with this extracellular pathogen. Because of the phenotypic similarities of the IL-4αr$^{-/-}$ strain when infected, C57Bl/6 mice were used as the control mice for the knockout strain in the experiments described here.

Addition of AZM to the co-culture increased production of the pro-fibrotic proteins arginase, TGF-β, and fibronectin (Chapter 2). Additionally, production of the protease MMP-9 was increased by AZM as well. These data show that AZM is capable of polarizing to an alternative-like, pro-fibrotic phenotype. The increase in MMP-9 levels, however, identifies this phenotype as distinct from the traditional AAM phenotype, and may be one reason that AZM has a positive effect on patient outcomes. Previous work has also established that AZM is capable of polarizing the macrophage to the alternative

phenotype *in vivo.* However, the impact that the AZM-polarized macrophage exerts upon the resulting fibrotic process in the mouse model has yet to be determined. Because of the increased concentrations of arginase, TGF-β, MMP-9, and fibronectin found *in vitro*, the same markers were evaluated in mice. To replicate chronic therapy in the CF patient, mice were pre-treated with AZM, and treatment was continued throughout the course of the infection, so that the AZM-polarized macrophages should persist. The aim of this chapter is to determine how the AZM-polarized macrophages affect fibrosis in a pulmonary model of infection. We hypothesize that AZM-induced alternative macrophage polarization in mice infected with *P. aeruginosa* promotes fibrosis development in the lungs.

B. Methods

Mice

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). BALB/c-Il4ratm1Sz/J strain, homozygous for the *Il4ra*^{tCAMSz} targeted mutation, was bred in-house. IL4rα is a common chain shared by IL4r and IL13r, and is required for transmitting signal from both cytokines.[132, 133] All animal studies were approved by the University of Kentucky Institutional Animal Care and Use Committee. Mice were housed under conditions of pathogen free isolation and were transferred to a biosafety level 2 housing unit after infection, at which time all mice were within 5 to 7 weeks of age.

Azithromycin Dosing

Tablets of AZM (Pliva Inc., Zagreb, Croatia) were triturated and the powder was suspended in 2% methylcellulose. Beginning 4 days before infection, mice were given

AZM at 0.16 g/kg of body weight in 150 µl or vehicle only via oral gavage in order to reach a steady-state concentration of drug exposure at the time of infection.[114] AZM and vehicle administration was continued once daily until the time that the mice were killed. Groups of three to six mice per time point per group were used.

Intratracheal Infection

The clinically derived mucoid strain *P. aeruginosa* M57-15 was utilized in these experiments. The bacteria were grown in Trypticase soy broth (TSB) to mid to late log phase before being incorporated into agarose beads.[134, 135] Utilizing growth curve information and the optical density of the culture, we established the appropriate starting number of bacteria required to produce a bead preparation that would result in an inoculum that fell within the desired range at the time of infection.[114] Bacteria were mixed with agarose at a temperature of 55°C. That mixture was then added to rapidly stirring and frothing mineral oil at a temperature of 50°C. The mineral oil/bacterial/agarose mixture was rapidly cooled by adding ice to create formation of bacteria-filled agarose beads. Beads were washed with PBS and allowed to settle for retrieval of beads in the range of 10-100µm. The beads were immediately homogenized, and the number of CFU was determined after overnight growth on Trypticase soy agar. The *P. aeruginosa* laden agarose beads were then diluted to achieve the desired inoculum for each strain infected. The beads were instilled intratracheally by using a blunted 24-gauge curved inoculation needle while the animals were under isoflurane anesthesia. To confirm that the mice received the desired inoculum, an aliquot of the bead preparation used was homogenized and plated on *Pseudomonas* selection agar immediately after infection and the numbers of CFU were counted after overnight incubation.

Tissue Harvest

The mice were humanely euthanized on day 0 and on post-infection days 3, 7, and 14. Bronchoalveolar lavage was performed to obtain a representative sample of the airway compartment. The lungs were lavaged with 5 ml of buffered solution containing 0.3 mM EDTA in 1-ml aliquots. The first 1 mL, called "first wash," was collected separately and centrifuged, and the supernatant was saved and frozen at -80°C for cytokine measurements. Pelleted cells from the first washes were added back to the remainder of each lavage fluid sample. The lungs were removed, with lobes saved for histological analysis. The remainder was digested in RPMI medium containing 5% heat-inactivated fetal calf serum with 1 mg/mL collagenase A and 50 U/mL DNase for 1 h at 37°C. The lung fragments were then pushed through 70 μm mesh screens to create a single cell suspension, and the red blood cells were lysed in a hypotonic solution. These preparations were analyzed as the lung interstitium. An aliquot was plated to assess the bacterial burden by manual counting of the numbers of CFU on *Pseudomonas* selection agar. *Pseudomonas* specific agar was utilized to avoid contamination from the upper airway flora. A second aliquot was saved in RIPA buffer for later protein analysis.

Adoptive Transfer

Bone marrow-derived monocytes and splenic T cells from BALB/c mice were purified using negative selection, with magnetic beads (Stem Cell Technologies, Vancouver, BC, Canada). Cell numbers transferred and timing of transfer were chosen based on preliminary experiments. Mice were anesthetized and 1 x 10^6 T cells or 1 x 10^6 T cells plus 1 x 10^6 monocytes were injected retro-orbitally one day before infection.

Flow cytometry

Surface protein expression and intracellular cytokine secretion were characterized by flow cytometry. Lavage and digest cell aliquots were incubated with combination panels of fluorescently labeled antibodies (Abs) for CD11b, CD11c, GR1, and CD4. The cells were washed thoroughly and resuspended in phosphate-buffered saline for analysis. The labeled cells were analyzed with an LSRII flow cytometer system (BD Biosciences), and 50,000 events were routinely acquired per sample. In figures in which cell numbers are given, the percentage of the gated cell subset was multiplied by the number of cells manually counted in each sample. FlowJo software (Tree Star, Ashland, OR) was used to analyze the data.

ELISA assay

Cell culture supernatants were analyzed and the MMP-9 and fibronectin concentrations quantified by indirect ELISA. Samples were diluted in coating buffer and incubated at 4°C overnight for adherence to the ELISA plate. Wells were blocked, then incubated with an antibody specific to MMP-9 (Abcam, Cambridge, MA, USA) and fibronectin (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA), followed by an anti-rabbit HRP conjugated secondary antibody (Millipore, CA, USA). Wells were analyzed by OD reading at 450nm, using OptEIA detection reagents (BD, CA, USA). Readings were compared to a standard curve using MMP-9 or fibronectin recombinant protein.

Histopathology

The mouse lung tissue was excised and immediately fixed in 10% buffered formalin. After an overnight incubation at 4°C, sections were washed three times with 0.1 M phosphate buffer and were then transferred into 2 ml sterile 20% sucrose (pH 7.2)

and stored at 4°C. The samples were then transferred into optimal cutting temperature (OCT) embedding media and incubated at 4°C for 24 h. The fixed cryoprotected tissues were sectioned at a thickness of 10μm (Thermo Fisher Scientific cryostat) and mounted on slides to dry overnight. The slides were stained with hematoxylin-eosin (H&E), Van Gieson TriChrome, or immunohistochemistry, by standardized protocols, and photographs were taken with an AxioCam HRc camera mounted on a Zeiss Imager.Z1 microscope.

Immunohistochemistry

Slides were washed with PBS+0.02% TritonX-100 (PB:X) for 10 minutes to wash OCT mounting media. When media washed off, slides were blocked with 5% normal goat serum (NGS) + 5% bovine serum albumin (BSA) in PBS+0.02% TritonX-100 for at least 1 hour at room temperature. Primary antibody was added in 1% NGS & 1% BSA in PB:X overnight at 4°C, then washed off with PB:X. Secondary antibody was added in 0.5% NGS & 0.5% BSA in PB:X at a 1:160 dilution for 1 hour at room temperature, and subsequently washed off. Slides were mounted with 20% glycerol and taken to the microscope within one hour. Pictures were obtained using an AxioCam HRc camera mounted on a Zeiss Imager.Z1 microscope. Primary antibodies used were specific for MMP-9 (Abcam, Cambridge, MA, USA), fibronectin (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA), arginase-1 (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA), and iNOS (Cayman Chemical, Ann Arbor, MI, USA). Secondary antibodies used were goat-anti rabbit IgG-FITC (Sigma Aldrich, St. Louis, MO, USA), and goat-anti rabbit IgG-Texas Red (Abcam, Cambridge, MA, USA). Intensity of staining was quantified using the program ImageJ (National Institutes of Health). All images for each mouse were averaged, and the median fluorescence intensity is represented graphically.

TriChrome

Slides were stained with Weigert's hemotoxylin for 3 minutes, washed, and followed by Van Gieson's solution staining, consisting of 0.05% acid fuschin in saturated aqueous picric acid. Slides were then dried with ethanol and cleared with xylenes to remove excess dye. Twenty-four hours after being coverslipped, images were obtained as described above.

Pathology Scoring

All slides were assessed for severity of four markers of lung damage; peribronchiolitis, bronchitis, alveolitis, and interstitial pneumonitis. Peribronchiolitis is identified as inflammatory cells surrounding a bronchiole; bronchitis occurs when the inflammatory cells are within the epithelium of the bronchial epithelium; alveolitis is the presence of inflammation within the alveolar spaces; and interstitial pneumonitis is defined as increased thickness of the alveolar walls, due to inflammatory cells or accumulation of fluid. Damage was scored on a scale of 0 to 4, with 4 being the maximum inflammation and 0 being no inflammation.[136] Scores were averaged for each marker, and graphed for statistical analysis in GraphPad Prizm (GraphPad Software, La Jolla, CA, USA).

Statistical Analysis

Results are reported as mean ± SD, except where indicated, and compared using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Data were compared via one- or two-way ANOVA where appropriate, followed by Bonferroni's post-test for individual comparisons. Differences were deemed statistically significant at a p value <0.05.

C. Results

AZM alters macrophage infiltration

C57Bl/6 mice were dosed with AZM and then infected with *P. aeruginosa* to analyze the infiltration of immune cells into both the lung and the airway spaces. Cells were analyzed by flow cytometry for markers identifying infiltrating macrophages (CD11b+), resident alveolar macrophages (CD11c+), neutrophils (CD11b- GR1+), and T cells (CD4+). Importantly, macrophage numbers were increased at day 7 in the lavage samples in mice treated with AZM compared to controls, and the neutrophils in the lung digest had a corresponding decrease in the AZM group (Figure3.1a, b). CD11c+ was used to identify resident alveolar macrophages, although this marker is also on dendritic cells[137]; these cells were unchanged in either the lung digest or in the lung lavage. CD4+ T cell numbers were increased at day 7 in the lung lavage as well as macrophages in mice who received AZM. This is followed by an increase in T cells at day 14 in the lung digest in the same group of mice. Therefore, in addition to previous data showing that AZM is altering the phenotype of the macrophages *in vivo* to the AAM [138], the drug is also increasing the presence of the infiltrating macrophages and T cells as infection is beginning to resolve.

However, in the IL-4rα$^{-/-}$ mice, AZM had different effects on cellular infiltration. Total cell numbers were decreased in mice that received AZM at day 3 compared to the control group. At the same timepoint, infiltrating macrophages, identified as CD11b+ cells, were decreased in both lung digest and lavage (Figure3.1c, d). There were no differences between the treatment groups in any of the other cell types, including neutrophils. It is important to remember that the T cells in these mice are also affected by the IL-4rα$^{-/-}$ mutation, and this may be why there were no differences between the groups. Nevertheless, while the IL-4rα$^{-/-}$ mice do not maintain the neutrophilic response to infection, the mice still show increased monocytic infiltrates with AZM treatment.

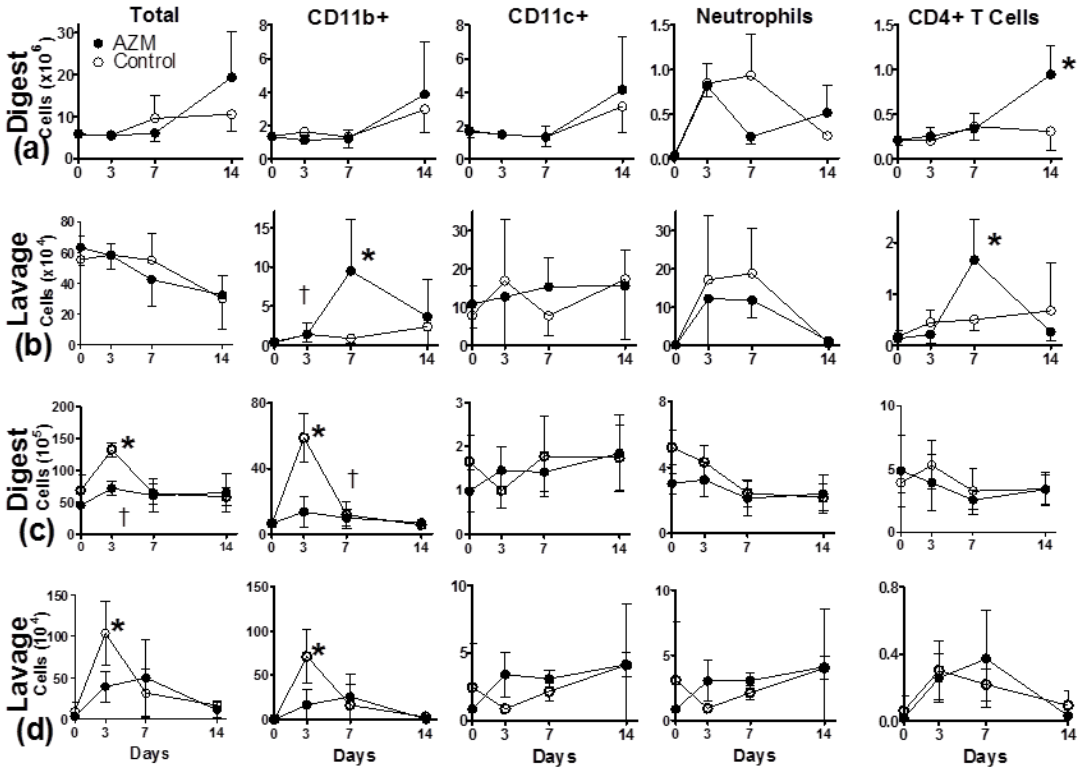

Figure 3.1. Immune cell infiltration in to the lungs. C57Bl/6 or IL4rα$^{-/-}$ mice, 4 per timepoint per treatment group, were infected with agarose beads containing 1 x 10^5 CFU of *P. aeruginosa* intratracheally and dosed with either AZM or methylcellulose vehicle. Immune cells were analyzed by flow cytometry in lung digest (a) and lung lavage (b) samples of C57Bl/6 mice and digest (c) and lavage (d) samples of IL-4rα$^{-/-}$ mice. Data is representative of 3 replicated experiments. Mean ± SD are reported, with $p < 0.05$ for the timepoint (*) and overall between groups (†).
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Arginase and iNOS Expression

In order to determine the impact of AZM treatment upon kinetics of AAM and CAM in the lungs infected with *P. aeruginosa*, and to address whether the drug elicits the same effect in the absence of IL-4 and IL-13 signaling, sections from lungs were stained by immunohistochemistry for arginase (FITC) and iNOS (Texas-Red). The intensities of the slides were measured by ImageJ (NIH), and median fluorescence intensity is represented graphically for each group. Representative slides are shown in panels Figure 3.2a and Figure 3.2d. WT mice showed increased expression of arginase

at day 3 in the AZM treatment group compared to control ($p < 0.0001$, Figure 3.2b), and a decrease of iNOS expression in the AZM treatment group compared to control at days 0 and 3 ($p < 0.01$, Figure3.2c). This indicated a reduction of the inflammatory effector protein both four days after AZM treatment began (day 0) and at peak of infection (at day 3). In contrast, IL4rα$^{-/-}$ mice had no differences in arginase expression in the AZM treatment group compared to vehicle control at any day post-infection (Figure 3.3b). IL4rα$^{-/-}$ mice treated with AZM had similar iNOS expression kinetics compared to vehicle control, until day 14 - at which point iNOS remained upregulated in the controls, and was downregulated in the treatment group ($p < 0.01$, Figure 3.3c). Overlays of day 14 are represented in Figure 3.4, which show co-staining and some co-localization of arginase and iNOS in the control group of the IL4rα$^{-/-}$ mice, but no co-localization in the AZM treatment group of the same mice.

Lastly, the lack of difference in arginase production in the IL4rα$^{-/-}$ mice suggested that while baseline levels of arginase were higher than those observed in the C57Bl/6 mice, the production was not changed by either infection or administration of AZM (Figure3.3c). Upon further analysis, arginase appeared to be produced by cells other than macrophages. Those sections were stained for both arginase and F4/80, a marker of macrophages. Indeed, we found that arginase appeared to be more prevalent than F4/80, especially at day 7, which is represented below (Figure 3.5). This demonstrates that macrophages are not the only producers of arginase in the lung in our model.

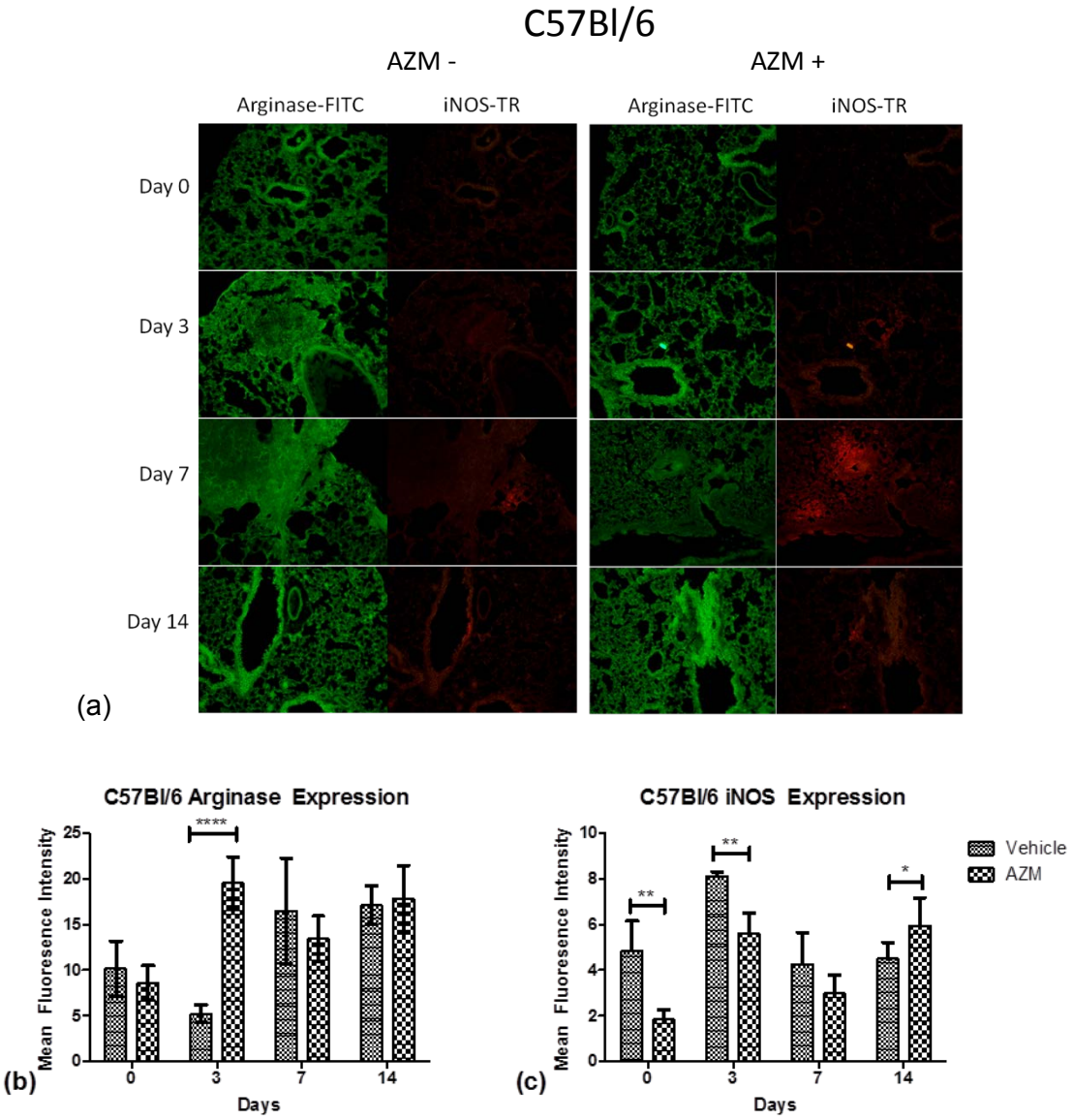


Figure 3.2. Arginase 1 and iNOS expression in C57Bl/6 mice treated with methylcellulose vehicle or AZM. Four mice per treatment group per timepoint were infected with agarose beads containing 1.5 x 10^5 CFU of bacteria. Lung sections from mice 0, 3, 7, and 14 days post infection were stained with primary antibodies to both arginase 1 and iNOS, with either FITC or texas red secondary staining. Representative slides of at least 3 replicates from C57Bl/6 controls in (a), with mean fluorescence intensity quantification of arginase (b) and iNOS (c). Samples were run in triplicate. Significance is indicated for p values < 0.05 (*), < 0.01(**), and < 0.0001(****).

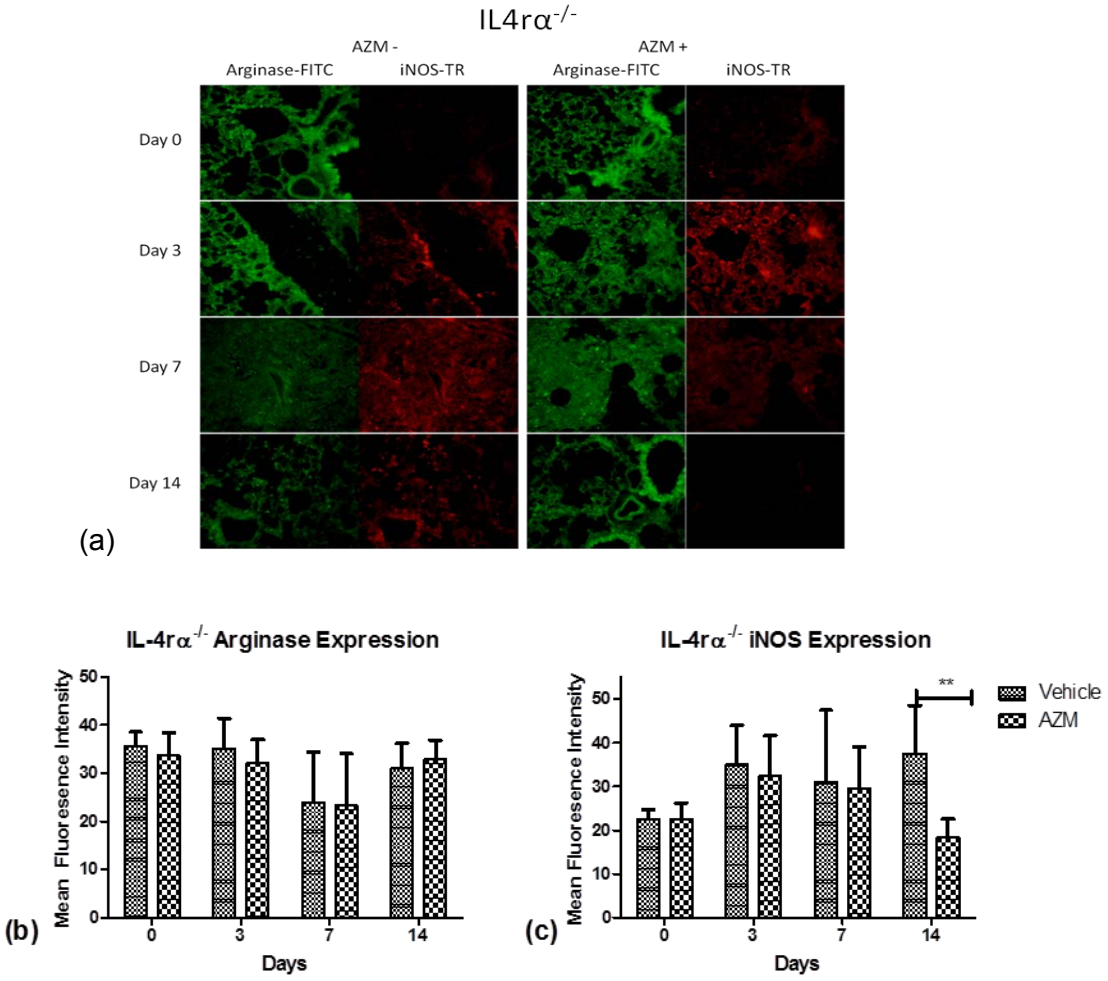


Figure 3.3. Arginase 1 and iNOS expression in IL-4rα$^{-/-}$ mice treated with methylcellulose vehicle or AZM. Four mice per treatment group per timepoint were infected with agarose beads containing 1.5 x 10^5 CFU of bacteria. Lung sections from mice 0, 3, 7, and 14 days post infection were stained with primary antibodies to both arginase 1 and iNOS, with either FITC or texas red secondary staining. Representative slides from IL-4rα$^{-/-}$ mice in (d), with mean fluorescence intensity, ± SD, quantification of arginase (e) and iNOS (f). Localization of arginase and iNOS in IL4rα$^{-/-}$ mice at day 14 (g). Panels represent arginase 1 localization, iNOS localization, and merged data, from left to right. Samples were run in triplicate. Significance is indicated for p values < 0.05 (*), < 0.01(**), and < 0.0001(****).

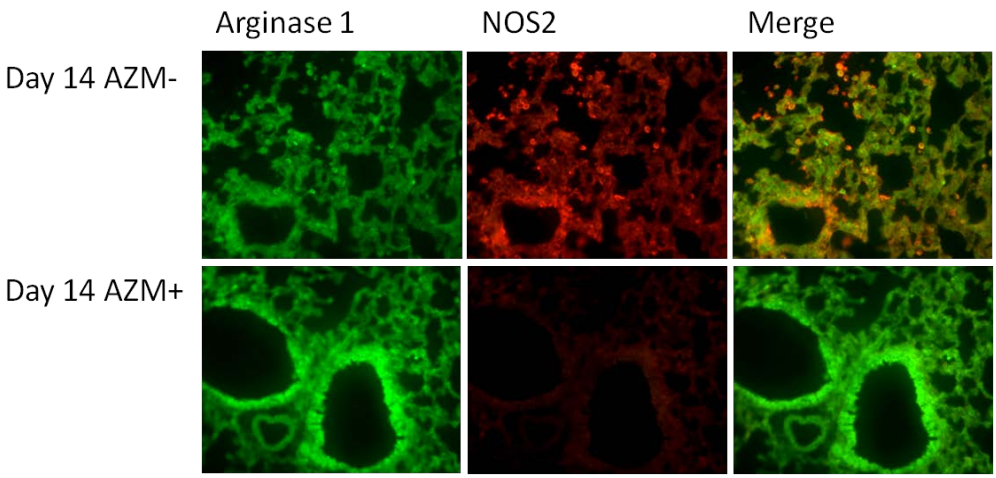


Figure 3.4. Arginase 1 and iNOS co-expression in IL-4rα$^{-/-}$ mice treated with methylcellulose vehicle or AZM. Four mice per treatment group per timepoint were infected with agarose beads containing 1.5 x 10^5 CFU of bacteria. Lung sections from mice 0, 3, 7, and 14 days post infection were stained with primary antibodies to both arginase 1 and iNOS, with either FITC or texas red secondary staining. Localization of arginase and iNOS in IL4rα$^{-/-}$ mice at day 14 (g). Panels represent arginase 1 localization, iNOS localization, and merged data, from left to right. Samples were run in triplicate. Significance is indicated for p values < 0.05 (*), < 0.01(**), and < 0.0001(****).[124]

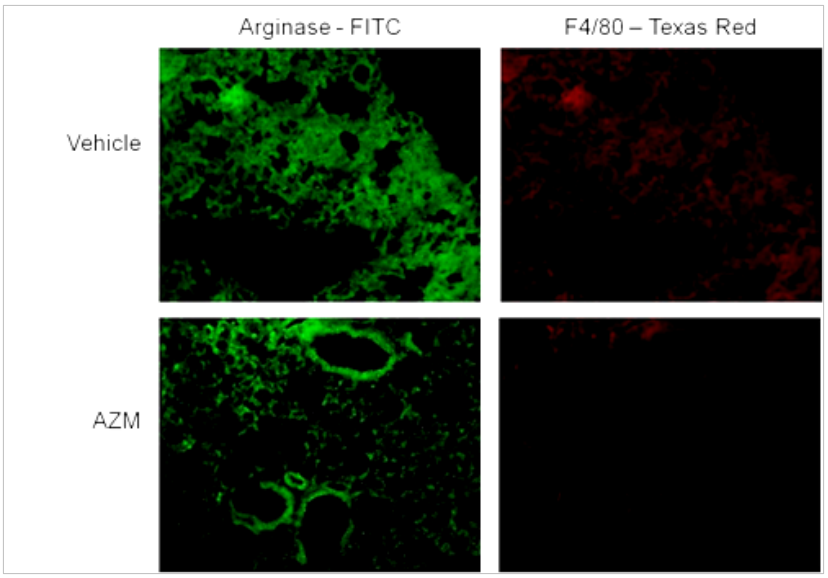


Figure 3.5. Arginase 1 and F4/80 expression in IL-4rα$^{-/-}$ mice treated with methylcellulose vehicle or AZM. Lung sections from mice at day 7 post infection with agarose beads containing 1.5 x 10^5 *P. aeruginosa.* Representative slides of at least 3 replicates from IL-4rα$^{-/-}$ mice lung sections stained with arginase-FITC and F4/80-Texas Red.

MMP-9 Expression

Next, we examined the effect of AZM on MMP-9 production in the lungs during immune response to *P. aeruginosa*, in order to determine the effect of the drug on mediators of ECM turnover. First, MMP-9 concentrations were measured in BALF samples via ELISA. WT mice had increased concentrations of MMP-9 in the BALF at day 7 in the lung lavage of AZM-treated mice as compared to the control group ($p < 0.01$, Figure 3.6a). However, by day 14, MMP-9 concentration had decreased compared to vehicle control. Interestingly, this increase in BALF concentrations follows an increase in arginase production at day 3. In the IL4rα$^{-/-}$ mice, the differences in MMP-9 concentration between the treatment groups were not statistically significant. The mice also had higher MMP-9 concentrations at day 0, which then decrease after infection in both treatment groups. These mice also had significantly higher concentrations of MMP-9 in the BALF throughout the duration of the infection.

Next, to determine MMP-9 expression in the lung, immunohistochemistry was performed on lung sections from both C57Bl/6 and IL-4rα$^{-/-}$ mice, using an antibody against MMP-9 (FITC). Representative slides from each mouse are shown in Figure3.4, panels c and e. Intensity of the fluorescence was measured using ImageJ, and median fluorescence is shown graphically below. In WT mice, AZM decreased the MMP-9 expression in the lung at day 3 compared to control ($p < 0.0001$), with no statistically significant differences at the later timepoints (Figure 3.7b). In contrast to the WT controls, IL4rα$^{-/-}$ mice had similar expression of MMP-9 until day 14, at which point the AZM treatment group had decreased MMP-9 compared to control ($p < 0.01$, Figure 3.7d). Furthermore, both treatment groups of C57Bl/6 mice had higher concentrations of MMP-9 in the lung, even at baseline, than did the IL-4rα$^{-/-}$ mice. Taken together, these data suggest that the C57Bl/6 mice treated with AZM, as well as the IL-4rα$^{-/-}$ mice of both treatment groups, are secreting MMP-9 into the airways.

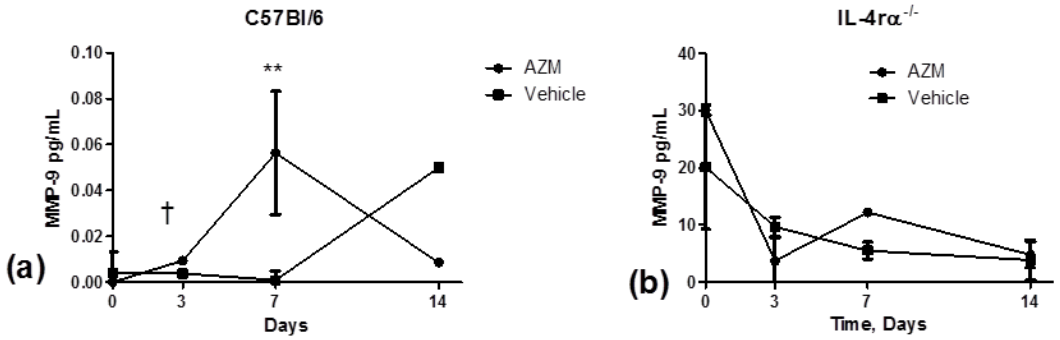


Figure 3.6. Expression of MMP-9 in the BALF in response to *P. aeruginosa* infection. Four mice per treatment group per timepoint were infected with agarose beads containing 1.5 x 10^5 CFU of bacteria. MMP-9 concentrations were measure by indirect ELISA from BALF in C57Bl/6 (a) and IL-4rα$^{-/-}$. Significance is indicated for p values < 0.01(**) and < 0.0001(****), and 0.05 overall between groups (†).

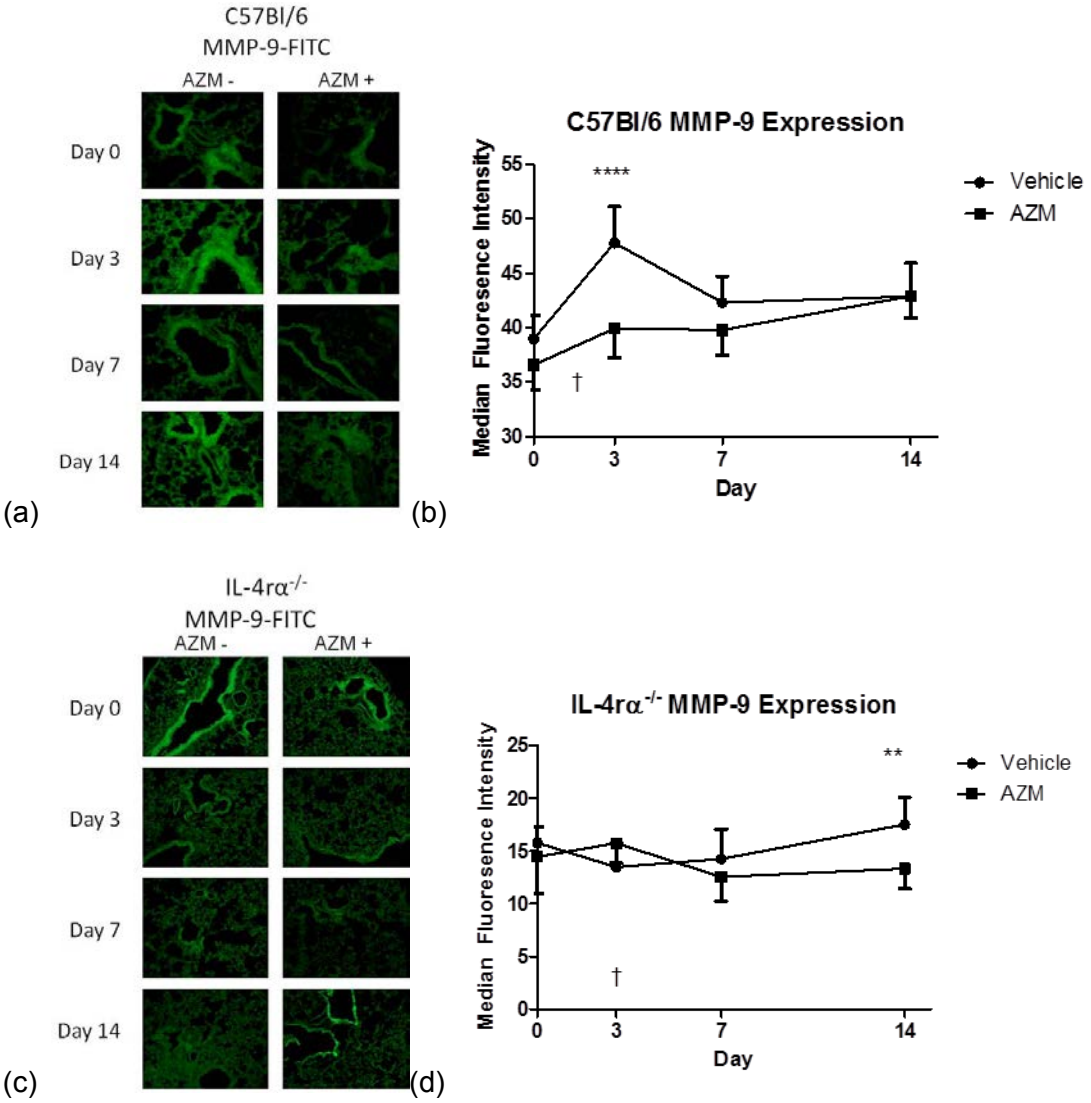


Figure 3.7. Expression of MMP-9 in the lung in response to *P. aeruginosa* infection. Four mice per treatment group per timepoint were infected with agarose beads containing 1.5 x 10^5 CFU of bacteria (b). Lung sections from mice 0, 3, 7, and 14 days post infection were stained with primary antibody to MMP-9 and secondary antibody tagged with FITC. Representative slides from C57Bl/6 controls are shown (c). Median fluorescence intensity is represented quantitatively (d). Representative slides of at least 3 replicates from IL-4rα$^{-/-}$ mice are shown (e). Median fluorescence intensity, ± SD, is represented quantitatively in (f). Samples were run in triplicate. Significance is indicated for p values < 0.01(**) and < 0.0001(****), and 0.05 overall between groups (†).

Fibronectin Expression

Next, we set out to determine the effect of AZM on changes in fibronectin concentration over time in the response to *P. aeruginosa* infection, and whether IL-4rα$^{-/-}$

mice would respond similarly. ELISA was performed on BALF to measure fibronectin concentration in the alveolar space. WT mice treated with AZM had increase fibronectin concentration in the BAL at day 7 ($p < 0.01$) but decreased concentration at day 14 post-infection compared to control mice ($p < 0.0001$, Figure 3.8a). IL4rα$^{-/-}$ mice had higher levels of fibronectin at both day 3 and day 7 compared to control mice. Though this difference was not statistically significant at any given timepoint, the overall difference has significance over time (Figure 3.8b).

To determine fibronectin expression in the lung, immunohistochemistry was performed on lung sections from each mouse strain. Representative slides from each group are shown in Figure3.5, panels c and e. Quantification of median fluorescence intensity from ImageJ is represented graphically. WT mice treated with AZM had increased fibronectin expression at day 3 ($p = 0.05$), but decreased expression at days 7 and 14 ($p = 0.05$ and $p < 0.0001$, respectively, Figure 3.9b), compared to vehicle control. The control group had a decrease in concentration at day 7, but the subsequent increase at day 14 left the fibronectin concentrations roughly at the level of baseline. In contrast, the mice that were treated with AZM had significantly lower levels of fibronectin expression in their lungs at day 14. This may be indicative of reduced fibrotic changes secondary to lessened damage.

IL4rα$^{-/-}$ mice had no differences in fibronectin expression in the lung at any day post infection between the treatment groups (Figure 3.9d). However, both groups of IL4rα$^{-/-}$ mice had an initial decease in fibronectin expression from day 0 to day 7, and then rebound expression, with day 14 fibronectin production similar to day 0 levels. There was also much less fibronectin in the lungs of the IL4rα$^{-/-}$ mice compared to C57Bl/6 controls. Lastly, fibronectin expression corresponds to the pattern of arginase expression, shown above, especially at days 3 and 7. This data suggests that arginase

is increasing fibronectin expression in the *in vivo* model as was also the case in our *in vitro* co-culture work (Chapter 2).

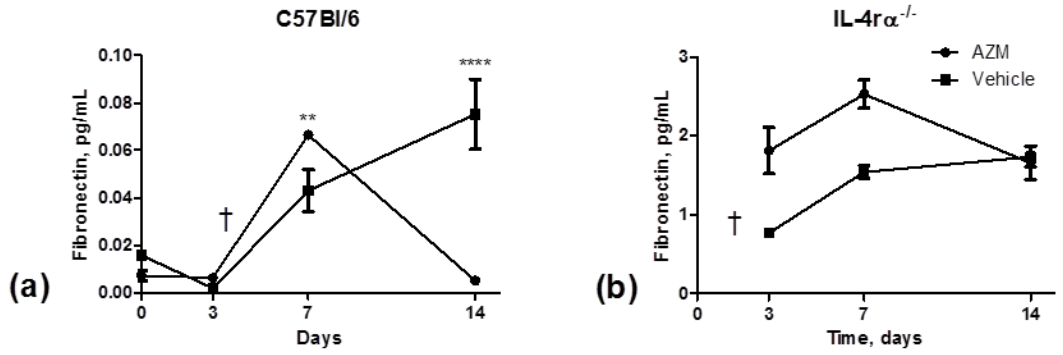


Figure 3.8. Expression of fibronectin in BALF in response to *P. aeruginosa* infection. Four mice per treatment group per timepoint were infected with agarose beads containing 1.5 x 10^5 CFU of bacteria. Fibronectin concentrations were measure by indirect ELISA from BALF in C57Bl/6 (a) and IL-4rα$^{-/-}$. Samples were run in triplicate. Significance is indicated for p values < 0.5 (*), < 0.01(**) and < 0.0001(****) and 0.05 overall between groups (†).

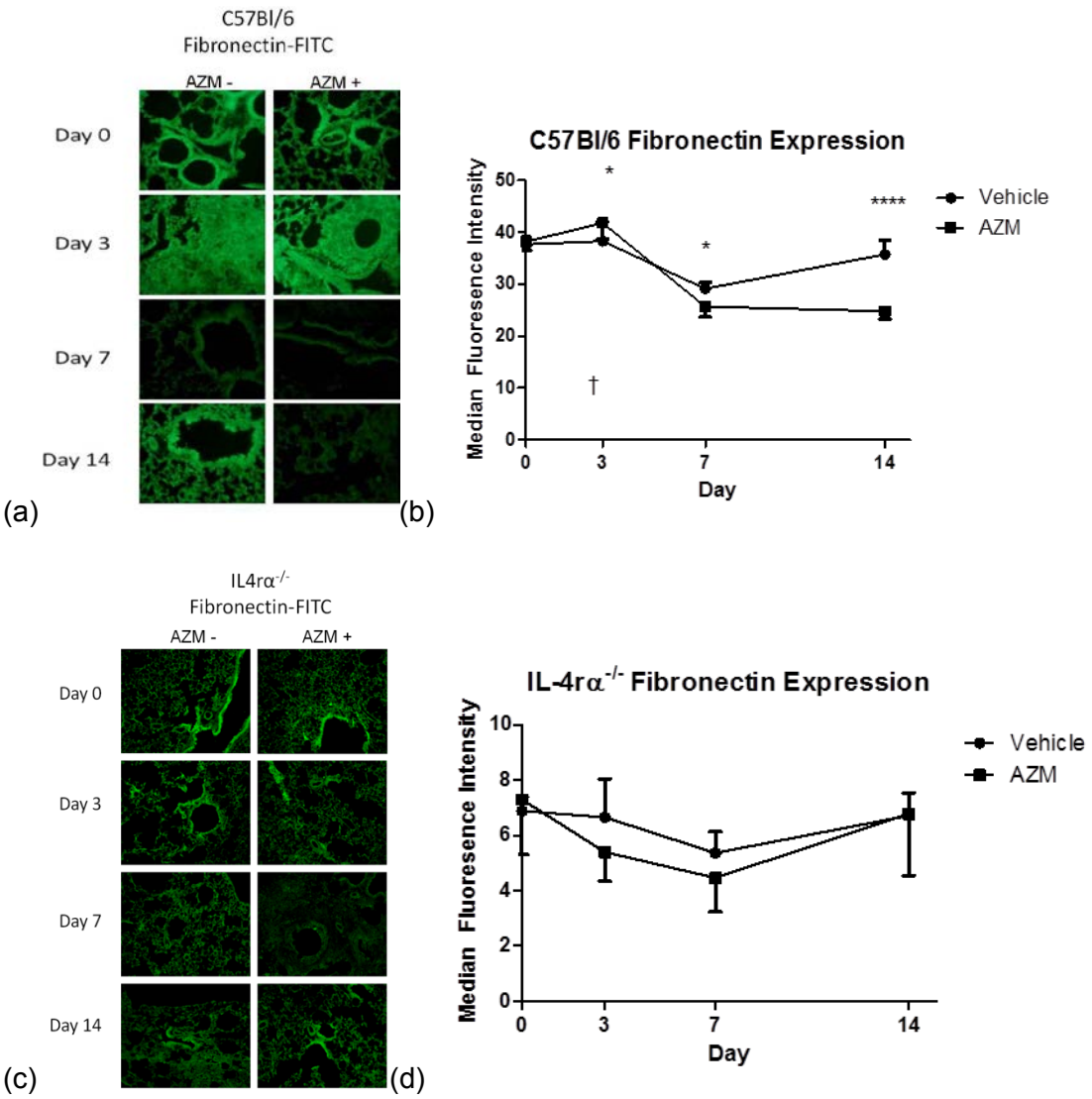


Figure 3.9. Expression of fibronectin in the lung in response to *P. aeruginosa* infection. Four mice per treatment group per timepoint were infected with agarose beads containing 1.5 x 10^5 CFU of bacteria. (b). Lung sections from mice 0, 3, 7, and 14 days post infection were stained with primary antibody to fibronectin and secondary antibody tagged with FITC. Representative slides from C57Bl/6 controls are shown (c). Mean fluorescence intensity, ± SD, is represented quantitatively (d). Representative slides of at least 3 replicates from IL-4rα$^{-/-}$ mice are shown (e). Median fluorescence intensity is represented quantitatively in (f). Samples were run in triplicate. Significance is indicated for p values < 0.5 (*), < 0.01(**) and < 0.0001(****) and 0.05 overall between groups (†).

Collagen Accumulation

Another important fibrotic marker, the ECM protein collagen, was analyzed in our infection model. Lung lobes from C57Bl/6 and IL4rα$^{-/-}$ mice infected with *P. aeruginosa* were sectioned and stained with Van Gieson's Tri Chrome reagent specific for all isoforms of collagen. Representative slides for each group are shown in Figure 3.a and c. Compared to vehicle control, C57Bl/6 mice treated with AZM had less lung damage early in the infection, but slightly more collagen accumulation around the airways at day 14 post-infection (Figure 3.10a). While AZM treatment was able to prevent much of the damage associated with the infection, the increased collagen in the treatment group at day 14 is a significant finding that could represent an overall lack of long-term impact of AZM treatment in terms of limiting lung damage. In the IL4rα$^{-/-}$ mice, all animals had increased lung damage compared to both treatment groups of C57Bl/6 mice at all timepoints (Figure 3.10b). However, AZM-treated mice showed decreased damage compared to their own (IL4rα$^{-/-}$) vehicle control group at day 14 (Figure 3.10b).

Each slide taken of Van Gieson stained tissue was assessed for lung damage. Sections were scored for severity of four pathologies; peribronchiolitis, bronchitis, alveolitis, and interstitial pneumonitis for C57Bl/6 mice (Figure 3.11) and IL4rα$^{-/-}$ mice. Peribronchiolitis indicates inflammation and cell infiltration surrounding a bronchiole; bronchitis occurs when the inflammatory cells are within the epithelium of the bronchi; alveolitis is the presence of inflammation within the alveolar spaces; and interstitial pneumonitis is defined as increased thickness of the alveolar walls, due to inflammatory cells or accumulation of fluid. While these pathologies are often localized to specific areas of the lung, leaving normal or near-normal tissue in between, each slide was given a score of the average damage throughout the specific section. In the C57Bl/6 mice treated with AZM, there was increased peribronchiolitis at day 7 post-infection, as well as significantly less severe bronchitis at day 3 post-infection. This was accompanied by

decreased alveolitis in the AZM-treated mice at both days 0 and 3, although this marker was then increased in these mice at day 7 post-infection compared to controls. These changes indicate that treatment with AZM may alter the type of damage in the lung, as well as the time post-infection when the damage occurs. In contrast, the IL4rα$^{-/-}$ mice which were treated with AZM had less severe peribronchiolitis and alveolitis at days 7 and 14 post-infection, accompanied by less severe bronchitis at day 7 post-infection. Interestingly, interstitial pneumonitis was more severe than in the C57Bl/6 animals, and was unchanged with AZM treatment. This suggests that IL-4 signaling may have a protective effect during infection. Further, while AZM may protect against lung damage, it may also contribute to collagen accumulation.

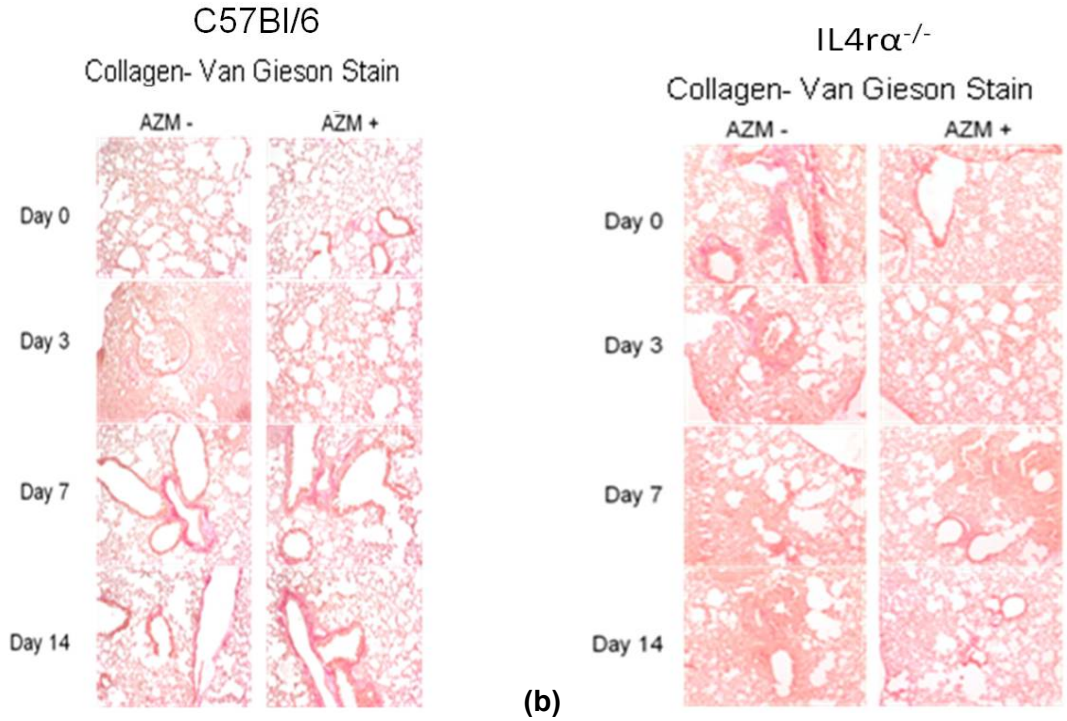


Figure 3.10. Expression of collagen in response to *P. aeruginosa* infection. Four mice per treatment group per timepoint were infected with agarose beads containing 1.5 x 10^5 CFU of bacteria. Lung sections from mice 0, 3, 7, and 14 days post infection were stained with Van Gieson's Tri Chrome. Representative slides of at least 3 replicates from C57Bl/6 controls (a) and IL-4rα$^{-/-}$ mice (c) are shown. Pink color of the Tri Chrome stains positively for collagen deposition.

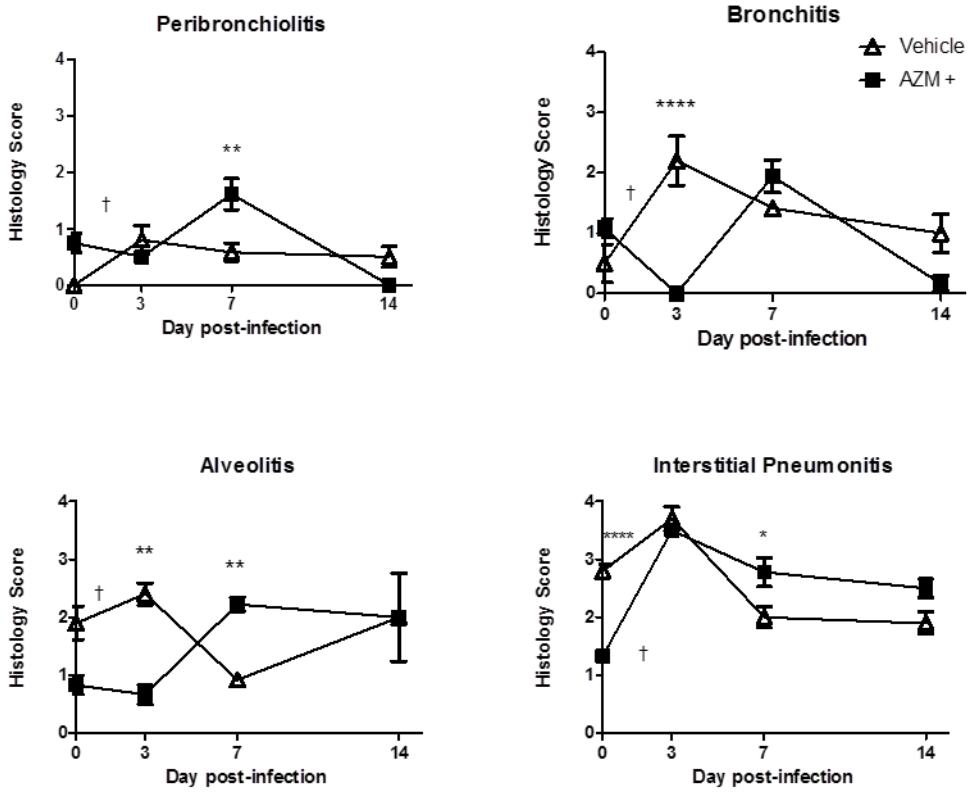


Figure 3.11. Pathology scoring of C57Bl/6 lungs infected with to *P. aeruginosa*. Four C57Bl/6 mice per treatment group per timepoint were infected with agarose beads containing 1.5 x 10^5 CFU of bacteria. Lung sections from mice 0, 3, 7, and 14 days post infection were stained with Van Gieson's Tri Chrome. Slides were scored for markers of pathology; peribronchiolitis bronchitis, alveolitis, and interstitial pneumonitis. Data is represented as mean ± SEM. Samples were run in triplicate. Significance is indicated for p values < 0.5 (*), < 0.01(**), < 0.001(***), and < 0.0001(****), and 0.05 overall between groups (†).

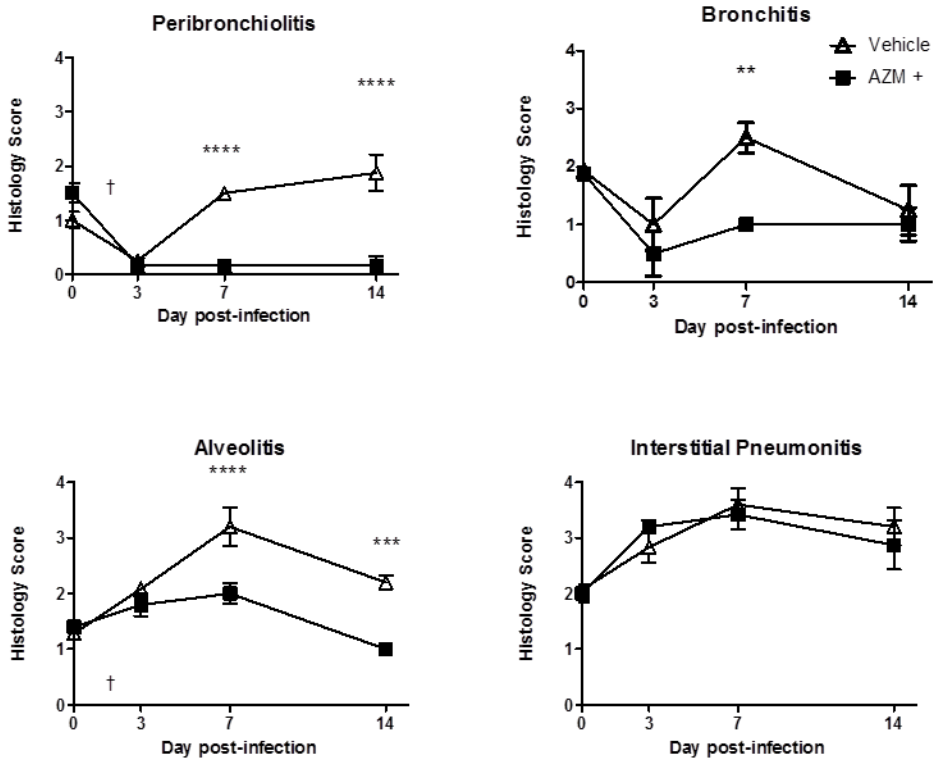


Figure 3.12. Pathology scoring of IL-4rα$^{-/-}$ lungs infected with *P. aeruginosa*. Four IL-4rα$^{-/-}$ mice per treatment group per timepoint were infected with agarose beads containing 1.5 x 10^5 CFU of bacteria. Lung sections from mice 0, 3, 7, and 14 days post infection were stained with Van Gieson's Tri Chrome. Slides were scored for markers of pathology; peribronchiolitis bronchitis, alveolitis, and interstitial pneumonitis. Data is represented as mean ± SEM. Samples were run in triplicate. Significance is indicated for p values < 0.5 (*), < 0.01(**), < 0.001(***), and < 0.0001(****), and 0.05 overall between groups (†).

Monocyte Rescue

Because the IL-4rα$^{-/-}$ mice had more severe histological damage than the C57Bl/6 controls, we decided to do an adoptive transfer of normal monocytes to attempt to correct this pathology in the post-infection timeframe. IL-4rα$^{-/-}$ mice received normal cells adoptively transferred from BALB/c mice. Mice received either T cells only or monocytes and T cells. All mice received adoptively transferred T cells, as the IL4rα$^{-/-}$ mice lack this receptor domain on T cells as well.

Previous data confirms that the experiments were successful, by detecting IL4rα on CD11b+ cells in the mice that received normal monocytes and T cells, though not on CD11b+ cells from mice that received normal T cells only. Additionally, mice that received both monocytes and T cells display a decreased neutrophilic influx compared to mice that only received adoptively transferred T cells. This data suggested that the addition of normal monocytes can alter the inflammatory response.

In order to determine if monocyte adoptive transfer would also modulate other parts of the inflammatory response, lungs were examined histologically. Lung sections were stained with Van Gieson's Tri Chrome and scored for four pathological markers; peribronchiolitis, bronchitis, alveolitis, and interstitial pneumonitis. The group that received both monocytes and T cells from normal mice had lower scores of alveolitis and interstitial pneumonitis at day 3 post-infection compared to mice who received only T cells from normal mice, although the difference was not statistically significant (Figure 3.13). Importantly, day 3 post-infection is at peak of inflammation response.

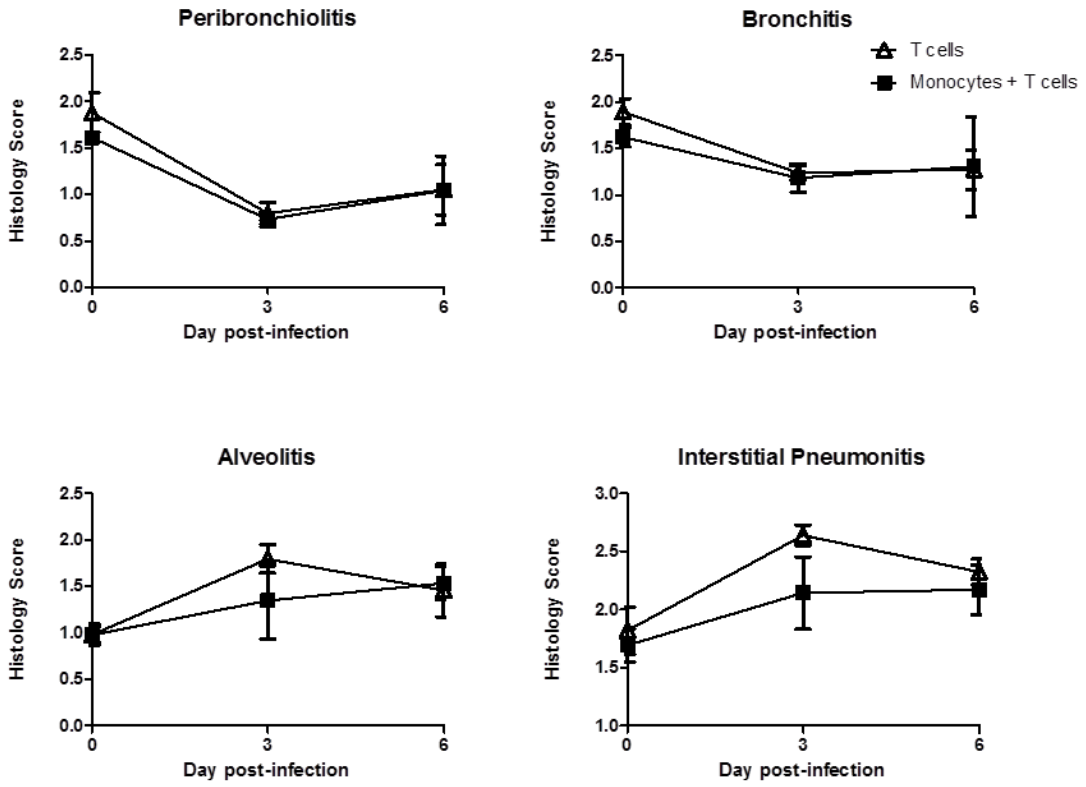


Figure 3.13. Scoring of damage in response to *P. aeruginosa* infection, with adoptive transfer of normal immune cells into IL-4α$^{-/-}$ mice. Cells were harvested from BALB/c mice and retro-orbitally injected into the transgenic strain. Four mice per treatment group per timepoint were then infected with agarose beads containing 1.5 x 10^5 CFU of bacteria. Lung sections from mice 0, 3, and 6 days post infection were stained with Van Gieson's Tri Chrome. Slides were scored for markers of pathology; peribronchiolitis, bronchitis, alveolitis, and interstitial pneumonitis. Samples were run in triplicate. Data is represented as mean ± SEM. Data were not statistically significant.

MMP-9 Deficiency

In light of the *in vitro* data suggesting that MMP-9 is a molecule of critical importance to the AZM-polarized macrophage phenotype, a preliminary experiment was conducted using MMP-9 deficient mice. Mice that are homozygous null for *Mmp9* gene, on a C57B/6 background, were used for these experiments. These mice have normal development, though may have decreased neutrophilic influx to sites of inflammation.[139] This strain of mouse was compared to the C57Bl/6 to determine if

the lack of MMP-9 would result in increased amount of fibrosis after infection with *P. aeruginosa.* We hypothesized that the lack of MMP-9 would result in a buildup of ECM, as the protease would be unavailable to degrade excess collagen and fibronectin. Previous studies utilizing the strain of MMP-9 deficient mice have primarily investigated the role of the protease in inflammation, in diseases such as allergic asthma, ventilator induced lung injury, and bronchopulmonary dysplasia.[140-142] The impact of MMP-9 deficiency on ECM buildup is currently unknown.

For this experiment, the mice were treated with AZM, infected with 1 x 10^5 CFUs of *P. aeruginosa*, and analyzed 14 days post-infection for the amount of fibrotic proteins in their lungs. The mice lacking MMP-9 tolerated the infection to the same degree as the C57Bl/6 mice; the weight loss between the groups was not significantly different at any of the timepoints, overall there was a significant different over time (Figure 3.14). In addition, none of the mice in either group grew bacteria on culture at day 14. This suggests that the MMP-$9^{-/-}$ mice did not have impaired inflammatory response significant enough to cause impaired bacterial clearance or prolonged infection.

When the lungs were sectioned and stained for presence of collagen, there were some differences noted between the two strains. The C57Bl/6 mice showed collagen accumulation at both the pre-infection and the post-infection timepoints (Figure 3.15). This is not surprising, given previous data with this strain. However, the MMP-9-/- mice had more collagen accumulating to the airways in both the uninfected mice and the mice at day 14 post-infection (Figure 3.15). This supports the hypothesis that lack of MMP-9 leads to increased accumulation of fibrosis.

However, when lung sections were stained by immunohistochemistry for fibronectin, there were no differences in expression of this protein between the two mouse strains. Representative pictures are shown in Figure 3.16a, and analyzed graphically in Figure 3.16b. This is an interesting finding, in light of the differences in

collagen accumulation. This data compliments the rest of the data gathered in this chapter and in Chapter 2 that shows collagen and fibronectin having different outcomes in response to the various stimuli. However, this preliminary experiment only examined a limited set of time after infection; a more detailed study is needed in the future to determine the role of MMP-9 in the response of fibrotic mediators to *P. aeruginosa* infection.

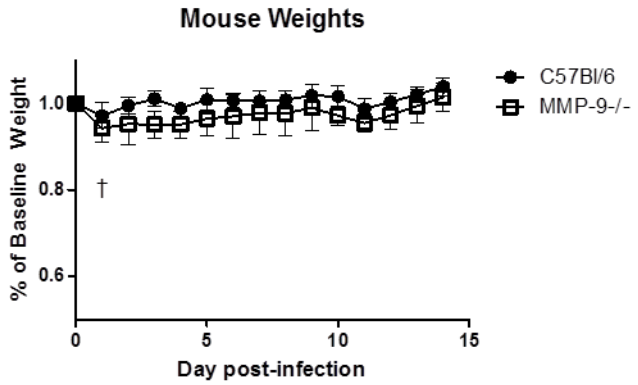


Figure 3.14. Lack of MMP-9 affects mouse weights in response to *P. aeruginosa* infection. Weight change of mice in each strain as percentage change from baseline weight. Four mice per group were infected with agarose beads containing 1.5 x 10^5 CFU of bacteria. Data is represented as mean ± SD, with four mice per group. Data were analyzed by two-way ANOVA, and $p < 0.05$ over time between groups (†).

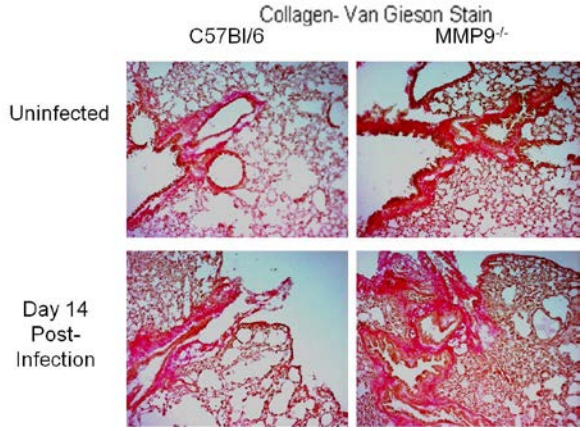
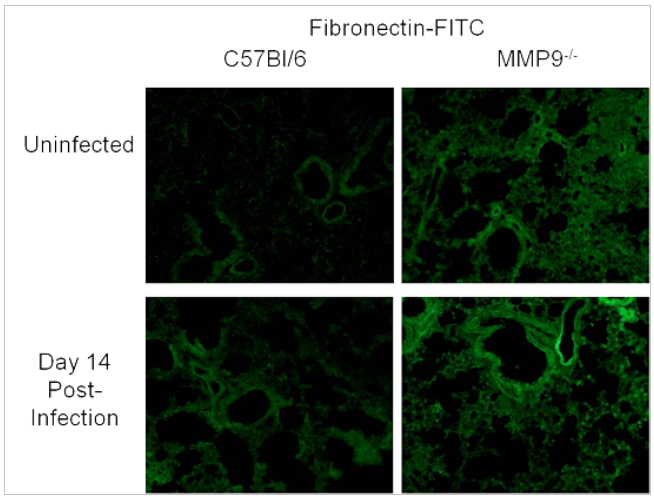
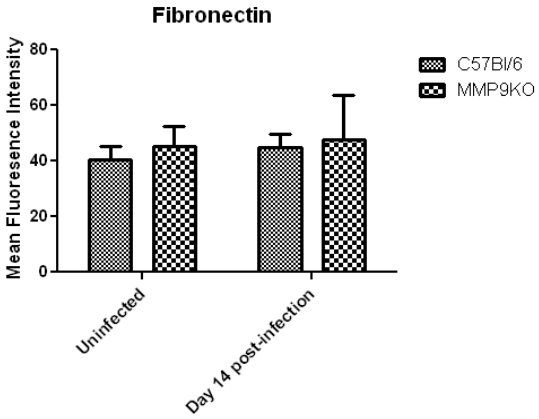


Figure 3.15. Lack of MMP-9 causes collagen accumulation. Expression of collagen in response to *P. aeruginosa* infection. Four mice per group were infected with agarose beads containing 1.5 x 10^5 CFU of bacteria. Lung sections from uninfected mice and mice 14 days post infection were stained with Van Gieson's Tri Chrome. Representative slides of at least 3 replicates from C57Bl/6 controls and MMP9$^{-/-}$ mice are shown. Pink color of the Tri Chrome stains positively for collagen deposition.



(a)



(b)

Figure 3.16. Lack of MMP-9 does not increase fibronectin accumulation. Expression of fibronectin in response to *P. aeruginosa* infection. Four mice per group were infected with agarose beads containing 1.5 x 10^5 CFU of bacteria. Lung sections from uninfected mice and mice 14 days post infection were stained with fibronectin-FITC by immunohistochemistry. (a)Representative slides of at least 3 replicates from C57Bl/6 controls and MMP9$^{-/-}$ mice are shown. (b) Mean fluorescence intensity is represented graphically. Data were analyzed by two-way ANOVA with Bonferroni's post-test. Data were not significant.

D. Conclusion

The hypothesis for this section of experiments, that fibrosis development in response to *P. aeruginosa* would depend on the alternative macrophage activation, turned out to be too simplistic for the interactions taking place in the immune response.

While these data did show that the AZM-polarized macrophage is affecting fibrosis, as well as influencing the response to infection, the effects appear to be multifaceted.

In C57Bl/6 mice, AZM caused increased numbers of CD11b+ macrophages to partition to the airway spaces at day 7 post-infection compared to the control group, while at the same time resulting in decreased numbers of neutrophils. This analysis by flow cytometry confirms previous analysis in our lab by histological methods.[138] This result also shows that macrophages are an important cell type in the immune response. Therefore, the *in vitro* data demonstrating AZM's polarizing effects has increased significance.

Staining for AAM and CAM markers showed that C57Bl/6 mice have significantly increased levels of arginase at day 3 in the AZM-treated group, with a corresponding decrease in iNOS. This shift in effector protein expression mimics the changes seen in the *in vitro* data, both in Chapter 2 and previously observed.[112] Interestingly, in this experiment, the AZM-treated group had a decrease in iNOS at day 0. At this timepoint, the mice had received four days of drug, but were had not yet infected. Furthermore, in the IL-4rα$^{-/-}$ mice, there was no difference in arginase expression between the vehicle control and the AZM treatment groups, but there was a significant decrease in iNOS at day 14 in the AZM-treated mice. This suggests that AZM may have a protective effect independent of IL-4/13 signaling.

Both MMP-9 and fibronectin concentrations in BALF mimics macrophage partitioning in C57Bl/6 mice. Importantly, MMP-9 and fibronectin concentrations are higher in the BALF in AZM-treated C57Bl/6 mice at day 7, which is when bacterial burden is highest in the course of this infection. The control mice don't secrete these proteins into their airways until day 14 post-infection. When lung sections are stained for these proteins, the relationships appear to be reversed; MMP-9 and fibronectin are both decreased in the lung at days 7 and 14 in the mice treated with AZM compared to

vehicle controls. Although fibronectin is increased at day 3 post-infection in the AZM treated mice, MMP-9 is always downregulated in these mice compared to vehicle controls. This could suggest that what is being made of these proteins is being directly secreted into the airways, at least at day 7, when the mice had received AZM. It is also important to note that MMP-9 is produced in other cell types in addition to macrophages. Pretreatment with AZM has been shown to prevent upregulation of MMP-9 in human airway epithelial cells.[143] Furthermore, fibronectin in the lung tissue may have been degraded by the presence of *P. aeruginosa.* Multiple studies have evidence to support the ability of this bacterium to bind and subsequently degrade fibronectin in order to establish infection.[144-146] Degradation of fibronectin by *P. aeruginosa* could be one reason for a decrease in fibronectin concentrations in the lung tissues over the course of the experiment.

When these proteins are examined in the IL-4rα$^{-/-}$ mice, there are little differences in MMP-9, and no differences in fibronectin between the two treatment groups. While, the knockout mice had higher concentrations of these proteins in the BALF compared to the control strain, they also produce less of these proteins in the lung tissues, as noted both in the representative pictures and in the scales of the median fluorescence intensity graphs. However, compared to the C57Bl/6 mice, the IL-4rα$^{-/-}$ mice had higher MMP-9 and fibronectin concentration in the BALF, yet lower concentrations of these proteins in the lung. This suggests that the IL-4rα$^{-/-}$ mice secrete these proteins into the airways to a higher degree than the C57Bl/6 mice.

Another important observation to make about the immunostaining is that the localization of these proteins is altered when AZM is given. Some of the proteins, such as arginase, move from the airways to the interstitium with the addition of AZM, and some, such as MMP-9, move in the opposite direction. While this is not represented

when the data is analyzed quantitatively, it may be of importance in the overall immune response to the infection and the subsequent fibrosis that progresses.

Along with reduced fibronectin in the lungs of mice treated with AZM, there was correspondingly less damage to the pulmonary tissue. This damage is also manifested by increased cellular infiltrates and fluid accumulation in sections stained with Van Gieson's Tri Chrome. Especially at day 3, which is when mice show the most dramatic weight loss[138], the damage to the vehicle control group is most significant in the bronchitis and alveolitis scores, which are both influenced by treatment with AZM. However, it appears that AZM may be shifting the kinetics of these types of damage to the later timepoints. Also, there is increased collagen accumulation in this group of mice at the latest timepoint. Because these experiments were unable to assess physiology or lung function, it is uncertain what effect the collagen had on the mice in this group.

When the IL-4rα$^{-/-}$ mice were assessed for lung damage and collagen accumulation, we found that even at baseline, before infection, these mice showed increases in cellular infiltration, peribronchiolitis, and collagen accumulation. Collagen was reduced in the AZM-treatment group, which had been receiving drug for four days by day 0. During the course of infection, both treatment groups of this strain developed more damage in response to infection that did the C57Bl/6. Again, AZM ameliorated the damage, with reductions in peribronchiolitis, bronchitis, and alveolitis. Interestingly, in the IL-4rα$^{-/-}$ mice that were treated with AZM, there was little collagen accumulation seen on histological examination.

Because damage was so severe in the IL-4rα$^{-/-}$ strain, even at baseline, we hypothesized that the absence of the alternative macrophage could be the reason. In fact, when adoptively transferred with normal cells, the group of mice that received normal T cells and normal monocytes had less damage at the peak of infection. While

these data were not significant, the sample size for the experiment was low and did not go to a 14 day timepoint, which might have shown even more differences in recovery.

The hypothesis proposed for this set of experiments was partially supported. Collagen was increased in AZM-treated mice at later timepoints, following an increase in arginase in these same mice. Interestingly, there was little collagen present at the day 3 timepoints in all experiments analyzed. This may be due to the high bacterial burden at this time; *Pseudomonas* elastases, an extracellular enzyme produced by the bacteria, has been shown to directly degrade collagen.[147] However, the other fibrotic marker analyzed, fibronectin, was decreased in AZM-treated mice, even after the increase in arginase. Furthermore, the increase in arginase in the C57Bl/6 mice was also accompanied by a decrease in damage, suggesting that blunting the inflammatory response is the main action of the AZM-macrophage, rather than fibrosis increase. Whether the decrease in fibronectin or the increase in collagen is more physiologically relevant to the mice remains unanswered.

Administration of AZM in the murine infection model appears to ameliorate some of the inflammation and damage associated with the bacterial infection, although it may also increase some of the fibrotic responses as well. In light of these data, it was important to investigate the mediators studied in Chapters 2 and 3 in human studies. Therefore, Chapter 4 will focus on the affect of AZM on fibrotic mediators in subjects with CF.

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Chapter 4: Azithromycin alters correlations between fibrotic mediators and inflammatory cytokines in sputum from patients with CF

A. Introduction

The final component outlined in this dissertation examines the fibrotic mediators studied in chapters 2 and 3 in human subjects. For this study, subjects were recruited from the University of Kentucky pulmonology clinic who were diagnosed with CF. These subjects were consented or assented, sputum samples were obtained, and clinical data was collected from medical records. The sputum samples were analyzed for the fibrotic mediators identified as important from our *in vitro* and *in vivo* animal studies, namely TGF-β, MMP-9, fibronectin, and MMP-2. MMP-2 was added because human studied have shown that MMP-9 and MMP-2 often have similar actions in disease states in the lungs. [148-150]

In addition, inflammatory cytokines were also profiled in these patients. IL-8 and IL-1β were the cytokines that were identified as the most important in these subjects. IL-8 is a classic inflammatory cytokine, and is elevated in the CF airway. [151, 152]. IL-1β, however, has a more complicated role in the CF disease process. IL-1β is traditionally considered an inflammatory cytokine, as its activity potentiates inflammatory processes in multiple pulmonary diseases, including CF. [153, 154] In fact, IL-1β can increase IL-8 in CF lung epithelial cells by activating NF-κB. [154] In general, IL-1β is cleaved from its pro-form downstream of inflammasome activation, usually in response to microbial infection.[155, 156] Once cleaved, IL-1β is secreted into the airways to coordinate inflammatory responses in the lung.[157, 158] However, IL-1β also initiates fibrotic mechanisms in addition to inflammatory processes.[159, 160] Kolb, et al, showed that intratracheal instillation with exogenous IL-1β resulted in early increases in inflammatory cytokine production, such as IL-6 and TNF. The increases in inflammatory proteins were

followed by late increases in TGF-β and hydroxyproline, the precursor to collagen. In fact, while inflammatory cytokines peaked at day 7 after IL-1β administration, hydroxyproline was still elevated by day 60 post IL-1β exposure. The delayed presence of hydroxyproline could indicate a continued production of collagen. IL-1β can also activate plasminogen activator inhibitor-1 (PAI-1), a conquence of which is the increase of collagen and lung fibrosis. [125] IL-1β has an additional direct effect on the CF airway; it can increase the *MUC5AC* gene, which encodes for mucin – a major component of mucus.[161] Upregulation of *MUC5AC* can cause increased accumulations of mucus in the airways. This effect also appears to be through NF-κB. Because IL-1β has such wide implications on the CF airway, and on mediators important for this study, we felt that this would be an important cytokine to include in our analysis.

Our lab has previously examined sputa from CF subjects for markers of alternative macrophage activation. This study[114] showed that subjects who were infected with *P. aeruginosa* had higher levels of mannose receptor and arginase activity compared to subjects who were uninfected. Arginase was inversely associated with FEV1, and the correlation was stronger in those subjects infected with *P. aeruginosa* ($r = -0.662$, $p = 0.001$). These subjects also had statistically significantly higher levels of the cytokines IL-12 and IL-1β in their sputa. In this previous work, the impact that AZM treatment had upon macrophage disposition was a secondary objective.

Based on the *in vitro* and *in vivo* data showing that AZM treatment causes increases in fibrotic mediator production, we set out to determine whether this relationship would be true in the lungs of patients with CF. For the current study, the focus was to determine whether fibrotic mediators in sputa of patients with CF are affected by AZM treatment, and whether their expression levels correlated with alternative macrophage activation. Therefore, we **hypothesized that AZM therapy in patients with CF increases markers of fibrosis buildup and decreases markers of**

ECM turnover in the lungs, and would be correlated with alternative macrophage activation.

B. Methods

Study design

Subjects were recruited from the adult and pediatric outpatient pulmonology clinics at the University of Kentucky Medical Center. Inclusion criteria were: diagnosis of cystic fibrosis, age 2 – 50 years, ability to extemporaneously produce sputum samples, and currently in a stable state of disease (non-exacerbation) as deemed by the treating physician. Exclusion criteria were presence of HIV, cancer, history or organ transplant, pregnancy or breastfeeding, or current IV antibiotic therapy. Subjects were consented if 18 or over and assented with parental consent if under 18. Sputum samples were collected, and clinical data from the time of the sample was obtained from the subjects chart. This included medication history, lung function parameters, and bacterial colonization status at the time of the sample. Subjects were considered positive for a given organism if that organism was reported to have grown from sputum sample/throat swab at the time of the sample collection. Microbiological data was obtained by the UK Medical Center. All processes were approved by the Internal Review Board at the University of Kentucky Medical Center.

Sample processing

Two to 5 mL of spontaneously expectorated sputum was collected and immediately placed on ice. Samples were digested in 0.1% diothiothreitol and DNase at 30 µg/mL in phosphate buffer for 30 min, with agitation. Supernatants were frozen at -80°C, while cells were washed, counted, and frozen in RNAlater (Applied Biosystems,

Foster City, CA, USA) for subsequent gene analysis. Because of the variability generated by this processing, analyses that follow were all normalized to either total protein or cell counts in each sample. This allows for normalization to volume of sample collected.

TGF-β Activity

Sputum supernatants were quantified by ELISA using the TGF-β_1 E_{max} ImmunoAssay System (Promega, Madison, WI, USA). Samples were diluted in PBS and processed with an acid wash per manufacturer's instructions. Plates were coated with a mAB specific for TGF-β, and samples were added to the wells along with a standard curve prepared using supplied TGF-β_1 standard. Polyclonal anti-TGF-β_1 antibodies were applied, followed by washing and incubation with horseradish peroxidase conjugate. Development solution was applied to the samples, and the OD readings were obtained at 450 nm and compared to the TGF-β_1 standard curve. TGF-β concentrations were normalized to cell count. Because the DTT processing of the sputum sample interfered with the assay, all the samples were acid washed, and thus represent total concentration of TGF-β. However, the TGF-β that is expectorated with the sputum is likely endogenously activated, as latent TGF-β is still bound to the matrix.

ELISA assay

Sputum supernatants were isolated and the MMP-9, fibronectin, and MMP-2 concentrations quantified by indirect ELISA. Samples were diluted in coating buffer and incubated at 4°C overnight for adherence to the ELISA plate. Wells were blocked, then incubated with an antibody specific to MMP-9 (Abcam, Cambridge, MA, USA), fibronectin(Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA), or MMP-2(Abcam, Cambridge, MA, USA) followed by an anti-rabbit HRP conjugated secondary antibody

(Millipore, CA, USA). Wells were analyzed by OD reading at 450nm, using OptEIA detection reagents (BD, CA, USA). Readings were compared to a standard curve using MMP-9, MMP-2, or fibronectin recombinant protein, and values were normalized to cell count.

Cytometric bead array

The levels of the following cytokines were measured in the sputum supernatant samples by using cytometric bead array assay kits (BD Biosciences): IL-8, IL-1β, IL-12p70, TNF-α, IL-10, and IL-6. Bead populations of distinct sizes that were coated with capture antibodies specific for each cytokine were incubated with a 5-fold dilution and a 50-fold dilution of each sample for 3 h at room temperature along with a phycoerythrin-conjugated detection antibody. The beads were then washed, and the fluorescence intensity was assayed by flow cytometry, as outlined above. Intensities were then compared to those on a standard curve generated for each cytokine to determine the concentration in each sample.

Statistical Analysis

Results are reported as mean ± SD and analyzed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Data were compared via ANOVA, followed by Bonferroni's post-test for individual comparisons, or unpaired student's t-test. Correlations were analyzed by linear regression. Nominal data was analyzed by Fischer's exact test. Principal component analysis (PCA) was performed as a method of data reduction to analyze multifactorial variability. PCA was run using Statview (Nesbit, MS, USA). Comparisons between PCA groups were done using Mann-Whitney Rank test. Differences for all statistics were deemed statistically significant at a p value <0.05.

C. Results

Subject Demographics

Thirty subjects were recruited for the study, with 66 samples collected from September 2009 until December 2010. For 37 of those samples, supernatants were saved and protein analysis was available. The subject demographics are reported in Table 4.1. Approximately 69% of the subjects were being treated with AZM. This was to be expected, as use of AZM is now wide-spread among CF treating physicians, especially for patients who are colonized with *P. aeruginosa.*[92] Although only 49% percent of the subjects were colonized with *P. aeruginosa,* nearly 80% of subjects were positive for *S. aureus.* This is not surprising, given that the majority of subjects were under 18, and younger than the typical age at which the incidence in *P. aeruginosa* colonization rises. This is an age when *S. aureus* infections are more prevalent.[5] Lastly, 62% of subjects were homozygous for the ΔF508 mutation. The remaining subjects had one ΔF508 allele and second mutation on their other allele.

Table 4.2 compares the demographics of the study population between subjects with and without AZM treatment, and between subjects with and without *P. aeruginosa* colonization. Of those subjects being treated with AZM, 73% were pediatric, while all of the subjects not being treated with AZM were pediatric. Additionally, only 56% of subjects positive for *P. aeruginosa*, while 95% of those subjects negative for *P. aeruginosa* were pediatric. Lung function was measured in these subjects as FEV1 and forced vital capacity (FVC) % predicted. While FEV1 is the most common method of tracking patients' lung function, FVC, which measures the total volume of air expired, also indicates lung compliance and function. Both lung function measurements, FEV1 and FVC % predicted, were significantly higher in the subjects who were not being treated with AZM. Lung function measurements were also significantly higher in the subjects who were not colonized with *P. aeruginosa* than those who were. Interestingly,

there was no significant difference between those subjects who were either treated with AZM or not who were colonized with *S. aureus*. There was also no significant differences between those positive and negative for *P. aeruginosa* who colonized with *S. aureus*.

The lung function measurement ranges for FEV1 and FVC % predicted were quite broad, although the age range was not. This suggests that we achieved our goal of including patients at all stages of disease. While FEV1 decline in patients with acute exacerbation indicates an increase in inflammation, FEV1 decrease in stable patients (such as our subjects) indicates and increase in amount of fibrosis accumulation, and therefore a change in lung architecture. In these subjects, when FEV1 % predicted is regressed against age, the lowest FEV1 values occur over five decades of age (Figure 4.1a). Despite this, there was a strong negative correlation of lung function and age. When compared by pediatric vs. adult subjects, FEV1 % predicted values were significantly higher in pediatric patients (Figure 4.1b). Similarly, FEV1 % predicted was higher in patients not taking AZM – most likely because these patients had less severe disease overall (Figure 4.1c).

Patient Age: median (range)	14 (8, 23)
Percent Male	40%
Percent under 18	75%
Percent taking azithromycin	69%
FEV1 % predicted: median (range)	62.5% (27% - 116%)
FVC % predicted: median (range)	72.5% (33% - 133%)
Percent colonized with P. aeruginosa	49%
Percent colonized with S. aureus	78%
Percentage receiving other antibiotics	68%
Percentage complaining of shortness of breath	54%
Percentage ΔF508 homozygous	62%

Table 4.1. Demographics of subjects enrolled in the study.

	AZM +	AZM -	p-value	*P. aeurginosa* +	*P. aeruginosa* -	p-value
% Pediatric	73%	100%	0.0074	36%	64%	0.0078
% Adult	27%	0%	0.0074	89%	11%	0.0078
% Male	63%	25%	0.0171	30%	60%	0.5077
FEV_1 (% predicted)	52.3%	90.7%	< 0.0001	45.7%	75.3%	0.0008
FVC (% predicted)	63.3%	100.8%	< 0.0001	57.5%	85.7%	0.0011
Colonized with *S. aureus*	82%	67%	0.3727	72%	84%	0.4470
Other Abx	57%	33%	0.2691	56%	47%	0.7459
Shortness of Breath	71%	0%	0.0002	78%	32%	0.0217

Table 4.2 Demographics analyzed by AZM treatment and *P. aeruginosa* colonization status.

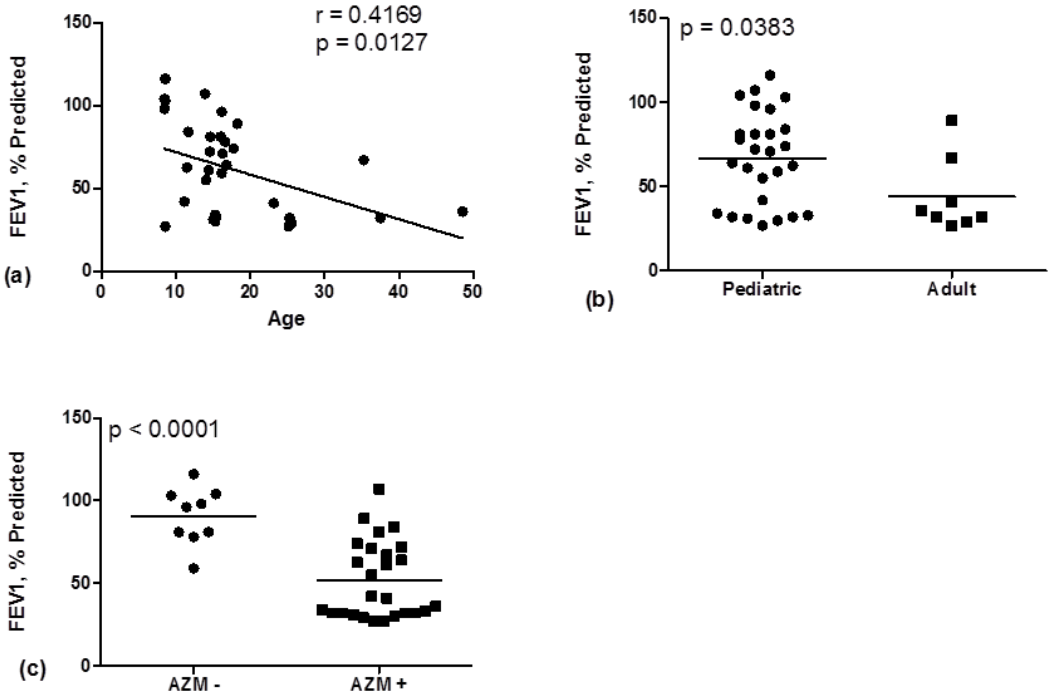


Figure 4.1. FEV_1 % predicted of subjects enrolled in the study. (a) FEV1 % predicted compared by linear correlation to age of subjects. Statistical analysis was performed using linear regression. (b) FEV_1 % predicted compared by pediatric, < 18 years of age, or adult subjects, ≥ 18 years of age. Data was analyzed by student's t-test. (c) FEV_1 % predicted compared by AZM treatment. Data was analyzed by student's t-test. Statistical significance indicated on the graphs.

Fibrotic markers and AZM

Concentrations of TGF-β, MMP-9, and fibronectin protein were measured in sputum samples collected from these subjects. MMP-2 concentration was also assessed to determine if the activity of this protease was similar to MMP-9 in this subject population. When protein concentrations were compared between subjects who were receiving AZM and those who were not, TGFβ- was the only fibrotic marker that was differed statistically. Interestingly, subjects on AZM had decreased levels of TGF-β in their sputum (Figure 4.2, p = 0.02). Concentrations of MMP-9 and MMP-2 had nearly identical means between the two groups (Figure 4.2b and d, p = 0.65 and 0.98 respectively), suggesting that treatment with AZM does not affect these markers.

Fibronectin trended toward a higher mean value in the group of subjects receiving AZM than those not receiving AZM, though it the difference was not statistically significant (Figure 4.2c, p = 0.10). Although this is only a trend toward higher concentrations in subjects receiving AZM, it is possible that more samples would detect a greater difference between the two groups.

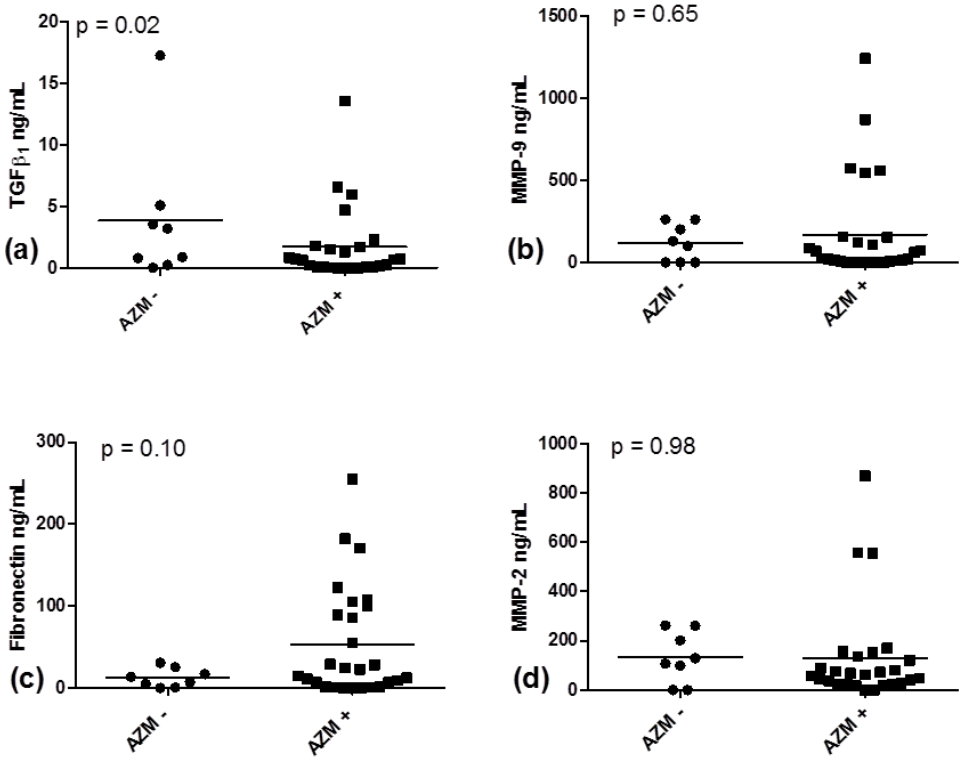


Figure 4.2. Fibrotic mediators from CF subjects' sputa. Subjects were analyzed with regard to their AZM treatment status. TGF-β (a) was measured by TGF-β_1 E_{max} ImmunoAssay System, while MMP-9 (b), fibronectin (c), and MMP-2 (d) were measured by indirect ELISA. to the subjects' dose (mg) of AZM. Data were analyzed by student's t-test and considered significant at $p < 0.05$.

Fibrotic markers and *Pseudomonas*

Because AZM has traditionally been used in patients with CF when they become colonized with *P. aeruginosa*,[50, 103] colonization status for this organism was determined at the time of the sample collection. The same proteins, TGF-β, MMP-9,

fibronectin, and MMP-2, were analyzed with regard to subjects' infection status. Interestingly, subjects that were positive for *P. aeruginosa* had decreased concentrations of TGF-β in their sputa compared to subjects whose cultures were negative (Figure 4.3a, $p = 0.03$). MMP-9 and fibronectin concentrations had very little difference in mean between the group positive for *P. aeruginosa* and the group that was not (Figure 4.3b and c, $p = 0.53$ and 0.44, respectively), indicating that the presence of this pathogen did not affect these two proteins. MMP-2 showed a trend toward increased concentrations in subjects with positive cultures, though the difference was not statistically significant (Figure 4.3d, $p = 0.09$).

In an effort to determine if AZM or PA status was more influential on fibrotic marker concentration, especially in the case of TGF-β, subjects on AZM who were positive for *P. aeruginosa* were compared to those who had negative cultures. Subjects not treated with AZM and negative for *P. aeruginosa* were also included. There were no subjects who were positive for *P. aeruginosa* but not treated with AZM. Because the subjects were recruited without prior knowledge of their AZM treatment status or their microbiological culture results, the lack of an AZM-PA+ subject cohort is indicative of prescribing practices at the our clinic sites. There were no statistical significances between the groups receiving and not receiving AZM. However, there was a significant difference in TGF-β concentrations between the AZM+PA+ subjects and the AZM-PA- subjects (Figure 4.3e, $p< 0.05$). There was no significant difference in MMP-9, MMP-2, or fibronectin concentrations between these groups of subjects (Figure 4.3 f, g, h). This data suggests that AZM may be more influential to TGF-β concentrations than the presence of *P. aeruginosa.*

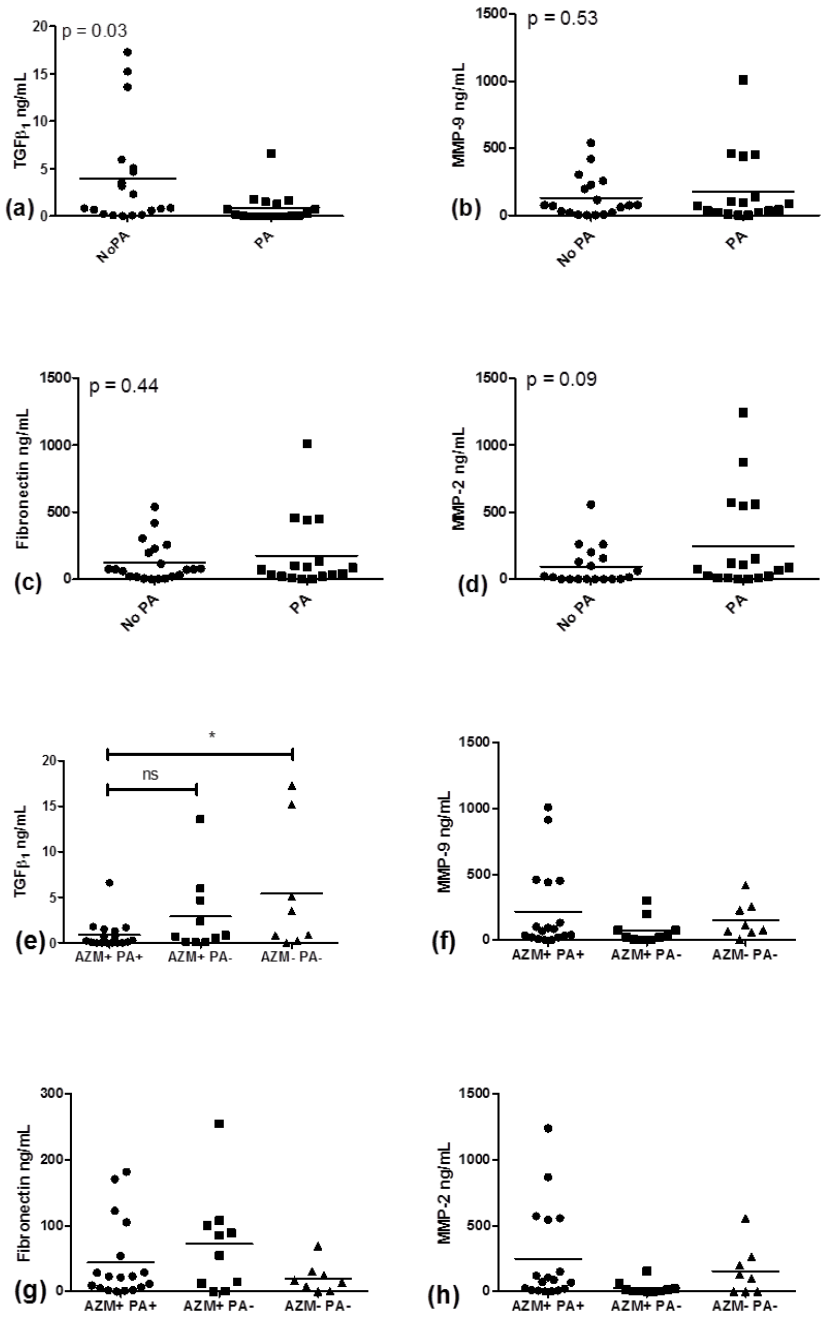
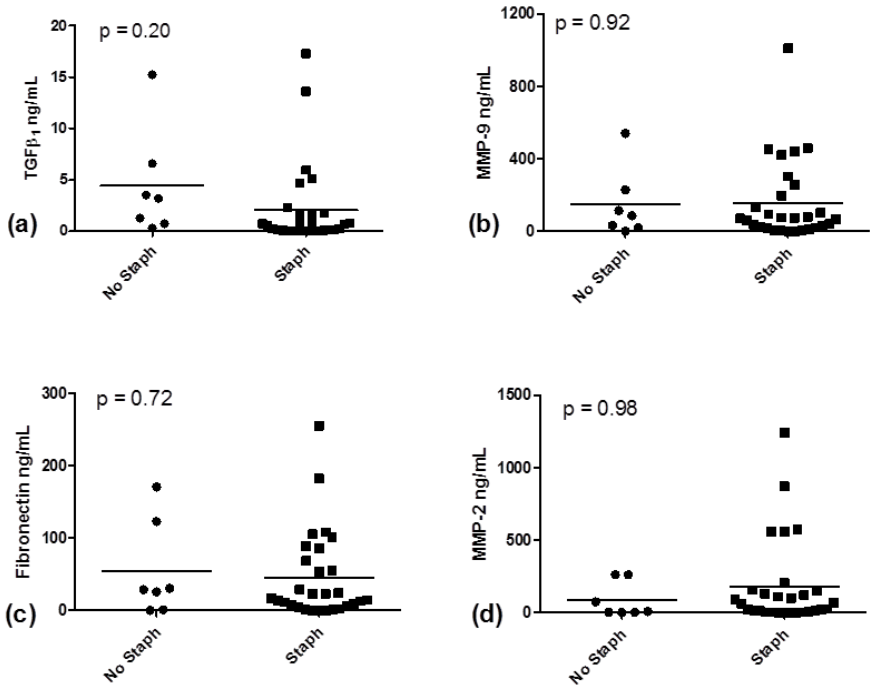


Figure 4.3. Fibrotic mediators from CF subjects' sputa and *P. aeruginosa*. Subjects were analyzed with regard to colonization status of *P. aeruginosa* (PA). TGF-β (a) was measured by TGF-β_1 E_{max} ImmunoAssay System, while MMP-9 (b), fibronectin (c), and MMP-2 (d) were measured by indirect ELISA. to the subjects' dose (mg) of AZM. Data in graphs a-d were analyzed by student's t-test and considered significant at $p < 0.05$. Data in graphs e-h were analyzed via student's t-test for group AZM+PA+ vs group AZM+PA- and via ANOVA with Bonferroni's post-test for all data. Data were considered significant at $p < 0.05$.

Fibrotic Markers and Staphylcoccus

Because such a higher percentage of subjects grew positive cultures for *S. aureus*, it was important to determine if the presence of this pathogen was influencing the concentration of these proteins in the subjects' sputa. This is particularly important considering that most patients are of the age in which *S. aureus* is a prevalent infection, and because of the emerging nature of this pathogen in this population. However, when the subjects were grouped on the basis of presence or absence of a positive *Staph* culture, there were no statistically significant differences in concentrations of TGF-β ($p = 0.20$), MMP-9 ($p = 0.92$), fibronectin ($p = 0.72$), or MMP-2 ($p = 0.98$, Figure 4.4). This suggests that *S. aureus* is not influencing the concentration of these proteins in this subject population.



Figureure 4.4. Fibrotic mediators from CF subjects' sputa and *S. aureus*. Subjects were analyzed with regard to infection with *S. aureus* (Staph). TGF-β (a) was measured by TGF-β_1 E_{max} ImmunoAssay System, while MMP-9 (b), fibronectin (c), and MMP-2 (d) were measured by indirect ELISA. to the subjects' dose (mg) of AZM. Data were analyzed by student's t-test and considered significant at $p < 0.05$.

Inflammatory Cytokines

Next, inflammatory cytokines were analyzed in the subjects' sputa. A cytometric bead array was executed for a group of pro- and anti-inflammatory cytokines. The cytokines TNF, IL-10, IL-6, and IL-12p70 were measured at below the limit of detection in all or nearly all the sputa samples. This is interesting, although not entirely unexpected, as subjects were recruited specifically when in a stable disease state. Therefore, they would not be expected to be expressing copious amounts of these inflammatory cytokines in extemporaneously expectorated sputum. However, the cytokines IL-8 and IL-1β were detectable in all the samples assessed. When compared between subjects receiving and not receiving AZM, there were no differences in cytokine concentrations between the subject groups (Figure 4.5a, b, $p = 0.22$ for both). When cytokine concentrations were compared with regard to infection status, *P. aeruginosa* colonization made no difference to concentration of either IL-8 or IL-1β (Figure 4.5c, d $p = 0.13$ and 0.61, respectively). However, subjects with positive *S. aureus* cultures had significantly lower concentrations of IL-1β (Figure 4.5e, $p = 0.04$) compared to those subjects who were negative for *S. aureus.* Levels of IL-8 were no different between subjects positive and negative for *S. aureus* (Figure 4.5f, $p = 0.82$).

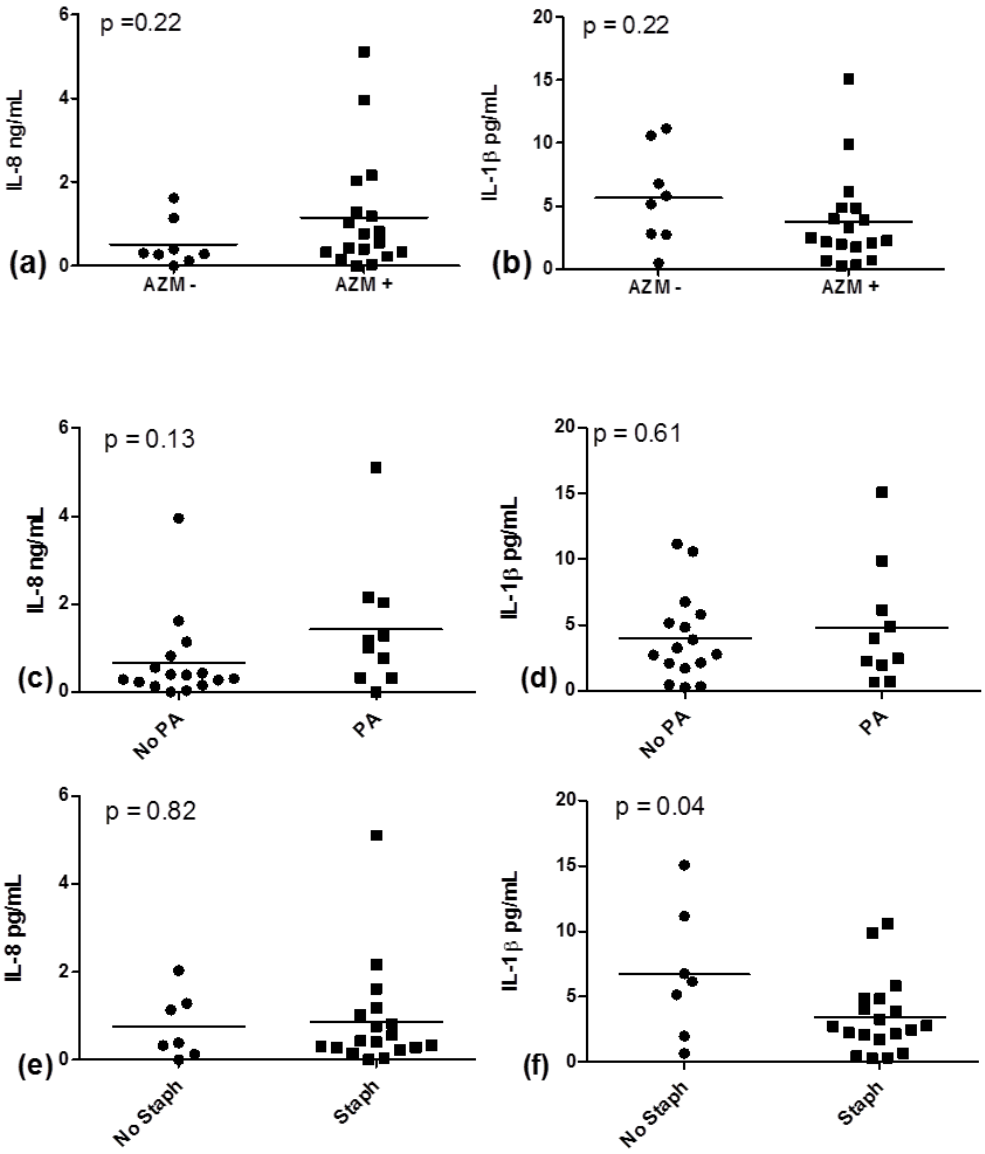


Figure 4.5. Inflammatory cytokines from CF subjects' sputa. IL-8 and IL-1β were measured by cytometric bead array. Data was analyzed with regard to subjects AZM treatment status (a) and (b), colonization with *P. aeruginosa* (PA) (c) and (d), and infection with *S. aureus* (Staph) (c) and (d). Data were analyzed by student's t-test and considered significant at $p < 0.05$.

Correlation between lung function, fibrotic, and inflammatory markers

We then examined the correlations between protein concentration and disease severity. We also examined the correlation of fibrotic proteins among themselves and with the inflammatory cytokines that were detected in the sputum samples. These

analyses were important to investigate the relationship between markers of fibrosis and inflammation. We hypothesized that the markers of fibrosis would be positively correlated to each other, indicating a fibrotic environment in those subjects being treated with AZM. We also expected to find that inflammatory cytokines would have inverse correlations to the fibrotic markers.

We initially assessed whether the proteins measured from the sputum samples of the subjects in the study were linearly correlated to age and markers of lung function. In Table 4.3, regression coefficients and p values are listed for each protein versus age, FEV1 % predicted, and FVC % predicted. A positive linear correlation existed between TGF-β and age, which was statistically significant. This indicates that the older subjects had higher concentrations of TGF-β in their sputum. TGF-β concentration was also positively correlated with FEV1 % predicted, with a p value of 0.0011. When age and FEV1 % predicted were compared, the correlation was negative; therefore some other factor must be influencing TGF-β. The correlation between MMP-9 and FEV1 % predicted was also statistically significant, and in this case the correlation was negative. This is a confirmation of a relationship shown in induced sputum samples, in a study performed in 20 subjects with CF.[90] . In this study, MMP-9 concentrations were increased in CF subjects compared to healthy controls, as well as negatively correlated to FEV1% predicted, as well as neutrophil count. It is, however, interesting that in the current study MMP-9 is not significantly correlated to age (Table 4.3), although FEV1% predicted and age are correlated in these subjects. Interestingly, fibronectin is the only protein significantly correlated to FVC % predicted. While FEV1 is the primary lung function measurement that is collected and monitored for CF disease progression, FVC % predicted is also monitored, as this marker begins to decline in late disease[162]. Therefore, the fact that fibronectin is correlated to age and FVC, but not FEV1, is interesting. This could indicate that fibronectin is also a marker of late disease decline.

Protein	Age	FEV1 % Predicted	FVC % Predicted
TGF-β	r = 0.5401 p = 0.0010	r = 0.5346 p = 0.0011	r = 0.3343 p = 0.0533
MMP-9	r = 0.1575 p = 0.3737	r = -0.4233 p = 0.0126	r = -0.0960 p = 0.5980
Fibronectin	r = 0.4009 p = 0.0188	r = -0.2779 p = 0.1115	r = -0.3419 p = 0.0478
MMP-2	r = 0.07346 p = 0.6797	r = -0.1283 p = 0.4697	r = -0.1806 p = 0.3068
IL-8	r = 0.3541 p = 0.0759	r = -0.3630 p = 0.0745	r = -0.2986 p = 0.1563
IL-1β	r = 0.1991 p = 0.3294	r = 0.3934 p = 0.0517	r = 0.2041 p = 0.3388

Table 4.3. Proteins measured from subjects sputa compared to age and two different markers of lung function; FEV1 % predicted and FVC % predicted. Data was analyzed by linear regression. Regression coefficients and p values reported for each comparison.

Next, the fibrotic markers were compared to each other to see which were linearly correlated. As expected, TGF-β and MMP-9 were highly correlated, with a p value of < 0.0001 (Table 4.4). The correlation between them was a positive one, which was interesting. This is surprising, given that TGF-β and MMP-9 have opposite effects on ECM, and would not have been expected to both be increased in the same subjects. Of additional importance was the finding that MMP-9 and MMP-2 were highly correlated, with a p value of < 0.0001 (Table 4.4). This is to be expected, given data from other studies, which suggest that MMP-9 and MMP-2 are upregulated in the same disease processes in human studies.[68, 163] It is not surprising because the two MMPs are generally produced at similar levels as demonstrated in the previous figures that assess the effects of AZM, *P. aeruginosa,* and *S. aureus* status in the subjects.

	TGF-β	MMP-9	Fibronectin	MMP-2
TGF-β	—	r = 0.7749 p < 0.0001	r = -0.3045 p = 0.1305	r = 0.4531 p = 0.0201
MMP-9	r = 0.7749 p < 0.0001	—	r = -0.2068 p = 0.3108	r = 0.7414 p < 0.0001
Fibronectin	r = -0.3045 p = 0.1305	r = -0.2068 p = 0.3108	—	r = -0.3592 p = 0.0726
MMP-2	r = 0.4531 p = 0.0201	r = 0.7414 p < 0.0001	r = -0.3592 p = 0.0726	—

Table 4.4. Fibrotic proteins measured from subjects sputa compared to markers of ECM turnover. Data was analyzed by linear regression. Regression coefficients and p values reported for each comparison.

Lastly, fibrotic mediator concentrations were analyzed against inflammatory cytokines. We were interested to know if the markers of inflammation were linearly correlated to markers of fibrosis or ECM turnover. This was important to help determine if inflammation is associated with fibrosis, or if the shift toward fibrosis includes a shift away from inflammation. IL-8 was weakly correlated to all four makers of fibrosis, with very high p values (Table 4.5). IL1-β, however, was strongly positively correlated to both TGF-β and MMP-9, with p values < 0.0001. IL-1β was correlated with MMP-2 as well, had a correlation, with a p value < 0.05 (Table 4.5). Interestingly, neither cytokine was significantly correlated with fibronectin (Table 4.5).

	IL-8	**IL-1β**
TGF-β	r = 0.2256 p = 0.2678	r = 0.8222 p < 0.0001
MMP-9	r = -0.1438 p = 0.4835	r = 0.7448 p < 0.0001
Fibronectin	r = 0.1653 p = 0.4198	r = -0.2272 p = 0.2643
MMP-2	r = 0.1653 p = 0.4198	r = 0.5314 p = 0.0052

Table 4.5. Fibrotic proteins measured from subjects sputa compared to inflammatory cytokines measured in subjects' sputa. Data was analyzed by linear regression. Regression coefficients and p values reported for each comparison.

Effect of azithromycin

The linear correlations above were analyzed for all the subjects grouped together without regard to their status of AZM treatment. This analysis is important in light of our data presented previously showing no significant difference in the mean concentrations of these protein concentrations between subjects who are and are not receiving AZM. In the CF population, AZM is dosed on a daily basis, although there is no standard dosage recommendation.[92] Traditionally, patients with CF are prescribed AZM once they are positive for *P. aeruginosa*, although recent studies have given evidence for physicians to prescribe this drug for colonization.[103] Because subjects with more progressive disease should have increased levels of fibrosis, AZM may be functioning to ablate these effects. We report in Figure 4.6 that certain markers were found to be have different correlations in subjects receiving AZM compared to subjects not receiving AZM. TGF-β had a more moderate correlation with FEV1 % predicted in subjects on AZM, with an $r = 0.4058$, compared to subjects without the treatment, who had an $r = 0.7376$. In this case, the statistical significance remained the same (Figure 4.6a, b). However, subjects who were not receiving AZM had a very strong correlation of IL-1β to FEV1 % predicted, with an $r = 0.8926$ and a statistical significance of $p = 0.0029$. When subjects treated with AZM were analyzed similarly, the correlation was found to be far weaker, $r = 0.1964$, and there was no statistical significance (Figure 4.6c, d).

Similarly, subjects not receiving AZM treatment demonstrated a weak correlation of $r = 0.3458$ between TGF-β and MMP-9, a relationship that was not statistically significant. When these markers were analyzed in subjects on AZM, a regression coefficient of 0.5973 was observed, which is stronger, and the p value dropped below 0.05 (Figure 4.6e, f). While the study is not able to determine causality, it would appear that AZM therapy is impacting relationships between proteins and lung function, and between some proteins among themselves.

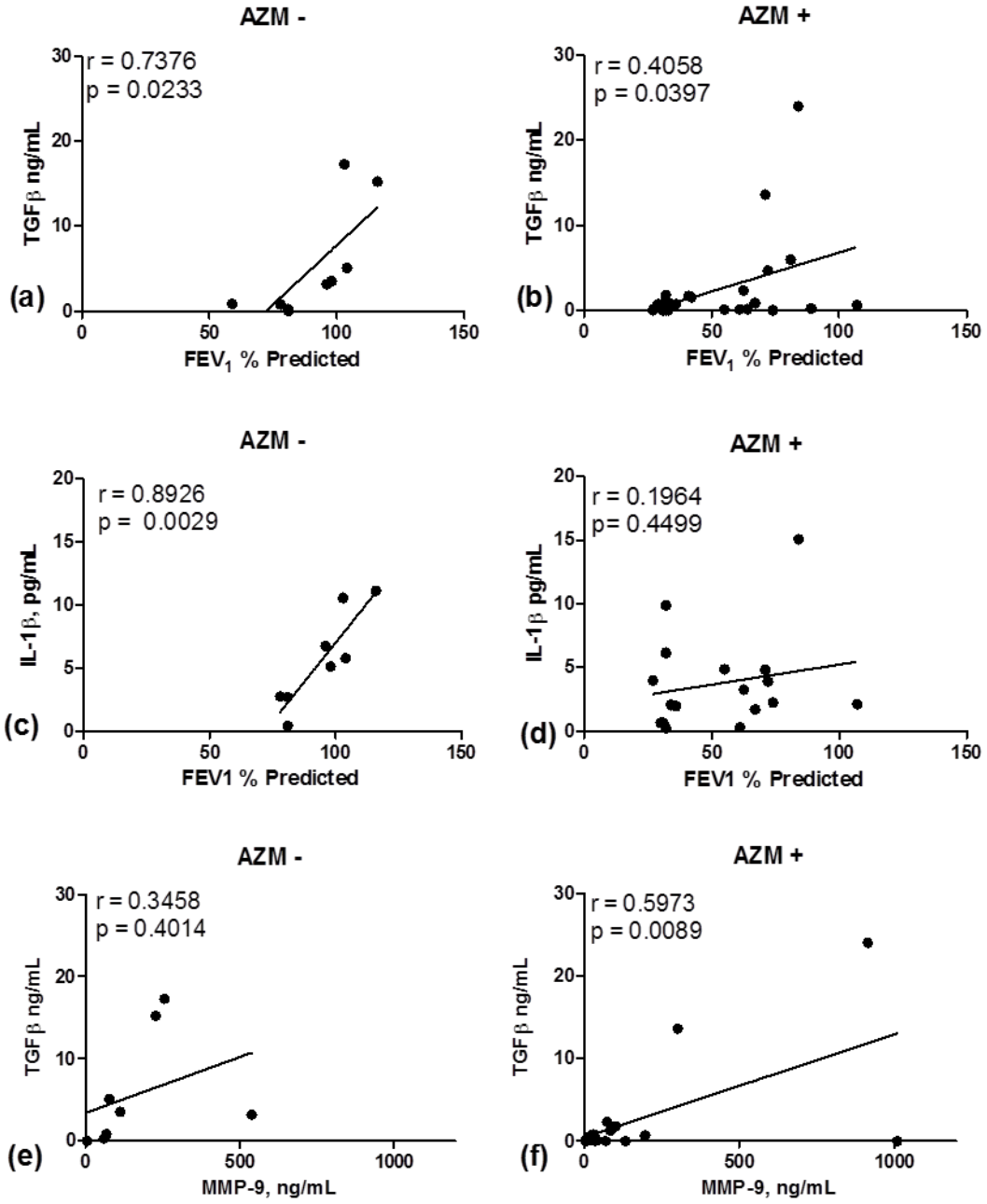


Figure 4.6. Proteins correlated by linear regression to lung function or other proteins. TGF-β concentrations correlated to FEV1 % predicted compared by linear regression in subjects either not on AZM (a) or on AZM therapy (b). IL-1β concentrations correlated to FEV1 % predicted compared by linear regression in subjects either not on AZM (c) or on AZM therapy (d). TGF-β concentrations correlated to MMP-9 concentrations compared by linear regression in subjects either not on AZM (e) or on AZM therapy (f) Statistical analysis was performed with linear regression. Regression coefficients and statistical significance indicated on the graphs.

Multifactorial Analysis

In order to better determine the relationships between these proteins, we utilized principal component analysis as a data reduction method. This allows for simultaneous evaluation of protein expression variability between the subjects. When the data are analyzed this way, two primary components appeared in the subjects, The first component, named PC1, was heavily comprised of TGF-β, MMP-9, and MMP-2, and accounted for 53% of total variance of the protein expression. (Figure 4.7). The second component, PC2, was primarily determined by fibronectin and IL-1β concentrations, and comprised and additional 19% of variability between the samples. Interestingly, IL-8 contributed to both groups(Figure 4.7). When the populations are represented graphically, the subjects who were not receiving AZM had higher expression of PC1, while subjects who were receiving AZM had higher expression of PC2. These associations confirm that fact that TGF-β is highly correlated to both MMPs, as well as confirming a potential pro-fibrotic role for IL-1β, as it is correlated with fibronectin. The data also highlight the fact that AZM treatment appeared to be creating two different populations of protein association.

Additionally, each component was analyzed according to AZM treatment status and *P. aeruginosa* colonization status. Subjects receiving AZM treatment are less likely to be expressing PC1 associated proteins that subjects who are not receiving AZM (figure 4.8). This is not surprising, when remembering that subjects on AZM had lower mean TGF-β expression. However, subjects receiving AZM were more likely to be expressing PC2 associated proteins (Figure 4.8). This suggests that the drug may be shifting protein expression from PC1 associated proteins toward PC2. However, colonization with *P. aeruginosa* did not alter the expression of these proteins to statistical significance (Figure 4.8). This also suggests that AZM treatment is more influential than *P. aeruginosa* colonization.

a)

	Factor 1	Factor 2	Factor 3
TGFb	.884	.026	.176
MMP-9	.918	.087	.193
MMP-2	.782	-.105	-.099
Fibronectin	-.438	.455	.755
IL-1b	-.180	.878	-.427
IL-8	.862	.390	-.001

b)

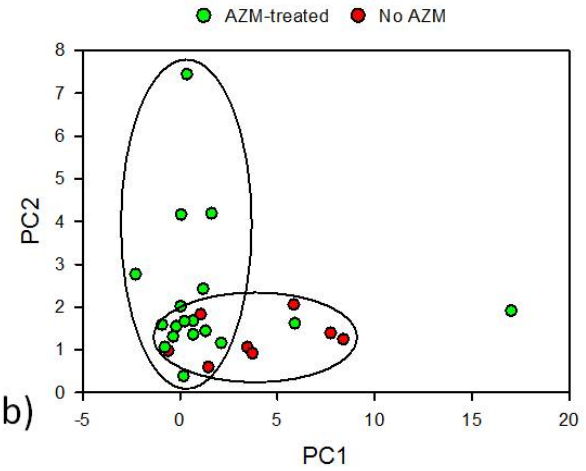


Figure 4.7. Principal component analysis of protein expression. a) component matrix for proteins assessed. Components 1 and 2 accounted for 72% of total variance in protein expression. b) Component expression in AZM treated subjects (green) and non-treated subjects (red).

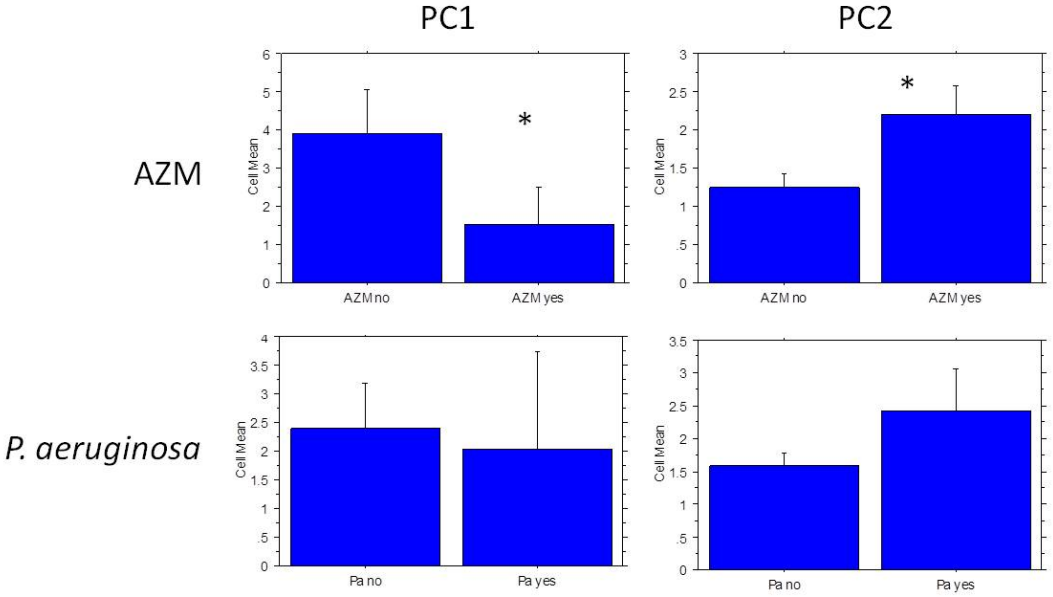


Figure 4.8. PC1 and PC2 expression. Expression of each component was compared to AZM treatment status and *P. aeruginosa* colonization status. Data are expressed as mean + 1 SE, and were analyzed with a Mann-Whitney Rank test. * indicates $p < 0.05$.

Arginase activity

The last goal for the human study was to determine if arginase, the effector molecule of alternative macrophage phenotype, would be correlated with other markers of fibrosis or cytokines in subjects with CF. This data is presented in Table 4.6.

Arginase concentration, assayed by arginase activity normalized to total protein, was measured in the subjects' sputa, and analyzed by linear regression with regard to the markers of fibrosis, as well as cytokines IL-8 and IL-1β. There were no significant correlations between arginase and any of the proteins measured, nor were these values changed when the data was analyzed with regard to AZM treatment status.

	Arginase		Arginase
TGF-β	r = - 0.2903 p = 0.1503	IL-8	r = 0.2639 p = 0.2024
MMP-9	r = - 0.2218 p = 0.2761	IL-1β	r = -0.0749 p = 0.7218
Fibronectin	r = - 0.2023 p = 0.3216		
MMP-2	r = -0.0112 p = 0.9566		

Table 4.6. Proteins measured from subjects sputa compared to arginase activity. Data was analyzed by linear regression. Regression coefficients and p values reported for each comparison.

We also analyzed arginase concentrations with regard to subjects' AZM treatment status, colonization with *P. aeruginosa,* and infection with *S. aureus.* When arginase activity levels were compared between subjects who were and were not receiving AZM therapy, the difference between the two groups were not statistically significant, Figure 4.7a (p = 0.11). However, there is a trend toward higher arginase activity in the AZM therapy group, and more subject samples may make this difference more significant. When arginase concentrations were compared between subjects who were colonized with *P. aeruginosa* and those who are not, there was a statistically significant increase in any concentration in subjects colonized with *P. aeruginosa* (p = 0.0009, Figure 4.7b). Interestingly, when the comparison was made for subjects with positive *S. aureus* cultures vs. subjects with negative *S. aureus* cultures, there was no

statistical difference, although there was a trend in the positive group toward higher arginase activity (Figure 4.7c). Because many of the subjects who were positive for *P. aeruginosa* were also positive for *S. aureus*, we compared arginase concentration between subjects positive for both organisms, subjects who were positive for only one, and subjects who were negative for both (Figure 4.7d). In this analysis, the double positive group had strikingly higher arginase activity levels, statistically significantly higher than subjects with *P. aeruginosa* only (p value < 0.01), *S. aureus* only (p value < 0.0001), and patients colonized with neither pathogen (p value < 0.01). While the sample sizes of these groups are small, this data is highly suggestive that the combination of the two pathogens may shift the environment in the lung so that it is conducive to alternative macrophage polarization. It is also interesting to note that the other proteins examined in this chapter where analyzed with regard to double infection, and none of them were found to be affected in this manner.

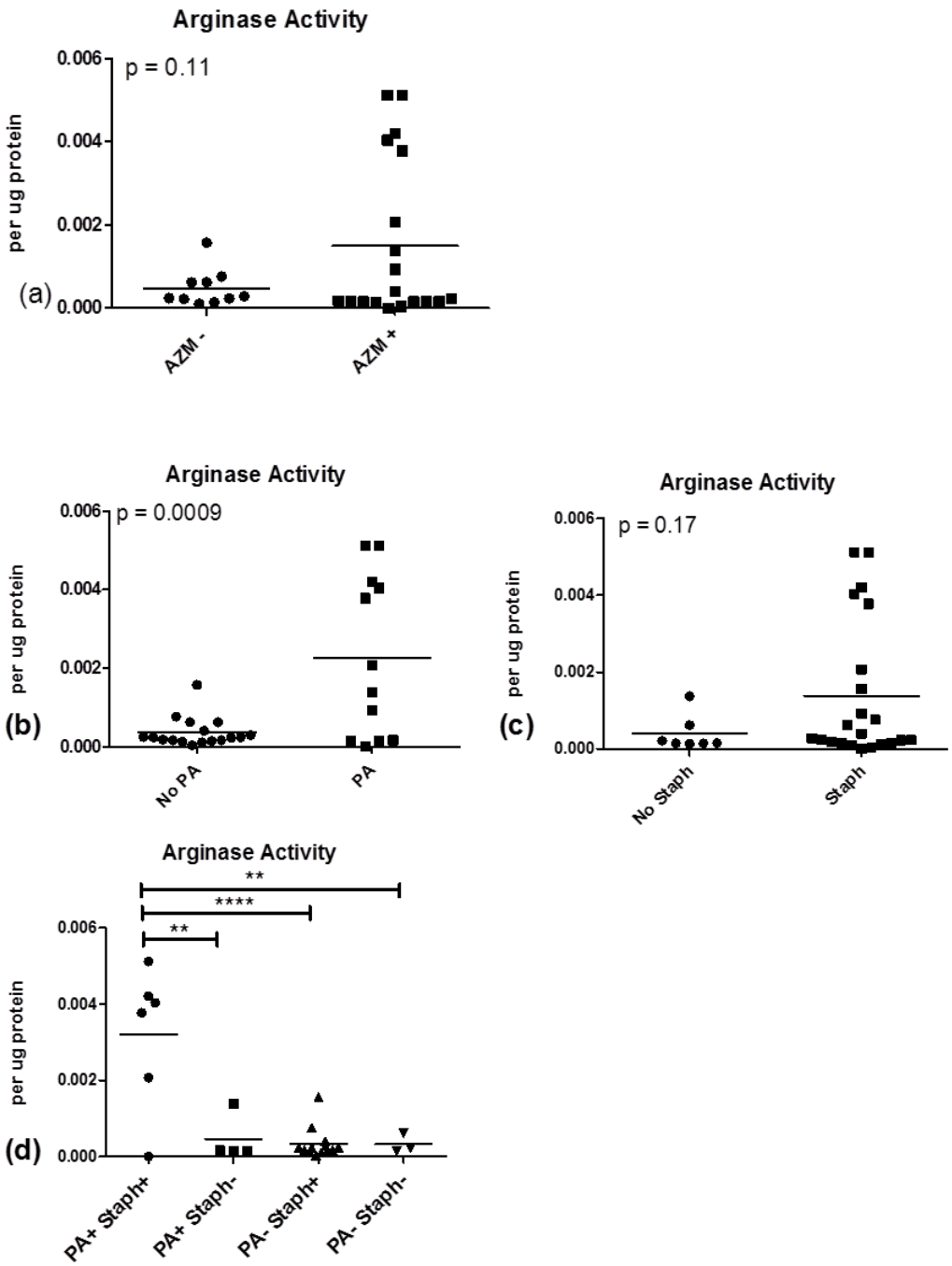


Figure 4.9. Arginase production in CF subjects' sputa. Arginase activity was measured as described in Methods. Subjects were analyzed with regard to AZM therapy (a), colonization status of *P. aeruginosa* (PA) (b), and *S. aureus* (c). In (d) subjects were split into PA+Staph+, PA+Staph-, PA-Staph+ and PA-Staph- groups. Data a-c were analyzed by student's t-test and considered significant at $p < 0.05$. Data in graph d was analyzed via ANOVA with Bonferroni's post-test for all data. Significance is reported on graphs, or as $p < 0.01$ (**) or < 0.0001 (****).

D. Conclusions

The human study allowed us to draw some interesting conclusions about the use of AZM in the CF population. While this study did not prove the proposed hypothesis to be correct, as subjects receiving AZM did not have higher levels of fibrotic protein concentrations overall, our data indicated that AZM may have a protective role. First, there were no overall increases in fibrotic mediators associated with AZM therapy as predicted by our *in vitro* data; in fact TGF-β was decreased in subjects on AZM. Because there was also no difference in fibronectin concentrations between the two groups, this data indicated that AZM may not contribute to increases in fibrosis development. This data was not surprising in combination with the gene data collected on the same subjects; fibronectin gene expression also was not increased in subjects treated with AZM. Additionally, the lack of difference in the two different MMPs measured shows no effect on ECM turnover when stratifying by AZM treatment. While presence of *P. aeruginosa* infection also resulted in lower TGF-β concentrations, further analysis suggested that this was more reliant on the fact that those patients infected with *P. aeruginosa* were being treated with AZM concurrently, confirmed by the PCA data. In the multifactorial analysis, AZM appeared to be an influential factor in altering protein expression, while *P. aeruginosa* colonization status did not significantly alter any proteins.

Because the subjects recruited were in a non-exacerbated state, inflammatory cytokines were expected to be of less importance. However, IL-1β exhibited some interesting differences in these subjects. While apparently not affected by AZM treatment, there were decreased concentrations of this cytokine in subjects positive for *S. aureus* infection compared to subjects who had negative cultures for this pathogen. This is particularly interesting, given evidence that suggests *S. aureus* can activate the inflammasome and induce secretion of IL-1β in monocytes and macrophages.[164, 165]

Furthermore, both TGF- β and IL-1β concentrations were highly correlated to FEV1 % predicted in subjects not being treated with AZM, but had markedly reduced correlations to FEV1 % predicted in subjects on AZM therapy. The alteration in these relationships suggests that AZM treatment may be affecting the interactions of these molecules in the mechanism of fibrosis development. Because of IL-1β's dual role in inflammation and fibrosis,[160] these correlations become significant.

While this human study was similar to the previous human study performed by our lab, in that we were unable to significantly associate increased arginase levels with AZM therapy,[114] similar results were obtained in the association between *P. aeruginosa* infection status and increased arginase activity. This subject group showed a striking trend toward increased arginase concentrations in subjects who were receiving AZM compared to those subjects who were not. Here, however, we show that subjects co-cultured with both *P. aeruginosa* and *S. aureus* have increased arginase activity, suggestive of the importance of an alternative macrophage phenotype in these subjects. This piece of data is quite important in light of the rising incidence of *S. aureus* infection in both the CF and the general population. Patients with CF are becoming more and more likely to be infected with both pathogens. The combination of AZM treatment and the alternative macrophage phenotype may be compensating to limit damage as lung function gets work. *In vitro* data from our lab has demonstrated that AZM does not have an effect on macrophage phenotype unless the cells are stimulated with LPS.[112] This may be why there is less arginase activity in subjects with neither pathogen.

Samples from subjects in the current study had been previously analyzed for gene markers indicative of macrophage phenotype. The inflammatory gene NOS2, which encodes for iNOS, was decreased in subjects treated with AZM, corroborating evidence that AZM and the alternative phenotype may be preventing damage, although arg1, the gene that encodes for arginase, was unaffected. Inflammatory cytokine genes

IL-1a and TNFα were positively correlated with FVC% predicted, as was the anti-inflammatory cytokine IL-10. Furthermore, principal component analysis was used to identify groups of patients with CAM and AAM-associated genes. Interestingly, MMP-9 was grouped with CAM-associated genes, while TGF-β was grouped with AAM-associated genes. Given that the proteins are so tightly correlated to each other in the same patients, their gene groupings are interesting. This may suggest that TGF-β and MMP-9 are upregulated together in order to prevent a universal shift toward either fibrosis or damage; neither fibrotic accumulation nor tissue damage is greater.

There are two possible scenarios for the function of AAMs in subjects with CF. In the first scenario, AAMs are upregulated compared to CAMs, which results in increased accumulation of fibrosis and lung structure change. In the second scenario, the increase in AAMs compared to CAMs causes decreased inflammation, which prevents the need to upregulate fibrosis, and therefore prevents further change in lung structure. The data collected in the human study, when combined with the data from chapters 2 and 3, suggest that the second scenario Is present in subjects with CF who are treated with AZM.

Limitations

There were several limitations to this study. First, since the study focused on subjects with stable disease, most of the CF patients recruited were pediatric, and all the subjects included in the protein analysis were under 25 years of age. While it is certainly possible for a patient with CF to have advanced disease at this age, we could be excluding a subset of the CF population that might be responding to AZM differently due to their age or disease process. Furthermore, the younger patients, regardless of lung function, were likely to have experienced fewer exacerbations and fewer types of infection, which also may be influencing the response of the immune system. Further,

because we were unable to control how long the subjects had been receiving AZM, there may be variation in the mediators measured, and may or may not reflect long-term treatment status.

Importantly, because subjects were recruited from the same clinic, they were all subject to the same prescribing practices. In this instance, that meant that these subjects are likely to be on AZM before they are infected with *P. aeruginosa*, and all of the subjects that were positive for *P. aeruginosa* were on AZM therapy. While this is becoming a national trend, it becomes a complication factor when distinguishing an effect of the drug vs. and effect of the pathogen. In addition, the effect of AZM seen in the PCA data may be confounded by disease severity. In the future, we may be able to separate the subjects into groups based on disease severity, to determine if this has any effect.

Lastly, because we collected sputum samples, there was some variability inherent in the study. This was due to variation in the amount of sample collected from each subject and the processing required. In addition, while subjects receiving oral glucocorticoids were excluded from the study, the subjects included were still on a range of medications, including inhaled glucocorticoids, which may confound results.

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Chapter 5: Discussion

A. Overview of Results

The goal of this project was to determine the role of the AZM-polarized macrophage in the process of ECM protein accumulation through the increased production of TGF-β and MMP-9. This was pursued by combining *in vitro, in vivo,* and human studies to determine the impact of AZM on remodeling and fibrosis mediators in response to *P. aeruginosa*. We investigated the impact of the AZM-polarized macrophage on fibroblasts using *in vitro* experiments, as well as the role of the AZM-polarized cell in the immune response to *P. aeruginosa* pulmonary infection. Extending our studies to human subjects with CF, our work contributes to a more global understanding of the immunomodulatory impact that AZM therapy can impart during the progression of CF pathology.

B: Significance

The fibrotic effects of AZM-polarized macrophages

One important function of the macrophage is to secrete chemokines and cytokines that alter other cell types. Therefore, one goal of the project was to determine how the AZM-polarized macrophage would affect other cell types, particularly concerning fibrotic mediator production. The *in vitro* work helped to identify some of the interactions between two types of cells involved in the upregulation of fibrotic mediator production. The single cell line controls run along with the co-culture experiments showed that the combination of the two cell types together was necessary for the increases in TGF-β, MMP-9, and fibronectin production.

The response of the macrophage to the drug AZM has, in part, been elucidated.[112] Our first goal was to continue this work by examining the production of

molecules from the macrophages polarized with AZM, as well as to investigate the effects of this macrophage on fibrotic mediators produced from fibroblasts. In the lung, macrophages move to the site of injury, as part of the initial immune response, where they must interact with the resident cells. In this initial investigation, we found that the interaction with fibroblasts allowed the AZM-polarized macrophage to increase production the pro-fibrotic molecules arginase, TGF-β, and fibronectin. From the studies outlined in Chapter 1, the associated increases of these three molecules were not unexpected. However, the shift toward fibrosis was not universal; AZM treatment did not increase collagen I or III, and it also increased the production of MMP-9, a protease. This shows that the AZM-polarized macrophage does not share all the characteristics of the typical IL-4/IL-13 stimulated AAM.[27]

The increase in MMP-9 concentrations caused by the addition of AZM was an unexpected result. Of importance, increased MMP-9 concentrations were not dependent on either TGF-β (as shown in the TGF-β neutralization experiments) or arginase (as shown in the arginase inhibitor experiments), while increased fibronectin production was dependent on both TGF-β and arginase. Because MMP-9 was not dependent on either, it was hypothesized that TGF-β may in fact be activated by the protease as an important mechanism in this setting as AZM drives macrophage polarization; the ability of MMP-9 to activate TGF-β has been shown in a model of tumor invasion.[127] However, this was not shown to be the case when MMP-9 itself was inhibited. A possible role for MMP-9 in fibronectin upregulation, however, was observed. MMP-9 and fibronectin have been linked in previous studies; fibronectin production can induce MMP-9 upregulation. When fibronectin is directly added to human monocytes in the presence or absence of TNF, MMP-9 protein is secreted; in addition, greater amounts of MMP-9 are produced when the fibronectin is added as specific fragments.[77] This indicates that already degraded fibronectin may initiate a positive-

feedback loop to accelerate its own degradation. In a wound healing assay *in vitro*, cells were treated with fibronectin, causing an increase of MMP-9 mRNA and protein activation, compared to cells not treated with fibronectin. Cell migration was observed in these wells.[166] This suggests that MMP-9 is degrading fibronectin in order to facilitate migration to sites of wound healing. Addition of a fibronectin neutralizing antibody prevented the increase in MMP-9 production, suggesting that fibronectin itself is the molecule that triggered the increased production of the protease.[167] However, the ability of MMP-9 to affect fibronectin is a functional role that has not previously been published.

The *in vitro* experiments also confirmed a possible mechanism for AZM's ability to polarize macrophages to an alternative-like phenotype. Previous results from our lab suggest that AZM reduces arginase production when the molecule IKK-β is inhibited. Here we extend these results to show that production of downstream effector molecules are also reduced when this inhibitor is added. AZM-polarized macrophages do not appear to have the same effect on fibroblasts when IKK-β cannot signal properly. Multiple studies have now shown that AZM has the ability to decrease NF-κB activity. In epithelial cells with a mutated CFTR, AZM treatment reduces DNA binding by NF-κB *in vitro.*[101] When epithelial cells are treated with AZM, there is less nuclear translocation of NF-κB, resulting in less upregulation of IL-8 in response to LPS.[168] The effect of AZM on NF-κB has been shown in human cells as well; in human tracheal aspirates taken from premature infants, AZM suppressed NF-κB activation and subsequently IL-8 secretion when the cells were cultured *ex vivo*.[169] In dendritic cells, inhibition of NF-κB activation by AZM also inhibits maturation and function of these cells.[170]

Previous data from our lab has isolated IKK-β as an important molecule in this pathway; AZM treatment dramatically increases expression of this protein. Work done by Fong, et al, has demonstrated that IKK-β can inhibit inflammatory functions in

macrophages by preventing Stat-1 activation.[113] Our previous data has shown both an increase in IKK-β expression and a decrease in cytokines downstream of Stat-1 signaling.[112] Possible IKK-β/Stat-1 cross-talk may help explain how AZM is able to downregulate the inflammatory cytokines and effector proteins, such as iNOS, and allow increase of alternatively activated-associated effector proteins instead. Current work is under way to investigate the interaction between these two signaling molecules in our AZM treatment model.

The AZM-polarized macrophage in the lung

When the AZM treatment during infection with *P. aeruginosa* was investigated *in vivo*, there was added complexity to the results obtained upon examining the interactions of the fibrotic mediators and cellular responses. This was not unexpected, given that there are many more cell types involved in the pulmonary immune response, and that the resolution of the infection occurs over weeks rather than days. The complexities included movement of the important mediators and cells from the lung interstitium into the alveolar space, as measured by bronchoalveolar lavage. Treatment with AZM has been shown to decrease neutrophilic influx in a model of endobronchial infection with *P. aeruginosa*, although this study did not examine the effect on macrophages in response to either infection or AZM. [171] The response of macrophages, however, is important, because $Cftr^{-/-}$ mice have been shown to respond to *P. aeruginosa* with an increase in macrophage influx when treated with AZM.[172] Furthermore, this shift from neutrophilic to monocytic response is also observed when erythromycin is used for pre-treatment during *P. aeruginosa* infection.[173] Therefore, it was important to determine the function and phenotype of these macrophages. The most striking result was that the increased numbers of macrophages in the BALF in the C57Bl/6 mice occurred at the same timepoint as the increased concentrations of MMP-9 and fibronectin in BALF in the

mice treated with AZM. Given that MMP-9 is so often strongly associated with neutrophil count [85, 142, 148, 174], it is an important distinction that MMP-9 is increased when neutrophils are decreased in this model. Lastly, these changes temporally followed a shift in the lung of expression from the CAM marker iNOS toward the AAM marker arginase in the same mice, as was also observed *in vitro*.

The mice that produce more iNOS had more evidence of pulmonary damage as a result of *P. aeruginosa* infection after resolution. The IL-4rα$^{-/-}$ mice that did not receive AZM continued to express iNOS until day 14 post-infection, and had significantly higher amounts of peribronchiolitis and alveolitis at day 14 compared to the same mice treated with AZM. This group did not have higher arginase concentrations in the lung but did have decreased iNOS at day 14, indicating that AZM's effect on iNOS may be the important factor in decreasing damage. Similarly, the C57Bl/6 mice of both treatments have less iNOS expression at day 7 compared to day 3, and the damage in these mice was less than in the IL-4r-/- mice. Increase in iNOS expression, and a general switch to a more CAM-like phenotype in the absence of IL-4 and IL-13 signaling, has been previously shown in the IL-4rα$^{-/-}$ mice.[175, 176] These studies utilized parasitic infection models to drive macrophage polarization toward AAMs. The adoptive transfer experiment confirmed that the alternative macrophage can be protective - the shift away from iNOS expression may be one of the protective effects of this phenotype. The shift from iNOS to arginase has been shown in multiple models as macrophages become alternatively activated, in response to cytokines, tumors, or infection.[26, 176, 177] The identification of decreasing iNOS as the protective event is confirmed by a study of iNOS knockout mice that were resistant to injury and fibrosis secondary to bleomycin administration.[178] Likely the contribution to inflammation made by iNOS is initiating the repair processes causing the disease. When iNOS is decreased, less damage

occurs, requiring fewer repair processes. This illustrates one way by which AZM may decrease damage caused by infection and inflammation.

The effect of AZM in the lungs of mice infected with *P. aeruginosa* was examined as well. By histological analysis, lungs of mice treated with AZM before and during infection showed increases of arginase expression accompanied by a decrease of iNOS expression; suggesting a shift of the macrophages toward an AAM phenotype. The increases in arginase in the lung at day 3 were followed by an increased concentration of both MMP-9 and fibronectin in the BALF of the AZM-treated mice at day 7. This is significant, because the increases in arginase preceded the increases of MMP-9 and fibronectin observed *in vitro*. Interestingly, the opposite effect was seen by histology; MMP-9 and fibronectin were reduced in the lung tissue in AZM-treated mice. This may be because the histological analysis stains for all the cell types in the lung, while the *in vitro* studies were limited to macrophages and fibroblasts. It has been shown by others that AZM can decrease MMP-9 production from epithelial cells. [143] Fibronectin is also made from many cell types, including epithelial cells, [179], and its production may be affected differently by AZM in these cells. In contrast, the majority of cells in the BALF tend to be immune cells, which may be why this fraction was affected by AZM in the way that was predicted by the *in vitro* experiments.

However, when examining the histology of the lungs to evaluate damage caused by the infection, there are some observations to be made beyond the pathologies that were compared. The mice that were treated with AZM had histological changes at day 14 that were not inflammatory in nature. This may indicate a type of pathology that is more common in another disease state. For instance, chord length (the measurement between alveoli), was not measured in our studies. This is a pathology that appears in certain types of COPD, but is not indicative of inflammation, infection, or fibrosis,[180] and yet may be altered by the treatment. Because we were unable to examine

physiological endpoints in the mice, and because the experiments lasted only 14 days, we were unable to determine whether the AZM-treated mice experienced any other consequences of treatment, such as change in elasticity or compliance of the lung.

When the lungs of each group of mice were examined for damage and markers of pathology, there were some interesting differences. Overall, the lungs of the mice treated with AZM appeared to have less damaged, but the architecture was altered compared to baseline. There was overall increased alveolar size in the AZM treated mice, especially at later timepoints. However, the drug did reduce bronchitis and alveolitis at early timepoints – yet alveolitis and interstitial pneumonitis were increased by the AZM at later timepoints. The AZM-treatment group also showed increased collagen accumulation at the latest timepoint compared to the vehicle group. However, the collagen was accumulated to the bronchi, and there was no difference in bronchitis at day 14 between the two groups. Therefore, it is unclear if the collagen accumulation is physiologically significant.

This increase in collagen observed in the mouse model of infection was not present during the *in vitro* experiments. This may be due to the fact that the increase happens late – 14 days post-infection. This type of chronic infection is difficult to replicate in cell culture, as cell viability becomes an issue. This may account for the difference observed in the co-culture – 48 hours may still be too early for any increase in collagen to appear.

Evaluation of pathologic processes in the IL-4rα$^{-/-}$ mice were quite different between the treatment groups. Peribronchiolitis and alveolitis were less severe in the AZM treatment group at days 7 and 14 compared to the vehicle groups, and bronchitis was less severe in the AZM group at day 7. Based on this difference, IL-4rα$^{-/-}$ mice were given either T cells or monocytes and T cells by adoptive transfer. In this experiment, the mice that received both types of cells had less alveolitis and interstitial pneumonitis,

although the differences were not statistically significant. The damage in the mice that received T cells alone mimicked the damage seen in IL-4rα$^{-/-}$ infected with *P. aeruginosa,* but not treated with AZM. Those mice that receiving monocytes and T cells more resembled the IL-4rα$^{-/-}$ mice that were infected but also received AZM; however the addition of monocytes did not reduce the damage to the degree that the drug did. This could indicate that the IL-4rα$^{-/-}$ which receive normal T cells and monocytes may be able to polarize to an alternative phenotype in response to infection. However, the presence of AAMs does not reduce inflammation as much as the administration of AZM.

Fibrotic mediators in human subjects

For our human study, we were unable to enroll subjects who were infected with *P. aeruginosa* and simultaneously not treated with AZM. This is due to the current practice of having all or most patients with CF who are infected with *P. aeruginosa* on AZM. For this reason, we did not have the comparison group that we had in the cell culture and mouse studies. This heightens the importance of our complimentary evaluation in our *in vitro* and mouse models.

The study performed in human subjects with CF followed up on examination of the fibrotic mediators studied in the other models. We expected subjects treated with AZM to have increased markers of fibrosis compared with subjects who were not on AZM therapy. However, subjects receiving AZM had decreased concentrations of TGF-β in their sputum. This change was consistent in subjects with positive *P. aeruginosa* cultures compared to subjects who were negative for this pathogen. Because the subjects who were colonized with *P. aeruginosa* were all on AZM, it was difficult to elucidate which was the underlying factor causing the decreased TGF-β concentrations. There are no published reports of changes in sputum TGF-β concentrations associated

with the presence of *P. aeruginosa,* although there is some evidence that plasma TGF-β levels are increased in the presence of the pathogen.[72] This was shown in a study of 40 children, comparing those with and without *P. aeruginosa* positive cultures. In this same study, BAL concentrations of TGF-β were not affected by presence of *P. aeruginosa.* However, the authors argue that plasma concentrations of TGF-β are a reliable indicator of lung environment, as they correlated with BAL concentrations.

Interestingly, more of the subjects in the study were infected with *S. aureus* than *P. aeruginosa,* yet the presence of this pathogen did not appear to affect the presence of any of the fibrotic mediators. The only protein that was different between subjects based on their *S. aureus* infection status was IL-1β; its concentrations were lower in subjects infected. Simultaneously, IL-β concentrations were not different when analyzed in terms of AZM treatment or *P. aeruginosa* infection status. Previously, we found that IL-1β concentrations are increased in subjects who are positive for *P. aeruginosa* compared to subjects who are not. [114] Subjects from these two studies had remarkably similar demographics, yet the current study excluding patients who were on systemic glucocorticoids, while the previous study included them. Also, the subjects in the previous study were not analyzed by presence or absence of *S. aureus.* These issues may be the source of variation between the two.

The proteins in the sputa that correlated to each other and to degree of lung function depict interesting characteristics associating macrophage function and fibrosis in these subjects. TGF-β was positively correlated to both lung function and MMP-9. IL-1β was positively correlated with TGF-β, MMP-9, and lung function. This suggests that these molecules may interact during lung function decline. Furthermore, many of the correlations were different when the data was analyzed separately by AZM treatment group. TGF-β and IL-1β have weaker correlations with FEV_1 in subjects receiving AZM compared to subjects who are not. This data indicates that treatment with the drug may

be changing some of the interactions of the fibrotic and inflammatory mediators we are examining. Furthermore, that TNF, IL-10, IL-6, and IL-12 were below level of detection are significant findings. There is evidence that these inflammatory cytokines are typically elevated chronically in CF,[181] and it was expected that we would have detectable levels of these cytokines in our subject population. Although the FEV1 measurements would suggest otherwise, the subjects in our study may have been on average young enough to have mild to moderate disease. Additionally, the above study did not specify whether the subjects were being treated with AZM. It may be that the high percentage of AZM treatment in our subjects may be the reason for the decreased inflammatory cytokines.

Lastly, the AAM effector protein arginase was measured in these subjects. As in our previous work, arginase concentrations were not different in subjects when analyzed for their AZM treatment status, although there was a trend toward higher arginase in those who were treated with AZM. Arginase was, however, increased in subjects positive for *P. aeruginosa,* compared to subjects who were negative for this pathogen. This corroborates conclusions of our previous study.[114] However, in the current study, subjects were analyzed for double colonization with *P. aeruginosa* and *S. aureus* versus single colonization with either pathogen. Traditionally, patients with CF tend to culture *S. aureus* less frequently as they become positive for *P. aeruginosa.*[5, 182] However, as *S. aureus* becomes more prevalent, this appears to be changing,[9] making those subjects that are culture-positive for both pathogens an important sub-population. It is important to know how the immune response changes in those patients, and how this affects their lung function. Those subjects who were positive for both pathogens had significantly higher arginase activity compared with subjects who were colonized with a single bacterium. There was no difference between subjects who were singly colonized and subjects who were negative for both. While we were unable to measure any other

marker of AAM in these subjects, the increased arginase activity may indicate a shift toward the alternative phenotype in these subjects. This may not be the case for the subjects who are not colonized with either bacteria because cells must be stimulated with a bacterial trigger, like LPS, in order for AZM to have an effect.[112] Additionally, AZM treatment in subjects with double colonization may be causing and IKK-β-dependent reduction in Stat-1 signaling, which would further reduce inflammatory macrophage products.

Important observations were also made regarding the subjects who were colonized with *S. aureus.* While this infection is quite common in both the young CF population and the general population, there is a current shift from methicillin-senstive *Staphylcoccus aureus* (MSSA) to methicillin-resistant *Staphylcoccus aureus* (MRSA) in both populations. [5] The current study collected information about which strain the subjects were infected with, but did not analyze them by resistance profile due to small sample sizes. Therefore, MRSA status may be a confounder to our results. Furthermore, as mentioned in Chapter 1, *S. aureus* is becoming an organism that can cause chronic colonization in patients with CF. [8] The subjects in this study were not identified as having transient or chronic *S. aureus* infections. This may be another confounder.

It is also important to note that in the *in vitro* and *in vivo* studies conducted previously by our lab, it is evident that AZM does not completely inhibit inflammatory cytokine production, it merely reduces it. [112, 138] While the *in vitro* evidence showed general upregulation of fibrotic mediators from macrophages and fibroblasts by AZM, those trends were ameliorated in the murine and human studies. These studies also showed decreases in inflammatory products, such as iNOS, with administration of AZM. Therefore, the anti-inflammatory properties of the drug may outweigh the pro-fibrotic ones. In addition, both the *in vitro* and *in vivo* studies demonstrated an increase in

MMP-9 production in AZM treated cells. The decreased inflammation, coupled with the increased MMP-9, may be why the subjects on AZM in our study do not have any statistically significant upregulation of fibrotic mediators in their sputa, and may be grounds for why AZM is effective at decreasing morbidity in patients with CF.

Importantly, the subjects who participated were receiving multiple medications in addition to AZM. In order to reduce as much variability as possible, the study excluded known modulators of the immune system, such as systemic glucocorticoids, but all patients with CF are receiving multiple medications on a daily basis. These range from mucolytics to digestive aids.[92] While we did not find any statistical significance in the data, one drug of interest was inhaled tobramycin. This is given to patients who are positive for *P. aeruginosa* cultures, but on a 28-day on, 28-day off cycle.[87] Therefore, the 28 days in which the patients are on both AZM and inhaled tobramycin may produce different immune responses than the 28 days in which the same patients are on only AZM. While tobramycin does not have immunomodulatory properties, the drug may be altering organism burden and indirectly affecting the immune response. This is a facet of treatment that we were unable to examine in our study, but may warrant further investigation.

Furthermore, the subjects were specifically chosen for their status of stable disease, as AZM is given to CF patients as part of maintenance treatment intended to decrease frequency of exacerbation. However, because AZM affects the inflammatory cytokine response, it is still unknown how the relationships between the fibrotic mediators and those cytokines might change during an acute exacerbation. This is a difference from the *in vitro* and *in vivo* models, which studied the initial and resolution phases of the response to the bacterium. These types of comparisons are not possible in our human studies as designed. Future work will investigate these issues.

Future Directions

The conclusions made in the experiments outlined here provide the foundation for further investigation into mechanism of fibrosis development and turnover. While we examined the interactions of several mediators in depth, there are some interactions that warrant future study.

MMP-9 was identified during the *in vitro* experiments as having direct impact upon the development of fibrosis, and was increased by the addition of AZM in the absence of any other stimuli. However, the inflammatory response early after infection in MMP-9$^{-/-}$ mice was not examined, nor was macrophage phenotype. There may be differences in macrophage and neutrophil influx in mice that lack MMP-9, and therefore differences in upregulation of iNOS and arginase. More *in vivo* experiments to evaluate these parameters and determine the exact mechanism of MMP-9 in the response of *P. aeruginosa* could be important. Most of the *in vivo* work done with MMP$^{-/-}$ mice have studied diseases of pulmonary structure, such as bronchopulmonary dysplasia, or ventilator associated lung injury.[141, 142] These studies demonstrate that lack of MMP-9 protects against ventilator induced lung injury, but also induces alveolar hyperplasia and promotes eosinophilic inflammation, suggesting a more complex role in inflammation for the molecule.[140] The preliminary work outlined in Chapter 3 indicates that the long-term effects of MMP-9 deficiency could also increase fibrosis. The data collected was not sufficient to determine the full nature of MMP-9's role in the response to bacterial infection, however. This could be important, especially since the control mice exhibited a neutrophilic response to *P. aeruginosa* infection.[114] Neutrophils are pre-loaded with MMP-9 filled granules – without MMP-9, response of these cells to *P. aeruginosa* may not be the same, nor may the resultant damage and repair.[79]

Investigation early in the course of infection may help elucidate the importance of MMP-9 in neutrophilic influx, and therefore, subsequent pulmonary damage.

IL-1β was shown to be affected by AZM treatment in the human subjects, but we did not investigate this molecule in the mouse models of infection. It is known that IL-1β can have effects on both inflammatory and fibrotic processes by increasing TNF and IL-6, as well as TGF-β and fibrotic mediators.[160] IL-1β can affect macrophages directly – inducing production of IL-8 and TNF, as well as later increases in fibrotic proteins such as TGF-β in diseases like idiopathic pulmonary fibrosis.[15, 119, 183] Given this dual role, it is likely that IL-1β production is increased in response to *P. aeruginosa* infection, and may be subsequently decreased by treatment with AZM. Because our human study did not include subjects who were positive for *P. aeruginosa* but not receiving AZM, we were not able to assess this affect on IL-1β accurately. In future experiments, our group will investigate this in our murine infection model.

Lastly, a major limitation of the clinical study was the limited availability of subjects who were not being treated with AZM. The imbalance of subjects in each treatment group made the statistics difficult to assess. Next, we plan to do a crossover study, in which subjects are taken off of AZM, a suitable washout period is observed, and the same markers measured during and AZM-negative course. This would not only allow us to generate more AZM-negative samples, but also to have more rigid, matched controls. We would observe the same subject both on and off the drug; therefore the changes that occur in the production of proteins examined would be more confidently attributed to AZM.

C. Conclusions

This research provides insights into the mechanisms by which AZM can influence fibrosis development in the setting of *P. aeruginosa* infection. We have identified one potential mechanism by which AZM is shifting macrophages toward an AAM phenotype, as well as the resultant functional characteristics through which these macrophages are then increasing fibrogenesis. This appears to be occurring through the increased production of IKK-β, which then leads to increased production of arginase, increased activation of TGF-β, and increased production of fibronectin. Additionally, AZM-polarized macrophages are different from typical IL-4/13 stimulated AAM, in that MMP-9 protein production is increased as well.

In vivo, we confirmed the impact exerted by AZM on some of these pathways, but also showed that the presence of the alternative phenotype may be globally protective. When faced with an acute infection, the presence of this AZM-polarized macrophage may blunt the damage associated with neutrophil-driven inflammation and prevent the need for later upregulation in airway repair. This is supported by the decrease in iNOS production in the AZM-treated mice. Therefore, the AZM-polarized macrophage may still be producing some fibrotic proteins, but the need to incorporate them into fibrotic tissues may be lessened. Interestingly, the two fibrotic proteins studied, fibronectin and collagen, were impacted oppositely when mice were treated with AZM; fibronectin concentration in the lung was decreased, while collagen concentration around the airways was increased. Because the increased collagen was accompanied by decreases in lung damage, it is difficult to ascertain what the physiologic consequences of the increased collagen might be.

The human study provided us with insights into how the fibrotic markers and inflammatory cytokines interact with each other. The proteins studied reflect not a global shift toward either fibrosis or inflammation, but rather a possible return to homeostasis

when the drug AZM is present. Additionally, treatment with AZM altered relationships between fibrotic mediators and inflammatory cytokines, as well as between specific proteins and lung function. Interestingly, arginase production was only increased in subjects who were positive for both *P. aeruginosa* and *S. aureus*, indicating that double colonization may drive the lung away from a highly inflammatory state as a protective mechanism. While subjects on AZM did not have significantly higher arginase production compared to subjects not on AZM, there was a trend that might grow stonger with more subjects.

Altogether, the three approaches used in this study gave us complementary insights into the mechanism of AZM's impact on fibrosis development, allowing us to reach a better understanding of how AZM-polarized macrophages affect fibrosis development during *P. aeruginosa* infection.

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Publications

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Presentations

"*Alternative Macrophage Phenotype Correlates with Fibrotic Markers in* Pseudomonas *Infection."* Susan Birket, Ted Cory, Brian Murphy, Dave Feola. Autumn Immunology Conference. November, 2011. Chicago, IL.
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"*Azithromycin impacts production of TGFβ and MMP-9."* **Susan E Birket**, Ted J Cory, David J Feola, and Brian S. Murphy. Autumn Immunology Conference. November, 2009, Chicago, IL.